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Toxic Gene Expression in Escherichia coli Using trpL as a Repressor

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Toxic Gene Expression in *Escherichia coli* Using *trpL* as a Repressor

by

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Abstract

Many research enzymes, proteins, and medical products are produced by genetic amplification of artificially engineered genes encoded in plasmids, which are inserted into bacterial cells. This process allows scientists to quickly and cost-effectively produce a large amount of valuable product. Unfortunately, some products must be produced in an alternative method due to their inherent toxicity to bacterial cells. Commercially available plasmids used for expression of artificially engineered genes produce product even in the absence of an inducer, i.e. "leak". In the event of a toxic product leaking, the bacterial cells die quickly before any harvestable amount of protein is produced. If this process is used today with a toxic product, many cell batches must be made and miniscule amounts of protein can be harvested from each. Alternative methods, such as *in vitro* transcription and translation, are more effective but also more expensive and require a greater investment of time than standard bacterial expression.

The goal of my project was to investigate the effectiveness of *trpL* DNA in suppressing leaky expression. I inserted *trpL* into the pET28 plasmid upstream of a Lac Z reporter gene in order to assay expression under a variety of conditions. The *trpL* gene has been observed to produce viable downstream mRNA only in the absence of tryptophan. The pET28 plasmid was chosen because it provides a promoter for T7 polymerase that is induced by IPTG. With tight regulation of the toxic gene, the bacterial cells would be allowed to grow robustly before toxic gene expression occurred. Upon induction by IPTG, the cells would express the toxic gene downstream of *trpL*. Surprisingly, my results showed that *trpL* stimulated expression of Lac Z, regardless of whether tryptophan was present or absent. Furthermore, this robust expression also

occurred when the plasmid was cloned into a cell line that does not contain T7 polymerase.

Literature Review

In order to use *Escherichia coli* to successfully express toxic proteins, a researcher must first regulate leaky expression of the toxic gene during the bacterial growth process. A number of methods may be used to accomplish gene regulation at this critical stage (Saïda et. al, 2006).

One method is to inhibit the T7 polymerase responsible for leaky expression using a lysozyme. Several cell lines containing pLysS use this method. Also, rather than inhibiting overall polymerase activity, some success has been observed by repressing the gene responsible for encoding T7 RNA polymerase by introducing competing DNA binding proteins at the promoter region; however, this has obvious repercussions on cell protein expression during induction (Saïda et. al, 2006).

The toxic gene can be regulated using a repressor/activator DNA sequence upstream of the toxic gene of interest. The arabinose operon, commercially found in pBAD plasmid, can repress leaky expression of downstream genes in the presence of glucose and can promote expression in the presence of arabinose. A similar technique utilizes the full-length *lac* operon that is tightly but not fully repressed by the lactose repressor. Regulation by the lactose repressor can be reversed in the presence of lactose and the *lac* operon can be induced using IPTG. The P(Ltet-O-1) gene can be placed upstream of a toxic gene. It can be repressed using the Tet repressor and induced by anhydrotetracycline. P(lac/ara-1) is a hybrid promoter developed from the naturally occurring P(*lac*) promoter and *araC* promoter. The P(lac/ara-1) promoter can be placed upstream of a toxic gene, repressed by glucose and induced using a combination of IPTG

and arabinose (Saïda et. al, 2006). Research projects such as these inspired this thesis project to test the *trp* operon.

A researcher can insert a palindromic DNA sequence of the toxic gene into the cell. As the toxic gene is transcribed the palindromic sequence forms an RNA helix that cannot be translated. Care must be taken to insure that the palindromic sequence is transcribed in quantity rivaling leaky toxic gene transcription during both during the growth phase and induction. If palindromic sequence transcription is inappropriately raised during the induction phase then no toxic gene will be translated (Saïda et. al, 2006).

Multiple plasmids can be used to provide creative toxic expression techniques. One researcher transformed a high copy number second plasmid containing arbitrary DNA sequences with T7 polymerase promoters into a cell already containing a T7 transcribable plasmid containing toxic gene of interest. This Trojan horse strategy limits T7 RNA polymerase present during the growth phase since it is more frequently bound to the second plasmid promoter rather than the first, thus decreasing leaky expression of the toxic protein (Saïda et. al, 2006).

The depth of complexity of expressing toxic genes is apparent in the next example. It is possible that a highly toxic gene, located on a T7 RNA polymerase plasmid, can be expressed in miniscule amounts by the *E. coli's* own polymerase. This phenomenon is called "readthrough." The quantity of RNA produced is much lower than in leaky expression. It is unusual that a miniscule amount of the toxic gene will be transcribed effectively, but in some cases toxic genes contain highly effective ribosome binding sites and if any toxic gene is transcribed the cell will die. In order to block

efficiency of transcription during the growth phase several termination sites can be placed upstream of the toxic gene. Upon induction transcription is obviously not optimal, but is efficient enough to express acceptable quantities of protein (Saïda et. al, 2006). Many plasmids used for protein expression contain no genes in the same orientation as the inserted gene in an effort to suppress readthrough.

A "mRNA switch" can be employed by inserting special DNA sequences directly upstream of the toxic gene. These DNA sequences are special because they will form a loop structure that effectively blocks ribosomal binding (and subsequently translation) at temperatures below 30 °. At 37 ° the DNA sequence does not form this loop structure and translation occurs normally. The PrfA factor found in *Listeria monocytogenes* behaves this way. Perhaps there are other DNA sequences still undiscovered which are thermally regulated (Saïda et. al, 2006).

Several of the above methods can be used in combination to increase protein recovery. Once the cells are fully grown, an induction process can be used to promote high expression of the toxic gene of interest and the cells will survive long enough to produce a harvestable amount of protein (Saïda et. al, 2006).

The cell line BL21(DE3) is commercially available and used for its high cloning efficiency and high protein expression characteristics. Recently two new cell lines have been developed using BL21(DE3) competent cells that are able to accept plasmids with high efficiency, but more importantly express toxic genes with high efficiency. C41(DE3) and C43(DE3) have been shown to express toxic genes and some membrane proteins in a soluble form. These cell lines do not contain pLysS, but they are commercially available with pLysS. While the T7 RNA polymerase is inhibited little to

no leaky or basal level expression of the toxic gene is observed. Upon induction with IPTG, T7 RNA polymerase is expressed which then transcribes the toxic gene in efficient quantities (Lucigen, 2008).

The *trp* operon in *Eschrichia coli* is well regulated by a combination of repression at the level of initiation of transcription by attenuation. Attenuation takes advantage of the fact that transcription and translation take place in the same time frame in bacteria. Upstream of the open reading frames for the enzymes responsible for synthesis of tryptophan is a short open reading frame designated *trpL* which encodes a short leader peptide. The leader contains two adjacent trp codons. As RNA of the *trp* operon is actively being transcribed by a polymerase, a ribosome begins to translate the RNA sequence while transcription is still taking place. If tryptophan is scarce the ribosome will stall at each of two UGG codons, which encode tryptophan, and the RNA sequence already produced can base pair to itself forming a secondary RNA loop structure. This RNA loop structure prevents a different RNA loop structure from forming that would terminate further transcription by the polymerase. In the presence of excess tryptophan there is an abundance of tryptophan charged tRNA and the ribosome reads through each UGG codon quickly, not allowing the first loop structure to form. The second RNA loop structure is allowed to form and transcription of the *trp* operon is halted by way of a typical *Eschrichia coli* ρ-independent termination signal (Oxender et. al, 1979).

Materials and Methods

Insertion of *trpL* and Lac Z gene into pET 28 Plasmid

Lac Z DNA was obtained from competent cells transformed with a pLexA plasmid containing Lac Z (pLexA/Lac Z). Cells were grown on plates containing 30 mg/ml kanamyacin for positive selection. DNA was isolated from cells using essentially the procedure found in *Molecular Cloning: A Laboratory Manual* (Maniatis et al, 1982). Breifly the cell culture was pelleted, lysed with sodium dodecyl sulfate in NaOH, extracted with phenol-chloroform, and the DNA was pelleted by addition of one volume of isopropanol to the aqueous solution. DNA was purified using the Wizard DNA membrane column system (Promega). The DNA concentration was determined by measuring the absorbance at 280nm. The pLexA plasmid was designed with Hind III and Bam HI restriction sites. Lac Z DNA was isolated using electrophoresis, extracted from the gel, and purified using the Wizard system (Promega). Lac Z DNA was digested with Hind III and Bam HI. The ligation was transformed into α-Select *Eschrichia coli* cells. Colonies were picked, grown overnight, and DNA was isolated. The DNA was digested with Nco I and Bam HI to confirm presence of the insert. pET 28/Lac Z was then transformed into *Escherichia coli* BL21(DE3) cells. Cells were grown on plates containing kanamyacin for positive selection (Figure 1).

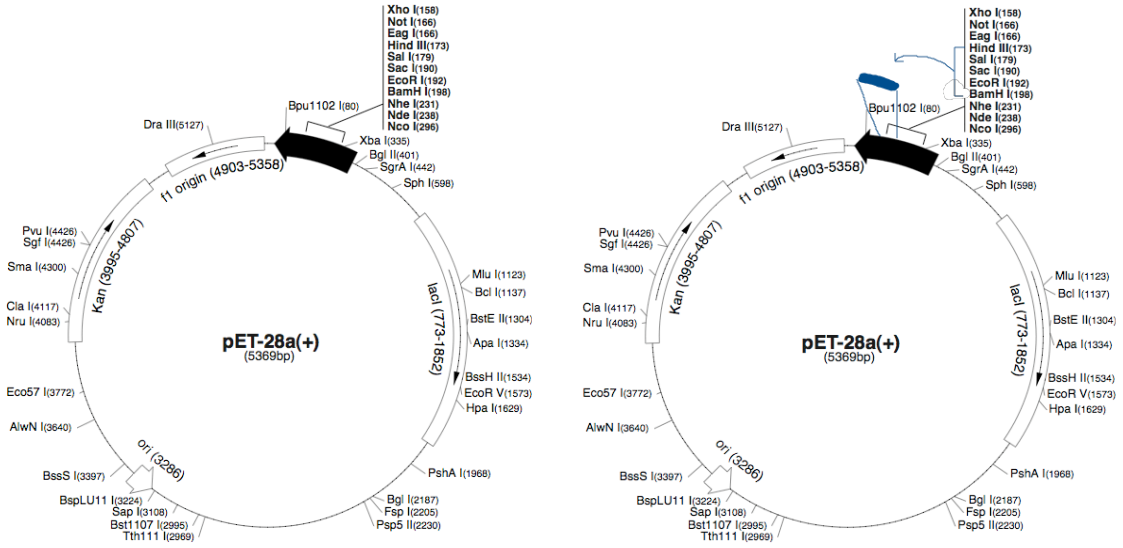

Figure 1 – The left image is an unmodified pET 28 plasmid. The right image shows Lac Z in blue ligated into pET 28. This is the control plasmid used later in the experiment (pET 28/Lac Z).). (Novagen pET28 vector map)

5' and 3' primers for the *trpL* sequences were designed to contain Nco I and Xba I restriction sites. The primers were purchased from Operon (Figure 2).

5' – CTTTCTAGAAGTTCACGTAAAAAGGGTATCG – 3'
5' – CTTCCATGGTTATTCTCTCTAATTTTGTTCA – 3'
5' – GCACCACAGATGAAACGCCG – 3'

Figure 2 – PCR primer sequences with corresponding primers in color. The 5' primer is indicated in blue and contains an Xba I restriction site. The 3' primer is indicated in red and contains an Nco I restriction site. The 3' primer used to identify *trpL*/Lac Z sequence is indicated in black.

DH-1 *E. coli* cells were grown in liquid media. The genomic DNA was isolated by pelleting the cells, resuspending them in distilled water, and boiling them. The genomic DNA was used as the template in the PCR reaction to produce the *trpL* sequence used throughout this experiment. Pfu Ultra II polymerase (Stratagene) was used for this PCR reaction. The DNA from the PCR reaction was isolated using electrophoresis and the *trpL* sequence was cut out and purified using a DNA membrane column system. An A-tail was added to the *trpL* sequence using Taq polymerase. The A-tailed *trpL* was ligated into pGEM-T Easy vector system (Promega) using T4 DNA ligase. The pGEM-T/*trpL* plasmid was transformed into competent *Eschrichia coli* cells that were grown on

plates containing the chromogenic β-galactosidase substrate X-gal. X-gal allows selecting β-galactosidase active colonies that turn blue and β-galactosidease inactive colonies that remain white. White colonies were chosen as candidates possibly containing the *trpL* sequence. The pGEM-T/*trpL* plasmid was isolated (a concentration of 89.9 ug/ml) and digested with Nco I and Xba I restriction enzymes. The *trpL* sequence was isolated using electrophoresis and purified using the Wizard system (Promega).

The pET 28/Lac Z plasmid previously described was digested using Nco I and Xba I restriction enzymes in preparation for receiving *trpL* DNA. Alkaline phosphatase was added to pET 28/Lac Z digestion after one hour to minimize vector religation. The *trpL* sequence was ligated into pET 28/Lac Z plasmid described using T4 DNA ligase (pET 28/*trpL*/Lac Z) (Figure 3). Using restriction enzymes and gel electrophoresis proved difficult to confirm correct plasmid DNA content. In the interest of time, a different process was employed. To insure presence of the *trpL* the more sensitive method of PCR amplification employing the original *trpL* 5' primer with a 3' primer designed to base pair with a region of LacZ DNA. Digested DNA and PCR products were subjected to electrophoresis through agarose gels.

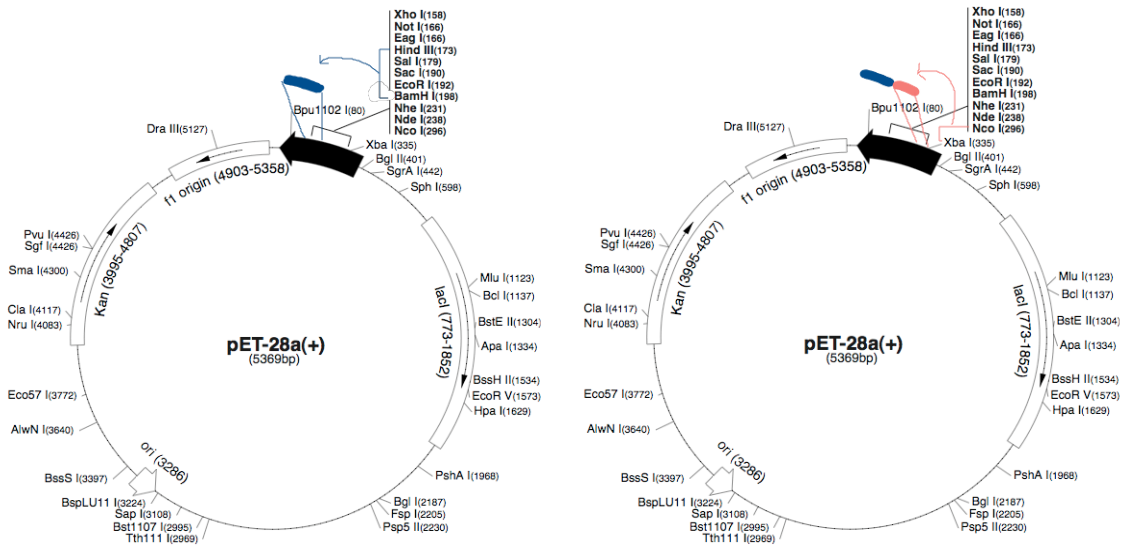

Figure 3 – The left image is the control pET 28/Lac Z plasmid. The right image shows the *trpL* sequence in orange ligated into pET 28/Lac Z. This is the experimental plasmid used later in the experiment (pET 28/*trpL*/Lac Z). (Novagen pET28 vector map)

pET 28/*trpL*/Lac Z plasmid was transformed into BL21(DE3) competent cells. A control plasmid, for later β-galactosidase activity assay use, of pET 28/Lac Z plasmid was also transformed into BL21(DE3). These cells were grown in standard media. Glycerol stocks were made from these cultures using 8% glycerol and kept at -80 °. These cells were used to analyze Lac Z expression under several growth conditions.

Minimal media used to grow cells

A non-standard media was made for this experiment. During preliminary studies the media ingredients were changed slightly. A 100 mL batch of minimal media contained 79 mL H_20, 20 mL M9 salt solution, and 64 ug of CSM (Complete Supplement mixture) purchased from BIO 101 which contains no tryptophan. After autoclaving this solution the following were added: 200 ul 1M magnesium sulfate, 10 ul 1M calcium chloride, 100 ul 1M potassium chloride, 20 ul trace elements, and 500 ul 40% glucose. Prior to 1957, it was reported that *Escherichia coli* does not require potassium, but in fact growth is retarded by a factor of over thirteen fold in the absence of potassium. Although

the original minimal media did not call for potassium, I added it to promote cell growth (Lester, 1957). For each culture of cells grown, a 3 mL aliquot of this media was used. To gain further insight into the regulation of the *trpL* sequence, for each β-galactosidase activity assay described below, a full second set of cultures was used in which sterile tryptophan was added into the minimal media to a final concentration of 10 mg/ml.

Rich media used to grow cells

Rich media was used in place of minimal media for an additional experiment. This media contains 0.75 grams of powdered yeast extract, 1.50 grams powdered Tryptone, and 0.75 grams sodium chloride dissolved into 150 mL of water.

β-galactosidase assay measuring leaky expression

β-galactosidase units were determined as described by Miller (Miller, 1972). Initial cell culture density was determined by measuring OD600 using an Eppendorf BioPhotometer spectrophotometer. To insure accuracy according to Beer's Law, absorbance was measured in a 50 ul sample of cell culture in 450 ul water.

The assay calls for 0.5 ml Z-buffer and 0.5 ml cell culture for detection of small amounts of β-galactosidase. Z-buffer is adjusted to pH 7 and contains 0.06 M disodium phosphate, 0.04 M monosodium phosphate, 0.01 M potassium chloride, 0.001 M magnesium sulfate, and 0.05 M 2-mercaptoethanol. This concentration was used when examining leaky expression. In addition two drops of chloroform and one drop of 0.1% SDS was added to each sample. O-nitrophenyl-β-D-galactopryanoside (ONPG) was added to each sample. The assay calls for the reaction to run until there is a distinct color change, but the reaction should be stopped quickly after the color change. In most cases the reaction was allowed to run from two to five minutes. To stop the reaction from

proceeding further and thus stopping the color change, 0.5 ml of sodium carbonate was added. Samples were assayed for ONPG using an Agilent Technologies 8453 light scattering spectrophotometer at OD420 and OD550 for use in a calculation to determine units of β-galactosidase present.

β-galactosidase assay measuring induced expression

β-galactosidase units were determined as described by Miller (1972). Cells were grown for an additional hour at 37 ° with 1 mM IPTG. Initial cell culture density was determined by measuring OD600 using an Eppendorf BioPhotometer spectrophotometer as described above. β-galactosidase units were also determined as described above with the following exception: 0.9 ml Z-buffer and 0.1 ml cell culture were used.

Results and Discussion

My initial research involved preparing a control plasmid (pET 28/Lac Z) and an experimental plasmid (pET 28/*trpL*/Lac Z) for insertion into BL21(DE3) cells. The process involved many steps. I first harvested pLexA/Lac Z DNA from cells prepared by Dr. Sue G. Bartlett. I then ligated Laz Z DNA into digesting pET 28 plasmids, which had been treated with alkaline phosphatase to prevent self-ligation. I set some of the resulting DNA aside as my control plasmid for use in the control cells, but also opened the plasmid directly upstream of the Lac Z sequence with Nco I and Xba I. I ligated *trpL* sequence DNA, isolated from PCR amplification with *trp* leader sequence primers and DH1 genomic DNA as a template, into the plasmid. This would be my experimental plasmid.

BL21(DE3) cells require large concentrations of plasmid DNA for effective transformation. To prepare for this demand I first transformed both plasmids into α-Select blue competent cells. I allowed the cells to grow overnight and performed a mini-prep to extract DNA. To insure the DNA plasmids isolated were the desired plasmids, I used digestion or PCR amplification on a small aliquot (Figure 4). I then used an aliquot of the plasmid DNA to transform into BL21(DE3) cells for use in β-galactosidase assays.

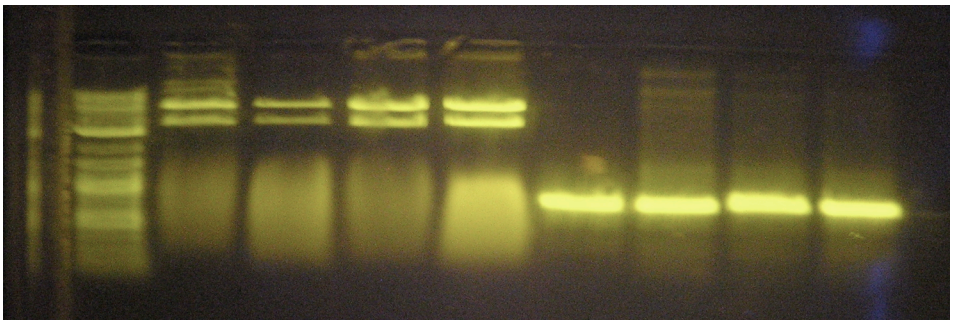

Figure 4 – This electrophoresis gel confirms DNA integrity of both plasmids: pET 28/Lac Z digestion (Lanes 2-5) and pET 28/*trpL*/Lac Z PCR amplification (Lanes 6-9). Lane 1 contains 2-Log Marker (New England BioLabs).

Two sets of cell cultures of both plasmids were grown at 37° for one hour to insure low cell density. The first set of cultures was used to detect leaky expression. IPTG was added to the second set of cultures, which were allowed to grow for an additional hour, and were used to detect induced expression. The β-galactosidase assay involves gently lysing the cells to release any β-galactosidase contained in the cytoplasm into solution. ONPG, which is converted by β-galactosidase into a yellow product, was then added to the solution. The results were quite puzzling because cells containing the pET 28/*trpL*/Lac Z plasmid expressed substantially larger leaky quantities of β-galactosidase than cells containing the pET 28/Lac Z plasmid. The change in rate expression of β-galactosidase in cells from leaky to induced conditions varied drastically between the two plasmids. In cells containing pET 28/Lac Z I observed an expected increase of about five hundred fold units of β-galactosidase with an average leaky expression of 8 Miller units to an average induced expression of 4162 Miller units. The cells containing pET 28/*trpL*/Lac Z showed an increase of only about 4 fold units of β-galactosidase with an average leaky expression of 868 Miller units to an average induced expression of 3061 Miller units. The ONPG reaction was halted using sodium carbonate and spectrophotometer readings were taken at OD420, to measure absorbance by ONPG, and OD550, to correct for light scattering by the lysed cells. The data was used in a calculation to determine β-galactosidase Miller units/OD600 in each culture (Figure 5, Tables 1-6, Figure 6, and Figure 7). Please note that there is an error associated with the data in which Miller units of β-galactosidase is sometimes reported in a negative quantity. The error may have occurred because the light scattering of the cells was high in

contrast to the low detection of yellow color produced by ONPG. Miller units in these cases should be considered close to zero.

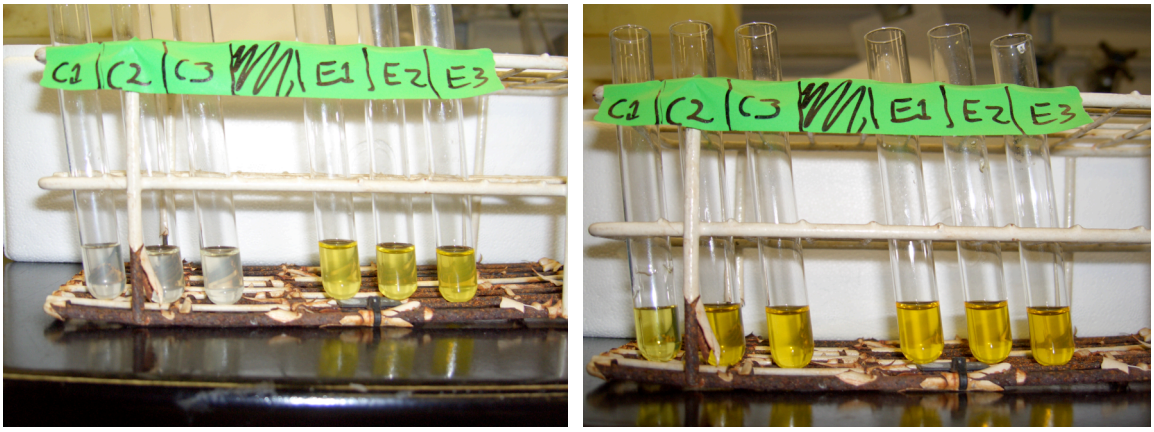

Figure 5 – β-galactosidase assay results. C1-C3 of left photograph are cells grown and exhibiting low leaky expression (pET 28/Lac Z). E1-E3 of left photograph are cells grown and exhibiting high leaky expression (pET 28/*trpL*/Lac Z). C1-C3 of right photograph are cells grown for an additional hour in presence of IPTG, and are exhibiting high expression (pET 28/Lac Z). E1-E3 of right photograph shows cells grown for an additional hour in presence of IPTG, and are exhibiting high expression (pET 28/*trpL*/Lac Z).

Table1 – Leaky β-galactosidase assay in M9 media containing tryptophan. *

sample	OD_{600}	OD_{420}	OD_{550}	units β-galactosidase
pET 28/*trpL*/Lac Z 1	1.47	2.16	0.39	1005.10
pET 28/*trpL*/Lac Z 2	0.9	1.54	0.31	1108.33
pET 28/*trpL*/Lac Z 3	1.41	2.72	0.43	1395.39
pET 28/Lac Z 1	1.45	0.88	0.51	-8.62
pET 28/Lac Z 2	1.59	0.87	0.44	62.89
pET 28/Lac Z 3	0.83	0.49	0.27	21.08

* Reaction used 0.5mL cell culture and 2 minute run time.

Table 2 – Leaky β-galactosidase assay in M9 media lacking tryptophan. *

sample	OD_{600}	OD_{420}	OD_{550}	units β-galactosidase
pET 28/*trpL*/Lac Z 1	1.63	2.47	0.49	991.10
pET 28/*trpL*/Lac Z 2	1.22	2.19	0.44	1162.13
pET 28/*trpL*/Lac Z 3	1.59	3.08	0.48	1410.26
pET 28/Lac Z 1	1.15	0.64	0.37	4.23
pET 28/Lac Z 2	1.28	0.73	0.40	21.55
pET 28/Lac Z 3	1.97	0.87	0.48	19.91

* Reaction used 0.5mL cell culture and 2 minute run time.

Table 3 – β-galactosidase assay induced with IPTG in M9 media containing tryptophan. *

sample	OD_{600}	OD_{420}	OD_{550}	units β-galactosidase
pET 28/*trpL*/Lac Z 1	3.03	2.94	0.25	4116.36
pET 28/*trpL*/Lac Z 2	4.02	3.03	0.26	3201.82
pET 28/*trpL*/Lac Z 3	3.52	2.93	0.29	3441.76
pET 28/Lac Z 1	0.81	1.55	0.09	8609.11
pET 28/Lac Z 2	3.78	2.99	0.26	3350.73
pET 28/Lac Z 3	3.31	3.01	0.26	3854.32

* Reaction used 0.1mL cell culture and 3 minute run time.

Table 4 – β-galactosidase assay induced with IPTG in M9 media lacking tryptophan. *

sample	OD_{600}	OD_{420}	OD_{550}	units β-galactosidase
pET 28/*trpL*/Lac Z 1	2.41	3.04	0.19	5594.72
pET 28/*trpL*/Lac Z 2	2.82	2.97	0.24	4520.19
pET 28/*trpL*/Lac Z 3	3.44	2.96	0.26	3640.35
pET 28/Lac Z 1	1.53	2.73	0.15	8058.49
pET 28/Lac Z 2	2.05	3.03	0.17	6655.30
pET 28/Lac Z 3	2.89	2.99	0.22	4500.69

* Reaction used 0.1mL cell culture and 2 minute run time.

Table 5 – Leaky β-galactosidase in LB media. *

sample	OD_{600}	OD_{420}	OD_{550}	units β-galactosidase
pET 28/*trpL*/Lac Z 1	1.19	1.67	0.58	372.78
pET 28/*trpL*/Lac Z 2	5.31	3.80	1.77	88.31
pET 28/*trpL*/Lac Z 3	3.38	3.83	1.37	280.57
pET 28/Lac Z 1	1.44	1.26	0.68	36.69
pET 28/Lac Z 2	1.66	1.87	1.19	-86.60
pET 28/Lac Z 3	3.03	2.19	1.27	-5.76

* Reaction used 0.5mL cell culture and 3 minute run time.

Table 6 – β-galactosidase assay induced with IPTG in LB media. *

sample	OD_{600}	OD_{420}	OD_{550}	units β-galactosidase
pET 28/*trpL*/Lac Z 1	5.74	3.44	0.50	1494.52
pET 28/*trpL*/Lac Z 2	8.66	3.33	0.74	783.55
pET 28/*trpL*/Lac Z 3	9.73	3.73	0.87	756.50
pET 28/Lac Z 1	10.54	3.37	0.95	539.84
pET 28/Lac Z 2	5.64	3.22	0.53	1355.07
pET 28/Lac Z 3	10.8	3.23	0.86	532.86

* Reaction used 0.1mL cell culture and 3 minute run time.

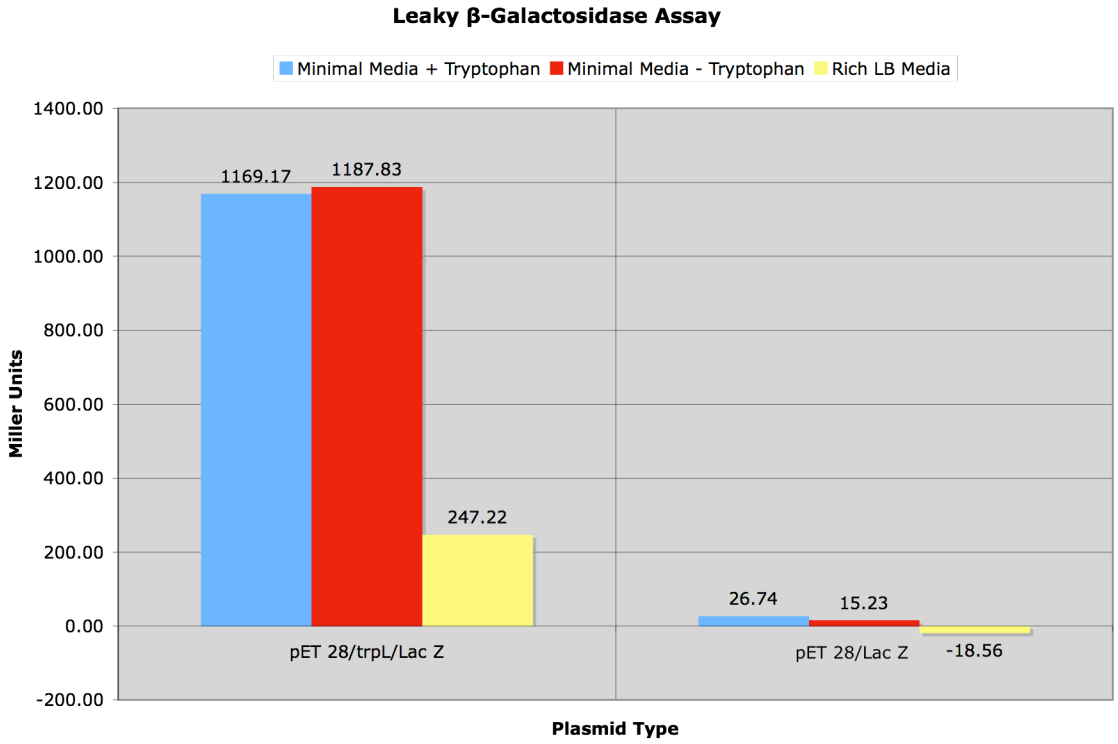

Figure 6 – Plotted results for leaky expression of β-galactosidase.

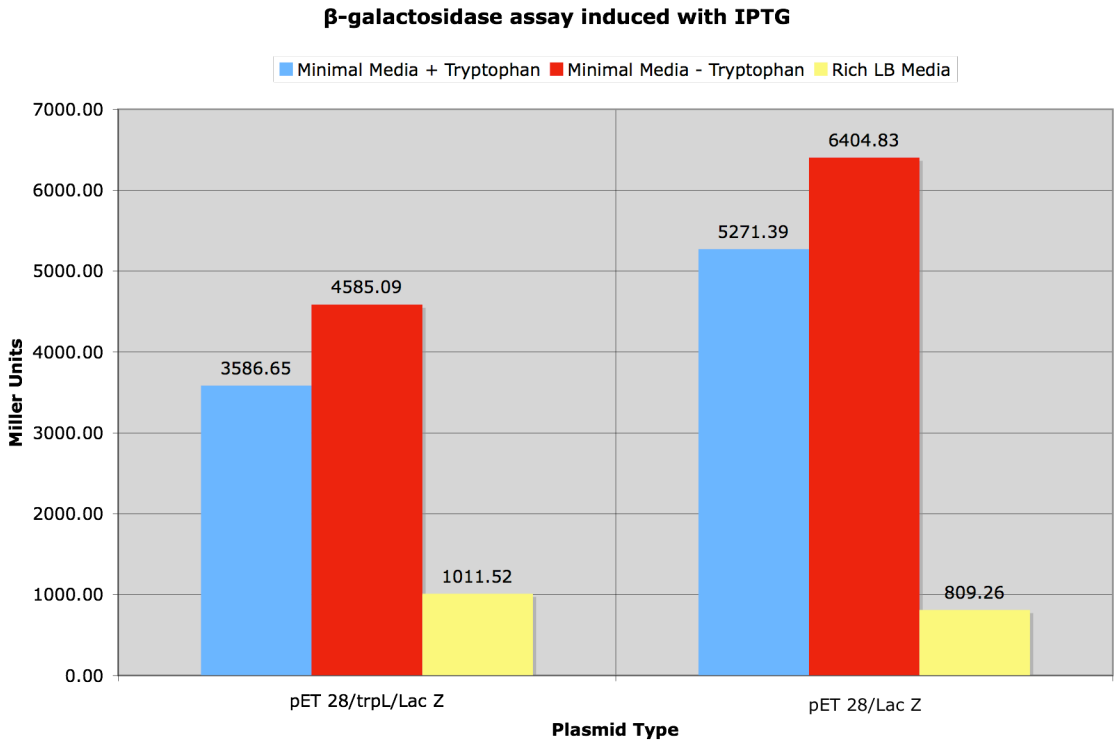

Figure 7 – Plotted results for induced expression of β-galactosidase.

At this point, PCR amplification was run on a few of the cultures to ensure that no error took place in the labeling of these tubes. No error was detected.

I grew α-Select blue competent cell cultures containing each plasmid on rich media containing kanamycin and also observed strong expression of β-galactosidase in cells transformed with pET 28/*trpL*/Lac Z, but not in cells transformed with pET28/Lac Z. This suggests that the promoter like function of *trpL* seen in the β-galactosidase assays is tied to the DNA sequence of the *trpL* because α-Select blue competent cells do not contain a T7 polymerase. The *trpL* or perhaps the *trpL* in conjunction with *lac* repressor appears to be behaving as a normal *Escherichia coli* promoter sequence. We can also note that expression seems to occur regardless of use of minimal or rich media, thus ruling out the possibility that the minimal media contains some activating species (Figure 8).

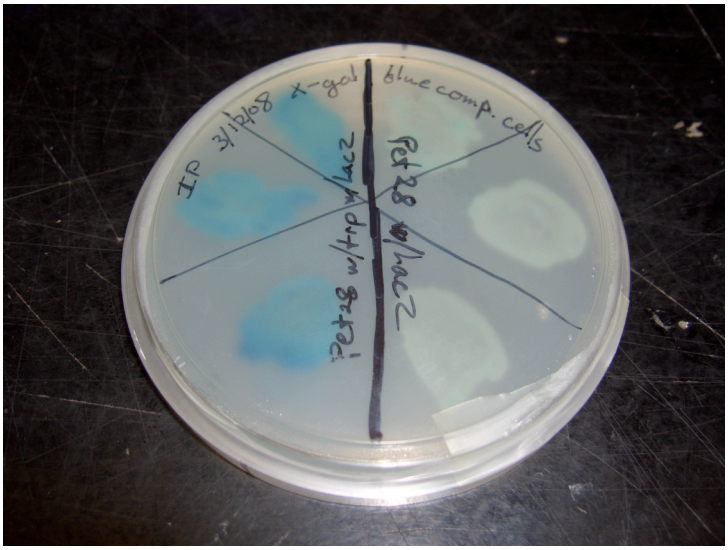

Figure 8 – α-Select blue competent cells grown on rich media kanamyacin antibiotic plate with X-gal. pET 28/*trpL*/Lac Z cells exhibit leaky expression while control cells exhibit no blue X-gal reaction with β-galactosidase indicating no leaky expression.

One explanation for these surprising results is that the *trp* leader is acting like a translational switch, much as is seen for the viral leader in the pTRC series of plasmids.

Another possibility is that when out of the context of the entire *trp* operon, *trpL* acts as a standard *Escherichia coli* promoter or part of one.

Conclusion and Future Research

The first portion of my thesis project involved creating two plasmids: pET 28/Lac Z, and pET 28/*trpL*/Lac Z. Both plasmids were transformed into BL21(DE3). The experimental plasmid with the *trpL* sequence upstream of Lac Z was expected to function in media containing tryptophan similarly to its well-documented role as an attenuator and prevent leaky expression of Lac Z. The control plasmid was to be used to measure relative Lac Z expression compared to the experimental plasmid.

I grew cells in both minimal media and rich LB media. An additional set of cells was allowed to grow for additional time with IPTG. These cells were then gently lysed open and ONPG was used to visualize β-galactosidase presence quantitatively. A color change from clear to yellow was observed in the presence of β-galactosidase. Absorbance of these samples was taking using a spectrophotometer and calculations were employed to determine exact unit numbers of β-galactosidase present.

My results showed something quite unexpected. In the experimental pET 28/*trpL*/Lac Z plasmid, Lac Z exhibited leaky expression of about one hundred times greater than the control pET 28/Lac Z plasmid. During induction by IPTG, both plasmids produced high amounts of Lac Z, but the factor of expression increase was different between the two plasmids. When induced with IPTG, cells containing pET 28/Lac Z expressed β-galactosidase by a five hundred fold increase, while pET 28/*trpL*/Lac Z expressed β-galactosidase by a 4 fold increase.

Rather than repressing Lac Z production, the *trpL* sequence appears to promote expression. I immediately wondered if the control and experimental plasmids were merely mislabeled and switched which would yield the expected results. I ran a PCR test

using primers for the *trpL* with Lac Z sequence and confirmed that the cells were not mislabeled and therefore the test results were indeed correct.

In order to rule out that T7 polymerase was involved in these unexpected results, I transformed pET 28/*trpL*/Lac Z and pET 28/Lac Z into α-Select cells exposed to X-gal. I observed the same pattern of leaky expression. Cells that contained pET 28/*trpL*/Lac Z were dark blue while those containing the control plasmid were white. Since α-Select cells do not contain T7 polymerase, expression clearly correlated with presence of *trpL*. Notably, the *trpL* sequence is very A-T rich at its 5' end. It is possible that *trpL* resembles an *Escherichia coli* promoter, or at least the -10 region of one in the context of pET 28 and Lac Z.

It is possible that *trpL* attenuates gene expression only in the context of the *trp* gene. In the *trp* operon, the *trp* operator is immediately upstream of *trpL*. It is possible that in the context of the *trp* operon, the *trp* repressor overlaps a portion of *trpL*. In the context of the pET 28/Lac Z construct *trpL* clearly enhances expression regardless of whether tryptophan is present or absent in the media.

Many toxic gene expression techniques similar to the one discussed here are yet unexplored. For example, the histidine operon leader also functions using the attenuation mechanism similar to *trpL* and therefore may be a candidate for toxic gene expression. Perhaps this work can provide evidence that those similar techniques should be studied. Just as previous works inspired this thesis, this document's true value lies in its promise to inspire and subsequently shed light on even more scientifically significant realizations.

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