Mistranslation and stress tolerance in *Escherichia coli*

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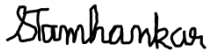
Certificate

This is to certify that this dissertation entitled “Mistranslation and stress tolerance in *Escherichia coli*” towards the partial fulfillment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Sharvari Tamhankar at the National Centre for Biological Sciences under the supervision of Dr. Deepa Agashe, Principal Investigator, during the academic year 2018-2019.



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Declaration

I hereby declare that the matter embodied in the report titled “Mistranslation and stress tolerance in *Escherichia coli*” are the results of the work carried out by me at the National Centre for Biological Sciences, under the supervision of Dr. Deepa Agashe and the same has not been submitted elsewhere for any other degree.

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Abstract

Mistranslation i.e. translation errors are much more frequent than errors at the replication or transcription level. These errors take place despite having many proofreading mechanisms in place. Thus it is debated whether mistranslation is harmful or beneficial to the cell and there has been evidence on both sides. Recent studies have shown that mistranslation can be beneficial especially under stress. Work in the lab has shown that strains with depleted initiator tRNA content mistranslate by -1 frameshifting and non AUG initiation, and have benefit under short term stress. In this study we are trying to see whether this mistranslating strain has any benefit as compared to the WT strain in long term constant and fluctuating antibiotic stress. To do this we evolved these two strains under antibiotic stress. We then looked at the growth rate increase, resistance increase and the pattern in mutations in the target genes of the evolved strains. We saw differences in the mutations that the WT and the mistranslating mutant show across stress regimes. From this data we can say that the WT and mutant rapidly show an increase in their growth rate and in this course they follow different mutational trajectories. Currently we are studying if there are differences in frameshifting between the two strains. We have created a data set with several controls (hyperaccurate, hypoaccurate and kasugamycin) with the evolution lines and further sequence analysis will reveal the mechanisms used by mistranslation in adaptation to long term and fluctuating stress.

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Introduction

Protein translation is an important step for the functioning of the cell. Therefore it is important to maintain its accuracy. There are several proofreading mechanisms in place at the level of tRNA charging, amino acid selection (performed by the aminoacyl tRNA synthases) and decoding codons on the mRNA (Mohler et al., 2017). Despite having these mechanisms in place to maintain fidelity of translation, translation errors occur frequently. Mistranslation is the incorporation of incorrect amino acids into the peptide chain during translation form the mRNA.

Mistranslation can be of various types like miscoding, misacylation, frameshifting and misinitiation. Schematics for the same are given in Figure 1.

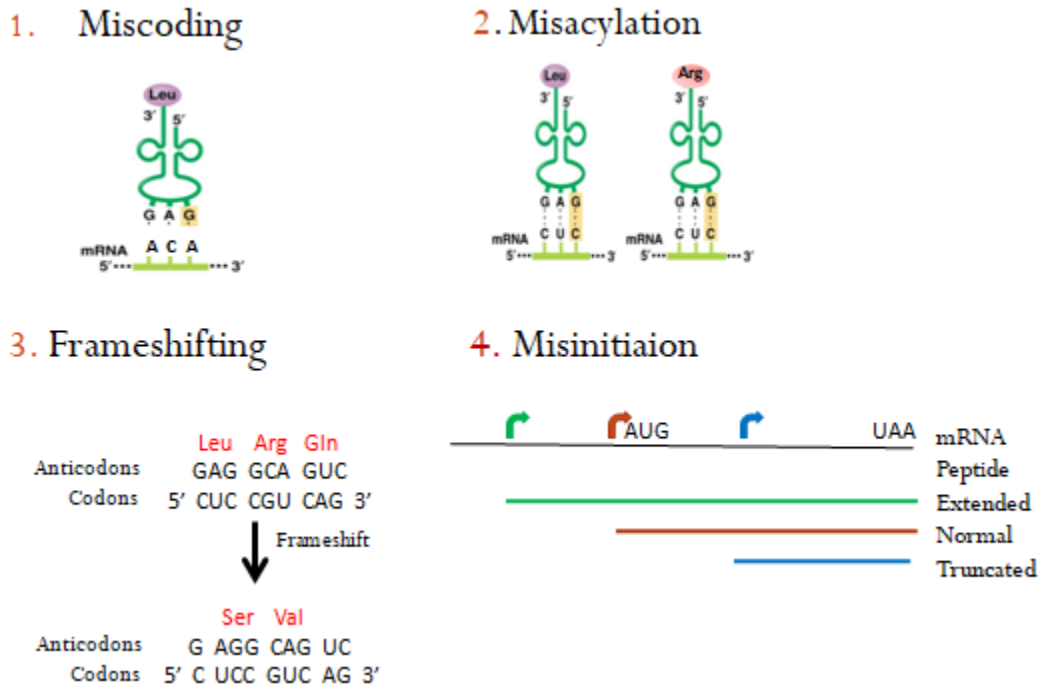

Figure 1: Types of mistranslation. Miscoding, misacylation, frameshifting and misinitiation. In miscoding, the aminoacyl tRNA complex binds to a non cognate codon resulting in the misincorporation of an amino acid in the peptide chain. In misacylation, the codon-anticodon pairing is correct. However, a wrong amino acid is loaded to the tRNA. Frameshifting causes the reading frame to change during translation resulting in a complete change in the amino acid sequence of the protein. In misinitiation, instead of translation starting at AUG, it starts at some other position upstream or downstream of the start codon which leads to an extended or truncated protein which may or may not be in the same frame as the original protein.

Mistranslation can occur as frequently as one in 10^3-10^5 codons. This is 10^4 times more likely to occur than point mutations in the DNA strand (Gomes et al., 2007; Ackermann et al., 2015). This can lead to diversity in the proteome which then comprises proteins having altered sequences (Gomes et al., 2007; Miranda et al., 2013). A high frequency of translation errors is maintained both in prokaryotes and eukaryotes and this could be explained by multiple hypotheses: there are tradeoffs between accuracy and efficiency for protein production; the selection for reducing rates of mistranslation rates would be reduced if there are mechanisms which increase the robustness of protein (improved folding of mistranslated proteins with the help of chaperones); mistranslation can lead to better survival in certain conditions due to the generation of a novel proteome.

The effects of mistranslation may not be as long lasting as those of mutations (which can be passed on to the next generation). Despite of this mistranslation may aid in differential survival of cells especially under stress. This could lead to change in the evolutionary trajectories of the population of cells.

It has been investigated whether mistranslation is harmful or beneficial and there is evidence on both sides. It is traditionally thought that mistranslated proteins are harmful to the cell. This is because they tend to misfold and aggregate which can in turn cause several neurodegenerative disorders like Parkinson's disease, Alzheimer's disease and Amyotrophic Lateral Sclerosis (ALS) (Ross et al., 2004). In mice, proteins misfold due to mischarged tRNAs. These misfolded proteins cause degeneration and death of the Purkinje neurons (Lee JW et al., 2006).

However, recent studies have shown that having mistranslation can have survival benefit for the organism, especially under stress conditions. Babak et al., 2014 created mistranslating mutants of *Mycobacterium smegmatis* in which aspartate was substituted for aspargine and glutamate for glutamine in all proteins. They found that mistranslation provided better survival under rifampicin stress but not under certain other antibiotic stresses because of alteration of specific aspargine and glutamate residues in RNA Polymerase protein, mutations which are responsible for rifampicin resistance (Babak et al., 2014). Mistranslation can also influence the success of pathogenesis. In *Candida albicans* and *Saccharomyces cerevisiae* it can increase cell surface antigen diversity in

a short period of time due to incorrect recognition of tRNA by aminoacyl tRNA synthases and incoroporation of serine instead of leucine respectively (Gomes et al., 2007; Miranda et al., 2013). In some *Mycoplasmatacae,* aminoacyl tRNA synthases have poor proofreading capability which leads to misacylation and proteome diversity (Yadavalli SS et al., 2013; Jaffe et al., 2011). This provides survival advantage by helping in evasion of host immune response. Sinisa B et al., 2017 showed that mistranslation can help in more efficient and faster purging of deleterious mutations which would otherwise have remained in the population for a longer time (Ackerman et al., 2015). These studies have made us understand that mistranslation is not completely disadvantageous for the cell but having diversity at the proteome level can actually be helpful, especially for resisting stressful conditions.

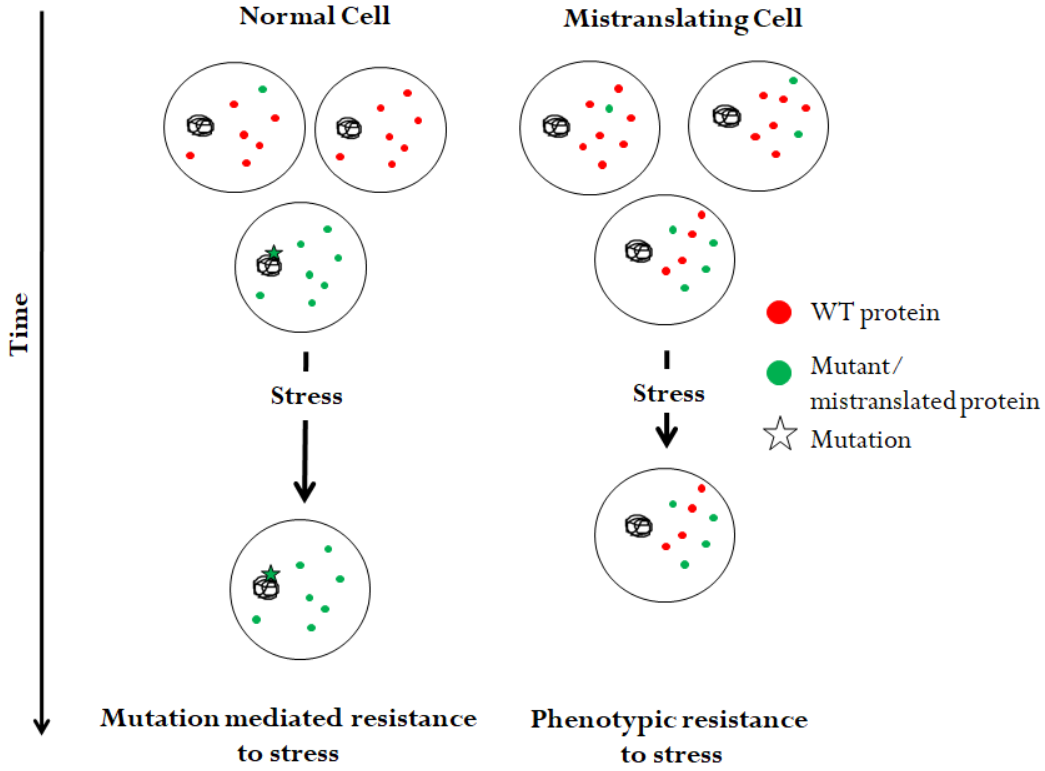

Figure 2: **Response to stress in a normal and mistranslating cell.** Proteins in a normal cell are represented in red colour while protein variants which provide resistance to stress are shown in green colour. A normal cell which has a low level of mistranslation produces only red proteins, while a mistranslating cell has diversity in its proteome and produces red and green proteins. A normal cell would survive only if mutations which facilitate the production of green proteins arise and are selected. However, the mistranslating strains already have a low level of the green proteins which can enable it to survive the stress without having mutations. Phenotypic resistance can be faster than mutation mediated resistance to stress.

Errors in translation can occur due to changes in the levels of factors involved in translation (Kramer et al., 2007; Ulrich et al., 1991). In particular these errors can occur if tRNA levels in the cell vary. There are two kinds of tRNAs involved in translation: initiator tRNA and elongator tRNA. Initiator tRNA helps in decoding the start codon AUG while the elongator tRNAs help in decoding codons in the rest of the mRNA. In *E.coli* the initiator tRNA is present in four copies in the genome: *metZ, metW, metV* and *metY* (Kenri et al., 1994). It was thought that the functions of the initiator and elongator tRNAs were mutually exclusive. However upon reducing the level of initiator tRNAs by deleting three of the four genes (Δ*metZWV*) in the cell, it was observed that the role of translation initiation can partially be taken over by the elongator tRNAs (Samhita et al., 2013). Translation initiation by elongator tRNAs could lead to initiation from non-AUG codons which can happen in the same open reading frame. Alternatively it is thought that initiation may happen at a new position on the gene where alternate SD like sequences are present but instead of an AUG codon an alternate codon is present in the vicinity. This can lead to the production of novel peptides due to misinitiation and frameshifting (Samhita et al., 2013)

Unpublished work done in the lab has shown that the ΔZWV strain does better than the wild type in several stresses like heat stress and nutrition starvation. Using a Biolog assay which is a phenotypic screen, it was identified that the mutant has higher survival than the wild type in two antibiotics: novobiocin which affects the DNA topology and Carbenicillin which affects cell wall synthesis. When both the strains were plated on inhibitory concentrations of Ciprofloxacin which is an inhibitor of DNA gyrase, it was observed that more colonies appear on the plate for the mutant than the wild type. In the short term (24 hours of exposure on Cip 25 plates), the WT was found to carry mutations in *gyrA* which is the primary target of Ciprofloxacin, while the mutant mistranslating strain carried mutations in a different gene, *marR* which is a regulator of efflux pump activity. Using lacZ reporter constructs described later it was seen that the mutant -1 frameshifts. Introduction of hyperaccurate ribosomes reverses this phenomenon of -1 frameshifting as well as the benefit the mutant has under Ciprofloxacin and heat stress.

Aims:

1) To determine the effect of mistranslation on mutational trajectories under constant and fluctuating antibiotic stress.

2) In the second part, I am trying to confirm that peptide sequences are altered in mistranslating cells. The presence of mistranslation in our system has previously been inferred through gene reporter assays and not through direct analysis of the protein sequence.

In this thesis I will primarily be focusing on the first aim. It can be said that mistranslation in the mutant, leads to better short term phenotypic resistance to stress. However, it is not known whether this short term benefit that the mutant experiences under stress is applicable under long term stress. The long term stress regimes included constant and fluctuating conditions. Fluctuating regimes were used to mimic natural conditions where the external environment usually does not remain constant. We hypothesize that in fluctuating conditions the mistranslating strain, owing to its ability to have phenotypic resistance to stress (Figure 2), may be able resist stress better and in a shorter time span as compared to the WT strain, without having the need to have mutations which provide resistance to stress. In the constant stress regime, the effect of phenotypic resistance may not be as apparent and could be used as control regimes for the fluctuating stress.

To test the hypothesis given above, we evolved the WT and the mistranslating strains in constant and fluctuating sub lethal antibiotic stress for 28 days (~500 generations) with 12 hour transfers (12 hour transfers allowed for rapid growth. At the same time this strategy selected against slow growing cells like persistors and small colony variants (SCV)).

As a control for the evolution experiment mentioned above, we have also set out to evolve hyperaccurate (makes translation more accurate) and hypoaccurate (increased mistranslation in this strain) variants of the WT and the mutant strain in the same regimes as before. The aim is to see whether the mutational trajectories of the Mut

hyperaccurate strain is similar to the WT and that of the WT hypoaccurate is similar to that of the Mut.

All the evolved lines showed an increase in growth rate. As growth rates and protein production rates are correlated, we wanted to know if the degree of mistranslation, in particular frameshifting has changed over the course of evolution. This was done using *lacZ* reporter constructs. The aim was to know if the same degree of mistranslation is maintained over the course of evolution or whether mistranslation levels changed according to the stress regime.

We had a few predictions regarding the outcome of the evolution experiment:

1) Mutational trajectories of the WT and Mut may differ especially under fluctuating stress
2) If mistranslation is beneficial under stress, it should be maintained or increase, especially in fluctuating conditions
3) If mistranslation is costly in the absence of stress, its levels should decrease over the course of the evolution experiment

Material and methods

Part 1

Media and antibiotics

LB Broth (Difco) was used for liquid cultures while LB Agar (Difco) was used as solid media. Unless mentioned otherwise the cultures were incubated at 37°C at 180 rpm and plates were incubated at 37°C. MacConkey Agar (Difco) was used to differentiate strains based on lactose utilization. The following antibiotics were used in the study: Ciprofloxacin, Kanamycin, Ampicillin and Kasugamycin (Sigma). The antibiotics that were chosen as stressors were Ciprofloxacin (Cip), Kanamycin (Kan) and Ampicillin (Amp). The choice of antibiotics was such that all three would have non overlapping modes of action (Cip acts by the inhibition of DNA gyrase, Kan acts at the level of protein synthesis while Amp prevents cell wall synthesis). All of the antibiotics were used at sublethal concentrations so that the cells experience stress but the antibiotics are not lethal to the cells. Antibiotic concentrations were chosen after carrying out prior growth rate analysis. I chose the highest concentration of antibiotic at which both the strains grew without being completely inhibited. The antibiotic concentrations were as follows: Cip at 10 ng/mL, Kan at 2 µg/mL and Amp at 4.75 µg/mL.

Strains

The following strains have been used in this work: *E.coli* KL16 (referred in this study as WT) (Low, 1968), *E.coli* KL16ΔZWV (this strain which lacks three of the four initiator tRNA genes was generated in the lab) (referred in this study as Mut) were used for the evolution experiment. These specific strains were chosen as previous work in the lab has been done on these strains. *E.coli* TG1 strain was used for cloning purposes in the second part of the project.

Evolution experiment

WT and Mut strains were used in the evolution experiment. The experiment had three treatment regimes: No stress (only LB), Constant antibiotic stress (LB+Cip, LB+Kan, LB+Amp) and fluctuating stress in which any of the four conditions (LB,LB+Cip, LB+Kan, LB+Amp) can randomly occur every 12h. 1% culture (5 µL)

was transferred in every cycle. The evolution experiment involved six replicates each of the WT and the mutant in the constant conditions (Cip, Kan, Amp and LB) and eight replicates of the same in the fluctuating condition. A schematic of the experiment is shown in Figure 3.

Glycerol stocks were made every 4-5 days during the course of evolution. Growth curves were generated through Optical Density (OD) measurements which were taken at 600nm every few days in an optical reader (Tecan). This gave an estimate for increase in growth rate if any as a measure of adaptation to the medium. Glycerol

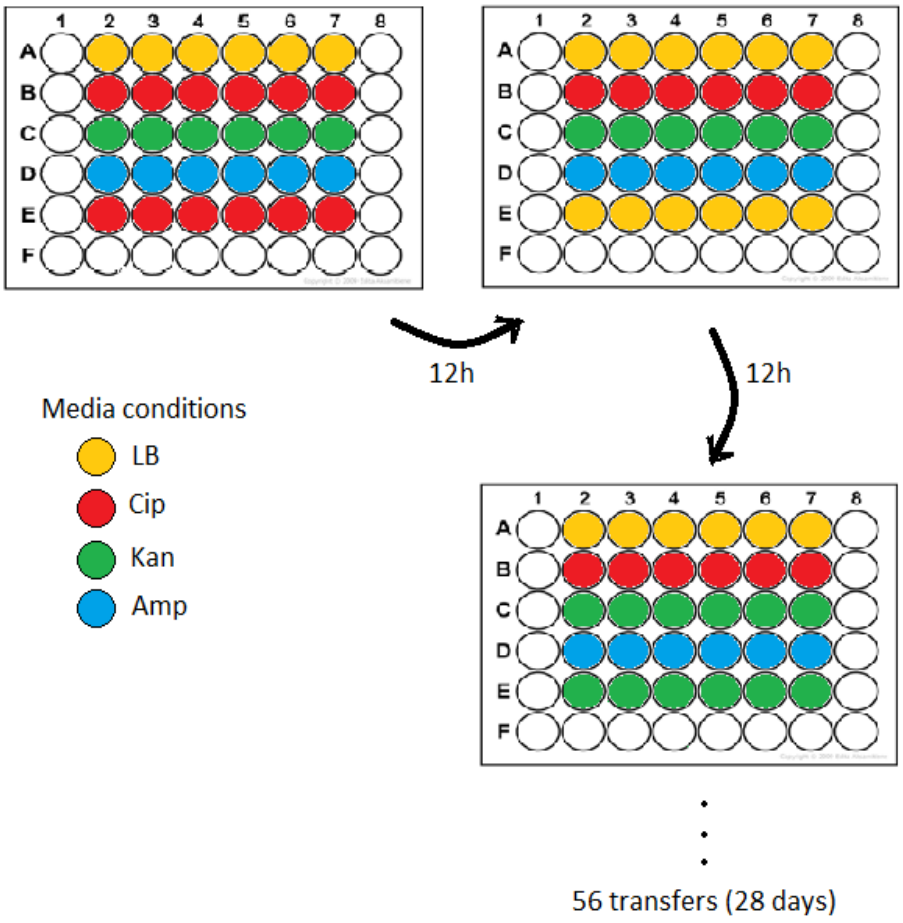

Figure 3: A schematic of the evolution experiment. Each colour represents different media conditions. Rows A-D are constant regimes while Row E represents the fluctuating regime where the media condition changes every 12 hours. The transfers were performed 56 times (28 days).

stocks were made every 5 days. Contamination check was done on the day of making the stocks. First the 64 lines were streaked on LA plates to confirm the absence of any

non-E.coli growth. Followed by this colony PCR of a few selected lines was done using the *metZWV* primers. The WT and the Mut gave bands of different sizes which allowed us to confirm the absence of any cross-contamination between the lines.

Qualitative resistance check

The OD readings gave an idea whether the strains were adapted to the conditions that they were evolved in, but to know whether an ability to grow at higher antibiotic concentrations had evolved, the strains were streaked on LA plates supplemented with antibiotics. Concentrations of antibiotic used were as follows: Cip in ng/mL (10, 25, 50), Kan in µg/mL (2, 10, 25) and Amp in µg/mL (4.75, 10, 50). It was seen that many of the strains (except those evolved in Amp) were able to grow at higher antibiotic concentrations. Hence, we decided to amplify and sequence the genes known to provide resistance to these antibiotics.

PCR amplification and Sanger Sequencing of Target Genes

Genes which were reported in literature to be providing resistance to the antibiotics of interest were chosen. They are as follows- Cip: *gyrA* (encodes a subunit of DNA gyrase) and *marR* (regulator of a drug efflux pump), Kan: *fusA* (encodes for elongation factor G), Amp: *envZ-ompR* (encodes for a sensory histidine kinase) (Lisa Yun Song et.al, 2016, Aalap Mogre et. al, 2014, Sun S et. al,2014, Okusu H et. al,1996). Both for the WT and the Mut, three colonies from three replicate lines from each regime were chosen for sequencing. This meant that 9 colonies each were chosen for the WT and Mut for each regime (there were a total of 4 regimes as plain LB regime was not included here). gDNA was isolated from each colony using Promega kit. Primers were designed for PCR amplification and for sequencing of these genes as indicated in Table1. After amplification PCR purification was performed using Genetix kit. Sanger sequencing was done at the NCBS sequencing facility. Sequencing results were analyzed using Snapgene. All alignments were done using the genes from the ancestral strains as the reference sequence.

Whole Genome Sequencing of the evolved populations

Genomic DNA was isolated from populations of the evolved replicate lines using GenElute Bacterial Genomic DNA Kit (Sigma). Sequencing was done using Illumina HiSeq Platform by the NCBS Sequencing Facility. Whole Genome Sequence analysis was done by Dr. Laasya Samhita.

Primers used in this study

Table 1: List of primers used in this study

Name of primer	Sequence (5' to 3')	Purpose
fusA.fl.rp	CGGTCAGAGTAGTTTTACCGTGG	To amplify fusA gene from E.coli MG1655
fusA.fl.fp	GGTTATCCCTTCGGAGTTTTAGTCACCAG	To amplify fusA gene from E.coli MG1655
acrB.fl.rp	GTTTTCGTATGAGATCCTGAGTTGGTGG	To amplify acrB gene from E.coli MG1655
acrB.fl.fp	TGGTGTCCAGGTAAAAGCACAAGAAG	To amplify acrB gene from E.coli MG1655
ompR,EnvZ.fl.rp	TGGTCCGAAACTGTAATGATTTGAAG	To amplify ompR and envZ gene from E.coli MG1655
ompR,EnvZ.fl.fp	CGCACATTGGGTATAACGTGATCATATC	To amplify ompR and envZ gene from E.coli MG1655
envZ_fp1	CCGGACGGCTCTAAAGCATGAGG	Sequencing primer (internal) of envZ gene
fusA_fp1	GTGTTCTCGATGGTGCGGTA	Sequencing primer

	ATGG	(internal) of fusA gene
gyrA.fl.fp	GACAAACGAGTATATCAGGCATTG	To amplify gyrA gene from E.coli MG1655
gyrA.fl.rp	TGCCACATTCCTTGTGTATAGCCAGCC	To amplify gyrA gene from E.coli MG1655
marR.fl.fp	CGATTTAGCAAAACGTGGCATCGGT	To amplify marR gene from E.coli MG1655
marR.fl.rp	GTGGCGATTCCAGGTTGTCCTCGAT	To amplify marR gene from E.coli MG1655
metZWV.fp	CACCGGCGTTAATCGGCGCGCCAA	To differentiate between WT and Mut during contamination check
metZWV.rp	GTCATTATGCTTGACCCTGGCCACGGTGGC	To differentiate between WT and Mut during contamination check

P1 transduction of evolved lines

To obtain strains in which the lacZ gene from the genome was knocked down. P1 transduction is used for the transfer of bacterial DNA from one donor cell to another recipient cell using the bacteriophage P1. Here P1 transduction was used to replace the lacZ gene with a Kanamycin cassette. A cell pellet from an overnight culture was resuspended in an equal amount of MC buffer(MC Buffer: 0.1M $MgSO_4$, 0.005M $CaCl_2$). 100µL of the cells was then mixed with 50 µL of the P1 lysate (raised on a strain containing the Kan cassette instead of the *lacZ* gene) and 50µL of MC buffer

(1:2 dilution). This mixture was incubated for 20 minutes at 37°C to allow for phage adsorption. The cells were then pelleted and resuspended in 0.1M citrate buffer. This step was repeated two times to remove any remaining phage from the cell surface. After the third wash, the cell pellet was resuspended in 1mL LB and 20mM Na citrate. This was incubated for 1 hour at 37°C to allow for expression of the Kanamycin resistance cassette. After the incubation time, the cell pellet was resuspended in 100uL of 0.1M citrate buffer. This was then plated with 5mM sodium citrate on LA+Kanamycin (25µg/µL) and incubated overnight at 37°C. The subsequent screening steps were done on LA+ Kanamycin (50µg/mL) (to confirm presence of Kan cassette) and MacConkey plates (to confirm the loss of *lacZ* gene)

Preparation of competent cells

Overnight culture of the desired strain was subcultured (1%) into fresh media (LB). After the OD reached to around 0.6, the cells were pelleted down at 4°C. The pellet was resuspended in 1/4th volume (of original volume) of RbCl I. It was allowed to stand in ice for 20 minutes. Followed by this the cells were pelleted again at 4°C and resuspended in 1/20th volume (of original culture volume) of RbCl II. Aliquots of the cells were made and flash freezing using liquid nitrogen was done.

Table 2: Composition of RbCl solutions

RbCl Solution I	For 200 mL
$RbCl_2$	2.4g
$MnCl_2$	1.98g
Potassium acetate (pH7.5, 1M)	6 mL
$CaCl_2$	0.3g
Glycerol	30mL
pH 5.8	
RbCl Solution II	For 100mL
3-(N-Morpholino)-Propanesulfonic Acid (MoPS) (0.5M, pH 6.8)	2mL
RbCl	0.12g

$CaCl_2$	1.1g
Glycerol	15mL
pH 6.8	

Transformation of the plasmids containing the *lacZ* reporter construct

2-3 µL of the plasmid was mixed with 100 µL of the competent cells. This mixture was kept on ice for 30 minutes. Heat shock was given at 42°C for 30 seconds after which the tube was moved back to ice for 5 minutes. 1 mL LB was added to the tube and it was incubated with shaking at 37°C for 1 hour. This allows for the expression of the antibiotic resistance marker. The cells were then plated on LA containing the desired antibiotic and incubated overnight at 37°C.

Generating cell extracts of the evolved lines for β galactosidase assay

Overnight culture was subcultured into LB containing Tet 7.5µg/mL . The cells were pelleted down at 4°C after 3 hrs of growth. The pellet was resuspended in soluble protein buffer containing protease inhibitor. The suspension was sonicated until the suspension became clear. The lysate was then centrifuged at 18000rpm at 4°C for 20 minutes. The supernatant was transferred into a new tube and was used for the subsequent assays.

lacZ reporter construct

The *lacZ* reporter constructs are a series of plasmids containing variants of the *lacZ* gene [O'Connor M et. al, 1992]. The WT version expresses β-galactosidase normally while protein expression happens in the +1 and -1 version only on +1 and -1 frameshifting respectively. A schematic for the constructs is given below.

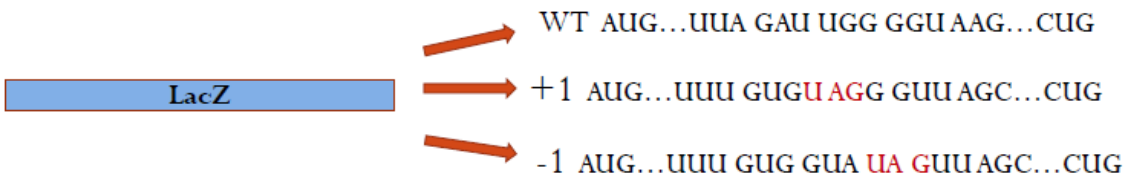

Figure 4: *lacZ* reporter construct. The three variants of the construct are WT, +1 and -1. B galactosidase expression happens in WT normally, in +1 only after +1 frameshifting and in -1 only after -1 frameshifting.

Measuring mistranslation using β galactosidase assay

Using Qubit fluorometer (Invitrogen), the amount of protein in the samples was quantified. 0.5µg, 10µg and 40µg of the protein were used in the assay for the strains containing psG lacZ WT, psG lacZ+1 and psG lacZ -1 respectively (This was previously standardized in the lab). In this assay ONPG (o-nitrophenyl-β-D-galactopyraniside) is the substrate which is used. β galactosidase cleaves ONPG and o-nitrophenol which is a coloured compound which absorbs at 420 nm is generated. The required amount of protein was added to 400µL Z buffer Z buffer composition (for 500 mL) is: 100mL 0.2M NaH_2PO_4, 150 mL 0.2M Na_2HPO_4, 5mL 1M KCl, 5 mL 0.1M $MgSO_4$. 35µL β-ME was freshly added to10mL Z buffer. 100µL ONPG (4mg/mL) was added to this mixture. The reaction mixture was incubated at 30°C. After yellow colour developed within the linear range of one hour the reaction was stopped by adding 250µL Na_2Co_3. The time of colour development was recorded. OD measurements were then taken at 420nm. This absorbance value is used to quantify the degree of frameshifting.

Part 2

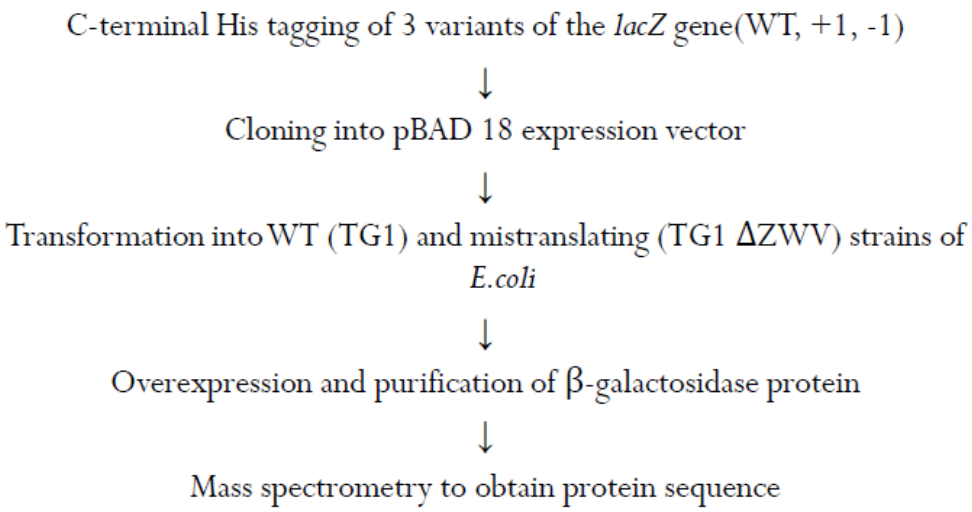

Figure 5: Protocol for overexpression and purification of the *lacZ* protein

In the first part of the project, variants of the l*acZ* gene (WT, +1 and -1) (O'Connor M et. al, 1992) have been used which upon frameshifting give rise to the production of functional beta-galactosidase protein. The reporter construct has been described in Figure 4. We have started by C terminal His tagging of the l*acZ* construct using the following oligonucleotides: forward primer 5' AGCGGCTAGCAACAGAATTCACCATGATTACG 3' and reverse primer 5' GCACCCCGGGTTAATGGTGATGGTGATGATGTTTTTGACACCAGACCA 3' in which NheI and SmaI restriction sites were included. This construct was cloned into pBAD18 expression vector and clones have been obtained which were confirmed by sequencing. The constructs were transformed into the WT and mistranslating strains.

Results and Discussion:

Part 1:

Growth rates of the evolved lines

We measured growth rate for the evolution lines every few days during the course of the experiment. Figure 6A shows the growth rates of the Wt and the mistranslating (Mut) strain under the four conditions mentioned before: LB, Cip, Kan and Amp, normalized to the growth rate of the WT ancestral strains in LB. Data for both the ancestral and the final growth rate has been included in this figure. The growth rate of the wild type in LB has not changed over the course of evolution as is expected (The wild type strain is already optimized to grow in LB). The mistranslating strain has a growth defect to start off with which is rescued in part during the course of evolution. The growth defect may be due to lower protein production caused by decrease in tRNA content in the cell or due to the cost of mistranslation. In all the stress conditions an increase in growth rate is seen both in the WT and the Mut strains across the course of evolution. The extent of increase in growth rate is different in the different conditions and is indicated in the figure below. The relative increase in growth rates is shown by normalising the final growth rates by the ancestral growth rates in all the conditions in Figure 5B. The increase in growth rates for the WT strain in the stress conditions is 24% for Cip, 58% for Kan and 10% for Amp on average. The growth rate of the mistranslating strain in LB increased by 14% on average, probably to compensate for the growth defect it had at the beginning. The mutant strains evolved in Kan and Amp showed an increase in growth rate compared to Mut LB (24% and 17% respectively), but those evolved in Cip shows comparable final growth rate increase (average14.8%)

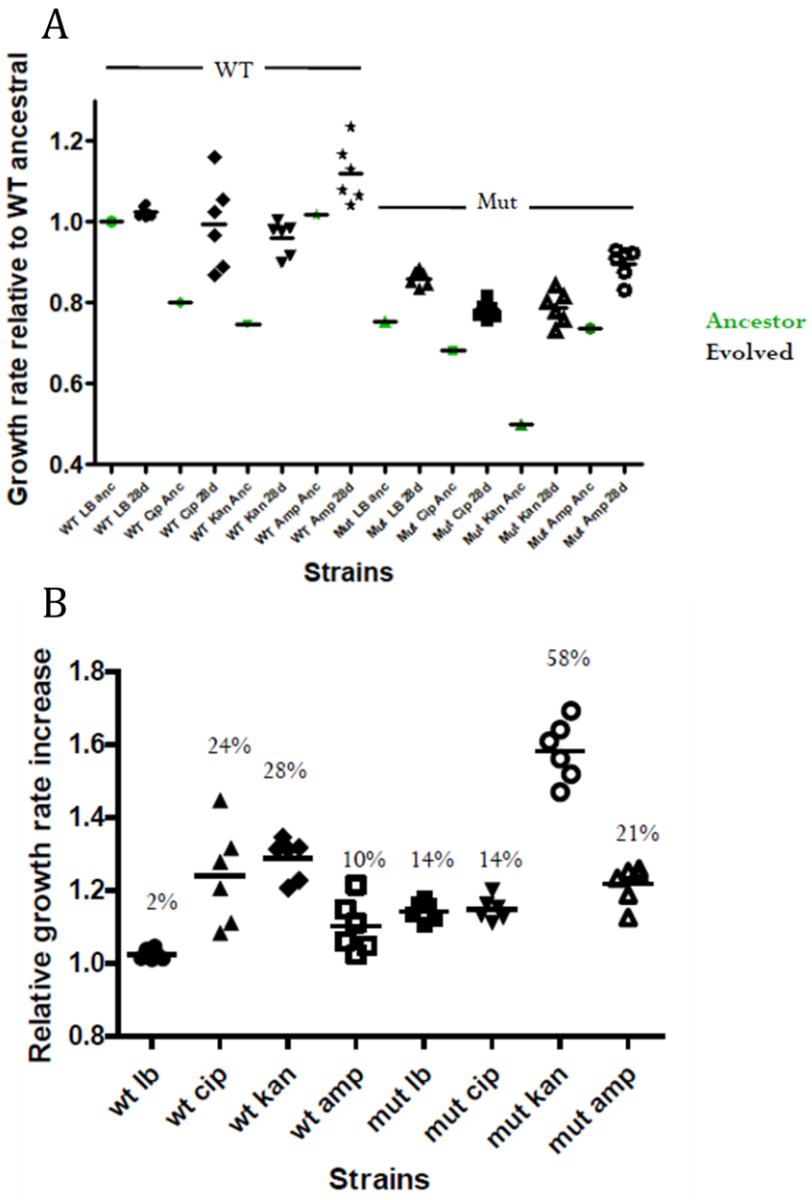

Figure 6: (A) Growth rates of the unevolved and evolved strains in the different antibiotic regimes normalized to the WT ancestral growth rate in LB. (B) Relative increase in growth rate for strains evolved in different antibiotics normalized to its ancestral growth rate in its respective antibiotics.

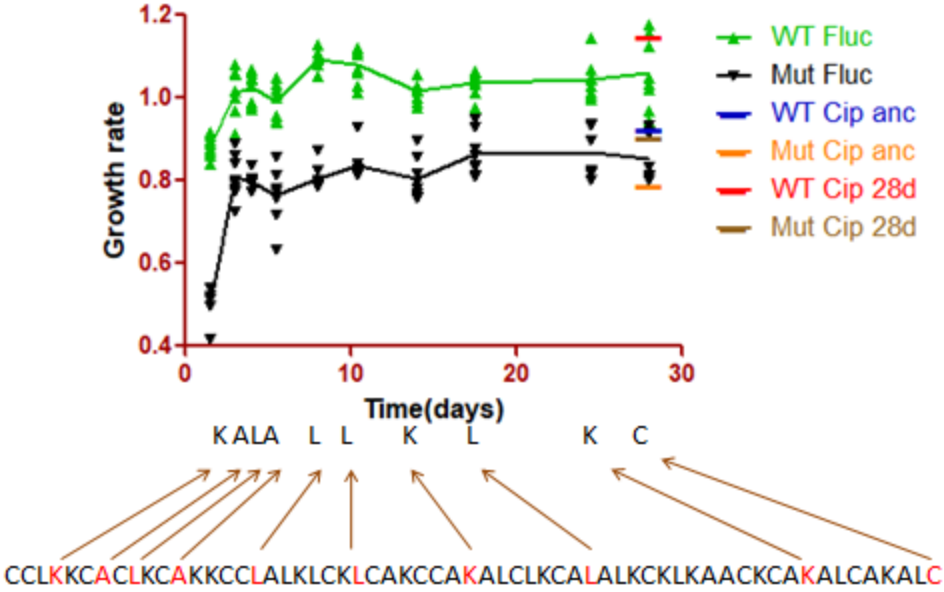

Figure7: Growth rates for the WT and Mut in fluctuating stress over the course of the evolution experiment. The stress conditions at which growth measurements were taken have been highlighted in red. Growth rates for the WT and Mut in constant LB and Cip stress have been plotted for reference. (C: Cip, K: Kan, A: Amp, L: LB)

The growth rates of the WT and the Mut in fluctuating stress over the course of the evolution experiment are plotted in Figure 7. The mean growth rate within the strains in fluctuating stress seems to stabilize to a constant level which may suggest the presence of a common resistance mechanism like efflux pumps for all the antibiotics. Interestingly the growth rate in LB for the mutant in fluctuating stress has not increased as it does for the mutant evolved in constant LB condition.

Qualitative check for resistance

To know whether the strains have developed resistance to the antibiotics that they were evolved in, I streaked them on LA plates with antibiotic concentrations used during the evolution experiment and higher concentrations. All the strains grew on the concentration of antibiotic they were evolved in. In Table 3, I have shown data for the highest concentration on which the strains were able to grow.

Table 3: Results of qualitative resistance check. (There are 6 replicate lines in constant stress regime and 8 in fluctuating stress regime. Blank cells indicate no replicates)

(++ high growth, + low growth, - no growth)

Cip	WT	Mut	WT fluc	Mut Fluc
Concentration (ng/mL)	50	50	50	25
Replicate no				
1	++	++	++	+
2	++	++	++	+
3	+	++	++	+
4	++	++	++	++
5	+	++	++	++
6	++	++	++	+
7			+	+
8			++	
Positive control	++	++	++	++
Negative Control	-	-	-	-
Kan	WT	Mut	WT fluc	Mut Fluc
	25	25	25	25
Replicate no				
1	++	+	+	-
2	++	++	+	+
3	++	+	+	-
4	++	++	+	+
5	+	++	+	-
6	++	+	+	-
7			+	+
8			+	
Positive control	++	++	++	++
Negative Control	-	-	-	-
Amp	WT	Mut	WT fluc	Mut Fluc
Concentration (µg/mL)	10	10	10	10
Replicate no				
1	+	-	++	+
2	-	-	++	+
3	+	-	++	+
4	+	-	++	++
5	-	-	++	++
6	-	-	++	+
7			++	++
8			++	
Positive control	++	++	++	++
Negative Control	-	-	-	-

The strains evolved in Cip and Kan have evolved the ability to grow on antibiotic doses 5 times and 10 times of the concentration that they were evolved in respectively. For Cip and Kan, the strains evolved in fluctuating stress show lower resistance than those which were evolved in constant stress. But it seems that overall the mistranslating strain

has lower resistance than the WT strain in the constant and fluctuating regimes. The strains evolved in Amp have not developed increased resistance.

Sanger Sequencing results:

We wanted to test whether the evolved lines had obtained any mutations in the genes that were common targets for the antibiotics used in the experiment. To do this the genes *gyrA* and *marR* were PCR amplified for the Cip evolved lines, *fusA* for the Kan evolved lines, *envZ* for the Amp evolved lines and the genes *gyrA*, *marR*, *fusA* and *envZ* for the lines evolved in fluctuating stress. The gene sequences were aligned to those in the unevolved ancestral strains. The sequence alignments were done in Snapgene. The results for the mutations have been shown in the tables below. *gyrA* mutations have been highlighted in blue, *marR* mutations in red, *fusA* mutation in green and *envZ* mutation in yellow.

Table 4: *gyrA* and *marR* mutations identified from Sanger sequencing of Cip and fluctuating stress evolved strains

Stress		Replicate line	Colony Number	*gyrA* mutation	*marR* mutation
Cip	WT	1	1	241 GGT-TGT (G81C)	
			2	241 GGT-TGT (G81C)	
			3	241 GGT-TGT (G81C)	
		2	1	247-249 deletion	
			2	247-249 deletion	
			3	No mutation	251 GTA-GAA (V98E)
		3	1	No mutation	251 GTA-GAA (V98E)
			2	No mutation	251 GTA-GAA (V98E)
			3	No mutation	No mutation
	Mut	1	1	No mutation	60bp insertion at nt 234

			2	No mutation	60bp insertion at nt 234
			3	No mutation	60bp insertion at nt 234
		2	1	No mutation	No mutation
			2	No mutation	No mutation
			3	No mutation	No mutation
		3	1	No mutation	Deletion at nt 88,89
			2	No mutation	Deletion at nt 88,89
			3	No mutation	Deletion at nt 88,89
Fluctuating	WT	1	1	No mutation	293 GTA-GAA (V98E)
			2	No mutation	293 GTA-GAA (V98E)
			3	No mutation	293 GTA-GAA (V98E)
		2	1	No mutation	293 GTA-GAA (V98E)
			2	No mutation	293 GTA-GAA (V98E)
			3	No mutation	293 GTA-GAA (V98E)
		7	1	No mutation	No mutation
			2	No mutation	No mutation
			3	No mutation	No mutation
	Mut	3	1	No mutation	No mutation
			2	No mutation	No mutation
			3	No mutation	No mutation
		4	1	No mutation	No mutation
			2	No mutation	No mutation
			3	No mutation	No mutation
		5	1	No mutation	No mutation
			2	No mutation	No mutation
			3	No mutation	No mutation

To summarize the results in Table 4, the WT strain in Cip stress had a combination of *gyrA* and *marR* mutations while the Mut had only *marR* mutations in two lines while the third line had neither *marR* or *gyrA* mutations. In fluctuating stress, 2/3 lines have only *marR* mutations. None of the Mut lines have either *marR* or *gyrA* mutations.

Table 5: *fusA* mutations identified from Sanger sequencing of Kan and fluctuating stress evolved strains

Stress	Strain	Replicate line	Colony Number	Mutation in *fusA*
Kan	WT	1	1	2033 GCA-GTA(A678V)
			2	2033 GCA-GTA(A678V)
			3	1310 G-C
		2	1	No mutation
			2	2033 GCA-GTA(A678V)
			3	2033 GCA-GTA(A678V)
		3	1	No mutation
			2	No mutation
			3	No mutation
	Mut	2	1	1823 GCG-GAG (A608E)
			2	1823 GCG-GAG (A608E)
			3	1823 GCG-GAG (A608E)
		3	1	No mutation
			2	No mutation
			3	No mutation
		4	1	No mutation
			2	No mutation
			3	No mutation
Fluctuating	WT	1	1	No mutation
			2	No mutation
			3	No mutation
		2	1	No mutation

			2	No mutation
			3	No mutation
		7	1	No mutation
			2	No mutation
			3	No mutation
	Mut	3	1	No mutation
			2	No mutation
			3	No mutation
		4	1	No mutation
			2	No mutation
			3	No mutation
		5	1	No mutation
			2	No mutation
			3	No mutation

In Kan stress 2/3 WT lines have fusA mutations while only 1/3 Mut lines have fusA mutations (Table 5). In the fluctuating stress regime neither the WT nor the Mut have any fusA mutations.

For ampicillin, *envZ* mutations were found in only one replicate line. All other lines had no mutations or sequencing quality for these lines was not good. The observed mutations have been shown below.

Table 6: *envZ* mutations identified from Sanger sequencing of Amp and fluctuating stress evolved strains

		Replicate line	Colony Number	
Amp	WT	1	1	No mutation
			2	No mutation
		2	1	No mutation
			2	No mutation
			3	No mutation
		3	1	No mutation

			2	152 GCG-GTG (A51V)
			3	152 GCG-GTG (A51V)
	Mut	1	3	No mutation
		2	1	No mutation
			2	No mutation
			3	No mutation
		3	1	No mutation
			2	No mutation
Fluctuating	WT	1	1	No mutation
			2	No mutation
			3	No mutation
		2	1	No mutation
			2	No mutation
			3	No mutation
		7	2	No mutation
	Mut	4	1	No mutation
		5	3	No mutation

In many of the replicates, especially those evolved in the fluctuating conditions, there were no mutations in the target genes. This may be because the mutations may have occurred in other genes or else mutations may have occurred in the region of the gene other that those chosen for sequencing. This was especially true for longer genes. Yet there was an increase in resistance to the antibiotics. Thus to identify such mutations whole genome sequencing of all the replicate lines was performed. Analysis and compilation of the whole genome sequencing data was done by Dr. Laasya Samhita. Figure 8 summarizes the sequencing data obtained from Sanger sequencing and Whole Genome Sequencing across all the lines. In constant Cip condition, the WT lines mostly have *gyrA* and *marR* mutations, while the mutant has no *gyrA* mutations but only *marR*/*gyrB* mutations. In Kanamycin all the WT lines have *fusA* mutations while in the Mut only 2/6 lines have this mutation. In the fluctuating condition, majority of the WT

lines have *marR*/*gyrB* mutations while others have *gyrA* and *fusA* mutations. In the Mut lines evolved in fluctuating stress, none of the lines have any of the mutations for the target genes. Instead it has mutations in *cpxA* gene which is a sensory histidine kinase

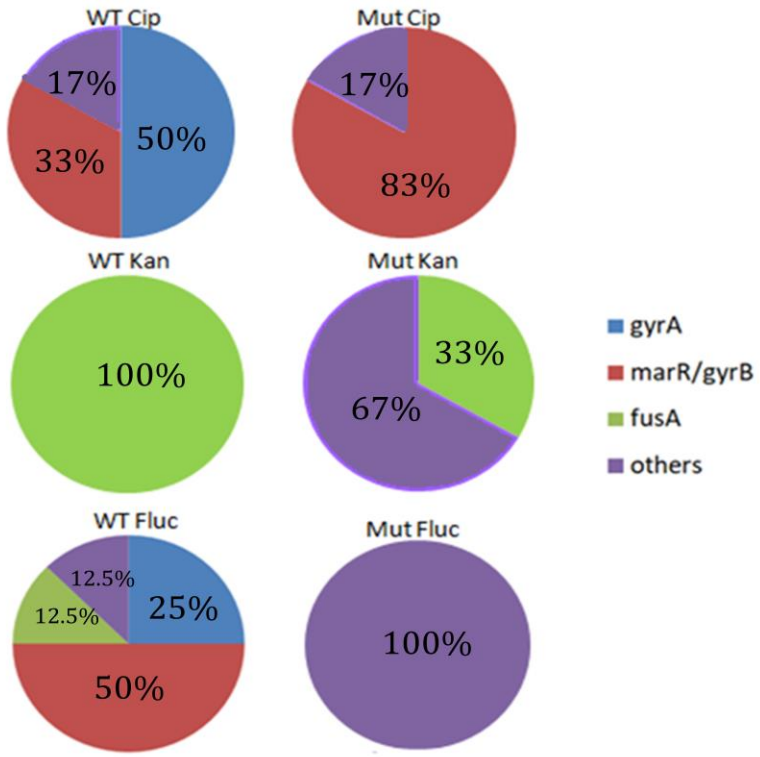

Figure 8: Comparison of distribution of mutations in WT and Mut in Cip, Kan and fluctuating stress across lines. In constant Cip condition, the WT lines mostly have gyrA and marR mutations, while the mutant has no gyrA mutations but only marR/gyrB mutations. In Kanamycin all the WT lines have fusA mutations while in the Mut only 2/6 lines have this mutation. In the fluctuating condition, majority of the WT lines have marR/gyrB mutations while others have gyrA and fusA mutations. In the Mut none of the lines have any of the mutations for the target genes.

In the growth rate measurements we had observed that the relative increase in growth rate for the WT strain evolved in LB across the course of the evolution experiment was negligible (Figure 6B) but the mutant strain showed an appreciable increase in fitness across replicate lines. This may be because the mutant started at a lower fitness. Growth rates are positively correlated with protein production rates. So we had speculated that Mut in LB may have obtained mutations in the remaining initiator tRNA gene or the promoter region upstream to it because initiator tRNA level is the limiting

factor in protein production rate in the mutant. Sequencing results for two of the WT strains did not reveal mutations in any of the target genes as is expected. However, the mutant strain evolved in LB had large portions of its genome deleted (~70000bp). We still have to confirm this deletion by PCR amplification.

β galactosidase assay to measure mistranslation assay for the evolved strains:

Over the course of the evolution experiment we saw that the growth rates of the strains increased. It was of interest to see whether the levels of mistranslation had decreased (because mistranslation can slow down growth) or increased (mistranslation could be advantageous under stress). In the ancestral strains as the mutant shows -1 frameshifting as compared to the WT, we tested frameshifting levels in the evolved strains. For this we first knocked out the *lacZ* gene from the genomes of the evolved strains and then transformed the 3 *lacZ* constructs described before into them. β galactosidase assay was performed using ONPG as the substrate. We observed that the absolute β galactosidase activity with WT *lacZ* had increased in the evolved strains. So we normalized the β galactosidase activity for each strain with that of the psG *lacZ* WT activity in that strain.

For the mutant lines evolved in fluctuating stress we had three biological replicates. The β-galactosidase activity in these lines was normalized to psG WT activity in the WT fluctuating lines because we were not able to obtain psG WT strains for the latter. We find that the degree of -1frameshifting in two of the lines has increased significantly while it has decreased in the third line (Figure 9). The third line (Mut f7) is the one which has a mutation in the S12 +1 frameshifting has decreased in all the lines. It has been shown in literarure that S12 mutations can lead to hyperaccurate translation (Uwe von Ahsen, 1998). Mutation in the same protein has been made to obtain the hyperaccurate strain used in our study. We used two tailed unpaired t test to calculate significance (n=6).

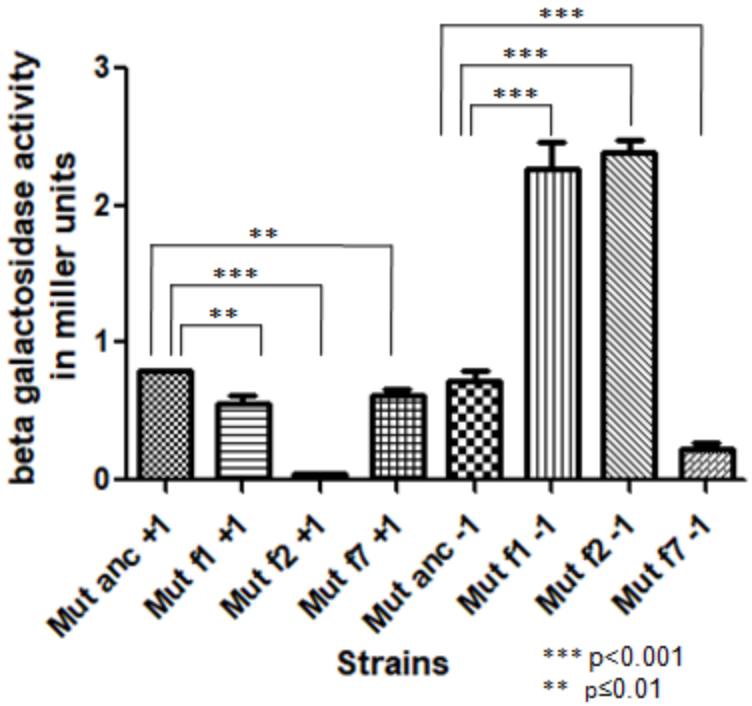

Figure 9: Relative levels of +1 and -1 frameshifting in the Mut evolved in fluctuating stress normalized to the WT *lacZ* activity. Unpaired two tailed t test. α=0.99, n=6

For rest of the evolved lines we have only one biological replicate. The +1 frameshifting activity in one of the Mut strains evolved in LB has increased. Interestingly, this is the only strain of the 6 which doesn't have the large deletion present. The +1 frameshifting activity in one of the WT and the Mut fluctuating stress evolved strains seem to have increased. Right now we only have one biological replicate for each strain. Thus we cannot comment on the significance of these results until we obtain results from biological replicates.

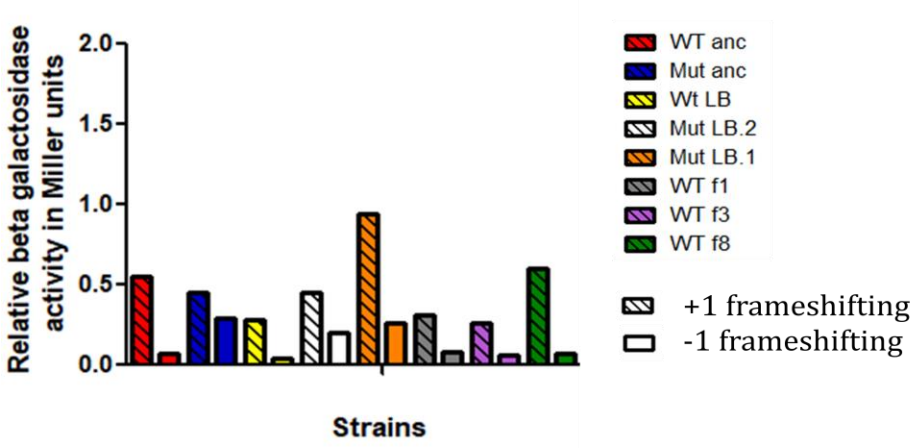

Figure 10: Levels of +1 and -1 frameshifting in the evolved strains.

Part 2:

The three variants of the *lacZ* gene were cloned with a C terminal His tag into the pBAD18 vector. The construct was then transformed into the WT and mistranslating (mutant) *E.coli* TG1 strain. We tried to overexpress β galactosidase protein by induction with L-arabinose. However we were unable to visualize overexpression on a polyacrylamide gel. However on a western blot we were able to observe expression of the protein of interest. Next we plan is to purify the His tagged protein from a large volume of culture so as to obtain enough protein for mass spectrometry.

Discussion:

The frequency of mistranslation in the cell is high as compared to replication errors even though there are several proofreading mechanisms in place to limit this occurrence. Every cell faces tradeoffs between accurate protein production and the efficiency or the speed with which translation happens (Magnus Johansson et al. 2012).

In our study we have used a strain with depleted initiator tRNA content. This strain mistranslates by -1 frameshifting and non AUG initiation. Unpublished work in the lab has shown that this mistranslating strain has higher survival under certain stresses, particularly Ciprofloxacin, heat stress and nutrient starvation. However, this phenotypic resistance was observed in a short time scale. We wanted to know whether this short term advantage could hold under long term constant and fluctuating stress.

We chose to use antibiotics stress in this study because it is pervasive in clinical settings. Sublethal concentrations of antibiotics may be physiologically relevant because during treatment, irregular or incomplete antibiotic regimes can lead to the development of resistant bacteria. In *E.coli* it has been shown that long term exposure to sub inhibitory antibiotic concentrations could lead to increased levels of ROS and thus higher mutation rates (Dwyer et al., 2009; Sengupta et al., 2013). This can lead to multi drug resistance and the selection of low cost mutations which may provide high resistance.

We used subinhibitory concentration of antibiotics Cip, Kan and Amp. The WT and the Mutant were evolved in constant and fluctuating stress regimes as described before. We saw an increase in growth rates for all replicate lines across the course of evolution which suggested that the replicate lines were adapted to the stress.

There was an increase in the level of resistance to Cip and Kan. The level of resistance for strains evolved in fluctuating stress was lower than that evolved in constant stress which may suggest that evolving in fluctuating stress impedes the development of resistance which has been shown in previous reports (Li L et. al, 2011, Kramer et. al, 2007). This may also be because of evolution of a common resistance mechanism to all antibiotics (such as efflux pumps) which provides low level resistance to multiple antibiotics.

Sequencing results revealed that in constant Cip conditions the WT mostly showed *gyrA* and a few *marR* mutations. This is expected as gyrA is the first target of choice to obtain ciprofloxacin resistance. In contrast to this, the mutant had no *gyrA* mutations but only *marR* and *gyrB* mutations. A similar pattern has been observed under short term Cip stress. Mutations in *marR* which is a regulator of efflux pumps can have large fitness costs and the increase in resistance due to these mutations is not as high as *gyrA* mutations (Marcusson et al., 2009). But efflux pump mutations could give resistance to multiple antibiotics. *marR* mutations are costly because *marR* is a transcriptional regulator of many genes and thus mutations in this gene are bound to affect a lot of genes. Also due to upregulation of efflux activity many compounds which are essential for the cell may be pumped out along with the antibiotics. Considering these facts, the occurrence of marR mutations in the mutant, especially in constant stress conditions is surprising.

Mutations can also have different fitness effects in different genetic backgrounds in a phenomenon known as epistasis (Sackton et al., 2016). This may also explain why different mutations arise in the two strains. Thus it may be possible that *marR* mutations are epistatic with the ΔZWV genotype or that the effect of marR mutations and phenotypic diversity due to mistranslation are responsible for the increased resistance of the Cip evolved strains.

For the Kan evolved strains, all the WT strains had *fusA* mutations while only one third of the Mutant lines harboured these mutations. The increase in resistance was similar for both the WT and Mut. Thus some of the mutant lines are able to survive on similar Kan concentrations as the WT without having any *fusA* mutations.

The Amp evolved strains did not show an increase in resistance and only one evolution line had target gene (*envZ*) mutations. The Ampicillin was later found to be degraded. I have repeated the evolution of the WT and mutant strains in Amp and fluctuating stress. The sequencing of these strains is ongoing.

In fluctuating stress, the difference in the pattern of mutations is even starker. The WT has mutations in the target genes (*gyrA, marR, fusA, gyrB*) while the mutant has no mutations in any of these genes. However from the resistance data it can be seen that the mutant has an increase in resistance as compared to its ancestor. Thus looking at

the mutation trajectories and the resistance data it may be possible that mistranslation is helping the mutant to adapt to the fluctuating antibiotic stress without having to have any target mutations.

Thus the first prediction that the WT and the mutant will follow different mutational trajectories under stress was confirmed.

It could also happen that the mutation spectrums of the two strains under study are different. This will cause different mutations to arise in the two strains in the different regimes. This hypothesis is now being tested in the lab.

We are further testing the hypothesis that mistranslation is causing the difference in mutation patterns by evolving hyperaccurate (mutant will have lower translation errors) and hypoaccurate (WT will have more translation errors) strains of the WT and by doing similar sequencing analysis. It will also be interesting to sequence strains from intermediate time points, especially for the Cip evolved and fluctuating strains to see which mutation occurred first and whether there was any competition between *gyrA* and *marR* mutations in the beginning such that eventually only one of the mutations got fixed in the population.

The growth rate of the mutant evolved in LB also increased. It is important to see whether any mutations have come up in Mut LB which, are involved in its fitness increasing and whether similar mutations are seen in the strains evolved in stress conditions. This will help in dissecting the adaptation for increase in growth rate versus adaptation to an antibiotic. Sequencing results showed that these strains had large deletions (~60,000 bp). The position and the size of these deletions were consistent across the replicate lines, which suggests that this may be an adaptive response to reduce the fitness cost of the *metZWV* deletion.

From the β galactosidase assay we observe that -1 frameshifting in two of the mutant strains evolved in fluctuating stress has increased more than two fold. Therefore in mutant strain evolved fluctuating stress the absence of target gene mutations and increase in mistranslation (-1 frameshifting) co-occur. This data agrees with the second prediction which is that mistranslation levels should be maintained or should increase under stress. However more work needs to be done to establish this causation.

From our study it seems that the adaptive trajectories followed by the different replicate populations varied because of mistranslation especially in fluctuating stress regime. However further work with the hyperaccurate and hypoaccurate strains and checking levels of mistranslation in the evolved lines will help in making this claim stronger.

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