Environmental Selection of Phenotypic Switching of the RpoS-dependent Response in *Escherichia coli*

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Abstract

Understanding the adaptive mechanisms of large regulatory networks can provide insight into long-term survival of bacterial populations in nature. The RpoS master stress regulator found in *E. coli* controls the expression of nearly 10% of the genome when cells enter stationary phase or in response to general stress conditions. Despite its important role in stress protection, mutations in the *rpoS* gene are frequently selected in laboratory strains, pathogenic strains and natural isolates. Loss-of-function mutations are beneficial in long-term stationary phase cultures and in steady state glucose- limited chemostat cultures. Although these mutants have increased utilization of an extensive set of substrates, selection for loss of RpoS function occurs at the cost of reduced stress resistance. Previous studies have demonstrated that highly reversible mutations occur within the *rpoS* gene (for example, transversions and nonsense mutations) when selected on succinate minimal media; however, no study has yet identified a natural compound that can select for restoration of RpoS function. In this study we identify a natural compound allowing restoration of $RpoS^+$ cells from a succinate selected $RpoS^-$ culture. Using an RpoS-dependent *osmY-lacZ* fusion reporter strain carrying a loss-of-function point mutation in the *rpoS* gene, we demonstrate that growth on 6% NaCl results in selection of mutants with restored RpoS function occurring at a mutation frequency of 10^{-9} mutants per cell plated. This is confirmed by RpoS protein detection, and *rpoS* sequencing results show transversion or transition mutations. These results are the first to demonstrate that selection for restoration of RpoS function can be mediated by a single

condition/compound and are consistent with the idea that mutations in the *rpoS* gene may act as a physiological molecular switch to control the expression of the RpoS regulon.

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"*Research is to see what everybody else has seen and to think what nobody else has thought*"

- Albert Szent-Gyorgi (1893-1986) U.S. biochemist

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Chapter 1.0: Introduction

For many years, findings that support the theory of evolution have emerged from comparative studies of living organisms and fossil records. However this methodology is limiting in that it can help gather data to infer what might have occurred but it does not allow us to witness the actual processes of descent with adaptation and modification by natural selection. The emergence of experimental evolutionary systems using microorganisms and in some cases plants and vertebrates has provided us with the opportunity to observe directly the dynamic processes of evolution. Earlier studies in microbiology mainly focused on developing and applying molecular techniques, but with the vast amount of genetic information and the need to further understand long-term sustainability of bacterial cultures, the mechanisms and dynamics of microbial evolution has become a heavily focused field of study.

Bacteria are the most abundant microorganisms found on earth, occupying some of the most extreme environments consisting of high temperature, radiation and heavy metals. Bacterial stress can be defined as cellular damage caused by environmental stimuli or perturbations from optimal growth resulting in decreased growth rate [1]. The continuous existence of bacterial populations over several million years suggests that overtime they have developed effective mechanisms of self- protection allowing them to not only compete against other bacterial strains but adapt to adverse changes in the environment. It is advantageous to study these single celled microorganisms to model systems of experimental evolution. The ability to reproduce quickly, allowing for

observation over several generations, and asexual reproduction, resulting in clonality which enhances the precision of experimental replication, are key factors that promote the study of bacterial evolutionary systems. Such model systems can help us to gain further understanding of how factors such as natural selection can shape or help regulate mechanisms of stress protection.

Two types of stress responses operate in bacteria; specific stress response or general stress response. A specific stress response enables the cell to cope with a single stress but does not provide protection against other stresses, e.g., heat shock response in *Escherichia coli*. Heat-induced protein damage triggers the increase in the master regulator σ^{32} [1-4] which specifically directs RNA polymerase to transcribe heat shock promoters only [1]. The general stress response in contrast, can be induced by multiple stress challenges (discussed in detail below) and is often regulated by a single master regulator. Even if only a single stress challenge stimuli is present in the environment response to protection against other stresses that may be experienced at a later time point, occurs. Therefore, general stress response regulators play a key role in providing not only protective measures but also preventative measures. In this thesis the general stress response sigma factor RpoS found in *Escherichia coli* is used as a model system to gain further understanding of the regulatory mechanisms of bacterial stress adaptation at the cellular and population level.

1.1 RpoS a master regulator

RpoS (also called σ^S or σ^{38}) is a 38 kDa sigma factor responsible for either directly or indirectly regulating approximately 10% of the *E. coli* genome, comprising what is known as the RpoS regulon [5-9]. By binding to core RNA polymerase and forming a holoenzyme it enhances expression of RpoS-dependent promoter specific genes. As a master regulator of general stress response, RpoS is involved in stress protection during stationary phase , as well as heat stress [10], oxidative stress [11], acid stress [12] nutrient deprivation [13], and near-UV exposure [11]. During optimal growth, levels of RpoS are attenuated by tight regulation at the transcriptional, translational and post-translational levels.

RpoS has been identified in several bacterial species that are known to infect humans, plants, insects and animals. For example, RpoS is required for the invasion of brain microvascular endothelial cells responsible for causing neonatal meningitis by *E. coli* K-1 strains [14]. In *Samonella* RpoS plays a role in virulence and stress resistance. During invasion in host macrophages, RpoS is needed for the resistance to stresses such as starvation, DNA damage, low pH and oxidative stress [15]. RpoS is critical for the persistence of *Samonella* in lymphoid organs such as the spleen [16, 17] and liver [17] because it positively regulates the expression of plasmid-borne *spvR* and *spvABCD* genes, which are required for intracellular growth and systemic infection in humans and mice [15, 18]. In the plant pathogen *Erwinia carotovorn* RpoS is needed for survival under stresses such as acid pH, starvation, and exposure to H_2O_2 [19]. Although RpoS has not been found in gram positive bacteria, they exhibit a similar stress response that is

physiologically similar to that of *E. coli* but under the control of a different master regulator known as σ^B. For example activation of σ^B results in increased resistance to acid, alkali, ethanol, heat, osmotic, or oxidative stress in *Bacilius subtilis* [20-23].

Therefore it is of interest to study this particular master regulator because of its existence in many different bacterial species and the possible role it may play in pathogenesis. Also, in nature bacteria often spend the majority of their time in sub-optimal growth conditions, so the study of RpoS can help understand natural populations as well.

1.2 The RpoS Regulon: Physiological functions of RpoS-controlled genes

The important role of RpoS in *E. coli* is emphasized by the several different functions it helps regulate within the cell. Not only does it help induce the expression of genes involved in stress protection, but it also inhibits the expression of genes whose products in excess might be either harmful or costly during nutrient limitation [24] in order to reduce unnecessary costs to the cell. Examples of key functional genes regulated by RpoS, is discussed below.

Oxidative stress occurs in *E. coli* due to the buildup of partially reduced reactive oxygen species (ROS) that carry a single electron making them highly unstable and more easily able to take away electrons from other biomolecules. Such ROS molecules include superoxide anion radical (O_2-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (HO-). Two genes that are regulated by RpoS and involved in coping with oxidative stress in *E. coli* are *katE* and *katG* which encode for catalase HPI and HPII respectively

(reviewed by Schellhorn [25]). KatE is highly dependent on RpoS for its expression [26]. RpoS activates the transcription of *katE* by a factor of 7-fold in exponential phase and 40-fold in stationary phase in rich media as found through β-galactosidase fusions [27].

The availability of water and solute concentration in the surrounding environment can drastically effect growth of *E. coli* if osmotic balance is not maintained. The sudden influx of water can result in bursting of the cell in hypertonic conditions while a significant loss of water can result in dehydration in hypotonic conditions. Resistance to osmotic stress is critical for survival and some genes known to be osmotically induced are found to be under the control of RpoS. Thus in addition to being osmotically induced, they are expressed when cells enter stationary phase of growth as well. The hyperosmotically induced *osmY* gene which encodes for a periplasmic protein of unknown function is highly RpoS dependent [28]. The *otsA* and *otsB* genes, which are required to synthesize the disaccharide trehalose; serves as a general stress protectant in bacteria, are both positively regulated by RpoS and have found to play a role in RpoS-dependent stationary phase thermotolerance [10]. Similarly, two other dual controlled genes are *treA* which encodes for a periplasmic glycoside hydolaze enzyme, trehalase [29, 30] and *osmB* which encodes for an outer membrane lipoprotein [31]. Both these genes are regulated by RpoS[1, 10] and inducible under hyperosmotic conditions as well as stationary phase dependent.

Acid resistance (AR) is important for survival of *E. coli* in acidic foods and may be required for passage through the gastrointestinal tract. Three AR systems exist in *E. coli*, one system is highly RpoS-dependent while the other two systems (arginine

dependent and glutamate dependent) are only partially dependent on RpoS [12]. When mice and calves were ingested with wild type and mutant *rpoS* forms of *E. coli* 0157:H7, the survival of wild type strain in feces was much higher than that compared to the *rpoS* mutant strain, this is likely due to the RpoS-regulated acid resistance response needed to survive the gastrointestinal tract [32]. Two genes found to play a role in extreme acid resistance (pH 2.5), are the *gadA* and *gadB* genes which encode for glutamate decarboxlases, both these genes are RpoS regulated.

Along with positive regulation of many genes, RpoS also negatively regulates particular sets of genes. There are three major classes of negatively regulated genes and these are; 1) genes encoding for functions in energy metabolism , 2) genes encoding for functions of flagellar biosynthesis which is responsible for motility and 3) genes encoding the Rac prophage region [6]. The first class, energy metabolism, predominately includes genes that encode for enzymes of the TCA cycle (for example; *pykA*, *acnB*, *fumA*, *sucAB* and *sdhA*). Flagellar genes include genes involved in chemical signaling such as those that are involved in chemotaxis and also genes that encode for structural components of the flagella such as *fliC* and *fliE*. Negative regulation of flagellar gene expression by RpoS is through the master regulator FlhCD, or through the flagellar sigma factor FliA [6] .

The involvement of RpoS-dependent genes in different pathways of stress resistance; including oxidative stress, osmotic stress, and acid stress, speaks to the multilayered and valuable function of the RpoS regulon for *E. coli*. As a single master

regulator, RpoS, can orchestrate the expression of the many layers of this regulon in response to environmental stimuli.

1.3 Acquisition of mutations in the *rpoS* gene

Natural selection acts on phenotypic changes in an organism, and mutations provide a means by which such changes can occur. In many important regulatory genes, mutations result in deleterious effects, for example, mutations in the housekeeping sigma factor RpoD results in unviable cells. Despite the important role RpoS plays in stress protection, mutations in the *rpoS* gene resulting in loss of RpoS function are selected for [33] . A summary of the experimental findings of sweeps of *rpoS* mutants across bacterial populations is discussed in detail below.

In studying the survival of *E. coli* during stationary phase it was observed that even after cultures had reached saturation, cells remained viable for several days and even weeks. Competition experiments between aged (10-day old) and young (1 day old) cultures of the same strain incubated for 2 weeks results in viable counts of the aged culture during progressive time points but death of the young culture [33]. Even after several generations of exponential growth of the aged cultures, the aged cultures repeatedly out-compete the young cultures. Sequencing of the *rpoS* gene from the wild type and from an out-competed aged cell revealed a 46-base pair duplication in the out-competed aged cell resulting in the addition of 39 amino acid residues in the RpoS protein; affecting its ability to recognize promoter sequences [33]. Although similar *rpoS* mutants with large duplications have not been found in other studies, this was the first study to demonstrate that the **g**rowth **a**dvantage during **s**tationary **p**hase (GASP)

phenotype observed in aged cells was a phenomena due to a mutational inheritance and not due to a reversible physiological response to starvation [33].

When wild type *E. coli* strains are inoculated into glucose-limited chemostats, after 4 days of incubation majority of the population is established by *rpoS* mutants [34]. These mutants exhibit reduced gas evolution with hydrogen peroxide indicative of reduced RpoS-dependent *katE* gene expression. Sequencing of 18 isolates exhibiting growth advantage in glucose limited chemostats revealed 10 different types of mutations in the *rpoS* gene (among the 18 isolates) resulting in attenuated RpoS function [34]. Similarly, *rpoS* mutants take over wild type populations during growth in nitrogen-limited chemostats. Within 5 days of incubation more than 90% of the population consisted of *rpoS* mutants [34].

Growth of wild type *E. coli* K-12 on succinate, a poor carbon source, selects for attenuated RpoS function as determined by Chen *et al*. [35]. Using strains harboring a highly RpoS-dependent *osmY-lacZ* reporter fusion it was demonstrated that large colonies on succinate minimal media (Suc^+) have acquired mutations in the *rpoS* gene [35]. The mutational frequency of Suc^+ phenotype was found to be 10^{-8} mutants per cell plated. These mutants exhibit reduced RpoS protein levels compared to the wild type strain and reduced RpoS-dependent *katE* gene expression. The generation time of wild type in succinate minimal media is 1027 ± 128 min where as the generation time of *rpoS* mutants in succinate minimal media is 155 ± 4 min [35].

In addition to laboratory strains, the spread of *rpoS* mutants among pathogenic strains of *E. coli* may also be explained by positive selective agents in the environment. Ten of

seven representative verocytotoxin-producing *E. coli* (VTEC) strains have large *rpoS* mutant colonies (Suc^{++}) occurring at a frequency of 10^{-8} mutants per cell plated when grown on succinate minimal media plates [36]. Approximately 90% of the Suc^{++} mutants of strain 0157:H7 EDL933 are impaired in RpoS-dependent catalase activity [36]. Similarly fumarate, another TCA cycle intermediate, has been identified as another selective agent that selects for *rpoS* mutants in pathogenic strains [36].

The findings from these studies reveal the types of environmental selection pressures influencing the spread of *rpoS* mutants among a population. There is a need for expression of the RpoS regulon in stress challenging environments but successful growth in nutrient limited conditions requires attenuation or loss-of-function of the RpoS regulon. This suggests that survival of *E. coli* populations in nature maybe increased by cells possessing effective on/off expression control of the RpoS regulon.

1.4 Mechanisms of regulation of RpoS

Since RpoS controls the expression of several genes in response to stress protection, unnecessary expression of the regulon can be costly and therefore levels of RpoS must be highly regulated. Regulation occurs at the transcriptional, translational and post-translational levels. Transcription of *rpoS* is positively regulated by polyphosphate; a common inorganic molecule found in many bacterial species that has multiple functions [37], guanosine 3’5’-bisphyrophosphate (ppGpp) which is necessary for transcription elongation , acetate, and growth rate reduction as experienced in stationary phase. Negative transcription regulation takes place using Fis, a global transcription factor that

can bind to the *rpoS* promoter region to block transcription during exponential growth but diminishes during onset of stationary phase to allow for *rpoS* induction[38]. The cAMP-CRP molecule regulates transcription of the *rpoS* gene both positively and negatively. During stationary phase cAMP-CRP positively regulates transcription but during exponential phase it is a negative regulator of transcription [39]. Built into the *rpoS* mRNA itself is a hair pin structure composed of567 nucleotides of the untranslated region at the 5' end that renders the mRNA inaccessible to ribosomes under non-inducing conditions, thereby preventing translation [40, 41]. Under inducing conditions, translation occurs after binding of the *rpoS* mRNA to positive regulator molecules. For example, DsrA is a small regulatory RNA that partially compliments *rpoS* mRNA and initiate translation by revealing the ribosomal binding site. RprA is another regulatory RNA similar to DsrA and functions as a positive regulator of *rpoS* mRNA translation. Negative regulation may also occur through H-NS, a histone-like protein, that binds to *rpoS* mRNA and changes its secondary structure [42].

When *E. coli* is growing under optimal growth conditions, RpoS can still be expressed but levels are kept low due to rapid degradation [39, 43]. The ClpXP protease is responsible for this degradation serving as a mechanism of post-translational regulation. RpoS binds to a specific recognition factor, RssB [44, 45] before being transferred to the ClpXP protease, where it is unfolded and completely degraded by an ATP-hydrolysis mechanism. RssB is then released and reused again [46].

1.5 Sigma factor competition

The need for on/off expression control of the RpoS regulon may stem from the inherent competition existing between sigma factors for limiting RNA polymerase core subunits [47]. *E. coli* has seven known sigma factors; RpoD , RpoN, RpoS , RpoH, RpoF, RpoE and FecI and with the exception of RpoS and RpoD, each sigma factor is responsible for regulating response to a specific stress (see [48] review). RpoD, also known as the housekeeping sigma factor, is responsible for the expression of genes involved in maintaining vegetative growth. The concentration of available core polymerase and the levels of RpoD remain relatively constant in *E. coli* K-12 under different growth conditions, but levels of RpoS and the other sigma factors vary in concentration depending on the type of environmental signals present [47]. Increased expression of RpoS during a general stress response or during entry into stationary phase reduces the number of available core polymerase for RpoD binding, creating competition between RpoS and RpoD (Figure1). However, in nutrient limiting environments such as growth on a poor carbon source, mutations in the *rpoS* gene alleviates this competition and improves nutrient scavenging by allowing an increase in RpoD-dependent gene expression [49]. Cells with increased RpoD –dependent gene expression have an increased ability to grow on an extensive set of substrates.

Stress protection and nutritional competence both involve the expression of large sets of genes controlled by two different master regulators that are competing for binding of limited RNA polymerase. A tradeoff between **s**elf- **p**reservation **a**nd **n**utritional **c**ompetence referred to as SPANC balance [50] occurs to help compromise between the

two physiological responses. SPANC balance suggests that within the cell active and inactive states of the RpoS regulon is needed to facilitate effective transition between two physiological responses. Because acquisition of mutations in *rpoD* are more likely to be lethal, the sweep of *rpoS* mutants; mutations in the *rpoS* gene resulting in attenuated or loss of RpoS function, maybe the only available mechanism by which a population can quickly turnover in RpoS levels.

1.6 Dynamics of rpoS gene status

The spread of *rpoS* mutants in a population of *E. coli* may be due to selection of a beneficial mutation. However, despite the strong fitness advantage in nutrient limited environments, it does not result in 100% fixation within the population. *E. coli* growth in glucose limited chemostat cultures showed that a stable subpopulation of wild type *rpoS* consisting of less than 1% of the cells is maintained. After 100 generations, this sub-population recovers to > 30% [51, 52]. This demonstrates that the status of the *rpoS* gene can be dynamic; populations fluctuate between wild type and mutant forms of the allele over a period of long term growth. The mechanism of the dynamic nature of the gene within the population is not well understood. Whether the re-emergence occurs because it consists of cells that did not acquire mutations in the *rpoS* gene, but developed alternative mechanisms to increase RpoD-dependent nutrient scavenging without hindering RpoS, or whether, this subpopulation represents the selection for restoration of RpoS function is not known. The re-emergence of $RpoS^+$ cells within the population was observed in all

eight independently grown chemostat cultures [51]. Additionally, analysis of *rpoS* status in natural isolates reveals both wild type and *rpoS* mutants within the population. For example, in a recent survey of 2040 natural isolates, 6 *rpoS* mutants were found (Chiang, S., *et al* 2011 – unpublished data), suggesting that in nature there is a strong selection pressure to maintain *rpoS*$^+$ populations. However, no studies have yet demonstrated a possible mechanism to explain the maintenance or re-emergence of RpoS$^+$ populations.

1.7 Bacterial genetic switches

Under constantly changing environments phenotypic variation based on genotypic variation such as an increase in genome-wide mutation rate may be advantageous due to an increased chance for adaptation [53, 54]. However, when environments are less variable, and bacterial growth is found to cycle between fixed environments, then genome-wide mutation rates can become disadvantageous. Because most mutations are more likely to result in deleterious effects, the costs associated with a deleterious mutation maybe greater than the benefit of a rare beneficial mutation [53, 54].

Selection for increased mutation rates at specific sites can be a mechanism of adaptation for growth between previously encountered environments. An increased mutation rate at a specific site can increase the rate of switching between alternating phenotypes resulting from that loci. An example of this in *E. coli* is phase-variable switching of the type 1fimbriae. The “on” expression of type 1 fimbriae promotes attachment to eukaryotic cells, but “off” expression is advantageous during pathogenisis

due to the high immunogenic response elicited by type 1 fimbriae . Phase-variable switching is associated with inversion of a 314-bp nucleotide element that contains the promoter for the *fimA* gene, which encodes for the main structural subunit of the fimbriae [55, 56]. The orientation of the element determines "on" or "off" expression of type 1 fimbraie which is regulated by environmental factors such as nutrient availability and temperature [53, 57-59]. Switching is found to occur at rates as high as 10^{-3} per cell per generation [55].

Additionally, phenotypic switching is also found in *Haemophilus influenza* and *Staphylococcus aureus*. *Haemophilus influenza* is a common inhabitant of the upper respiratory tract in humans but can become pathogenic in some hosts [60, 61]. The existence of contingency loci defined as loci containing tandem DNA repeats of unit sizes of 1-8 bp is used to vary expression of many virulence molecules. Tracts of nucleotide repeats that are prone to undergoing strand slippage mediated by the mis-match repair system (MMR), results in an increased mutation rate [53, 62]. These tracts are found either within or near to a coding region of a gene. The "on" or "off" expression of genes encoding for virulence molecules in *Haemophilus influenza* is mediated by mutations at contingency loci resulting in either the disruption or establishment of a functional operator region.

Cyclic exposure of *Staphlococcus aureus* between gentamicin and an antibiotic free environment demonstrates the ability for phenotypic switching between antibiotic sensitive and resistant phenotypes [63]. Resistance to gentamicin results from point mutations in genes encoding target proteins. The rate of switching from wild type to

gentamicin-resistant small colony variants (SCV) is about 0.07%-0.3% cells/per generation and the rate of switching from SCV to revertants (back to wild type) is 0.0009%-0.04% cells/generation [63]. The ability to switch resistance on and off is adaptive because it allows the bacteria to circumvent negative fitness costs associated with permanent resistance [53, 63]. However the mechanism behind antibiotic resistant phenotypic switching is not yet known.

The development of genetic switches mediated by environmental factors in these organisms suggests that environmental selection for increased mutation rates at specific sites is common and may occur in nature. Whether this form mutation mediated phenotypic switching applies to RpoS-dependent response found in *E.coli* is not yet known.

1.8 Mechanism of alterations to bacterial genomes

The genome evolves in response to selection pressures in the environment. Alterations to bacterial DNA can occur through natural process such as horizontal gene transfer and mutation. Horizontal gene transfer is the acquisition of foreign DNA either through transformation, conjugation or transduction. Mutations which occur spontaneously in nature can either be beneficial, neutral or deleterious. The most common source of mutations is due to errors in DNA replication. The error rate of DNA polymerase III is about 10^{-6} to 10^{-7}mutations per nucleotide per replication [64, 65]. The DNA mismatch repair mechanism fixes 99% of the errors made by DNA polymerase III, but 1% escapes this repair resulting in a mutation. The overall rate of mutation during

replication is 10^{-8} ~10^{-10} mutations per base-pair replicated. The measured mutation rate for *E. coli* is 5.4 x 10^{-10} mutations per nucleotide per generation [66]. Given that the size of the *E. coli* genome is 4.2 million base pairs or 8.4 million nucleotides [67] and that *E. coli* grows exponentially, it requires at least 11 generations (2028 cells) before 1 mutation can occur. The majority of mutations result in deleterious effects, and the frequency of beneficial mutations is low. In *E. coli* K-12, the rate of deleterious mutations per genome per generation is 2-8 x 10^{-4} [68, 69] while the rate of beneficial mutations is 2 x 10^{-9} [70].

Back mutations (reverse mutations) can restore phenotypes resulting in revertants, which occurs in nature and may play a crucial role in the cycling of gene functions between environments. There are two mechanisms by which phenotypes may be restored, the first mechanisms is by true reversions. True reversions result in restoration of not only the original phenotype but also restoration of the original DNA sequence. The second mechanism is by suppressor mutations (or pseudo-reversions), this is due to a mutation occurring within the gene that restores the original gene function but not the original DNA sequence. Intergenic suppressor mutations (mutations that occur elsewhere in the genome), can restore the phenotype but not the gene function and thus are considered to result in compensation for original function. The chance of obtaining a revertant depends on the nature of the original mutation. Point mutations revert at the highest frequency because they only require a single base change in the sequence to restore the original sequence. In contrast, deletion mutations cannot revert because the chance of a mutation resulting in the exact DNA sequence is highly unlikely.

1.9 Mutation rates

The Luria and Delbruck fluctuation test described 68 years ago, was the first method to determine rates of spontaneous mutations in cultured bacterial cells [71]. This established that mutations arise prior to selection and not as a response to selection. Knowing the rate at which spontaneous mutations occur can yield beneficial information regarding bacterial adaptation, for example commensal *E. coli* strains have significantly lower mutations per genome than pathogenic strains [72, 73] suggesting increased mutation rate may enhance selective adaptation to different host environments. Polymorphisms in the *rpoS* allele are common in laboratory strains, natural isolates and pathogenic strains [50, 74, 75]. Although it has been determined that the frequency of *rpoS* mutants selected on succinate (1%) minimal media is 10^{-8} mutants per cell plated [35], the rate at which mutations occur within the *rpoS* gene has not yet been determined.

The mutation rate is the probability of the number of mutations a cell will acquire per generation. This is different from the mutation frequency, which refers to the proportion of cells in a population that are mutant. In the literature when discussing mutations, the frequency is often reported however it is important to know that when properly determined the mutation rate is more accurate and reproducible than the mutation frequency [71]. The reason being, after a cell acquires a mutation it continues to divide and produce more mutants that accumulate within the population. The size of the mutant population will vary depending on when during the growth of the population the mutation occurred. Because cell growth is exponential, mutations acquired during early stages of growth will result in “jack pot”, cultures that contain many mutants, where as

mutations occurring late in population growth will produce fewer clones. Therefore, the frequency or proportion of mutants in the population is a reflection of an underlying stochastic process.

In a fluctuation test a small number of cells are inoculated into several parallel cultures and allowed to grow to saturation (see [76] for review) . Each culture is plated onto the selective condition that enhances the growth of the mutants. Total cell number is determined by plating dilutions onto non-selective media. The mutation rate is determined from the distribution of the number of mutants among parallel cultures. The number of mutations that occur among parallel cultures has a Poisson distribution, that is, mutations occur with an average known rate and independently of the time when they last occurred. The number of mutants among parallel cultures has what is known as the Luria-Delbruck distribution [76].

1.10 Rationale and project objective

Stress resistance is critical for survival and because RpoS is a single master regulator of multiple stress responses that not only aids in survival in nature but also contributes to survival within human and animal host environments (see [77] for review), it may be considered a huge loss in fitness if a population evolves to exist without it. Currently, our understanding of the RpoS regulon is that its expression is promoted during entry into stationary phase or during stress challenges but its expression becomes costly when cells are experiencing extended nutrient limitation. This suggests that there exists a need for an effective method of control of expression of the RpoS regulon for

long term survival between fixed environments. Many studies have focused on identifying the type of environmental conditions under which "off" expression of the RpoS regulon is selected for [33-35, 78], and although it is predicted that RpoS function cycles between stressful and nutrient limited environments in nature [79], no studies have yet demonstrated whether restoration of an $RpoS^+$ population ("off" to "on" expression) is possible when there is a change in growth condition from nutrient limitation to stress challenging. If *rpoS* function is important in cycling between environments, then there must be a natural compound that selects for restoration of function. To determine this, a high through-put screen examining nearly 2000 compounds was conducted. It is hypothesized that mutations in the *rpoS* gene may act as a physiological molecular switch to control on and off expression of the RpoS regulon. If this is true, then recovery of an $RpoS^+$ population is expected via selection for reverse mutations in the *rpoS* gene. This was done by examining the growth of an $RpoS^-$ population on the identified selective agent(s).

Furthermore, because mutations commonly occur within the *rpoS* gene, it is possible that the mutation rate at the *rpoS* locus is higher than the expected mutation rate for *E. coli* making the *rpoS* locus a potential "hotspot" within the genome. Thus far, only mutational frequencies have been reported, however they are not a true reflection of the number of mutations occurring per generation per cell. Therefore, the second objective of this study is to determine the rate at which loss-of-function mutations occur within the *rpoS* gene.

Chapter 2.0: Materials and Methods

2.1 Bacterial strains and growth conditions

All bacterial strains and mutants selected in this study are listed in Table 1. The *rpoS* mutant strain SS53 used for salt selection was selected on M9 (1.0% succinate)/agar plate by S. Sullivan (Schellhorn lab, McMaster University). All bacterial strains were initially streaked out onto Luria-Bertani (LB)/agar plates as described by Miller (1992) [80] supplemented with kanamycin (50µg/ml) when appropriate and incubated overnight at 37^{o}C. To compare sensitivity to NaCl, independent colonies were inoculated (in triplicate) into 50ml flasks containing 10ml of LB broth lacking NaCl (1g/100ml Bacto tryptone and 0.5g/100ml Bacto yeast extract only) grown overnight at 37^{o}C with shaking at 200rpm. Cultures were then sub-cultured the following day into 50ml of fresh media and maintained in exponential phase for at least 8 generations of growth prior to experimentation in high salt (NaCl) media. Growth was monitored by measuring optical density at 600nm using Shimadzu UV-1201 spectrophotometer. When appropriate, plates were supplemented with 5-bromo-4-chloro-3-indoyle-β-D-galactopyranoside (X-gal) (50 ug/ml, final concentration) to utilize blue/white screening methodology.

2.2 Phenotypic microarray analysis

To determine chemical compounds that can potentially select for the restoration of RpoS function, growth comparison between the wild type strain MG1655 and isogenic

rpoS-null mutant strain HW2211 was conducted. Phenotypes were compared by using a high-throughput phenotype screening service, Phenotypic Microarray (PM) analysis (Biolog, Hayward, CA), that tests for approximately 2000 cellular phenotypes. This analysis included the testing of stress and repair functions, utilization of carbon, nitrogen, phosphorus and sulfur, cellular respiratory functions, biosynthesis of small molecules, and cell surface structure and transport functions. The phenotypic tests were preconfigured into sets of arrays and each well of the array was designed to test a different phenotype. Cell response to growth condition in each well is measured based on levels of cellular respiration. If the cells have a positive response to the phenotype being tested, then cells respire actively, resulting in the reduction of tetrazolium dye and formation of a strong color in the well. If the cells have a weakly positive response, then there is a decrease in cellular respiration and less color present in the well, and if cells have a negative response then no color is formed in the well. Using OmniLog software, graphs are produced based on amount of color present in each well, and differences in phenotype between the wild type and *rpoS* mutant are determined based on quantitative differences in overlapping graphs.

2.3 Phenotype confirmation using RpoS reporter fusion strains

To verify results obtained by Bio-log phenotypic array analysis with our RpoS-dependent *osmY-lacZ* reporter fusion strains, replica plating was done on different concentrations of NaCl. Independent colonies were inoculated into wells containing 200µl of LB broth in a 96-well microtiter plate, using a sterilized toothpick. The

microtiter plate was incubated overnight at 37^{o}C. Before transfer of cultures from LB to selective media plates, cultures were washed twice in M9 salts. To transfer cultures from microtiter plate to selective media plates, a replica plating tool (metal-prong block) was used, between each transfer, the prong was sterilized in 95% ethanol.

2.4 Selection for NaCl-resistant mutants

To easily identify mutants having restored RpoS function, a strain harboring a highly RpoS dependent *osmY-lacZ* reporter fusion was employed. The *lacZ* gene encodes for the enzyme β-galactosidase which can cleave the chromogenic substrate X-gal to produce an insoluble blue product. Expression of RpoS-dependent *osmY-lacZ* results in blue colonies indicating possible gain of RpoS function. Lack of RpoS-dependent *osmY-lacZ* expression results in white colonies indicating no gain in RpoS function. Single colonies were inoculated into LB broth and grown over-night, approximately 10^{9} cells were plated onto LB/agar plates containing 6% NaCl and supplemented with X-gal. After 3 days of incubation at 37^{o}C, large dark blue colonies (NaCl-resistant) indicative of putative gain of function mutants along with white mutant colonies indicative of possible compensation of RpoS function mutants were purified and further characterized. Fifteen independent cultures were grown to ensure independent mutational event.

2.5 Purification and Qualitative Catalase Assay

All isolated NaCl-resistant mutants were streaked for purity three times on to LB (6% NaCl)/agar plates containing X-gal. After purification, plate catalase assays were

used to qualitatively test for RpoS-dependent catalase activity since *katE*, which encodes catalase, has a highly RpoS-dependent promoter [26]. Strains were replica plated onto LB/agar plates and grown overnight at 37^{o}C. Ten microlitres of 30% H_2O_2 was dropped onto each individual growth patch and intensity of bubbling was compared between control and NaCl-resistant strains.

2.6 Western blot analysis of RpoS in NaCl-resistant mutants

Cultures were grown in LB broth media at 37^{o}C while shaking at 200rpm and growth was monitored periodically. Stationary phase samples were collected (OD_{600} = 1.5) and centrifuged at 15,000g for 2 minutes. Pellets were washed three times in PBS buffer (pH = 7.0) to remove salts from growth media and resuspended in SDS-PAGE loading buffer to OD_{600} = 1.0 and boiled for 5 minutes. Twenty microlitre samples were loaded and resolved on 10% SDS-PAGE gels at 100 V for 2 hours. Samples were transferred to PVDF membrane (Millipore) at 20V for 25 minutes. After transfer, PVDF membrane was blocked with 5% skimmed milk and incubated with primary mouse monoclonal antibody to RpoS (NeoClone – Product # SP002-W0009) followed by incubation with secondary Goat anti-mouse antibody (Bio-Rad- Product # 170-6516). After, the membrane was incubated with ECL solution (Amersham) and exposed to Hyperfilm-ECL (Amersham). To confirm equal protein loading, identical SDS-PAGE gels were run in parallel and stained by Coomassie Blue R-250.

2.7 Amplification of the *rpoS* region and sequencing

The *rpoS* region of the NaCl-resistant catalase positive and negative strains was amplified using forward primer ML-08145 (CACAAGAAGTGAAGGCGGG) (synthesized byMOBIX lab, McMaster University) and reverse primer ML-08146 (TGGCC TTTCTGACAGATGCT TAC) (synthesized by MOBIX lab, McMaster University) by whole colony PCR. A single colony from each strain was inoculated into 20µl of ddH_20 and heated at 95^{o}C for 5 minutes. After boiling, 2µl was used in a standard 25µl PCR reaction. PCR conditions were 2 minutes at 95^{o}C, followed by 30 cycles of heating at 95^{o}C for 30 seconds, 57^{o}C for 30 seconds, and 72^{o}C for 1 minute followed by final extension at 72^{o}C for 2 minutes. PCR products were purified using Nucleospin Extraction II Kit and sequenced by MOBIX lab (McMaster University).

2.8 Fluctuation test

To determine the rate at which mutations occur in the *rpoS* gene, a fluctuation test was performed. A single colony of wild type *rpoS* strain HS1091 was inoculated into 10ml of LB broth supplemented with Kanamycin and grown overnight at 37^{o}C with shaking at 200rpm. After overnight incubation, the batch culture was sub-cultured 1/10 000 into fresh LB broth supplemented with Kanamycin and 200µl aliquots of the diluted cultures were distributed per well into 30 wells of a 96-well microtiter plate. Cultures were grown to saturation by incubation at 37^{o}C with shaking at 200rpm. After cultures

reached saturation, 100µl of culture (per well) was collected in a 1.5ml eppendorf tube and spun for 2 minutes at 10 000 x g. Pellet was washed three times in M9 salts. After washing, cultures were diluted (10^5 fold dilution) and plated onto M9 (1%succinate)/agar plates containing X-gal. Plates were incubated for 3 nights at 37^{o}C. To determine total number of CFU in saturated cultures, 3 representative cultures were serially diluted (100 folds per dilution) in M9 salts and plated onto LB/agar media and incubated overnight at 37^{o}C. The mutation rate was calculated using the Ma-Sandri-Sarkar Maximum Likelihood Estimator (MSS-MLE) implemented by the FALCOR Web tool [81]. This method uses an initial estimate of the number of mutational events, *m*, to calculate the probability of observing *r* mutants on the selective medium. The likelihood function is the product of the probability for each observed value of *r*. The mutation rate is calculated as *m/Nt*, where *Nt* represents the total number of cells in each culture [81].

Chapter 3.0: Results

3.1 Identification of NaCl as a selective agent for restoration of RpoS function

To identify chemicals that can potentially select for mutants that have restoration of RpoS function, a phenotypic microarray analysis was employed to exhaustively determine which chemicals favor the growth of wild type *rpoS E. coli* K-12 compared to an *rpoS* null mutant. Cell growth was indirectly measured by monitoring rates of cellular respiration. Out of approximately 2000 chemical compounds that were tested, 126

compounds were identified that allowed increased growth of the *rpoS* null mutant strain compared to the wild type, while 14 lead compounds (Table 2) were identified that allowed increased growth of the wild type strain compared to the *rpoS* null mutant. All phenotypes tested are associated with nutrient utilization or sensitivity to various stresses, affecting pathways of cellular respiration. A loss of RpoS function affected carbon and nitrogen source utilization but not phosphorous, or sulfur. Out of the 14 compounds identified to allow for increase in wild type growth compared to the mutant, the largest measured difference in growth was observed from testing oxytetracycline (PM = -202), however the increased sensitivity of the *rpoS* mutant is likely due to the replacement of the oxytetracycline resistant gene in this strain. The smallest measured difference in growth was observed from testing propionic acid as a carbon source (PM = -51). The *rpoS* mutant strain was also hypersensitive to two types of fungicide, totlylflunid and dichlofluanid, as well as increased sensitivity to protamine sulfate, a chemical involved in membrane ATPase activity [82]. The most common phenotype identified in the mutant strain was increased sensitivity to osmolytes (osmotic stress) specifically NaCl suggesting that NaCl might serve as a strong selective agent. However testing a range of concentrations of NaCl alone was done by PM services, but their data doesn't show a significant difference in levels of sensitivity between the wild type and *rpoS* null mutant (see Table 2). To confirm their findings, strains, HS1091 (WT) and HS1091p (*rpoS* null mutant) of the GC4468 background were replica plated onto LB/agar plates containing concentrations of NaCl ranging from 2% to 8% (see Table 3). Sensitivity/resistance to NaCl was determined based on intensity of growth patch on each plate (indicated by

number of " +" in table). The control plate has full patches of growth of both strains when plated on LB/agar lacking NaCl. Replica plating onto plates containing 2% and 4% NaCl has growth similar to the control plate. At 6% NaCl the *rpoS* mutant shows sensitivity to NaCl but the wild type strain is resistant to the stress imposed by NaCl. At 8% NaCl, there is very little growth of the wild type strain and no growth of the *rpoS* mutant strain. These results confirm that 6% NaCl can be used to preferentially select for the growth wild type *rpoS* which is contrary to what was found by PM services analysis.

To effectively identify mutants with restored RpoS function a blue/white screening methodology utilizing a *lacZ* reporter fusion was employed. The *osmY* gene encodes for a periplasmic protein serving as an osmoprotectant and is highly RpoS-dependent [28].The expression of RpoS dependent *osmY-lacZ* reporter gene indicates functional RpoS within the cell, resulting in blue color colonies when plated on X-gal. To confirm that the use of this reporter fusion or the addition of X-gal in the plates does not affect growth on 6% NaCl, replica plating was conducted using isogenic strains lacking the reporter fusion on LB + 6% NaCl plates. The control (Figure 2a) shows that all strains grow equally well when plated onto LB with no NaCl and no X-gal. When X-gal is added to the plate (Figure 2b) there is no difference in the amount of growth between strains harboring a reporter fusion (HS1091, H1091p, and SS53) and strains lacking a reporter fusion (GC4468 and GC122). Because the strain HS1091 has a functional wild type RpoS, the growth patches produced by this strain are dark blue in color. The growth patches produced by the strains HS109p and SS53 are white and light blue in color, respectively. HS1091p is an *rpoS* null mutant so it completely lacks expression of the

RpoS dependent *osmY-lacZ* reporter fusion, but SS53 has a point mutation in the *rpoS* gene resulting in attenuated RpoS function, thus SS53 has some expression of the RpoS dependent *osmY-lacZ* reporter fusion. Growth on 6% NaCl is not affected by the reporter fusion or X-gal (seen Figures 2c and 2d). As expected, both wild type *rpoS* strains, HS1091 and GC4468 have the same level of growth.

3.2 Growth curve

To determine the effect that 6% NaCl stress has on growth, the wild type and *rpoS* mutant strain were grown in liquid LB + 6% NaCl (see Figure 3). When strains are grown in LB lacking NaCl (Figure 3 a), both the wild type and mutant have generation times of approximately 43 minutes during exponential growth. When strains are grown in LB + 6% NaCl (Figure 3 b), the wild type has a generation time of approximately 112 minutes but the mutant is too sensitive to grow (generation time could not be determined). Growth in NaCl has slowed down the generation time of the wild type strain by approximately 69 minutes compared to growth in the control experiment.

3.3 Selection of NaCl-resistant mutants

Selection for restoration of RpoS function was on LB + 6% NaCl plates containing X-gal. Approximately 10^9 cells, of strain SS53 harboring a loss of function mutation in the *rpoS* gene, were plated per plate, for a total of 15 biological replicates.

NaCl-resistant mutants growing after 3 days of incubation were purified and characterized. Growth of both, blue and white mutants (Figure 4) occurred on seven of the fifteen plates, while five of the plates had only white mutants growing (see Table 4). Blue NaCl-resistant mutants are indicative of putative $RpoS^+$ mutants because they are expressing the highly RpoS-dependent *osmY-lacZ* reporter fusion. While the white NaCl-resistant mutants are putative $RpoS^-$ mutants because, they lack expression of the *osmY-lacZ* gene. To confirm RpoS-dependent gene expression in the NaCl-resistant mutants, the activity of another highly RpoS-dependent gene function was tested. Mutants were tested for presence of catalase (KatE) activity by dropping 30% hydrogen peroxide onto each mutant colony. All blue NaCl-resistant mutants tested were catalase positive, indicated by high gas evolution similar to wild type strain. All white NaCl-resistant mutants tested were catalase negative, indicated by reduced gas evolution similar to the *rpoS* mutant strain. The mutation frequency of NaCl-resistant catalase-positive phenotype is 10^{-9} mutants per cell plated. The mutation frequency of NaCl-resistant catalase-negative phenotype is 10^{-8} mutants per cell plated (see Table 4). These mutants were further characterized to confirm restoration or identify compensation of RpoS function.

3.4 Identification of *rpoS* mutations

To confirm that the phenotypic changes seen in the NaCl-resistant catalase-positive mutants were the result of sequence changes in the *rpoS* gene, the *rpoS* gene of three independent mutants was amplified by PCR and sequenced. The *rpoS* gene of three independent NaCl-resistant catalase-negative mutants was also PCR amplified and

sequenced to identify any silent mutations. Whole cell PCR was conducted to amplify the *rpoS* region of each mutant (see Figure 6) and products were sequenced and aligned with wild type *rpoS* gene (see Figure 7). Sequencing confirms all three NaCl-resistant catalase-positive mutants (SS53- BA, SS53- BB, and SS53- BC) acquired transition or transversion mutations in codon 265 of the *rpoS* gene. The predicted effect of these mutations on the amino acid sequence is that there is a change from stop codon → Glu in SS53- BA, stop codon → Gln in SS53- BB and stop codon → Trp in mutant SS53- BC. These mutations confirm restoration to $rpoS^+$. As expected, the three NaCl-resistant catalase-negative mutants (SS53- WA, SS53- WB and SS53- WC) did not acquire mutations in codon 265 of the *rpoS* gene, confirming that these mutants are $rpoS^-$ and therefore may have acquired compensation for RpoS-dependent NaCl-resistance function.

3.5 RpoS protein levels in NaCl-resistant mutants

To confirm that the mutations acquired in the NaCl-resistant catalase-positive strains result in restored RpoS protein levels, stationary phase samples were obtained for Western blotting (see Figure 5). There is no RpoS protein detected in the *rpoS* null mutant HS1091p (lane 1). An RpoS protein product smaller in size (truncated RpoS) than the wild type RpoS protein from strain HS1091 (lane 2), is detected in strain SS53 (lane 3). The truncated RpoS is the result of a stop sequence (UAG) mutation at codon 265. Wild type RpoS is detected in all three mutants identified to be NaCl-resistant catalase-positive (lanes 4-6). As expected, only truncated RpoS is present in the NaCl-resistant catalase-negative mutants.

3.6 Mutation rate at *rpoS* locus

To determine the mutation rate, a fluctuation test was performed. A small number of cells from a batch culture were inoculated into 30 parallel cultures and grown to saturation. Cells were plated onto M9 (1% succinate)/agar plates supplemented with X-gal. Only a fraction of the saturated cultures (~1.99 x 10^4 cfu) was plated because, otherwise, the background growth occurring on the plates was too dense resulting in non-countable colonies. One of the underlying assumptions is that the initial inocculum in each culture contains no preexisting mutants and is relatively small compared to the final number of cells. Although it cannot be insured that each starting inocculum contains no preexisting mutants, it has been established that most methods to calculate mutation rate are valid if the initial inocculum is at least 1/1000 of the final cell density [83]. In this fluctuation test the initial inocculum is 1/10000 of the final cell density. To determine the mutation rate, the number of mutations occurring in each culture was first determined. There are several methods available (see [76] for review on methods) to calculate this value but the standard method that is valid over an entire range of mutation rates is the Ma-Sandri-Sarkar Maximum Likelihood Estimator (MSS-MLE) Method [76]. The number of white *Suc*$^+$ mutants, indicative of loss of RpoS function, occurring after 3 days of incubation is shown in Table 7. The total number of cells in each culture was found to be about 3.99 x10^8 cfu. The average number of mutants per culture is 2.83. Because only a fraction of each culture was plated in this experiment, a correction factor was applied (equation obtained from [84]) to correct for errors in low plating efficiency. The equation

corrects for the number of mutations in each culture adjusting for what it would be if the entire culture was plated. The number of mutations per culture was determined to be 1.05, and after applying the correction factor, the number of mutations per culture was found to be 2128. The mutation rate was found to be 5.3×10^{-6} mutations per generation per cell. Taking into account the growth of wild type cells (3.6×10^{10} cells per plate) that may give rise to mutations post-selection, a second mutation rate was calculated. The second mutation rate obtained was 0.3×10^{-10} mutations per generation per cell.

Tables

Table 1. List of strains used in this study.

Table 2. List of chemical tests showing penotypes lost due to mutation in the *rpoS* gene

Strain	**Genotype**	**Source**
GC4468	Δ*lac*U169 *rpsL*	(Schellhorn and Stones, 1992) [85]
GC122	As GC4468 but *rpoS :: Tn10*	(Schellhorn and Hassan, 1988) [26]
MG1655	*ILvG-rfb*50 *rpH1*	(Blattner *et al*., 1997) [67]
HW2211	As MG1655 but Δ*rpoS*	Schellhorn Lab- unpublished
HS1091	AS GC4468 but omY-lacZ	(Schellhorn *et al*., 1998) [86]
HS1091p	As HS1091 but *rpoS :: Tn10*	(Schellhorn *et al*., 1998) [86]
SS53	As HS1091 but C to T mutaion in *rpoS*	Schellhorn Lab- unpublished
SS53 - BA	$NaCl^R$ mutant derivative of SS53	This study
SS53 - BB	$NaCl^R$ mutant derivative of SS53	This study
SS53 - BC	$NaCl^R$ mutant derivative of SS53	This study
SS53 - WA	$NaCl^R$ mutant derivative of SS53	This study
SS53 - WB	$NaCl^R$ mutant derivative of SS53	This study
SS53 - WC	$NaCl^R$ mutant derivative of SS53	This study

of *E. coli* K-12.

Test[a]	Mode of Action	Phenotype[b]	Difference[c]
α-Hydroxy-Butyric Acid	Carbon source	Decreased utilization of α-hydroxy-butyric acid	-110
α-Keto-Butyric Acid	Carbon source	Decreased utilization of α-keto-butyric acid	-86
Glycyl-L-Proline	Carbon source	Decreased utilization of Glycyl-L-Proline	-69
Propionic Acid	Carbon source	Decreased utilization of propionic acid	-51
Tolylfluanid	Fungicide	Increased sensitivity to tolylfluanid	-138
Dichlofluanid	Fungicide	Increased sensitivity to tolylfluanid	-91
Protamine Sulfate	Membrane ATPase agent	Increased sensitivity to protamine sulfate	-73
Ser-Gln	Nitrogen source	Decreased utilization of Ser-Gln	-67
6% NaCl +Dimethyl Sulphonyl Propionate	Osmolarity	Increased sensitivity to 6%NaCl + dimethyl sulphonyl propionate	-69
6% NaCl + N-N Dimethyl Glycine	Osmolarity	Increased sensitivity to 6%NaCl + N-N dimethyl glycine	-65
6% NaCl + Glycerol	Osmolarity	Increased sensitivity to 6%NaCl + glycerol	-78
6% NaCl + KCl	Osmolarity	Increased sensitivity to 6%NaCl + KCl	-88
6% NaCl + Trimethylamine-N-Oxide	Osmolarity	Increased sensitivity to 6%NaCl + Trimethylamine-N-Oxide	-61
1% NaCl – 10% NaCl	Osmolarity	No difference in sensitivity	-

[a] Chemicals were tested in 96-well PMs

[b] Phenotypes present in wild type *E. coli* K-12 strain MG1655 but lost in *rpoS* mutant strain HW2211.

[c] Respiration curves are generated using OmniLog-PM software based on reduction of tetrazolium dye. PM Values represent differences in areas of respiration curves between the wild type and *rpoS* mutant. The units are arbitrary. Negative indicates that the wild type showed greater rates of respiration than the mutant.

Table 3. Comparison of osmotic stress sensitivity between wild type *E. coli* and *rpoS* mutant.

	LB medium	
Concentration[a] of NaCl (w/v)	**WT[b]**	***rpoS***
0%	++++	++++
2%	++++	++++
4%	++++	++++
6%	++++	NG
8%	+	NG

NG -no growth
Sensitivity/resistance was determined based on size of growth patch
+ indicates level of growth, where ++++ indicates full patch of growth while, + indicates faint growth patch
[a] Final concentration in plate.
[b] Growth comparison was between wild type *rpoS* strain HS1091 and *rpoS* null mutant HS1091p.

Table 4. Frequency of mutants arising from growth of *rpoS* mutant strain SS53 on 6% NaCl.

Biological Replicate	**Number of white mutants[a]**	**Mutation frequency[b] (mutants per cell plated)**	**Number of blue mutants[c]**	**Mutation frequency (mutants per cell plated)**
1	42	4.2E-08	2	2.0E-09
2	10	1.0E-09	0	-
3	4	4.0E-09	0	-
4	21	2.1E-08	4	4.0E-09
5	20	2.0E-08	0	-
6	16	1.6E-08	1	1.0E-09
7	12	1.2E-08	1	1.0E-09
8	24	2.4E-08	5	5.0E-09
9	0	-	0	-
10	43	4.3E-08	3	3.0E-09
11	4	4.0E-09	0	-
12	0	-	0	-
13	6	6.0E-09	0	-
14	22	2.2E-08	1	1.0E-09
15	0	-	0	-
Average	**15**	**1.4E-08**	**1**	**1.1E-09**

[a] Number of white$^-$ mutants refer to lack of *osmY-lacZ* expression mutant colonies appearing on selection plate (putative *rpoS*$^-$ mutants)
[b] Number of blue mutants refers to *osmY-lacZ* expressing mutant colonies appearing on selection plate (putative *rpoS*$^+$ revertants)
[c] Mutation frequency refers to the number of mutants observed divided by the number of cells plated (10^9 cells plated).

Table 5. Summary of types of *rpoS* mutations and predicted effects on amino acid sequence of catalase-positive NaCl- resistant strains.

Strain	**Type of Mutation**	**Nucleotide Position[a]**	**Predicted effect on Amino acid sequence**
HS1091	Wild type	-	-
SS53	nonsense	793 (C to A)	Glu to stop codon UAG
SS53- BA	transversion	793 (A to C)	Stop codon UAG to Glu
SS53- BB	transition	793 (A to G)	Stop codon UAG to Gln
SS53- BC	transition	794 (T to C)	Stop codon UAG to Trp

[a] Nucleotide position with respect to translation start site (1st nucleotide in start codon = +1)

Note: SS53 is a succinate selected strain that has a loss of RpoS function mutation, while SS53-BA, SS53-BB and SS53-BC are NaCl-selected derivatives of SS53.

Table 6. Number of Suc^+ mutants from wild type *E. coli* K-12 strain HS1091 resulting from fluctuation test used to determine mutation rate.

Biological Replicate	**Number of Suc^+ Mutants**	**Biological Replicate**	**Number of Suc^+ Mutants**
1	1	16	0
2	3	17	0
3	0	18	1
4	0	19	8
5	0	20	11
6	0	21	3
7	16	22	2
8	11	23	1
9	2	24	1
10	0	25	4
11	1	26	1
12	0	27	2
13	4	28	2
14	1	29	0
15	1	30	9
		Average	**2.83**

Note: An equal number of cells were grown in each biological replicate (final density is approximately 4.0 x 10^8 cfu/culture).

Figures

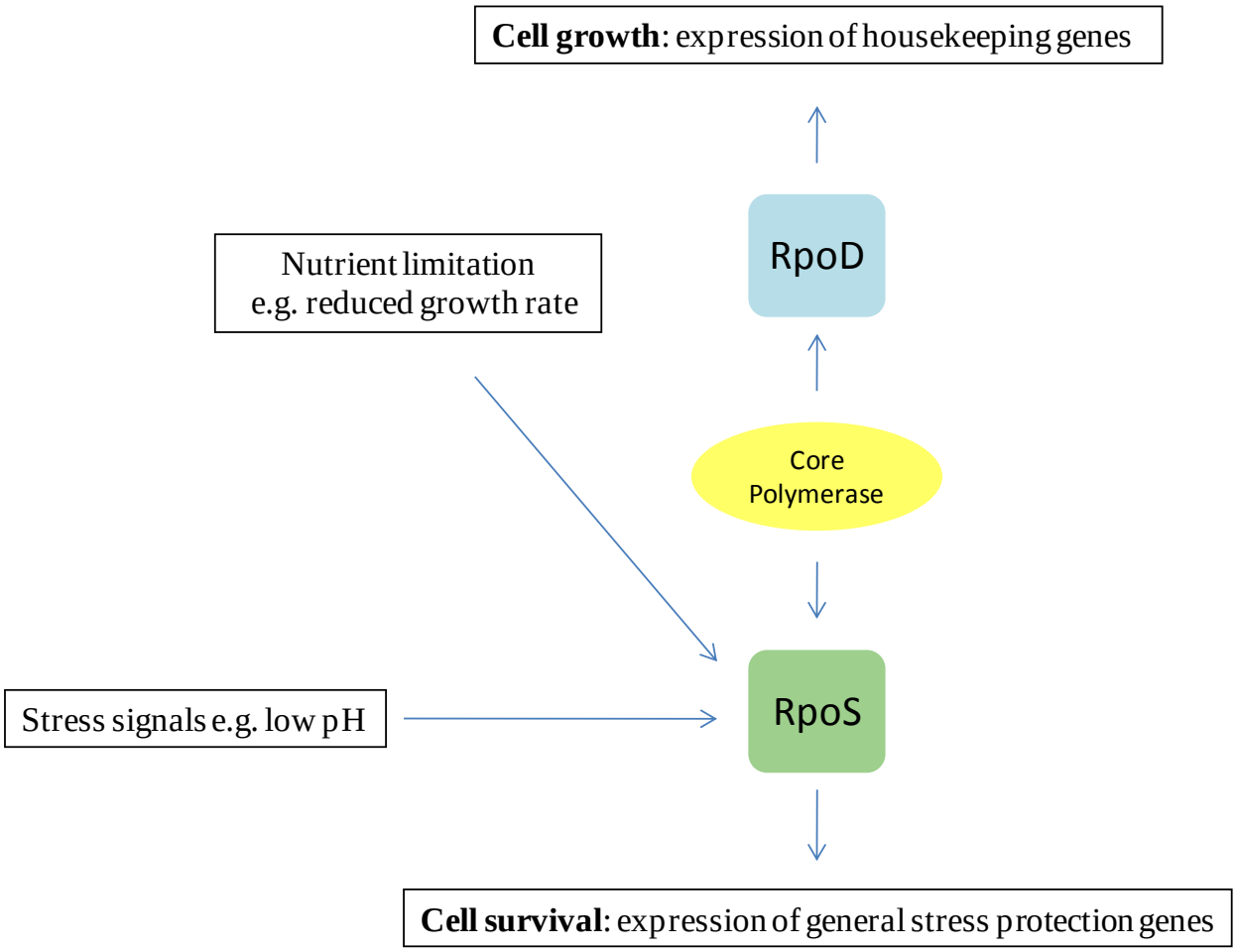


Figure 1. Sigma factor competition between RpoS and RpoD for limited number of available core polymerase.

During optimal growth, RpoS levels are low due to transcriptional, translational and post-translational control mechanisms and RpoD-dependent gene expression is dominant within the cell. Stress or the onset of stationary phase induce expression of RpoS. High levels of RpoS reduce available core polymerase for RpoD binding. Figure was modified from [79].

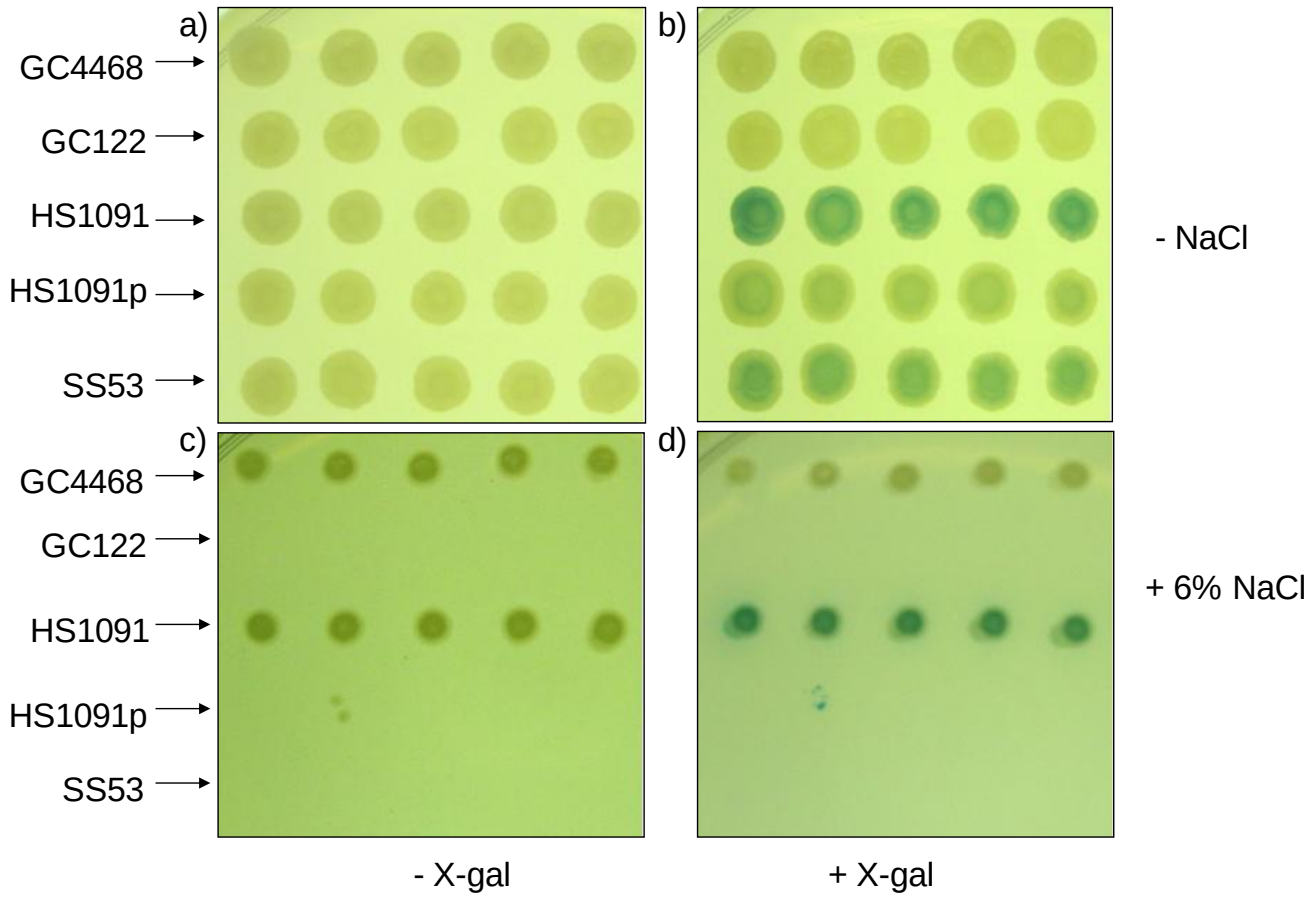


Figure 2. Effect of osmY-lacZ fusion and X-gal on sensitivity response to 6% NaCl.

Growth of wild type strain GC4468, isogenic *rpoS* null mutant GC122, wild type *rpoS* harboring *osmY-lacZ* fusion HS1091, isogenic *rpoS* null mutant harboring *osmY-lacZ* fusion HS1091p, and succinate-selected attenuated RpoS function mutant SS53 (tranversion mutation in *rpoS*) on a) LB without NaCl, b) LB without NaCl + X-gal, c) LB + 6% NaCl and d) LB + 6%NaCl + X-gal plates incubated at 37^{o}C.

a) b)

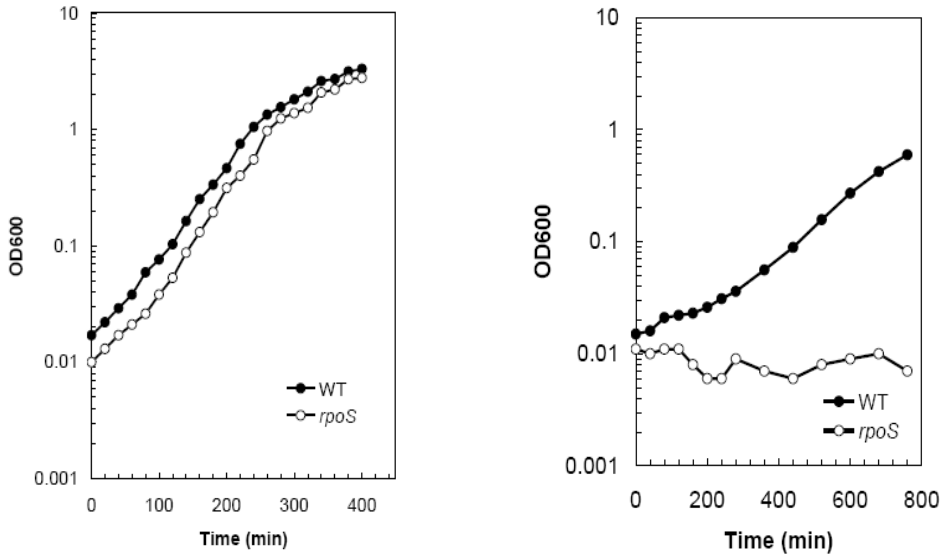


Figure 3. Growth comparison of wild type and *rpoS* mutant in 6% NaCl liquid medium.

Graph shows growth comparison of wild type (WT) strain HS1091and isogenic mutant (*rpoS*) strain HS1091p in a) LB with no NaCl (control) and graph b) LB + 6% NaCl. Cultures were inoculated from overnight cultures to starting OD_{600} = 0.01, and incubated aerobically at 37^{o}C at 200rpm.

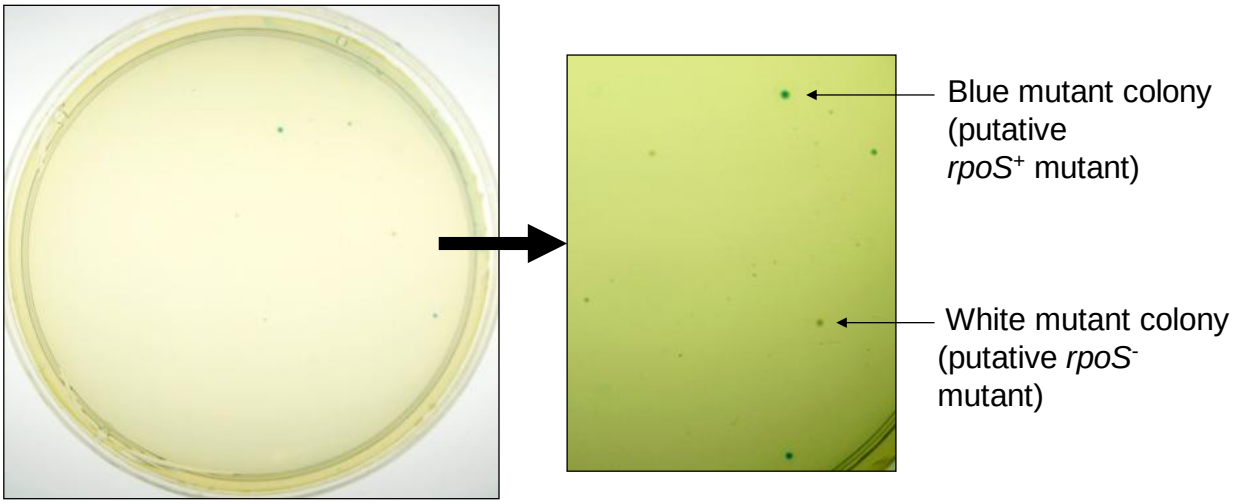


Figure 4. Isolation of NaCl-resistant mutants.

Selection on LB+6% NaCl plates resulting in growth of putative *rpoS* revertants from strain SS53, indicated by large blue colonies. Both blue and white mutants were purified and tested for RpoS-dependent catalase activity.

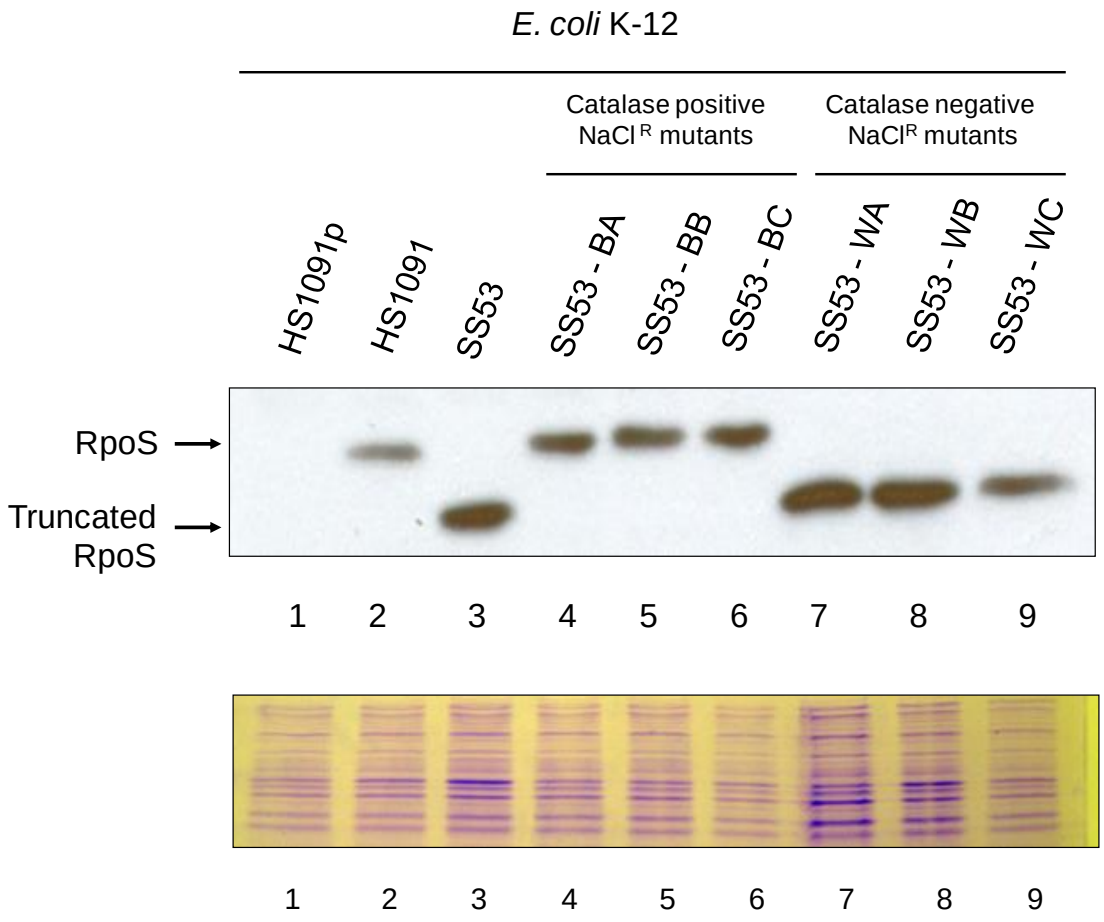
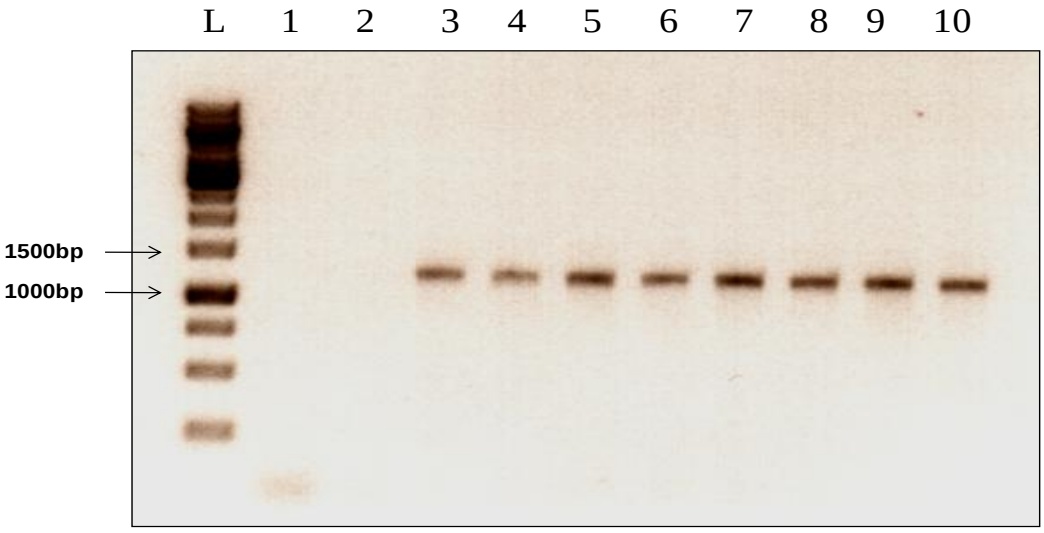


Figure 5. Western blot analysis of RpoS protein levels.

Samples were prepared from early stationary phase (OD_{600} = 1.5) and detected with primary RpoS antibody and secondary anti-mouse antibody. Truncated RpoS refers to mutant form of product due to C->A transversion mutation in the *rpoS* reading frame at codon 265. *E. coli* null *rpoS* mutant (lane 1) wild type RpoS (lane 2) succinate selected *rpoS* mutant (lane 3) and six representative NaCl-resistant mutants (catalase positive mutants (lanes 4-6) and catalase negative mutants (lanes 7-9)). To confirm equal protein loading, identical gels were run in parallel and stained with Coomassie Blue R-250 (bottom image).



Lanes:

L – ladder 1kb
1 – water
2 – HS1091p
3 – HS1091
4 – SS53
5 – SS53-BA
6 – SS53-BB
7 – SS53-BC
8 – SS53-WA
9 – SS53-WB
10 – SS53-WC

Figure 6. PCR amplification of *rpoS* gene for sequencing.

Amplification products were run on 1% agarose gel and stained with ethidium bromide. The wild type *rpoS* gene is identified as a 1400bp fragment. Lanes 1-2 represent negative controls. Lanes 3-4 represent positive controls. Lanes 5-7 represent NaCl-resistant catalase positive mutants. Lanes 8-10 represent NaCl-resistant catalase negative mutants.

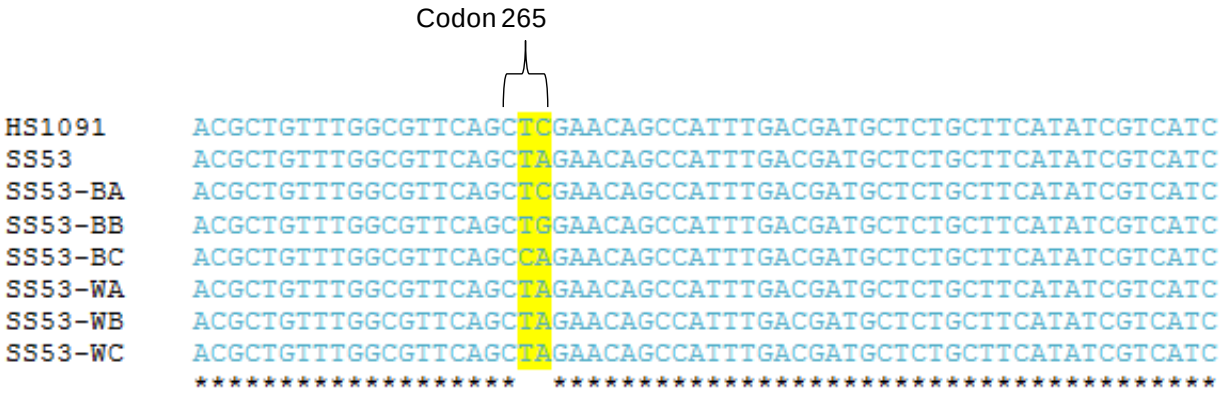


Figure 7. Sequence alignment of *rpoS* gene fragment.

Alignment of wild type HS1091 and attenuated RpoS function mutant SS53 with NaCl resistant mutants identified to be putative *rpoS*$^{+}$ (SS53-BA, SS53-BB, SS53-BC), and putative *rpoS*$^{-}$ (SS53-WA, SS53-WB and SS53-WC). Reverse mutations are seen in codon 265 of strains SS53-BA, SS53-BB and SS53-BC confirming restoration to wild type *rpoS*.

Chapter 4.0: Discussion

Stress protection is one of the primary factors affecting bacterial survival in nature and in human/animal host environments. It is of interest to study this response in *E. coli* because the general stress response network is under the control of a single master regulator, RpoS, and natural populations of *E. coli* contain a high frequency of polymorphisms in this gene. Expression of the RpoS regulon is needed in stress challenging environments, but it becomes costly during long term nutrient limitation since this creates a greater need for allocation of resources toward RpoD - dependent gene expression; RpoD and RpoS both compete for binding sites on core RNA polymerase. In this study we demonstrated that a succinate selected *rpoS*$^{-}$ culture has poor growth when transferred to a high-osmolarity environment, and we identified 6% NaCl to select for restored RpoS function via reversible mutations in the *rpoS* gene.

4.1 Selection for restoration of RpoS function

For the identification of a selective agent, growth on 2000 compounds was compared between the wild type *E .coli* K-12 strain and its isogenic *rpoS* mutant. Sensitivity to growth on 14 chemical compounds in the *rpoS* mutant compared to the wild type strain suggests that RpoS is needed for maximal growth in these conditions. The wild type strain exhibits increased cellular respiration from the utilization of propionic acid, glycyl-L-proline, alpha-ketobutyric acid, and alpha-hydroxybutyric acid as carbon sources. Genes involved in either the uptake or break down of these compounds maybe

either directly or indirectly regulated by RpoS. It is interesting to note that previous studies demonstrate growth of *E.coli* on succinate, a TCA cycle intermediate, confers a growth advantage for *rpoS* mutants [35]. However based on PM services analysis, the ability to grow well on alpha-keto-butyrate, a compound that can be incorporated into the TCA cycle by conversion to succinyl Co-A, is lost in the *rpoS* mutant. Succinate is a direct intermediate in the TCA cycle and is converted to fumarate by the enzyme succinic dehydrogenase encoded by the *sdh* operon. Expression of the *sdh* operon is three-fold higher in an *rpoS* mutant background compared to wild type cells [87]. Identification of *rpoS* mutants sensitive to growth on alpha-ketobutyric acid suggests that RpoS may play a role in increasing expression of certain TCA cycle genes however further testing will need to be done to confirm if this result is due to differences in growth conditions used by PM services.

Increased resistance to osmolytes, particularly NaCl, in wild type cells compared to *rpoS* mutants is consistent with the findings that RpoS plays a role in osmotic stress protection [5, 10, 88]. In this study, we found that the growth of *E. coli* on 6% NaCl selects for mutants with restored RpoS function, and mutants that are NaCl-resistant but posses a non-functional RpoS. This suggests that compensation for RpoS function is possible in the presence of NaCl selection. Mutants with restored RpoS function are isolated at a frequency of approximately 10^{-9}, which is consistent with the mutation rate for spontaneous beneficial mutations [70]. The impaired *rpoS* NaCl-resistant mutants are isolated at a frequency of approximately10^{-8}, similar to the frequency at which loss of RpoS function mutants are isolated on succinate [35]. This suggests that in general,

mutations affecting the *rpoS* gene are rare, and therefore constitute a small portion of the population unless prolonged selective conditions are maintained. Also we now know that for the *rpoS* gene, loss-of-function mutants are isolated at a higher frequency than restoration of function mutants. There is still a high frequency of NaCl-resistant mutants, but largely through an RpoS independent mechanism. This may reflect the underlying limitation in the number of possible mutations that can result in restoration of function compared to the number of possible mutations that can result in loss-of-function. Strain SS53 carries a C -> A point mutation at codon 265 resulting in a truncated RpoS protein incapable of binding to either RNA polymerase or promoter sites (exact mechanism of regulation inhibition not yet known). Restoration to wild type RpoS can only happen if codon 265 is restored back to wild type sequence or if there is a change in sequence from stop codon to a different amino acid that restores function, thus, there is a limitation to the number of mutations that can restore function. Mutations downstream codon 265 will have no effect, and mutations upstream may result in a truncated RpoS protein with higher affinity for binding to either RNA polymerase or promoter site, but this is highly unlikely; all three independent restoration of function mutants sequenced had mutations only in codon 265. In contrast, mutations resulting in loss-of-function are more likely to occur because these mutations can occur within a larger region of the *rpoS* gene. Therefore, demonstrating that restoration of RpoS function is possible and that it can be selected on a single naturally prevalent chemical compound, NaCl, is highly significant given the limited number of possible mutations that can result in restoration of function.

This is the first study to demonstrate the adaptation of a Suc^+ *E. coli* K-12 population in a stressful environment. Although Suc^+ mutants grow well on poor carbon sources [35], they are not able to grow well when transferred to an osmotically stressful condition. This again emphasizes the importance of RpoS function in stress conditions. Previous studies show all Suc+ mutants isolated from laboratory K-12 strains have mutations in the *rpoS* gene resulting in attenuated or loss of RpoS function [35]. Interestingly, when the same succinate selected K-12 mutants are grown on NaCl only 7% of the isolated mutants have restored RpoS function while the majority (93%) remain $RpoS^-$. In the case of growth on succinate, it is predicted that mutations in the *rpoS* gene help alleviate sigma factor competition between RpoD and RpoS. This allows for increased expression of TCA cycle genes under RpoD control, which leads to increased utilization of succinate. In the case of growth on high NaCl, a greater frequency of mutants with restored RpoS function would be expected given the important role RpoS plays in adaptation to osmotic stress but the number of mutations that can result in restoration of function plays a significant role. The results from this study suggest that when such limitations are present, alternative mechanisms of adaptation occur to compensate for loss of RpoS dependent osmotic stress protection. However, the larger size of the $RpoS^+$ (restored function) mutant colonies suggests that there is an increased growth rate compared to the RpoS-independent NaCl resistant mutants (Figure 4). Therefore with continuous NaCl selection pressure, it is possible that over time the $RpoS^+$ cells will reach majority within the population.

The selection of RpoS-independent NaCl- resistance mutants suggests the development of a compensatory mechanism. Because sigma factors direct RNA polymerase to specific promoter sequences for gene specific expression, changing patterns of expression from RpoS-dependent to RpoS-independent requires either alterations in the promoter region so that it can be recognized by one of the other six sigma factors found in *E.coli* or it requires alterations in the sigma factor proteins themselves so they can now recognize new promoters. Recent reports by Stobel et *al* [89] conclude strains lacking RpoS evolve in a osmotically stressful environment by the insertion of IS10 into the promoter of the *otsBA* operon . This operon is essential for the synthesis of trehalose, a carbohydrate that aids *E. coli* in the maintenance of osmotic balance. In the wild type background, the expression of the *otsBA* operon is under RpoS control, but in strains lacking *rpoS* the restoration of wild type response to osmotic stress is achieved by making the *otsBA* operon RpoS-independent, since the IS10 insertion carries its own promoter. In strains lacking *rpoS* and free of IS10 elements, the adaptation to high osmotic stress is by mutations occurring at other loci that do not affect the expression of the *otsBA* operon [90]. These proposed mechanisms of evolution resulting in RpoS-independent osmotic stress response may help explain the growth of NaCl-resistant mutants with non-functional RpoS from this study. However, further testing, such as sequencing of the promoters of genes associated with osmotic stress resistance would have to be done to confirm this correlation. Furthermore, the *rpoS*$^{-}$ strain examined by Stobel et *al*, have a deletion of the *rpoS* locus unlike the strain used in this study which as a loss-of-function point mutation. Deletion of the *rpoS* locus completely eliminates the

possibility of restoring RpoS function when grown under osmotic stress which does not reflect what may occur in nature. Since natural isolates of *E. coli* have a high frequency of polymorphisms in the *rpoS* gene [79]; they are more likely to have acquired mutations that result in attenuated or loss of RpoS function which allow for the possibility of restoration. Thus, this study is the first to determine the adaptation trajectory of an RpoS$^-$ population, carrying a revertable mutation, when grown under osmotic stress.

Due to the high cost associated with unnecessary expression of its regulon, RpoS is tightly regulated at the transcriptional, translational and post-translational level. When a population of *E. coli* is growing under stressful conditions, "on"expression of the regulon is favored in majority of the cells, but once stress is no longer a primary concern, and nutrient limitation is experienced such as depletion of glucose in the surrounding, "off" control of the regulon is favored. Results from this study suggest an additional level of control exists at the population level. Our findings support the idea that mutations in the *rpoS* gene may act as a physiological molecular switch to control the expression of the RpoS regulon [35]. The type of mutation that accumulates within the *rpoS* gene in *E. coli* K-12 strains is mainly transversions [35, 75] and approximately 50% of K-12 strains possess a common amber mutation in codon 33 of the *rpoS* gene [75]. In this study we show that growth of an RpoS$^-$ culture, carrying a transversion mutation, on 6% NaCl selects for restored RpoS function. Selection for restored RpoS-dependent NaCl-stress resistance occurred in 7 independently grown cultures. This is the first study to demonstrate the ability for a population to "turn on" RpoS regulon expression in cells that were once selected as loss-of-RpoS function via a reversible mutation. Furthermore, our

results infer environments high in NaCl, such as sea water, may promote the recovery of $RpoS^+$ *E. coli* populations in the nature. Whether natural isolates are subject to NaCl selection for restoration of RpoS function is not yet known.

Although compensation for RpoS-dependent osmotic stress resistance is possible, fixation of *rpoS* mutants in a population may be detrimental in the long term since $RpoS^+$ populations are more variable in their fitness increase compared to *rpoS* mutants [89] . Increased variation in fitness within a population suggests increased evolvability. Selection for $RpoS^+$ cells among an $RpoS^-$ dominant population may increase potential to generate better-adapted descendants. Therefore, selection for restored $RpoS^+$ sub-population not only demonstrates that mutations may act as a molecular switch to control expression of the RpoS regulon but it could also demonstrate second-order selection.

4.2 Mutation rate at the *rpoS* locus

Loss of function mutations in the *rpoS* gene commonly occur in laboratory, natural isolates and pathogenic strains. It has also been reported that the region between the *rpoS* gene and the *mutS* gene is highly polymorphic [91]. However no studies have tried to determine whether mutations occur at a higher rate at the *rpoS* locus than elsewhere in the genome, making it a target site for evolutionary adaptation. To determine the mutation rate at the *rpoS* locus succinate selection was employed; mutants carrying attenuated or loss of *rpoS* function mutations are selected for on succinate [35]. The determined mutation rate, 5.3×10^{-6} mutations per cell per generation, is much higher than the spontaneous mutation rate for *E. coli*. This suggests that mutations do occur at a higher

frequency within the *rpoS* gene than elsewhere in the genome. However it is important to consider possible factors that may contribute to the high mutation rate. An important requirement for obtaining reliable results in a fluctuation test is that no mutants arise after selection is imposed. This means only pre-existing mutants will grow on the selective condition succinate medium, and all other cells die. The number of mutants was counted after 3 days of incubation for all 30 parallel culture plates but it was noted that there was an increase in the frequency of mutants over subsequent days even on plates that contained no mutants on day 3. This suggests mutations were arising after exposure to the selection condition. There are two possible reasons for mutations arising after plating cells on selective condition. They can either arise because non-mutant cells are slowly growing on the selective succinate medium due to non-lethal growth conditions or they can arise as a result of adaptive mutations; mutations that occur in response to succinate selection. Because stringent selection conditions is not possible given that wild type *rpoS* cells do grow on succinate but at a slower rate compared to *rpoS* mutants [35], it is very likely that mutations are arising in wild type cells after plating onto the selection condition (post-selective mutants). About 10^4 cells were plated, and after 3 days of incubation the total number of cells on a plate containing no mutants is about 10^{10} cells. Thus approximately 20 generations of growth occurs post-selection giving opportunity for new mutations to arise. The second possibility is that mutations are occurring as a direct response to succinate selection and not independently of succinate selection. Although previous experiments with Lac^- strains have demonstrated that mechanisms responsible for adaptive mutations do exist in *E. coli*. For example, adaptive mutations occur in Lac^-

strains of *E. coli* carrying a frameshift mutation when plated onto lactose minimal media [92]. However, the molecular mechanism that gives rise to point mutations in response to selection is partly dependent on RpoS function. For example, polymerase IV which is RpoS controlled is required for adaptive point mutations but not growth dependent lac^+ reversion mutations (reversion mutations that occur prior to exposure to selection condition) [93-95] . Therefore, in this study it is more likely that the high mutation rate can be accounted for by mutations arising in slowly growing wild type cells on the succinate media plates. With such deviations from ideal conditions for a fluctuation test, the distribution of mutant numbers is a combination of both the Poisson and Luria-Delbruck distribution. This may have resulted in an inflation of the number of mutations that occur during optimal growth (growth prior to selection exposure) resulting in a high mutation rate. Taking into account the number of post-selective mutants the mutation rate is 0.3×10^{-10} mutations per cell per generation which is similar to the measured mutation rate for *E. coli* [66]. Further experimentation will need to be done to confirm these results.

Chapter 5.0: Conclusion and Future Work

Environment regulated phenotypic switching mediated by mutations is common among organisms cycling between fixed environments [96]. Survival of *E. coli* in the lower intestine of human host environments may demand transition between expression of RpoS-dependent stress protection and RpoD-dependent nutrient acquisition. Osmolarity in the intestinal lumen due to bile production and other sodium salts is thought to be equivalent to 300mM NaCl or higher [97, 98], requiring expression of stress protective genes, but with the large number of bacteria residing in the gut, competition for carbon sources becomes important during periods of nutrient deprivation [99]. In this case, elevations in genome wide mutation rates may not be as advantageous as the development of a phenotypic switch mediated by mutation.

Growth in a nutrient limited environment results in a reduced growth rate that is detected as stress even though no physical or chemical danger surrounds the bacterium [79], therefore the cell is signaled to express RpoS-dependent genes when in fact there may be a greater need for RpoD-dependent gene expression. At this point any control mechanisms of RpoS expression at the cellular level are no longer effective and competition between RpoD and RpoS for limited RNA polymerase creates an inherent need for "off" expression of the RpoS regulon. Therefore, any possible mechanism of control at this stage may only be mutational control of the RpoS regulon. The results from this study strongly support the possibility of phenotypic switching of RpoS-dependent response in *E. coli* mediated by mutations at the *rpoS* loci. This is the first study to

identify a natural selective agent and demonstrate selection for "off" to "on" expression of the RpoS regulon in 7 independently grown cultures. The selection for these mutants not only represents restored RpoS function via reversible mutations, but it may represent selection for mutants that have increased mutation rates at the *rpoS* locus. Selection for increased mutation rates at the *rpoS* locus may help overcome the barrier of limited number of mutations that can result in restored RpoS function. Thus increased mutation rates, may increase the possibility of switching and the direction of switching may be regulated by environmental factors.

Future experiments involving repeated cycling of growth of *E.coli* between a poor carbon source environment and osmotic stress challenging environment resulting in a pattern of "on" and "off" expression mediated by mutations in the *rpoS* gene can confirm the existence of a genetic switch. In this study, we tried to determine the mutation rate at the *rpoS* locus in wild type cells by testing for the rate of loss-of-funtion. However, once ideal conditions for a fluctuation test can be established, an experiment comparing the mutation rate at the *rpoS* locus between the RpoS-dependent and independent NaCl-resistant mutants can determine whether selection for site specific increase in the mutation rate at the *rpoS* locus has occurred or not.

In conclusion, data from this study heavily supports the idea that mutations may act as a phsyiological molecular switch to control expression of the RpoS regulon, which may help explain the fluctuation of $RpoS^+$ and $RpoS^-$ cells observed in glucose limited chemostat cultures, as well as the findings of both $RpoS^+$ and $RpoS^-$ populations in natural isolates.

Appendix

SOP:

Media Preparation and Growth Conditions:

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

LB broth:

- LB is prepared by adding 25g of powder to 1L of distilled water to give a final concentration of 10g of bacto-tryptone, 5g of bacto-yeast extract and 10g of sodium chloride per liter.
- Broth is sterilized by autoclaving at 121^0C.
- Unless otherwise indicated, all cultures grown in LB broth were inoculated from single colonies.
- Cultures were grown in Erlenmeyer flasks at a ratio of 1:5.
- All cultures were grown at 37^0C and shaking at 200rpm.

LB plates:

- To make LB plates, add 250ml of 2X LB broth to 250ml of 2X Agar.
- When appropriate media is supplemented with Kanamycin (50ug/ml, final concentration) and/or X-gal (50ug/ml, final concentration).
- Dry plates under the fume-hood for about 20minutes and store at 4^oC.
- All cultures taken from -80^0C storage freezer are first streaked onto LB plates for isolation of individual colonies.

Salt Media:

- Different concentrations of salt media are prepared by adding 1g/100ml Bacto tryptone and 0.5g/100ml Bacto yeast extract to either 2g , 4g, 6g, or 8g/100ml of NaCl in distilled H_2O_2.
- Media is sterilized by autoclaving at 121^0C.

- When appropriate media is supplemented with Kanamycin (50ug/ml, final concentration) and/or X-gal (50ug/ml, final concentration).
- For preparation of salt plates, the concentration of Bacto tyrptone, Bacto yeast extract and NaCl described above was doubled to produce 2X LB salt media.
- 1X LB salt plates were made by adding 250ml of 2x LB salt media to 250ml of 2X Agar.

1% Succinate Plates:

- Prepare 10X M9 salts by adding 60g of Na_2P0_4, 30g of KH_2P0_4, 5g of NaCl and 10g of NH_4Cl in distilled H_20_2 and brining the volume to 1000ml.
- 1M $CaCl_2$ is made by adding 14.70g of $CaCl_2 \cdot 2H_20$ to distilled H202 and brining the volume to 100ml
- 1M MgS04 was made by adding 17.04g of (anahydrous) $MgS0_4$ to distilled H202 and bringing the volume to 100ml
- 10% Succinate was made by adding 10g of succinate to distilled H_20_2 and bringing the final volume to 100ml. Solution was brought to a pH of ~7.0.
- 100mL of 10X M9 salts, 2mL of 1M $MgS0_4$, 50ml of 20% succinate (1% final concentration), and 0.1ml of 1MCaCl2 were added to distilled H_20_2 and the volume was brought to 500ml.
- To make plates, 250ml of 2X M9 (2% succinate) media was added to 250ml of 2X Agar and supplemented with 50ul of 1% thymine and X-gal (50ug/ml).

<u>Growth curve</u>:

1. Inoculate in triplicate single colonies into 50ml Erelenmyer flask containing 10ml of LB without NaCl supplemented with kanamycin and grow overnight at 37^0C with shaking at 200rpm.
2. Take overnight cultures and sub-culture 1:10000 into fresh LB media containing no salt and grow for at least 8 generations at 37^0C with shaking at 200rpm.

3. Transfer exponential phase cultures into either 50ml LB without NaCl or LB + 6% NaCl (final concentration) in 250ml Erlenenmyer flasks to an initial OD = 0.01 .

4. Using the Shimadzu UV-1201 spectrophotometer, periodically measure OD_{600} by taking 1ml samples in a cuvette and placing it within the spectrophotometer. After samples reach OD_{600} = 0.5 dilute the readings 1/100 by taking 10ul of culture and adding it to 990ul ofH_2O_2.

Selection for NaCl-resistant mutants:

1. Inoculate single colonies into 10ml of LB broth and grow to saturation at 37^0C with shaking at 200rpm.

2. To determine cell count in saturated culture, serially dilute (100 fold per dilution) of left over culture in M9 salts and plate onto LB plates. Incubate plates overnight at 37^0C and colonies were counted the next day.

3. For selection, plate approximately 10^9 cells onto LB/agar plates containing 6% NaCl (w/v, final concentration) supplemented with X-gal.

4. To plate the cells, pour 1 ml of culture onto the surface of the plate and swirl around until evenly spread, remove the remaining liquid off the plate using a pipette. Determine volume plated by deducting amount of volume removed.

5. Incubate plates for 3 days at 37^oC.

6. Purify mutant colonies by re-streaking onto LB + 6%NaCl plates and incubate at 37^0C. Re-streak mutants at least 3X before using for experimentation.

Catalase Assay:

1. Into a sterilized 96-well microtittre plate, add 200ul of LB to each well and cover plate in parafilm

2. Using a sterilized toothpick, inoculate single into each well (5 biological replicates per culture).

3. Remove parafilm and incubate plate overnight at 37^0C.

4. Replica plate cultures onto LB media using a replicating prong that is sterilized by dipping in 95% alcohol followed by passing through a flame.

5. Dip sterilized cooled prong into the wells of the microtitre plate then gently onto the surface of the media plates to be grown on. Incubate plates overnight at 37^0C.

6. After overnight growth, remove plates from the incubator and apply 10ul of 30% hydrogen peroxide onto each growth patch and record bubbling intensity.

Western Blot:

1. Inoculate single colonies into 10ml of LB containing kanamycin and grow overnight at 37^0C with shaking at 200rpm.

2. Take overnight cultures and sub-culture 1:10 000 into fresh LB. Monitor growth by measuring OD_{600} and collect 0.2ml samples when OD_{600} = 1.5 (onset of stationary phase)

3. Collect samples in a 1.5ml centrifuge tube and spin at 13,000 g for 1min. Discard the supernatant was pellets 3x in PBS buffer (pH=7.0) to remove salts.

4. Resuspend the washed pellets in 1X SDS-PAGE loading buffer to obtain a final OD = 1.0.

5. Place samples in boiling water for about 5 minutes then allow to cool at room temperature. Centrifuged the cooled samples for 1min at 10 000g.

6. Prepare 10% SDS-PAGE stacking gels according to the table below:

   Combine the following reagents in this order in a 20ml flask while swirling with each addition to avoid bubbles.

   Bottom Layer

Reagent	**Volume needed for 2 gels**
ddH20	4 ml

1.5M Tris-Cl (pH 8.8)	2.5 ml
10% SDS	0.1 ml
30% Acry/Bis	3.3 ml
10% APS	0.1 ml
TEMED	0.01 ml

Top Layer

Reagent	**Volume needed for 2 gels**
ddH20	3.6 ml
1 M Tris-Cl (pH 6.8)	0.63 ml
10% SDS	0.05 ml
30% Acry/Bis	0.66 ml
10% APS	0.05 ml
TEMED	0.005 ml

7. Load 20ul of sample and resolve on 10% SDS-PAGE gels at 100 V for 2 hours. Add running buffer with SDS (5ml 10% SDS to 500ml 1X running buffer).

8. Transfer protein to PVDF membrane at 20V for 25 minutes. Stain one gel for protein loading by placing it in 0.1% Coomassie Blue for 1 hour on a shaker followed by destaining for 30 min and overnight.

9. After transfer, incubate membrane in 5% milk (made in 1X TBST) for 2 hours.

10. Cut membrane down to the size of a small petri dish and incubate in 10ml of primary mouse monoclonal antibody to RpoS (1:10000 dilution made in 5% milk) overnight in cold room.

11. Wash membrane 3X with 1XTBST for 10min per wash on a shaker. Then incubate membrane in 10ml of secondary goat anti-mouse antibody (1:30 000 dilution made in 5% milk) for 2 hours.

12. Again, wash membrane 3X with 1XTBST for 10min each wash on a shaker. Using tweezers, carefully remove the membrane and drain off TBST before placing it in Amersham detection solution for 1min.

13. Wrap membrane in saran wrap and use paper towels to absorb excess liquid. Develop membrane in dark room by exposing it to GE Health care Amersham Hyperfilm ECL.

PCR using a colony:

1. Inoculate a single colony from each strain into 20ul of ddH_20 and heat at 95^0C for 5minutes.

2. Add 2ul of each sample to a standard 25ul PCR reaction using the conditions listed in the table below:

Sample preparation conditions:

Reagent	**Volume**
ddH20	17ul
10x buffer	2.5ul
25uM fw	1ul
25uM rv	1ul
10mM dNTP	0.5ul
1 Unit/ul Taq	1ul
DNA template	2ul
Total Volume	25ul

PCR conditions:

Step	**Duration**	**No. of Cycles**	**Temperature (^{0}C)**
Denature	2min	1 cycle	95^{0}C
Denature	30sec	Repeat for 30 cycles	95^{0}C
Anneal	30sec		57^{0}C
Extend	1min		72^{0}C
Extend	7min	1 cycle	72^{0}C

3. When PCR is complete remove the tubes and run samples on a 1% agarose gel.

4. Visualize band size using a UV-trans-illuminator.

Fluctuation Test:

1. Inoculate a single colony into 10ml of LB broth supplemented with Kanamycin and grow overnight (batch culture) at 37^0C with shaking at 200rpm.
2. Take overnight culture and sub-culture 1/10 000 into fresh LB broth and distribute 200ul aliquots into 30 wells of a 96-well microtiter plate.
3. Incubate plate at 37^0C with shaking at 200rpm until cultures reach saturation.
4. Place 100ul of each biological replicate sample into a 1.5ml eppendorf tube and spin for 2 minutes at 10 000g.
5. Wash pellet three times in M9 salts and resuspend in 100ul of M9 salts.
6. Dilute cultures (10^5 fold dilution) in M9salts and plate onto M9 (1%succinate)/agar plates containing X-gal using the pour plate method as described before.
7. Incubate plates for 3 nights at 37^0C.
8. To determine the cell count in each well, serially dilute (100 folds per dilution) of 3 representative biological replicate cultures in M9 salts and plate onto LB/agar plates.
9. Incubate plates overnight at 37^oC and count colonies the next day.

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