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The Role of transcription in stationary phase mutagenesis in *Bacillus subtilis*

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THE ROLE OF TRANSCRIPTION IN STATIONARY PHASE
MUTAGENESIS IN *BACILLUS SUBTILIS*

by

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Bachelor of Science
Morningside College
2007

A thesis submitted in partial fulfillment of
the requirements for the

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School of Life Sciences

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Holly Anne Martin

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ABSTRACT

The Role of Transcription in Stationary Phase Mutagenesis in *Bacillus subtilis*

by

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Stationary phase mutagenesis, also known as stress-induced or adaptive mutagenesis, is defined as the accumulation of mutations during conditions of no net growth or conditions of stress. This process has been implicated in acquiring antibiotic resistance and evasion of host immune responses in microbial pathogens and in the generation of mutations that lead to neoplasia in animal cells. Previous work has shown that defects in DNA synthesis and repair systems contribute to the formation of adaptive mutations; however the role of transcription in the accumulation of mutations is still being examined. It is speculated that transcriptional derepression leads to an increase in the accumulation of mutations. We tested the transcription associated mutagenesis hypothesis by modulating the level of transcription of a point-mutated *leuC* allele. We modulated transcription by altering growth conditions which physiologically altered transcription and genetically through the use of inducible and repressible promoters. We found that increases in transcription correlated with the accumulation of mutations that confer leucine prototrophy. Since transcription is a ubiquitous biological process, transcription-associated mutagenesis may influence evolutionary processes in all organisms.

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CHAPTER 1

INTRODUCTION

Stationary phase mutagenesis, also known as adaptive or stress-induced mutagenesis, is the process by which cells in non-dividing conditions acquire mutations. These processes take place under conditions of stress, such as amino acid or carbon source starvation, and are implicated in the acquisition of antibiotic resistance and evasion of host immune responses in microbial pathogens, as well as the generation of mutations that lead to neoplasia in cells of differentiated eukaryotic tissue (Foster, 2005).

Francis Ryan first observed evidence of stationary phase mutagenesis in the early 1950s when studying mutations in a histidine auxotrophic strain of *E. coli*. When cells were placed under starvation conditions, more bacteria became prototrophic than when placed under growth conditions (Ryan, 1954). Almost four decades later, in 1988, the origin of mutations in stationary phase was again speculated about when Cairns *et al*, observed a *lac⁻* *E. coli* strain that accumulated mutations which allowed growth on lactose when it was provided as the only carbon source. This report was highly controversial because of the notion that bacterial cells “directed mutation”. Many experiments later, this notion has long been dispelled and several of the factors that influence stationary phase mutagenesis in *E. coli* have been elucidated.

From studies using *E. coli* as a model, it has been proposed that mutations arising in conditions of non-lethal stress accumulate via the activation of error prone polymerases, the repair of DNA double-stranded breaks, or amplification of the gene under selection (Galhardo *et al*, 2007). The measurement of stress-induced mutations in bacterial systems often relies on the use of a strain that has either a point or a frameshift

mutation that renders cells auxotrophic for a growth factor, such as an amino acid or a carbon source. Cells are then placed under conditions in which that growth factor is limiting. The first revertant colonies appear on growth limiting conditions after two days and are considered to have originated by growth-dependent processes. Those colonies appearing after cells are incubated under conditions of prolonged stress, four days and after, are considered the result of stress-induced mutagenesis. Later experiments have demonstrated that the vast majority of stress-induced mutants are not affected in growth or the ability to faithfully repair DNA (Longerich *et al*, 1995).

In *E. coli*, the most commonly used system to measure stationary phase mutagenesis relies on measuring reversion to lactose degradation. The strain used in these studies has a deletion of the chromosomal *lac* operon and a *lac+1* frameshift allele in *lacI* on an F conjugative plasmid and is placed on agar medium in conditions which lactose is the sole carbon source (Cairns and Foster, 1991). It is important to note that this *lac+1* frameshift allele is transcriptionally fused to a functional *lacZ* gene. Therefore, transcriptional read-through of the allele will generate a functional β -gal protein.

The use and study of this assay has allowed the elucidation of two processes leading to the accumulation of revertants, one process generates point mutations and the other that generates amplification of the test allele. The majority of stress-induced mutants that occur early are proposed to arise from point mutations and up to 40% of the mutants appearing on later, days 8 and 9, are due to amplification (Galhardo *et al*, 2007). Hersh *et al*, 2004, suggest that point mutation reversions are generated during the repair of double strand breaks, because the loss of the proteins, RecA, RecBD, and RuvABC, needed to repair double strand breaks, decreased the accumulation of revertants in

stationary phase but do not affect generation-dependent mutations. The origin of these double strand breaks is endogenous. Other components of adaptive point mutations are the stress and SOS response. When the stress response and SOS response are induced, the error-prone DNA polymerase, PolIV/DinB, is upregulated and shown to generate up to 85% of adaptive point mutations in the lactose assay (McKenzie *et al*, 2001). It has been observed that Pol IV makes -1 frameshift mutations in both prokaryotic and eukaryotic systems (Kim *et al*, 2001 and Ohashi *et al*, 2000).

Tegova *et al* studied the effect of Pol IV in stationary phase mutagenesis in *Pseudomonas putida* at a +1 frameshift mutation in the *pheA* gene, which codes for the phenol monooxygenase gene (2004). The frameshift mutation renders the cell unable to degrade phenol to catechol. Therefore, when these cells are plated on minimal media containing phenol, there are unable to grow unless they acquired a mutation that allows phenol degradation. They found that Pol IV was needed for most Phe⁺ reversions.

Pol IV, as well as Pol V, belongs to the Y family of polymerases. This group of polymerases replicate through damaged DNA, and hence, there are referred to as translesion synthesis polymerases. The synthesis through the damage can be either error-free or error-prone, but these polymerases have low fidelity on undamaged templates. As noted above, Pol IV has been shown to be involved in stationary phase mutagenesis, while less is known on the role of Pol V; there is evidence that suggests that PolV also has a role in the stress-induced phenomenon (Yeiser *et al*, 2002, and Bhamre *et al*, 2001).

Stumpf and Foster (2005) showed that Ppk interacts with Pol IV and alters the accumulation of mutations in stationary phase. In *E. coli*, Ppk catalyzes the polymerization of inorganic phosphate into long chains of polyphosphate. These chains

of polyphosphate have been found to be complexed with RNAP in stationary phase, but not in exponential phase (Kusano *et al*, 1997). Polyphosphate also regulates the activity of *E. coli*'s Y-family polymerases. 65 to 80% of the Pol IV-dependent Lac⁺ revertants disappear in a *ppk*⁻ strain. Ppk acts independently of the regulation of PolIV administered by RpoS or RecA. Stumpf and coworkers suggest that the accumulation of polyP stimulates the mutagenic and transcriptional bypass activity of the Y-family polymerases.

Lac reversions, in the *E. coli* system, are also originated via amplification of the *lacI-lacZ* frameshift allele. This frameshift mutation is located within the *lacI* region of the construct, while the *lacZ* is wildtype. Therefore, transcriptional read-through of the test allele generates a full length *lacZ*, the gene coding for beta-galactosidase, message and non-revertant cells show 1-2% lactose-degrading activity. Therefore, if the gene is amplified twenty to fifty times, this would allow for growth and the sequential selection of cells carrying the test allele that was significantly amplified. While point mutations are stable, the process of amplification is not, as evidenced by the presence of sectorized colonies on media containing X-gal. The mechanism for which amplification occurs is not known, but it is hypothesized that repair of doubled strand ends could lead to non homologous end joining and ultimately amplification (Hersh, *et al*, 2004).

Another phenomenon seen in stationary phase is the generation of growth advantage in stationary phase (GASP) mutants. GASP mutants obtain the ability to outcompete other bacteria during stationary phase. They were found when an *E. coli* culture was incubated for 30 days without the addition of fresh medium or the dilution of the culture. The resulting bacteria were phenotypically different from the original bacteria. When minimal amounts of "old" bacteria were inoculated with the original

strain, the “old”, or adapted strain, numerically dominated the original strains in culture. This shows that the bacteria adapted and were fitter than the parental strain in that environment (Finkel and Kolter, 1999).

It has been postulated that transcription promotes the accumulation of mutations in genes that are under selective pressure. To explain Cairns’ finding, Bernard Davis (1989) proposed that “...by stimulating selective transcription the environment can impose a bias on the ‘random’ process of mutation, thus increasing the frequency of adaptive mutations.” In other words, DNA coding for genes placed under selection, which is highly transcribed in conditions of stress, maybe more prone to accumulate pre-mutagenic lesions because of the process of transcription than DNA that is non-coding or transcriptionally repressed. The mutations that arise during stationary phase are an important component to the evolutionary process because bacteria in natural environments, such as soil, are often nutrient deprived. This process could also explain changes in the rate of evolution and the development of complex traits (Foster, 2005).

The phenomenon of stationary phase mutagenesis is not limited to *E. coli*. Similar findings have been seen in other microbial systems as well as in eukaryotic cells (Kasak *et al*, 1997, and Halas *et al*, 2002). In 2002, Sung and Yasbin developed a system that tests the reversion of mutant cells to amino acid prototrophy to show that stationary phase mutagenesis occurs in *Bacillus subtilis*. They also showed that the transcription factors, ComA and ComK, affected the accumulation of revertants. ComK activates genes involved in competence and ComA is the master regulator of the induction of the competence regulon. The involvement of these proteins suggests that a subset of the bacterial population becomes hypermutable, which could lead to escape from arrested

growth because these genes are involved in cell fate in *Bacillus subtilis* (Sung and Yasbin, 2002).

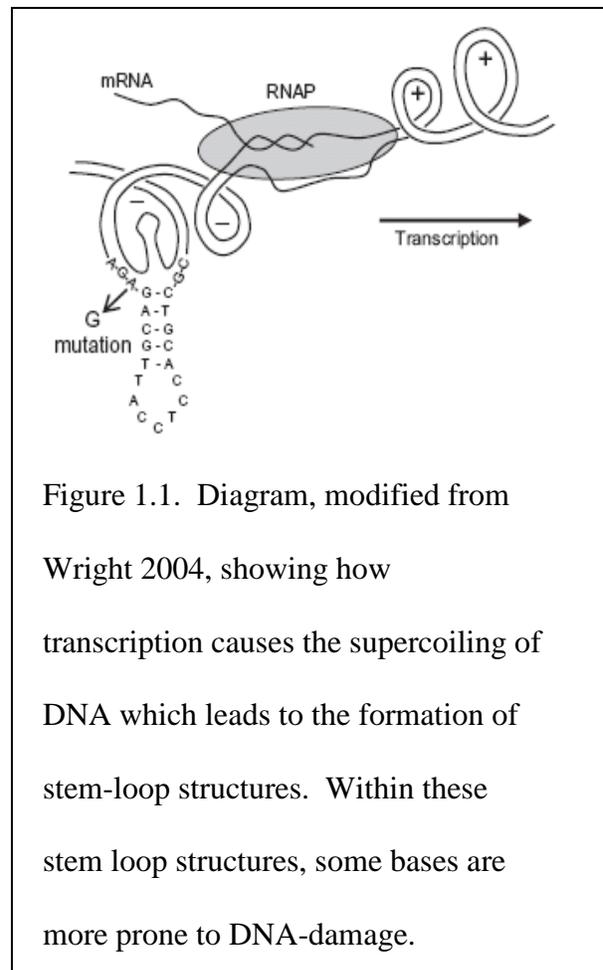
The transcription elongation factor, Mfd, has also been shown to affect stationary phase mutagenesis in *B. subtilis*. Mfd is the transcription coupling repair factor and removes RNAP stalled at DNA lesions and facilitates the localization of DNA repair proteins to the lesion. In an Mfd⁻ background, there is a reduction in the accumulation of revertants (Ross *et al*, 2006). Moreover, Mfd has also been shown to influence the accumulation of mutations to fluoroquinolone resistance in *Campylobacter jejuni* (Han *et al*, 2008). The concept that transcription factors influence stationary phase mutagenesis is interesting because it provides a link by which cells may bias mutation to regions of highly transcribed regions. This concept fits within the view that was first postulated in the late 1980s (Davis, 1989). In support of this concept, Mfd homologs in *E.coli* and HeLa cells have been linked with the concept denoted as transcriptional mutagenesis (reviewed by Saxowsky and Doetsch, 2006; Charlet-Berguerand *et al*, 2006).

Transcriptional mutagenesis is a process that takes place in stationary phase cells and can lead to mutations. It has been established that RNA polymerase (RNAP) can easily transcribe over DNA lesions, a process that may generate a mutant message and protein that allows for enough growth and DNA replication to occur. If the DNA lesion is not repaired before DNA replication occurs, the mutated DNA would then be passed on to a daughter cell's DNA (reviewed by Saxowsky and Doetsch 2006).

One other proposed molecular mechanism by which transcription mediates mutagenesis is the formation of single-stranded, stem-loop structures in the non-templated DNA strand that occur during transcription. Transcription causes positive supercoiling, and the resulting stem and loop structures likely occur behind the transcription bubble. Furthermore, the stability and likelihood of formation of these stem-loop structures (SLS) may be determined by sequence and thermodynamic instability. With increased transcription, SLS are formed and stabilized, nucleotide-altering agents are more active on the unpaired bases of SLS, which would then lead to an increase in mutation frequency (see figure 1.1). *In vitro* studies have shown that single-stranded DNA has a >100 fold

increase in the rate of depurination (Lindahl and Neiberg, 1972) and deamination (Lindahl and Neiberg, 1974) compared to double stranded DNA. Hence, genes that are highly transcribed due to selective pressures are more likely to accumulate mutations than those that are repressed or transcribed at basal levels (Wright, 2004).

Transcription-associated mutagenesis is an alluring explanation because it fits within the view of



Darwinian evolution while offering a solution to the question of how cells accumulate beneficial mutations, increase genetic diversity and avoid genetic load. Evidence that supports this concept has been reported in experiments that study the stringent response, a phenomenon that takes place during the transition from exponential growth to stationary phase. The stringent response is characterized by changes in gene expression and results in repression genes involved in translation and activation of genes that facilitate the escape from stationary phase conditions, such as amino acid biosynthesis genes (Bernhardt *et al*, 2003). (p)ppGpp, synthesized by RelA, is the signaling molecule that triggers the stringent response. Rudner *et al*, 1999, studied the effect of *relA* on rates of reversion to prototrophy and found that the strains that were unable to accumulate (p)ppGpp had lower reversion rates than those that could. They concluded that reversion rates to prototrophy appear dependent on the rate of transcription of genes for amino acid biosynthesis in stationary phase mutagenesis.

The work described here tests the concept of transcription-induced mutagenesis by altering transcription of genes under selection and measuring accumulation of mutations at those genes. The experimental hypothesis to be tested here is that modulating transcription of a point-mutated allele, *leuC427*, in conditions of leucine starvation leads to changes in the accumulation of mutations in *leuC*. We use *Bacillus subtilis*, a Gram positive organism, as a model which provides a useful paradigm for studying the creation of mutations in cells under conditions of stress. Our experiments show that in cells under non-growing conditions the rate of transcription of a gene under selection directly correlates with the accumulation of mutations in that gene.

CHAPTER 2

TRANSCRIPTION-ASSOCIATED MUTAGENESIS

(Part of this chapter has been accepted for publication in the J. Bacteriology)

Introduction

The generation of mutations has been traditionally ascribed to spontaneous processes affecting actively growing, dividing cells. Nevertheless, by the mid-1950's, several reports describing mutagenesis in non-dividing cells of bacteria, plants, flies, and fungi appeared in the scientific literature (Ryan, 1955 and references therein). Much of the initial characterization of this process in bacteria took place in the laboratory of Francis Ryan, where he observed *Escherichia coli* mutants capable of synthesizing histidine arising from *his*⁻ (auxotrophic) cells undergoing prolonged starvation (Ryan, 1955), while cell turnover remained undetectable and DNA replication slowed with increasing time (Nakada and Ryan, 1961). Renewed interest in adaptive mutation was generated when Cairns and coworkers published their work on the generation of Lac⁺ reversions in *E. coli* cells which were previously unable to use the lactose provided as the sole carbon source in a minimal medium (Cairns *et al*, 1988). This work demonstrated that adaptive mutations can arise as a result of stress rather than from selection of pre-existing mutations. The generation of stress-induced Lac⁺ reversions, assayed via a plasmid-borne system, has been studied intensively by several laboratories (reviewed in Roth *et al*, 2006; Foster, 2007; Galhardo *et al*, 2007); Matsumura, 2004), and is dependent on activation of the SOS and stress responses as well as genetic amplification. Further studies have also suggested that a subpopulation within the Lac⁻ stressed cells engage in a exquisitely regulated transient state of hypermutation limited in time and to

DNA sites near double stranded breaks (reviewed in Galhardo *et al*, 2007). Collectively, the results from studies on this system have provided interesting insights into the acquisition of beneficial mutations and demonstrated the role of several genetic factors in the adaptive mutation phenomenon.

A significant question in the study of adaptive mutagenesis centers on what processes would allow arrested cells under stress to acquire beneficial mutations before the accumulation of deleterious mutations resulted in cell death. One intriguing and seldom explored possibility is the role of transcription in this phenomenon. While cells are under stressful conditions, stochastic processes acting upon derepressed genes could bias the accumulation of mutations to highly transcribed alleles. Selection would favor mutations enhancing cell survival, while a transcription-associated mutation bias would aid the population in avoiding a lethal genetic load. In support of this idea, several studies on adaptive mutagenesis have noted the appearance of mutations principally in chromosomal alleles under selection (Steele, 1992, and Hall, 1998).

Other experiments have directly illuminated the role of transcription in mutagenic processes. For instance, using actively dividing *E. coli* cultures, Wright and coworkers established a correlation between rates of transcription and of reversion to prototrophy in the *argH* and *leuB* alleles (Wright and Minnick, 1997) and led Wright to hypothesize a stress-directed mutagenesis model (Wright, 2004): Essentially, as a response to stress, a small percentage of the genome is derepressed with respect to its transcription. During the transcription process the potential exists for single-stranded DNA to supercoil and form secondary stem-loop structures, exposing vulnerable bases to mutagenic processes. Subsequent lack of high fidelity repair would then generate a heritable mutation. The

resulting genetic variants are selected for an adaptive advantage, facilitating escape from arrested growth. Further support for this concept was shown by recent experiments in our laboratory that demonstrated a connection between transcriptional derepression and mutagenesis by examining how defects in Mfd, a transcription elongation factor, influences the accumulation of mutations in three different chromosomal loci. Those studies showed a dramatically depressed mutagenesis in genes under selection (Ross *et al*, 2006) in the Mfd-deficient background. Further examination of the Mfd deficiency indicated that a decrease in transcription in a gene under selection preceded the formation of stress induced mutations at the same gene (Pybus *et al*, accepted). The Mfd effect was not observed in constitutively expressed genes or in the genes tested in growing conditions.

The stress-induced mutagenesis phenomenon is not exclusive to bacteria. Specifically, in growing cells of yeast and mammalian stem cells it has been hypothesized that high transcription rates promotes the formation of mutations (Kim *et al*, 2007, and Hendriks *et al*, 2008). So, while the role of transcription in mutagenic processes has been demonstrated in conditions of active DNA replication, whether transcription mediates the formation of mutations in non-growing conditions remains largely unknown.

Here we show compelling evidence that supports the role of transcription in adaptive mutagenesis. We used several approaches, either by alteration of physiological conditions or by genetic manipulation, to alter the transcription of a point mutated allele. The correlation between transcription and mutagenesis was genetically tested by decoupling transcription and selection. To further increase transcription of a gene under

selective pressure, we placed a defective *leu* allele under the control of an IPTG-inducible promoter and compared the accumulation of Leu⁺ revertants in conditions of transcriptional induction and repression. Leu⁺ reversions increased significantly in parallel with the induced increase in transcription levels. We also examined the condition in which a gene is placed under selection but transcriptionally repressed, by placing the defective *leu* allele under the control of riboswitches that repress transcription in response to levels of either methionine or tyrosine. Exogenous addition of tyrosine or methionine resulted in different levels of expression of *leu* that directly correlated with accumulation of mutations at this locus. Lastly, we used effectors that regulate transcriptional activity of the native *leu* allele and showed that moderately decreased transcription preceded a lower accumulation of stationary phase mutations than the one observed in conditions of derepression. These results further support the concept that cells engage in programs that increase genetic diversity in conditions of stress through the process of transcription. Because transcription is a ubiquitous cellular function, these findings have profound implications in the evolutionary process of all organisms.

Materials and Methods

Bacterial strains and media. Strain YB955 is a prophage-“cured” derivative of *B. subtilis* strain 168 containing point mutations in three alleles, *metB5* (ochre), *hisC952* (amber), and *leuC427* (missense) (Sung and Yasbin, 2002).

B. subtilis strains were routinely isolated on tryptic blood agar base (TBAB) (Acumedia Manufacturers, Inc., Lansing, MI), and liquid cultures were grown in Penassay broth (PAB) (antibiotic medium 3; Difco Laboratories, Sparks, MD)

supplemented with 1X Ho-Le trace elements (Gerhardt *et al*, 1994). Spectinomycin was added to a final concentration of 100 µg/ml where appropriate. *E. coli* strain XL1-Blue, used for plasmid subcloning and transformation was maintained on Luria-Bertani agar containing 100 µg/ml ampicillin. All solid media for maintenance and tests were grown at 37°C in a humidified incubator. Liquid cultures were aerated at 250 rpm at 37°C.

For the experiments in which culture conditions were manipulated to alter transcription of the native *leuC* allele, we used exogenous additions of isoleucine 200 ng/ml and norvaline 500 ug/ml. Expression of the *ilv-leu* operon has been extensively studied and is affected by many factors. CodY is a global repressor, interacts with branched chain amino acids (BCAAs) and represses the *ilv-leu* operon. When BCAAs are present CodY binds them and increases its affinity for the *ilv-leu* promoter region (Tojo *et al*, 2005). Therefore, we used this cellular response, supplementing culture conditions with differing levels of BCAA, to modulate the transcription levels of the *ilv-leu* operon.

Experiments that used genetic constructs to alter transcription of *leuC427* were constructed by fusing this allele to either an IPTG-inducible promoter or promoters in which transcriptional repression of *leuC427* is mediated by riboswitches that respond to an abundance of methionine and tyrosine. Riboswitches are mechanisms within the cell that regulate transcription. When the leader sequence is transcribed, it will fold into different secondary structures based on the presence or absence of an effector molecule. This secondary structure will then either allow transcription to continue or terminate it (Grundy and Henkin, 2003). This mechanism effectively decouples transcription and selection in a gene.

Strain construction. To construct the IPTG-inducible gene in YB955, the 1419 bp *leuC427* allele (GenBank accession number Z99118) and its Shine-Dalgarno sequence were amplified by PCR using primers hsleuC F Sall 5'-AAAAGTCGACTAGAGGGAGGAAAT AAAA GATG ATG C-3' and hsleuC R SphI 5'-AAAAGCATGCCGCACTCCTTACACA ACTG T-3'. The PCR mix contained 1.2 units Vent^R DNA Polymerase (New England Biolabs, Ipswich, MA), 20 mM Tris-HCl, 10 mM (NH₄)₂SO₄, 10 mM KCl, 2 mM MgSO₄, 0.1% Triton X-100, 0.2 mM deoxynucleotide triphosphates, and 500 ng YB955 genomic DNA. To simplify subcloning, the PCR product was ligated into pGEMT Easy (as recommended by the manufacturer, Promega, Madison, WI) following the manufacturer's protocol, digested with Sall and SphI, gel purified and ligated to the similarly digested pHyperspank vector (a kind gift from Dr. David Rudner). The resulting construction was confirmed by PCR, sequenced and denoted as YB955 *amyE::pHS* and YB955 *amyE::pHS-leuC427*. Details are shown in figure 2.1. The pHyperspank-*leuC427* vector was transformed into strain YB955 by natural competence (Boylan *et al*, 1972). Integration was confirmed by screening for spectinomycin-resistant colonies and by PCR. IPTG-induction of this construct was confirmed by SDS-PAGE gel electrophoresis (Data not shown).

To construct a defective *leuC* under the transcriptional control of a riboswitch, the S-box was amplified from the gene *yitJ* of YB955 with the following the primers, *yitJsboxF*, ACCCAAGCTTATTATTGTCTTTATTTTCGGTAAATTTGGAGAAA, and *yitJsboxR*, TAGAGTCGACTGTCTGCCTCCTTTATTCACATCA. The T-box was amplified from the gene *tyrS* of YB955 with the primers DIRTB-TyrS AAAGATAAGCTTCCGAAAACCAGAA and Rev TyrS Box CGGTCGACAAGATA

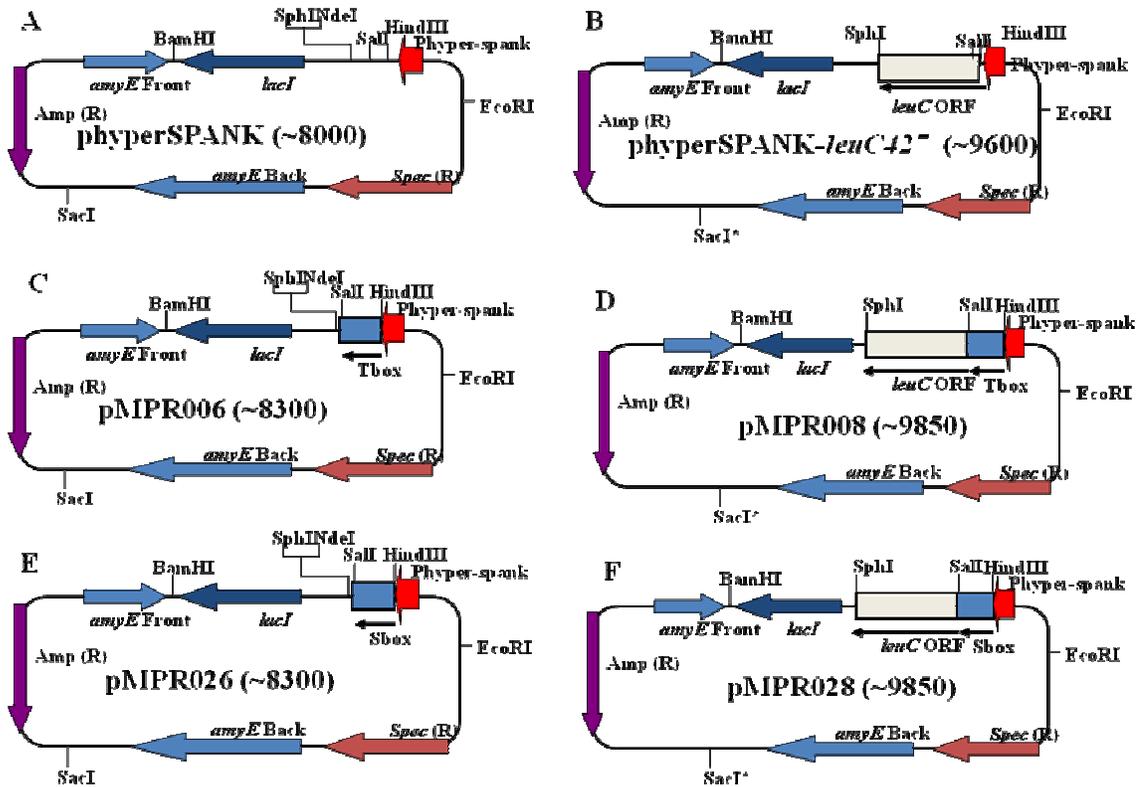


Figure 2.1. Diagrams of the plasmids used in this study.

AAGCTCCTTTTTTA. The plasmids, pHyperSPANK and pHyperSPANK-*leuC427*, and PCR amplified riboswitches were digested with the restriction enzymes HindIII (Promega, Madison, WI) and SalI (NEB, Ipswich, MA) according to the manufacturers' recommendations. The PCR fragments were ligated into pHyperSPANK using T4 Ligase (Promega). The ligation reaction was then transformed into chemically-competent *E. coli* DH5 α (Invitrogen, Carlsbad, CA) using the manufacturer's instructions. Transformants were then selected on Luria Bertani (LB) plates containing 100 μ g/mL ampicillin. This procedure generated plasmids MPR006, MPR008, MPR026, and MPR028. Plasmid DNA were extracted from transformants using the Qiagen Mini-Prep Kit (Carlsbad, CA) and verified by size and restriction digestion. Details of plasmid construction are shown

in Figure 2.1. The plasmids were then transformed into YB955 as previously described. Briefly, YB955 was grown to ninety minutes after the transition from exponential to stationary phase (T90), in GM1 broth (0.5% dextrose, 0.1% yeast extract, 0.2% casein hydrolysate, essential amino acids 50ug/mL, 1X Spizzien Salts) and then diluted 10-fold into GM2 broth (GM1 broth plus 50 uM CaCl₂, 250 uM MgCl₂). After one hour of incubation at 37°C with aeration, plasmid DNA is added. After plasmid addition, the culture was incubated for one hour supplemented with 0.1 mL of 10% Yeast Extract and incubated for an additional hour. Cultures were then plated onto Tryptone Blood Agar Base (TBAB) containing 100 ug/mL spectinomycin. Transformants were verified by PCR using the primers amyEF, GATCAAAAGCGGAACCATCTTC, and amyER, AATGGGGAAGAGAACCGC.

Because YB955, the background strain used in this study, is Met⁻ and the S-box riboswitch system demands methionine prototrophy, we transformed HAM101 and HAM102 with DNA from a Met⁺ strain and created HAM103 and HAM104. To do this, genomic DNA was isolated from the methionine prototrophic strain, YB007 (Luniatti, unpublished). The sample was grown overnight in 2.5 mL of PAB. Cells were harvested by centrifugation, washed with 1.0 ml of lysis buffer (50 mM EDTA, 100mM NaCl, pH 7.5), and resuspended in 0.3 ml of lysis buffer. Cell suspensions were treated with Lysozyme by adding and 0.1 ml of lysozyme (10mg/ml in lysis buffer), delivered in a dropwise manner, vortexing after each drop, and incubating at 37°C for 15 minutes. Proteinase K (1 mg/ml) is added to the sample and incubated at 37°C for 30 minutes, combined with 30 uL of 20% SDS, mixed and incubated at 37°C for 5 minutes. The nucleic acids were extracted with an equal volume of buffer-saturated 25 phenol:24

chloroform:1 isoamyl alcohol until aqueous-phenol interface is clear, then extracted once with 24 chloroform:1 isoamyl alcohol. The DNA was precipitated with the addition of 45 uL of 3M sodium acetate (pH 5.2) and 2.5 volumes of ice cold 95% ethanol. The sample was inverted several times until a visible pellet formed. The supernatant is removed and the remaining pellet was rinsed with 95% ethanol, air dried, and resuspended in 0.1 ml of 10mM Tris HCl (pH 8.0), 1mM EDTA.

This DNA was then transformed into HAM101 and HAM102 as described above without the addition of 10% yeast extract. The cells were plated onto minimal plates lacking methionine (containing 1X SMS, 0.5% glucose, and a concentration of 50 ug/ml of leucine, histidine, isoleucine, and glutamic acid per ml). Transformants were verified by sequencing the *metB* gene, which was amplified with primers, metBf, GCGAGAGAACACCTTGACG and metBr, AAACCCCCGGCTTCCTTT. The PCR fragments were purified with the Qiagen PCR clean-up kit. Sequencing was performed by Biotechnology (Madison, WI). Analysis of the sequences indicated that HAM103 and HAM104 had acquired the *metB*⁺ allele from YB007.

Stationary Phase Mutagenesis Assay. The procedure for the stationary phase mutagenesis assay has been previously described (Sung and Yasbin, 2002). Strains were grown overnight in 2 ml of PAB (BD) in a 37°C shaking incubator. 0.6 ml of the overnight cultures was transferred into 10 ml of PAB with 10 µl of Ho-Le trace elements (Gerhardt *et al*, 1994) into 125 ml neploflasks. Growth was tracked using a Klett-Summerson colorimeter (no. 66 filter; Klett MFG Co., Inc.; 1 kU 106 CFU/ml). When cells reached T90, the cells were harvested by centrifugation and resuspended in 1X Spizizen Salts (SMS). 0.1 ml of the cell suspensions were plated onto minimal medium

containing 1X SMS, 0.5% glucose and either 50 ug or 200 ng of the required amino acid per ml and 50 ug of both isoleucine and glutamic acid per ml. Over nine days, the plates were incubated at 37°C and scored for revertant colonies. For the experiments that used isoleucine to modulate transcription of the *ilv-leu* operon, addition of 50 ug/ml or 200ng/ml of isoleucine were included in the stationary phase assay. The initial amount of cells used in the assay is determined by serially diluting and plating an aliquot of the cell suspension onto medium containing all the required amino acids. This experiment was replicated at least five times.

Survival rates of the non-revertant background cells were determined by removing agar plugs from areas of agar that contained no colonies and plating on medium containing all the essential amino acids. These plates were incubated at 37°C for two days and then counted.

Stationary phase mutagenesis soft agar overlay assays with IPTG induction.

There were two types of experiments that employed soft-agar overlays to detect Leu⁺ mutations in the strains carrying the *ilv-leu leuC427* and either the *amyE::Phs* or the *amyE::Phs-leuC427* alleles. In the first type of experiment, strains YB955 *amyE::Phs* and YB955 *amyE::Phs-leuC427* were grown to T₉₀ in 10 ml PAB with trace elements. Cultures were centrifuged and resuspended in 1X Spizizen Minimal Salts (SMS) (Spizizen, 1958) to 10 ml. Aliquots of 100 µl of this suspension were plated on minimal media with trace (200 ng/µl) leucine and methionine either containing 0.07 mM IPTG or no IPTG. The initial titer was determined from the 10 ml culture. Starting from 48 hours of incubation, a set of plates was overlaid with soft agar (0.7 % agar and pre-warmed at 42° C) lacking leucine, and containing 0.07 mM IPTG and 50 µg/ml methionine. Of note,

adjustment of the final concentrations for IPTG and methionine considered the volume and IPTG concentration in the media dispensed previous to performing the overlay. The plate was incubated for two days and the number of revertant colonies was scored. For the second type of experiment, we used soft-agar overlays to deliver the test cells onto agar medium with trace (200 ng/ μ l) amounts of leucine and methionine and either 0.07 mM of IPTG or none, instead of spread plating. The test cells were initially grown and prepared as described above and added to 3-ml aliquots of soft agar lacking leucine and methionine and containing none or 0.07 mM IPTG. The viability of non-revertant cells was assayed as described for the plate assay. This experiment was replicated at least five times.

Stationary phase mutagenesis soft agar overlay assays using amino acids to repress transcription. This assay is similar to the one described above and modified to include the use of soft agar overlay containing methionine or tyrosine as effector molecules. Cell suspensions from cultures grown as previously described, were spun down, resuspended in 1X SMS, and added to 3 ml semi-solid overlay, which consisted of Spizizen minimal media supplemented with 0.5% glucose and trace amounts of leucine and histidine (200 ng/ml). To control for transcription derepression in HAM103 and HAM104, 200 ng/ml (derepressed) or 50 μ g/ml (repressed) of methionine, were used to amend the overlay. For the experiments involving HAM201 and HAM202, transcription of *leuC* was repressed by supplementing the experimental medium with 50 μ g/ml of tyrosine.

Starting two days after the initial overlay was added and every other day from then until the tenth day, a top overlay, which allowed for growth of leucine prototrophs,

was added. This semioverlay consisted of Spizizen minimal media supplemented with 0.5% glucose and 200 ng/ml of histidine and methionine. Revertant colonies were counted two days after the top overlay was added. This experiment was replicated at least five times.

Plugs were taken from the minimal plates to determine the viability of the non-revertant background. Agar plugs were diluted in ten-fold and plated on media containing all the essential amino acids. Plates were incubated at 37°C for two days and then counted.

Fluctuation test. The growth-dependent reversion frequencies for the His⁻, Met⁻, and Leu⁻ alleles were measured by fluctuation tests which has been previously described (Sung and Yasbin 2002). Cultures were grown in the absence or presence of IPTG (YB955 *amyE::Phs* and YB955 *amyE::Phs-leuC427*), tyrosine (HAM201 and HAM202), or methionine (HAM103 and HAM104). Bacterial cultures were grown to saturation (roughly 14 to 16 hours) at 37°C with aeration in PAB. The saturated cultures were then diluted 10⁻⁴-fold in PAB and 1 ml aliquots were dispensed into 38 18-mm test tubes. Test tubes were incubated to saturation at 37°C with aeration. The cultures were harvested by centrifugation and resuspended in 0.1 ml of 1X Spizizen salts. The cells were then plated onto the same selective minimal media described in the stationary phase mutagenesis assay section. After 48 hours of incubation, the plates were scored for revertant colonies. The Lea-Coulson formula, $r/m \cdot \ln(m) = 1.24$, which uses the median (r), was used to estimate number of mutations per culture (m). To determine the total number of CFU plated (N_t), three cultures run in parallel were serially diluted and spread

onto minimal agar-media containing all the required amino acid. Mutation rates were calculated with the formula $m/2Nt$.

RNA extraction and preparation. RNA was isolated, as previously described (Silvaggi *et al*, 2005), from experiments in which cells were subject to conditions of transcriptional derepression or activation by supplementing cultures with either of the following effector molecules, tyrosine or methionine. To determine the effects of norvaline on the transcription of *leuC*, YB955 was grown in 10 ml of PAB with either none or 500 ug per ml _{DL}-norvaline (Eymann *et al*, 2002). YB955 cultures were grown in 10 ml of minimal broth (1X SMS, 0.5% glucose, 50ug of the required amino acids per ml, 50 ug of glutamic acid per ml, 0.005M MgSO₄, Ho-Le trace elements, and 50 ug/ml or 200ng/ml of isoleucine) to determine the rate of transcription of *leuC* when under limiting conditions of branch-chained amino acids. Similar conditions were used for YB955 *amyE::Phs-leuC427* without norvaline but in the presence or absence of IPTG. Culture conditions for HAM202 were similar to the ones described above with the exception that tyrosine, 50 ug per ml, instead of IPTG was used to control transcription of the *leuC427* allele. For the experiments with HAM104, 50 ug per ml of methionine were used to derepress and repress transcription of *leuC427*, respectively. Cells were harvested during mid-exponential phase or at T90 by mixing the culture with an equal volume of methanol and centrifuging for 10 minutes at 4,000 X g at 4°C. The pelleted cells were stored at -20°C to be processed later. Pellets were resuspended in 3.2 ml of LETS buffer (10mM Tris-HCl pH 8, 50 mM LiCl, 10 mM EDTA, 1% SDS). The cells were lysed and nucleic acids precipitated by vigorously vortexing the sample in vials containing 2 ml of glass beads (Sigma, P-4682, St. Louis, MO) and 2.4 ml of acid-

phenol. An equal volume of chloroform was added to the sample and centrifuged for 10 minutes at 3,200 X g at 4°C. The aqueous phase was added to a fresh tube containing 3.2 ml of 5:1 phenol-chloroform and centrifuged for 10 minutes at 3,200 X g at 4°C. The aqueous phase was divided into 0.7 ml aliquots and added to an equal volume of isopropanol. After a 10-minute incubation at room temperature, the samples were centrifuged at 14,000 rpm for 25 minutes at 4°C. The pellets were then washed with 75% ethanol, centrifuged for 5 minutes, resuspended in 20 uL of DEPC-treated water and vortexed upon the addition of 1.2 ml of TRIzol reagent. The sample was incubated at room temperature for 5 minutes, combined with 0.24 ml of chloroform, mixed by inversion for 15 seconds and kept at room temperature for 2 minutes. Then, samples centrifuged at 14,000 rpm for 15 minutes at 4°C to separate the aqueous phase, which was transferred to isopropanol at a 1:1 (v/v) and allowed to precipitate at -20° C overnight. The RNA pellet was collected by centrifugation at 14,000 rpm for 15 minutes at 4°C and air dried for 2 minutes. Lastly, pellets were resuspended in 20 uL of DEPC-treated water, incubated at 55°C for 2 minutes and stored at -20° C until further use. The resulting RNA was quantified on the General Electric NanoVue Plus Spectrophotometer (Piscataway, NJ) which was graciously provided by the Wing lab.

DNase treatment. To remove any residual DNA, the Ambion TURBO DNA-*free* (Austin, TX) kit was used according to manufacturer's instructions.

16S rDNA PCR. To verify that RNA samples were free of any contaminating DNA, the 16S rRNA gene was PCR amplified using primers 16S RT F, TCGCAAGACTGAACTCAAAGGA, and 16S RT R, TCAGAGGATGTCAAGACCTGGTAAG.

cDNA synthesis/Real-Time PCR. For each RNA sample, we conducted three independent cDNA synthesis reactions. 200 ng of RNA was used in the Quanta Biosciences qScript One-Step SYBR GREEN qRT-PCR Kit (Gaithersburg, MD) to synthesize cDNA and perform the real-time PCR. The following primers were used to amplify the *leuC* gene, leuC RT F, GACCCGGGCGCTGTTTACG, and leuC RT R, GTTAATGCCCATGTCACCATAGG. The primers used to amplify the housekeeping gene *veg* were veg RT F, TGGCGAAGACGTTGTCCGATATTA, and veg RT R, CGGCCACCGTTTGCTTTAAC.

Test for slow-growing revertants and for multiple mutations. Leu⁺ colonies were further tested by patching the colonies on SMM media lacking leucine and in conditions of transcriptional derepression. Plates were incubated at 37°C and scored after 48 hours. A portion of the colonies from these plates was patched to SMM lacking methionine or histidine and scored similarly.

Results and Discussion

General and specific DNA repair systems, mismatch repair, and oxidative damage repair (GO system), have been shown to be repressed or inefficient in cells under conditions of stress in eukaryotic and prokaryotic systems (Mihaylova *et al*, 2003, Pedraza-Reyes and Yasbin, 2004, Hara *et al*, 2005, Vidales *et al*, 2009), while error-prone polymerases are active in stationary cells (Sung *et al*, 2003, Tegova *et al*, 2004; Hara *et al*, 2005, Duigou *et al*, 2005, and Duigou *et al*, 2004). Thus, one can speculate that the combination of transcriptional derepression and DNA repair inadequacies in non-growing conditions biases mutations to transcribed regions. In support of this concept,

recent experiments involving Mfd have shown that the ability to transcribe a gene under selection is correlated with the accumulation of mutations occurring at that gene (Pybus *et al*, accepted). Thus, we hypothesize that transcription-associated mutagenesis mediates adaptive mutation. In this study, we test this hypothesis by modulating transcriptional activity and measuring the accumulation of mutations at *leuC427* in conditions of stress. Transcriptional activity of the *leuC427* was modulated by i) physiological culture conditions of test cells and genetically altering the promoter to ii) increase transcription and iii) repress transcription in conditions of nutritional stress.

To determine if increases in transcription correlate with increases in the accumulation of revertants during stationary phase, we subjected cells to physiological conditions that lead to increases of transcription of the *ilv-leu* operon which contains the defective *leuC* allele and measured reversions to leucine biosynthesis. In the experiments that used isoleucine to alter transcription of this gene, real-time polymerase chain reaction (RT-PCR) indicated that in stationary phase cells supplemented with 200 ng/ml of this amino acid had transcript levels of *leuC427* that were four-fold higher than in cells exposed to 50 µg/ml of isoleucine (table 2.1). Significantly, stationary phase mutation assays conducted under the same conditions showed that transcriptional derepression correlated with an increase the number of revertants to leucine biosynthesis. However, the observed increase in reverants did not amount to significant differences when compared to the values obtained in repressed conditions (figure 2.2). The moderate differences in the number of revertants were not due to the viability of the cells (figure 2.3).

Table 2.1. The difference in *leuC* expression measured by RT PCR. The method $\Delta\Delta Ct$ was used to calculated fold increase with the *veg* gene serving as the housekeeping gene.

Strain	Repressed condition	Derepressed condition	<i>leuC</i> transcription fold increase
YB955	50 μ g/ml isoleucine	200ng/ml isoleucine	4
YB955	no norvaline	500 μ g/ml norvaline	2.6
HAM201	50 μ g/ml tyrosine	no tyrosine	13
HAM104	50 μ g/ml methionine	no methionine	58

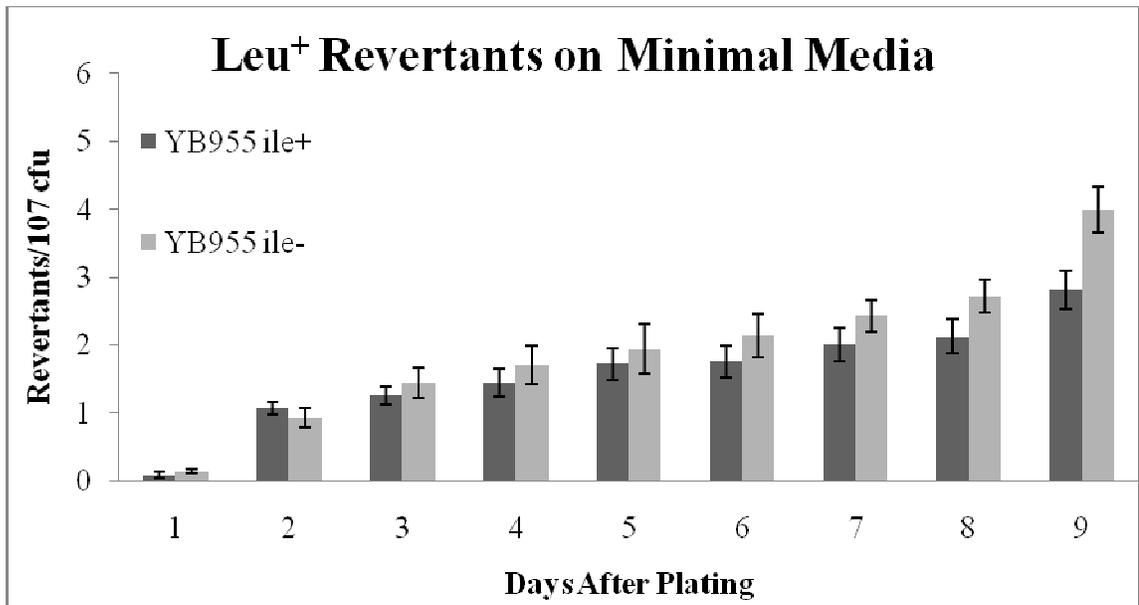


Figure 2.2. The acculumation of Leu⁺ revertants over time in YB955 under high, 50 ng/ml, and low, 200 ng/ml, concentrations of isoleucine. The error bars are representative of the standard error.

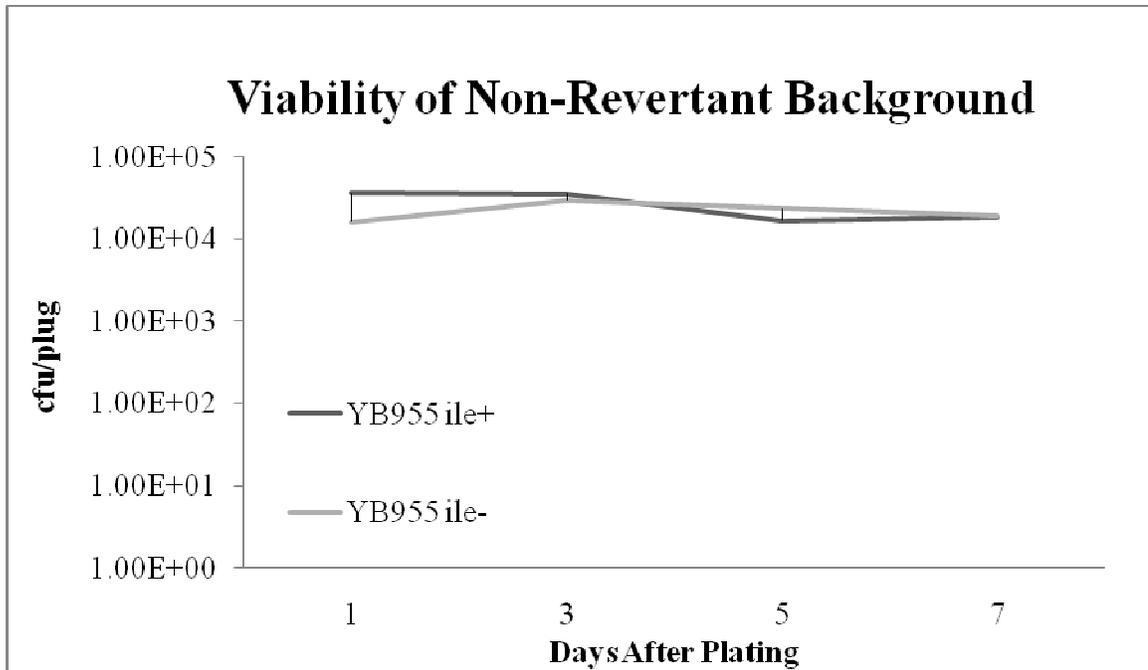


Figure 2.3. The viability of the non-revertant background of the cultures in figure 2.2.

We also compared the levels of leucine reversion in culture conditions supplemented with two BCAAs, isoleucine and valine (repressed condition), or isoleucine (derepressed condition). Interestingly, similar results were observed, accumulation of leucine revertants was higher in derepressed conditions than in repressed conditions, but the differences were not significant (figure 2.4). There were no differences in viability (figure 2.5). Taken together, these experiments showed that increases in transcription of *leuC427* mediated moderate increases, albeit not statistically significant, in the accumulation of leucine revertants. It is possible that for transcriptional responses to influence stationary phase mutagenic processes higher than four-fold increases in levels of transcription are required. In the context of a mechanism that promotes the formation of stem-loop structures and considering the Mfd effect on transcription (see above), an increase of a four-fold in transcription is not enough to form

or stabilize such structures, which would lead to only moderate increase in mutations. As such, the results presented here provide inconclusive supporting evidence for the transcription-associated mutagenesis concept.

Since the experiments described above only resulted in moderate increases in transcription levels, we examined the effects of norvaline to determine if such treatment further stimulated increases (more than four-fold) in transcription of *leuC427*. mRNA, collected from stationary phase cells grown in the absence or presence of norvaline, measurements showed a 2.6-fold increase in *leuC* transcription in the presence of norvaline (table 2.1). Since the increase in transcription was lower than the increase seen in the isoleucine experiments, we opted not to study the effects of norvaline in stationary phase mutagenesis.

Since the previous experiments that manipulated culture conditions to modulate transcription only resulted in a suggestive trend between transcriptional derepression and stationary phase mutagenesis, we decided to further investigate how transcription influences stationary phase mutagenesis of a single gene through the use of an inducible genetic *leuC427* construct. For these experiments, we used a derivative of YB955 that contains two identical defective *leuC* alleles: one allele located within the *ilv-leu* operon (normal locus), and another identical allele recombined into the *amyE* locus. The latter *leuC* allele is under the transcriptional control of the IPTG-inducible promoter, pHyperspank (Figure 2.1B). In this genetic background, mutations that result in Leu⁺ revertants may be acquired in either allele; however, mutations within the allele controlled by the hyperspank promoter require the presence of IPTG to be detected (since

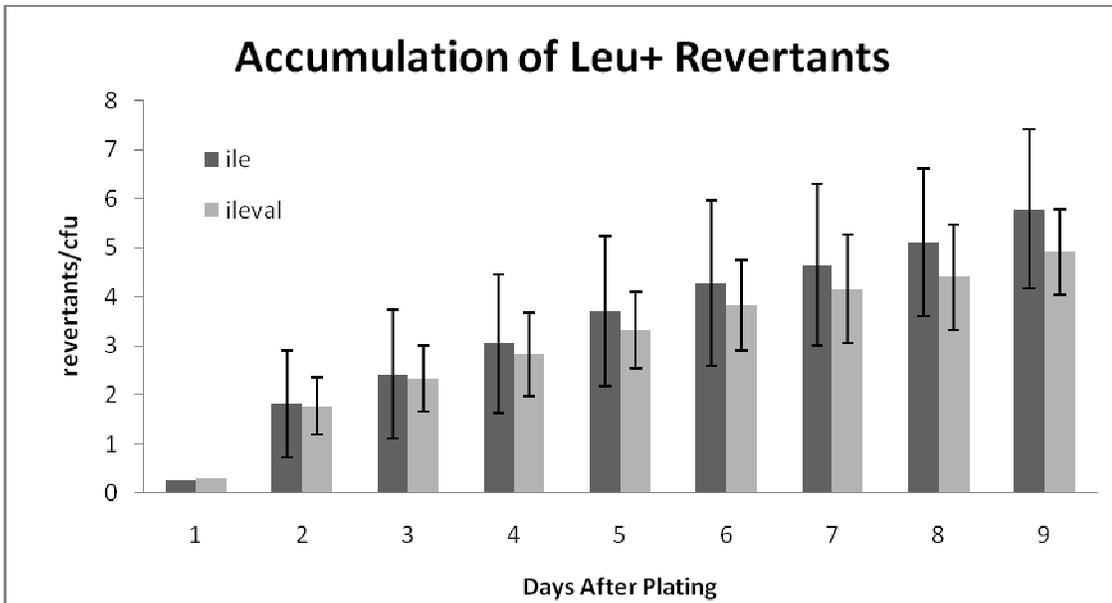


Figure 2.4. The acculuation of Leu⁺ revertants over time in YB955 in the presence of isoleucine, derepressed conditions, or isoleucine and valine, repressed conditions. The error bars are representative of the standard error

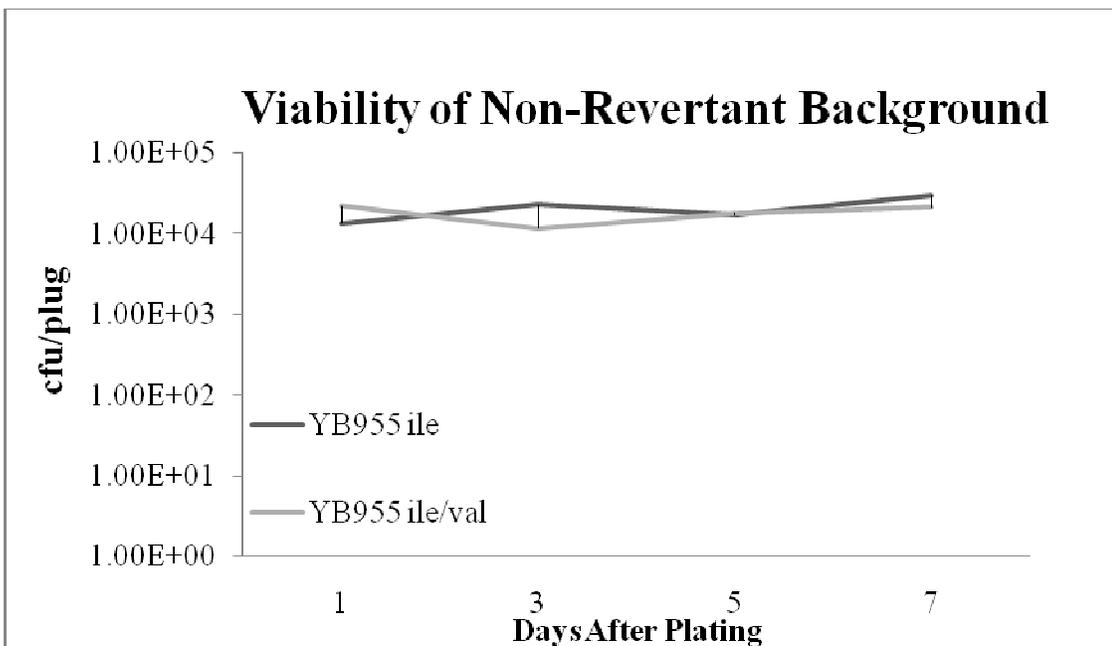


Figure 2.5. The viability of the non-revertant background of the cultures in figure 2.4.

transcription of this gene is dependent upon this inducible system). By using this two-allele approach, we can differentiate between genetic events occurring during growth and in non-growing conditions, as well as those events that are promoted by experimentally increased transcription in stationary phase conditions. Thus, we compared levels of mutagenesis in the strain containing this construct, designated YB955 *amyE::Phs-leuC427*, with an isogenic strain containing only the inducible promoter (YB955 *amyE::Phs*) on leucine-deficient media with and without inducer.

We conducted stationary phase mutagenesis assays in two ways. In the first experiment, we spread plated 100 μ l aliquots of cells onto media where induced and uninduced cells were starved for leucine and methionine and subsequently shifted to conditions that relieved methionine starvation and induced transcription of *Phs*-constructs (see material and methods). In the second experiment, induced and uninduced cells were added to soft-agar overlays containing no leucine or methionine and dispensed on top of regular agar medium of the same composition as the soft-agar. The initial absence of essential amino acids methionine and leucine negates the growth of Leu^+ revertant cells while allowing the application of leucine biosynthesis selective pressure as well as the IPTG-controlled induction of the specific allele. For both experiments, and beginning the second day of incubation and daily thereafter, a set of plates for both strains were overlaid with soft agar containing minimal medium with IPTG, methionine and no leucine.

As shown in figures 2.6 and 2.7, the accumulation of Leu^+ colonies in YB955 *amyE::Phs-leuC427* was increased over six-fold in the presence of inducer as compared to YB955 *amyE::Phs* at day 10. Mutagenesis in YB955 *amyE::Phs* was comparable in

induced and non-induced conditions. Since the appearance of colonies in this strain reflects reversions at the *ilv-leuC427* allele only, it is clear that increased transcription of *leuC427* from *Phs* is linked to the majority of revertants in YB955 *amyE::Phs-leuC427*. Significantly, cell viability was similar for each strain (figure 2.8). While both experiments show the dramatic effects of increased transcription on stationary phase mutagenesis, the second experiment rules out the possibility that the overlay process spreads cells of microcolonies formed during starvation. Another important consideration was whether presence of the inducer would affect mutagenesis at other alleles. YB955 contains two additional point-mutated alleles, *metB5* and *hisC952*, which have been previously studied during stationary phase (Sung and Yasbin, 2002). We assayed YB955 *amyE::Phs* and YB955 *amyE::Phs-leuC427* for reversion to methionine and histidine prototrophy in the presence and absence of IPTG. No change was observed in mutation level (figures 2.9 and 2.10) or in cell viability (data not shown). This indicated that the effect of IPTG-based induction was specific to our construct. We also examined the effect of increased transcription on the rate of mutation of *leuC427* in exponential growth and found no striking differences in rates of mutation between the wild type and the strains carrying the inducible constructs in the presence or absence of IPTG (figure 2.11), suggesting that the influence of transcription on mutagenesis is exclusive, or dramatically pronounced in stationary phase conditions.

Since it may be argued that the experiments described above only increased transcription in conditions of selective pressure and no effective decoupling between transcription and selection has been shown, we conducted stationary phase mutagenesis (SPM) experiments that placed *leuC427* under promoters that respond to other selective

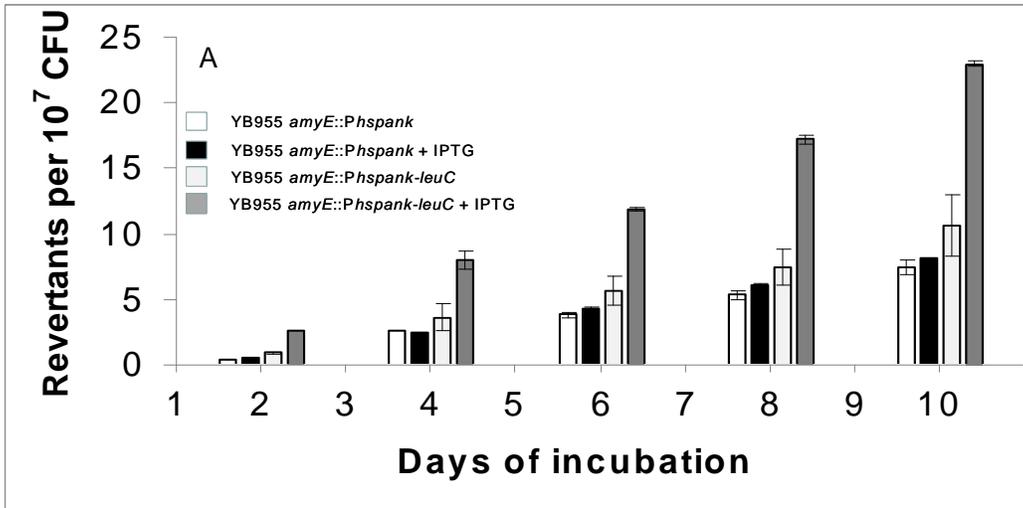


Figure 2.6. The accumulation of Leu⁺ revertants in the stationary phase under conditions of transcriptional repression (no IPTG) and induction (IPTG). Test cells were spread plated. Error bars represent standard error.

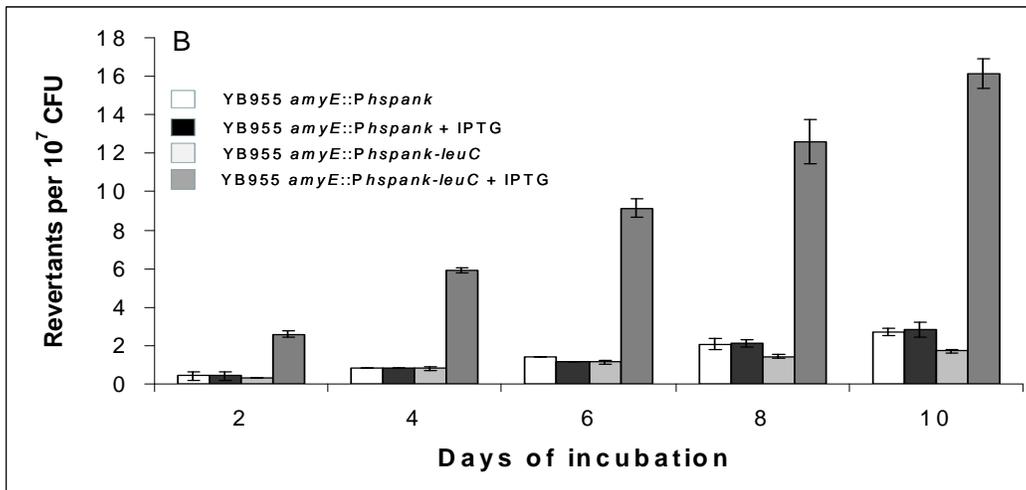


Figure 2.7. The accumulation of Leu⁺ revertants in the stationary phase under conditions of transcriptional repression (no IPTG) and induction (IPTG). Test cells were immobilized with soft agar. Error bars represent standard error.

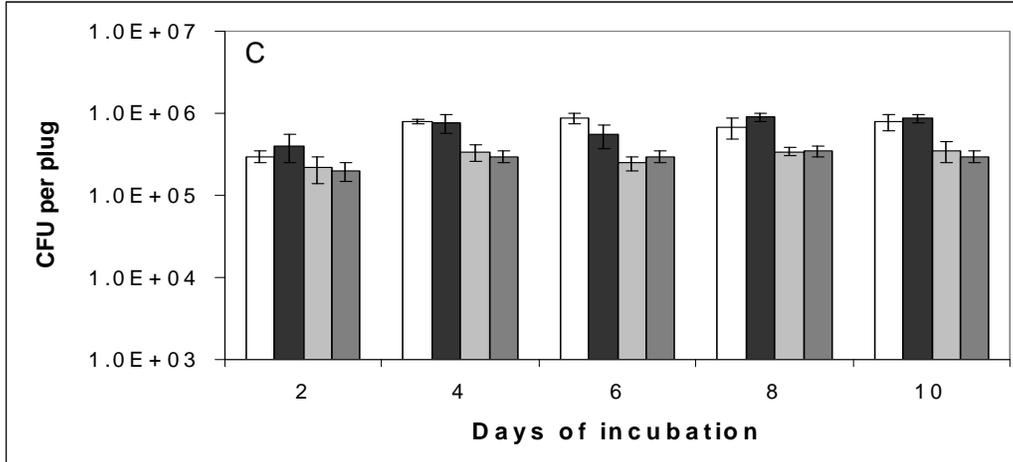


Figure 2.8. Viability of non revertant test cells of the cultures from figures 2.6 and 2.7.

The white bars are YB955 *amyE::pHs*, black bars are YB955 *amyE::pHs* with IPTG, the light gray bars are YB955 *amyE::pHs-leuC*, and the dark gray bars are *amyE::pHs-leuC* with IPTG. Error bars represent the standard error.

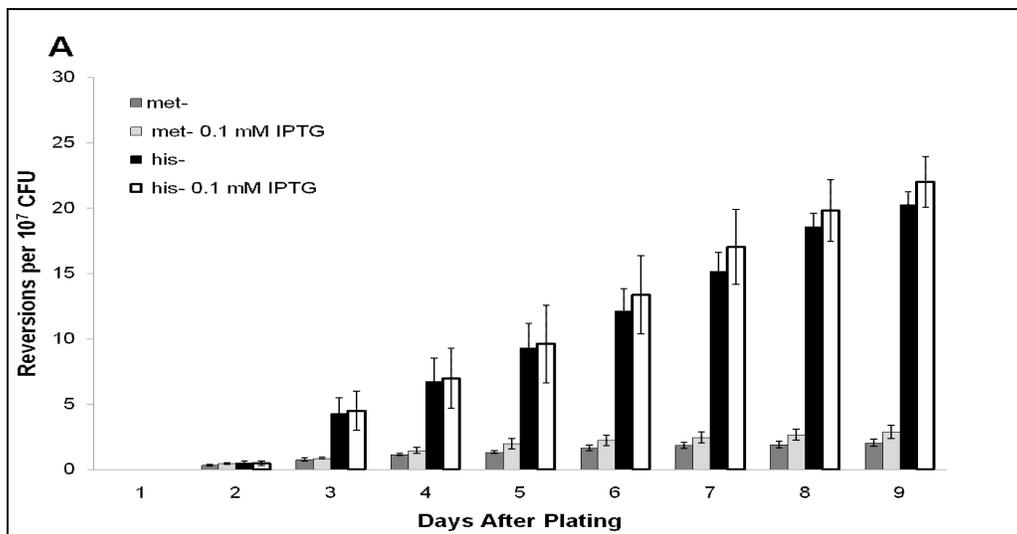


Figure 2.9. Accumulation of *Met*⁺ and *His*⁺ revertants during the stationary phase mutagenesis plate assay in YB955 *amyE::pHs*. Results represent levels of mutation in YB955 strains on selective media with and without inducer containing the *P_{hS}* promoter. Error bars represent the standard error.

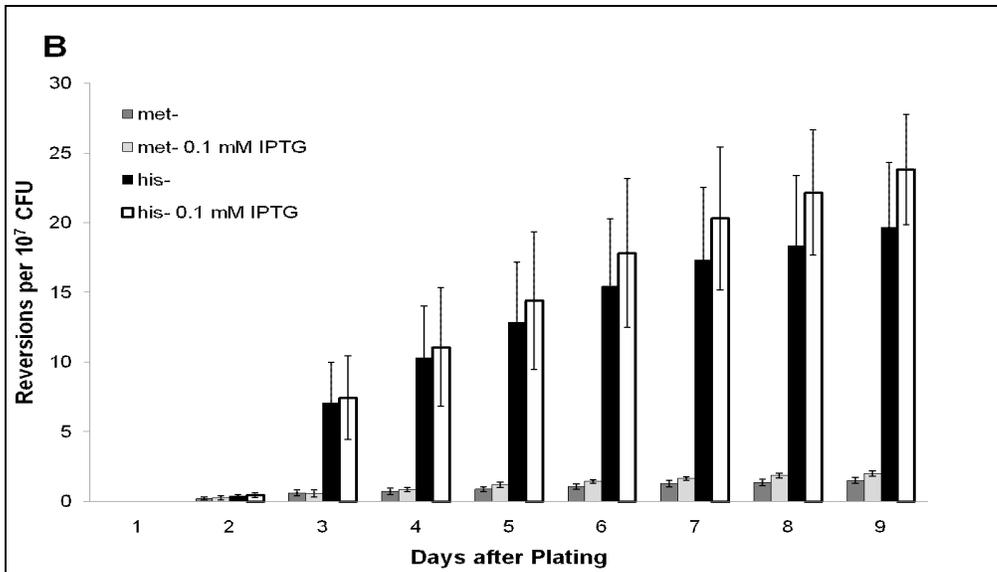


Figure 2.10. Accumulation of Met⁺ and His⁺ revertants during the stationary phase mutagenesis plate assay. Results represent levels of mutation in YB955 strains on selective media with and without inducer containing the *Phs-leuC427* inducible construct. Error bars represent the standard error.

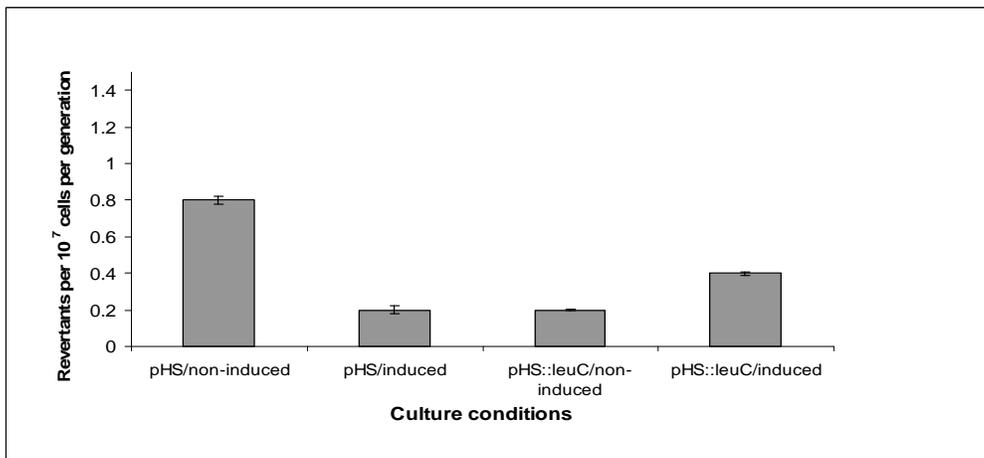


Figure 2.11. Fluctuation test for leucine biosynthesis revertions in strains containing the *Phs* promoter or the *Phs-leuC427* inducible construct in the presence and absence of IPTG.

pressures, such as methionine or tyrosine starvation. This was accomplished by transcriptionally fusing *leuC427* to the T-box and S-box riboswitches which progressively terminate transcription when increasing amounts of tyrosine or methionine, respectively, are exogenously added in culture conditions.

We examined the response in transcription to conditions of derepression and repression as affected by addition of tyrosine (strain HAM202) and methionine (HAM 104). RT-PCR of HAM102 *leuC* mRNA showed that transcription was increased 13-fold in derepressed conditions, whereas the same analysis for HAM104 indicated a 58-fold increase in transcription of the *leuC* gene from repressed to derepressed conditions (table 2.1). Stationary phase mutagenesis assays, shown in figure 2.12, in the strain that derepresses the *leuC427* in response to limiting amounts of tyrosine resulted in about six times (days 8 and 10 of incubation) as many revertants compared to conditions of transcriptional repression. Also, the strains that only contained the *ilv-leu* version of *leuC427* remained at background levels. The differences in the number of revertants were not due to the viability of the cells as seen in figure 2.13. The effect of transcriptional derepression was even more striking in the strain that responds to methionine limitation, there were approximately 60 times more revertants in the HAM204 when the *leuC* was transcribed the entire time the cells were starved for leucine compared to the number of revertants accumulated in the same strain when *leuC* transcription was repressed (as seen in figure 2.14). There were no differences in culture viability as seen in figure 2.15. To establish, if the increase in revertants seen was due to a general or specific effect, we also performed the traditional stationary phase mutagenesis on the strains, HAM101 and HAM102, for histidine prototrophy, and HAM203 and HAM204, for methionine

prototrophy. There were no differences between the strains or in the presence or absence of either tyrosine or methionine (figures 2.16 and 2.17).

We also determined the rate of spontaneous mutations for the methionine, histidine, and leucine markers under repressed and derepressed conditions for the strains, HAM101, HAM102, HAM203, and HAM204. The rates seen for all strains and conditions were similar (table 2.2).

Leucine revertant colonies from each strain were tested in order to assess their phenotype on minimal medium lacking leucine and in conditions of transcriptional repression. While the colonies from YB955 *amyE::Phs (ilv-leuC427)* revertants were opaque and robust within 48 hours, growth of close to 90% of colonies from YB955 *amyE::Phs-leuC427* on this medium was impaired, indicating that these colonies arose from adaptive reversion in the *Phs-leuC427* construct and were dependent upon the presence of IPTG (Table 2.3 and Table 2.4). Similar results were observed in the experiments that used riboswitches (data not shown). Finally, the Leu⁺ colonies were tested on minimal media, devoid of either methionine or histidine, for the presence of secondary mutations at the *metB5* and *hisC952* alleles, respectively. In the strain with S-box constructs, only histidine reversion was tested. Consistent with previously reported results, no additional Met⁺ or His⁺ phenotypes were observed (Sung and Yasbin, 2002 and Ross *et al*, 2006). Taken collectively, these results further support the hypothesis that transcription mediates a synergistic effect on mutagenic processes in cells under stress. Significantly, the results from the soft agar overlay experiments also demonstrate

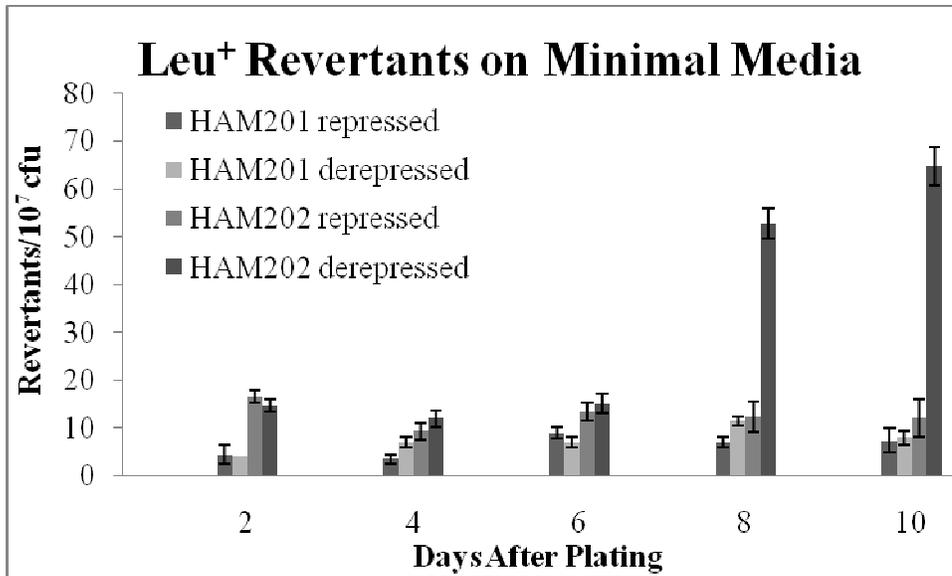


Figure 2.12. The accumulation of Leu⁺ revertants over time in HAM201 and HAM202 under repressed and derepressed conditions. Error bars represent the standard error.

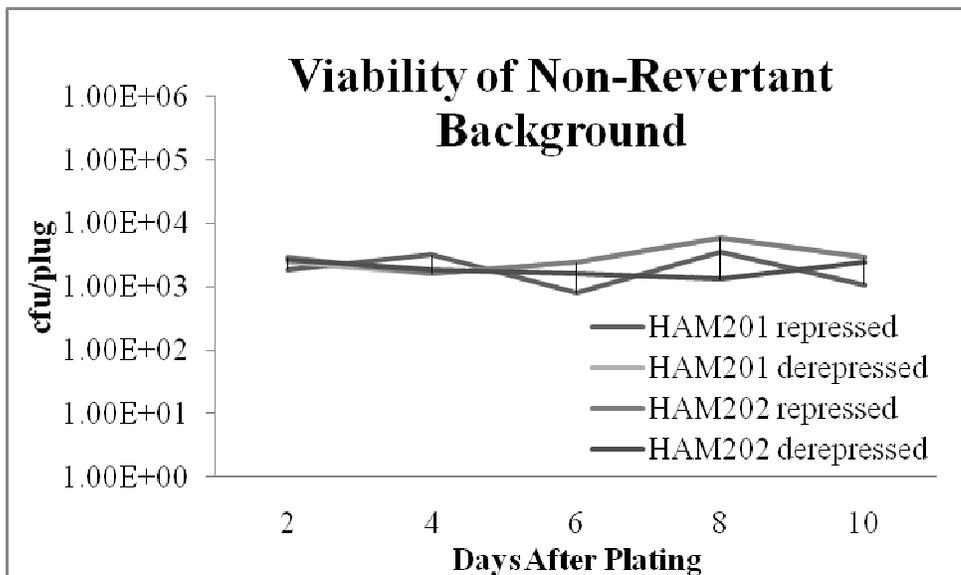


Figure 2.13. The viability of the non-revertant background of the cultures from figure 2.12. Error bars represent the standard error.

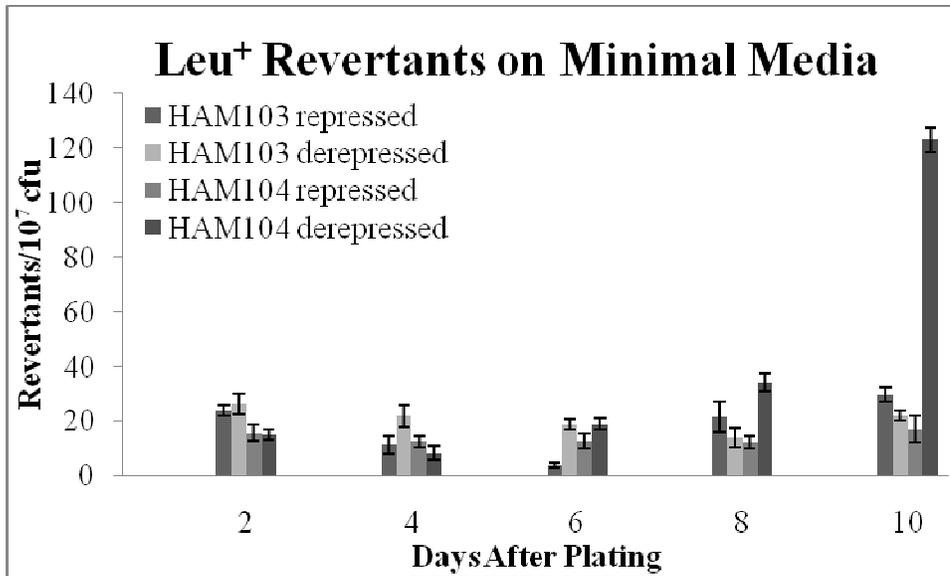


Figure 2.14. The acculumation of Leu⁺ revertants over time in HAM103 and HAM104 under repressed and derepressed condntions. Error bars represent the standard error.

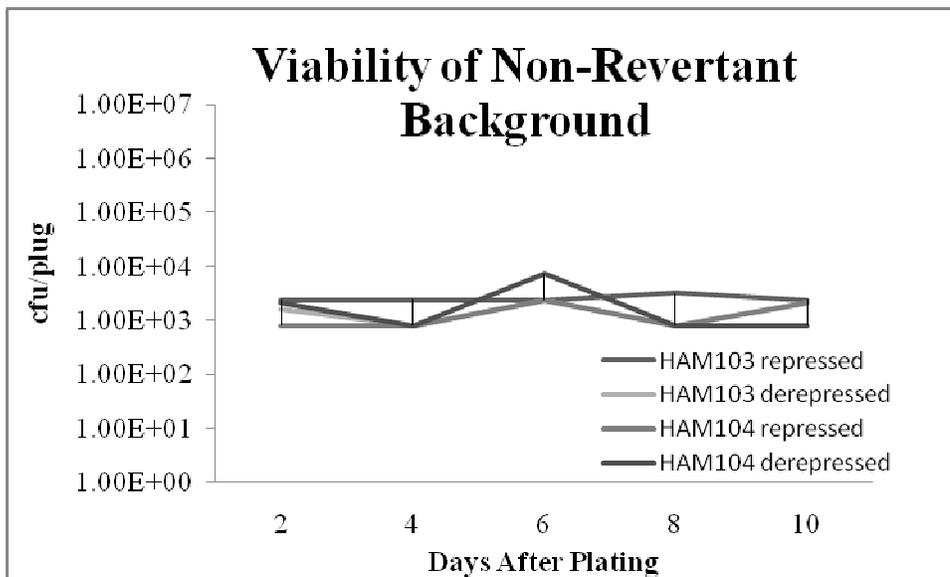


Figure 2.15. The viability of the non-revertant background of the cultures in figure 2.14. Error bars represent the standard error.

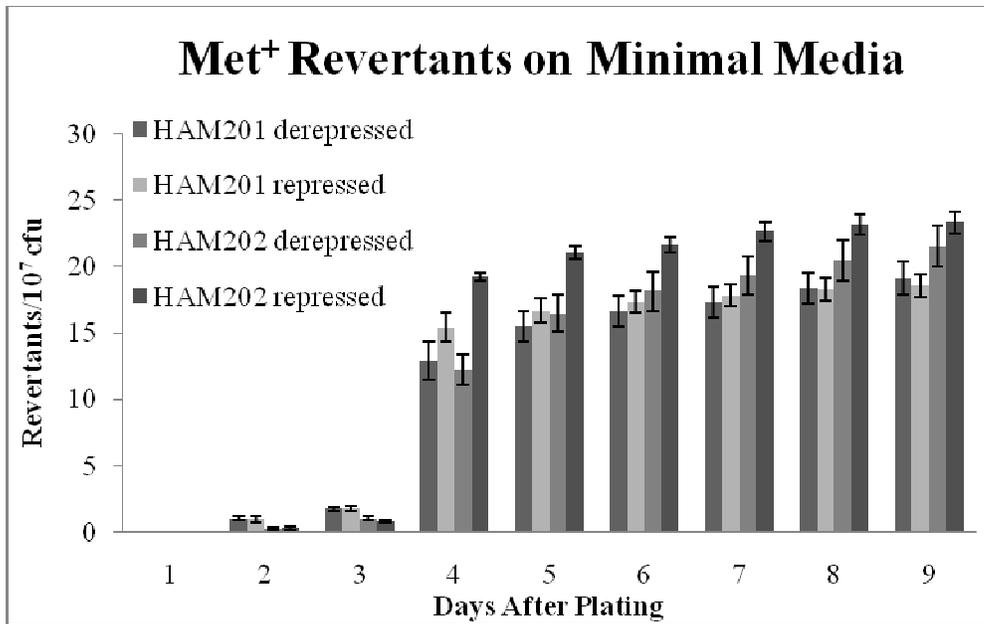


Figure 2.16. The accumulation of Met⁺ revertants over time in HAM201 and HAM202 in the presence or absence of tyrosine. Error bars represent the standard error.

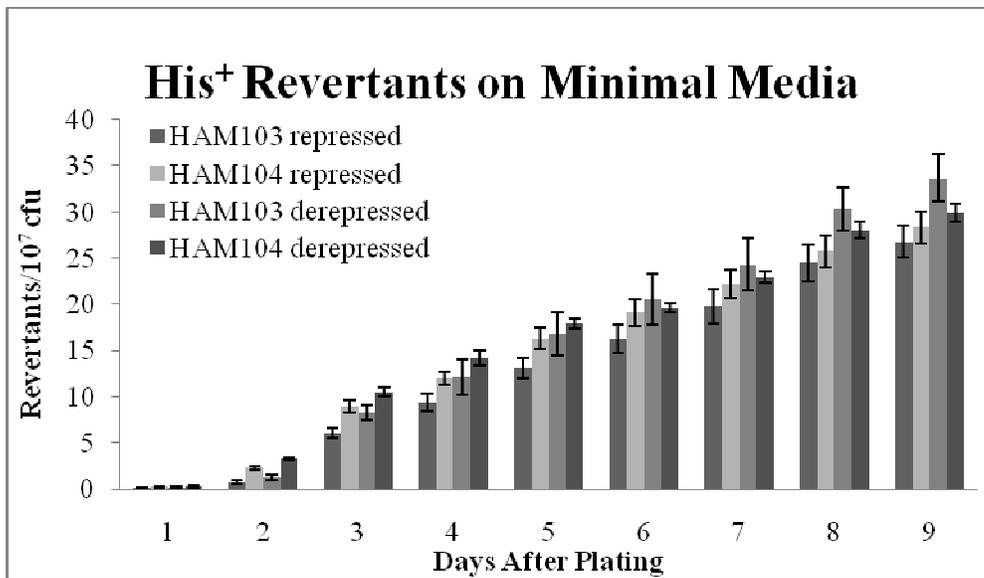


Figure 2.17. The accumulation of His⁺ revertants over time in HAM103 and HAM104 in the presence or absence of methionine. Error bars represent the standard error.

Table 2.2. The spontaneous His and Met reversion rates for HAM101, HAM102, HAM203, and HAM204. Error is representative of the 95% confidence value.

Revertant Allele	Strain	Condition	Rate	Error (\pm)
Histidine	HAM103	repressed	1.35E-09	1.12E-09
Histidine	HAM103	derepressed	4.47E-09	1.33E-09
Histidine	HAM104	repressed	1.73E-09	8.10E-10
Histidine	HAM104	derepressed	6.34E-10	5.90E-11
Leucine	HAM103	repressed	1.06E-09	9.80E-10
Leucine	HAM103	derepressed	5.68E-09	9.80E-10
Leucine	HAM104	repressed	1.75E-09	1.12E-09
Leucine	HAM104	derepressed	3.49E-09	1.12E-09
Histidine	HAM201	repressed	9.41E-10	9.80E-10
Histidine	HAM201	derepressed	3.61E-09	5.90E-11
Histidine	HAM202	repressed	1.21E-09	8.10E-10
Histidine	HAM202	derepressed	1.5E-09	8.10E-10
Leucine	HAM201	repressed	2.07E-10	7.10E-11
Leucine	HAM201	derepressed	1.39E-10	7.10E-11
Leucine	HAM202	repressed	3.03E-09	8.10E-10
Leucine	HAM202	derepressed	1.47E-09	9.80E-10

Table 2.3. Growth Phenotypes of YB955 *amyE::Phs* Leu⁺ Revertants
(from two experiments) on Minimal Media Lacking Leucine.

Day after initial selection	No. revertants with phenotype/No. revertants tested (% revertants with phenotype)			
	Revertants with opaque growth		Revertants with faint growth	
2 ML ⁻	8/8	(100)	0/8	(0)
2 ML ⁻ IPTG	15/15	(100)	0/15	(0)
3 ML ⁻	7/7	(100)	0/7	(0)
3 ML ⁻ IPTG	2/2	(100)	0/2	(0)
4 ML ⁻	7/7	(100)	0/7	(0)
4 ML ⁻ IPTG	9/9	(100)	0/9	(0)
5 ML ⁻	4/4	(100)	0/4	(0)
5 ML ⁻ IPTG	7/7	(100)	0/7	(0)
6 ML ⁻	5/5	(100)	0/5	(0)
6 ML ⁻ IPTG	1/1	(100)	0/1	(0)
7 ML ⁻	8/8	(100)	0/8	(0)
7 ML ⁻ IPTG	10/10	(100)	0/10	(0)
8 ML ⁻	10/10	(100)	0/10	(0)
8 ML ⁻ IPTG	6/6	(100)	0/6	(0)
9 ML ⁻	16/16	(100)	0/16	(0)
9 ML ⁻ IPTG	18/18	(100)	0/18	(0)

Table 2.4. Growth Phenotypes of YB955 *amyE::Phs-leuC427* Leu⁺ revertants
(from two experiments) on Minimal Media Lacking Leu.

Day after initial selection	No. revertants with phenotype/No. revertants tested (% revertants with phenotype)			
	Revertants with opaque growth		Revertants with faint growth	
2 ML ⁻	2/34	(5.9)	32/34	(94.1)
2 ML ⁻ IPTG	3/39	(7.7)	36/39	(92.3)
3 ML ⁻	6/26	(23.1)	20/26	(76.9)
3 ML ⁻ IPTG	6/32	(18.8)	26/32	(81.2)
4 ML ⁻	1/9	(11.1)	8/9	(88.9)
4 ML ⁻ IPTG	2/18	(11.1)	16/18	(88.9)
5 ML ⁻	6/29	(20.7)	23/29	(79.3)
5 ML ⁻ IPTG	3/16	(18.8)	13/16	(81.2)
6 ML ⁻	2/24	(8.3)	22/24	(91.7)
6 ML ⁻ IPTG	5/26	(19.2)	21/26	(80.8)
7 ML ⁻	1/21	(4.8)	20/21	(95.2)
7 ML ⁻ IPTG	1/32	(3.1)	31/32	(96.9)
8 ML ⁻	1/23	(4.3)	22/23	(95.6)
8 ML ⁻ IPTG	2/8	(25)	6/8	(75)
9 ML ⁻	3/24	(12.5)	21/24	(87.5)
9 ML ⁻ IPTG	1/16	(6.2)	15/16	(93.8)

that the mutations accumulate, following transcriptional derepression, without being coupled to growth. One other explanation for the differences in the accumulation of mutations observed between derepression and repressed conditions could be due to high expression of variant mutant enzymes with lower activities that allow wild-type growth. However, this is not the case in our system because our assay starved cells for two amino acids and the presence of an overexpressed less efficient enzyme that generates leucine for wild-type growth would not relieve cells from methionine or histidine limitation. Thus there is no selective advantage to overexpressing a “crippled” enzyme in our system. Previous experiments from our laboratory have not detected mutations that confer prototrophy to both amino acids. Lastly, examination of several leucine revertants showed that the vast majority of mutations are A to G changes that restore the wild-type glycine at position 143 (Sung and Yasbin, 2002) of the *leuC* gene.

One interesting set of experiments that would further test the notion that transcriptional derepression mediates the formation of mutations in stationary phase conditions would be to compare the accumulation of mutations of an unselected allele in conditions in which transcription is either activated or repressed. Our current system features different auxotrophic markers (Met^- and His^-) which facilitates these type of experiments because cells can be subject to biosynthesis of methionine or histidine selection while in the presence of leucine and either derepression or repression of transcription. Methionine or histidine revertants can then be screened for leucine biosynthesis or sequencing of *leuC427*. However, reversions at *leuC427* are acquired through rare gain-of-function mutations (roughly 1 in 10^7 cells) and screening for such events would require an impractical amount of effort. We are currently constructing an

inducible allele system, similar to that described by Petrosino *et al* (2009), in which loss-of-function mutations or activity at the single cell level are easily detected in combination with the methionine, histidine, and leucine auxotrophic markers.

These experiments clearly demonstrate an influence of transcription on the adaptive mutation phenomenon. The molecular mechanisms responsible for this influence still need to be elucidated. As previously mentioned, the formation of stem-loop structures by single stranded DNA exposed during transcription is one possibility (Wright, 2004). Higher levels of transcription would further expose bases prone to mutation to mutagenic conditions in the intracellular environment. Wright *et al.* tested this hypothesis *in silico* using an algorithm predicting the mutability of individual bases (Wright *et al*, 2002). They observed that highly mutable bases in the *p53* tumor suppressor gene were associated with the predicted degree of base exposure in stem-loop structures (SLS) associated with a high negative free energy of folding (Wright *et al*, 2002). Mechanistically, from observations in *E. coli* and yeast, the presence of lesions during transcription may result in pauses that may recruit unfaithful repair systems by interacting with error-prone DNA polymerases (Cohen *et al*, 2009, and Kim *et al*, 2007). It is also possible that lesions or secondary structures prompted by transcription or its pauses block replication, which is subsequently rescued by recombination functions (Kim *et al*, 2007, and Cohen and Walker, 2010).

Another transcription-associated process potentiating the generation of mutations is the ability of RNA polymerase to bypass lesions present in DNA, resulting in base misinsertion in the transcript. Spontaneous cellular processes such as deamination, oxidation and base loss generate uracil, 8-oxo-7,8-dihydroguanine (8-oxoG) and

apurinic/aprimidinic (AP) sites in DNA, respectively (reviewed in Saxowsky and Doetsch, 2006). Of these DNA insulting processes, those causing oxidative damage have been shown to be significant in *B. subtilis* cells in conditions of starvation (Vidales *et al* , 2009). The ability of RNA polymerase to bypass these and perhaps other types of lesions may result in base misinsertion in the transcript, generating mutant proteins resulting in the appearance of pseudo-prototrophs in non-dividing cells (Bridges, 1997, Holmquist, 2002, and Saxowsky and Doetsch, 2006). This process was shown to occur in non-dividing *E. coli* cells, where Bre`geon *et al* demonstrated that RNA polymerase can efficiently bypass uracil and 8-oxoG lesions engineered into a luciferase reporter gene (Bre`geon *et al*, 2003). Analysis of the resulting transcripts indicated that RNA polymerase has the ability to insert adenine opposite to uracil, and adenine or cytosine opposite to 8-oxoG (Bre`geon *et al*, 2003). Interestingly, lesion bypass during transcription has also been implicated in the generation of mutations in *p53* (Rodin *et al*, 2002). Further, recent *in vitro* studies have demonstrated that the human Mfd homolog, CSB, and other transcription elongation factors mediate transcriptional bypass in HeLa cells (Charlet-Berguerand *et al*, 2006). Hence, transcription-associated mutagenesis appears well conserved across the domains of life.

In the strain containing two *leuC427* loci, growth of the majority of leucine revertants was depressed in conditions of transcriptional activation which suggests that the effect of transcription on stress mutagenesis is *cis* acting (Table 2.3 and 2.4). In yeast cells, a similar *cis* acting effect of transcription on mutagenesis has been observed in assays with growing cells. It is proposed that transcription road blocks collapse replication forks that are subsequently reassembled by recombination functions and the

recruitment of error-prone DNA polymerases (Kim *et al*, 2007). Since recombination functions do not influence the generation of stress induced mutations in *B. subtilis* (Sung and Yasbin, 2002), the *cis* acting effect reported here is mediated by a yet-to-be uncovered mechanism. Future experiments studying transcriptional induction in combination with DNA repair systems and error-prone polymerases should further improve our understanding of the mechanisms producing genetic diversity in non-dividing cells.

In this study, we have demonstrated the association of transcription with mutation in an allele with transcriptional controls that are independent of selective pressure in stationary phase cells. Such an association could explain why adaptive mutations often appear directed and how cells in these conditions avoid the accumulation of lethal mutations resulting in genetic load, thus resolving this biological conundrum. Transcription exposes DNA strands to potentially mutagenic processes, and conditions influencing these processes are prevalent in non-dividing cells. Yet an even more thought-provoking concept, brought about by reports on transcription bypass, is the possibility that stationary cells engage in the testing of transient alleles for their potential to allow cells to resume growth. This potential could be subsequently locked into the cell's DNA by error-prone repair. In summary, transcription-associated adaptive mutation may provide an evolutionary strategy to escape from growth-limiting conditions by supporting the diversification of metabolic profiles, the utilization of xenobiotic compounds, and the development of antibiotic resistance (Han *et al*, 2008). In human cells, however, adaptive mutation in arrested cells could have potentially deleterious effects, where a cell-selfish strategy for growth results in the formation of cancer or the

onset of degenerative disease, as well as in resistance to certain cancer treatments (Holmquist, 2002, and Charlet-Berguerand *et al*, 2006). Since transcription is a process occurring in all living cells, including resting cells, transcription-associated mutagenesis may well be relevant to the evolutionary process throughout all the domains of life.

CHAPTER 3

CONCLUSION AND FUTURE DIRECTIONS

The aim of this study was to determine if transcription played a role in the generation of mutations during stationary phase. Through the use of physiological modification, an inducible promoter, and riboswitches, we modulated transcription of a defective *leuC* allele and tested for reversion to leucine prototrophy. As transcription was derepressed there was an increase in the accumulation of revertants. The evidence gathered is compelling and supports our hypothesis that increased transcription will lead to an increase in the accumulation of stationary phase mutagenesis.

Future research will address the mechanism of how transcription promotes mutagenesis. One line of future research may aim at elucidating which of the two proposed models, previously discussed, explains the influence of transcription on mutagenic processes in stationary phase. In summary, one model posits that transcription per se is mutagenic, while the other invokes the formation of a transient phenotype/protein and thus requires translation of mutated messages. Furthermore, these two models may be tested in several ways. In the presence of two identical alleles that are differentially expressed and if transcription per se is mutagenic, one would expect a cis effect in the generation of mutations. That is, the majority of mutations would take place at the allele that is transcribed the most. In contrast, mutations would be equally distributed to both alleles if the generation of mutations depends on the production of a transient phenotype. Alternatively, these models may be differentiated by generating inducible alleles that contain sequence targets for ribonucleases or by altering translational activity.

The stem-loop structure concept may be tested by using an inducible defective allele that contains a stable structure. The mutation site to be tested would be engineered in to the susceptible region of the structure, subject to transcriptional induction and assay for the accumulation of mutations. This construct would then be compared to a less-stable variant. Should stem-loop structures, created during transcription, mediate the formation of mutations, then those structures that are stable would accumulate more mutations than their less-stable variants. One other way to test this notion would be to alter supercoiling activity, as it has been postulated to influence the formation and stability of such structures.

It is also important to identify the molecular factors that mediate the formation of adaptive mutations. So, what other factors beside Mfd are involved in the generation of stress-induced mutations? This question may be addressed by genetically testing factors, previously postulated to be involved in this process in other models, for their influence in SPM in *B. subtilis*. Alternatively, this could be approached by conducting genetic searches in an Mfd⁻ background since it has been shown that leucine reversion mutagenesis is abolished by this factor (Ross et al, 2006). Gain-of-function mutations or loss-of-function mutations may then be probed for their effect on the accumulation of mutations under leucine starvation conditions. This could also be complemented with microarray/proteomic analyses of wt and Mfd-deficient backgrounds.

Research that would also contribute to understanding the mechanism behind the generation of diversity in conditions of stress is to uncover what DNA transactions are taking place in stationary phase conditions. A system based on detection of forward mutations (loss-of-function mutations), expected to be more frequent than point

mutations (our current system), combined with deficiencies in repair factors would be tremendously informative of DNA insulting agents that contribute to the generation of adaptive mutations.

Lastly, one interesting question that has only been barely addressed here is whether the whole population of cells or a subset of them engages in programs of genetic diversity in conditions of stress. This requires the use of cell separation techniques in combination with labeling cells that have entered conditions of stress. In conclusion, these future studies promise to provide a less fussy picture of the dynamic cellular functions that give rise to genetic variants in conditions of stress. More importantly, they will contribute to our understanding of novel ways in which stochastic evolutionary processes generate beneficial mutations.

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