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In Vivo Analysis of Riboswitch Activity Using Luciferase Assays

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Abstract

Riboswitches are a type of RNA structure found on messenger RNAs that function in gene expression regulation. The preQ1 riboswitch is particularly interesting to study because it contains a relatively small aptamer binding region and it is not essential in *E. coli* for the organism's survival, allowing *in vivo* experimentation in a viable organism. Upon ligand binding to the aptamer, a hairpin-type RNA pseudoknot forms, which functions to turn off the gene using a premature transcriptional termination mechanism. However, in some species studied, this terminator hairpin possesses enough thermodynamic stability that it is doesn't unfold upon release of the ligand. Based on this, it has been shown that the terminator and anti-terminator hairpins are capable of coexisting in the absence of the ligand. Thermodynamics play a large role in determining the actual degree of the translational control. It was found in previous experiments that the greater the stability of the terminator hairpin, the less reporter gene (firefly luciferase) activity recorded. When the aptamer-anti-terminator hairpin conformation is present, luciferase activity was found to be roughly double. Since it is possible for both hairpins to be present, the gene is not either turned off or on completely. This allows for the ability for partial effects (differential degrees of control). These different expression patterns have been explored for different species that contain this riboswitch.

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Introduction

In the original central dogma of molecular biology, Ribonucleic Acid (RNA) was thought to act primarily as an intermediary message between the DNA genetic code and the enzymatic and functional proteins. However, RNA has since been recognized to exhibit many different functions and structures, adopt countless three-dimensional structures, and to act as a catalyst in biochemical reactions (Cech, 2014). The synthesis of RNA based on the DNA template is the process of transcription. It is followed by the synthesis of proteins by using the RNA as a template sequence, which is the process of translation (Berg et al., 2007). The genetic information that is stored in DNA is expressed through this process of transcription and translation in the form of proteins. RNA is a single stranded molecule made up of the nucleotides Adenine (A), Guanine (G), Cytosine (C), and Uracil (U) and is synthesized by the enzyme RNA polymerase (Berg et al., 2007). Since RNA is a single stranded molecule, the nucleotides are capable of forming classic Watson and Crick base pairs, which can give rise to the RNA secondary and tertiary structures. These structures are responsible for the various activities of RNA molecules in forming structures, binding to small molecules, DNA, RNA, proteins, and functioning in catalysis.

RNA synthesis (transcription) is catalyzed by a large enzyme called RNA polymerase. This process is carried out in three stages, initiation, elongation, and termination. During initiation, RNA polymerase binds to promoter sites on the DNA template. Promoter sequences are upstream from the gene being transcribed and signal the start of transcription for the RNA polymerase. The RNA polymerase then unwinds the DNA and begins to add the template's complementary nucleotides together with phosphodiester bonds. This process is known as elongation, Figure 1A. In bacteria, sequences within the newly transcribed RNA signal

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termination. One common method is the formation of a G-C rich hairpin structure followed by a sequence of four or more uracil residues, Figure 1B (Lee, 2011). RNA polymerase pauses immediately after it has synthesized a stretch of RNA that folds into a hairpin. The RNA polymerase is then able to scan its recently transcribed regions to determine if it contains a terminator sequence (Berg et al., 2007).
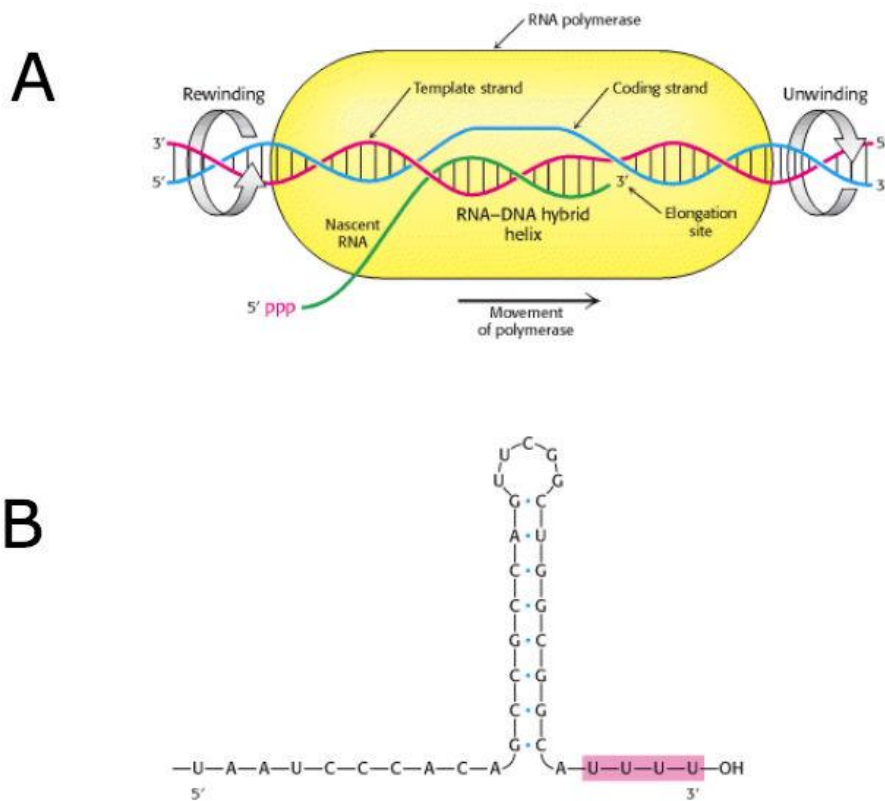
Figure 1: (A) Transcription by RNA polymerase. (B) Termination signal found at the 3' end of an RNA transcript. Figure adapted from Berg et al., 2007.

Riboswitches are small segments of non-coding mRNA located in the untranslated regions (UTRs) of genes that function in gene expression (Rieder et al., 2010). A tight control of gene regulation is seen as an advantage for bacteria because it allows them to conserve resources and reserve its energy for cellular necessities. Expressing genes unnecessarily can deplete

valuable resources that could be utilized in other pathways. Riboswitches are found widespread among distinct evolutionarily distinct microorganisms, which indicate an early evolutionary history. This control of gene expression is not as advanced as some methods seen in higher organisms today, but riboswitches may have provided an advantageous method of gene regulation in the ancient hypothesized RNA-based world (Winkler, 2003).

Riboswitches control gene expression by binding small ligands, which results in a conformation change of the RNA structure and leads to gene repression or activation (Atkins et al., 2001). Riboswitches are found in the 5' untranslated regions of mRNA in bacteria and in 5' and 3' untranslateed regions and introns of pre-mRNA's of plants and fungi (Villa, 2009). Riboswitches generally bind their prospective ligand with high affinity and with high specificity.

The ligand-binding portion of the riboswitch is referred to as the aptamer domain. The binding of the ligand in this region induces structural changes further downstream in the RNA in a region known as the expression platform, Figure 2. In transcriptional control riboswitches, the ligand binding causes a formation of a terminator hairpin loop similar to the hairpin loop in Figure 1B. This hairpin loop prevents the transcription of the complete protein-encoding mRNA (Villa, 2009). In this manner, the system functions like a negative feedback loop. The binding of the ligand induces a conformational change that results in the formation of a terminator hairpin, which effectively stops gene expression, Figure 2 (Liberman, 2012).

In the absence of ligand binding to the aptamer region, the expression platform folds to form an anti-terminator hairpin. The anti-terminator hairpin sequence overlaps with that of the terminator hairpin. As a result, both cannot exist simultaneously. When the anti-terminator hairpin is present, the G-C rich terminator hairpin cannot form and the oligo U tail cannot signal termination. Consequently, transcription continues along and the whole RNA is transcribed.
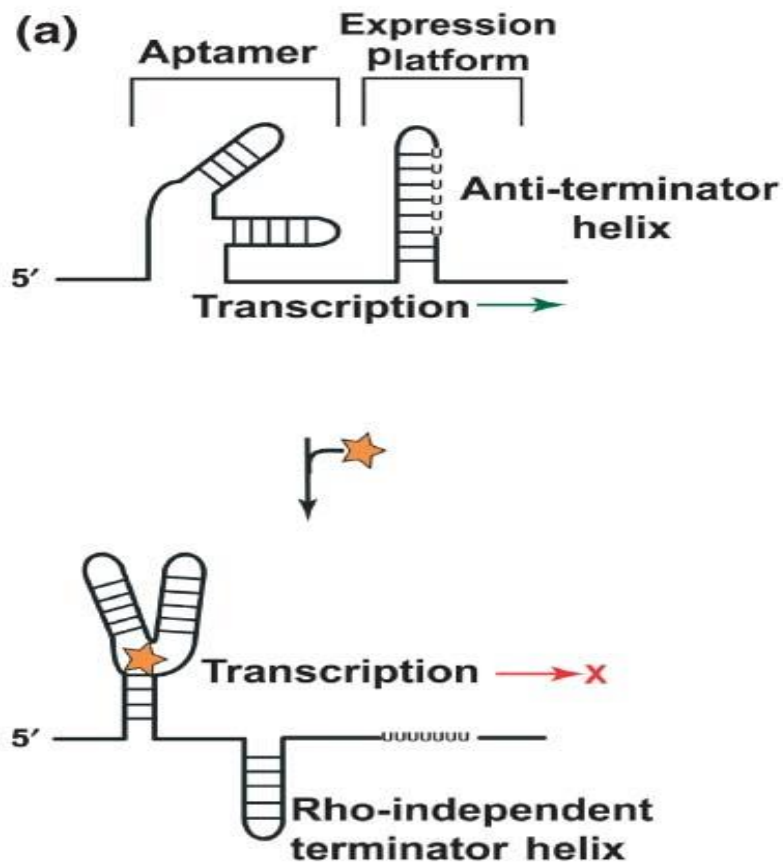
Figure 2. Formation of terminator hairpin as a result of ligand binding. The star represents the binding of the riboswitch specific ligand. This figure was taken, without modification from Liberman, 2012.

One riboswitch system that is of particular interest to study is the preQ$_1$ riboswitch. The preQ$_1$ riboswitch is a part of the biological pathway for the production of queuosine (Kang et al., 2009). Queuosine is a modified guanine nucleotide that occurs in the wobble position in some anticodons of tRNA's. The presence of the queuosine at this position is believed to improve translational accuracy (Kang et al., 2009).

Although queuosine is present in both mammals and bacteria, only bacteria are able to synthesize queuosine. Eukaryotes acquire queuosine from their diet and/or from bacteria in the

intestines and insert the queuosine directly into tRNA using an enzyme called tRNA-guanine transglycosylase (TGT) (Iwata-Reuyl, 2003). Bacteria are able to synthesize queuosine although the complete details of the biological pathway have not been completely determined, Figure 3.
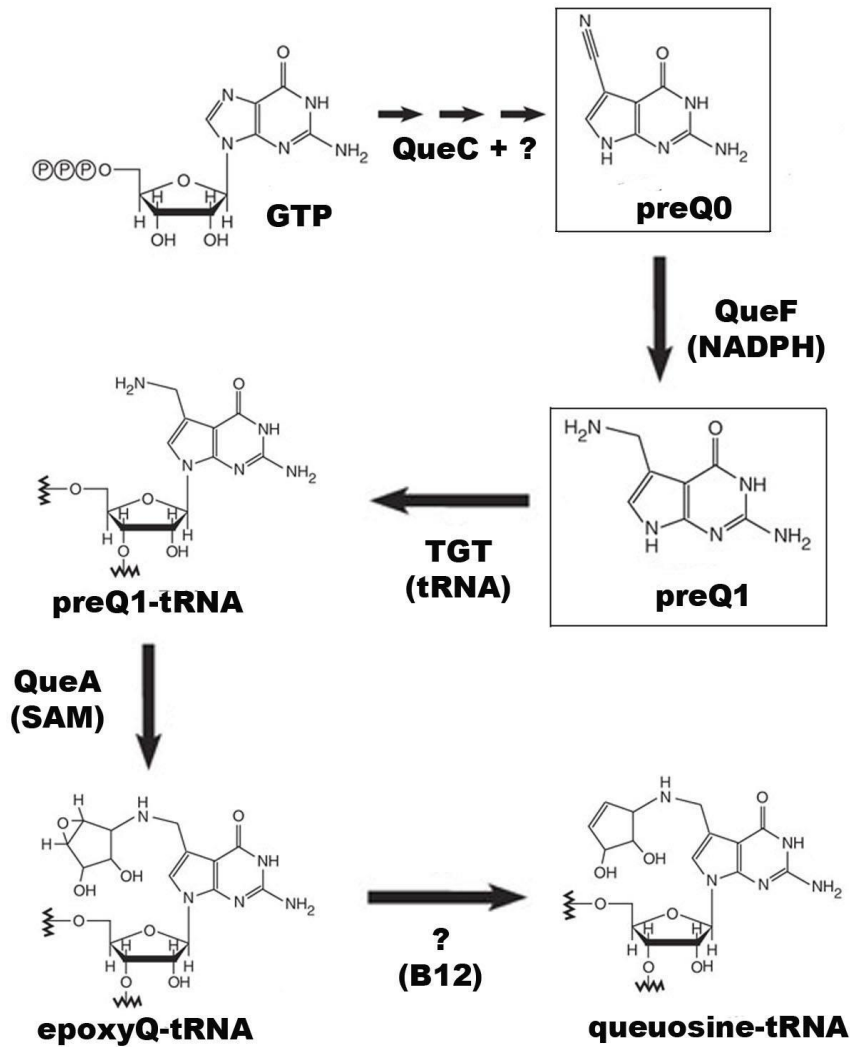

Figure 3. Pathway of queuosine biosynthesis in bacteria. This figure is adapted from Roth, 2007.

The preQ$_1$ riboswitch is responsible for the production of the queuosine precursors, preQ$_0$ and preQ$_1$, by modifying the gene expression of the *queC* gene in the *queCDEF* operon (Reader

et al., 2003).   First, GTP is converted to 7,8-hydroneopterin triphosphate (H2NTP) by GTP cyclohydrolase I (GCYH-I), the first enzyme of the folate biosynthesis pathway, providing a link between the queuosine-tRNA synthesis and primary metabolism (Kang et al., 2009).   Next, 7-cyano-7-deazaguanine (preQ$_0$) is converted to preQ$_1$ by an NADPH dependent oxidoreductase, QueF (Lee et.,al 2007).   Free preQ$_1$ is then inserted into the wobble position of the appropriate tRNA anticodon by a tRNA-guanine transglycosylase (TGT), replacing the unmodified guanine (G) in that position (Roth et al., 2007).

An abundance of preQ$_1$ in a cell is a signal that queuosine biosynthesis as currently not necessary, and results in preQ$_1$ binding to the riboswitch.   The binding of the preQ$_1$ ligand to the aptamer region results in rearrangement of the aptamer to the ligand-bound state which abolishes the anti-terminator hairpin structure, and allows the terminator hairpin to form, which prevents the production of more preQ$_1$ and the downstream product, queuosine (Kang et al., 2009).

The preQ$_1$ riboswitch is a good model system to study riboswitch-mediated transcription termination for a number of reasons.   First, the aptamer domain of the preQ$_1$ riboswitch consists of only 34 nucleotides, which makes it the smallest naturally known occurring aptamer region.   Second, both NMR and X-ray crystal structures are available for the preQ$_1$ bound aptamer domain (Roth et al., 2007).   The preQ$_1$ riboswitch is not essential for *E. coli* and thus allows *in vivo* characterization to be carried out.   These features make it easier to study the preQ$_1$ riboswitch compared to other larger and more complicated riboswitches.

One example of the individual riboswitch for *B. subtilis* is shown below in Figure 4.
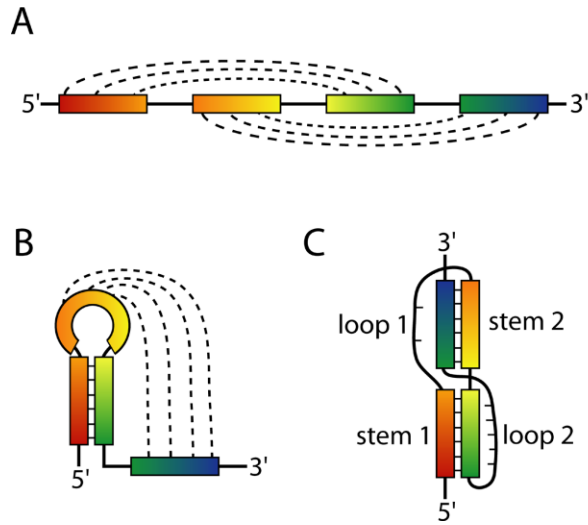
Figure 4. Secondary structure of *B. subtilis* riboswitch.

The riboswitch consists of the P0 hairpin, P1 (aptamer) hairpin and the terminator hairpin. The anti-terminator hairpin is shown above the anti-terminator hairpin (Roth 2007). The solution and crystal structures of the aptamer regions of the preQ$_1$ riboswitch from *Bacillus subtilis* have revealed that, upon the binding of preQ$_1$, the aptamer domain folds into an H-type RNA pseudoknot (Kang et al., 2009). In an H-type pseudoknot, the bases in the loop of a hairpin form intramolecular pairs with bases from outside of the stem (Figure 5A and 5B). This causes a second stem and loop, resulting in a pseudoknot with two stems and two loops (Figure 5C). In a pseudoknot, the stems and loops are labeled in sequential order (Staple et al., 2005).

The preQ1-binding pseudoknot from *B. subtilis* consists of the binding of preQ1 with a number of important base pairs in the riboswitch including C60 and A72, Figure 4 and 6 (Gong et al., 2014). This pseudoknot sequesters a portion of a 3' A-rich tail that is involved in the structure of the transcriptional anti-terminator (Kang et al., 2009). In the absence of preQ$_1$ the 3' A-rich tail remains unfolded and the RNA folds to form the anti-terminator hairpin.

Figure 5. RNA H-type pseudoknot. (A) Linear arrangement of base-pairing elements within an H-type RNA pseudoknot. (B) Formation of initial hairpin within pseudoknot sequence. (C) Classic H-type pseudoknot fold. This figure was modified from Staple et al., 2005.
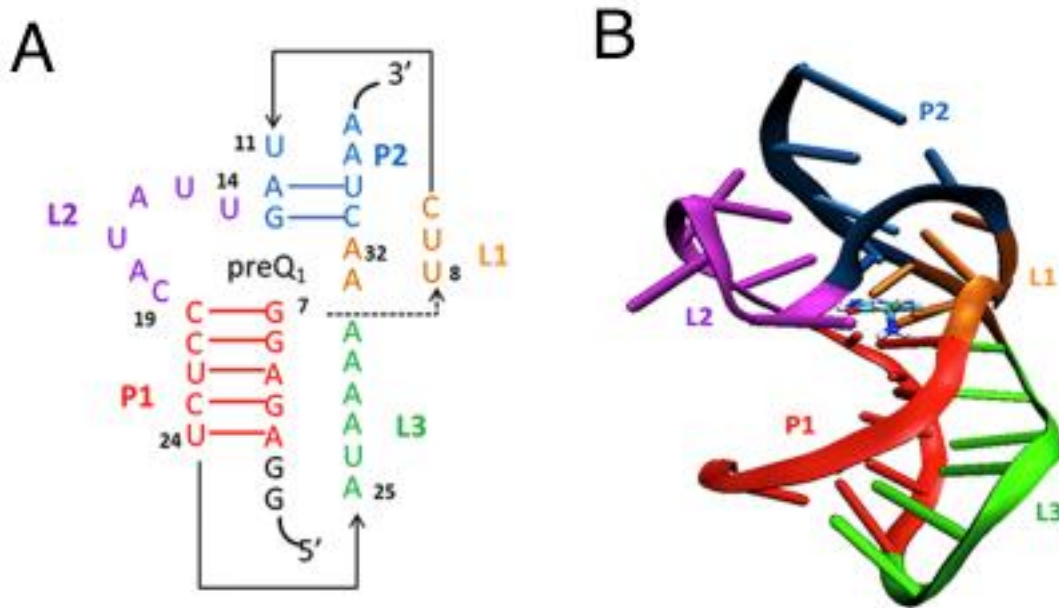

Figure 6. Structure of $preQ_1$ bound riboswitch. (A) Secondary structure of riboswitch. (B) Tertiary structure of riboswitch. This figure was adapted from Gong et al., 2014.

Thus far, studies on a single riboswitch of a certain type have been used to characterize entire families of riboswitches. While this is a good starting point, it ignores how species

variability could influence the degree to which riboswitch-mediated gene expression control could vary within a family, particularly when the ligand-binding family includes BOTH transcriptional control and translational control riboswitches. The sequences of the preQ$_1$ riboswitch in a number of different bacterium can be seen in Figure 7 (Roth, 2007).

From Figure 7, the aptamer domain regions of the riboswitches between species are highly conserved, while the expression platform is not as strictly conserved. Since the aptamer regions between species are highly conserved, they all will bind preQ$_1$ fairly similarly. The almost completely conserved C's from nucleotides 25-27 and the highly conserved A's from nucleotides 32-38 can be related to the C60 and A72 binding nucleotides in *B subtilis*.

The expression platform region is not as highly conserved. As a result, it is not safe to characterize the activity of the riboswitch based on studying one type of bacteria. Some conservation is still seen. There is a high conservation of groups of green thymine nucleotides. These can relate to the sequence of U's following the terminator hairpin that is necessary to terminate transcription. However, since conservation within the expression platform is not seen as clearly it is important to study the riboswitch from each species of bacteria independently.
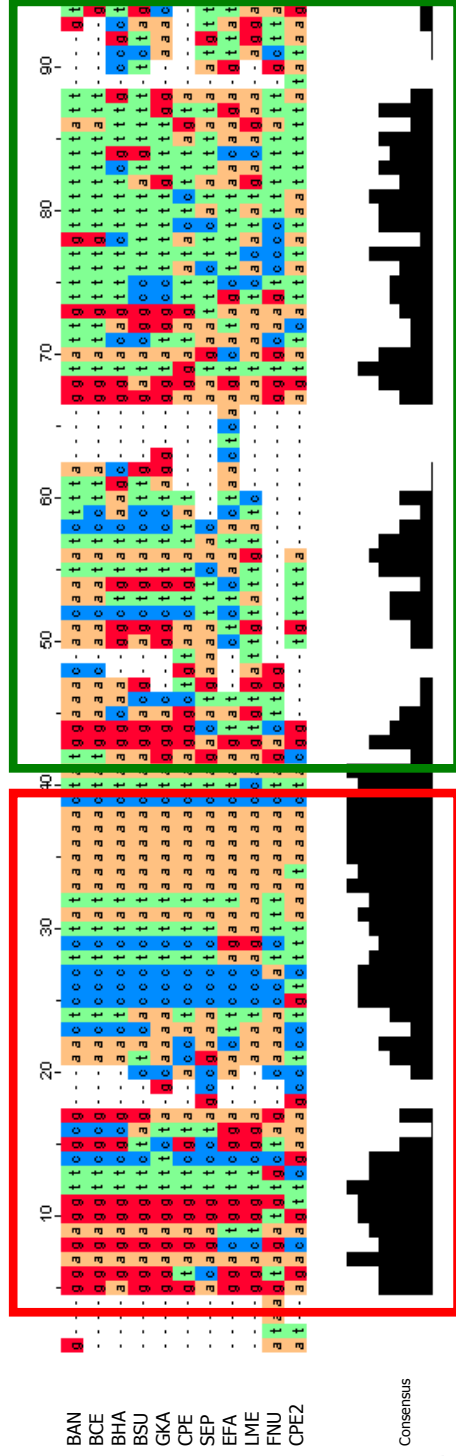
Figure 7. Alignment of 11 transcriptional control preQI-I riboswitches. Regions boxed in red and green correspond to the highly conserved aptamer domain sequence and the poorly conserved expression platform region, respectively. Nucleotides are colored by type: orange- adenine, blue-cytidine, red-guanine, green-thymine. The sequences aligned were from *Bacillus anthracis (Ban), Bacillus cereus (Bce), Bacillus halodurans (Bha), Bacillus subtilis (Bsu), Geobascillus kaustophilus (Gka), Clostridium perfringens (Cpe),Staphylococcus epidermis (Sep), Enterococcus faecalis (Efa),Leuconostoc mesenteroides (Lme), and Fusobacterium nucleatum (FNU). Adapted from Roth et al., 2007*

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Materials and Methods

In previous work by Dr. Nakesha Smith, a graduate student at the University at Albany, the riboswitch constructs of five species of bacteria were chosen to study. The species that were chosen to study in more detail were *Bacillus cereus* (BCE), *Bacillus halodurans* (BHA), *Geobacillus kaustophilus* (GKA), *Bacillus subtilis* (BSU), and *Fusobacterium nucleatum* (FNU). In all the species, the terminator hairpin was predicted to be more stable than the anti-terminator hairpin by a range of 0.6 to 7.1 kcal/mol. Since the terminator hairpin is predicted to be more stable, it is possible that the terminator hairpin remains even when the ligand unbinds. This gives the bacteria a range of expression as previously discussed.

In order to study the preQ1 riboswitch from different species of bacteria, the Dual-Glo Luiciferase Assay from Promega was utilized. This assay uses Recombinant Firefly Luciferase in a reaction that produces light. The amount of light produced can be measured by a lumometer and correlated to how much Recombinant Firefly Luciferase that was produced by the cell. The reaction for the assay can be seen below in Figure 8, which was taken from Promega's Dual-Glo protocol.
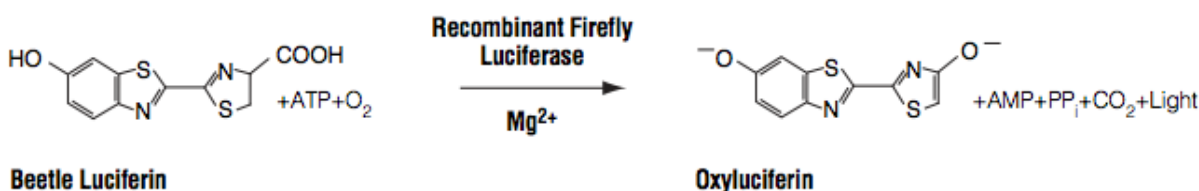

Figure 8. Recombinant Firefly Luciferase assay reaction.

A series of test vectors were constructed by Dr. Nakesha Smith designed to produce recombinant firefly luciferase based on the riboswitch system. First, the reporter gene, firefly luciferase, was inserted into an expression vector. The pRSF.1b expression vector from Novagen was chosen for a number of reasons. It is a high copy number plasmid, confers Kanamycin resistance, contains an RSF origin and has an inducible T7 promoter. The plasmid map of the pRSF.1b vector is shown in Figure 9.
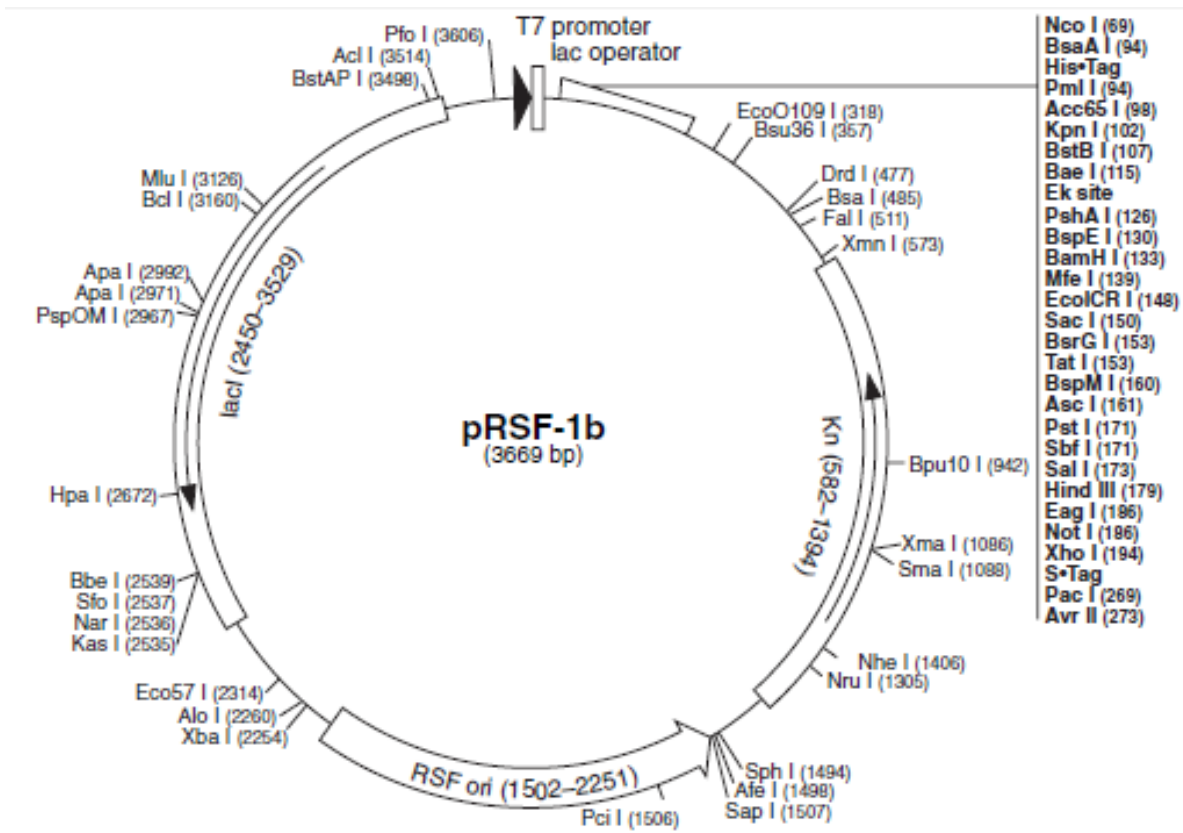

Figure 9. Plasmid map of the pRSF.1b vector.

The gene for firefly luciferase was cloned into the expression vector from the pSp-luc+NF fusion vector from Promega. This vector is shown in Figure 10 below.
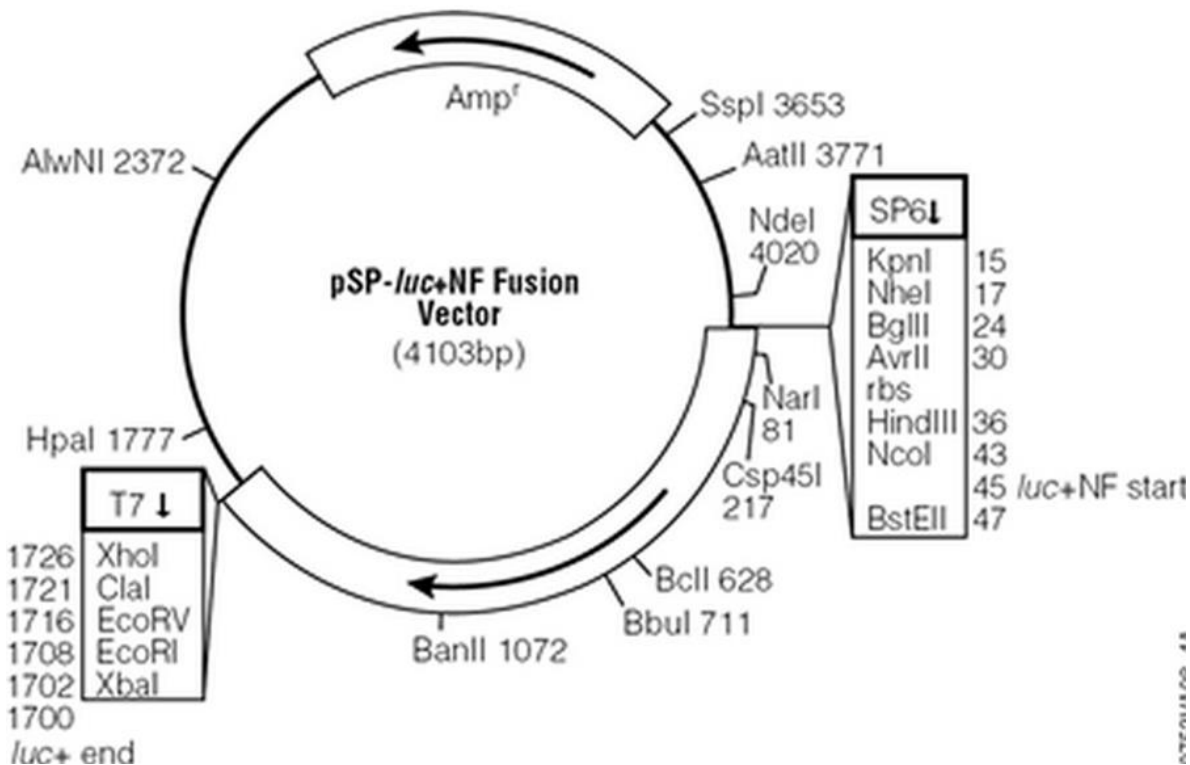
Figure 10. Plasmid map of the pSP-luc+NF fusion vector.

The resulting vector was the pRSF-Luc plasmid. The riboswitch constructs for the chosen bacteria were cloned into the 5' untranslated of the pRSF-Luc vector using a megaprimer-based polymerase chain reaction mutagenesis strategy (Tyagi, 2004).

Two vectors for each riboswitch system were constructed, a riboswitch construct and an expression platform construct. The riboswitch construct contains the whole riboswitch system, while the expression platform construct only contains the anti-terminator and terminator hairpin region. In the expression platform constructs, the aptamer region is removed to study the activity of the expression platform in the absence of ligand binding. As previously discussed, in all the bacteria studied so far, the terminator hairpin has a higher thermodynamic stability than the anti-terminator hairpin. It was predicted that these constructs would have less expression than the

riboswitch constructs because in the absence of ligand binding, the terminator hairpin is thermodynamically favored.

Work over the past few semesters has been dedicated to creating a glycerol stock of transformed BL21(DE3) cells that can be used in Luciferase assays. Once in a stock, the cells can be easily recovered and used in the assay without having to transform the cells each time.

Making competent cells

The first step in order to transform the cells is to grow a batch a competent cells. Cells must be made competent in order for them to uptake the pRSF-luc plasmid that will express the gene for the production of Recombinant Firefly Luciferase. First, a tube of frozen liquid BL21(DE3) cells were taken from the -80ºC freezer and streaked on an LB agar plate. The plate was incubated at 37ºC and allowed to grow overnight. One colony was selected from each plate and placed in a Falcon tube with 5 mL of LB broth. The tube was shaken overnight at 225 RPM at 37ºC. The following day, the overnight culture was inoculated into 500 mL of LB broth and allowed to grow at 37ºC, while shaking at 225RPM.

Once the OD_{600} measured near 0.6, the cells were placed into two centrifuge tubes and balanced. The cells were spun down at 4,000 RPM at 4ºC for 10 minutes. The supernatant was removed and the pellet of cells was re-dissolved in 25 mL of ice cold 0.1 M $CaCl_2$. The cells were placed on ice for 30 minutes and spun down again at 4,000 RPM at 4ºC for 10 minutes. The supernatant was poured off and the cells were dissolved in 3 mL of 0.1M $CaCl_2$ and 450 µL of 99% glycerol. The cells were pipetted into 500 µL aliquots and placed in the -80ºC freezer for long term storage.

Transformation

The expression platform (THP) and riboswitch (RS) constructs for *Bacillus subtilis* (BSU) and *Bacillus cereus* (BCE) were the first two sets of constructs to be transformed. A tube of the competent cell stock was allowed to thaw on ice for 10 minutes. It was flicked a few times to ensure thawing and proper mixing. First 3 μL of each plasmid DNA was placed into an epitube. Next, 50 μL of thawed competent cell stock was mixed with the plasmid DNA in the tube. The tube was flicked to mix the cells and DNA. The cells were placed on ice for 30 minutes. After, each tube was heat shocked at 42ºC for 90 seconds. Following the heat shock, the cells were recovered on ice for 1 minute. Next, 250 μL of pre-warmed SOC was added to each tube. The tubes were then shaken at 225 RPM at 37ºC for 1 hour to ensure the expression of the antibiotic resistance conferred from the plasmid in the cells that had taken up the plasmid DNA. The cells were plated onto LB agar plates that contained 35 mg/L of Kanamycin in 100 μL and 150 μL amounts. The plates were then incubated at 37ºC overnight.

Making Bacterial Stocks

Once the transformed cells were grown on plates, a stock of the transformed cells was made to keep the transformed cells for long-term use. A single colony from each plate was selected and placed into a Falcon Tube, which contained 5 mL of LB broth with 35 mg/L of Kanamycin. The cells were allowed to grow overnight at 37ºC. The next day, the overnight culture was inoculated into 500 mL of LB broth also containing 35 mg/L of Kanamycin. The cells were grown by shaking at 225 RPM at 37ºC until the OD_{600} was close to 0.6. The cells were harvested by centrifuge at 6,000 RPM at 4ºC for 4 minutes. After, the cells were re-

dissolved in 3 mL of LB containing 35 mg/L of Kanamycin and 450 μL of 99% glycerol. The cells were placed in 500μL aliquots for long-term storage in the -80ºC freezer.

Luciferase Assays of Riboswitch Mediated Gene Expression Control

The transformed cells were removed from the -80ºC, thawed on ice for 10 minutes and mixed by flicking. First, 50 μL of thawed cells were added to an epitube. Next, 250 μL of LB with 35 mg/L of Kanamycin was added to the tube. The tube was shaken at 37ºC at 225 RPM for 1 hour. After, the 150 μL of each tube was plated on an LB agar plate containing 35 mg/L of Kanamycin. The plates were placed into the incubator at 37ºC and allowed to grow overnight.

The next day a single colony was selected from each plate and placed into a Falcon Tube containing 5 mL of LB with 35 mg/L of Kanamycin. Each tube was placed in the incubator at 37ºC and allowed to grow overnight, while shaking at 225 RPM. The next day, the overnight cultures were spun down at 4,000 RPM at room temperature for 12 minutes. After spinning, the supernatant was carefully removed and the cell pellet was re-dissolved in 10 mL of fresh LB containing 35 mg/L of Kanamycin. The OD_{600} was measured to ensure there was enough growth of the cells.

Each culture was induced with 20 μL of 0.5 M IPTG for a final concentration of 1 mM in each culture. The cultures were placed in the incubator at 37ºC for 4 hours while shaking at 225 RPM to induce the production of Recombinant Firefly Luciferase from the pRSF-luc plasmid. After 4 hours, the cultures were removed from the incubator and the OD_{600} was measured for each culture. Using the OD_{600} measurements, approximately 1 mL of each culture were pipetted out and placed in an epitube. Each tube was spun at room temperature at 6,000 RPM for 10 minutes. After spinning, the LB broth supernatant was removed from each tube and the cell

pellet was re-dissolved into 100 µL of lysis buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM EDTA, 2 mM DTT, 10% glycerol, and 2.5 mg/mL of egg white lysozyme. The cells were incubated on ice for 30 minutes with the lysis buffer and lysozyme. After 30 minutes, the culture was spun again at room temperature at 10,000 RPM for 5 minutes and the cell lysate was collected.

The Dual-Glo Luciferase (Promega) assay was removed from the freezer and allowed to thaw. The bottle of Dual-Glo Luciferase Buffer was added to the bottle of Dual-Glo Luciferase Substrate to create the Dual-Glo Luciferase Reagent. The reagent was mixed by inversion until it was fully dissolved. The reagent was placed in 1 mL aliquots and placed back into the -80ºC freezer to prevent freeze-thaw cycles for the whole reagent. One tube of reagent was kept out and allowed to equilibrate to room temperature. Next, 50 µL of cell lysate was added to a 96-well plate followed by 75 µL of Dual-Glo Luciferase Reagent. Two wells of lysate were used for each construct. The mixture was shaken gently to ensure mixture and allowed to react for 20 minutes at room temperature. After 20 minutes, the luminescence of each reaction was measured using a Biotek Synergy HT microplate reader. The instrument was set to a read time of 10 seconds for each well.

Results and Discussion

Making competent cells for the first part of the experiment was successful. The cells grew nice colonies on the LB agar plate and the overnight culture produced a nice cloudy mixture, which indicated growth over night. Once inoculated, the cells were allowed to grow for 6 hours until the OD_{600} measured 0.527. The cells were spun down and placed into storage according to the protocol above.

There was more difficulty when trying to transform the cells for the next part. At first no growth of transformed cells was being seen after a number of transformation attempts. It was determined that the wrong antibiotic was being used to select for the transformed cells. Ampicillin was in the LB agar plates so when the transformed cells were being plated after shaking, the Ampicillin was killing all the E. coli cells. Once it was discovered that Ampicillin was being used instead of Kanamycin, the transformations for BCE (RS and THP) and BSU (RS and THP) were completed. The number of colonies grown on each plate is described in Table 1 below.

Table 1. Number of colonies grown from transformation of BCE and BSU plasmid constructs.

Plasmid Construct	Plated amount (µL)	Number of colonies seen
BCE THP	100	1
BCE THP	150	2
BCE RS	100	1
BCE RS	150	3
BSU THP	100	4
BSU THP	150	5
BSU RS	100	6
BSU RS	150	8

After transformation, the plates were stored at 4ºC until the stocks could be produced. First, the BCE THP and BCE RS constructs were grown up according to the protocol above. Both overnight cultures grew cloudy indicating growth overnight. After inoculation into the 500 mL of LB and the culture was allowed to grow, the OD_{600} for BCE THP was measured at 0.634 and the OD_{600} for BCE RS was 0.574. The cells were then spun down and made into stocks without any problems.

Next, the BSU THP and BSU RS constructs were grown up from the transformed plates. Again the overnight culture was cloudy indicating that growth had occurred. After inolculation and further growth the OD_{600} was measured for each culture. The OD_{600} measurement was 0.684 for the BSU THP culture and 0.738 for the BSU RS culture. Both cultures were spun down and the stocks were created without any issues.

The transformed cells were able to be retrieved out of the glycerol stocks without any problems. Colonies grew on all the plates without any problems. When a colony was selected from each plate and allowed to grow overnight, each culture was cloudy the next day indicating growth. After spinning and re-dissolving in fresh LB, the OD_{600} measurement was taken for each culture. The measurements can be seen in Table 2.

Table 2. OD_{600} reading of each culture prior to induction with IPTG.

Plasmid Construct	OD_{600} measurement
BCE RS	0.712
BCE THP	0.717
BSU RS	0.717
BSU THP	0.701

As shown in Table 2, the optical density for each culture is fairly close, which indicates approximately the same number of cells for each culture. Since the OD_{600} reading was above 0.6, the cultures were ready to be induced with IPTG and grown for another 4 hours.

After induction and growth, the OD_{600} for each culture was measured once again. Using this measurement as a relative estimate for the number of cells present, approximately 1 mL of each culture was taken out. Table 3 shows the optical density measured and the volume of cell culture used.

Table 3. OD_{600} of cultures after induction and volume of culture used.

Plasmid Construct	OD_{600} Measurement	Volume of culture used (µL)
BCE THP	0.916	970
BCE RS	0.919	967
BSU THP	0.889	1000
BSU RS	0.891	997

By taking less amount of culture from the tubes with a high optical density, it is possible to approximate that they have the same number of cells. This method was double checked by taking two samples of culture from each tube to measure the luminesce.

The cells were lysed according to the protocol above and loaded into the well plate with the reagent mixture. The BSU RS construct was in wells A1 and A2, BSU THP was in B1 and B2, BCE RS was loaded into C1 and C2, and BCE THP was pipetted into D1 and D2. The light produced from the luciferase reaction was measured by microplate reader and the results are shown in Table 4.

Table 4. Luminescence measurements for plasmid constructs.

Well	Plasmid Construct	Luminescence (RLU)
A1	BSU RS	49,633
A2	BSU RS	50,677
B1	BSU THP	2,563
B2	BSU THP	2,345
C1	BCE RS	70,991
C2	BCE RS	68,249
D1	BCE THP	50,868
D2	BCE THP	46,819

The average luminescence for each construct can be determined and compared to each other. This is shown in Table 5.

Table 5. Average firefly luminescence in RFU for each plasmid construct.

Plasmid Construct	Luminescence (RLU)
BSU RS	50,155
BSU THP	2,454
BCE RS	69,620
BCE THP	48,843

From Table 5, both RS constructs had a higher expression of Firefly Luciferase than did their prospective THP constructs. This was expected because in the THP constructs, the aptamer binding region was removed. As a result, the expression platform was predicted to mostly consist of the terminator hairpin, which would result in a down regulation of the production of Firefly Luciferase. The RS constructs showed a greater amount of expression because the aptamer region in the construct allowed for full activity of the riboswitch.

Despite more assays needing to be completed to ensure consistency of results, these preliminary results can lead to some minor conclusions. Previously Dr. Nakesha Smith found the stabilities of terminator and anti-terminator hairpins in a number $preQ_1$ riboswitch systems. The values are summarized below in Table 6, which was adapted from her thesis.

Table 6. Experimental free energies of BCE and BSU terminator and anti-terminator hairpins.

Riboswitch system	ΔG_{ATHP} (kcal/mol)	ΔG_{THP} (kcal/mol)	$\Delta\Delta G^a$ (kcal/mol)
B. cereus	-3.08	-6.46	-3.38
B. subtilis	-4.56	-10.94	-6.38

[a] - $\Delta\Delta G = (\Delta G_{THP} - \Delta G_{ATHP})$

As seen in the table, both systems' terminator hairpins have a lower free energy value, which means a greater stability for the terminator hairpin. The table also shows that *B. subtilis* has both a more stable terminator hairpin and anti-terminator hairpin than that of *B. cereus*. The *B. subtilis* riboswitch system also has a larger difference in free energy between the anti-terminator hairpin and the terminator hairpin than the *B. cereus* riboswitch system. This property may explain the difference in the Firefly Luciferase expression between the THP constructs. Since the BSU THP construct has a more stable terminator hairpin than the BCE THP construct, the expression of Firefly Luciferase in the BSU construct is lower.

A similar comparison can be made in the RS constructs as well. In the BSU RS construct, the $preQ_1$ is able to bind to the aptamer region to turn gene expression off. However, when the $preQ_1$ unbinds from the construct, the greater stability in the terminator hairpin of BSU allows for the terminator hairpin to exist longer than when BCE riboswitch unbinds $preQ_1$. This relationship can be seen by a lowered amount of expression from the BSU RS construct than

from the BCE RS construct. Further assays with these constructs should be carried out to ensure consistency of results and in order to draw firmer conclusions.

Conclusions

Riboswitch systems are small segments of untranslated RNA that function in gene expression. The study of riboswitches is extremely relevant because they offer insights that can help us to further understand transcriptional control in bacteria. Further work can be completed on this project not only by continuing to study *B. subtilis* and *B. cereus*, but also by expanding and studying *B. halodurans*, *G. kaustophilus*, and *F. nucleatum*. Stocks of these bacteria transformed with the test vector can be produced as described above in order to have a source of readily available transformed bacteria. Those bacterial riboswitch systems can also be studied using the Dual-Glo Luciferase assay. Predetermined stabilities of the hairpins in the systems can also be compared to the assay results to see if further patterns emerge.

The riboswitch systems can also be studied under different environmental factors. For this experiment, the cultures were grown in rich medium. Other assays can be carried out with cells grown in poor growth medium in order to see how the cell responds to more challenging environments. $PreQ_1$ can also be added to the culture in order to see how the riboswitch responds to external sources of $preQ_1$.

Riboswitch systems play an important role in gene expression. Their ability to recognize small molecules and their abundance in a number of bacterial species make them a potential new target for anti-bacterial drug design. In order to identify riboswitches that can function as anti-bacterial targets it is necessary to continue the study of other riboswitch systems, such as the $preQ_1$ riboswitch to further understand their composition and function.

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