Increased mistranslation protects *E. coli* from protein misfolding stress due to activation of a RpoS-dependent heat shock response

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The misincorporation of an incorrect amino acid into a polypeptide during protein synthesis is considered a detrimental phenomenon. A mistranslated protein is often misfolded and degraded or nonfunctional and results in an increased cost to quality control machinery. Despite these costs, errors during protein synthesis are common in bacteria. Here, we report that mistranslation in *Escherichia coli* increase the protein level of the heat shock sigma factor RpoH and protect cells against heat stress. Surprisingly, this increase in RpoH due to mistranslation is dependent on the presence of the general stress response sigma factor RpoS. This report provides evidence for a protective function of mistranslation and suggests a novel regulatory role of RpoS in the heat shock response.

**Keywords:** heat shock response; mistranslation; RpoH; RpoS

Errors during protein synthesis can be the result of external stresses, such as aminoglycoside treatment [1], or failures in proofreading and protein quality control [2]. Each of these mechanisms result in the production of proteins with incorrect amino acids within the polypeptide. These mistranslated proteins often result in nonfunctional and misfolded protein products and an inherent cost to quality control mechanisms within the cell. Under severe mistranslation stress, this can ultimately result in growth inhibition or cell death [3,4].

In order to handle the stress of misfolded protein accumulation, bacteria activate a highly conserved alternative sigma factor, sigma 32 (RpoH) [5,6]. RpoH production and activation is complex and highly regulated due to the expensive nature of the heat shock response [5–8]. RpoH activation results in the production of proteases and chaperones, including DnaK and GroEL, that collectively refold or degrade misfolded proteins [7]. Once the misfolded protein pool has been appropriately managed, these proteases degrade RpoH, resulting in a dynamic and transient regulation of the heat shock response [9–12].

Interestingly, natural isolates of *Escherichia coli* have a wide range of ribosomal error-rates, indicating that, despite their cost, errors during translation may be beneficial under some conditions [13]. Recent studies have found examples of direct mechanistic benefits of mistranslation and broad physiological changes due...
to mistranslation that can be beneficial to cells [14–18]. In the case of mechanistic benefits, increased methionine misincorporation into proteins can increase oxidative stress tolerance by sequestering oxygen radicals away from functional proteins [19–21]. Also, increased mistranslation is sufficient to generate gain-of-function heterogeneous protein pools that can be resistant to antibiotic binding [15]. Physiological changes to cells as a result of mistranslation can protect cells by pre-activating stress responses. For example, increased mistranslation rates activate the general stress response sigma factor, RpoS, which protects the cells from future lethal oxidative stress [17,22].

In this report, we show that RpoH is activated in E. coli cells with a moderately increased mistranslation rate. This response allows cells to survive lethal heat stress and manage misfolded protein stress better than cells with a lower mistranslation rate. Surprisingly, this protective heat shock response is dependent on the general stress response sigma factor, RpoS. This indicates a novel regulatory role for RpoS for mistranslation-induced heat shock response activation and a second benefit RpoS provides due to increased mistranslation rates in addition to protection against oxidative stress [16].

Materials and methods

Bacterial strains and growth conditions

Escherichia coli cultures were grown in Luria broth (LB) and plated onto Luria agar plates. Cultures were grown at 30 °C shaking unless otherwise indicated. Bacteria were pre-grown at 30 °C to lower base level of heat shock response as described [7]. The following antibiotics were used for plasmid maintenance: 100 µg·mL⁻¹ ampicillin, 25 µg·mL⁻¹ chloramphenicol.

Heat killing assay

Escherichia coli cultures were grown overnight in LB from individual colonies and then diluted 1:100 or 1:50 (ΔrpoS strains) in LB. The diluted cultures were grown for 3 h at 30 °C to mid-logarithmic phase. To test the effect of canavanine on heat killing, MG1655 cultures were grown in LB supplemented with 3 mg·mL⁻¹ canavanine for 1 h. To control for the decrease in growth due to canavanine treatment, separate MG1655 cultures were treated with 0.5 µg·mL⁻¹ chloramphenicol. Then, 500 µL of the cultures was centrifuged at 1500 g for 8 min and washed with phosphate buffer. After a second wash, the cultures were resuspended in 500 µL of phosphate buffer. The absorbance (A600) of the cultures in phosphate buffer was determined via spectroscopy in a microplate reader (Synergy HT, BioTek, Winooski, VT, USA) and normalized to A600 = 0.5. After normalization, the cultures were further diluted to A600 = 0.1 and placed in a 96-well plate (Corning Inc., Corning, NY, USA). Samples were subjected to 50 °C heat shock in a water bath. A 20 µL aliquot of each sample was taken every 30 min, and 5 µL of serial dilutions was spotted onto an LB agar plate, and the survival was determined by the colony forming units (CFU).

Fluorescent aggregation assay and image analysis

Escherichia coli strains were grown overnight in LB with 100 µg·mL⁻¹ chloramphenicol. Then, they were diluted 1:100 in LB with 100 µM IPTG to induce sfGFP-ClpB expression and grown for 3 h at 30 °C. To induce aggregation, mid-logarithmic phase cultures were treated with 100 µg·mL⁻¹ streptomycin or incubated at 42 °C for 1 h. After treatment, cells were treated with 500 µg·mL⁻¹ spectinomycin to stop protein synthesis and incubated at 30 °C. Immediately after spectinomycin treatment and 2 h after stopping protein synthesis, respectively, aliquots of cells were placed on 1.5% agarose phosphate buffer pads to visualize aggregate formation and clearance. The quantification of aggregate numbers in each cell was done manually in IMAGEJ (National Institutes of Health, Bethesda, MD, USA).

This protocol was altered for tracking aggregate clearance in single cells. Overnight cultures were additionally grown in ampicillin if the cells contained the pZS*11-Ptee-mCherry plasmid. Once the cultures reached mid-logarithmic phase, they were incubated at 42 °C for 30 min. After treatment, cells were treated with 500 µg·mL⁻¹ spectinomycin to stop protein synthesis and immediately transferred to a 200 µL agarose LB pad containing 500 µg·mL⁻¹ spectinomycin. Then fluorescence and DIC images were taken immediately and at 3 h. The quantification of aggregate numbers was done manually in IMAGEJ, > 70 cells in one image and three different images were selected for qualification.

Determination of RpoH protein level

Overnight cultures were diluted 1:100 or 1:50 (ΔrpoS strains) in LB and grown for 3 h in LB at 30 °C. One milliliter of the culture was treated with 100 µL of cold trichloroacetic acid, and then incubated on ice for at least 10 min. Samples were centrifuged at 15 000 g for 10 min at 4 °C. After discarding the supernatant, the pellet was washed in 500 µL of 80% cold acetone. The samples were centrifuged and the supernatant discarded. The samples were dried in open air at room temperature for 30 min, then resuspended in 50 µL of 7 M urea to resuspend the pellet. To facilitate complete resuspension of the pellet, the samples were incubated at 95 °C while shaking. Once the pellet was completely resuspended, the samples were stored at −80 °C.
The lysate protein concentration was determined via bicinchoninic acid assay (BCA assay) according to manufacturer’s directions (ThermoFisher Scientific, Waltham, MA, USA). The RpoH protein level was determined using western blotting.

Gene expression analysis via E. coli promoter collection strains

Strains containing a low-copy number plasmid with selected promoters fused to gfp were grown at 30 °C shaking in a 96-well plate and fluorescent measurements taken every 20 min. The values used for analysis were at the timepoint where the OD600 was closest to 0.4. For data analysis, expression of the RpoH-target promoters was normalized to ppa expression to account for potential changes to overall transcription levels between MG1655 and rpsD*

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 [23,24]. Mistranslation results in the production of misfolded proteins; therefore, we hypothesized that increased levels of mistranslation would protect populations from heat stress by pre-activating the heat shock response. To model cells experiencing a high mistranslation rate, we used the error-prone E. coli strain, rpsD*. This strain contains a ribosomal point mutation, I199N, which decreases ribosomal accuracy [17,25].

To determine whether mistranslation protects against heat stress, we performed a heat killing assay with rpsD*

![Fig. 1. The rpsD* strain is protected from heat killing and has an activated heat shock response.](image)

(A) MG1655 and rpsD* strains were grown to mid-logarithmic phase at 30 °C, then treated at 50 °C for 90 min. Serial dilutions were made every 30 min and CFU counts were used to determine the rate of killing (N = 3). (B) MG1655 and rpsD* strains expressing promoter fusions to gfp on a low-copy plasmid were grown to mid-logarithmic phase. The fluorescence in each culture was determined using fluorescence spectroscopy (N = 3). *P-value < 0.05. **P-value < 0.01 by unpaired t-test.

In addition to heat killing, we determined whether rpsD* cells have an advantage in nonlethal protein misfolding stress conditions. To do this, we created a fluorescent reporter, sfGFP-ClpB. This reporter is a fusion between superfolder-GFP (sfGFP) and the primary protein chaperone disaggregate, ClpB [26]. With this reporter, protein aggregates formed in cells can be visualized as green fluorescent foci as ClpB binds to the aggregates. We used this reporter to determine the ability of cells to clear aggregates after being exposed to protein misfolding stresses – nonlethal heat or streptomycin treatment – by visualizing the clearance of aggregates after stress treatment.

In response to incubation at the nonlethal heat stress, 42 °C, both MG1655 and rpsD* formed aggregates in every cell (Fig. 2). 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 h, 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 h of recovering (Fig. 2A,B). This assay...
was repeated with the antibiotic stress, streptomycin treatment. This aminoglycoside antibiotic binds directly to the ribosome and drastically increases mis-translation, resulting in the accumulation of misfolded proteins. After recovery from streptomycin treatment, rpsD* cells were able to clear streptomycin-induced aggregates more effectively than MG1655 cells (Fig. 2C,D).

To ensure that the increased disaggregation is not a rpsD* strain-specific phenotype, we tested whether increasing mistranslation in MG1655 via canavanine treatment during growth would recapitulate the protective phenotypes seen in rpsD*. Canavanine is an arginine analog that is misincorporated by the ribosome into proteins in place of arginine and results in the production of proteins that misfold [27]. 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 was quantified. As with the rpsD* strain, MG1655 cells grown in canavanine were able to clear heat-induced aggregates from cells better than MG1655 cells grown without canavanine (Fig. 2E).

In the experiments described above, samples of the population were assayed at different time points to determine how the population was recovering from stress. We aimed to visualize single cells recovering immediately after treatment until they had fully recovered. This allows us to more accurately determine how cells were able to recover and the differences between MG1655 and rpsD* cells. To accomplish this, we 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, we visualized the recovery of MG1655 and rpsD* cells together on an LB agarose pad and directly compare their recovery rates (Fig. 3A). Using this method, we found that over 90% of rpsD* cells were able to completely clear their heat-induced aggregates in 3 h, while ~70% of MG1655 cells still had at least one aggregate in the same timeframe (Fig. 3B).

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

We hypothesized that the benefits to the rpsD* cells against misfolded protein stress were due to the pre-activation of the heat shock response. We measured the levels of the heat shock sigma factor, RpoH, under normal growth conditions to determine whether mistranslation was sufficient to activate the heat shock response in rpsD*. To measure RpoH levels, we performed western blotting on lysates using an anti-RpoH antibody. Under normal growth conditions, rpsD* increases RpoH protein levels over twofold higher than in MG1655 cells (Fig. 4A, lanes 1 and 2). This appears to be an incomplete activation of the heat shock response, as MG1655 cells under heat conditions increase their RpoH levels over fivefold (Fig. 4A, lane 4).

**RpoS is necessary for the protective mistranslation-induced heat shock response**

The levels of the general stress response sigma factor, RpoS, are increased in the rpsD* strain [17]. The increase in RpoS is responsible for the protective effect
of mistranslation against oxidative stress [17]. Additionally, there is some evidence that RpoS could play a role in the protection of cells from heat stress, although no conclusive mechanism has been identified [28].

We tested whether RpoS plays a role in the survival of rpsD* in 50 °C lethal heat treatment. The deletion of rpoS in the rpsD* background (rpsD* ΔrpoS) decreases the heat survival of rpsD* (Fig. 5A). This phenotype is not rpsD*-specific. MG1655 grown with canavanine survives lethal heat stress better than MG1655 grown without canavanine (Fig. 5B). Consistent with our model, canavanine does not protect MG1655 ΔrpoS cells the same as MG1655 against heat killing (Fig. 5B).

To determine whether RpoS affects the activation of the mistranslation-induced heat shock response, we 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 of rpsD* is ~ twofold higher than in MG1655 cells. In the rpsD* ΔrpoS strain, the RpoH level decreases to the level of MG1655 (Fig. 4A). This indicates that mistranslation-induced RpoS increases heat protection by affecting heat shock response activation.

**Discussion**

We used two model systems to examine the physiological effect of mistranslation, an endogenous source of mistranslation via ribosomal mutation and an exogenous source via treatment with an antibiotic, canavanine. Using these model systems, we found that increasing the mistranslation rate above wild-type levels resulted in protection from heat killing. This led us to a model by which cells may alter their mistranslation rate in order to increase their survival in conditions which may otherwise be lethal. This benefit of increased translation error rate may help explain the observation that ribosomal mutations that increase mistranslation are common in natural isolates of *E. coli* [13]. It is interesting to consider that the ribosome and protein synthesis machinery may act as internal stressors that can prepare cells for future stresses. The heat protection is another benefit to add to the growing list of mistranslation-induced benefits including oxidative stress resistance [17], antibiotic resistance [15], and facilitating more efficient adaptive evolution [29].

During heat shock, the production of RpoH is independent of RpoS; however, the activation of RpoH in the absence of heat is not well understood. In response to misfolded protein production, the presence of oxygen is required for RpoH activation, indicating that solely the production of misfolded proteins, which should result in an increase in RpoH stability via sequestration of the protease machinery, is not sufficient to activate the response [30]. Here, we show that RpoS is necessary to increase RpoH protein levels in response in increased mistranslation rates. It is appealing to hypothesize that oxidative damage to misfolded proteins results in the activation of RpoS, which is well-known to be involved in the oxidative stress response. The activation of RpoS in combination with the accumulation of misfolded proteins and sequestration of chaperones and proteases is then sufficient to
activate the RpoH heat shock response. Further, the mechanism by which RpoS increases the levels of RpoH remains a mystery. Direct transcriptional regulation by RpoS is unlikely since we were unable to detect any transcriptional changes to rpoH in the presence or absence of RpoS. RpoH plays a central role in the heat shock response [5–8], leading us to speculate that RpoS protects rpsD* cells from heat killing through increasing the RpoH protein level. However, we cannot completely rule out the possibility that RpoS enhances the heat shock response in an RpoH-independent pathway. A direct test is challenging due to the essential nature of the rpoH gene.

In wild isolates of E. coli, purified ribosomes have been found to have a range of accuracy [13]. However, upon growth and selection within a laboratory setting, the strains mutated their ribosomes to have an accuracy rate comparable to a known laboratory strain. Clearly, evolutionary pressure can impact ribosome activity and accuracy to broadly affect cell physiology to adapt to changing environmental conditions. Our study supports a model that ribosomal mutations that decrease accuracy in natural isolates may increase strain fitness by protecting cells from environmental stresses, such as increased heat or conditions that stress the proteome by increasing misfolding.

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Author contributions

CRE, YF, and JL designed the study, performed data analyses, and wrote the manuscript. CRE and YF performed the experiments.

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Protection against heat stress by translational errors

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