INVESTIGATING THE SINGLE CELL HETEROGENEITY AND PHYSIOLOGICAL IMPACT OF MISTRANSLATION

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INVESTIGATING THE SINGLE CELL HETEROGENEITY AND PHYSIOLOGICAL IMPACT
OF MISTRANSLATION

A

DISSERTATION

Presented to the Faculty of
The University of Texas
MD Anderson Cancer Center UTHealth
Graduate School of Biomedical Sciences
in Partial Fulfillment
of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

By
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Houston, TX
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INVESTIGATING THE SINGLE CELL HETEROGENEITY AND PHYSIOLOGICAL IMPACT OF MISTRANSLATION

Christopher Ryan Evans, B.S., M.S.

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Bacterial populations grow clonal populations; however, individual cells have a variety of phenotypes. The physiological heterogeneity observed in populations has been attributed to variations in the processes of gene expression. For example, promoter expression has been shown to be heterogeneous within a population and contribute to increased stress tolerance in a subpopulation of cells. In comparison to transcription, the influence of translation on single cells is unclear. In this study, my collaborators and I have developed a dual-fluorescence reporter system that allows us to measure mistranslation rates in single cells in vivo. Using this reporter, we found that mistranslation rates are heterogeneous in bacterial populations. Additionally, our work has provided insights into the mechanisms that affect mistranslation rates in vivo, such as overall protein synthesis rates and the activity of release factor proteins.

The accuracy of protein synthesis has a significant effect on bacterial physiology. Severe increases in mistranslation result in the accumulation of misfolded proteins that can be detrimental and lethal to the cell. Despite the cost of errors during translation, the process of translation is error-prone in comparison to other processes of gene expression. Surprisingly, a number of benefits have been found as a result of mistranslation including increased oxidative stress and antibiotic tolerance. My research has revealed that the
heterogeneity of mistranslation in a population results in a subpopulation of cells that recover quickly from starvation. Additionally, in a population-based study, I have found that the mistranslation-induced heat shock response is not detrimental and, instead, protects cells from future lethal heat stress. Together, this study characterizes the heterogeneity of mistranslation in single cells for the first time and identifies the beneficial role mistranslation can have in single cell and population-based physiology.
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Chapter I

Introduction
Protein synthesis is a critical component of cellular life. As the final step in turning genetic information into functioning proteins, translation is a heavily regulated process with many functional components. Despite the clear importance of translation to the cell, the process of translation has flaws that result in the misincorporation of non-cognate amino acids into nascent peptides. This introduction provides a brief overview of translation, how errors in translation are generated, and the effect this has on the cell.

1.1 Errors during translation

During the elongation and termination phases of protein synthesis, the ribosome must successfully recognize the mRNA codon, select the cognate tRNA with a matching anticodon, and move to the next codon while staying in-frame. Once the ribosome encounters a stop codon, release factors signal the release of the nascent peptide and ribosome recycling. Additionally, aminoacyl-tRNA synthetases must correctly charge their target tRNA with the cognate amino acid. Errors during these processes result in the incorporation of non-cognate amino acids, frameshifting, or readthrough mistakes which usually result in the production of non-functional and potentially detrimental proteins (Figure 1-1).

1.1.1 Missense Errors

In order to be able to decode mRNA sequences into proteins, the ribosome has the unique responsibility to recognize and differentiate between dozens of aa-tRNA substrates. During elongation, the ribosome selects aminoacyl-tRNA (aa-tRNA) based on codon-
Figure 1. Mechanism of errors in translation. (A) During aminoacylation of tRNA substrates, the aa-tRNA synthetase can charge the cognate tRNA with the incorrect amino acid (pink spike). (B) During translation, the ribosome is capable of selecting the wrong tRNA during elongation, resulting in the incorporation of the wrong amino acid (pink circle) into a growing peptide. (C) During the termination of translation, the ribosome can misread the stop codon (red arrow) and use a near-cognate tRNA to continue elongation. This figure was created with BioRender.
anticodon pairing. In *E. coli*, this process is fairly accurate with an error rate of $3 \times 10^{-4}$(1). In comparison to transcriptional errors however, this is surprisingly high, as errors in transcription occur at every $4 \times 10^{-5}$ nucleotides(2). The selection of incorrect aa-tRNA by the ribosome is usually due to the promiscuity of selectivity at the third nucleotide position, or ‘wobble’ position, of the codon(3). Structural studies have shown that the ribosome directly monitors the structure of the first and second nucleotide pairs during codon-anticodon interaction(4). Conversely, the ribosome does not monitor base pairing at the third position as closely and will allow for the formation of non-cognate pairings.

Ribosomal accuracy can be directly targeted by antibiotics. Aminoglycoside antibiotics, such as kanamycin and streptomycin, bind directly to the ribosome and prevent proofreading activity during translation(5–8). This results in misincorporation of non-cognate amino acids and the formation of protein aggregates. Ultimately, treatment with these antibiotics results in cell death, although the mechanism by which cells die is up for debate. The detrimental effects of mistranslation will be discussed in further detail below.

Amino acids are charged onto their corresponding tRNA by aminoacyl-tRNA synthetases (aaRSs). aaRS activity occurs in two steps: 1) the ATP-dependent charging of an amino acid, then 2) ligation of the amino acid onto the 3’ end of the cognate tRNA. These enzymes function to charge a specific amino acid onto the range of tRNAs that correspond to that amino acid. Due to the range of potential amino acid and tRNA substrates and the importance of correct charging, aaRS editing can occur at the pre-transfer or post-transfer stage of tRNA charging. The mechanism of pre-transfer editing can vary between enzymes but results in the release of non-cognate or misactivated amino acids before attachment to the tRNA due to decreased affinity for the amino-acid-binding
pocket or active hydrolysis by the aaRS(9–12). Only around half of aaRSs have post-transfer editing activity(13). In these cases, the aaRS is able to deacetylate non-cognate aa-tRNAs via translocation to an editing domain of the aaRS – a mechanism called cis editing. Additionally, trans editing can occur where separate non-synthetase proteins hydrolyze misacylated tRNA. For example, the AlaXp family of proteins is a group of trans editing proteins that hydrolyze misacylated Ser-tRNAAla (14).

Environmental factors impact the ability of aaRS enzymes to accurately charge cognate tRNAs. In particular, specific aaRSs are sensitive to oxidative stress(15, 16). For example, E. coli threonyl-tRNA synthetase (ThrRS) has a cysteine residue that becomes oxidized in the presence of reactive oxygen species (ROS), which results in the production of Ser-tRNAThr(15). This phenomenon is not limited to bacterial aaRSs, as MetRS in eukaryotes is also sensitive to oxidative stress(16). In mammalian systems, MetRS becomes promiscuous under oxidative conditions and charges methionine onto a wide range of non-cognate tRNA substrates. The increased incorporation of Met into proteins at random sites due to mischarging results in increased cell survival under oxidative conditions(16).

1.1.2 Ribosomal Frameshifting

Frameshifting errors during translation occur when the ribosome shifts to the +1 or -1 position of the mRNA during elongation. This process is largely deleterious and results in premature termination of translation; however, frameshifting is rare. On average, the E. coli ribosome has been shown to undergo +1 frameshifting once per 30000 amino acids(17). This rate can be increased depending on the coding context. ‘Slippery’ mRNA sequences, such as ones containing CC[C/U]-[C/U], increase the frameshifting rate because the 0-frame
and +1 frame look identical to the ribosome(18). In comparison, programmed frameshifting, in which the ribosome must shift in order to complete protein synthesis, can reach up to 80% efficiency(19). Although examples of this mechanism of gene expression are limited, as recently as 2017 a new example of programmed frameshifting that produces the metallochaperone, CopA, was found(20, 21). In contrast to the genomic contexts that induce ribosomal frameshifting, environmental factors that impact frameshifting rates have not been heavily studied.

1.1.3 Stop Codon Readthrough

Translation termination is essential for the production of active proteins and recycling of the ribosome. When the ribosome encounters one of the stop codons (UAA, UAG, or UGA), release factor proteins facilitate ribosome termination and recycling. There are two classes of release factors: class 1 release factors recognize the stop codon and direct release of the nascent peptide, and class 2 release factors which promote recycling of class 1 factors. In bacteria, the class 1 release factors, Release Factor 1 (RF1) and Release Factor 2 (RF2), have overlapping activity where they are responsible for recognition of UAA/UAG or UAA/UGA stop codons, respectively. During termination, RF1 or RF2, which are structural mimics of an aa-tRNA, recognize the stop codon and enter the ribosomal A site. Then, catalyze release of the newly synthesized protein via a conserved GGQ motif(22, 23). After peptide release, the class 2 release factor, Release Factor 3 (RF3), uses GTP hydrolysis to catalyze the dissociation of RF1 or RF2 from the ribosome.

Readthrough of the stop codon occurs when the ribosome uses a near-cognate aa-tRNA during elongation over a stop codon. This is a biased process where a specific amino acid is used for readthrough of each stop codon. For example, during readthrough events of
the UGA stop codon, tryptophan is inserted into the nascent peptide(24). This is the result of decreased selectivity of the ribosome at the third nucleotide position of the codon. In the case of tryptophan, Trp-tRNA is mistakenly used by the ribosome due to its UGG anti-codon. Alternatively, glycine is inserted during UAG readthrough and either glycine or tyrosine is inserted during UAA readthrough(24).

The rate of readthrough is not identical between the three stop codons. Both UAA and UAG stop codons have very low error rates of ~0.4%(25). In contrast, UGA readthrough has been measured to have a readthrough rate of ~3%(26). The rate of readthrough can be influenced by environmental conditions like nutrient availability and antibiotic treatment. For example, increased availability or production of Trp-tRNA_Trp leads to increased UGA readthrough due to the competition between Trp-tRNA_Trp and RF2 for the ribosome at UGA codons(27). Additionally, treatment with ribosome targeting antibiotics, such as chloramphenicol, paromomycin, and erythromycin, have all been shown to increase stop codon readthrough(23, 28).

1.2 Physiological Effects of Mistranslation

Errors during protein synthesis usually result in the production of non-functional and potentially detrimental protein products. Individually, these products can negatively impact cell function and the accumulation of these products can be severely detrimental or deadly. Despite these effects, studies have also found that, in some cases, errors can benefit the cell.
The accumulation of mistranslated and misfolded proteins can be detrimental, or even lethal, in bacteria(29–33). This outcome of mistranslation has been used to develop antibiotics that decrease ribosome accuracy in order to control infections. Aminoglycoside antibiotics are bactericidal and directly target the ribosome(5). These antibiotics, such as streptomycin and kanamycin, induce cell death due to membrane damage by insertion of misfolded proteins and an increase in oxidative stress(32). The overproduction of alkyl hydroperoxide reductase subunit F, a hydrogen peroxide scavenger, has been shown to prevent the protein aggregation caused by aminoglycoside treatment, highlighting the role of oxidative damage in aminoglycoside-induced mistranslation(34).

Editing mechanisms of aa-tRNA synthetases have been shown to be important for normal bacterial growth. Mutations in the isoleucyl-tRNA synthetase, leucyl-tRNA synthetase, and phenylalanyl-tRNA synthetase editing domains have been shown to decrease growth in *E. coli*(29, 31, 33). In addition, oxidative stress has been shown to directly lead to mischarging by threonyl-tRNA synthetase by oxidizing a cysteine in the editing site, although the impact of this modification on the proteome is unclear(15, 35).

The detrimental effects of mistranslation are not limited to prokaryotes. Increased mistranslation has been shown to lead to mitochondrial dysfunction in yeast and apoptosis in mammalian cells(36, 37). At the organism level, mutations found in editing domains of aaRS genes result in cardiac, developmental, and neurological diseases(38, 39).
1.2.2 Positive Impact of Mistranslation

Despite the negative consequences of mistranslation, protein synthesis is not perfect. In fact, natural *E. coli* isolates were found to have a wide range of ribosomal accuracies *in vitro*[40]. Additionally, when given the time to adapt to laboratory conditions, these strains accumulated ribosomal mutations that altered ribosomal accuracy to match the accuracy of a laboratory strain[40]. *E. coli* strains have also been constructed that reach extremely high rates of mistranslation, but are able to still maintain growth, indicating that they are equipped to handle translational errors[41]. This evidence all suggests that there may be conditions where mistranslation may be advantageous.

The misincorporation of methionine has been shown to be advantageous in both human cells and bacteria. In response to oxidative stress, the methionyl-tRNA synthetase becomes highly promiscuous and methionylates a range of tRNAs[42, 43]. The increased methionine incorporation into the protein pool is protective to the cells, as methionine acts as an oxidative radical ‘sink.’ The authors of these findings argue that this mechanism of mistranslation should be labeled ‘adaptive translation’ (44). This is a conserved programmed cellular response to a stress where increasing the misincorporation of an amino acid results in increased cellular fitness.

Mistranslation can also increase cellular fitness by directly altering the function of proteins in the cellular proteome. In this case, mistranslation would act similarly to random mutagenesis where occasionally proteins would be created with a non-cognate amino acid that results in a gain-of-function. This has directly been shown in mycobacterium resistance to rifampicin[45]. Rifampicin binds RNA polymerase and inhibits RNA synthesis[46]. They showed that a strain with a high ribosomal error rate during translation had higher resistance
to rifampicin than a low error rate strain (45). Additionally, they showed that the RNA polymerase protein pool of the error-prone strain had higher activity in vitro compared to the low error rate strain, indicating that a heterologous protein pool is the most likely explanation for increased rifampicin resistance (45). To date, this is the only specific example of the heterologous target protein pool offering a concrete benefit to cells via transient gain of function.

Increases in mistranslation have been linked to increased DNA mutagenesis (47, 48). In one case, the expression of mutant glycine tRNA resulted in a constitutive mutator phenotype that was RecA dependent (48). In another, a mutation in the isoleucyl-tRNA synthetase increased mutation frequency due to an increase in error-prone DNA repair (47). Both of these cases are thought to be due to the statistical increase in non-functional DNA repair proteins due to misincorporation of non-cognate amino acids during translation. This increase in mutation rate could be beneficial in highly adaptive environments, providing another example of the potential for increases in mistranslation to be beneficial.

The pre-activation of stress responses by a non-lethal stress has been shown to be protective against future lethal stresses. For example, bacterial cells exposed to non-lethal heat shock survive lethal heat stress significantly better than naïve cells. Increases in mistranslation have been shown to activate the general stress response and production of heat shock proteins (41, 49, 50). Activation of the general stress response by mistranslation protects cells from hydrogen peroxide killing (51). A ribosomal point mutation that increases errors during translation results in the production of the general stress response sigma factor, RpoS, and downstream peroxide scavengers KatE and OsmC. Heat shock proteins have been shown to be increased in cells with increased mistranslation rates; however, a protective effect has not been directly shown (41, 50). In these cases, there must be a clear trade-off between the level of mistranslation and the future benefits to the cell. In order to
be protective, the mistranslation levels must be high enough to activate stress responses, but not high enough to overwhelm the cellular machinery.

1.3 Bacterial Heterogeneity

1.3.1 Heterogeneity of Gene Expression

Bacteria live in large populations of genetically identical groups. Interestingly, the individual cells within populations do not behave identically and can have unique quantitative and qualitative traits. The sources of these differences have been a topic of interest for ~20 years. Most importantly, in 2002 a landmark study found that gene expression is stochastic within bacterial cells(52). In this study, cells expressing two fluorescent proteins under control of identical promoters found that the total fluorescence levels and ratio in each cell of the two fluorescent proteins was heterogeneous(52). The mechanism of gene expression heterogeneity has primarily been focused on transcriptional heterogeneity. More detailed analysis of transcription in single cells has found that transcription occurs in bursts which contribute to differences between cells(53, 54). Despite the stochasticity of the process of transcription, not all genes are equally heterogeneous. In fact, in a study of the heterogeneity of every known E. coli promoters, some promoters are expressed more heterogeneously than others(55). Further, sets of promoters, such as stress response promoters, were more heterogeneous than others, such as promoters for housekeeping genes(55). This indicated that the heterogeneity of promoters could have a physiological impact and be selected upon during natural selection. In support of this idea, synthetically developed promoters de novo exhibit very little noise in expression(56).
authors argue that increased heterogeneity of natural genes is advantageous to populations and is actively selected for as a mechanism of increased regulation.

The study of heterogeneity of translation has lagged behind the studies of transcription. Transcriptional noise is very impactful and occurs upstream of translation, leading to difficulties in developing reliable control experiments where transcriptional noise is eliminated. However, attempts have been made to determine noise in translation. An early study used single fluorescent reporters of transcription and translation to quantitatively determine that translation initiation rates is heterogeneous in single cells and contribute to phenotypic heterogeneity in a population (57). This study was limited by the fact that their conclusions were the result of mathematically comparing populations expressing different reporters, so were unable to identify these traits in individual cells. Only recently has progress been made to visualize translation in single cells. A technique that involves tethering fluorescently labeled mRNA allowed for the visualization of translation in real time in mammalian cells (58). Like transcription, the authors found that the translation rate of each mRNA was heterogeneous and occurred in bursts (58).

1.3.2 Physiological Heterogeneity

The heterogeneity of gene expression has a dramatic impact on cell physiology. This observation has been labeled ‘phenotypic heterogeneity’ (59). These are physical characteristics of cells within a population that are not due to changes in their genome or changes in the environment of individual cells. The most well-established benefit of phenotypic heterogeneity is a ‘bet-hedging’ mechanism within a bacterial population (53, 59, 60). This model proposes that a sub-population of cells within a larger population sacrifice fitness at normal conditions in order to increase survival under stressful conditions. This has
been shown to be true in persister cell formation (61). Persister cells are a small subpopulation of cells within a larger population that are highly tolerant of antibiotics without acquiring any antibiotic resistance genetic mutations. They are characterized by their unusually slow growth under normal conditions and ability to fully recover after antibiotic treatment that wipes out the rest of the population. Interestingly, the rate of persister cell formation, and thus the heterogeneity of gene expression, within a population can be directly affected by nutrient availability (62).

In addition to the ‘bet-hedging’ benefit of phenotypic heterogeneity, cooperative subpopulations have also been identified. In these systems, the behavior of a subpopulation benefits the entire population. For example, *Bacillus subtilis* can secrete a protease capable of extracellular protein degradation, subtilisin E (63). Under nutrient starvation, only a subpopulation of cells actually express the subtilisin E gene, produce, and secrete the protease (63). However, because the degradation happens in the extracellular space, the resulting amino acids are available to the entire *B. subtilis* population.

The benefits of phenotypic heterogeneity extend beyond cultures grown in a laboratory setting. During infections, the behavior of subpopulations of cells has been shown to influence the outcome of infections. In *Salmonella enterica* gut infections, only a subpopulation of the infectious population expresses a type III secretion system (64). This system is designed to increase the invasion of the bacterium and trigger inflammation. The subpopulation of *Salmonella* that expresses the secretion system invades the epithelium while the type 3 secretion system-negative subpopulation stays in the gut and has increased proliferation based on the inflammation induced by the invasive group.
1.4 The Heat Shock Response

The accumulation of misfolded proteins is detrimental to the cell. The formation of misfolded proteins can result in membrane damage, a decrease in the levels of active proteins, protein aggregation, and cell death. In order to maintain the homeostasis of the protein pool, cellular proteases and chaperones unfold, refold, or degrade damaged proteins. In situations where there are increased levels of protein misfolding in the cell, the heat shock response is activated. This highly conserved response increases the pool of chaperones and proteases, as well as a host of other proteins, to prevent the damage caused by the accumulation of misfolded proteins (65).

In bacteria, the heat shock response is activated by an increase in an alternative sigma factor, $\sigma^{32}$ (RpoH) (66). As a sigma factor, RpoH is responsible for the transcriptional activation of genes involved in the heat shock response (65, 66). Due to the costly nature of the heat shock response, regulation of RpoH is multi-faceted. The increase in RpoH levels due to heat shock is regulated in primarily three ways: increased $rpoH$ mRNA, increased translation of RpoH, and decreased degradation of RpoH (67–69). The transcriptional regulation of RpoH is complicated by the fact that it is controlled by four promoter regions. However, under non-lethal heat shock conditions, such at 42°C, the transcription of $rpoH$ slightly increases (67, 70, 71). Under lethal heat shock conditions, such as 50°C, the transcription of $rpoH$ is increased via increased activity of one of its promoter regions, P3. This increase in transcription is driven by a different alternative sigma factor, $\sigma^E$, which responds to membrane damage (70).

The translational efficiency of producing RpoH is controlled by an mRNA secondary structure that occludes the ribosome binding site (68, 69). This hairpin structure forms upstream of the start codon and suppresses protein synthesis. Under heat conditions, the
hairpin melts and increases the production of RpoH(68). When this hairpin melts, the production of RpoH protein is increased 3.5-fold(68). Destabilization of the hairpin structure is sufficient to increase RpoH synthesis, as shown by single point mutations within the mRNA hairpin sequence(69). To date, heat is the only known factor responsible for the melting of the rpoH mRNA hairpin(72). There is evidence that the small RNA chaperone Hfq mediates RpoH activity in a post-transcriptional manner, although no direct mechanism has been elucidated(73).

Lastly, degradation of RpoH tightly regulates its level in the cell. Under normal growth conditions, the half-life of RpoH is ~1 minute(69). When exposed to heat, the half-life of decreases and RpoH becomes very unstable for a brief period, followed by stabilization of the protein(69). RpoH is degraded by an essential membrane-bound ATP-dependent protease, FtsH(74). The current model for RpoH degradation indicates that intracellular chaperones DnaJK and GroEL/ES transport RpoH to FtsH for degradation(74–76). The role of these chaperones in RpoH degradation presents an interesting dynamic between the state of protein folding in the cell and activation of the heat shock response. In this titration model, the ratio of chaperones to unfolded proteins determines the rate of RpoH degradation(69). When unfolded protein levels are low relative to chaperone levels, the chaperones are free to bind RpoH and deliver it to FtsH for degradation. However, when unfolded protein levels are high compared to chaperone levels, the chaperones are sequestered by the unfolded proteins and unable to bind to RpoH. This decreases RpoH degradation and results in an increase in RpoH protein levels in the cell. When RpoH is stabilized, it promotes transcription of heat shock response proteins, including DnaJK and GroEL. When chaperone levels are increased, their relative levels match that of unfolded proteins in the cell, and thus they are able to bind to RpoH and lead to its degradation(69).
This mechanism is responsible for the transient stabilization, then destabilization, of RpoH seen in cells under heat shock.

Induction of the heat shock response under non-heat conditions is not as clearly understood. Despite the role of heat in the increased translation efficiency of RpoH, the overexpression of a misfolded protein construct under normal growth conditions is sufficient to activate the heat shock response (77). In addition, an ribosomal-error-prone E. coli strain with a ribosomal mutation that decreases accuracy could only activate the heat shock response under oxidative conditions, but the heat-induced heat shock response was unaffected by the oxidative state of the cells (78). This indicates that the production of misfolded proteins is not sufficient to activate the heat shock response under normal temperature conditions. Additionally, this supports the idea that induction of the heat shock response in the absence of heat occurs through a different pathway, although the components and mechanisms of this pathway have not been described.

1.5 Gaps in knowledge and significance of research

Due to their stochastic nature and programmed variability between cells, gene expression is heterogeneous within bacterial populations. This has been quantitatively measured for transcription and translation initiation; however, other processes of gene expression have not been effectively measured. This is due to the difficulty of developing a reporter system capable of measuring heterogeneity of specific molecular mechanisms while controlling for other sources of heterogeneity. In Chapter 3, I have developed a dual-fluorescence reporter capable of measuring the accuracy of translation in vivo in single cells. With the development of this system, we can measure the heterogeneity of translation accuracy for the first time. We found that stop codon readthrough is heterogeneous in
bacterial populations. Additionally, we unexpectedly found that decreased protein synthesis levels in cells leads to increased stop codon readthrough – a previously unknown phenomenon.

Bacterial cells display a range of phenotypes within a population. The molecular mechanisms contributing to the phenotypic heterogeneity observed in cells have not been fully described. In Chapter 3, we used newly-developed stop codon readthrough reporter to measure how the heterogeneity of stop codon readthrough could influence phenotypic heterogeneity. We found that increased stop codon readthrough levels in single cells correlated with increased recovery rates from starvation. Currently, the mechanism connecting stop codon readthrough to recovery from starvation is still unknown.

Stop codon readthrough can occur due to changes in ribosomal accuracy, tRNA levels, and release factor activity. I hypothesized that environmental factors that affect these components of stop codon readthrough will affect the heterogeneity of stop codon readthrough and its impact on phenotypic heterogeneity. In Chapter 4, I discovered an environmental condition – low pH due to growth in excess glucose- 1) increased the rate of stop codon readthrough due to decreasing release factor activity, 2) increased the heterogeneity of stop codon readthrough in the population, and 3) eliminated the role of stop codon readthrough heterogeneity on the phenotypic heterogeneity of starvation recovery. This work provides new insights into the release factor function and how stop codon readthrough and heterogeneity can be affected in vivo.

Although the production of mistranslated proteins is generally considered detrimental to the cell, recent studies have demonstrated potential benefits to errors generated during translation. These benefits include increasing mutation rates, pre-activation of stress responses, and the generation of a heterogeneous protein pool containing gain-of-function
protein variants. In Chapter 5, I demonstrate that increasing mistranslation can induce protection from protein misfolding stress, including heat and aminoglycoside treatment. Using fluorescence microscopy and single cell tracking, I was able to determine that increased mistranslation rates in cells leads to faster clearance of protein aggregates.

The mechanism of activation of the heat shock response under normal growth temperature is unclear. The production of misfolded proteins alone can only activate the heat shock response under oxidative conditions, despite oxidative conditions having no effect on the heat-induced heat shock response (78). In Chapter 5, I show that the mistranslation-induced heat shock response is dependent on the general stress response sigma factor, RpoS. This is the first study demonstrating a role of RpoS in the induction of RpoH production. Additional studies will be needed to determine the molecular mechanism explaining how RpoS affects RpoH protein levels.
Chapter 2

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions

All the bacterial strains and main plasmids used in this study are listed in Table 2-1. All the oligos for making the mutant strains and plasmids are listed in Table 2-2. The mutant strains are derivatives of E. coli K-12 MG1655 (WT). To construct MG1655 prfB::3 3FLAG strains, a cassette (kan-ccdB) containing the toxin encoding gene ccdB under the control of araBAD promoter and a kanamycin resistance gene was amplified from template genomic DNA of CR201 strain (obtained from N. De Lay, UHealth), and introduced into the specific sites on the chromosome of the parental strain harboring plasmid pSIM6 by red recombinase-mediated gene replacement (Datta et al., 2006). Strains containing pSIM6 were induced for Red expression by growth at 42°C for 15, and electroporated with PCR fragments containing the kan-ccdB cassette. 1 mL LB was then added and cells were incubated at 32°C for 2 hr. The successful transformants were selected by kanamycin resistance. The kan-ccdB cassette was then replaced with respective DNA fragments. The positive clones were selected by growth on 0.5% arabinose LB plate, and verified by colony polymerase chain reaction (PCR). All mutant strains were verified by sequencing.

To construct plasmids pZS-Ptet-m-y, pZS-Ptet-m-TGA-y, pZS-Ptet-m-TAG-y, pZS-Ptet-m-y +1 fs, and pZS-Ptet-m-y -1 fs, the pZS-Ptet-29AA-y plasmid was generated first by
### Table 2-1. Strains and plasmids used in described studies.

<table>
<thead>
<tr>
<th>Strain or Plasmid</th>
<th>Source</th>
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<td><strong>Strains</strong></td>
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<tr>
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<td>Laboratory collection</td>
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<tr>
<td>rpsD*</td>
<td>Fan et al., 2015</td>
</tr>
<tr>
<td>BW25311 Δrmf</td>
<td>Baba et al., 2006</td>
</tr>
<tr>
<td>MG1655 ΔprfC</td>
<td>Fan et al., 2017</td>
</tr>
<tr>
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<td>Quan et al., 2015</td>
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<td>MG1655 Δ4 (ΔrrnGBAD)</td>
<td>Quan et al., 2015</td>
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<td>MG1655 Δ6 (ΔrrnGBADBHC)</td>
<td>Quan et al., 2015</td>
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<td>MG1655 prfB::3x FLAG</td>
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<td><strong>Plasmids</strong></td>
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</tr>
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<td>pZS-P_Tet-m-TAG-y</td>
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<td>pSIM6</td>
<td>Datta et al., 2006</td>
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ligating a 29 amino acid (LQTSAGEAAAKEAAAKEAAKEAAAKAAA) linker with plasmid pZS*11-yfp13 using In-Fusion HD Cloning according to manufacturer’s protocol. Fragments amplified from optimized mCherry gBlock by primer pZS-mCherry-29AA-IF paired with pZS-mCherry-29AA-IR, pZS-mCherryTGA-29AA-IR, pZS-mCherryTAA-29AA-IR, pZS-mCherryTAG-29AA-IR, pZS-mCherryTAAA-29AA-IR, and pZS-mCherryTA-29AA-IR, respectively, were ligated into the pZS- Ptet-29AA-Y plasmid through In-Fusion HD Cloning.

Unless otherwise noted, E. coli strains were grown in LB at 37°C. For fluorescence microscopy analysis, overnight cultures were diluted 1:1,000 and grown for 24 hr. 100 mg/ml Chl was added to immediately stop protein synthesis. After 4 hr incubation to allow full maturation of mCherry and YFP, samples were subjected to fluorescence microscopy analysis. The minimal medium contains 47.8 mM Na₂PO₄, 22.0 mM KH₂PO₄, 8.6 mM NaCl, 18.7 mM NH₄Cl, 4 mM MgSO₄, 0.2 mM CaCl₂, 40 mg/ml each of the 20 amino acids, and 0.4%–1% glucose or indicated sugar. For the preparation of low pH LB, concentrated HCl was added to LB and the pH measured with a pH probe. The LB + glucose media was prepared by adding filter-sterilized glucose to autoclaved LB.

**Measuring Cell Growth**

Overnight cultures were diluted 1:1000 in 100μl of LB or LB + glucose in a clear 96-well plate (Corning). Growth in a microplate reader (Synergy HT, BioTek) was set at 37°C shaking for 15 minutes, then stop for 5 minutes. The A600 absorbance of the culture was taken every 20 minutes.
Fluorescence Microscopy

Samples were placed on a 2 μL agarose (1.5%) phosphate buffer pad on 15-well Multitest Slides (MP Biomedicals, LLC.). Images were obtained on an Olympus IX81-ZDC inverted microscope using Slidebook imaging software. Fluorescent images were taken at 0.5 second exposure with 0 gain and exported as Tiff files.

Image Processing and Analysis

Background fluorescence was subtracted from each image using ImageJ Background Subtraction with a 50.0 pixel rolling ball radius. Single-cell fluorescence quantitation was completed with microbeTracker(79), a MATLAB-based software package. For microbeTracker analysis, inverted mCherry images were generated in ImageJ in place of phase contrast images. The parameter set used for microbeTracker analysis is ‘alg4ecoli’ which is included with the software. After completing the microbeTracker analysis of each image, individual cells were added, deleted, or modified using the ‘Add,’ ‘Remove,’ and ‘Modify’ functions within the microbeTracker client. Single-cell fluorescence data were exported from MATLAB using the command ‘>exportcells2xls’ and all further data analysis was completed in Microsoft Excel.

Time-lapse Microscopy

Overnight cultures were diluted 1:1,000 in LB and grown for 24 hours at 37°C. Cultures were placed on a 200 μL 1.5%agarose LB pad. Fluorescent images were taken at the initial time point for quantitation in microbeTracker. Cells were followed for 150 minutes
at room temperature with DIC images taken at regularly spaced intervals throughout the experiment. Image analysis and editing were performed using ImageJ.

**Error Rate Determination**

*E. coli* cultures were incubated at 37°C in a microplate reader (Synergy HT, BioTek) using 96-well black side plates (Corning). The signals of mCherry, YFP, and A600 were measured every 20 minutes with fluorescence spectrometry. To calculate translational error rates, the YFP/mCherry ratio of m-TGA-y, m-TAG-y, m-y +1 fs, and m-y -1 fs was normalized by the YFP/mCherry ratio of the control m-y reporter. Protein synthesis rates were calculated as described(80) with the following formula:

Protein synthesis rate; \( S = \frac{1}{\text{Absorbance}} \times \frac{d(\text{fluorescence})}{d(\text{time})} \).

To calculate translational error rates by western blotting, *E. coli* strains were grown in LB medium with and without addition of chloramphenicol at 37°C for 24 hours before 10 mL of culture was harvested. The cells were lysed by sonication, and whole protein samples were analyzed by western blotting with a primary antibody against mCherry. The signals were qualified by Image Lab (Bio-Rad), and the error rates were calculated as the percentage of mCherry-YFP fusion protein.

**Calculation of CV and Noise**

CV was calculated as the standard deviation (\( \sigma \)) divided by the mean (\( \mu \)) of the YFP/mCherry ratio of each cell in the same microscopic image frame. Noise was calculated as the ratio of the variance (\( \sigma^2 \)) over the square of the mean (\( \mu^2 \)).
RNA Isolation

To prepare total RNA for qRT-PCR, cells in LB medium with or without 10 μM IPTG were grown to mid-log phase (OD$_{600}$ ~0.6-0.8). 800 μL of culture was used for total RNA extraction using hot phenol, and residual chromosomal DNA was removed.

To isolate total RNA for acidic gel Northern blotting, overnight cultures were diluted 1: 1,000 in LB and grown for 14 hours at 37°C. 10 mL of cell culture was harvested at 4°C and resuspended with TRIzol reagent immediately. Cells were lysed by the beads beater with 0.1 mm glass beads (RPI). RNA sample was prepared according to manufacturer’s protocol, and was stored in 10mM sodium acetate buffer (pH 5.0) with 1 mM ethylenediaminetetraacetic acid (EDTA) at -80°C to preserve aminoacyl-tRNAs. Deacylated samples were obtained by incubating RNA in 200 mM Tris pH 9.0 at 37°C for 30 minutes.

qRT-PCR

1 μg of total DNA-free RNA was reverse transcribed using iScript cDNA Synthesis Kit (Bio-Rad) according to manufacturer’s protocol. cDNA was amplified with the corresponding primers (see Table S4). qPCR was performed using Bio-Rad CFX96 and SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) according to manufacturer’s suggestion. mreB transcript level was used for normalization. The ΔΔCt method was used to obtain the fold changes of target genes.
Acidic Gel Northern Blotting

Acidic Gel Northern Blotting was completed by Yongqiang Fan. Acid urea polyacrylamide gel electrophoresis was performed according to the procedures described in (81). Briefly, 12% Acid urea polyacrylamide gel was prepared freshly before use. 5 μg of RNA sample was loaded onto the gel, and subjected the electrophoresis with sodium acetate running buffer (100 mM sodium acetate pH 5.0, 1 mM EDTA) in cold room for 6 hours. Gel-separated RNA was transferred to GT membrane (Bio-Rad) by semi-dry electroblotting at 15 V for 40 minutes in 0.5x TBE. The membrane was cross-linked by UV and probed with 5’ end biotin labeled DNA oligonucleotide probes (see Table 2-2). Trp-tRNA$^{Trp}$, tRNA$^{Trp}$, Gly-tRNA$^{Gly}$ and tRNA$^{Gly}$ were detected by the Northern blotting, respectively, with SsrA as the loading control. Quantitation was performed with Image Lab (Bio-Rad).

Heat Killing Assay

MG1655 and rpsD$^+$ cultures were grown overnight from individual colonies then diluted 1:100 or 1:50 (ΔrpoS strains) in LB. The diluted cultures were grown for 3 hours at 30°C to mid-logarithmic phase. To test the effect of canavanine on heat killing, MG1655 cultures were incubated with 3 mg/mL canavanine for one hour. To control for the decrease in growth due to canavanine treatment, separate MG1655 cultures were treated with 0.5 μg/mL chloramphenicol. Then, 500 μL of the cultures were centrifuged at 4000 rpm for 8 minutes and washed with phosphate buffer. After another centrifugation, the cultures were resuspended in 500 μL of phosphate buffer. The OD600 of the cultures in phosphate buffer was determined by measuring the A600 absorbance via spectroscopy in a microplate reader (Synergy HT, BioTek) and normalized to OD = 0.5. After normalization, the cultures were further diluted to 1:5 (50 μL of culture into 250 μL of phosphate buffer) and placed in a 96-
well plate (Corning). Serial dilutions for time = 0 were taken by diluting 20 μL into 180 μL of phosphate buffer 4 times. 5 μL of each dilution were plated onto an LB agar plate. After the initial sample was taken, the 96-well plate was covered with an aluminum plate cover and placed into a 50°C water bath to float. New samples were taken for measurement every 30 minutes.

**Fluorescence Microscopy Aggregate Clearance Assay**

Overnight cultures were grown in LB with 100 μg/mL chloramphenicol. Then, they were diluted 1:100 in LB + chloramphenicol + 100 μM IPTG and grown for 3 hours at 30°C. Mid-logarithmic phase cultures were treated with 100 μg/mL streptomycin for incubated at 42°C for one hour. After treatment, cells were treated with 500 μg/mL spectinomycin to stop protein synthesis and incubated at 30°C. Immediately after spectinomycin treatment and 2 hours after treatment, aliquots of cells were placed on 1.5% agarose phosphate buffer pads. Fluorescence and DIC images were taken of cells as described above. The quantification of aggregate numbers in each cell was done manually in ImageJ.

This protocol was altered for tracking aggregate clearance in single cells. Overnight cultures were additionally grown in ampicillin if the cells contained the pZS-P_{Tet}-mCherry. Once the cultures reached mid-logarithmic phase, they were incubated at 42°C for thirty minutes. After treatment, cells were treated with 500 μg/mL spectinomycin and immediately transferred to a 200 μl agarose LB pad containing 500 μg/mL spectinomycin. Then fluorescence and DIC images were taken at time = 0 hours and time = 3 hours. The quantification of aggregate disappearance was done manually in ImageJ.
Cell Lysate Purification

Overnight cultures were diluted 1:100 or 1:50 ($\Delta rpoS$ strains) in LB and grown for 3 hours in LB at 30°C. 1 mL of the culture was treated with 100 μL of cold trichloroacetic acid, and then incubated on ice for at least 10 minutes. Samples were centrifuged at 13000 rpm for 10 minutes at 4°C. After discarding the supernatant, the pellet was washed in 500 μL of 80% cold acetone. The samples were centrifuged again and the supernatant discarded. The samples were dried in open air at room temperature for 30 minutes, then resuspended in 50 μL of 7M urea. To facilitate complete suspension of the pellet, the samples were incubated at 95°C while shaking. Finally, the samples were stored at -80°C.

The quantification of lysate protein concentration was determined via bicinchoninic acid assay (BCA assay) as per manufacturer directions (ThermoFisher Scientific). Briefly, 100 μL of Working Solution was added to 2.5 μL of the lysate sample or a protein standard in a 96-well plate. Then the samples were incubated at 37°C for 30 minutes. After incubating for 5 minutes at room temperature, the 562nm absorbance of the samples was measured with a spectrophotometer. The concentration of the lysate sample was determined by comparison to the protein standard curve.

Western blot analysis

Protein samples were mixed with 6x SDS protein loading buffer at 95°C. Equal amounts of protein were loaded into a 10% SDS-PAGE gel. Samples were run through the stacking gel for 10 minutes at 120V and then run to the bottom of the gel at 190V. The proteins were transferred to a nitrocellulose membrane with a semi-dry transfer apparatus at 15V for 30 minutes (1 gel) or 12V for 45 minutes (2 gels). Membranes were blocked with
5% milk in Tween-20 + Tris-bufferend saline solution (TBST) overnight at 4°C. Membranes were incubated with α-RpoH or α-RpoB (loading control, Santa Cruz) mouse antibodies at 1:1000 dilution in 3 mL of milk + TBST for one hour while rocking. After the primary incubation, the membrane was washed in ~40mL of TBST for 5-15 minutes three times. For the secondary incubation, the membranes were incubated with horse radish peroxidase conjugated goat-α-mouse antibody at 1:5000 dilution for one hour in 5 mL of TBST while rocking. After the secondary incubation, the membrane was washed in ~40mL of TBST for 5-15 minutes three times. The membrane was then incubated with Clarity™ Western ECL Substrate for 5 minutes (Bio-Rad). The signal imaged and quantified by Image Lab (Bio-Rad).
Chapter 3

UGA readthrough is heterogeneous and impacts single cell physiology

3.1 Introduction

Protein synthesis is a fundamental and essential process in all three domains of life. Accurate protein synthesis requires correct matching of amino acids and transfer RNAs (tRNAs) by aminoacyl-tRNA synthetases, proofreading of aminoacyl-tRNAs (aa-tRNAs) by trans-editing factors, precise decoding of mRNA codons by proper aa-tRNAs, and accurate translocation of mRNA by the ribosome(78, 82–85). High translational errors cause growth defects and cell death in bacteria(5, 31, 33), mitochondrial dysfunction in yeast(36), shortened lifespan in flies(86), and neurodegeneration and cardioproteinopathy in mammals(38, 87). Surprisingly, naturally isolated Escherichia coli strains display a wide range of ribosomal fidelity, suggesting that high translational errors may be favored under some natural habitats(40). Recent evidence suggests that increased translational errors paradoxically provides benefits to microorganisms under certain stress conditions(44, 51, 88, 89). For example, amino acid misincorporation in the β subunit of RNA polymerase increases resistance of mycobacteria to rifampicin(45), and translational errors improve bacterial tolerance to oxidative stress by activating the general stress response(51, 90). Interestingly, in such cases only subpopulations of genetically identical cells survive severe stresses(51, 91), suggesting that stress response activated by translational errors may be heterogeneous (noisy) in individual cells. However, the noise levels of translational errors have not been determined, and how such heterogeneity originates remains unknown.
Gene expression has been shown to be stochastic and noisy (53, 54, 92–97). The first experimental evidence came from pioneering work by Elowitz et al. (52) showing that transcription is intrinsically noisy. Later studies revealed that transcription is bursty and noncontinuous (53, 54, 98), and the promoter architecture regulates transcriptional noise (53). Noise in gene expression can be harmful by disrupting regulatory networks, but increasing evidence shows that such noise can also be beneficial by generating phenotypic heterogeneity that helps the population to quickly adapt to environmental changes through bet-hedging (53, 59, 60). Compared with transcriptional noise, translational noise is extremely poorly understood. Earlier studies used a single fluorescent reporter to determine the noise levels when translation initiation (57) or the codon context (98) is varied. It was suggested that in Bacillus subtilis, increasing the efficiency of translation initiation enhances translational noise (57). However, it remains a significant challenge to separate translational noise from transcriptional noise (99). Here, we have developed transcription-normalized dual-fluorescence reporters to quantitate the noise levels of stop codon readthrough and investigate the sources of such noise. Our work suggests that reduced translation promotes UGA readthrough by altering competition between the ternary complex (TC) and release factors and provides a model for the heterogeneity of UGA readthrough among single cells. We also show that increased UGA readthrough promotes growth of E. coli cells from stationary phase, indicating that UGA readthrough heterogeneity provides an advantage to the population during environmental shifts.
3.2 Results

Dual-Fluorescence Reporters to Quantitate Translational Errors

To separate the noise of translational errors from transcriptional noise, we have constructed a series of mCherry-YFP (red and yellow fluorescent proteins) fusion reporters that measure the errors of stop-codon readthrough and frameshifting (Figure 3-1). The mCherry and yfp genes are transcribed as a single mRNA and translated from the same start codon to yield a single polypeptide (Figure 3-1A), therefore minimizing the influence of the noise from transcription and translation initiation in single-cell analysis. With these reporters, 0.2%–3% error rates were detected in wild-type (WT) E. coli MG1655 grown in Luria-Bertani broth (LB) at 37°C using fluorescence spectrometry (Figure 3-1B). Such error rates were orders of magnitude higher than DNA mutational and transcriptional error rates(100, 101), suggesting that the observed errors directly result from translation. In support of this notion, a ribosomal ambiguity mutation (rpsD I199N or rpsD*), which affects the ribosomal de-coding center to reduce translational fidelity (51, 102), increased all four types of errors that we tested (Figure 1B).

To validate that the fluorescence of the reporters reflects the actual protein level, we used western blotting to detect and quantitate mCherry and mCherry-YFP fusion protein (Figure 3-1C). In WT cells expressing the m-TGA-y reporter, the mCherry-YFP fusion accounted for 2.5% of total mCherry proteins produced. This was in good agreement with the 2%UGA readthrough level determined by fluorescence spectrometry (Figure 3-1B) and consistent with previous reports(103, 104).
Figure 3-1: Dual-Fluorescent Reporters for Translational Errors. (A) Dual-fluorescence reporters that measure translational errors. *MCherry* (m) and *yfp* (y) genes are fused under the control of a constitutive promoter $P_{Ltet0-1}$. At the end of *mCherry* is a stop codon, a frameshifting (fs) codon, or no stop codon. Readthrough of the stop codon or frameshifting produces a single mCherry-YFP fusion protein and yields YFP signal. (B) The reporters on a low-copy-number plasmid were expressed for 24 hours in Luria-Bertani broth (LB) at 37°C in wild-type (MG1655) and ribosomal error-prone (*rpsD*) *E. coli* strains. Fluorescence was quantified by spectrometry on a plate reader. The error rate was calculated as the
YFP/mCherry ratio of the error reporter normalized by the YFP/mCherry ratio of the m-y control. (C) Western blotting showing that UGA readthrough of the m-TGA-y reporter. Anti-mCherry antibody was used to detect both mCherry-YFP fusion and mCherry. Data are represented as mean ± SD.
The fraction of mCherry-YFP in the rpsD* strain increased to 8%, which was again consistent with the UGA readthrough level determined by fluorescence (Figure 3-1B).

**UGA Readthrough Is Heterogeneous among Single Cells**

We next applied our dual-fluorescence reporters to determine the heterogeneity of stop codon readthrough among genetically identical single cells grown under the same condition. Using fluorescence microscopy, we visualized mCherry and YFP signals in single cells (Figure 3-2). Whereas the YFP signal of the m-TGA-y reporter was substantially higher than background, cells without reporters showed no detectable fluorescence signal, suggesting that the influence of auto fluorescence on signal quantification is negligible under our experimental setting. Our results showed that the control m-y reporter exhibited tight linear correlation between the YFP and mCherry signals, and the YFP/mCherry ratio was mostly homogeneous (Figures 3-2A and C). In contrast, the YFP/mCherry ratio of the m-TGA-y reporter (indicating UGA readthrough level) was more dispersed (Figures 3-2B and D), suggesting that the concentration of UGA readthrough products was heterogeneous among single cells.

Heterogeneity of gene expression is quantified with coefficient of variation (CV), calculated as the ratio of the SD (s) over the mean (μ), or noise (σ)(52, 96, 98). Our results revealed that the CV of YFP/mCherry ratio in cells with the control m-y reporter was ~0.1 (Figure 3-2E). Such a low level of heterogeneity may be caused by noise from fluorophore maturation, partial degradation of mRNA and protein, ribosomal drop-off during translation, missense errors, and the imaging system. The CV of YFP/mCherry ratio in cells with the m-TGA-y reporter increased to 0.2, corresponding to a 4-fold increase in noise (Figures 3-2E and F).
Figure 3-2: UGA Readthrough is Heterogeneous among Single Cells. (A and B) YFP and mCherry fluorescence in MG1655 cells carrying either the m-y (A) or m-TGA-y (B)
Cells were grown in LB for 24 hours. The YFP/mCherry ratio is the relative YFP signal normalized by the mCherry signal. Cells with high and low UGA readthrough are indicated by yellow and red arrows, respectively. (C and D) In the scatterplots of m-y (C) and m-TGA-y (D), each dot represents a single cell. The fluorescence is background-subtracted and arbitrary units are shown. (E and F) Heterogeneity of UGA readthrough among single cells is indicated by CV (E) and noise (F) of the YFP/mCherry ratio. μ, the mean of the YFP/mCherry ratio; σ, SD. Data are represented as mean ± SD. AU, arbitrary units.
It has been suggested that when the copy number of protein molecules is low (<1,000/cell), partitioning errors and finite-number effect would contribute to the overall protein noise, whereby reducing the fluorescence mean would increase the CV and noise (93, 105). However, the estimated concentration of mCherry-YFP fusion protein in cells with the m-TGA-y reporter is ~6,000 molecules per cell. Therefore, partitioning noise and finite-number effect is expected to have little contribution to the observed CV of the YFP/mCherry ratio. In support of this argument, we found that reducing the mean of mCherry by decreasing the promoter strength of the m-TGA-y reporter did not further increase the CV (data not shown). We thus reason that the higher CV and noise of the YFP/mCherry ratio in m-TGA-y compared to m-y is largely due to the heterogeneity of UGA readthrough events among individual cells.

**Reduced Protein Synthesis Increases UGA Readthrough**

To understand how the heterogeneity of UGA readthrough arises, we analyzed the correlation between UGA readthrough (indicated by the YFP/mCherry ratio) and various cell parameters (Figure 3-3). We found that cells with lower mCherry expression levels exhibited higher UGA readthrough (Figure 3-3A and B). Treating *E. coli* with low concentrations of chloramphenicol (Chl), which binds to the peptidyl transferase center (PTC) of the ribosome and impedes binding of aa-tRNAs and release factors (106), reduced protein synthesis and increased UGA readthrough, as determined by fluorescence spectrometry and western blotting (Figures 3-3C and D). To determine whether increased UGA readthrough is a specific effect of Chl, we further tested other ribosomal inhibitors tetracycline (Tet, inhibiting aa-tRNA delivery to the ribosome), spectinomycin (Spc, inhibiting translocation), and erythromycin (Ery, blocking the 50S exit tunnel). Tet, Spc, and Ery also significantly enhanced UGA readthrough as Chl (data not shown).
Figure 3-3: Reducing Protein Synthesis Increases the Level of UGA Readthrough. (A) In single MG1655 cells, higher UGA readthrough correlates (Spearman’s rank correlation) with lower mCherry protein level. The mCherry Intensity is the mCherry signal normalized by cell volume with arbitrary units. Relative UGA readthrough is calculated from the YFP/mCherry ratio. Experimental conditions were the same as in Figure 3-2. (B) The y axis (R_{10\%}) is the ratio of the average UGA readthrough in the bottom 10% of cells divided by the average UGA readthrough in the top 10% of cells ranked by mCherry intensity from (A). (C) MG1655 cells were grown in LB with and without Chl for 24 hours, and UGA readthrough and protein synthesis rates were determined by fluorescence spectroscopy. Treating
MG1655 with low doses of Chl decreases protein synthesis rate and enhances UGA readthrough. (D) Western blotting confirms that Chl increases UGA readthrough of the m-TGA-y reporter. The analysis of mCherry-YFP levels by western blotting was performed by Yongqiang Fan. (E) Chl treatment decreases the CV of UGA readthrough. Data are represented as mean ± SD. AU, arbitrary units.
The next question is how reduced translation may increase UGA readthrough errors, which is surprising given previous kinetic studies showing that there is a trade-off between translational speed and accuracy (107). Using acidic gel northern blotting, we show that the overall Trp-tRNA_{Trp} level increases in the presence of Chl due to an increase in total tRNA_{Trp} (Figure 3-4A). Further time course analysis shows that tRNA_{Trp} is stable both in the presence and absence of Chl (Figure 3-4B), indicating that the increased level of tRNA_{Trp} with Chl is not caused by increased tRNA stability, but rather by enhanced transcription. This results indicate that reduced translation increases the effective concentration of EF-Tu:Trp-tRNA_{Trp}:GTP complex to more efficiently compete against RF2, thereby promoting UGA readthrough.

A recent study shows that Chl and Tet decrease the active fraction of ribosomes (Dai et al., 2016). To test the effects of active ribosomes on UGA readthrough, we used mutant strains lacking several copies of the rrn operon that encodes ribosomal RNAs (108). Deleting six out of seven copies of the rrn operon significantly increased UGA readthrough (Figure 3-5A), suggesting that reducing the cellular ribosome concentration promotes UGA readthrough. We also tested a strain that lacks RMF (ribosome modulation factor), which promotes 70S ribosomes to form inactive dimers during stationary phase and stress conditions (109, 110). Deleting rmf is expected to increase the concentrations of active ribosomes. Indeed, deleting rmf mitigates the effect of Chl to enhance UGA readthrough (Figure 3-5B), supporting that Chl increases UGA readthrough by reducing the fraction of active ribosomes.

Next, we investigated how reduced translation affects the heterogeneity of UGA readthrough. Growth in Chl decreases the CV of YFP/mCherry ratio (Figures 3-3I), suggesting that UGA readthrough heterogeneity is reduced when translation is attenuated across the population.
Figure 3-4: Chloramphenicol Increases Trp-tRNA<sup>Trp</sup> Level. (A) Chl treatment increases the level of Trp-tRNA<sup>Trp</sup> shown by acidic northern blotting. Total RNA was isolated from MG1655 cells grown in LB with or without 2 μg/mL Chl under acidic conditions and treated with or without alkaline (OH<sup>-</sup>) before acid gel electrophoresis and northern blotting. Alkaline treatment causes deacylation of aminoacyl-tRNAs. Almost 100% of tRNA<sup>Trp</sup> was aminoacylated without alkaline treatment. SsrA was used as an internal standard to calculate the relative concentration of Trp-tRNA<sup>Trp</sup>. (B) Stability of tRNA<sup>Trp</sup> with and without
Chl. MG1655 cells grown in LB in the presence of absence of Chl were treated with a high concentration of rifampicin to stop transcription. RNA samples were prepared at indicated time points following addition of Rif and subjected to northern blotting analysis. Chl increases the overall level of tRNA$_{Trp}$, but not the stability. (C) The level of Gly-tRNA$_{Gly}$ was determined using acidic gel northern blotting as in (A). (D) The protein levels of EF-Tu and FLAG-RF2 with and without Chl revealed by western blotting. A FLAG tag is fused to the C terminus of RF2 at the native chromosomal site. Data are represented as mean ± SD. This analysis of tRNA charging and levels was performed by Yongqiang Fan.
Figure 3-5: Ribosomal Availability impacts the Rate of UGA Readthrough. (A)
Reducing ribosome copy number increases UGA readthrough. WT MG1655 and its ribosomal operon deletion mutants (Δ2, ΔrrnEG; Δ4, ΔrrnGBAD; and Δ6, ΔrrnGADBHC) were grown in LB with and without Chl for 24 hours, and UGA readthrough levels were determined with fluorescence spectroscopy. (B) Deleting *rmf* partially suppresses the effect of Chl to increase UGA readthrough. Data is represented as mean ± SD.
Collectively, these results suggest that reduced translation enhances the level of UGA readthrough, and various levels of protein synthesis among single cells contribute to heterogeneous UGA readthrough in a bacterial population.

**Defective Translation Termination Increases UGA Readthrough Heterogeneity**

We next tested how RF2 fluctuations affect UGA readthrough. In *E. coli*, the coding region of the *prfB* gene (encoding RF2) contains a TGAC frameshifting codon, which autoregulates the production of RF2 (111, 112). A high level of RF2 decreases the frameshifting efficiency at the TGAC site by promoting early termination, therefore reducing RF2 translation. To test how RF2 autoregulates UGA readthrough heterogeneity, we used a genome engineering tool (113) to change the chromosomal site of TGAC in *prfB* to TAGC. RF2 does not terminate translation at UAG codons. Therefore, the TAGC mutation in *prfB* is expected to abolish autoregulation of RF2 production and increase fluctuations of RF2 protein levels among single cells. Over expressing RF2 in WT cells did not decrease the level of UGA readthrough (Figures 3-6A and B). However, when *prfC* (encoding RF3 that facilitates RF2 during termination) was deleted, overexpressing RF2 decreased the level of UGA readthrough, suggesting that the RF2 activity was no longer saturating in the absence of RF3. Consequently, deleting *prfC* increased both the level and heterogeneity of UGA readthrough (Figures 3-6C and D). These results suggest that RF2 fluctuations do not significantly contribute to UGA readthrough heterogeneity in WT *E. coli* cells, but defects in translation termination enhance the sensitivity of UGA readthrough to RF2 fluctuations among single cells, thereby increasing readthrough heterogeneity.

**UGA Readthrough Promotes Cell Growth from Stationary Phase**

Altering translational fidelity results in various phenotypic changes (44, 114).
Figure 3-6: Defective Termination Increases UGA Readthrough Heterogeneity. (A) ASKA-prfB (Kitagama 2005) leads to overproduction of prfB mRNA as determined with quantitative reverse transcriptase PCR. In the absence of isopropyl β-D-1-thiogalactopyranoside (IPTG), the promoter of the ASKA plasmid is leaky (Kitagama 2005). (B) The m-TGA-y reporter was tested in strains with and without the RF2 overexpression plasmid. In MG1655, increasing the RF2 level does not further decrease UGA readthrough, suggesting that the RF2 activity is close to saturation. In contrast, excess levels of RF2 decrease UGA readthrough in the RF3 (encoded by prfC) deleted strain, indicating increased sensitivity of UGA readthrough to RF2 fluctuations when the release factor is compromised. The ΔprfC result also suggests the RF2 protein is successfully overproduced from the plasmid. (C) Deleting RF3 and introducing the rpsD* mutation both increases UGA readthrough of the m-TGA-y reporter. (D) RF3 deletion increases the CV of UGA.
readthrough. In contrast, the rpsD* mutation decreases the CV. In addition to recycling release factors, RF3 also maintains fidelity during translation elongation\(^{(85)}\). The rpsD* result suggests that increased UGA readthrough heterogeneity upon RF3 deletion is caused by defective termination rather than reduced fidelity during elongation. Data are represented as mean ± SD.
To provide insights into how UGA readthrough heterogeneity affects readthrough in bacterial physiology, we used time-lapse fluorescence microscopy to monitor division of individual cells with various levels of UGA readthrough. Stationary-phase WT cells carrying the m-TGA-y reporter were tested on agar pad with minimal glucose medium in the chamber of an automated fluorescence microscope. We found that cells with high levels of UGA readthrough required a shorter time to reach the first division than cells with low UGA readthrough levels (Figure 3-7A). To test whether increased UGA readthrough is sufficient to cause faster growth from stationary phase, we expressed the WT and suppressor tRNA^{Ser} in MG1655. The suppressor tRNA indeed improved regrowth of stationary-phase cells in minimal media with various carbon source (Figures 3-7B and C). However, growth in rich media LB was not affected by the suppressor tRNA (data not shown), suggesting that the growth advantage provided by UGA readthrough is manifested under poor-nutrient conditions. Collectively, these results suggest that heterogeneous UGA readthrough allows a subpopulation of cells to grow faster from the stationary phase, thereby improving the overall fitness of the bacterial population.
Figure 3-7: UGA Readthrough Promotes Cell Growth from Stationary Phase. (A)

Percentage of cells with high and low error levels that require different time periods to reach the first cell division. Stationary-phase MG1655 cells were placed on M9 glucose agar pad and monitored for fluorescence and growth. High and low error cells are the top and bottom quartiles of individual cells ranked by the YFP/mCherry ratio of the m-TGA-y reporter, respectively. A significantly higher percentage of cells with high GUA readthrough levels divide within 120 minutes compared with cells with low UGA readthrough levels. (B) Stationary-phase cultures of MG1655 carrying the WT or UGA suppressor tRNA<sup>Ser</sup> were diluted 50-fold, and growth was monitored over time. (C) UGA suppressor tRNA<sup>Ser</sup> improves growth in minimal media with various carbon sources (1% each), but not in LB. Data are represented as mean ± SD.
3.3 Discussion

Noise in gene expression has been extensively studied at the transcriptional level, but the levels and sources of noise during translation are poorly understood. Here, we have developed a dual-fluorescence reporter system to determine the noise of stop codon readthrough with minimal influence from noise produced during transcription, translation initiation, and protein degradation. Such reporters will be broadly useful to determine the levels and heterogeneity of translational errors in single cells in their native environments, e.g., within biofilms and during host-microbe interactions. We have further provided in-depth analyses of the regulation of UGA readthrough noise. The sources of gene expression noise include intrinsic sources that result from stochastic transcription and translation of individual genes and extrinsic sources caused by fluctuations of global resources among single cells (52, 59, 98). The heterogeneity of UGA readthrough over long periods of growth under our experimental conditions is likely dominated by extrinsic sources of noise, such as fluctuations of the concentrations of nutrients and translational components among individual cells. For example, amino acid levels may reduce or deplete in some cells after 24 hr of growth in LB. UGA readthrough results from occasional recognition of the stop codon by the EF-Tu:Trp-tRNA^{Trp}:GTP ternary complex, which competes with RF2 that releases the elongating peptide (Figure 3-8). Fluctuations of TC and RF2 among single cells would lead to heterogeneity of UGA readthrough and increase its noise. We have shown that reduced translation increases the level of UGA readthrough and Trp-tRNA^{Trp} (Figures 3-3 and 3-4). Because tRNA^{Trp} is stable (Figure 3-4B), the increase in tRNA^{Trp} in the presence of Chl is likely due to enhanced transcription. In addition to tRNA^{Trp}, the level of tRNA^{Gly} is also increased by Chl (Figure 3-4C). It is possible that some unknown protein factor is involved in repressing the tRNA promoters, and Chl reduces synthesis of the repressor to upregulate tRNA transcription.
Figure 3-8: Model for UGA Readthrough Heterogeneity.  (A) The near-cognate Trp-tRNA\textsuperscript{Trp} forms a ternary complex (TC) with EF-Tu and GTP to compete with RF2 for the UGA stop codon. Recognition of UGA by RF2 leads to release of the growing peptide from the ribosome, whereas Trp-tRNA\textsuperscript{Trp} suppresses UGA by adding Trp to the growing peptide. The UGA readthrough level in a single cell is determined by the effective concentrations of TC and RF2. (B) The concentration of active ribosomes may vary among single cells due to fluctuations of global resources and ribosome maturation/inactivation. A low concentration of active ribosomes decreases global protein synthesis, which increases the effective concentration of TC due to its reduced usage during translation. (C) UGa readthrough is sensitive to TC fluctuations. An increase in effective TC leads to enhanced UGA
readthrough. (D) WT cells with effective translation termination exhibit nearly saturated RF2 activity, and UGA readthrough is insensitive to RF2 fluctuations. Defects in RF2 activity, e.g., due to RF3 deletion, enhance the sensitivity of UGA readthrough to RF2 fluctuations and thus increase UGA readthrough heterogeneity. Dashed lines indicate arbitrary ranges of concentrations. AU, arbitrary units.
We also show that the protein level of EF-Tu is not significantly affected by Chl (Figure 3-4D). Given that EF-Tu is very abundant and in 6-fold excess relative to the ribosome(115), an increase in Trp-tRNA_{Trp} is expected to drive the formation of TC. Another factor that may affect UGA readthrough is the RF2 protein level. We show that the FLAG-tagged RF2 level increases in the presence of Chl (Figure 3-4D), which is not expected to enhance UGA readthrough. Our data thus suggest that reduced protein synthesis promotes UGA readthrough by increasing the effective concentration of TC.

Recently, Dai et al.(115) have shown that some ribosome-targeting antibiotics, including Chl and Tet, reduce the active fraction of ribosomes rather than decreasing translation elongation rates). Our results suggest that reducing the concentration of active ribosomes promotes UGA readthrough (Figures 3-3 and 3-5). Several lines of evidence indicate that protein synthesis is intrinsically heterogeneous: (1) the mCherry protein level is highly heterogeneous among single cells even with the chromosomal reporters, indicating that overall protein production is heterogeneous; (2) the components of the protein synthesis machinery, including ribosomal proteins and elongation factors, vary among single cells at the protein level in E. coli(96); (3) translation initiation has been shown to be noisy in bacteria(57); and (4) ribosome hibernation alters gene expression heterogeneity(116). Collectively, such evidence suggests that the concentration of active ribosomes may vary among genetically identical single cells due to heterogeneity during expression of ribosomal proteins and rRNAs, ribosome maturation, and inactivation. This would exemplify fluctuations of TC and contribute to the heterogeneity of stop codon readthrough (Figure 3-8).

Our proteomic analysis identified Trp to be the major amino acid that suppresses UGA in E. coli, which is in agreement with previous studies(27). E. coli tRNA_{Trp} contains a modification at A37 that facilitates C-A pairing at the wobble position(117), which may
explain why tRNATrp suppresses UGA better than other near-cognate tRNAs such as tRNA\textsubscript{Cys}. The suppression efficiency of UAG appears to be lower than UGA, presumably because of the stronger termination activity of RF1. Indeed, in an RF1 deletion strain, multiple amino acids have been detected to suppress UAG(118).

In \textit{E. coli}, UGA is used by ~30\% of the protein-coding genes as the stop codon. Readthrough of stop codons may cause misfolded protein stress or production of protein isoforms with new functions(119, 120). For example, sequences following stop codon readthrough may contain novel localization signals(119). In this study, we show that increased UGA readthrough in \textit{E. coli} enhances growth under poor-nutrient conditions and promotes growth from stationary phase (Figure 3-7). The underlying mechanism remains to be elucidated. A pathway enrichment analysis suggests that genes with UGA as stop codons are most significantly enriched in ABC transporters, which are involved in nutrient uptake(121, 122). It is possible that UGA readthrough may alter the function of such transporters and provide growth advantage when nutrients are limited. In line with this notion, UGA suppression does not enhance growth in rich media (Figure 3-7C). Such advantage of high UGA readthrough on cell growth is also observed in single cells (Figure 3-7A). Heterogeneous UGA readthrough among single cells may thus provide benefits to the microbial population by enhancing phenotypic diversity and facilitating adaptation to ever-changing environments.
Chapter 4

Low pH Decreases Release Factor 2 Activity *in vivo*
4.1 Introduction

When the ribosome encounters a stop codon, two factors directly affect ribosomal release: 1) release factors and 2) charged near-cognate tRNAs. As shown in Chapter 3, the levels of charged tRNAs can change due to changes in environmental conditions and this results in changes in stop codon readthrough (Figure 3-4). Also shown in Chapter 3, increasing the expression of Release Factor 2 (RF2), the release factor responsible for recognizing the UGA stop codon, had no effect on the levels of stop codon readthrough in MG1655 cells (Figure 3-6). This supports the idea that, under normal growth conditions, the activity of RF2 is saturating. However, environmental conditions that may decrease release factor activity were not explored.

There are 3 release factors in *E. coli*, RF1, RF2, and RF3. RF1 is responsible for the recognition of the UAA and UAG stop codons. Alternatively, RF2 is responsible for the recognition of the UAA and UGA stop codons. In support of RF1 and RF2, RF3 assists the other two release factors to be released by ribosome and increases their effectiveness (123). Due to their direct role in translation termination, RF1 and RF2 are essential in *E. coli*; however, the deleterious mutations have been found that are not lethal(124, 125). This is especially relevant for K12 strains of *E. coli*, such as MG1655 used here, as they have an A246T mutation that decreases RF2 activity(126).

Along with mutations, environmental conditions have been shown to impact release factor activity. Release factors have been shown to have decreased activity in low pH conditions *in vitro*(127). Here, I have utilized a UGA stop codon readthrough reporter to assay how low pH conditions affect stop codon readthrough levels and how this impacts population heterogeneity. Additionally, I found that growth in high glucose conditions, which
results in a decrease in media pH as the cells produce lactic acid during glycolysis, impacts stop codon readthrough and heterogeneity.
4.2 Results

**Low pH increases stop codon readthrough *in vivo***

Previous studies have shown that release factor activity is decreased in acidic conditions *in vitro*(127–129). To determine whether release factor activity is affected by low pH *in vivo*, I used the dual fluorescence m-TGA-y stop codon readthrough reporter (Figure 3-1A). MG1655 cells expressing the m-y or m-TGA-y reporter were grown in buffered LB media with pH 6.0, 7.0, or 8.0 for 24 hours and their fluorescence was observed using fluorescence microscopy and quantitatively measured by image analysis using microbeTracker. Analysis of the cultures showed that cultures grown in pH 6.0 conditions had a significantly higher stop codon readthrough rate than those grown at pH 7.0 or 8.0 (Figure 4-1A).

Stop codon readthrough is heterogeneous within bacterial populations (Figure 3-2). Under normal growth conditions, release factor activity is thought to be saturating since increasing release factor levels does not decrease the rate of stop codon readthrough; and thus, may not be a factor in the heterogeneity of readthrough (Figure 3-6). However, in low pH conditions release factor activity may be decreased in cells and may affect heterogeneity by making release factor activity a limiting factor in stop codon readthrough. We found that the heterogeneity of stop codon readthrough increased in cultures grown at pH 6.0 compared to those grown at pH 7.0 (Figure 4-1B). This indicates that under low pH conditions, release factor activity levels in individual cells could contribute to the heterogeneity of the population.
Figure 4-1 Growth in Low pH Increases UGA Readthrough and Population

**Heterogeneity.** MG1655 cells expressing the m-y control reporter or m-TGA-y stop codon readthrough reporter were grown for 24 hours in M9 media at pH 6.0, 7.0, or 8.0. Cells were imaged using fluorescence microscopy and single cell fluorescence was analyzed using microbeTracker. (A) The UGA readthrough% was determined as a ratio of total YFP/total mCherry signal in cells expressing the m-TGA-y reporter compared to cells expressing the m-y reporter. (B) The heterogeneity of the population was determined by calculating the coefficient of variance (CV) of the single cells in the population. This is calculated as the standard deviation of the yfp/mCherry ratio divided by the mean yfp/mCherry ratio.
Growth in high glucose increases stop codon readthrough

To further characterize the effects of low pH on stop codon readthrough, we grew MG1655 cultures in LB supplemented with 1% glucose. In these conditions, bacteria will preferentially undergo glycolysis to generate energy. This process results in the overproduction of acetic acid and a low environmental pH (130). We confirmed that supplementing LB with 1% glucose decreases pH and affects bacterial growth in our conditions (Figure 4-2). Cultures grown with glucose had a lower pH and did not reach the same terminal OD600 as compared with cultures grown without glucose (Figure 4-2). Additionally, growth in this medium resulted in morphological changes in the bacteria. Cells grown in glucose are wider than cells grown in LB (Figure 4-3C). Interestingly, the cell width of cells grown in LB + 1% glucose was uneven. Some cells appeared to expand at one end of the cell or at midcell (Figure 4-3C indicated by blue arrows).

To determine whether growth in high glucose conditions affected stop codon readthrough, MG1655 cells expressing the m-y or m-TGA-y dual fluorescence stop codon readthrough reporter were grown in LB with or without 1% glucose for 24 hours.

Fluorescence images were taken of these cultures and analyzed with microbeTracker. Cells grown in the presence of glucose had a higher rate of stop codon readthrough (Figure 4-3A). To ensure that the fluorescence results were not significantly impacted by the change in media pH, we used western blotting to quantitate the presence of mCherry and mCherry-YFP fusion protein in the cultures. Consistent with our fluorescence data, the western blot showed an increase in stop codon readthrough, as seen by the increased level of mCherry-YFP fusion protein compared to mCherry alone (Figure 4-3B).
**Figure 4-2 Growth in LB + 1% Glucose Decreases pH.** Overnight MG1655 cultures were diluted in LB (in blue) or LB + 1% glucose (in green) and allowed to grow for 24 hours at 37°C. The OD600 was measured every 20 minutes. The pH of the cultures was semi-quantitatively determined using pH strips.
Figure 4-3 UGA Readthrough is Increased in LB + 1% Glucose. MG1655 or rpsD* cells expressing the m-y or m-TGA-y reporter were grown in LB or LB + 1% Glucose for 24 hours at 37°C. (A) Fluorescence was quantified by spectrometry on a plate reader. The error rate was calculated as the YFP/mCherry ratio of the error reporter normalized by the
YFP/mCherry ratio of the m-y control. (B) The amount of mCherry-YFP fusion protein was quantified and compared to the amount of mCherry protein by Western blot. (C) Representative images of MG1655 cells expressing the m-TGA-y dual fluorescence reporter after growth in LB or LB + 1% glucose. Individual cells with abnormalities in cell shape are indicated by blue arrows. **Note: Lysate purification and Western blot analysis was completed by Yongqiang Fan.**
We also assessed the heterogeneity of stop codon readthrough in populations grown in LB supplemented with 1% glucose. In cultures expressing the m-y control reporter, the CV of the YFP/mCherry ratio was unchanged in cultures grown in glucose compared to those grown without glucose (Figure 4-4A). In cells expressing the m-TGA-y reporter, cultures grown with glucose had a significantly higher CV (Figure 4-4A-B).

Decreased pH affects release factor activity in vivo

In cultures grown in glucose, we hypothesize that glycolysis results in lower environmental pH which affects release factor activity. To test whether this process is dependent on the cells undergoing glycolysis, we tested the stop codon readthrough in a strain without the gene for the glucose importer PtsG (ΔptsG). This strain was obtained from the Keio collection which has E. coli BW25113 as the parental strain. We measured the stop codon readthrough of the BW25113 and ΔptsG strains expressing the m-TGA-y reporter. In cultures grown in LB, the two strains have ~2% TGA readthrough (Figure 4-5). In cultures grown in LB with 1% glucose, the readthrough of BW25113 increases to ~5.5%, but the ΔptsG strain is unaffected (Figure 4-5). This supports the model that the stop codon readthrough is affected in LB with 1% glucose due to the increase in glycolysis.

We hypothesize that growth in glucose affects stop codon readthrough due to negatively impacting release factor activity. To rule out that pH broadly affects translational accuracy of the ribosome, we tested whether growth in glucose affects other ribosomal errors. To test the frameshifting rate of the ribosome, we used a frameshifting reporter, m-+1fs-y, which contain an additional adenine after the mCherry gene. MG1655 cultures grown in LB with or without 1% glucose showed no difference in frameshifting error rate (Figure 4-6A).
Figure 4-4 Growth in LB + 1% glucose increases UGA Readthrough Heterogeneity.

MG1655 cultures expressing the m-TGA-y dual fluorescence reporter were grown in (A) LB or (B) LB + 1% glucose for 24 hours. Fluorescence was captured via fluorescence microscopy and data quantified using microbeTracker. The ranges of YFP/mCherry ratio values are shown on histograms. (C) The heterogeneity of populations grown in LB or LB + 1% glucose are quantified by calculating the CV defined by the standard deviation of the YFP/mCherry ratio in single cells in the population divided by the mean YFP/mCherry ratio.
**Figure 4-5 Glucose Import is Necessary to Increase UGA Readthrough.** Strains from the Keio *E. coli* knockout collection were grown in LB or LB + 1% glucose for 24 hours. Both the parental strain (BW25113) and glucose importer knockout strain (ΔptsG) expressing the m-TGA-y dual fluorescence reporter had their mCherry and YFP signals measured with fluorescence spectroscopy. The Error Rate % was determined by the ratio of YFP-to-mCherry of cells expressing the m-TGA-y reporter compared to the m-y control reporter.
To test whether misincorporation of amino acids via selection of non-cognate tRNA was affected by pH, we used a missense reporter, m-G65R-y, which has a glycine substitution for a catalytic arginine. MG1655 cultures grown in LB with or without 1% glucose showed no difference in missense error rate with this reporter (Figure 4-6A).

Additionally, the rpsD* strain was tested for increases in stop codon readthrough during growth in 1% glucose. The rpsD* strain has a mutation that decreases proofreading by the ribosome and has an increased stop codon readthrough rate compared to MG1655 (Figure 4-2). When grown in LB supplemented with 1% glucose, the stop codon readthrough rate increased even further than when grown in LB alone. This indicates that the increase in stop codon readthrough rate due to decreased pH is additive to the decreased proofreading in rpsD* (Figure 4-2). This is consistent with our hypothesis that decreased release factor activity, and not ribosomal accuracy, is responsible for the increased stop codon readthrough observed in growth in LB + 1% glucose.

Lastly, we examined whether pH affects the rate and level of charged Trp-tRNA_{Trp}. As shown in Chapter 3, increasing charged Trp-tRNA_{Trp} levels is sufficient to increase TGA stop codon readthrough. To test this, use tRNA Northern analysis to determine the levels of Trp-tRNA_{Trp} in cultures grown in LB with and without 1% glucose. We found no difference in the overall level of Trp-tRNA (Figure 4-6B bottom bands) or the rate of aminoacylation (Figure 4-6C). This suggests that the increased stop codon readthrough observed in growth in LB +1% glucose is not the result of increased Trp-tRNA_{Trp}.

**Low pH increases ribosome availability**

In Chapter 3, I showed that the heterogeneity of stop codon readthrough affects phenotypic population heterogeneity.
Figure 4-6 Increase in UGA Readthrough is Due to Decreased Release Factor Activity.

(A) MG1655 cells expressing the m-TGA-y stop codon readthrough reporter, m-+1fs-y frameshift reporter, or m-G65R-y missense reporter were grown in LB or LB + 1% glucose for 24 hours at 37°C. The total mCherry and YFP fluorescence were quantified by fluorescence spectroscopy and the Error Rate % determined as the ratio of YFP-to-mCherry compared to a culture expressing the m-y control reporter. (B) The tRNA\textsuperscript{Trp} levels (bottom bands) in MG1655 cells grown in LB or LB + 1% glucose were assayed using acidic Northern blot analysis. SsrA levels were used as the RNA loading control. (C) The quantitation of tRNA\textsuperscript{Trp} levels from the acidic Northern blot.
Our data shows that an increase in stop codon readthrough in single cells results in a quicker average recovery from stationary phase than a cell with low stop codon readthrough (Figure 3-7). We hypothesized that this could be due to changes in ribosome availability during late stationary phase. A cell with high stop codon readthrough may have increased levels of active ribosomes during stationary phase due to increased time on mRNA during translation. This increased level of active ribosomes in the cell may result in faster recovery when the cells encounter nutrients in the future.

If increased stop codon readthrough increases ribosome availability in stationary phase, I expect for protein synthesis to be increased in populations with increased stop codon readthrough grown to late stationary phase. Our data shows that growth at low pH increases stop codon readthrough, so I expect these populations may have increased protein synthesis levels once grown to late stationary phase. To test this, I determined the protein synthesis levels of individual cells expressing the m-TGA-y reporter grown at pH 6.0, 7.0, or 8.0 in LB media for 24 hours. As a measure of protein synthesis in each cell, I quantified the ‘mCherry Intensity’ of each cell. The ‘mCherry Intensity’ is defined by the total mCherry signal divided by the cell volume as determined by microbeTracker. This provides a measure of protein synthesis in each cell normalized by cell size in order account for changes in cell dimensions. In MG1655 cultures grown at pH 6.0, the average ‘mCherry Intensity’ of each cell is significantly higher than cultures grown in pH 7.0 or 8.0 (Figure 4-7A). This supports the model that increased stop codon readthrough increases ribosome availability into late stationary phase. Therefore, I decided to test whether the physiological influence of stop codon readthrough is maintained in cultures grown in LB supplemented with 1% glucose.
**Figure 4-7 Growth in Low pH Increases Ribosome Availability in Stationary Phase.** (A) MG1655 cells expressing the m-TGA-y dual fluorescence reporter were grown for 24 hours in M9 media at pH 6.0, 7.0, or 8.0. The mCherry Intensity was calculated by dividing the total mCherry signal of a single cell by its cell volume as determined by microbeTracker. The average mCherry Intensity of all the single cells in the population is shown in the bar graph. (B) MG1655 cells expressing the m-TGA-y dual fluorescence reporter were grown in LB with 1% glucose for 24 hours before being transplanted to an M9 agarose pad. The mCherry and YFP signals were visualized by fluorescence microscopy and then cells were followed over time to determine how long it took each cell to complete cell division. The time it took for each individual cell to reach its first division is represented as a single dot on the plot.
Under normal growth conditions, the range of stop codon readthrough rates is capable of influencing the ability of each individual cell to recover from starvation (Figure 3-7). Under low pH conditions, the rate of stop codon readthrough is increased in every cell to a level higher than any cell under normal growth conditions (Figure 4-4). I found that in this condition, there was no correlation between the rate of stop codon readthrough and recovery from stationary phase in single cells, $R^2 \sim 0.03$ (Figure 4-7B).
4.3 Discussion

Responding to changes in environmental conditions is a key feature of all life. This is especially true for bacteria, as many of them encounter severe changes in environmental conditions regularly. Changes in the pH of the environment are common in almost every bacterial community including biofilms (131), soil (132, 133), marine environments (134), and within hosts during infection (135). There is a clear need to fully understand how bacterial physiology is affected by changes in pH. This study shows a previously uncharacterized molecular change in response to pH in vivo – an increase in stop codon readthrough in acidic conditions. As stop codon readthrough affects cell physiology, future work should be done to determine how the increase in readthrough due to low pH could affect bacterial behavior.

I have shown that low pH conditions increase TGA stop codon readthrough. Our data support a model by which glycolysis decreases the environmental pH, which negatively impacts the activity of RF2; and thus, increases TGA stop codon readthrough during protein synthesis (Figure 4-8). However, it is still not clear whether this is a consequence of growing in low pH or part of a response to low pH. Could this provide an advantage to the population? Growth in low pH increases the heterogeneity of stop codon readthrough, but it is difficult to determine if this provides any benefit to the population without knowing how physiology is affected. Although increasing translation errors may seem detrimental, studies are continuing to find benefits to changes in translation error rate (44, 45, 51, 89).

Analyzing translation accuracy in vivo has been a challenge. In past studies, biochemical techniques such as mass spectroscopy or ribosome profiling have provided some information about ribosome accuracy, but are unable to provide information about
Figure 4-8 Model describing how stop codon readthrough is increased in LB + 1% glucose. In our model, glucose (blue hexagons) is imported and undergoes glycolysis. This results in the production of protons (grey circles) which prevent RF2 activity. Due to decreased RF2 activity, UGA readthrough by the ribosome during protein synthesis is increased. This figure was created with BioRender.
single cell dynamics. Conversely, single fluorescent reporters with stop codon insertions or missense codon mutations fail to account for the impact on transcriptional heterogeneity or protein synthesis rate variability in populations. The use of dual fluorescent reporters, like the TGA reporter in this study, provides a great tool for the analysis of errors during translation in single cells. Currently, we have focused on TGA readthrough due to the relatively high error rate of TGA readthrough compared to other mistranslation events; however, errors, such as frameshifting, are possible to examine with this system. Further, with the development of more efficient and brighter fluorescent proteins and higher resolution fluorescent microscopes, the applications of this technique can go much further.
Chapter 5

Increased Mistranslation Protects *E. coli* from Heat Stress
5.1 Introduction

In order for protein synthesis to be successful, the incorporation of amino acids into the growing polypeptide must be accurate. Errors during protein synthesis can result in malfunctioning or misfolded proteins that are detrimental to the cell (5, 31, 33). In situations where mistranslation rates are significantly increased, such as during aminoglycoside treatment, increased errors in protein synthesis result in cell death (5, 32). Despite this clear advantage to accurate protein synthesis, translation is not perfect. In natural isolates of *E. coli*, purified ribosomes were found to vary in accuracy *in vitro* – some strains were found to have a higher accuracy than a traditional lab strain (40). It has been found that, in some cases, increasing the mistranslation rate in cells can be beneficial.

The accumulation of misfolded proteins activates the heat shock response. The heat shock response is characterized by the production of protein chaperones and proteases that disaggregate, refold, or degrade misfolded proteins in the cell. Accumulation of misfolded proteins is sensed by chaperones and initially results in the production of the heat shock sigma factor σ32 (RpoH) (65, 69, 71). RpoH directly activates transcription of a number of heat shock proteins, including the chaperone DnaK and protease complex GroEL (70). Under heat conditions, this is a transient response where RpoH is produced for a short time period, then degraded as the cell adapts to its environment.

Pre-activation of stress responses can be beneficial to protect cells from future stresses. For example, cells grown at a high, but permissible temperature, survive lethal heat treatment better than cells grown at lower temperature (136). I have applied this principle to cells with increased mistranslation rates. I hypothesize that increased
mistranslation rates pre-activate the heat shock response and can protect cells from heat killing – a previously uncharacterized benefit of mistranslation in bacteria.
5.2 Results

**Increased mistranslation protects against heat stress**

Increased levels of mistranslation have been shown to have a protective effect on cells by pre-activating stress responses that are beneficial when a population encounters lethal stress (16, 51). Mistranslation results in the production of misfolded proteins; therefore, I hypothesize that increased levels of mistranslation will protect populations from heat stress by pre-activating the heat shock response. In order to test this hypothesis, I used the error-prone *E. coli* strain, rpsD*. This strain contains a ribosomal point mutation, I199N, which decreases ribosomal accuracy.

To determine whether mistranslation protects against heat stress, I performed a heat killing assay with rpsD* and its parental wild type strain, MG1655. In this assay, cells were grown to mid-logarithmic phase in LB, resuspended in phosphate buffer, and then subjected to killing at 50°C. Cell survival was assayed by counting colony forming units. I found that the rpsD* strain survived heat killing better than MG1655 (Figure 5-1).

In addition to heat killing, I determined whether rpsD* cells have an advantage in non-lethal protein misfolding stress conditions. To do this, I created a fluorescent reporter, sfGFP-ClpB. This reporter is a fusion between superfolder GFP (sfGFP) and the primary protein chaperone disaggregase, ClpB. With this reporter, aggregates formed in cells can be visualized as green fluorescent foci as ClpB binds to the aggregates. I used this reporter to determine the effectiveness of cells to clear aggregates after being exposed to protein misfolding stresses – non-lethal heat or streptomycin treatment.
Figure 5-1: The rpsD* Strain is Protected from Heat Killing. MG1655 and rpsD* strains were grown to mid-logarithmic phase at 30 degree C, and then treated at 50°C. Serial dilutions were made every 30 minutes and colony counts used to determine the rate of killing due to heat stress.
In response to incubation at the non-lethal heat stress, 42°C, both MG1655 and rpsD* formed aggregates in every cell (Figure 5-2A). The number of aggregates formed were visually quantified and the rpsD* strain formed fewer aggregates per cell. In comparison, after recovering from heat stress for 2 hours there was a dramatic difference in the number of aggregates in rpsD* cells and MG1655 cells. On average, MG1655 cells still had ~2.5 aggregates per cell, whereas rpsD* cells had cleared most aggregates and had only ~0.5 aggregates per cell after 2 hours of recovering (Figure 5-2B). This indicates that the heat shock response machinery is more effective in the error-prone mistranslation strain rpsD* compared to MG1655. This assay was repeated with antibiotic stress, caused by streptomycin treatment. This aminoglycoside antibiotic binds directly to the ribosome and drastically increases mistranslation, resulting in the accumulation of misfolded proteins. After recovery from streptomycin treatment, rpsD* cells were able to clear streptomycin-induced aggregates more effectively that MG1655 cells (Figure 5-2C and D).

To ensure that the increased disaggregation is not an rpsD* strain-specific phenotype, I tested whether increasing mistranslation in MG1655 via canavanine treatment during growth would show the same phenotypes. Canavanine is an arginine analogue that is misincorporated by the ribosome into proteins in place of arginine and results in the production of proteins that misfold. MG1655 cells expressing the sfGFP-ClpB reporter were grown with or without canavanine treatment and treated at 42°C, then their ability to clear aggregates quantified. As with the rpsD* strain, MG1655 cells grown in canavanine were able to clear almost all aggregates from cells (Figure 5-3).

In the experiments described above, samples of the population were assayed at different time points to determine how the population was recovering from stress. I aimed to visualize single cells recovering from stress immediately after treatment until they had fully recovered.
Figure 5-2: The rpsD* Strain has Increased Aggregate Clearance Activity *in vivo*.

MG1655 and rpsD* strains expressing an IPTG-inducible sfGFP-ClpB fusion were grown to mid-logarithmic phase in LB + 100uM IPTG at 30 degrees C. Then, cells were treated at 42 degrees C (A and B) or with 100ug/mL streptomycin (C and D) for one hour. After treatment, cultures were treated with 100ug/mL spectinomycin to stop protein synthesis. Images were taken immediately after and two hours after spectinomycin treatment. (A and C) Representative images of cells after treatment. (B and D) The numbers of aggregates per cell were quantified manually using imageJ.
Figure 5-3: Increased Mistranslation Induces Aggregate Clearance. MG1655 cells expressing the IPTG-inducible sfGFP-ClpB construct were grown in the presence or absence of the arginine analogue, Canavanine, to mid-logarithmic phase in LB + 100uM IPTG. After growth, cultures were treated at 42 degrees C for one hour. After treatment, cultures were treated with 100ug/mL spectinomycin to stop protein synthesis. Images were taken immediately after and two hours after spectinomycin treatment. The numbers of aggregates per cell were quantified manually using ImageJ.
This would allow me to more accurately determine how cells were able to recover and the differences between MG1655 and rpsD* cells. To accomplish this, I expressed mCherry from a constitutive tetracycline-on promoter in either MG1655 or rpsD* strains along with the sfGFP-ClpB reporter. Then, after growth and heat stress, I could visualize the recovery of MG1655 and rpsD* cells together on an LB + agarose pad and directly compare their recovery rates (Figure 5-4A). Using this method, I found that over 90% of rpsD* cells were able to completely clear their heat-induced aggregates in 3 hours, while ~70% of MG1655 cells still had at least one aggregate in the same timeframe (Figure 5-4B).

**The heat shock response is pre-activated in rpsD**

I hypothesize that the benefits to the rpsD* cells against misfolded protein stress is due to the pre-activation of the heat shock response. In order to test this, I have measured the levels of the heat shock sigma factor RpoH in rpsD* cells under normal growth conditions. To measure RpoH levels, I performed Western blotting on lysates using an anti-RpoH antibody. I found that in cells grown in LB at 30°C to logarithmic phase RpoH protein levels are over 2-fold higher than in MG1655 cells (Figure 5-5A and B). This appears to be an incomplete activation of the heat shock response, as MG1655 cells under heat conditions increase their RpoH levels over 5-fold (Figure 5-5A and B).

If RpoH levels are higher in the rpsD* strain, the transcription of RpoH targets are also expected to be increased. To test this, I used transcriptional reporters with RpoH-dependent promoters fused to GFP. I measured the transcription of dnaK and groE in MG1655 and rpsD* strains in LB at 30°C. The expression of both dnaK and groE is 2-fold higher than in MG1655 (Figure 5-6).
Figure 5-4: Increased Mistranslation Increases Aggregate Clearance in Single Cells.

MG1655 and rpsD* cells expressing the IPTG-inducible sfGFP-ClpB construct were grown in LB + 100uM IPTG to mid-logarithmic phase. Additionally, the rpsD* cells constitutively expressed mCherry on a plasmid. After growth, cultures were mixed and incubated at 42 degrees C for 30 minutes. Then, spetinomycin were added to the culture to stop protein synthesis. Cells were transferred to a 1.5% agarose pad. (A) Aggregate formation was visualized using fluorescence microscopy and recovery was tracked for 3 hours. (B) The number of cells containing aggregates in the MG1655 and rpsD* cells were quantified manually using ImageJ.
Figure 5-5: RpoS is Necessary for the Mistranslation-Induced Increase of RpoH.

MG1655, rpsD*, rpsD* Δrpos strains were grown to mid-logarithmic phase in LB at 30 degrees C. As a control for RpoH induction, MG1655 cells were incubated at 42 degrees C. Lysates from each culture were purified via lysozyme incubation and sonication. (A) A western blot of cell lysates using an α-RpoH antibody to determine RpoH levels in each strain. RpoB protein levels were determined as a loading control. (B) Three independent repeats were quantified using volumetric analysis.
MG1655 and rpsD* strains expressing promoter fusions to gfp on a low copy plasmid were grown to mid-logarithmic phase at 30°C. The fluorescence in each culture was determined using fluorescence spectroscopy.

Figure 5-6: Mistranslation Increases RpoH Activity but Not rpoH Transcription.
The general stress response sigma factor, RpoS, plays a role in the mistranslation-induced heat shock response

The levels of the general stress response sigma factor, RpoS, is increased in the rpsD* strain (51). Increased levels of RpoS is responsible for the protective effect of mistranslation against oxidative stress (51). Additionally, there is evidence that RpoS could play a role in the protection of cells from heat stress, although no conclusive mechanism has been identified (137).

First, I tested whether RpoS plays a role in the survival of rpsD* in 50°C. In rpsD*, the deletion of rpoS (rpsD* ΔrpoS) decreases the survival compared to rpsD* (Figure 5-7A). This is the first evidence that RpoS may play a role in the mistranslation-induced heat shock response. This phenotype is not rpsD*-specific. I found that MG1655 grown with canavanine survives better than MG1655 grown without canavanine (Figure 5-7B). In contrast, in an MG1655 strain without rpoS (MG1655 ΔrpoS), growth in canavanine does not protect as well as MG1655 with rpoS and canavanine (Figure 5-7B).

Clearly, RpoS is playing a role in the protection from heat killing in the rpsD* strain. Next, I performed the single cell disaggregation analysis to see if RpoS is affecting the ability of the rpsD* strain to disaggregate proteins. To do this, I grew the rpsD* or rpsD* ΔrpoS strains with constitutively expressed mCherry to mid-logarithmic phase. Then, cells were heat treated and placed on an agarose pad. After 3 hours, I quantified how many cells retained aggregates (Figure 5-8). Surprisingly, the rpsD* cells with or without rpoS were able to equally clear heat aggregates. This indicates that the effect of RpoS on the RpsD* strain may be independent of its increased disaggregation activity.
Figure 5-7: Mistranslation-Induced Heat Protection is Dependent on RpoS. (A) RpoS deletion strains of MG1655 and rpsD* were grown to mid-logarithmic phase at 30 degrees C, then incubated at 50 degrees C. Heat killing was assayed via colony formation by serial dilution every 30 minutes. (B) The same experiment was performed with MG1655 with or without RpoS grown in the presence of canavanine.
Figure 5-8: RpoS does not Play a Role in Mistranslation-Dependent Aggregate Clearance. RprsD* and rpsD* ΔrpoS cells expressing the IPTG-inducible sfGFP-ClpB construct were grown in LB + 100uM IPTG to mid-logarithmic phase. Additionally, the rpsD* cells constitutively expressed mCherry on a plasmid. After growth, cultures were mixed and incubated at 42 degrees C for 30 minutes. Then, spectinomycin was added to the culture to stop protein synthesis. Cells were transferred to a 1.5% agarose pad. Aggregate formation was visualized using fluorescence microscopy and recover was tracked for 3 hours. The number of cells containing aggregates were quantified manually using ImageJ.
To determine whether RpoS affects the activation of the mistranslation-induced heat shock response or has some other role in heat protection, I determined whether the presence of RpoS affects the production of RpoH in the RpsD* strain. In cells grown to mid-logarithmic phase, the RpoH protein level is ~2 fold higher than in MG1655 cells. In an RpsD* ΔrpoS strain, the RpoH level decreases to the level of MG1655 cells. This indicates that RpoS may play a role in directly affecting the heat shock response activation, rather than some other protective mechanism such as increasing membrane integrity.
5.3 Discussion

I have shown that the increased rate of mistranslation can be beneficial to bacterial cells due to increased heat survival resulting from the pre-activation of the heat shock response. This protective benefit is similar to that seen in mistranslation-prone cells to oxidative stress \(51\). It is interesting to consider that the ribosome and protein synthesis machinery may act as internal stressors that can prepare cells for future stresses. Natural isolates of \textit{E. coli} have been identified that have acquired ribosomal mutations that decrease fidelity, indicating that the benefits given to cells by increasing ribosomal errors, such as protection from heat, could be selected for in a natural environment. It should be noted that the \textit{rpsD*} mutation used in this study is not one of the natural mutations found in \textit{E. coli} ribosomes; therefore, it would be interesting to study whether the benefits found in this study and others are found in those natural isolates.

The general stress response sigma factor, RpoS, had not previously been shown to affect the activation of the heat shock response, although there have been a couple studies that indicated it may play a role. RpoS has been shown to bind to the promoter region of the \textit{rpoH} gene \(138\); although the transcriptional regulation of RpoH has minimal impact during heat shock and was found to by unchanged in this study. Additionally, a food preservation study found that an \textit{rpoS} deletion strain was more sensitive to heat killing than the parental strain, although the molecular details were not investigated nor a mechanism proposed \(137\). My work shows that RpoS impacts the levels of RpoH under normal growth conditions in the RpsD* strain; however, I was unable to determine the mechanism of RpoH activation in this strain. My experiments showed no difference in RpoH transcription, translation, or degradation between the RpsD* strain and the parental strain, MG1655 (data not shown). Perhaps the mechanism of increased RpoH production is some other unknown mechanism, such as mRNA stability, or my experiments were not sufficiently sensitive to
measure the subtle difference that resulted in a 2-fold increase in RpoH in cells grown to logarithmic phase. Further study is needed to determine this molecular mechanism of RpoH induction due to mistranslation.

The production of misfolded proteins in the absence of heat is sufficient to activate the heat shock response(77). This finding was the result of inducing the expression of an unstructured protein mutant in a short time period. This is in contrast to the mistranslation-induced heat shock response. Under constitutive mistranslation conditions, such as ribosomal mutation in this study, the production of misfolded proteins is relatively low and occurs during the entire growth cycle of the cell. How does a cell cope with constitutive misfolded protein stress? How does this mechanism differ from acute stresses such as heat shock or the induced production of misfolded protein? The development of more sensitive assays may be required to determine the subtle changes in this system compared to the dramatic changes observed in cells responding to acute protein misfolding stress.
Chapter 6

Discussion and Future Directions
6.1 Dual-fluorescent reporters enable *in vivo* single cell measurements of translation errors

Studies investigating the heterogeneity of gene expression have largely been limited to analysis of transcription. In this study, we have developed a reporter system that allowed us to observe and investigate whether the fidelity of translation is heterogeneous and if this has any influence on the phenotypic heterogeneity in bacterial populations. With our dual-fluorescence reporter system, we can quantitatively measure the fidelity of translation *in vivo* in single cells for the first time. Using this system, in Chapter 3 we found that the accuracy of translation is heterogeneous and has an impact on single cell recovery from starvation. Additionally, in Chapter 4 this system was used to determine how components of the translation machinery, in this case release factor activity, is affected by altered pH *in vivo*.

The ability to use a dual-fluorescent reporter has been very beneficial to expanding our understanding of bacterial heterogeneity and the accuracy of translation *in vivo*. However, our current reporter system is limited to only measuring errors that occur frequently, such as UGA stop codon readthrough and ribosomal frameshifting. Errors that are rarer, such as ribosomal misreading or readthrough of the UAA stop codon, are more difficult to measure due to low fluorescence intensity. To expand the usefulness of the current reporter system, mechanisms to increase the fluorescence signal above background may be beneficial. For example, a localization tag at the end of YFP may increase the sensitivity of the reporter to lower error rates. If a periplasmic-localization tag was added to YFP, all of the YFP produced by the reporter would be concentrated in the periplasmic space. By concentrating the entire YFP signal in a smaller space, fluorescence quantitation may be high enough that it could accurately be differentiated from background cellular fluorescence. Additionally, this would have the benefit of allowing the quantification of the background of each cell individually, since the background fluorescence could be
determined from the middle of the cell and the reporter signal could be determined around the edge. Further advancements, such as this, may be needed to expand studies into errors that occur less frequently.

6.2 The mechanism and impact of UGA readthrough heterogeneity

Readthrough of the UGA stop codon is primarily due to two competing factors, Release Factor 2 and Trp-tRNA<sub>Trp</sub>. Release Factor 2 is responsible for recognition of the UGA stop codon by the ribosome and initiating termination. In comparison, Trp-tRNA<sub>Trp</sub> is a near-cognate tRNA that can be used by the ribosome to incorrectly decode the UGA stop codon(27). This study has shown that influencing Trp-tRNA<sub>Trp</sub> levels or RF2 activity can affect both UGA readthrough levels and UGA readthrough heterogeneity. Under normal growth conditions, RF2 activity seems to be saturating since increasing expression of RF2 does not result in decreased UGA readthrough (Figure 3-5). This suggests that variation in the Trp-tRNA<sub>Trp</sub> level within each cell may be the primary factor that influences the heterogeneity of observed UGA readthrough in a population, but this has not been directly confirmed. To test this, controlling Trp-tRNA expression from a tightly controlled, low-heterogeneity promoter may result in the ability to decrease UGA readthrough heterogeneity.

During our study of UGA readthrough, we found that increasing UGA readthrough results in faster recovery from starvation. In fact, increasing UGA readthrough via expression of a suppressor tRNA was sufficient to promote faster recovery (Figure 3-6). How could the increase in readthrough lead to faster recovery? In eukaryotic and viral systems, programmed stop codon readthrough has been shown to increase genetic diversity and provide additional functions to proteins(119, 120). In comparison, programmed
stop codon readthrough has only one good example in prokaryotes. In *E. coli*, RF2 has an in-frame UGA stop codon which results in a necessary frameshift for successful protein synthesis(111, 112). This acts as a mechanism of feedback inhibition for RF2 production. If UGA stop codon readthrough is too high, more RF2 is produced which results in a decrease in stop codon readthrough. Perhaps there are other currently unknown examples of programmed stop codon readthrough in bacteria which could explain the enhanced recovery phenotype of high-readthrough cells. One clue is that ABC nutrient transporters are significantly enriched among the genes that have a UGA stop codon(121, 122).

Investigation into the mRNA sequence after the UGA stop codon may provide insight into how increased readthrough could affect ABC transporter activity or localization.

Alternatively, the effect of UGA readthrough on recovery may not be direct. During periods of low nutrient availability or starvation, the active ribosome pool decreases(110). A decrease in protein synthesis in these conditions results in ribosome dimerization – a process called ribosome hibernation. This process occurs to non-active monomeric 70S ribosomal subunits(139, 140). Under conditions where UGA stop codon readthrough is high, the ribosome is located on its substrate mRNA longer than if termination is successful. In this case, hibernation may be less likely to occur; thus, the active ribosome pool may be larger and protein synthesis may occur further into stationary phase. This idea is supported by our data showing that cells grown at pH 6.0 – a condition that increases UGA readthrough - produce more mCherry from the dual-fluorescent reporter than cells grown at pH 7.0 (Figure 4-7). Additionally, growth at pH 6.0 eliminated the correlation between starvation recovery and UGA stop codon readthrough (Figure 4-7). This evidence for the ribosome hibernation model needs further testing to be confirmed. For example, strains that have defective ribosome hibernation pathways, such as Ribosome Modulation Factor or short Hibernation Promoting Factor deletions could be tested. With these strains in which
ribosome hibernation is decreased, the correlation between UGA readthrough and starvations recovery should be eliminated if the model is correct.

6.3 Environmental impact on UGA readthrough

Using our dual-fluorescence reporter system we are able to determine how various environmental conditions could affect UGA readthrough in vivo. It is possible for us to quantify and compare readthrough rates with our reporter because the normalization by mCherry is an effective control for changes in transcription and translation rates due to changes in environmental conditions.

Surprisingly, in Chapter 3 we found that decreasing protein synthesis rates increased UGA stop codon readthrough (Figure 3-3). In previous in vitro studies, the prevailing model for ribosomal accuracy was that an inverse relationship existed between the speed and accuracy of protein synthesis (107). Our initial finding in single cells was confirmed by observing an increase in UGA readthrough after treatment of a population with a ribosome inhibitor antibiotic, chloramphenicol. Growth in the presence of chloramphenicol resulted in an increase in Trp-tRNA^{Trp} and previous studies suggested that chloramphenicol treatment does not decrease the processivity of the ribosome (115); therefore, our results do not directly conflict with in vitro measurements of ribosomal speed and accuracy. Could other conditions that decrease protein synthesis increase UGA readthrough? Additionally, the mechanism by which chloramphenicol increases Trp-tRNA^{Trp} and other charged tRNAs is currently unknown. Our data indicate that the charging rate of the tRNA is unaffected after chloramphenicol treatment; therefore, it seems likely that chloramphenicol treatment increases the transcription of tRNAs (Figure 3-4). This could be a programmed response that increases charged tRNA availability to the ribosome in order to attempt to increase
protein synthesis, or chloramphenicol may prevent the synthesis of an unknown transcriptional inhibitor of tRNA synthesis.

Besides antibiotic treatment, a variety of naturally-occurring environmental stresses have been shown to decrease the accuracy of translation, such as low temperature and oxidative stress (15, 16, 141). In this study, we showed that growth in a low pH environment can decrease translational accuracy by decreasing Release Factor 2 activity. Previous studies had shown that RF2 activity could be affected by pH in vitro, but our reporter system allowed us to investigate this interaction in vivo. This is a particularly interesting interaction since bacteria can encounter acidic conditions in a variety of important environments. During infections, bacteria can encounter low pH in the stomach and during exposure to phagocytic cells, which use acids to neutralize invading pathogens. Additionally, the pH in the interior of biofilms can become acidic and directly affect stress tolerance (142). Is UGA stop codon readthrough increased during infections and within biofilms? We have shown that increasing UGA readthrough levels can significantly impact cell physiology and a physiological response, such as the ability to withstand and recover from starvation, could be beneficial in both infections and biofilms. Our dual-fluorescence reporter system would be a great tool for investigating how UGA stop codon readthrough is affected in these conditions.
6.4 The role of the UGA stop codon

The work in this study and previous studies has shown that the UGA stop codon is significantly more error-prone than the UAA or UAG stop codons. Despite this apparent disadvantage, ~30% of *E. coli* genes use the UGA stop codon. Is there a benefit for UGA to be more error-prone? We have shown that increased UGA readthrough can be beneficial by increasing starvation recovery; however, whether this is true for the other stop codons has not been investigated. Further investigations into the specific genes that utilize the UGA stop codon may find that readthrough could result in altered activity.

6.5 Mistranslation-induced heat shock response activation

The accumulation of misfolded proteins is largely detrimental to the cell. To protect against the stress of misfolded proteins on the proteome, cells activate the heat shock response. Despite its name, heat is not the only activator of this response. This study and other have found that increasing errors during translation can also activate the heat shock response; however, the mechanism by which mistranslation activates the heat shock response is not clear. Mistranslation can only activate the heat shock response under aerobic conditions, whereas heat activates the heat shock response under both aerobic and anaerobic conditions(78). My work has shown that RpoS, the general stress response sigma factor, is necessary for the mistranslation-induced heat shock response. In addition, another study found that RpoS was important for heat tolerance in food-borne pathogens(137). Our data indicates that mistranslation does not increase levels of the heat shock response sigma factor, RpoH, in the absence of RpoS. Interestingly, RpoS activity, especially its role in stress resistance, is altered under anaerobic conditions (King 2005). This could indicate that the activity of RpoS in aerobic conditions may be essential for the activation of the heat shock response, and this change in activity deters heat shock
response activation in anaerobic conditions. Despite its role as a sigma factor, it seems unlikely that any role of RpoS directly on rpoH transcription would significantly impact RpoH levels. First, the transcription of rpoH is not increased in the error-prone RpsD* strain compared to the wild-type strain (Figure 5-6). Second, increasing rpoH transcription is not a primary mechanism of heat shock response activation.

Stress tolerance can be phenotypically heterogeneous within populations(143). A sub-population that pre-activates stress responses in order to be protected from future stress is a bet-hedging mechanism used to protect the long-term fitness of the population. Our study shows that increasing mistranslation rates is sufficient to activate the heat shock response and protect cells from heat killing. Based on this data, increasing ribosomal errors in individual cells to pre-activate the heat shock response could be a mechanism that contributes to stress tolerance heterogeneity observed in bacterial populations. To test this, a fluorescent reporter, such as a dual-fluorescence mistranslation reporter, could be utilized in combination with a heat shock response fluorescence reporter. Investigations into a correlation between mistranslation and heat shock response activation in single cells could test this hypothesis. Additionally, single cell tracking during heat stress killing could be analyzed with cells expressing a mistranslation reporter.

In summary, the work presented in this study provides novel insights into the heterogeneity of mistranslation, the mechanisms that lead to mistranslation, and the physiological impact mistranslation can have on cells in both single cell and population-based contexts. The development of the quantifiable dual-fluorescence mistranslation reporter has allowed us to investigate the single cell dynamics and impact of mistranslation within bacterial populations. Further, I used this reporter system to investigate the
environmental factors that can affect stop codon readthrough *in vivo*. Finally, I found that increased mistranslation can be beneficial by protecting cells from future lethal misfolded protein stress.
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