Maximizing the expression of the MutT4 enzyme in *E.coli*

A Major Qualifying Project
Submitted to the Faculty of
Worcester Polytechnic Institute
In partial fulfillment of the requirements for the
Degree in Bachelor of Science

In

Biochemistry

By
Matthew Cochran

`

Date: 12Aug24

Project Advisors: Elizabeth Bafaro (CBC) and Louis Roberts (BBT)

Maximizing protein expression in bacterial systems requires a deep understanding of the regulatory mechanisms influencing mRNA expression and protein yield. This project focuses on optimizing protein expression by manipulating the lac operon system in *E. coli* and investigating the impact of various factors on protein stability and yield. The research primarily centers on the MutT4 enzyme from *M. smegmatis*, which was genetically modified to include a translational ramp sequence aimed at enhancing protein stability. Despite successful initial PCR reactions and Gibson Assembly, attempts to integrate the translational ramp sequence into the MutT4 plasmid were ultimately unsuccessful. However, optimal conditions for protein expression were determined through experiments involving temperature and IPTG concentration variations. The results indicated that a temperature of 28°C and IPTG concentration of 0.5 mM yielded the highest levels of MutT4 enzyme. Further attempts to improve expression through environmental factors such as metal-ion cofactors and pH adjustments were inconclusive. This project highlights the importance of optimizing both genetic constructs and environmental conditions to achieve high levels of protein expression in bacterial systems.

Table of Contents

1. Introduction

Understanding the regulatory mechanisms of protein expression in bacterial genomes is essential in determining the most prominent factors for maximizing mRNA expression and protein yield. The greatest challenges in maximizing protein expression arise in the structure of the bacterial operon, composition of the genetic sequence, and the chemical and cellular environments where proteins are expressed.[1] For most *E.coli*-based protein synthesis mechanisms, a traditional genetic modification of the *lac* operon is used to maximizing protein yield in *E.coli* colonies.[2] The genetic sequence of the *lac* operon allows for tightly regulated protein expression through inducible repressor control. Additionally, the T7 RNA polymerase promoter in the operon contains the sequence to bind the T7 RNA polymerase integrated elsewhere in the *E. coli* genome, (5′TAATACGACTCACTATAG 3′), allowing for the high fidelity binding and transcription of the T7 polymerase.[2] Additionally, the promoter region contains the binding sequence for the inducible LacI repressor protein, which binds to the promoter, blocking access for the T7 polymerase to initiate the transcription of the desired gene.[3] In a traditional bacterial cell, allolactose binds to the repressor in the presence lactose, inducing a conformational change on the repressor. This conformational change causes the repressor to detach from the promoter, allowing for the transcription of the LacZ, LacY, and LacA proteins.[3] While the allolactose-based repressor mechanism is optimal for the degradation of lactose, it is not ideal to use as an inducer for controlled expression of specific proteins in a laboratory setting due to its degradation by the cell.[2] Instead, isopropyl β-D-1-thiogalactopyranoside (IPTG) is used to regulate gene expression in *E.coli* cells, allowing for the controlled expression of desired protein through variations of IPTG concentration.[2]

In order to maximize protein expression at high levels, the bacterial cells must be in an environment where the mRNA is not easily degraded by the cell. Additionally, the expressed protein must be stable and exhibit high solubility in the medium in order to be expressed and detectable at high concentration. There are several different stress-response factors to consider which can affect the regulation and stability of mRNA in the cell post-transcription by increasing or decreasing the catalytic activity of RNase degradation enzymes. Among these factors include: temperature, mRNA abundance, oxygen limitation, and nutrient starvation.[4] Understanding how RNase activity changes due to specific stress conditions surrounding temperature is important for discovering the optimal temperature for maximizing mRNA expression and protein production. In one particular experiment, researchers cold shocked *E.coli* cells to determine the effects of low temperatures on RNase R expression.[5] The researchers discovered that there was a direct increase in RNase expression for samples that were cold shocked at 10°C compared to the cells that remained at 37°C.[5] Additionally, cells that were transferred to a 15°C and a 25°C environment also exhibited higher RNase R expression levels, suggesting lower temperatures increase mRNA degradation enzyme activity.[5]

Increasing protein stability and expressivity can also be achieved through genetic manipulation by the insertion of translational ramp sequences. Due to protein synthesis being a rate-limiting step for protein synthesis, incorporation of specific amino acids at codons 2-5 has shown to help increase the stability of protein elongation and increase the rate at which proteins are synthesized.[6] In particular, one experiment conducted shown that the incorporation of the amino acids, Phe, Ile, Gly, and Lys at codons 2-5 helped increase ribosome processivity demonstrated by increased fluorescence intensity at the fifth codon.[6]

The MutT enzyme is a catalytic bacterial protein expressed in the *M.tuberculosis* and *M. smegmatis* genome, responsible for recognizing and hydrolyzing the mutant nucleotides of 8-oxo-deoxy-guanosine triphosphate (8-oxo-dGTP) and 8-oxo-guanosine triphosphate (8-oxo-rGTP), preventing their insertion into the bacterial genome.[7] There are multiple mutant forms of the MutT enzyme ranging from MutT1 to MutT4. For this project, we examined the MutT4 gene isolated from the *M. smegmatis* genome and inserted into an E.coli bacterial plasmid to determine the optimal conditions for maximizing the enzyme's expression.[8] For this project, the variation of temperature and IPTG concentration were the two primary factors examined for determining the optimal conditions for limiting mRNA degradation and maximizing protein expression of the MutT4 enzyme. The translational ramp sequence will also be incorporated through a mutagenesis reaction in an attempt to increase the enzyme’s stability and enhance its expression.

2. Methods

2.1. Designing a set of primers to perform mutagenesis reaction

A set of primers was designed using Benchling to amplify the bacterial plasmid and insert the translational-ramp stabilization sequence at the transcription start site of the MutT4 gene. The primers are a set of forward and reverse primers containing annealing sequences along with the mutated translational-ramp sequence. The primers used for the PCR reaction are identified below. The annealing sequences are indicated by lower-case letters and the sequence to insert the translational ramp is indicated by capital letters. The red letters indicate the overlapping bases in the plasmid to direct NEB HiFi assembly.

Forward Primer: 5' - atgTTTATTGGCAAAcatcaccatcaccatcacattgagggac - 3'

Reverse Primer: 5' - tgTTTGCCAATAAAcatatgtatatctccttcttaaagttaaacaa - 3'

To calculate the annealing temperature of the primers, NEBioCalculator was used to determine the melting temperature of the annealing sequence. For the forward primer, the melting temperature was determined to be 71°C whereas the reverse primer had a melting temperature of 60°C.

2.2. PCR reaction to replicate MutT4 plasmid and insert the ramp sequence.

After the set of primers was designed, two 50 microliter PCR reactions were performed to replicate the MutT4 template plasmid and insert the mutated sequence. The PCR was initially assembled by adding 40ng of MutT4 template to each PCR tube. 0.5 micromolar concentrations of the forward and reverse primers were added to each tube along with NEB 2X Q5 Master Mix.

Two separate parameters were then programmed into the thermocycler to perform separate PCR reactions. Both PCR reactions ran with an initial denaturation temperature of 98°C for 30 seconds. Both reactions were programmed to run 30 cycles of denaturation, annealing, and extension. The denaturation conditions were set to 98°C for 30 seconds and extension at 72°C for seven minutes. The annealing conditions for the first PCR reaction used a touchdown approach, running at 60°C for the first five cycles and dropping down to 55°C for the remaining 25 cycles. The annealing temperature for the second reaction remained constant at 52°C for 30 cycles. A final extension was programmed for both reactions running at 72°C for 2 minutes.

When the PCR reactions were complete, an electrophoresis separation was performed on each sample to confirm if the PCR reactions were successful. The gel was then imaged using a BioRadimaging apparatus.

After the gel displayed the MutT4 PCR reaction was successful in replicating the 4.5kb plasmid using the touchdown approach, the PCR tube was prepared for Dpn1 restriction enzyme digestion. 4 microliters of Dpn1 enzyme along with 6 microliters of 10X CutSmart buffer was added to the tube. The digestion reaction was carried out in a thermocycler at 37°C for 30 minutes and 80°C for 20 minutes.

Upon completion of the digestion, the DNA was purified via PCR cleanup column purification using a MacheneryNagel PCR purification kit. The purified DNA product was then analyzed using a Nanodrop. The final concentration of the purified PCR product was determined to be 20.2 ng/uL.

2.3. Gibson Assembly reaction and transforming the mutated MutT4 plasmid

After PCR purification was completed, the mutated MutT4 plasmid was prepared to be assembled into competent cells. To ensure proper assembly, a positive and negative control sample were each assembled. For the positive control sample, 50 ng (2.5uL) of mutated MutT4 plasmid was added to a collection tube along with 2.5 microliters of nuclease free water and 5.0 microliters of 2X assembly master mix/ For the negative control sample, 50 ng of mutated MutT4 plasmid was added to a collection tube along with 7.5 microliters of nuclease free water. Both samples were incubated in a water bath at 50°C for 20 minutes. After the incubation was completed, 2 microliters of the experimental and negative control samples were transformed into separate tubes of competent *E.coli* cells. The tubes were then placed on ice for 5 minutes followed directly by a water bath at 42°C for 45 seconds. The samples were then placed back on ice for two minutes. 300 microliters of SoC outgrowth medium was added to both samples. The positive and negative control samples were then added to LB + kanamycin plates and were allowed to incubate overnight at 37°C. The following morning, 5 colonies from the positive control plate were sent for sequencing.

2.4. Determining the optimal conditions for expression and total protein assay.

In order to determine the optimal conditions for MutT4 protein expression, seven different cultures were developed with varying temperatures and induction concentration. The stock culture was developed by transforming the MutT4 plasmid into the BL21 DE3 pLysS E. coli cells. The *E.coli* colonies were grown overnight on LB + kanamycin plates at 37°C. The following morning, 7 colonies were selected and inoculated into a single overnight culture, that was then used to inoculate each flask (5 ml of culture was taken from the primary sample and was distributed into the 7 experimental flasks) and were allowed to grow at 37C in a shaking incubatoruntil the OD600 value reached 0.4 the following morning. The medium for the 7 experimental flasks was initially developed by preparing 4L of total medium volume. 31g of LB power per liter or 124g total was carefully mixed and dissolved into 4L of water. 500 ml of each medium was added to each individual flask followed by the placement of the colonies into the

flasks. Each culture was then placed into separate incubators at different temperatures and were induced with varying IPTG concentrations. The conditions for all 7 flasks are described on Table 1 below.

Table 1. **IPTG and Temperature Conditions for each flak.**

Flask #	1	2	3	4	5	6	*7*
[IPTG]	0.1mM	0.5mM	1mM	0.1mM	0.5mM	1mM	*Not induced*
Temp.	18°C	18°C	18°C	28°C	28°C	28°C	*28°C*

Upon induction with IPTG, the flasks were kept in the incubators overnight in order for the *E.coli* cells to express the MutT4 enzyme. The following morning, the flasks were removed from the incubator and the contents were poured into 50 ml conical tubes. The tubes were then centrifuged at 3,500 RPM for 10 min. The liquid supernatant was then removed from the tube and the cell pellets were placed on ice, or frozen at -20C for long term storage.

Following the centrifugation process, the cells were prepared for lysing by adding 2.5 ml of lysis buffer (20mM sodium phosphate, 300mM sodium chloride, 10mM imidazole, 16.36ml of water) to each pellet. Additionally, 0.1% of IGEPALdetergent was added to the buffer. The lysing process was continued by adding 25 microliters of DNase to the buffer along with 10 microliters of protease inhibitor and equal amounts of glass beads to each tube. The tubes were then individually vortexed for two minutes. The samples were then centrifuged at 3000 rpm for 10 minutes to separate the insoluble material from the cell lysate. 1 ml of the cell contents were removed and placed into separate 2 ml tubes. 80 microliters of cell contents was removed from each individual tube and placed into separate tubes. 20 microliters of 5X SDS buffer was then added to each tube.

An A660 total protein assay was performed on each sample to determine the total protein concentration in each sample using Pierce 660nm Protein assay reagent (Product Number - 22660). Seven columns in the well plate were loaded with 100 microliters each sample following a two-fold serial dilution ratio. Bovine serum albumin (BSA) and its serial dilutions were used as a positive and negative control. The well-plate setup can be found in Table 2 below.

Table 2. Well-plate setup for total protein assay displaying the locations of each sample and their serial dilutions.

Well	+	-	18 (.1M)	18 (.5M)	18 (1M)	28 (.1M)	28 (.5M)	28 (1M)	28 (Uninduced)
A	Blank	Blank	(1:1)	(1:1)	(1:1)	(1:1)	(1:1)	(1:1)	(1:1)
B	BS (1:1)	BS (1:1)	(1:2)	(1:2)	(1:2)	(1:2)	(1:2)	(1:2)	(1:2)
C	BS (1:2)	BS (1:2)	(1:4)	(1:4)	(1:4)	(1:4)	(1:4)	(1:4)	(1:4)
D	BS (1:4)	BS (1:4)	(1:8)	(1:8)	(1:8)	(1:8)	(1:8)	(1:8)	(1:8)
E	BS (1:8)	BS (1:8)	(1:16)	(1:16)	(1:16)	(1:16)	(1:16)	(1:16)	(1:16)
F	BS (1:16)	BS (1:16)							
G	BS (1:32)	BS (1:32)							

2.5. SDS-PAGE and western blot to determine which conditions were optimal for maximum MutT4 expression.

The initial SDS-PAGE and immunoblot gel was prepared by loading 1.1 micrograms of each individual sample protein into separate wells on both sides of the SDS-PAGE 4-20% gradient TBE gel (BioRad). The gel was allowed to run at 110 volts for 60 minutes, after which both gels were carefully removed from the casing. The gel was stained in Thermo Scientific GelCode Blue protein stain (Product number: 24594) and was allowed to soak overnight. The western blot gel was prepared by cutting out four pieces of filter paper and a nitrocellulose blotting membrane the same size as the gel. Two pieces of the filter were soaked in a transfer buffer and placed inside the western blot casing directly on top of a sponge. The gel was then placed on top of the filter paper followed directly by the placement of the membrane. Two more pieces of filter paper were soaked in the transfer buffer and placed on top of the membrane. The gel casing was then enclosed and inserted into the western blot apparatus and was run at 30 volts for 16 hours.

Following the completion of the transfer, the membrane was carefully removed from the casing. The antibody [Invitrogen MAS-15256-HBP] was prepared by creating a 0.1% solution by diluting 10 uL of the antibody in 10 ml of PBS buffer. A 1X PBS-T block solution was prepared by creating 300ml of a 5% dry milk buffer. 0.1% of Tween-20 detergent was added to the buffer to help the antibody bind specifically to the target. The membrane was then carefully added to a tightly sealed plastic bag as the antibody was carefully poured into the bag. The bag was then sealed shut and was placed on a shaker for 1 hour at room temperature to allow the antibody to bind. Following the binding of the antibody, the membrane was removed from the bag and was washed in the 1X PBS-T dry milk buffer solution for 5 minutes. Two more washes were performed with the dry milk buffer and a final wash with standard 1X PBS buffer was performed. 10 ml of the developing agent was poured on to the membrane. The membrane was imaged after sufficient color development was observed.

3. Results

3.1. PCR reaction and Gibson Assembly

A PCR reaction and gibson assembly was performed to incorporate the translation ramp sequence into the MutT4 plasmid. As seen in Figure 1, a 4.5kb fragment labeled “PCR Band,” confirms the reaction was successful using the touchdown approach. The presence of multiple colonies on the positive control experimental plates and no colonies on the negative control experimental plates indicates that the Gibson Assembly reaction may have been successful in assembling the new plasmid and transforming it into competent cells.

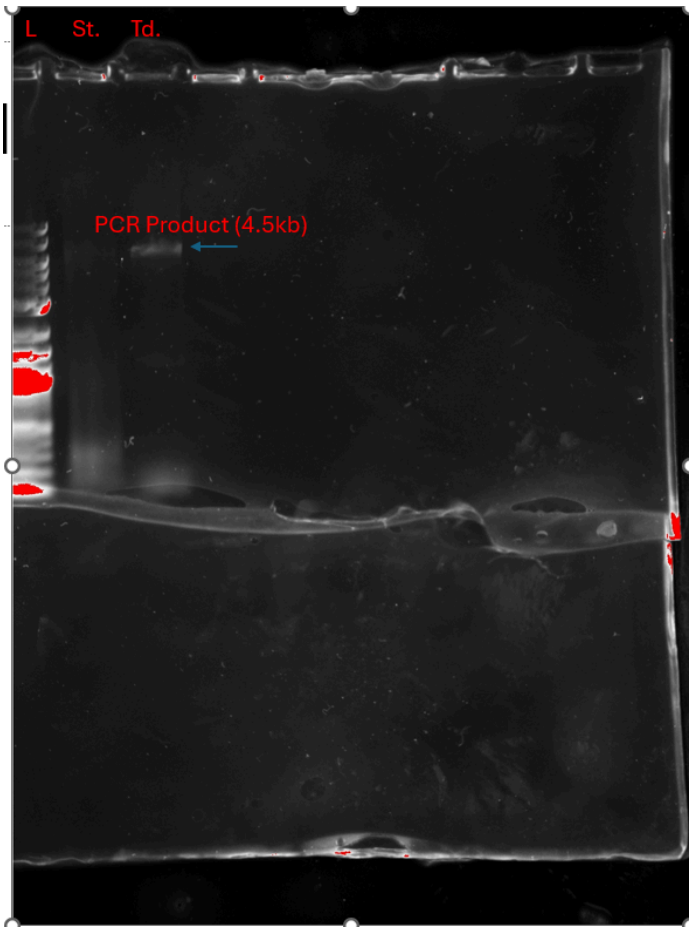

Figure 1. Gel electrophoresis image displaying that the PCR reaction was successful. Td = touchdown parameters and St = constant parameter, and L = 1 kb ladder. PCR product was 4.5kb.

3.2. Sequencing for translation ramp

The returned sequencing data confirmed that none of the five samples sent out produced the desired mutations in the MutT4 plasmid upon aligning to the original plasmid sequence in Benchling. A second PCR and assembly reaction were performed after the unsuccessful attempt. The sequencing data from the second reaction were also confirmed unsuccessful.

3.3. Total protein assay, western blot, and SDS-PAGE gel

Table 3: Concentration of total protein in each sample based on total protein assay.

Sample	28°C Uninduced	28°C 0.1M	28°C 0.5M	28°C 1M	18°C 0.1M	18°C 0.5M	18°C 1M
[protein] (ug/uL)	0.372	0.069	0.158	0.145	0.163	0.087	0.100

The SDS-PAGE gel allowed us to determine which conditions were optimal in yielding the greatest concentration of protein. The protein staining from the SDS-PAGE gel indicates that the samples inoculated with IPTG at 28°C produced the highest level of protein due to the indication of a more intense protein band. It is also apparent on the SDS-PAGE gel that the 28°C uninduced, 0.1M, and 0.5M had the highest level of protein.

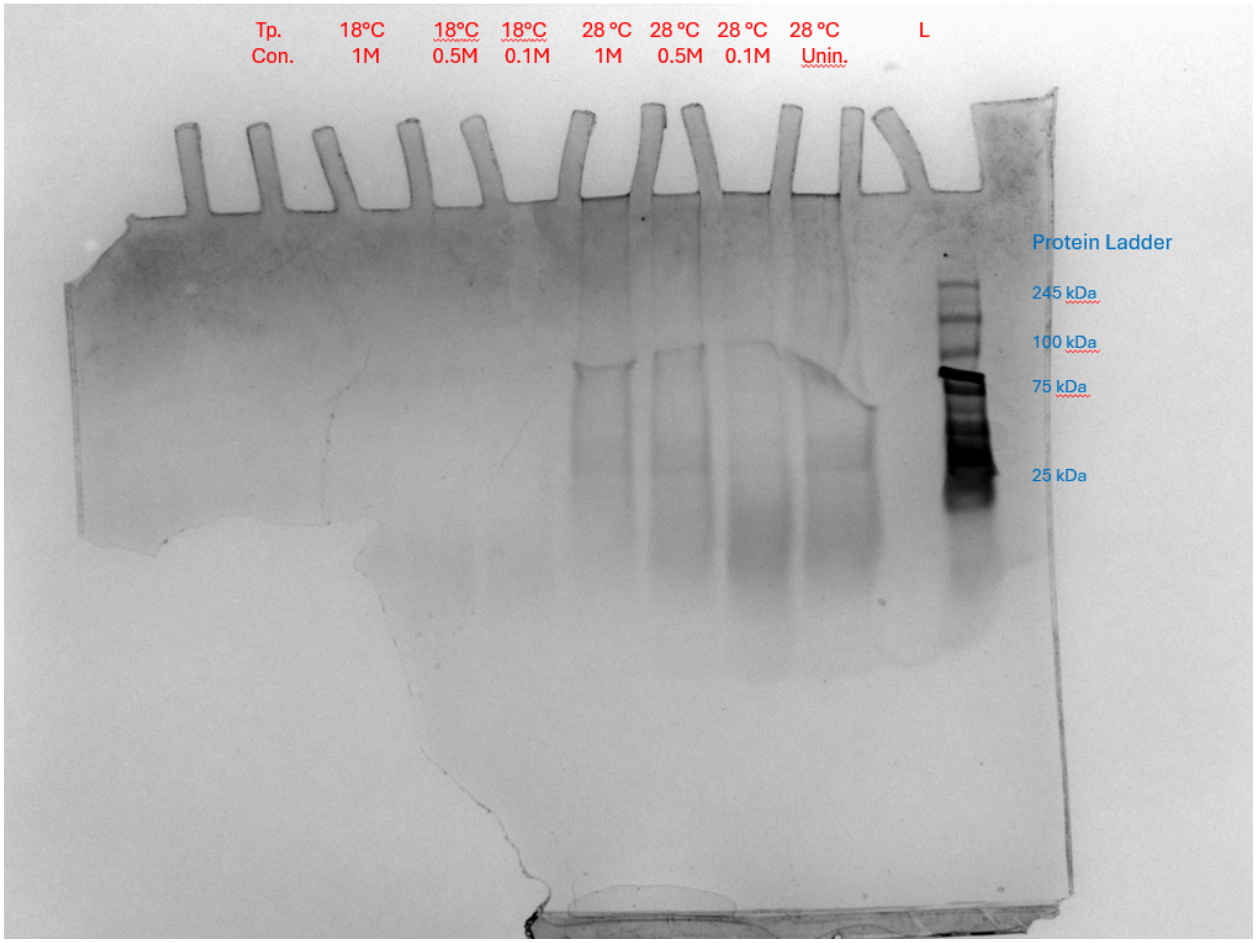

Figure 2. SDS-PAGE gel indicating the temperature and concentration of the samples along with each lane they were loaded into. For each lane, 1.1 micrograms of protein was loaded into each of the lanes. The gel was electrolyzed for 16 hours and stained in Thermo Scientific GelCode Blue protein stain for an additional 16 hours.

The imaging data from the western blot membrane allowed us to determine the optimal conditions for expressing the MutT4 enzyme. Based on the western blot imaging analysis, it was determined that the 28°C 0.1M and 0.5M induction concentration yielded the greatest concentration of MutT4 enzyme. It was decided that the conditions of 28°C and 0.5M would be best suited for expressing the greatest amount of MutT4 enzyme in the following experiments.

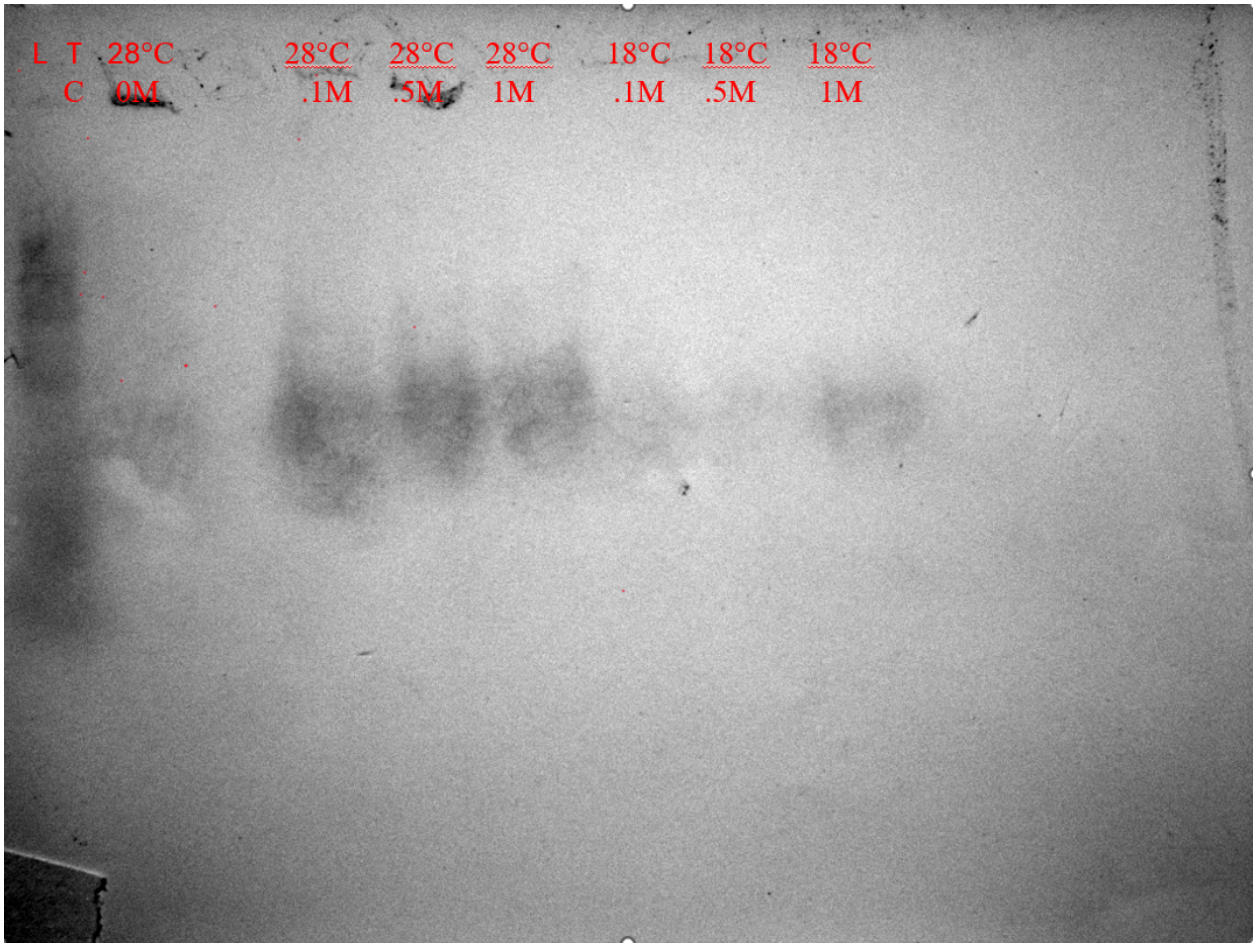

Figure 3. Western blot membrane indicating the temperature and concentration of each sample along with each lane they were loaded on to the gel. For each lane, 1.1 micrograms of protein was loaded. The western blot was performed overnight for 16 hours and the membrane was inoculated with the antibody, washed and developed the following day,

3.4. Testing environment factors under optimal conditions through a western blot and SDS-PAGE gel

With the unsuccessful insertion of the translational ramp sequence, we opted to test alternative environmental conditions to determine if MutT4 expression would be increased using metal-ion cofactors and acid catalysts. Four cultures were prepared by combining 250ml of LB medium, 1.25ml of kanamycin and 5ml of wild-type MutT4 *E.coli* cells. 50 ml of culture volume was then allocated into five separate flasks four of which were induced with 0.5M IPTG. 1mM of magnesium and manganese (sulfate salts) was added to two cultures. 2 ml of 1M HCl was added to one of the cultures to drop the pH of the medium down from 6.5 to 3. The final culture

was kept unchanged at 0.5M IPTG and no additional conditions. All four cultures were allowed to develop at 28°C overnight until the OD value reached 0.4 the following morning. One additional culture was left uninduced and was developed at 37°C overnight. The cells were then centrifuged, lysed, and a western blot and SDS-PAGE were performed on each sample. The results of the SDS-PAGE gel and western blot could not be developed, but all samples were saved at -20C for future analysis.

4. Discussion

The main objective of this project was to genetically manipulate the MutT4 protein-coding sequence in order to insert a translational ramp sequence to increase protein stability and solubility along with the ultimate outcome to increase the concentration of MutT4 enzyme expressed. Additionally, the optimal induction concentration and temperature for expressing the MutT4 enzyme were discovered through the development of multiple bacterial cultures and analysis of an SDS-PAGE gel and western blot for each culture.

From the initial PCR reaction, it was discovered that the reaction was successful using the touchdown approach as evidenced by expected product size on an agarose gel. Upon obtaining sequencing data for the successful insertion of the translational ramp sequence, when aligned with the wild-type MutT4 plasmid in benchling, it was determined that the insertion was unsuccessful for all five samples. While analyzing the imaging data from the SDS-PAGE gel and western blot, it was determined that a temperature of 28°C and IPTG concentration of 0.5M produced the greatest concentration of MutT4 enzyme.

The major challenge for this project was attempting to insert the translation ramp sequence into the MutT4 plasmid. The primary solution to overcome this problem would be to redesign the primers and utilize the NEB QuickChange procedure in order to lower the annealing temperature and increase the likelihood of a successful insertion of the translational ramp sequence. Analyzing the samples from the cultures treated with magnesium, manganese, and lowered pH via SDS-PAGE, western blot, and total protein assay would be recommended.

References

1. Makrides SC.1996.Strategies for achieving high-level expression of genes in Escherichia coli. Microbiol Rev60:.https://doi.org/10.1128/mr.60.3.512-538.1996
2. Lalwani, M.A., Ip, S.S., Carrasco-López, C. *et al.* Optogenetic control of the *lac* operon for bacterial chemical and protein production. *Nat Chem Biol* 17, 71–79 (2021). https://doi.org/10.1038/s41589-020-0639-1
3. S. Velazco *et al*., "Modeling Gene Expression: Lac operon," *2021 43rd Annual International Conference of the IEEE Engineering in Medicine & Biology Society (EMBC)*, Mexico, 2021, pp. 1086-1091, doi: 10.1109/EMBC46164.2021.9630940.
4. Vargas-Blanco DA and Shell SS (2020) Regulation of mRNA Stability During Bacterial Stress Responses. Front. Microbiol. 11:2111. doi: 10.3389/fmicb.2020.02111
5. Chen, Chengula., Deutcher, M.P. *et al.* Elevation of RNase R in response to Multiple Stress Conditions. *Journal of Biological Chemistry* 280, 34393-34396 (2005). https://www.jbc.org/article/S0021-9258(20)63871-6/fulltext
6. Manasvi Verma, Junhong Choi, Kyle A. Cottrell, Zeno Lavagnino, Erica N. Thomas, Slavica Pavlovic-Djuranovic, Pawel Szczesny, David W. Pisto , Hani S. Zaher, Joseph D. Puglisi & Sergej Djuranovic. *A short translational ramp determines the efficiency of protein synthesis*.
7. T. Dos Vultos, J. Bla´zquez, J. Rauzier, I. Matic, and B. Gicquel. Identification of Nudix Hydrolase Family Members with an Antimutator Role in Mycobacterium tuberculosis and Mycobacterium smegmatis†. *Journal of Bacteriology* 188, 3159-3161, (2006)
8. Cafiero and Shell, 2024 (Unpublished Results)