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Release Factor Mutants that Affect the Function of the TnaC Regulatory Peptide

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by

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Abstract

The present work used *Escherichia coli* strains SVS1144, PDG1158, and VK800, which expressed release factor mutant proteins to determine the effects of these proteins on the expression of *tnaCAB* operon induced by the amino acid L-tryptophan (L-Trp). In each cell line, three bacterial constructs were created containing the pET RF1, pET RF2, or pET RF2 R256T plasmids whose genes are expressed under control of the T7 promoter. T7 RNA polymerase was expressed from the pTARA plasmid, which was under the control of L-arabinose; inducing levels of L-arabinose cause release factor expression in these bacterial constructs. Bacteria cells were grown under several conditions: 1) in inducing levels of L-Trp with noninducing levels of L-arabinose, 2) inducing levels of L-Trp with inducing levels of L-arabinose, 3) noninducing levels of L-Trp with inducing levels of L-arabinose, and 4) noninducing levels of L-Trp with noninducing levels of L-arabinose. β-Galactosidase activity assays were then performed on extracts from these subcultures to find the rate of *tnaCAB* expression in each of the cell lines. The regulatory region of the *tnaCAB* operon, which responds to L-Trp, controls β-Galactosidase production. All cell lines had lower activity rates in the presence of inducing levels of L-arabinose compared to those grown in noninducing levels of L-arabinose because having more release factors helps the cell to overcome stalling. The RF2 R256T mutant behaved similarly to its wild type RF2 counterpart. Our data indicates that the L-Trp effects in the *tnaCAB* operon depend on the nature of the release factor present in the bacterial cells

Introduction

Tryptophanase is a multifunctional enzyme that degrades L-Trp to indole, pyruvate, and ammonia by a β-elimination reaction (Konan and Yanofsky 1999). In this way, L-Trp can be a source of carbon, nitrogen, and energy for the cell. Tryptophanase is produced from the *tnaCAB* operon, whose expression is dependent in L-Trp.

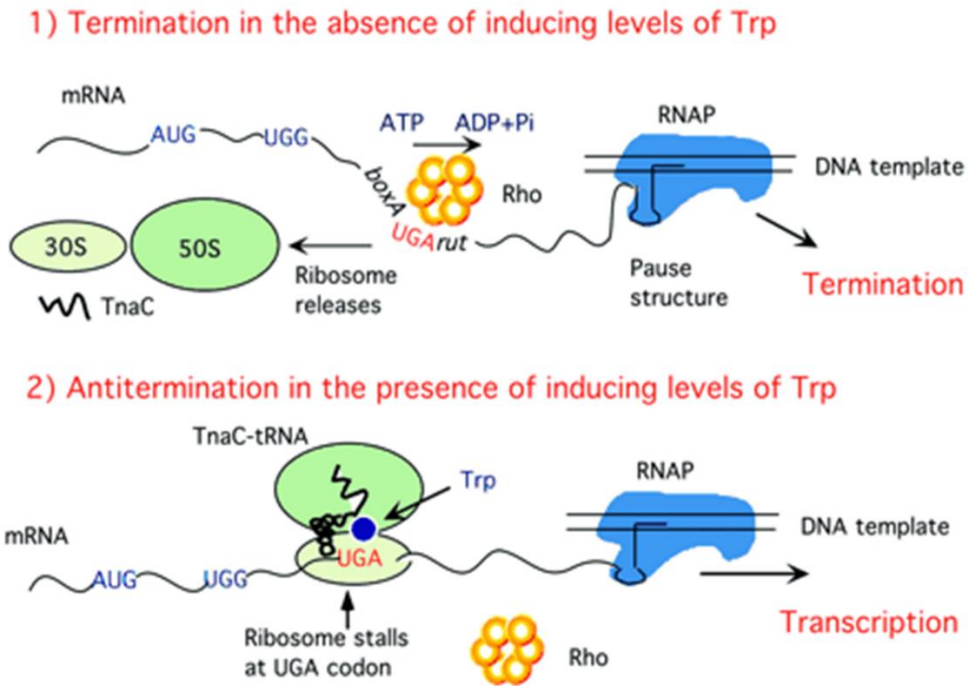

Figure 1: Regulation of operon *tnaCAB* in *E. coli* with and without L-Trp (Konan and Yanofsky 1999). The stop codon in this example is UGA but the process occurs with the other stop codons.

In bacterial cells grown under conditions of noninducing levels of L-Trp [Figure 1.1], the *tnaCAB* operon undergoes Rho-dependent transcription termination in the *tnaC* leader region of the operon (Gong and Yanofsky 2002). The stop codon is reached and the peptide is released from the ribosome. These last events induce the RNA polymerase to stop before reaching the *tnaA* (Tryptophanase) and *tnaB* genes of the operon, which therefore are not expressed.

The TnaC nascent protein chain creates two hydrophobic pockets where each L-Trp molecule can bind (Bischoff et al, 2014). When inducing levels of L-Trp are present [Figure 1.2], the ribosome stalls because L-Trp enters the exit tunnel of the ribosome and binds in the hydrophobic pockets, which cause conformation changes in the ribosome (Martinez et al 2011). The ribosome stalls at the *tnaC* stop codon, which blocks Rho's access to its binding site on the transcript. Transcription termination is blocked, RNA polymerase continues transcription, and the *tnaA-tnaB* coding region is expressed.

L-Trp inhibits the action of release factors in the ribosome. The release factors 1 and 2 cannot hydrolyze the peptidyl-TnaC-tRNA when L-Trp is bound within the ribosome. Release factors bind to ribosomes that have a stop codon in the A site of the ribosome (Martinez et al 2013). These factors catalyze ester bond hydrolysis by positioning a water molecule at the ribosome active site, named the peptidyl transferase center.

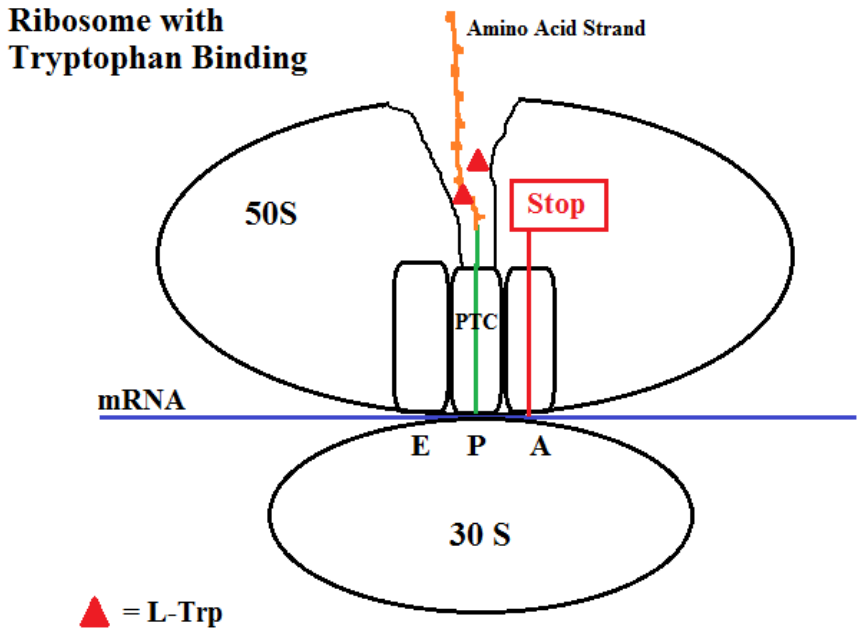

Figure 2: Tryptophan binding in the ribosome during transcription.

Release factors recognize stop codons located in the ribosomal A-site [Figure 2]. The SPF binding motif is specific for RF2 meanwhile the PVT binding motif is specific for RF1. The SPF motif interacts specifically with the U1 and G2 of the UGA stop codon, which discriminates between G and A at the third codon (Korostelev et al 2008). The PVT motif determines the U1 and A2 of the UAG stop codon, which discriminates between A and G at the third codon. Swapping the SPF and the PVT motifs has been shown to switch codon specificity (Korostelev et al 208).

Followed by recognition of the stop codons, the release factors induced hydrolysis of the last peptidyl-tRNA. The GGQ motif contributes directly to catalysis of peptidyl tRNA hydrolysis by breaking the tRNA ester linkage between the tRNA and the peptide at the peptidyl transferase center (Korostelev et al 2008). The GGQ motif is conserved in all release factors of all three primary domains of life. This motif is crucial in forming the catalytic pocket where the water cleaves the ester bond causing hydrolysis. If GGQ is deleted then RF2 is non-functional, which is likely why this motif is so highly conserved. Following protein release, RF1 and RF2 dissociate from the ribosome. RF3 hydrolyzes GTP to speed up this process along with the ribosome recycling factor (Cruz-Vera et al 2005).

It is unknown which amino acids of RF1 and RF2 are involved in the L-Trp inhibition of translation termination. However, there are several conserved regions that are known to be key for both release factors. To determine the importance of the other amino acids near the GGQ motif, an RF2 R256T mutant was created. This mutant is expected to act like RF1 if this arginine at position 256 is crucial to the mechanism of inhibition of L-Trp. RF2 is expected to induce ribosome stalling at the *tnaC* sequences if it is overexpressed.

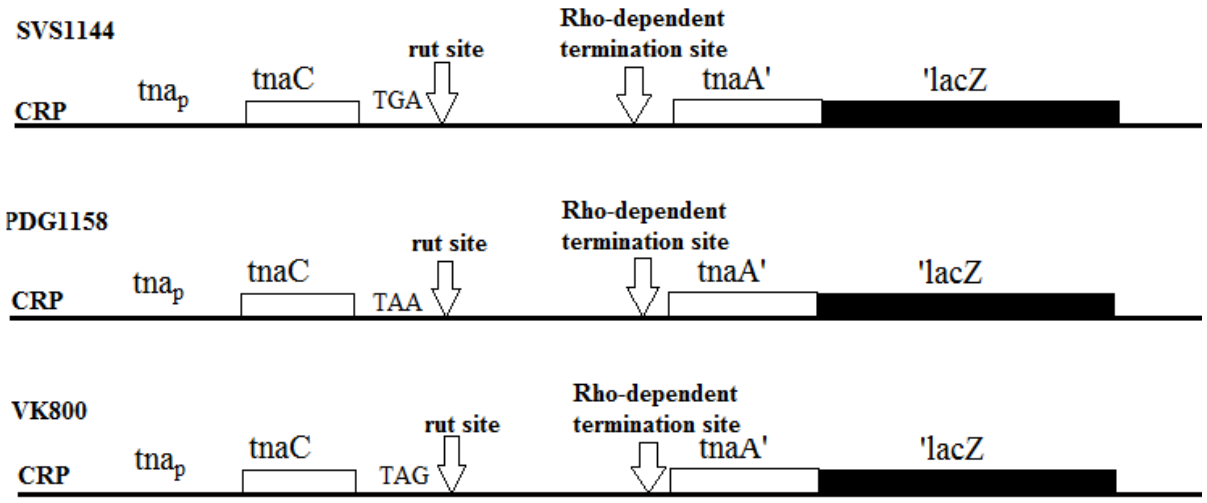

Figure 3: A schematic representation of the basic chromosomal construct *tnaCAB* operon – '*lacZ* fusions used in this study (Konan and Yanofsky 1999).

The constructs used in this work contain a *tnaA'*— '*lacZ* protein fusion in place of the original *tnaCAB* operon. Incorporation of the *tnaA'*— '*lacZ* fusion was achieved by inserting a lambda bacteriophage into the genome of *E. coli*. *lacZ* was fused to the first 20 codons of *tnaA* (Gollnick and Yanofsky 1990). The location of the *lacZ* region relative to the *tnaA* and *tnaC* regions can be seen in Figure 3. *TnaC* is the regulatory leader region with a 24-residue open reading frame. Induction requires translation of *tnaC*, which encodes a peptide with a single L-Trp residue at position 12. The *tnaC* region and the L-Trp residue are both crucial to L-Trp induction.

The rut site is thought to be right behind the *tnaC* region [Figure 3] which (overlaps with the stop codon of *tnaC*). *TnaA* encodes for Tryptophanase and 200 base pair spacer regions containing several transcription pause sites. The *lacZ* gene located in the spacer region between *tnaA* and *tnaB*, controls production of the β-Galactosidase enzyme, which should be synthesized at a proportional rate to Tryptophanase (Gollnick and Yanofsky 1990).

When the ribosome is stalled on a *tnaC* stop codon of the reporter gene TnaA' – '*lacZ*, then *lacZ* is produced by the cell. *LacZ* production can be measured with a β-Galactosidase assay, where this enzyme cleaves o-nitrophenyl-β-D-galactoside (ONPG) to yield D-galactose and o-nitrophenol, which production can be detected and quantitate by absorption at 420 nm. Therefore, a β-Galactosidase assay can be used to measure the expression of the reporter gene, under any growth condition (Konan and Yanofsky 1999).

There are three cell lines used in this project, VK800, SVS1144, and PDG1158. The VK800 cell line was derived from the CY15076 line. The SVS1144 and PDG1158 cell lines were both derived from the SVS1100 cell line. The SVS1144 cell line has a *tnaC* gene with a stop codon UGA (Konan and Yanofsky 1999). The VK800 cell line has a *tnaC* gene with the stop codon UAG. The PDG1158 cell line has a *tnaC* gene with the stop codon UAA. RF1 binds to UAG and UAA, while RF2 binds to UAA and UGA. UAA binds preferentially with RF1 (Grentzmann 1997).

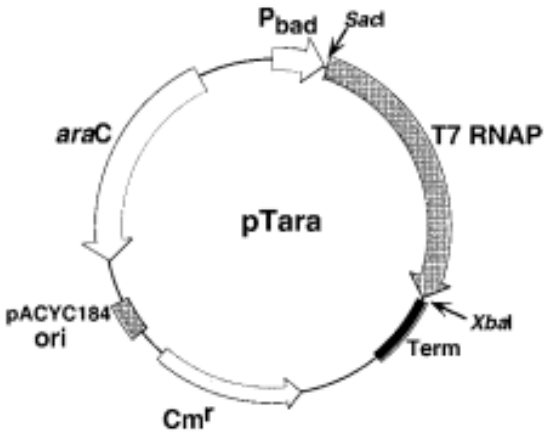

Figure 4: The pTARA plasmid encoding T7 RNA polymerase (Wycuff and Matthews 2000)

The T7 RNA polymerase is a monomeric protein that recognizes its own highly conserved promoter sequences with great specificity and efficiency. It catalyzes the formation of RNA in the 5' to 3' direction. It transcribes only DNA downstream of the T7 promoter in the

pET plasmids. Magnesium is a required cofactor for RNA synthesis in this promoter. In the pTARA plasmid the T7 gene is placed under the control of the Pbad promoter regulated by the AraC protein [Figure 4], the T7 gene is expressed from pTARA under the addition of L-arabinose (Wycuff and Matthews 2000). Co-transformation of the pTARA with each pET plasmids RF1, RF2, and RF2 R256T would induce the expression of the prfB, prfA, or prfB R256T genes which code for RF2, RF1, and RF2 R256T respectively [Figure 5], under the presence of L-arabinose.

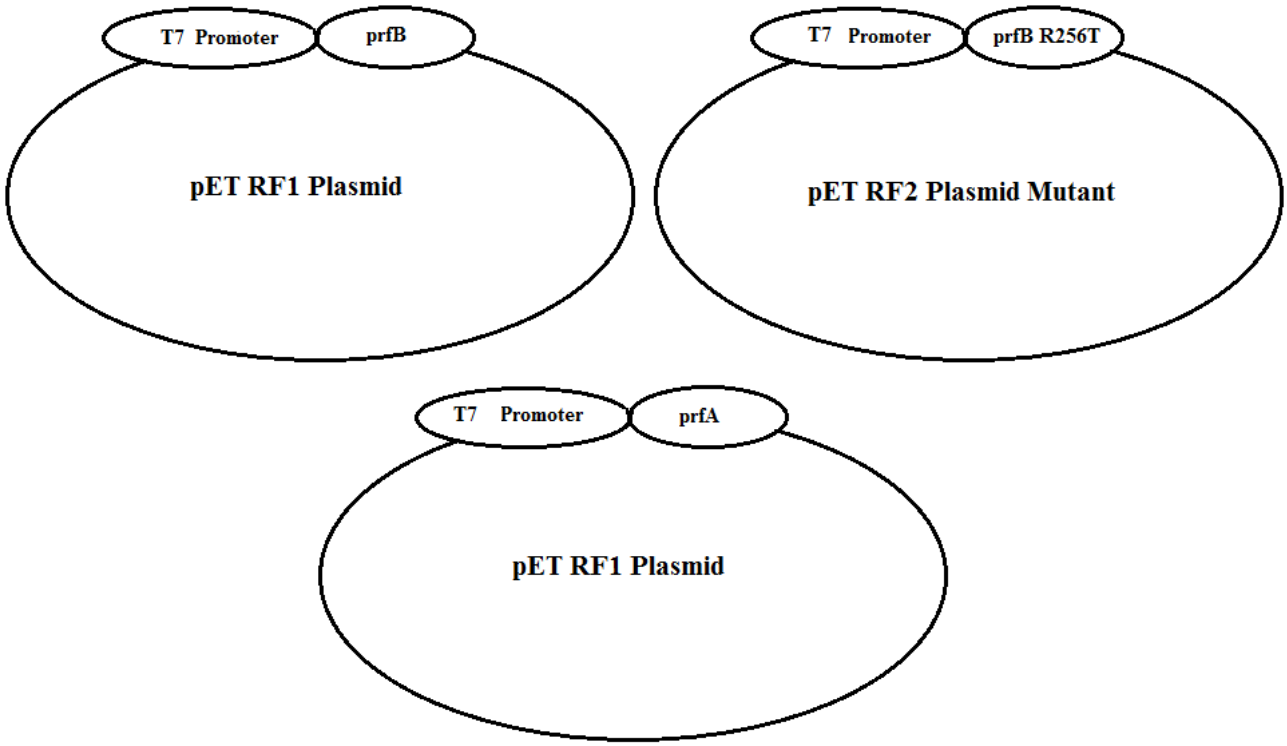

Figure 5: pET plasmids with T7 promoter. prfA codes for RF1 and prfB codes for RF2.

The expression of the T7 promoter potentially allows repression by glucose and induction by arabinose in the range of 0.5 to 20 mM sugar concentration (Wycuff and Matthews 2000). T7 RNA polymerase in pTARA is expressed in the presence of inducing levels of arabinose. Levels of arabinose are considered noninducing at less than 0.01-1mM in concentration, more is

considered inducing levels of arabinose, more than 1mM is considered toxic. The arabinose regulated system is used to deliver T7 RNA polymerase to the T7 promoter-driven, expression vectors (Wycuff and Matthews 2000). If noninducing levels of arabinose are present, then the T7 RNA polymerase is not expressed and the T7 promoter-driven, expression vectors are not active. In the presence of inducing amounts of arabinose, T7 RNA polymerase is expressed. Even low levels of arabinose are expected to result in high expression levels of the promoter. Protein expression can be adjusted based on arabinose concentrations; lower levels of arabinose might cause intermediate levels of release factor expression (Wycuff and Matthews 2000).

In each of the cell lines three different constructs were created, a total of nine constructs were obtained. Each of the following combinations (1) pET for RF1 and the pTARA plasmid, (2) pET for RF2 and pTARA plasmid, and (3) pET for the mutant RF2 R256T which changes arginine to threonine (the amino acid at this locus in the RF1 genome) and the pTARA plasmid were produced on each SVS1144, VK800 and PDG1158. In this work, we expected that the RF1 pET plasmid, unlike the RF2 and RF2 R256T plasmids, would affect the expression of the reporter gene of the VK800 cell line. The RF2 and RF2 R256T pET plasmids, unlike RF1, would affect the expression of the reporter gene of the SVS1144 cell line. Finally, all pET plasmids would affect the expression of the reporter gene of the PDG1158 strain. We expected also that the 256th residue may provide the crucial difference between RF1 and RF2 (Konan and Yanofsky 1999).

Materials & Methods

Media and reaction solutions

A solution of M9 media was made by mixing 200 ml of 5X M9 salts, 2 ml of 1M magnesium sulfate, 20 ml of 20% glycerol, 0.1 ml of 1M calcium chloride, and 10 ml of 5% ACH to a final volume of 1 liter. The M9 salts was made by mixing 64g of disodium phosphate heptahydrate, 15g potassium phosphate, 2.5g of sodium chloride, and 5 g of ammonium chloride in a liter of water. The media also contained 25μg/mL chloramphenicol and 50μg/mL kanamycin to maintain the pTARA and pET plasmids. High L-Trp media contained 50μg/mL of L-Trp.

Miller's reaction solution was made containing 14.25 ml of Z-buffer, 750μl 0.1% sodium dodecyl sulfate, and 60mg ortho-Nitrophenyl-β-galactoside (ONPG). For this a solution of Z-buffer was made containing 0.8g disodium phosphate heptahydrate, 0.28g sodium phosphate monohydrate, 0.5ml 1M sodium chloride, 0.05ml 1M magnesium sulfate, and 0.135ml of β-mercaptoethanol and the pH to 7.0.

B-Galactosidase Assay

Two colonies of each construct were transferred to 1 mL of M9 media supplemented with ACH and glycerol. The culture was then grown in a shaker water bath at 250 rpm at 37°C. After 16 hours, 40 μL of the culture was transferred into 1mL of the desired media (with or without tryptophan). 10 μL of 1 M L-arabinose was added to the final solution. The new culture was incubated for 5 hours in a shaker water bath at 250 rotations per minute at 37°C. 1mL of Miller's reaction solution was mixed with 100 μl chloroform by inverting the tube several times. The chloroform was added to break down the cells so that ONPG can interact with interior of the cell. The phases were then allowed to separate for 3 minutes. After 3 minutes 180μl of the final mix

was placed in a well of the microplate for all the experimental samples. 20-40µl of bacterial culture was then placed in the microplate and thoroughly mixed. The reaction was allowed to proceed for between 10 and 30 minutes until the formation of a yellow color occurred. The time of reaction was recorded. 100µl of sodium carbonate was added to each well and then mix thoroughly. The microplate was then placed in the microreader for analysis at 420nm, 550nm, and 600nm with Z-Buffer as the blank.

$$\text{Miller Equation:} \quad 1000 * \frac{OD420 - 1.75 * OD550}{\text{time (min)} * OD600 * \text{Volume (mL)}}$$

Figure 6: Miller equation used to obtain miller units of enzyme activity (Konan and Yanofsky 1999).

The activity of the cell was computed using the Miller equation [Figure 6]. OD550 is a measure of background by cell debris. OD600 is a measure of cell density. OD420 is a measure of the amount of o-nitrophenol the product of β-galactosidase catalyzed breakdown of ONPG.

This process was repeated three times with each strain of VK800, PDG1158, and SVS1144, inducing levels of L-Trp with noninducing levels of L-arabinose, noninducing levels of L-Trp with noninducing levels of L-arabinose, inducing levels of L-Trp with inducing levels of L-arabinose, and noninducing levels of L-Trp with inducing levels of L-arabinose.

Results

Reporter gene expression with a UGA codon

SVS1144 strains containing the *tnaC* gene with a UGA codon were grown under low and high L-Trp concentrations [Figure 7]. The presence of the pETRF1 plasmid induced high levels of β-Galactosidase under both low and high L-Trp concentrations comparing with the pET RF2 and the pET RF2 R256T mutant plasmids [Figure 7]. Interestingly, addition of L-arabinose (meaning overproduction of all RF1, RF2 and RF2 R256T mutant proteins) reduced the β-Galactosidase expression under high concentrations of L-Trp [Figure 7]. The expression of RF2 R256T protein did not make any differences in the β-Galactosidase expression comparing with the wild type RF2 under high concentrations of L-Trp [Figure 7].

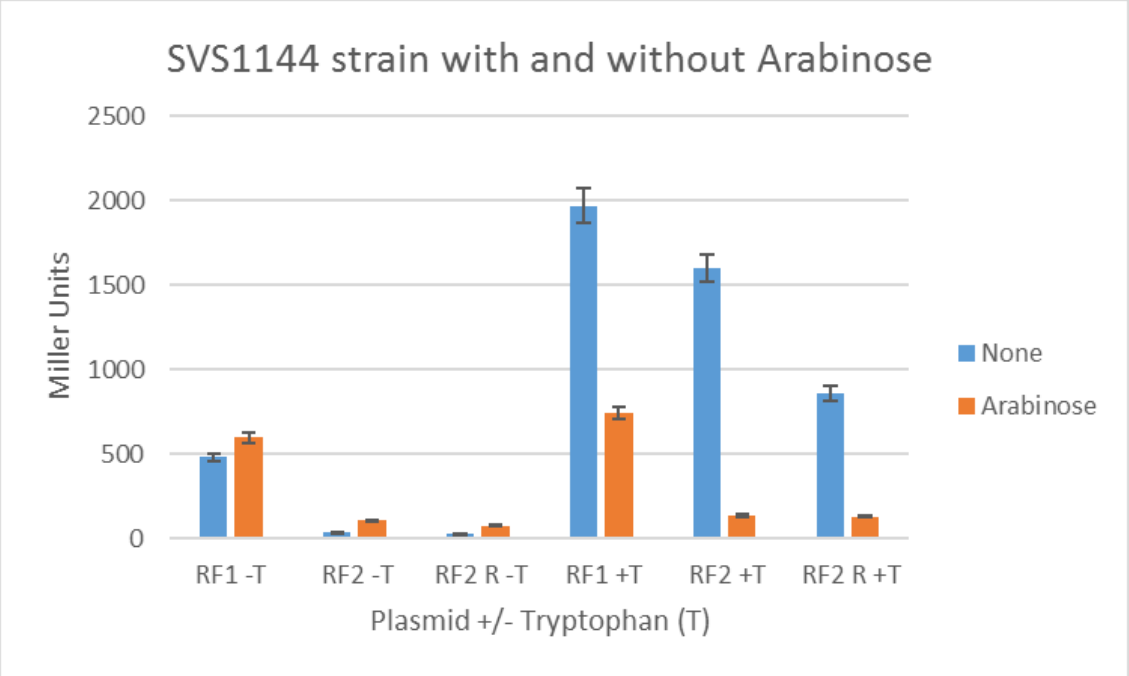

Figure 7: β-Galactosidase expression (Miller Units) from several SVS1144 constructs grown in inducing (orange bars) and noninducing levels of L-arabinose (blue bars). Wild type RF1 (RF1), RF2 (RF2) and R256T mutant (RF2 R) strains were grown in the presence (+T) or absence (-T) of L-Trp.

Reporter gene expression with a UAG codon

VK800 strains containing the *tnaC* gene with a UAG codon were grown under high and low increments of several release factors [Figure 8]. In general, we observed that addition of L-arabinose (meaning overproduction of all RF1, RF2 and RF2 R256T mutant proteins) reduced the β-Galactosidase expression under low and high concentrations of L-Trp [Figure 8]. Interestingly, the overexpression of RF1 produced lower β-Galactosidase expression under high concentrations of L-Trp than the overexpression of RF2 and the RF2 R256T mutant, as expected [Figure 8]. Also, the mutant RF2 R256T reduced the β-Galactosidase expression less under high concentrations of L-Trp [Figure 8].

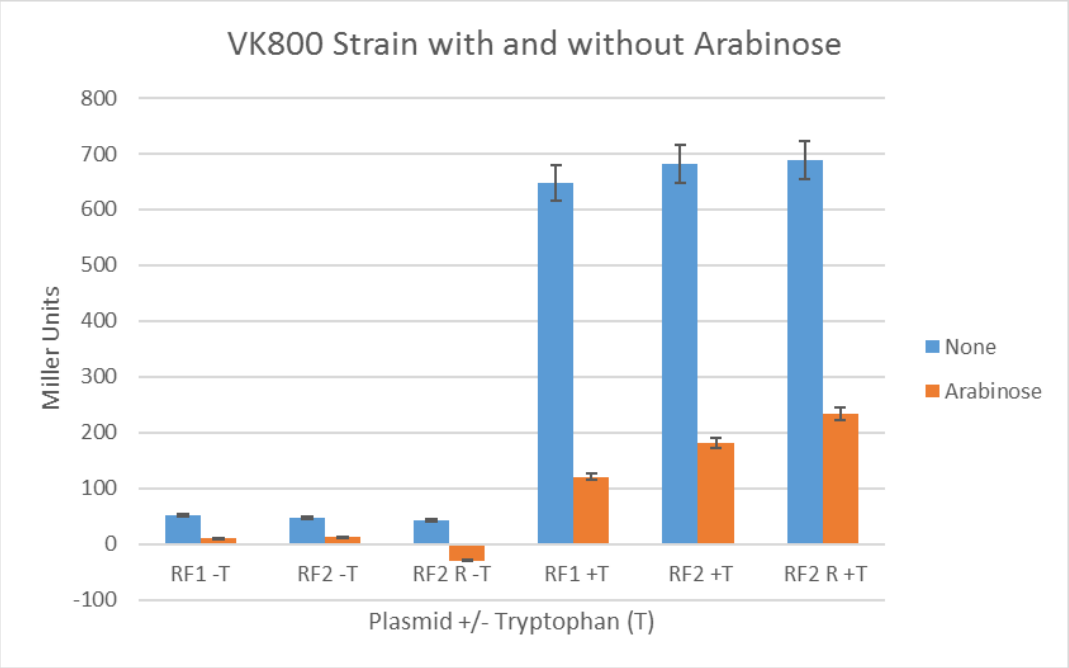

Figure 8: β-Galactosidase expression from several VK800 constructs grown in inducing and noninducing levels of L-arabinose. See Figure 7 for figure information.

Reporter gene expression with a UAA codon

PDG1158 strains containing the *tnaC* gene with a UAA codon were grown under high and low L-Trp concentrations [Figure 9]. In general, we observed that addition of L-arabinose reduced the β-Galactosidase expression under low and high concentrations of L-Trp [Figure 9]. The overexpression of all release factors reduced the β-Galactosidase expression under high concentrations of L-Trp [Figure 9]. Also, we did not observe any differences in the β-Galactosidase expression during the overexpression of the wild type RF2 and the RF2 R256T mutant proteins under high concentrations of L-Trp [Figure 9].

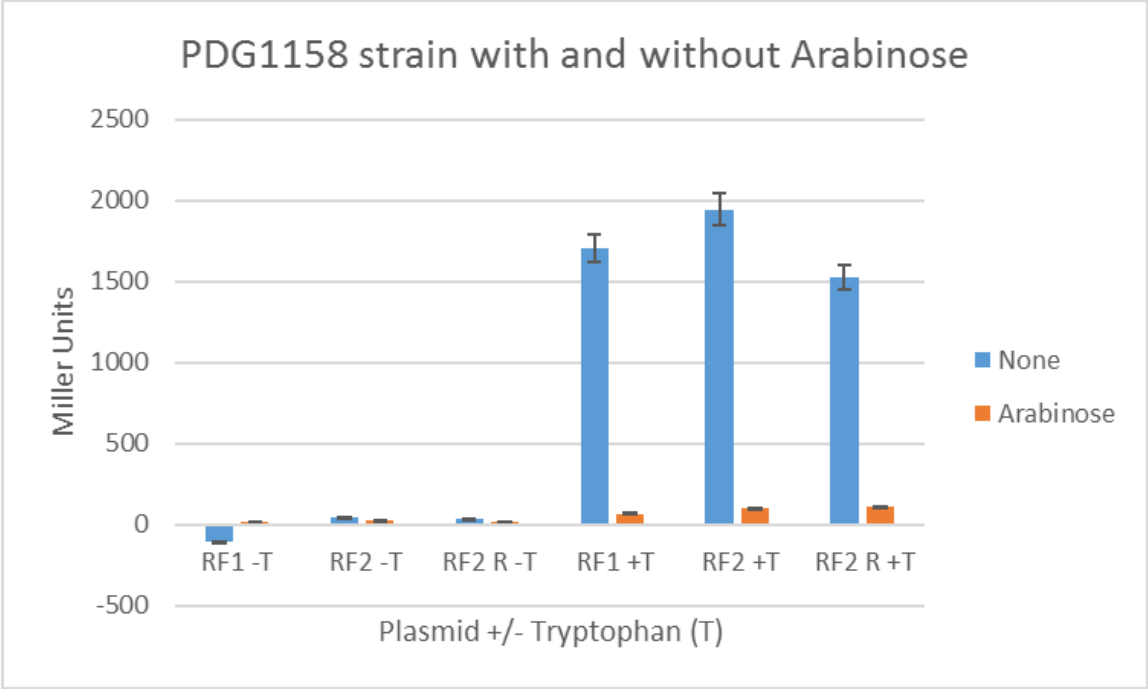

Figure 9: β-Galactosidase expression from several PDG1158 constructs grown in inducing and noninducing levels of L-arabinose. See Figure 7 for figure information.

Conclusion

In this work, we studied the results of the overexpression of release factors on the expression of the Tryptophanase gene, an important gene related with bacterial cell survival. To understand our data, it is important to recognize that inducing levels of L-Trp produce higher activity levels in the β-Galactosidase assay, because L-Trp blocks the action of release factors within the ribosome translating the *tnaC* gene (Martinez et al. 2011), which is located at the *tnaC* stop codon (Martinez et al. 2013). The L-Trp-arrested ribosomes allow the transcriptional expression of the reporter gene (Martinez et al. 2011), which expresses the β-Galactosidase enzyme. In this project, we analyzed the effect of the nature of the *tnaC* stop codon in the L-Trp regulation of the expression of the *tnaCAB* operon. We used a series of reporter genes containing any of the three known stop codons (UAG, AGA and UAA) to analyze the effects of release factors in the L-Trp action on translation termination.

As expected, all cell lines grown in inducing levels of L-Trp produced significantly higher β-Galactosidase production than their counterparts grown in noninducing levels of L-Trp because the cells grown in noninducing levels of L-Trp do not experience prolonged stalling (Martinez et al. 2013). Of the three strains VK800 had the least β-Galactosidase activity, compared to the other strains, when grown in inducing levels of L-Trp and noninducing levels of L-arabinose [Figure 10]. This implies that the action of RF1 on the UAG and UAA codons could be less inhibited by L-Trp then the RF2 action in the UGA and UAA codons. Contrary, the strains containing the R256T mutant plasmids behaved more similarly to the RF2 cells than to their RF1 counterparts in all cases except in the PDG1158 cell line. PDG1158 cell line likely had unexpected results because PDG1158 cell line has the stop codon UAA which binds preferentially to RF1 but can also bind to RF2.

In general, when more release factors are produced, under inducing L-arabinose conditions in the presence of L-Trp, then less activity occurs, the opposite of what was expected [Figure 7, 8, 9]. This is because the release factors are perhaps competing with L-Trp in releasing the arrested ribosomes.

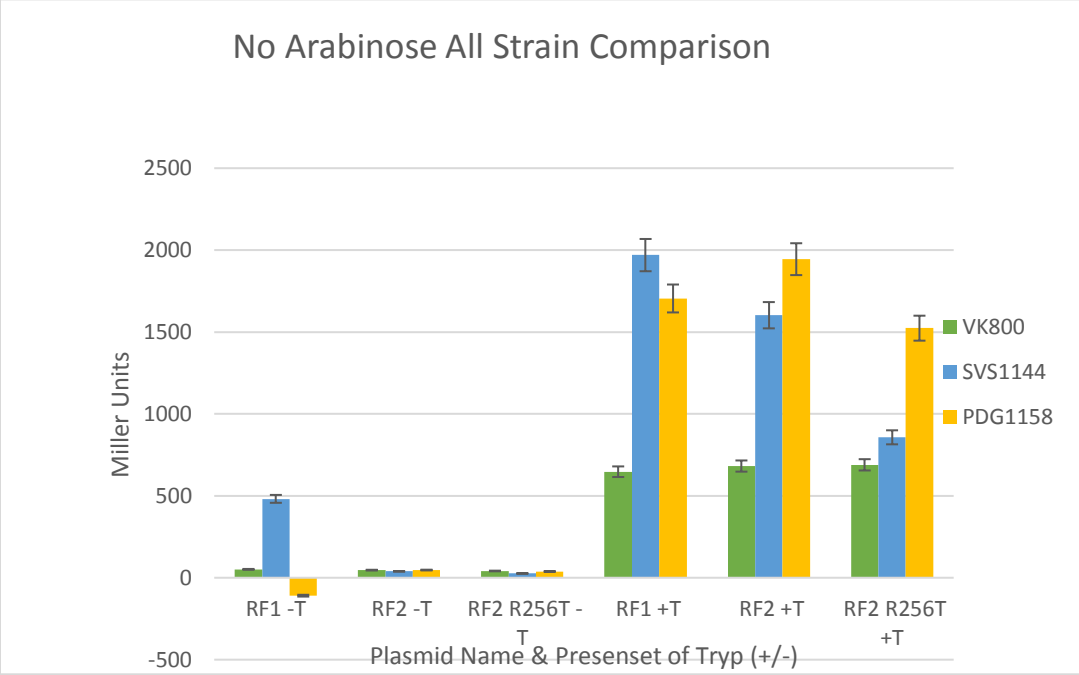

Figure 10: Comparison between all constructs grown in noninducing arabinose media. Constructs grown in inducing levels of L-Trp have higher galactosidase production. See Figure 7 for figure information.

PDG1158 and SVS1144 (except in the RF1 construct) showed the greatest decrease in activity under inducing levels of L-Trp when L-arabinose was added [Figure 11]. The VK800 cell line was affected less by expression of any of the T7 controlled plasmids.

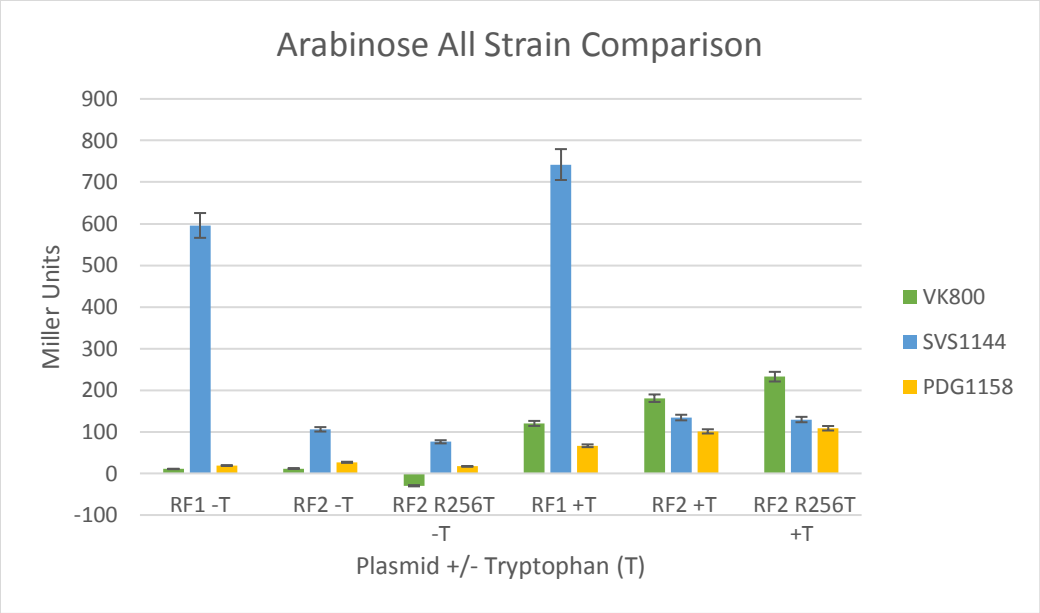

Figure 11: Comparison of all constructs grown in inducing L-arabinose conditions. Activation of the pET plasmid via inducing levels of L-arabinose, lowers the rate of β-Galactosidase production. SVS1144 was not susceptible to pET RF1 activity, as β-Galactosidase production did not decrease. See Figure 7 for figure information.

Of the three strains SVS1144 showed the least decrease in activity in the RF1 plasmid between noninducing and inducing levels of L-arabinose [compare Fig 10 and Fig 11]. This construct showed significantly more activity under all conditions than the RF2 and RF2 R256T mutant constructs in SVS1144 bacterial strains because RF2 and RF2 mutant are able to overcome the stalling under inducing levels of L-arabinose. Lower activity rates were also seen in all cell lines grown in inducing levels of L-arabinose compared to their counterparts grown in noninducing levels of L-arabinose [compare Miller units levels between Fig 10 and Fig 11]. Therefore, activating the pET plasmid under the T7 promoter (controlled by the pTARA plasmid) alleviates some of the stalling seen under inducing levels of L-Trp. Therefore, increasing the amount of release factors does decrease L-Trp stalling which reduces the expression of the reporter gene.

Still, this experiment lacks controls with expression of the release factor proteins. Therefore, future experiments could focus on vectors that contain no release factors to confirm that the decreased activity is not an effect of increased L-arabinose levels.

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Tables and Graphs

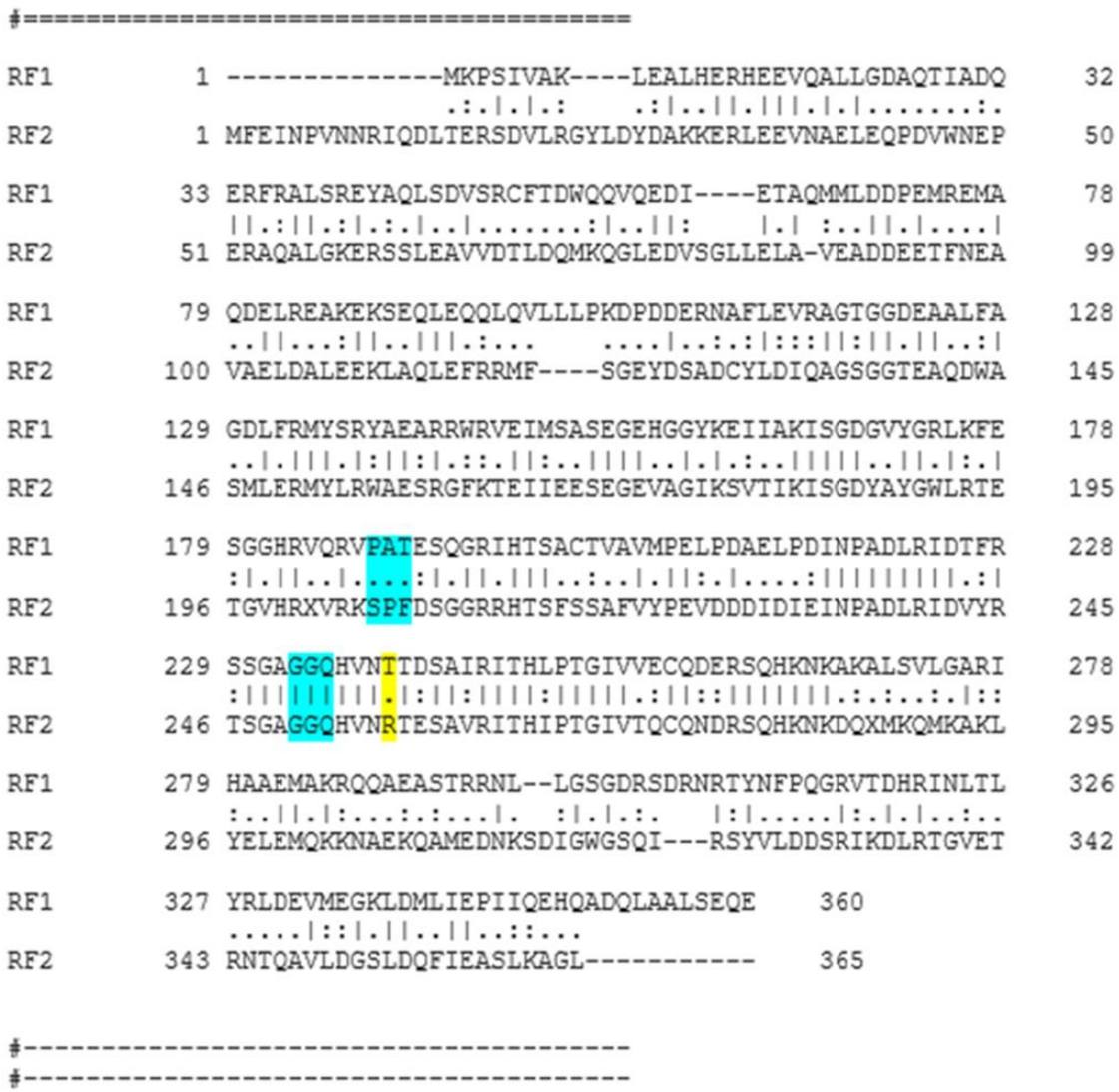

Figure 12: Amino Acid sequence of the operon. The mutation is highlighted in yellow. The critical conserved regions are highlighted in blue.