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Sophia Miranda M. Gonzalez April 10, 2023

Investigation of mRNA Recognition and Specificity by the Type II Toxin YoeB in *Escherichia coli*

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An abstract of
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Abstract

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Toxin-antitoxin (TA) modules are gene pair loci ubiquitous in bacterial cells that encode for a toxin, inhibitors of cell growth, and its cognate antitoxin which counteracts the toxin. In general, toxins are proteins and inhibit cell growth by targeting crucial processes such as DNA replication or protein translation, whereas the antitoxin can either be a protein or RNA. Currently, there are eight known types of TA modules, classified based on the nature of the antitoxin and its mode of repression of the toxin. The best studied TA systems are the type II TA modules; the main characteristic of these is that both toxin and antitoxin are proteins. Under normal physiological conditions, the antitoxin is directly bound to the toxin, inhibiting toxin activity. The addition of stress to the system releases the labile antitoxin from the toxin, resuming toxin activity. My research investigates the type II toxin YoeB, a ribosome-dependent endoribonuclease that targets mRNA actively involved in translation. YoeB selectively cleaves mRNA at the ribosomal aminoacyl (A) site between the second and third nucleotide and confers specificity for certain nucleotide sequences. This paper investigates the specificity and recognition mechanisms of YoeB to its mRNA substrates by generating variants of the YoeB protein. Through the systematic substitution of the YoeB Glu62 residue, we may better understand how this toxin targets mRNA and define how specificity for cleavage relates to its biological function.

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I thank Dr. Vince Conticello and Dr. Simbarashe Nkomo, my committee members who have supported me not only throughout this project, but also throughout my time at Emory.

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Introduction

Type II Toxin-Antitoxin Modules

Toxin-Antitoxin (TA) modules are widespread in bacteria and archaea, which are composed of a toxin capable of inhibiting cell growth, and a cognate antitoxin, which neutralizes the toxin. There are eight primary classes of TA modules which are classified based on either the mechanism of toxin inhibition by the antitoxin, the type of interaction between the antitoxin and toxin, or what biomolecule the antitoxin is (Singh, et al., 2021; Song and Wood, 2020). In general, toxins are proteins that affect crucial cellular processes such as DNA replication, RNA transcription and protein translation, while the antitoxin counteracts the toxin through a variety of mechanisms and can either be a protein or RNA (Cook, et al., 2015; Harms, et al., 2018; Page and Peti., 2016). The largest and best studied class of TA systems are type II TA modules, where the antitoxin is a labile protein that binds to the toxin to neutralize its activity (Figure 1) (Harms, et al., 2018; Page and Peti., 2016). Under stress conditions such as amino acid starvation, heat shock, and antibiotic stress, cellular proteases selectively degrade the antitoxin, allowing the toxin to resume its activity (Fraikin, et al., 2020; Takagi, et al., 2005). Despite the diversity of type II toxins, most of them target protein synthesis by cleaving RNAs and can be divided into two subclasses, ribosome-independent or ribosome-dependent toxins.

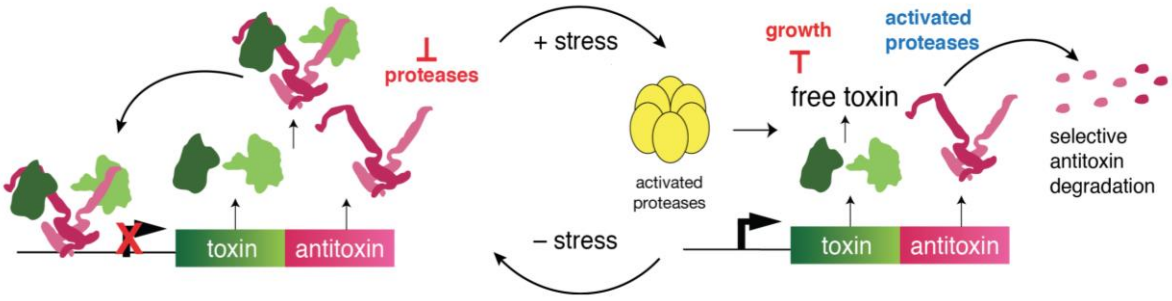

Figure 1. Schematic of type II toxin-antitoxin modules. Type II TA complexes act as transcriptional regulators by binding to the promoter upstream the toxin and antitoxin operons. The addition of stress to the systems leads to the degradation of the antitoxin by cellular proteases, allowing the toxin to resume its activity in inhibiting cell growth.

Ribosome-Dependent Type II TA Modules

The ribosome is the site of protein synthesis within cells and is responsible for the translation of RNA into proteins (Ramakrishnan, 2002). The ribosome has three tRNA binding sites: the aminoacyl (A) site, where aminoacylated-tRNA bind and mRNA is decoded; the peptidyl (P) site, the binding site for the tRNA that holds the extending polypeptide chain; and the exit (E) site, where uncharged tRNA are released from (Figure 2) (Ramakrishnan., 2002). The best studied ribosome-dependent toxins are the RelE-like toxins which include *E. coli* RelE, YoeB, and YafQ, and *Proteus vulgaris* HigB (Neubauer et al., 2009; Maehigashi et al., 2015; Schureck, et al., 2015; Zhang and Inouye., 2009). The RelE-family toxins heavily rely on the ribosome for mRNA recognition and bind to the ribosomal A site for the cleavage of the A-site codon, specifically between the second and third nucleotides (Jurėnas and Melderen, 2020; Neubauel et al., 2009; Pedersen et al., 2003). The cleavage of mRNA actively undergoing translation disrupts protein synthesis, and subsequently, cellular growth.

These toxins have similarities in their functions and target the same location on the ribosome. However, they differ in the amino acids in their active sites and in their interactions

with the ribosome (Neubauer et al., 2009; Maehigashi et al., 2015; Pavelich et al., 2019; Schureck et al., 2015). Type II ribosome-dependent toxins do not target all codons and vary in sequence specificity for mRNA cleavage. HigB preferably cleaves AAA (lysine) codons but can also cleave adenosine rich sites with an adenosine or cytosine in the second position, or codons with an adenosine in the third nucleotide (Hurley and Woychik., 2009; Schureck et al., 2015; Schureck et al., 2016). YafQ is like HigB in that it preferentially cleaves in-frame AAA codons but requires the following nucleotide to either be an A or G. (Hurley and Woychik., 2009; Prysak et al., 2009). The RelE toxin induces mRNA cleavage with a preference for the UAG stop codon, but also cleaves the UAA and UGA stop codons, as well as CAG (glutamine), UCG (serine), UGG (tryptophan), UUG (leucine), AAG (lysine), and GAG (glutamate) sense codons (Pederson et al., 2003; Neubauer et. al., 2009). YoeB exhibits broad codon specificity for the cleavage of sense codons AAU (asparagine), CUG (leucine), AAA (lysine), GCU and GCG (alanine), but also recognizes the UAA stop codon for cleavage (Kamada and Hanaoka., 2003; Zhang and Inouye, 2009). The RelE family toxins recognize different three-nucleotide codons at the ribosomal A site, but how these toxins demonstrate sequence specificity is not fully understood. The exact mechanism of mRNA recognition by these toxins remains unclear, and further studies of each toxin and their interactions with the ribosome merits further investigation.

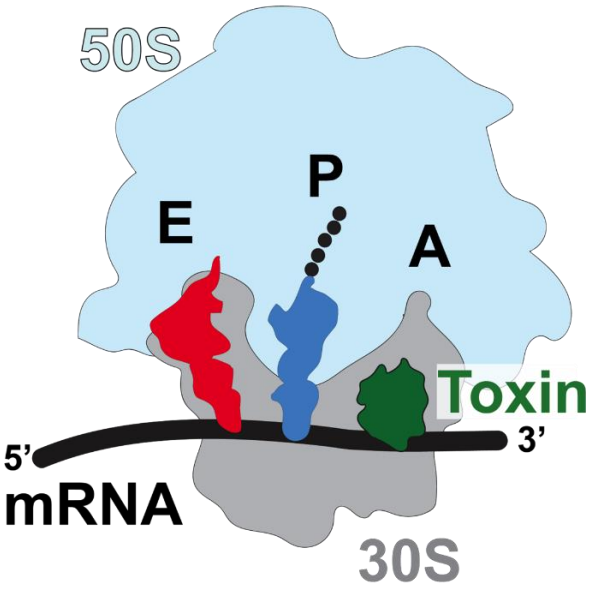

Figure 2. Protein translation by the ribosome. The ribosome is the site for protein synthesis within cells and is responsible for translating RNA into protein. There are three tRNA binding sites, the aminoacyl, peptidyl, and exit sites. Type II ribosome-dependent toxins (green) bind to the ribosome's aminoacyl (A) site during translation, cleaving mRNA and effectively inhibiting cellular growth.

Defining mRNA Specificity in Ribosome-Dependent Toxins

E. coli YoeB, YafQ, RelE, and *P. vulgaris* HigB generally adopt a conserved RelE-family RNase fold comparable to that of RNase T1, an endoribonuclease that degrades single-stranded RNA and uses conserved histidine and glutamate residues for catalysis (Jurėnas and Melderen, 2020; Kamada and Hanaoka, 2005; Schureck, et al., 2016). Even though these toxins share a conserved RNase fold, they have low sequence identities (15%) and contain different amino acids in their active sites, which may contribute to their varying degrees of codon specificity for mRNA cleavage. Despite these differences, RelE-lke toxins are accommodated within the A site for mRNA cleavage and coordinate with the three nucleobases of the target codon.

Previously, Schureck et al., investigated how the HigB toxin recognizes its mRNA substrate through the structural determination of a 70S ribosome-HigB complex, and later compared it

with the structures of the 70S ribosome-RelE and YoeB complexes (Figure 3). Further, they conducted mRNA cleavage assays of different A-site mRNA sequences upon HigB addition. From these assays, they determined that HigB demonstrates a preference for adenosine at the third A-site nucleotide (Schureck et al., 2015; Schureck et al., 2016). The structure of the 70S ribosome-HigB complex revealed HigB interacted with the 16S rRNA nucleotide C1054 and it was proposed that this interaction was important to define codon recognition at the third position of the A site (Figure 3A). HigB amino acid Asn71 stacks with C1054 and C1054 forms a *trans* Watson-Crick-Hoogsteen interaction with the third adenosine of the A-site codon. It was proposed that Asn71 may play a key role in HigB specificity because of its high conservation across HigB homologs (sequence identity of over 85%). To test this, Schureck et al., engineered a HigB N71A variant and found that the N71A variant is still active in the inhibition of cell growth. Interestingly, the HigB N71A variant cleaved the AAA codon roughly 10-fold less efficiently than that of HigB wild-type. So, while HigB N71A remains active, it no longer allows for the cleavage of its preferred codons suggesting that HigB Asn71 confers substrate specificity (Schureck et al., 2015; Schureck et al., 2016).

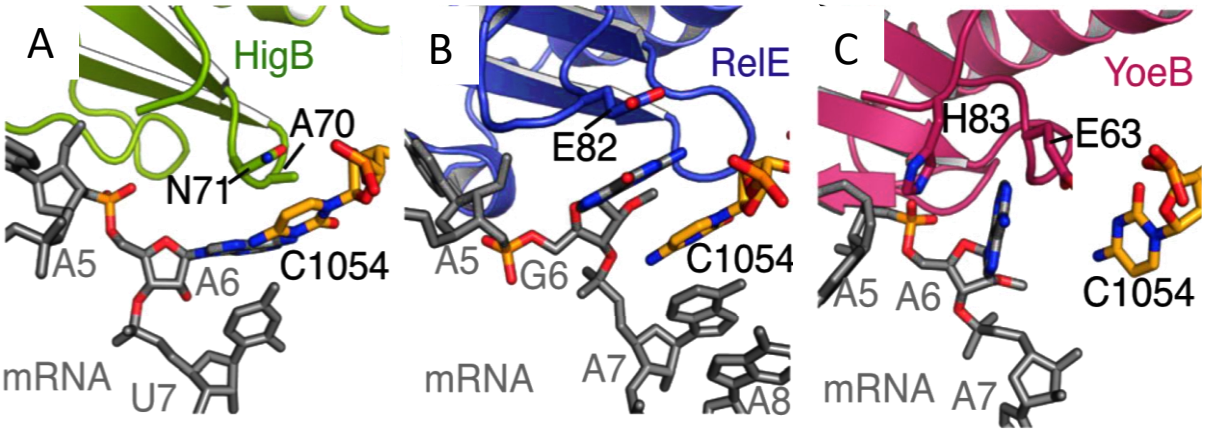

Figure 3. Structural comparison of HigB, RelE, and YoeB during nucleotide selection. Included are the C1054 residue of the 16s rRNA and the third mRNA nucleotide at the A site, which is located at the +6 position. (A) HigB N71 stacks with C1054, orienting the Watson-crick face of C1054 for interaction with the Hoogsteen edge of A6 (B) RelE E82 forms a continuous stack with G6 and C1054 (C) YoeB H83 and E63 stack around A6 with C1054 playing a minor role in nucleotide selectivity.

Investigation of YoeB Glu62

While it was shown that the HigB Asn71 residue mediates mRNA specificity, the mechanisms of specificity in the toxins RelE and YoeB are still poorly understood. The aim of my project is to investigate if specificity is conferred in a similar way in the YoeB toxin. The structure of the 70S ribosome-YoeB complex recognizing the AAU codon reveals the interaction between YoeB Glu62, the 16S rRNA nucleotide C1054, and the third position of the A-site codon (Figure 4) (Pavelich et al., 2019). I propose that the Glu62 residue may play a key role in nucleotide specificity for mRNA cleavage by YoeB. In this thesis, I determine the importance of YoeB Glu62 in mRNA toxin activity through the substitution of the glutamate residues to generate the YoeB variants E62A and E62D. Glutamate was converted into alanine to eliminate the charge and into aspartate to make the side chain shorter, both of which could potentially lead to the loss of

specificity by YoeB. Through this investigation of the E62 residue, we may better understand the mechanisms of specificity in mRNA cleavage by YoeB.

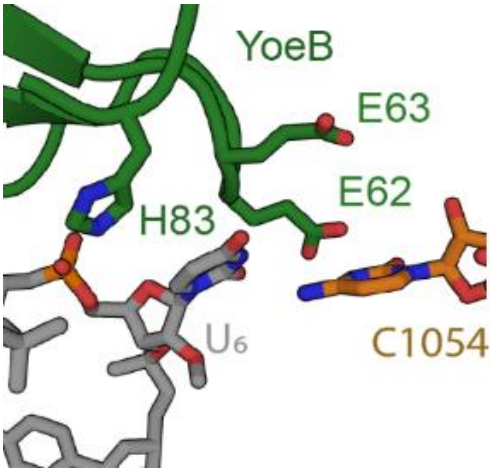

Figure 4. Remodeled structure of the 70S ribosome-YoeB complex in recognizing the AAU codon. This structure suggests that YoeB E62 may be interacting with the C1054 of the 16S rRNA and U6 of the mRNA. This differs from the previous structures that show minimal interaction between YoeB and the C1054 residue.

Significance: Antimicrobial Resistance and Bacterial Persistence

The discovery of antibiotics in the early 20th century played a crucial role in the advancement of modern medicine, but one of the problems that arises with the overreliance of antibiotics is that bacteria have developed mechanisms that render these medications ineffective, making the development of novel antimicrobials increasingly difficult. One of these mechanisms is antibiotic resistance, when bacteria evolve and mutate into new variants with genes that allow them to grow normally in the presence of an antibiotic (MacGowan and Macnaugton, 2017). Another way bacteria can become tolerant to antibiotics is through persistence. Unlike resistance, which is a genetic mutation, persistence is a phenotypic change that results from environmental changes. Under stress, bacteria can enter a state of persistence

in which cellular growth is halted until the stress has been resolved. As stress response modules, type II toxin-antitoxin systems are thought to be key players in bacterial persistence to antibiotics (Hayes and Van Melderen, 2011). The idea of persistence is relatively new in bacterial physiology and the studies on this are limited. However, the rise of antimicrobial resistance emphasizes the importance of investigating these mechanisms that allow bacteria to survive under stress for the develop on new antibiotics, most of which, target the bacterial ribosome (Figure 5) (Lin et al., 2018; Polikanov et al., 2018). Therefore, type II TA modules have become promising targets for antimicrobials because of their roles in the regulation of cell growth and bacterial persistence.

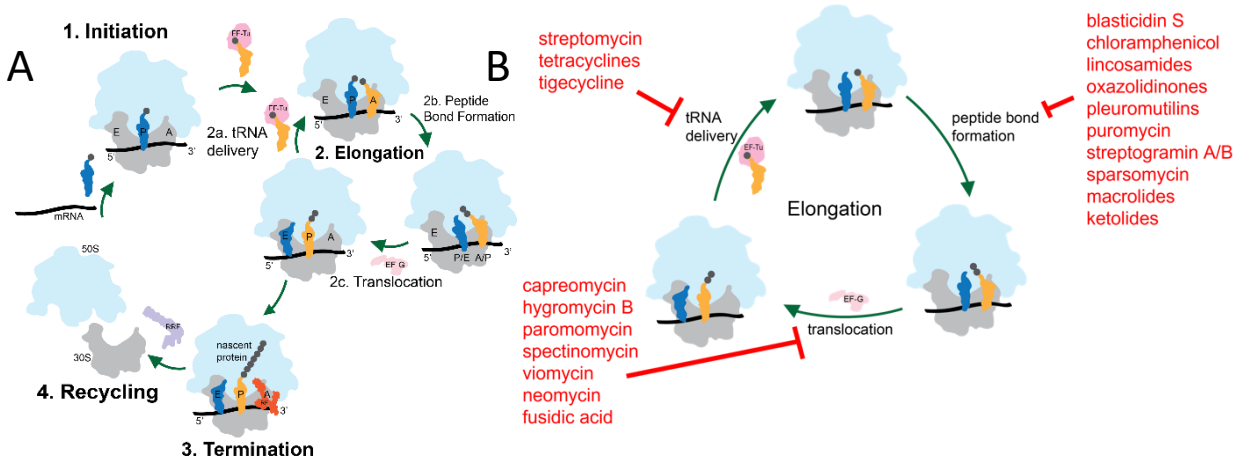

Figure 5. The ribosome in translation and ribosome-targeting antibiotics. (A) The ribosome is responsible for translating RNA into proteins, which occurs in 3 main steps: initiation, elongation, and termination. The important role that the ribosome plays in protein synthesis makes it one of the major targets for antibiotics, especially since translation occurs in several different steps, allowing for the development of diverse antibiotics. (B) A variety of antibiotics that target ribosomes during the elongation step of translation; over 60% of antibiotics that have been developed target the ribosome.

Materials and Methods

Strains and Plasmids

E. coli strains BW25113 and BL21 Gold (DE3) were used for protein expression, and DH5α $RbCl_2$ competent cells were used for cloning. The plasmids pBAD33-*yoeB,* pET21c-*yoeB* and pET21c-*yefMyoeB*$(His)_6$ were provided by Professor Masayori Inouye (Robert Wood Johnson Medical School, NJ, USA). These plasmids served as the backbone for the variants listed below and pBAD33 variants were made by Dunham Lab members at Emory University. LB broth and M9 media were used as the growth mediums, which were supplemented with the appropriate antibiotics (100 µg/ml ampicillin and 10 µg/ml chloramphenicol final concentrations) and carbon sources (0.2% glycerol, 0.2% glucose, and 0.2% arabinose final concentrations), when needed.

pBAD33	*yoeB WT*	*yoeB E62A*	*yoeB E62D*
pET21c	*yoeB WT*	*yoeB E62A*	*yoeB E62D*

Primer Design and Site-directed Mutagenesis

The plasmid pET21c-*yefMyoeB*$(His)_6$ was used as the backbone for the site-directed mutagenesis of *yoeB*-E62A and *yoeB*-E62D. The primer pairs were designed based on prior studies (Liu and Naismith *BMC Biotechnology*). Oligos are shown in detail in the table below.

A

Mutation	Primer Name	Sequence (5'-3')
pET21c YoeB E62A (GAG to GCG)	YoeB_E62A_F	**GACGGTGTTCCGCTGT**AATGCGTCGGGACCAGAAGC
	YoeB_E62A_R	**ACAGCGGAACACCGTC**TGGTATACGCGGTTACCGACG
pET21c YoeB E62D (GAG to GAC)	YoeB_E62D_F	**GACGGTGTTCGTCTGT**AATGCGTCGGGACCAGAAGC
	YoeB_E62D_R	**ACAGACGAACACCGTC**TGGTATACGCGGTTACCGACG

B

Reagent	E62A rxn (µL)	E62D rxn (µL)
5X Phusion HF buffer	10	10
Phusion (20 units/ ml)	0.5	0.5
5 mM dNTPs	1	1
10 µM F primer	2.5	2.5
10 µM R primer	2.5	2.5
DNA (5 ng/µl)	2	2
MQ H_2O	31.5	31.5
Total	50	50

Table 1. Oligos and PCR reagents for site-directed mutagenesis. (A) Oligos used in site-directed mutagenesis. Bolded areas correspond with the regions where the primer pairs overlap. The nucleotides in red are the site of point mutation and green indicates the mutant nucleotide. (B) Reagents used in PCR reactions.

The PCR was run according to the protocol below.

1. Initial cycle of 95°C for 5 min.
2. 12 cycles of 95°C for 1 min, Tm_{no} - 5°C for 1 min, and 72°C for 500 bp/min (12 min for 6 kb).
3. Final cycle of Tm_{pp} - 5°C for 1 minute and then 72°C for 30 minutes.
4. Hold at 4°C.

Afterwards, 1 µl of DpnI (NEB) was added to each reaction, incubated at 37°C overnight, and then heat inactivated at 80°C for 20 minutes the next day. Reactions were transformed into DH5α $RbCl_2$ competent cells, plated, and incubated at 37°C overnight. Randomly selected single colonies on the plate were inoculated in 5 ml LB with 100 µg/ml ampicillin and were left shaking at 200 rpm overnight in a 37°C incubator. The cultures were spun down, miniprepped following the QIAprep protocol, and sent for Sanger sequencing at Azenta/Genewiz.

Induction of YefMYoeB, YoeB, and YoeB variants

BL21 (DE3) Gold cells containing pET21c-*yoeB,* pET21c-*yoeBE62A*, pET21c-*yoeBE62D*, and pET21c-*yefMyoeB* were subcultured (1:100 dilution) into fresh LB media with ampicillin (final concentration of 100 µg/ml at 37°C to an OD_{600} of 0.4 to 0.7 and induced with 1 mM Isopropyl β-D-1 thiogalactopyranoside (IPTG). Cultures were grown for 3 hours and then cells were pelleted by centrifugation at 16,000 rcf for 5 minutes. The cells were resuspended in 25 µl lysis buffer (20 mM Tris-HCl pH 7.5, 250 mM KCl, 0.1% Triton X-100, 5 mM β-mercaptoethanol, 0.1 mM Benzamide, and 0.1 mM Phenylmethanesulfonyl Fluoride (PMSF)) and then put through a freeze thaw cycle 10 times (2 minutes freeze, 2 minutes thaw at 37°C, and 5 seconds vortex). The samples were spun down at 16,000 rcf for 5 minutes, then the soluble fraction was collected and diluted with 25 µl laemmli buffer (40% glycerol, 125 mM Tris-HCl, 4% SDS, 0.05% bromophenol and 0.05% BME added upon use). These samples were boiled for 10 minutes at 95°C and loaded with 2 µl of Precision Plus Protein Standards (Biorad) on a 4-20% gradient SDS-PAGE gel, which was run at 150 V for 60 minutes or until the dye front has nearly run off. The SDS-PAGE gel was then stained with a water based-Coomassie G-250 stain and then destained in water overnight for analysis.

Spot Dilution Assays

E. coli BW25113 strains transformed with either pBAD33*,* pBAD33-*yoeB*, pBAD33-*yoeB-E62A,* or pBAD33-*yoeB-E62D* were grown in 5 ml M9 minimal media supplemented with chloramphenicol and glycerol at 37°C with 200 rpm shaking to an OD_{600} of 0.2. The cultures were then serially diluted 10-fold (10^{-1} to 10^{-6} dilutions) with M9 minimal media in a 96 well plate (Figure 6). An 8x6 stamp was used to stamp the samples from the wells onto M9 agar plates

supplemented with chloramphenicol and either glucose or arabinose; the cells were grown at 37°C for over 48 hrs.

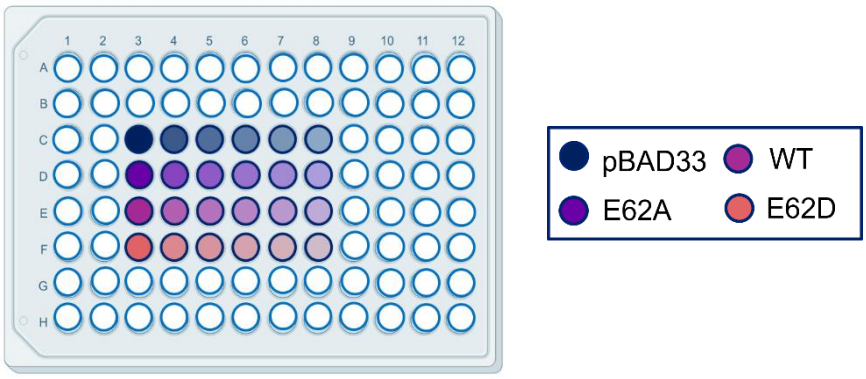

Figure 6. Diagram of 96 well plate for spot dilutions. Samples were prepared in the wells, which were serially diluted 10^{-1} to 10^{-6} dilutions with M9 minimal media.

Bacterial Growth Assays

5 ml M9 media with chloramphenicol and glycerol were inoculated with single colonies from *E. coli* BW25113 strains transformed with either pBAD33, pBAD33-*yoeB,* pBAD33-*yoeB-E62A,* or pBAD33- *yoeB-E62D.*The cultures grew at 37°C with 200 rpm shaking to an OD_{600} of 0.2, subcultured to an OD_{600} of 0.1 with M9 media, and then loaded into the 96 well plate (Figure 7). Glucose (0.2%) was added to half of the samples and the plate was left in the plate reader for 4 hours. After 4 hours, the plate was removed, and the other half of the samples were induced with arabinose (0.2%); the cells grew for 20 more hours for a total of 24 hours.

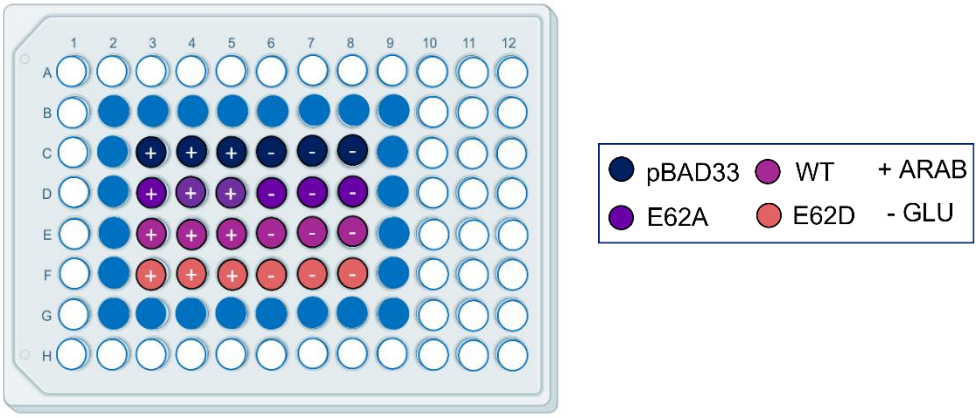

Figure 7. Diagram of 96 well plate for growth assays. Each variant was tested in triplicates, and the samples were treated with arabinose and glucose, which are notated as + and -, respectively. The blue circles represent the surrounding wells with Milli-Q H_2O.

Results and Discussion

The purpose of testing *in vivo* function is to determine the effect of toxin overexpression on bacterial cell growth, and to compare this activity between the wild-type and variants. Former lab members have previously solved the X-ray crystal structure of the YoeB toxin bound to the *Thermus thermophilus* 70S ribosome, giving insight into how the UAA codon is recognized by YoeB. This structure revealed that Glu62 may potentially be important for the selection of the third nucleotide of the A-site codon and likely interacts with 16S rRNA nucleotide C1054 (Pavelich et al., 2019). To better understand the role of E62 in YoeB function, I generated the variants YoeB E62A and E62D and tested for toxin activity through spot dilutions and bacterial growth assays. For both experiments, bacterial cultures were grown in M9 minimal media, instead of LB, to control the growth of the cells, allowing for better comparison between YoeB WT and the variants.

Spot Dilutions of YoeB Variants

To identify if the YoeB variants are still active, the *yoeB* gene was cloned into the pBAD33 vector to generate pBAD33-*yoeB.* The pBAD plasmid contains the araBAD promoter, allowing for the tightly controlled expression of the toxin protein through the addition of arabinose or glucose, which induce and repress protein expression, respectively (Guzman et al., 1995; Zhang and Inouye, 2009). Thus, the presence of bacterial colonies on the plates is an indication of cell growth, which can be related to toxin activity. The results show that when YoeB WT, E62A, and E62D are uninduced, the samples are similar and exhibit normal growth, as seen in the number of colonies for each dilution (Figure 8A). When plated on arabinose, all samples containing YoeB did not give any colonies after a 48-hour incubation period (Figure 8B). This indicates that YoeB was expressed, and that the toxin remains active even after the substitution of the glutamate to either alanine or aspartate.

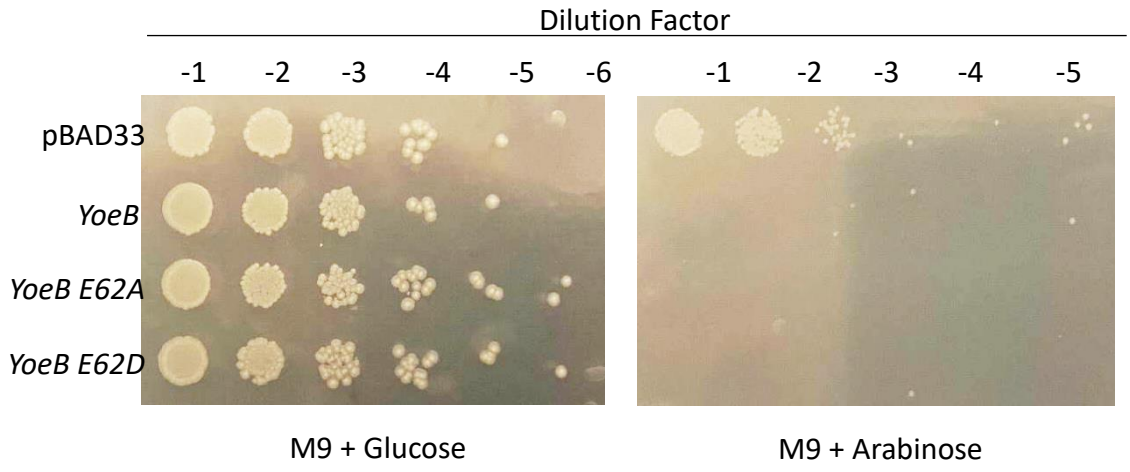

Figure 8. Spot dilution assay of YoeB variants. *E. coli* BW25113 was transformed with the plasmids pBAD33, pBAD33-*yoeB*, pBAD33-*yoeB-E62A*, and pBAD33-*yoeB-E62D.* Cultures were grown to a final OD_{600} of 0.2, then serially diluted 10-fold. The samples were spotted on M9-glycerol-CHL plates with either 0.2% glucose (repressing) or 0.2% arabinose (inducing), then grown for 48 hours at 37°C.

Growth Curves of YoeB Variants

Bacterial growth can be monitored through growth curves, which correlate the optical density (OD) at 600 nm, to the incubation time. The OD_{600} is used to quantify cell growth and is related to the light scattering properties of the culture; as cells grow, the turbidity of the sample increases, and so does the $OD_{600.}$ Since the overexpression of YoeB inhibits cell growth, we should expect a growth defect; therefore, the recovery of any growth may be an indication of a loss in toxin activity. In this bacterial growth assay, we can determine if Glu62 is important for YoeB function by comparing cellular growth upon the induction of YoeB WT, E62A, and E62D.

As expected, when YoeB is not expressed, there is no growth defect, and the samples exhibit normal growth (Figure 9A). However, the bacterial growth assays show that upon the expression of YoeB WT, there are minimal changes in the OD_{600}, indicating that the wild-type is active and stalls cellular growth (Figure 9B). The substitution of glutamate to alanine reveals a difference in growth suppression, which shows a slight restoration of bacterial growth unlike the samples where YoeB WT was induced (Figure 9B). For the substitution of glutamate to aspartate, the variant retains its acidic functional group but is shorter by a single C-C bond. This replacement greatly reduces the activity of the toxin and exhibits normal cellular growth (Figure 9B).

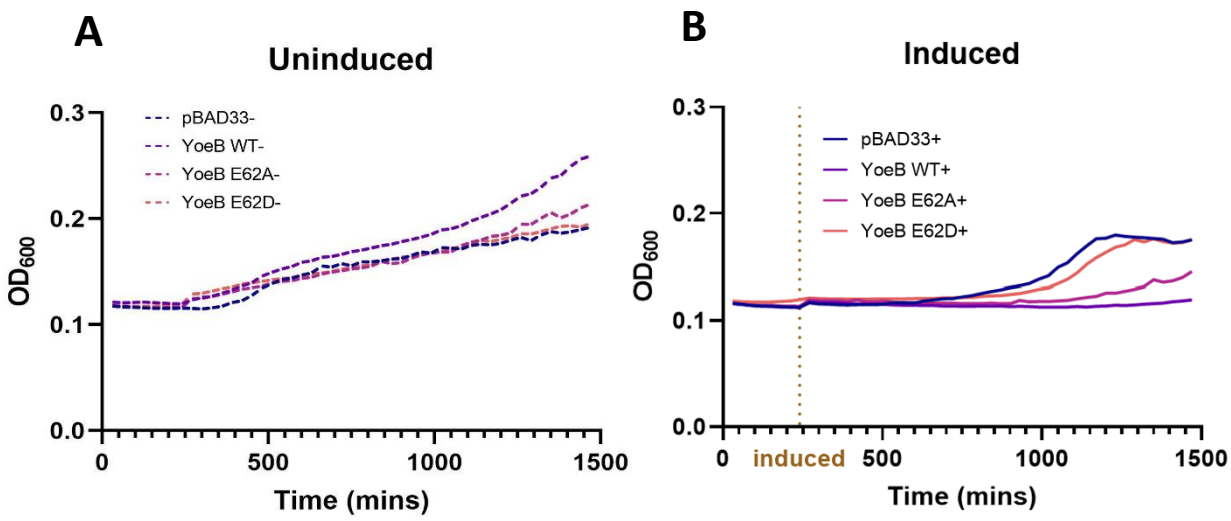

Figure 9. Bacterial growth assays of YoeB variants. Half of the samples are treated with glucose. After 4 hours, the other half were induced with arabinose, indicated by the brown dotted line. The cells were grown for another 20 hours for a total of 24 hours and monitored at OD_{600}. (A) Growth of the samples treated with 0.2% glucose which shows normal cellular growth. (B) Growth of the samples treated with 0.2% arabinose after 4 hours.

The growth assays revealed a difference in toxin activity upon the substitution of glutamate to either alanine or aspartate. It was hypothesized that the substitution of glutamate to alanine would have had a greater effect on toxin activity instead of aspartate since the degree of difference between glutamate and alanine is more significant than with aspartate. Yet, based on the growth assays, the YoeB E62D variant led to a significant decrease in toxin activity whereas the E62A variant retains toxin function and shows a slight decrease in activity. While the results obtained from the bacterial growth curves suggest that the E62 residue is important for YoeB *in vivo* function, this contradicts the spot dilutions, which show that both variants are active and inhibit cell growth. One potential reason for this discrepancy is that the spot dilutions and growth assays were tested in two different conditions, mainly that the spot dilutions were induced and grown for over 48 hours with a starting OD_{600} = 0.2, but the growth assays grew for 24 hours with a starting OD_{600} = 0.1, and the samples were induced after the first 4 hours. Therefore, whether

E62 plays a role in nucleotide recognition or specificity is inconclusive but warrants further investigation, and the optimization of the conditions for growth curves may be worthwhile to accurately determine if Glu62 is important for YoeB function.

Small-scale Induction of YoeB E62A and E62D for Protein Purification

After generating pET21c-*yoeB* E62A and E62D, a small-scale protein induction trial was completed to check for the overexpression of the YoeB variants.

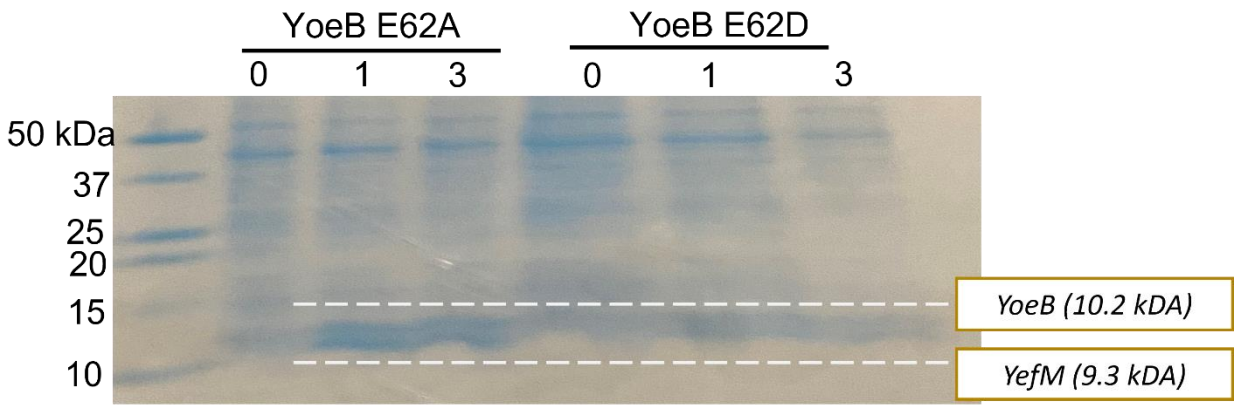

Figure 10. 4-20% SDS-PAGE of YoeB E62A and E62D. The loaded samples were taken before induction, 1 hour, and 3 hours post-induction with 1 mM IPTG. The gel reveals both YoeB and YefM were expressed after 1 hour.

Conclusion

In this thesis, I investigate the role of Glu62 in mRNA cleavage specificity by the endoribonuclease YoeB. Previously, it was determined that the specificity of mRNA cleavage by *P. vulgaris* HigB is conferred by a single amino acid, N71 (Schureck et al., 2015). Since HigB and YoeB adopt a similar fold and HigB exhibits selectivity around the third A site nucleotide of the mRNA, then specificity of YoeB may be conferred in a similar manner to HigB. The exact mechanism of nucleotide selection and mRNA cleavage by YoeB remains unknown, therefore,

the determination of the role of the E62 residue may give rise to new information to assist in answering these questions.

Effects of YoeB Variants on *In vivo* Function

The effects of the YoeB variants on *in vivo* function were tested through spot dilutions and bacterial growth assays. The spot dilutions reveal that both variants are still active and inhibit cell growth comparable to the wildtype. For YoeB E62A, the growth defect in the spot dilutions corroborated with the data from the growth curves which shows the overall decrease in cell growth with minimal recovery. This indicates that YoeB E62A is still active but is less active than the wild-type. For YoeB E62D, the spot dilutions showed a growth defect, but the growth curves contradict this data, which determined that the substitution to an aspartate neutralized toxin activity and showed normal bacterial growth. The results from the E62D variant are inconclusive, and the role of E62 in YoeB activity remains unknown, but these results may assist in future studies on the mechanisms of specificity by YoeB.

Future Directions

The future directions for this project include a large-scale protein overexpression and purification for YoeB E62A and E62D for downstream biochemical assays to examine toxin activity. One of these methods is a stopped-flow single-turnover kinetic assay developed by the Dunham lab that can determine the initial rate mRNA cleavage (k_{obs}) by a toxin through fluorescence. By testing YoeB against a panel of mRNA substrates, these experiments will establish which codon sequences are preferred for cleavage by YoeB. After determining which A-site codons are specifically cleaved, this technique can also compare the cleavage efficiency of the wildtype, to that of the variants which may give insight on the specific role of E62 in YoeB

specificity. It is worth noting that the results from the E62A variant showed a decrease in *in vivo* function and it would be worthwhile to compare the k_{obs} values between the wild-type and the variant.

In conjunction with single-turnover kinetic assays, which only establish the rate of a reaction with the toxin in excess, binding assays such as microscale thermophoresis (MST) assays can monitor interactions between the toxin to the ribosome-mRNA complex. MST monitors temperature induced change on protein-protein interactions and can track the movement of the complex. This technique may help determine if E62 plays a role in the recognition and binding of mRNA by YoeB.

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