Development of Ligand-Controlled Riboregulators in *E. coli*

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Abstract:

After seeing the capabilities of RNA regulators of protein expression in nature, synthetic biologists adapted the technology. The difference in the transition from transcription to translation between eukaryotes and bacteria resulted in the necessity of different technologies for eukaryotes and bacteria. Currently eukaryotes have the ligand-controlled antiswitches, but bacteria have transcription factor-controlled riboregulators. By combining antiswitch and riboregulator technology, a ligand-controlled riboregulator could be developed; thus, allowing riboregulators to be capable of responding to a much greater variety of molecules. Using a technique adapted from PCR mutagenesis, I have been able to generate full length self-complementary DNA parts from shorter segments. Using BioBricks to clone the parts into *E. coli*, I am replicating the original riboregulator device. Before attempting to make the device ligand controlled, I had to make sure a functional original riboregulator could be generated by our methods. Flow cytometry will be used in the future to determine functionality and efficiency. If the methods work, a ligand-controlled riboregulator will be built by substituting the taRNA complement to the crRNA into the antisense stem. If the ligand-controlled riboregulator can be developed, it will have applications for generating bacteria biological sensors.

Introduction:

Synthetic biology takes an engineering approach to biology. Using the basic building block of DNA, researchers build individual parts like promoters and coding sequences. These parts combine to make devices, which perform certain tasks. Systems employ multiple devices to carryout complex applications such as acting as a biosensor or destroying solid tumors. Through mathematical modeling, synthetic biologists design parts, devices, and systems and predict their behaviors before physical construction begins (Endy 2001). If testing shows the construct does not work as predict, then an assumption made about how the parts or devices work or interact was wrong. In these cases researchers learn more previously hidden nuances of biology. Synthetic biology research differs from genetic and microbiological research because it looks at multiple genes and how they work together or a whole process instead of just looking at one gene at time.

As development of new parts leads to new possible systems by combining different parts, synthetic biologists need to standardize parts so that a part that was originally developed for one device can easily be used in another. The BioBrick system uses a set of five restriction enzymes cut sites that are divided into the BioBrick prefix (EcoRI, NotI, XbaI) and the BioBrick suffix (SpeI, NotI, PstI). By cutting one part with EcoRI and XbaI, a researcher can put another part (such as a promoter) that is cut by EcoRI and SpeI upstream of this part (Figure 1, partsregistry.org 2005). A part can also be inserted downstream by cutting the receiving part with SpeI and PstI while the insert is cut with XbaI and PstI. Once put together, the parts cannot be separated from each other as the XbaI and SpeI fusion does not generate a viable restriction cut site.
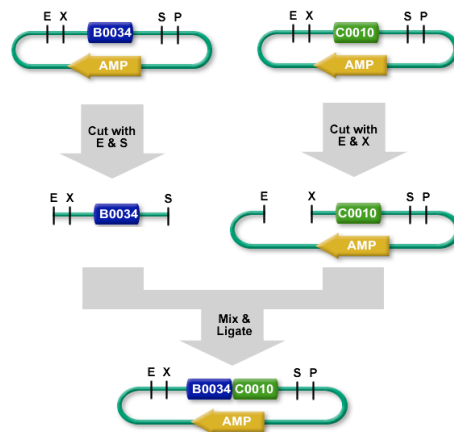
Figure 1: BioBrick Standard Part Assembly. Two parts are joined together to make a composite part. The insert part (cut E/S) is being inserted upstream of the vector part (cut E/X). (partsregistry.org 2005)

Using this standard assembly method and other principles of synthetic biology, practical biological systems and devices can be engineered. Biosensors are one such application. In a biosensor, the organism takes up the molecule. The cell recognizes the molecule by some mechanism and triggers a detectable response such as fluorescing or changing pH (Jacobson 2007; Edinburgh 2006). Biosensors have the potential to detect pollutants, toxins, and abnormalities in our body's chemical make-up such as those caused by cancer or other diseases.

Nature indicates translational regulation as promising for developing biosensors. Regulation of translation can provide an excellent tool for the production of different sensors by controlling the translation of specific genes depending on cellular conditions. Technologies that allow for regulation of translation provide promise for future research. One technology that regulates translation in response to ligands was developed in eukaryotes in 2005. Christina Smolke's laboratory originally developed antiswitches in *Saccharomyces cerevisiae* (Bayer and Smolke 2005) that either inhibited or permitted translation of RNA in the presence of particular ligands.

Antiswitches, ligand-controlled RNA molecules that regulate translation of mRNA, are made of an aptamer, an aptamer stem, and an antisense stem (Figure 2). An aptamer is a nucleotide sequence that binds to a particular ligand with high specificity while the aptamer stem is an RNA sequence that changes the antiswitch's structure in response to the aptamer binding to or releasing the ligand. Because the aptamer has such high specificity, the change in structure does not occur to a significant degree in the presence of molecules that are similar to the ligand. One of Smolke and Bayer's aptamers was so specific that it only bound to theophylline and not caffeine, which differs by a single methyl (Figure 2 c). Finally, the antisense stem contains a sequence that matches a targeted RNA transcript and a second sequence that sequesters this complementary sequence to keep it from binding the transcript (Figure 2 a). When the antisense stem is not duplexed with itself, it prevents translation by binding to the complementary mRNA (Figure 2 b).
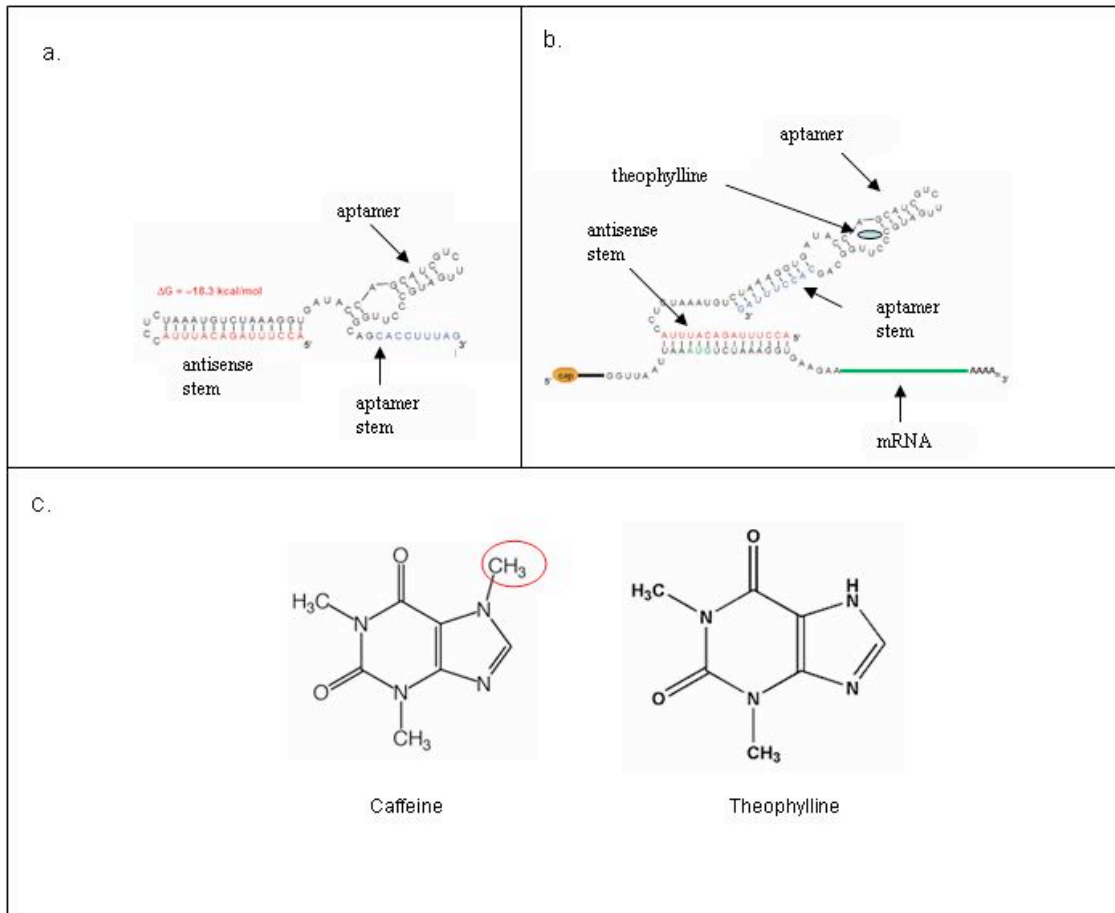
Figure 2. Antiswitch Mechanism a) An inactive off-switch. The antisense stem (red bases) is duplexed with itself (adapted from Bayer and Smolke 2005). b) The same switch after being activated by theophylline (blue ellipse). The antisense stem is duplexed with the mRNA (adapted from Bayer and Smolke 2005). c) Caffeine and theophylline differ by one methyl group (circled in red). This aptamer binds theophylline but not caffeine.

Although attempts have been made by both Smolke's lab and others to adapt Smolke's antiswitches to bacteria, Smolke's eukaryotic technology has not been successfully adapted to bacteria *in vivo*. Matt Gemberling's initial attempts of adapting antiswitches for use in *E. coli* (Gemberling 2005) suggested that the speed at which translation begins after transcription in bacteria is a major problem for Smolke's technology. In eukaryotes, translation occurs after post-transcriptional modifications, allowing antiswitches to bind to the mRNA. In bacteria, translation begins before the rest of the RNA is transcribed and thus before antiswitches can bind.

Isaacs *et al.* (2004) engineered a different mechanism that successfully controlled protein expression in bacteria but was not ligand-controlled (Figure 3). They engineered a system where an oligo of DNA that is complementary to the ribosomal binding site (RBS) is inserted into the genome between a gene's RBS and its promoter. When the DNA is transcribed into cis-repressing RNA (crRNA), a stemloop is created by the complementary sequence binding to the RBS sequence. This stemloop blocks the ribosome from accessing the RBS and translating the RNA (Figure 3 a). A second trans-activating RNA (taRNA) is regulated by a second promoter

and the taRNA binds to the crRNA. When the taRNA is transcribed, the taRNA interacts with the complementary portion of the crRNA stemloop and opens the RBS for the ribosome (Figure 3 b).
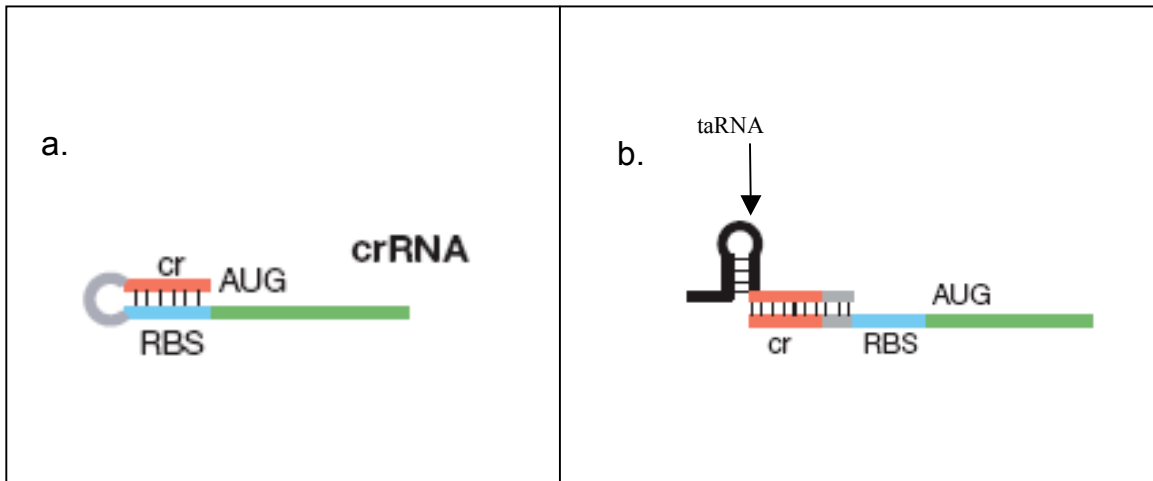
Figure 3. Riboregulator Mechanism a) Translation is prevented by sequestering the RBS by the red complementary (cr) sequence. b) Translation is activated by the taRNA (black, red, and gray) binding the crRNA and revealing the RBS. (Adapted from Isaacs *et al.* 2004)

While Isaacs *et al.* succeeded, the riboregulator system is controlled by transcription factor proteins and inducible promoters instead of being directly controlled at the RNA level with the addition of ligands. Transcription factors bind to sites on the promoter or sites upstream of the promoter called operators (Ptashne 1986) and have several limitations. First, control by these proteins can rely heavily on cooperativity that is multiple proteins binding to one site, in order to see an effect (Gardner *et al.* 2000). Second, while synthetic biologists could use the transcription factors and their binding sites that are found in nature, rational design of additional new transcription factors is difficult and limited by those available in nature. Finally, transcription factors affect gene expression prior to transcription instead of translation. When the stimulus changes and the gene is expressed, the time it takes for the phenotype to be expressed is longer because both transcription and translation must occur instead of just translation.

Using regulatory RNA molecules, instead of transcription factors, to control protein expression circumvents some of those limitations. First as discussed above, regulatory proteins that control promoters often require cooperativity that is multiple regulatory proteins need to bind to a single promoter in order to fully control the function. When using regulatory RNA molecules (*e.*g. taRNA) to control translation, only one molecule needs to bind to the target (*e.*g. crRNA). By choosing a promoter for the gene coding the regulatory RNA that has a transcription rate equal to the transcription rate of the promoter for the target RNA's gene, the RNA-based regulatory system should function properly. Second, RNA in a cell is generated by transcribing DNA. With DNA synthesis technology available today, RNA can be engineered that complements any other RNA sequence; therefore, we are not limited to sequences found in nature. Aptamers are also easily developed through rational design, and the number in existence is continually increasing and providing new molecules that can act as ligands (Ellington Lab 2006). Finally, regulatory RNAs halt protein translation after transcription. Once the stimulus is

removed, the target RNA already produced by the gene simply needs to be translated. Translation and modification are the final steps to producing a functional protein. Therefore, the RNA-regulated expression should have faster kinetics than transcription factor-based controlled gene expression.

To circumvent the problems presented by both bacterial transcription and translation and the absence of ligand-controlled versions of Isaacs *et al.'s* prokaryotic riboregulators, I have proposed three new designs for antiswitches that might work in bacteria: two on-antiswitches and one off-antiswitch. The first on-antiswitch combines the original antiswitch technology with riboregulators (Isaacs *et al* 2004) while the design for the other two antiswitches combines riboswitch and antiswitch concepts to allow ligand control of RNA translation. If these new designs can adapt antiswitch technology to bacteria, biologists would have tighter control of RNA regulation based on environmental cues (*e.g.*, specific ligands) than is currently possible with available technologies.

Before I can build the proposed ligand-controlled riboregulators, I first needed to duplicate the results generated by Isaacs *et al*. Because synthesis of full genes can take a very long time and is expensive, I needed to devise methods of generating the parts without simply having a company synthesize the full genes. Currently I have generated these parts and am testing functionality. If these parts are functional, the designed ligand-controlled riboregulator can be built. Through these experiments, I have taken the first steps to generating a ligand-controlled regulatory RNA in bacteria that could be employed in biosensors.

Methods and Materials:

Bacterial Strains
JM109 cells (#T3003 from Zymo Research) and *dam-/dcm- E. coli* competent cells (NEB C2925) were the two strains used in the experiments.

Growth and Fluorescence Assays
LB liquid media was made that had theophylline concentrations of 0 µM, 1 µM, 5µM, 10 µM, or 12.5 µM and 100 µL of an overnight culture of GFP containing cells, of YFP containing cells, or of media only were added to triplicate cultures. All cultures were placed in a shaking incubator at 37˚ C. 150 µL was sampled from each culture and placed in separate wells in a 96 well plate after 2.5 hrs, 4.5 hrs, 6.5 hrs, and 8.5 hrs. from the initial seeding. The ELx808 microplate reader (BioTek Instruments, Inc.) determined the optical density at 595 nm at every time point. GFP and YFP fluorescence was measured at excitation of 500/27 and emission of 540/25 and at excitation of 500/27 and emission of 528/20 for every time point with the FLx800 microplate fluorescence reader (BioTek Instruments, Inc.). Data was gathered by KC junior (BioTek) and analyzed with Microsoft Excel.

Secondary Structure Predictions
Using RNAstructure 4.5 (Mathews *et al.,* 2007), I typed in the sequences for Bayer and Smolke's antiswitches, Isaacs *et al.*'s riboregulators, and my designed structure and received the folding predictions. The window size was decreased to 2 and the max energy difference was increased to between 15% and 50% depending on the sequence.

Oligo Annealing

The sequences of full length constructs were copied into Lance Harden's oligo cut optimization program (Harden 2006) or had sticky ends designed instead of full restriction sites if the full sequence was small. Oligos were ordered from either Sigma Proligo or MWG Biotech. Davidson College's protocol for building dsDNA with oligos was followed to anneal the oligos (Davidson College Building 2006).

Ligation

DNA was cut as vector or insert following BioBricks standard assembly for upstream or downstream insertion (BioBricks Registry, 2007) using Promega enzymes EcoRI (Promega R601J), PstI (Promega R611B), XbaI (Promega R618G), and SpeI (Promega R659D) and the NEB salt buffers (NEB 1998) or Buffer H (R008A). Plasmid pSB1A2 originally containing RFP was cut as vector using EcoRI and PstI for crRNA, taRNA, and RBS.

After digestion or annealing, Promega's 2X rapid ligation protocol was followed using Promega 2X ligation Buffer (C671B 20537516) and Promega T4 DNA ligase M180B 19827731) (Promega 2006).

Transformations

Plasmids were transformed into JM109 cells (#T3003 from Zymo Research) using the Zymogen Zippy Transformation Protocol. SOC media (invitrogen 15544034) was used to bring the reaction up to 80 μL. (Zymogen 2006)

Plasmid containing crRNA was transformed into dam-/dcm- competent *E. coli* cells from New England Biolabs (NEB C2925) using the NEB heatshock protocol (NEB 2007).

Colony PCR to confirm insertions

Colony PCR was performed following the Colony PCR to Screen for Successful ligations protocol that was modified by Dr. Todd Eckdahl (Eckdahl 2007). Promega GoTaq Green Master Mix 2x (M712B 22007503) was used for the polymerase and buffer. BioBrick Registry Primers VR and VF2 were used. These primers add 238 bp to parts cloned in vector pSB1A2.

Gel Check for Part

To confirm colony PCR results, the colony was grown overnight in liquid LB + ampicillin. The plasmid was extracted from the cells using Promega Wizard Plus SV Miniprep kit (A1460) and digested with EcoRI (R601J) and PstI (R611B) in Buffer H (R008A). Cut DNA was then run on an agarose gel with Invitrogen 1 Kb ladder (10488-072). Universal Hood II (BIORAD 170-8062) was used to take a picture of the gel.

To confirm parts built through PCR, cut DNA was then run on an agarose gel with Invitrogen 1 Kb ladder (10488-072). Universal Hood II (BIORAD 170-8062) was used to take a picture of the gel.

Sequence Verification

At least 30 ng of plasmid are lyophylized in a tube using the Savant Speedvac. 10 µL of 1 pmol VF2 dilution is sent to Clemson. An order form was filled out at http://www.genome.clemson.edu. The tubes were then sent to Clemson for sequencing.

Primer Dimer Part Construction

Sequences were analyzed by hand, BLAST2 (NCBI), and ClustalW (EBI) to generate primers of no more than 80 bp. The oligos were ordered from Sigma-Proligo and MWG Biotech. The primers were used in individual PCR reactions. The products were gel purified and used in subsequent PCR reactions. This method was continued until the entire constructs were generated.

To generate the crRNA, I used the primers:
5'GCATGAATTTCGCGGCCGCTTCTAGATCTAGTTCACCTCTTGGATTTGGGTATTA 3'

5' GCATCTGCAGCGGCCGCTACTAGTTCTCCTCTTTAATACCCAAAT 3'

To generate the taRNA, I used the oligos:
5'GCATGAATTCGCGGCCGCTTCTAGAGACATTGATTATTTGCACGGCGTCACACTTTGCTATG
CCATAGCATTTT 3'

5'GTAGAGAGTTGCGATAAAAAGCGTCAGGTAGGATCCGCTAATCTTATGGATAAAAATGCTAT
GGCATAGC 3'

5'GCTTTTTATCGCAACTCTCTACTGTTTCTCCATACCGTTTTTTTGGGCTAGCACCCAAATCC
AGGAGGTG 3'

5'CTAGAGATATATGGTAGTAGTAAGTTAATTTTCATTAACCACCACTCTAGATCACCTCCTGG
ATTTGGGT 3'

5'CTACCATATATCTCTAGAGACTCCTGTTGATAGATCCAGTAATGACCTCAGAACTCCATCTG
GATTTGTTCAGAACGC 3'

5'GCATCTGCAGCGGCCGCTACTAGTAATTCTCACCAATAAAAAACGCCCGGCGGCAACCGAGC
GTTCTGAACAAATCC 3'

Gel Purification

PCR products or DNA part inserts are first run on agarose gels. The DNA is then extracted from the gel following the QIAquick Gel Extraction Kit (Qiagen 28704, Qiagen 2001).

Shrimp Alkaline Phosphatase

Shrimp alkaline phosphatase was used to remove the phosphates from cut vector and prevent self-annealing during ligation. (Davidson College Shrimp 2006)

Freeze and Squeeze Gel Extraction

Gel slices containing DNA are placed in a centrifuge tube in a -80° freezer until frozen solid. The tube is spun for ten minutes in a centrifuge at maximum speed. The liquid containing the DNA was removed for use.
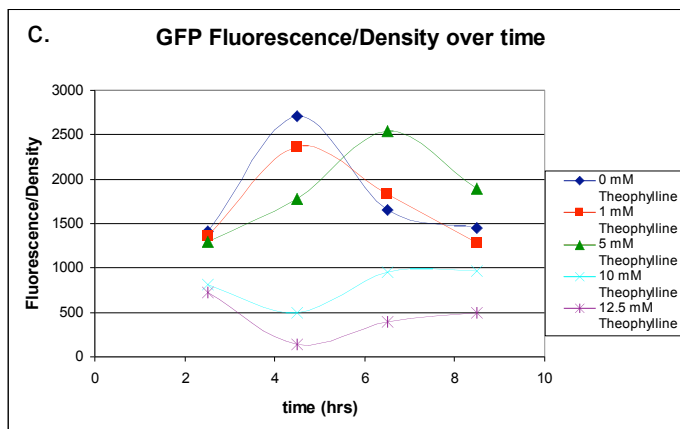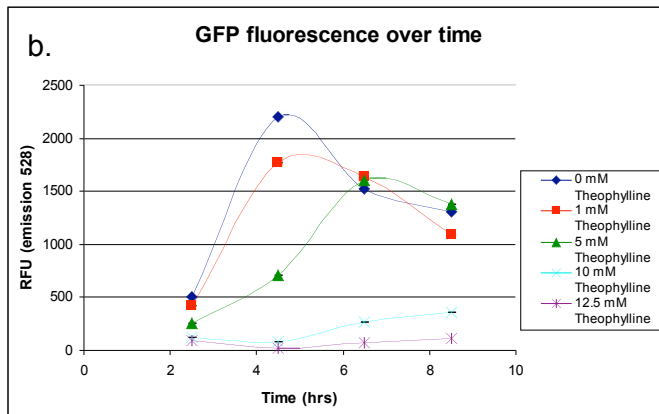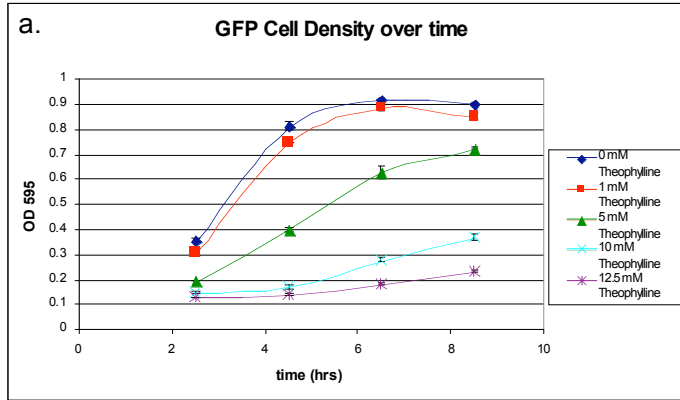
Results:

Design:

Before designing ligand controlled riboregulators, a reporter gene and ligand had to be chosen. During Matt Gemberling's attempts to adapt antiswitches to bacteria, theophylline was used. While no significant reduction in fluorescence was seen, there was a slight trend that fluorescence decreased as theophylline concentrations increased (Gemberling's Honor's Project 2005). In order to determine whether theophylline has a toxic effect on *E. coli* that results in a decrease in protein production in general or whether some antiswitches were working, I needed to test the effect of theophylline on wild-type JM109 *E. coli* cells. If theophylline showed a lethal toxic effect, it would not be a good ligand. Growth and fluorescence assays tested whether theophylline would be a viable ligand. While high concentrations of theophylline slowed the growth of cells, the cells were not affected with low doses (Figures 4 and 5). Cells exposed to theophylline also produced detectable amounts of GFP or YFP. Cells expressing GFP showed higher levels of fluorescence than cells expressing YFP at every concentration of theophylline (Figures 4 and 5). Not only was the level of fluorescence higher in GFP cells but also the fluorescence was less variable at a single time point with GFP cells. Based on these results, I proceeded to design the riboregulators with theophylline as the ligand and GFP as the reporter. To ensure that the fluorescence reflects the actual current translation of GFP, the GFP reporter gene will include an LVA tag, a nucleotide sequence that encodes an amino acid sequence that *E. coli* proteases recognize and causes the protein to be degraded quicker (BioBricks Registry).
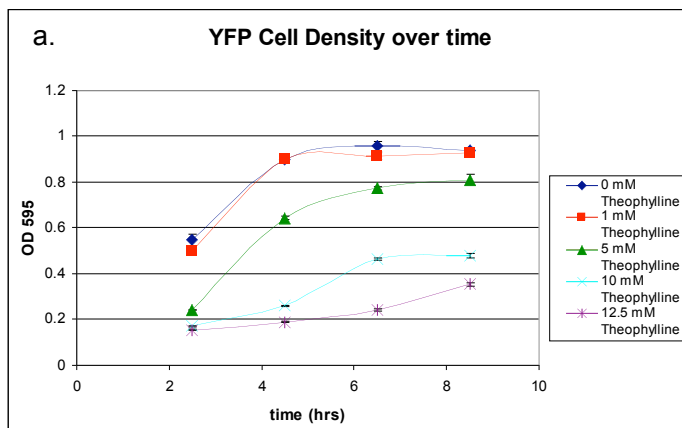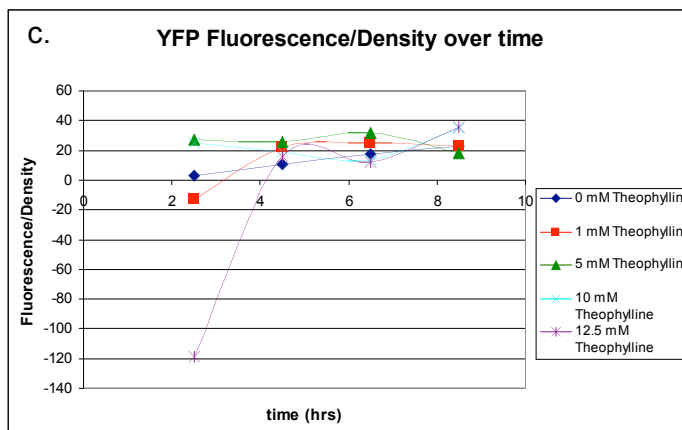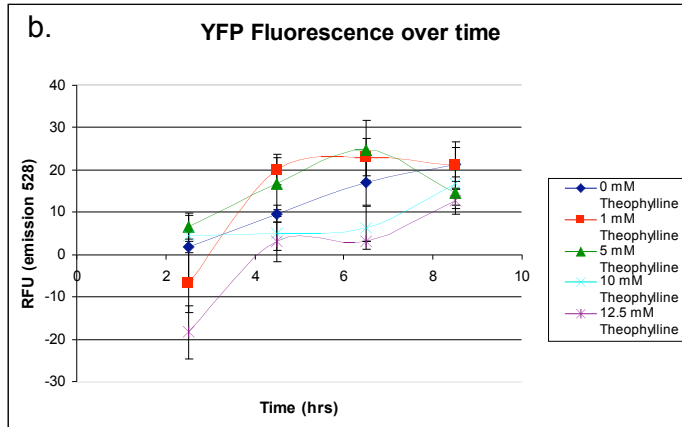
Figure 4. Effects of theophylline on YFP cells' growth and health. a. The Y-axis shows cell density by optical density at 595 nm. The X-axis represents the time after inoculation of the initial culture. Optical Density measurements of 150 µL samples of each culture were taken every 2 hrs. Error bars represent the standard deviation between the three samples of a single culture at that time point. **b.** The Y-axis represents fluorescence as measured by emission of light with wavelength centered at 528 nm. The X-axis represents the time after inoculation of the initial culture. Fluorescence measurements of 150 µL samples of each culture were taken every 2hrs. Error bars represent the standard deviation between the three samples of a single culture at that time point. **c.** The Y-axis represents the average fluorescence of samples normalized to OD (average fluorescence of three samples divided by average density of three samples).

Figure 5. Effects of theophylline on GFP cells' growth and health. a. The Y-axis shows cell density by optical density at 595 nm. The X-axis represents the time after inoculation of the initial culture. Optical Density measurements of 150 μL samples of each culture were taken every 2 hrs. Error bars represent the standard deviation between the three samples of a single culture at that time point. **b.** The Y-axis represents fluorescence as measured by emission of light with wavelength centered at 528 nm. The X-axis represents the time after inoculation of the initial culture. Fluorescence measurements of 150 μL samples of each culture were taken every 2hrs. Error bars represent the standard deviation between the three samples of a single culture at that time point. **c.** The Y-axis represents the average fluorescence of samples normalized to OD (average fluorescence of three samples divided by average density of three samples).

When designing the new antiswitch-riboregulator hybrid, the general structure should not deviate tremendously from the original antiswitch and taRNA. As software is updated, the predicted structure for sequences can change. Therefore, differences in folding among RNAstructure 4.5 (the new software), RNAstructure 3.7 (the software used by Bayer and Smolke 2005), and mFold (the software used by Isaacs *et al.*) must be determined and taken into consideration when designing the hybrid. When folding Smolke's sequences for antiswitches, RNAstructure 4.5 was typically only one base pair off (Figure 6). RNAstructure generated a perfect match of Isaac's predicted structure of the taRNA (Figure 7). The only real differences in the programs seem to be their choice of linear versus circular when drawing the structures (Figures 6 and 7).
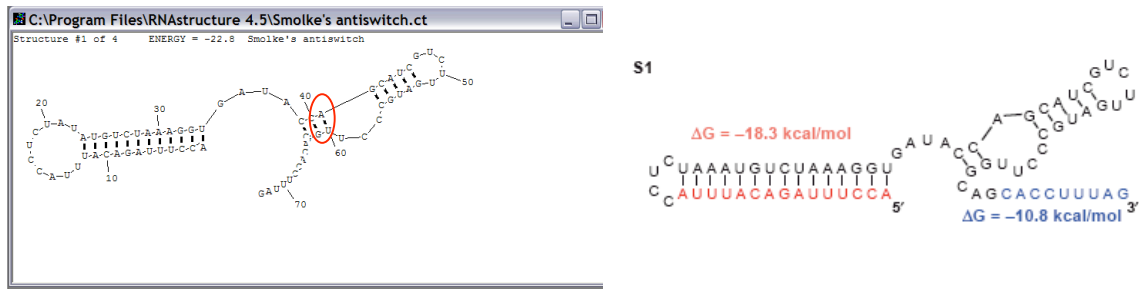

Figure 6. Comparison of RNAstructure 4.5 and RNAstructure 3.7 folding predictions. On the left is the structure generated by RNAstructure 4.5 for Smolke's first antiswitch sequence. On the right is Smolke's antiswitch as shown in Bayer and Smolke 2005 and generated by RNAstructure 3.7. The base pairing is almost exactly the same except RNAstructure 4.5 predicts one A-U (red circle) bond that RNAstructure 3.7 did not.
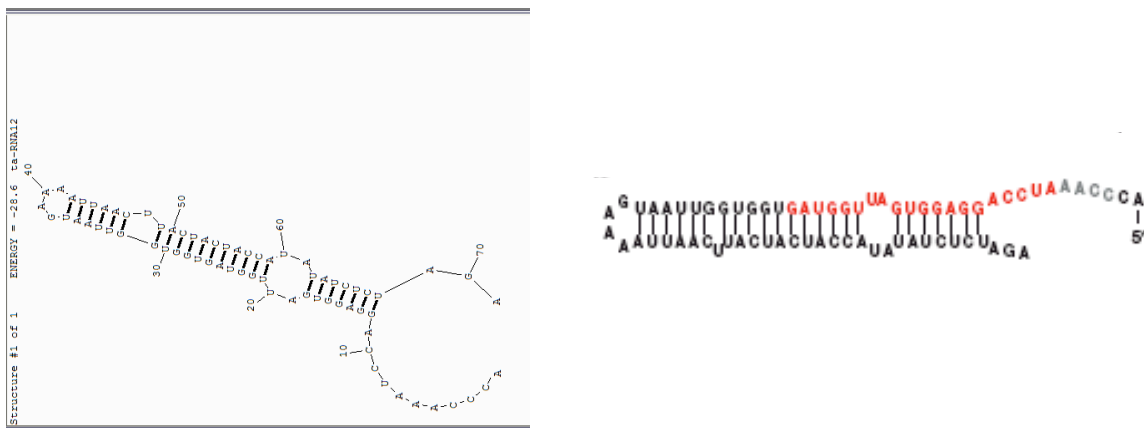
Figure 7. Comparison of RNAstructure 4.5 and mFold folding predictions. RNAstructure 4.5 can also duplicate structures generated by mFold. On the left is the RNAstructure 4.5 taRNA 12 predicted structure generated from the sequence provided by Isaacs *et al.* On the right is Isaacs *et al.'s* structure prediction of taRNA using mFold. All base pairing predictions are the same in the two structures. Note it is rotated 180 degrees around the horizontal axis.

The algorithms employed by RNAstructure 4.5, RNAstructure 3.7, and mFold generated nearly identical predicted RNA secondary structure for each sequence; therefore, I was able to use RNAstructure 4.5 to generate a predicted secondary structure of my designed riboregulator and then compare the secondary structure to Smolke's antiswitch and Isaac's taRNA to see if my design still folded similarly. To design a ligand-controlled riboregulator, I combined the main features of the antiswitch with the main features of Isaac *et al.'s* riboregulator (Figure 8). The structure of my designed riboregulator is predicted to fold like both the taRNA and the antiswitches. Before attempting to generate a modified taRNA system, I needed to make sure that I could duplicate the results Isaacs *et al.* produced with their inducible promoter controlled riboregulator.
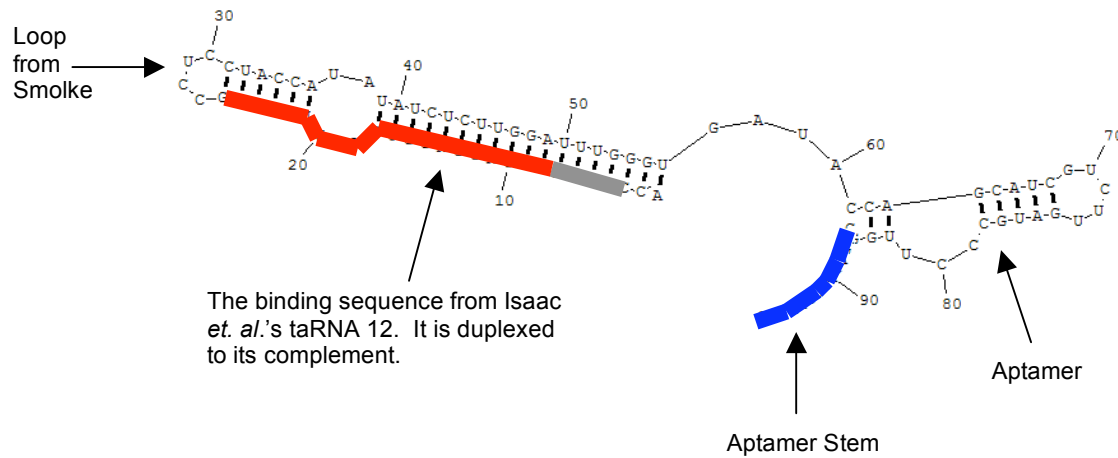
Figure 8. Predicted folding and design of proposed ligand-controlled riboregulator. Above is the design combining Smolke's antiswitch with Isaacs's taRNA. This structure should allow the taRNA sequence to be ligand controlled. Unlike the original antiswitch design, the taRNA only has to bind to the crRNA when it wants the gene to be expressed instead of first binding to the RNA to turn off translation and then releasing the transcript when the ligand is present.

Oligo Design:

When duplicating Isaac's work and building the ligand-controlled riboregulators, BioBrick ends needed to be added to produce standardized parts and allow many scientists easy access and use of these parts (Figure 1). In order to accommodate the BioBrick ends, several base pairs were changed from the original sequence to complement the spacer sequence created by the fusion of the XbaI and SpeI sites. When the reporter gene is inserted downstream of the crRNA, the reporter gene's BioBrick ends will be cut with XbaI and PstI. Cutting with these restriction enzymes remove the reporter gene from its plasmid. The crRNA will be cut with SpeI and PstI. Cutting with these enzymes opens the plasmid downstream of the crRNA. The PstI site reforms from the PstI sticky ends of the insert part and vector part when the two parts are ligated together. The cut section of SpeI and XbaI are complements and ligate together. The SpeI/XbaI "scar" sequence (ACTAGA) is not a palindrome; therefore, neither SpeI nor XbaI can cut at this site after they are ligated: this fusion site is called a scar (BioBricks Registry, 2007). The original spacer sequence separating the ribosomal binding site (RBS) from the coding region

has been replaced by the SpeI/XbaI scar (`ACTAGA`); therefore, the crRNA required the reverse complement to the scar at the beginning instead of the reverse complement to the original spacer. The scar then also needed to be added to the taRNA in order to allow it to base pair properly with the crRNA.

After confirming the modified sequence, Lance Harden's oligo optimization program was run to determine the optimal short oligos of crRNA and taRNA to have synthesized in order to generate the whole constructs through the annealing of shorter segments (Harden 2006). While the program was able to suggest oligos for the crRNA, the taRNA was to long for the program to work. Therefore, I needed to either expand the capabilities of the program or analyze the sequence by hand with the help of melting temperature calculators.

Since an automated program would be more efficient when generating good taRNA oligos than hand analysis, I attempted to expand the Perl program written by Harden to solve longer sequences. The original program was explicitly coded and adding more if statements to extend the capabilities would have been unwieldy and inefficient. Andrew Martens had made a recursive version of the program, but it was also unable to do the longer sequences. I read the code and attempted to follow what it was doing by hand, but the repetition of actions in the program made it easy to lose track of what action I should be doing. I then added print statements to see what information the computer actually had at different points in the program. I have not yet been able to reconcile the print statements to the program and identify the error. I needed to continue the lab portion of the project; therefore, hand analysis or a different protocol was needed to generate the taRNA.

crRNA construction:

While attempting to make the program work for the taRNA, the oligos for the crRNA (Figure 9) were synthesized. After following the protocol for generating double stranded DNA by annealing oligos, the product of the annealing was then ligated into the plasmid vector pSB1A2 and transformed using the transformation protocol. I used colony PCR to screen for the crRNA. During colony PCR with vector pSB1A2, 238 bp are added to the part; thus, a predicted crRNA should run at approximately 317 bp. Colonies 7 and 14 had bands of approximately the correct length of the primers plus crRNA (Figure 9). While colony 9 ran at a length smaller than predicted with these primers, the band did not indicate an empty plasmid like colonies 8, 10, 11, 12, 13, and 15 nor indicate that RFP remained in the plasmid. Therefore, I decided to check colony 9 for crRNA as well in case the primers actually add fewer base pairs than previously noted. These three colonies were grown overnight.


Figure 9. Schematic of crRNA construct. The full construct of the designed crRNA is represented above. The construct contains both the crRNA (red and grey) and corresponding RBS (blue). These sequences are flanked by the BioBrick prefix and suffix (green).
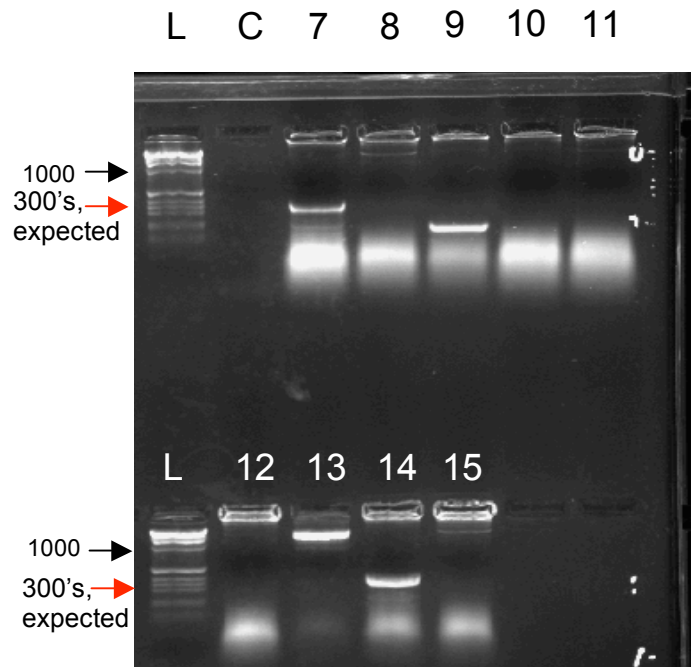
Figure 10. Results of Colony PCR of crRNA transformation. Above is a 2% agarose gel. Lanes marked L contain 5 µL of I Kb ladder. Lane C is the control of the colony PCR primers. It contains no template and shows that the visible bands are not from primer amplification. The other lanes are the products of colony PCR done on colonies from the transformation of plasmid potentially containing crRNA into JM109 cells. The primers used add 238 bp to the crRNA (79 bp); therefore bands of about 317 bp are most likely crRNA. Bands that have similar lengths to the expected length of crRNA appear in lanes 7, 9, and 14.

Although the colony PCR looked promising, a gel of the EcoRI and PstI digestion of the plasmids from these colonies gave no bands or bands of approximately 200 bp in length; thus, the insert was not true crRNA (Figure 11). VF2 and VR, the primers used for the colony PCR, have recently been shown to bind multiple places with certain parts. It is possible that they can also bind at different places on the vector. There could also have been DNA contamination of the vector, so it had some part that is in the 200's and binds VF2 and VR in a different place (partsregistry Problems 2008). The small band in lane 9 (Figure 10) might be a 238 base pair amplification of empty vector while the higher molecular weight band in lane 13 could be the RFP part that is originally in the vector. The empty lanes might not have had any plasmid but survived as satellite or random chance.
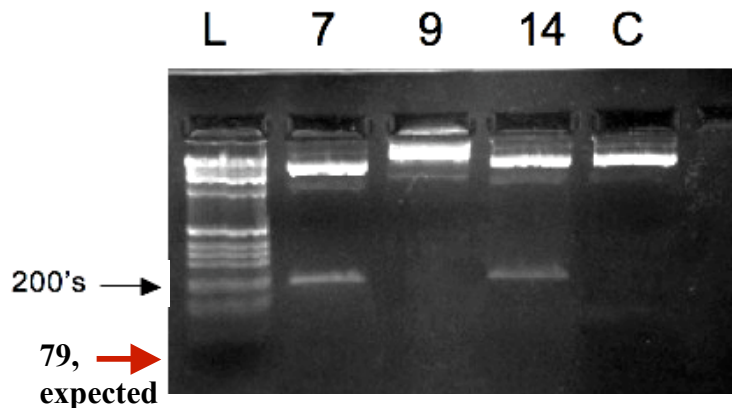
Figure 11. Restriction analysis of potential crRNA colonies. Above is a 2% agarose gel. Lane contains 5 µL of I Kb ladder. Lanes 7, 9, and 14 are EcoRI and PstI digestions of the minipreps of the plasmids from colonies 7, 9, and 14. The control (C) is an EcoRI and PstI digestion of Hix C (26 bp). Because the control is 26 bp, more DNA may have been needed to see the band. The bands for 7 and 14 are over 200 bp in length; therefore, the insert is not a 79 bp crRNA fragment. Colony 9 has no insert band.

Since annealing oligos into the full construct continually failed after multiple attempts, I tried a modified PCR mutagenesis protocol. I generated overlapping primers that together spanned the full length of the crRNA. No template was used. The primers were allowed to form dimers, which produce the crRNA (Figure 12).
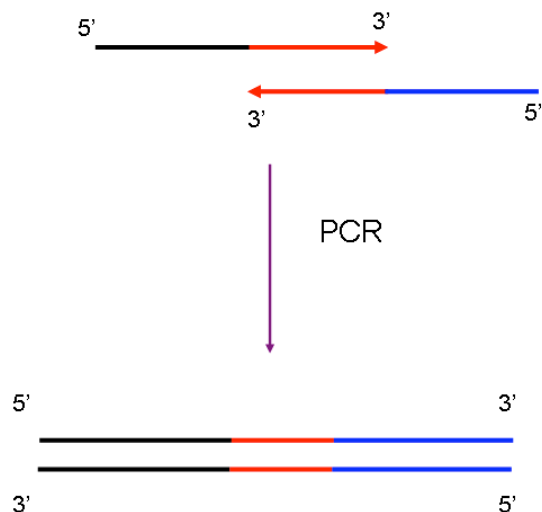
Figure 12. crRNA PCR construction protocol. Two primers with an overlapping sequence (red) are used without template in a PCR. The primers create dimers and are filled in by the Taq DNA polymerase. The result is the desired full length oligo.

The PCR protocol was new, so two different concentrations of primer were used: the traditional 1 μM of each primer and a higher 5 μM of each primer. The 1μM of each PCR primer generated a cleaner band of the correct size product and was the concentration used in further experiments (Figure 13). The reaction was scaled up to ensure that a usable amount of crRNA would be generated. The product was gel purified, cut with EcoRI and PstI, and ligated into vector pSB1A2. The plasmid was then transformed. Of the six colonies picked from the transformation to be grown up overnight, all six had bands that corresponded to the correct length for crRNA (Figure 13 b). The OD's of colonies 4, 5, and 6 had the best DNA to protein ratios; therefore, they were sent to be sequenced. All three colonies were confirmed to have the desired crRNA sequence (Figure 14).
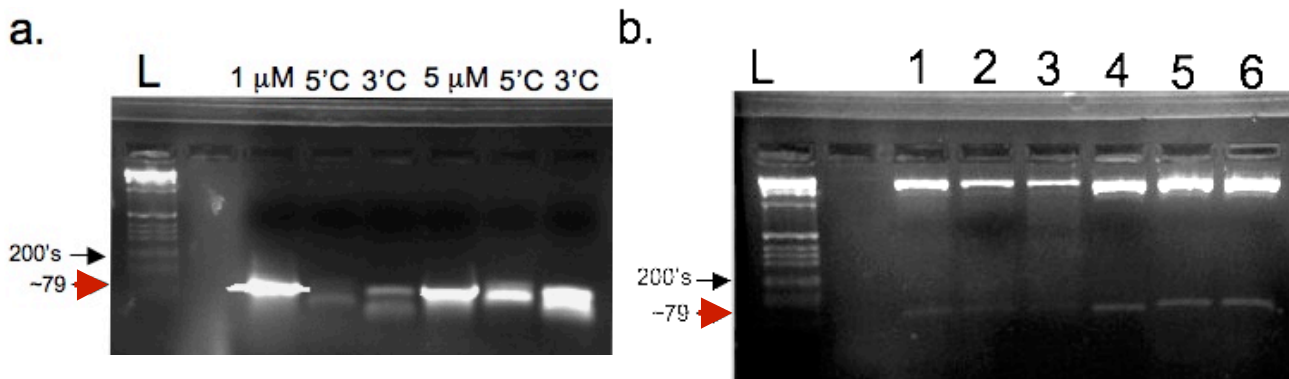
Figure 13. Gel analysis of potential crRNA generated through PCR. a. 2% agarose gel of the products of PCR using the crRNA primers. 1 μM contains the product of PCR using 1 μM of each primer. The two lanes (5'C and 3'C) immediately to the right are the controls for PCR using the 5' or 3' primer alone at 1 μM concentration. They demonstrate that both primers were needed to cause significant amplification. 5 μM contains the product of PCR using 5 μM of each primer. The two lanes (5'C and 3'C) immediately to the right are the controls for PCR using just the 5' or 3' primer at 5 μM concentration. Lane L contains 5 μL of 1 Kb ladder. b. The DNA from the 1 μM was gel purified, digested with EcoRI and PstI, and ligated into pSB1A2. The plasmids were transformed into JM109 cells. Lanes 1-6 represent EcoRI and Pst1 digested plasmid minipreps from six different transformed colonies. Each colony has a band at ~79 bp, the length of crRNA.


Figure 14. Sequence alignment of designed crRNA and potential crRNAs. Three colonies whose DNA was sequenced are aligned against the sequence of the designed crRNA (third line). The red box highlights the portion of the sequence that represents the crRNA. The four sequences align with a perfect match.

Now that the crRNA sequence was confirmed, it could be inserted upstream of the reporter gene GFP (Figure 15). The crRNA was cut to be the receiving vector while the GFP reporter coding sequence and terminator were prepared as the insert. These two parts were ligated, and the plasmids were transformed. Six colonies were grown up overnight and checked for crRNA-GVPLVA-tt. Five of the six colonies had the 975 bp insert (figure 16).

Figure 15. Schematic of crRNA-GFPLVA-tt construct. The crRNA part (red, grey, blue) has been inserted upstream of a GFPLVA-tt part (neon green, dark green, and pink) that lacks a promoter and RBS. The dark green represents BioBrick scars where two parts have been ligated together. The whole part is flanked by BioBricks.
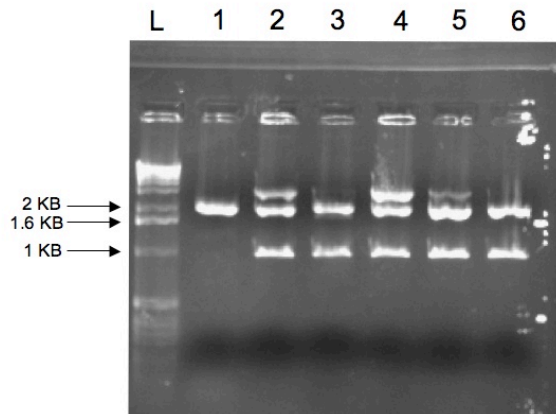
Figure 16. Gel analysis of potential crRNA-GVPLVA-tt colonies. 1.2% agarose gel of EcoRI and PstI digested plasmid from the transformation of the ligation of GVPLVA-tt downstream of crRNA in vector pSB1A2. Colonies 2-6 have bands a little less than 1 Kb; thus, they most likely contain crRNA-GVPLVA-tt (975 bp). L is a 1 KB ladder.

As a failed ligation would have generated a band in the three hundreds or no band and the individual parts were sequence confirmed, I continued without sequencing the new composite part. In order to test modularity as well as function, two promoters, pLac and pBad, were digested as vector to accept the crRNA-GVPLVA-tt insert downstream and generate the full crRNA reporter construct (Figure 17).

Figure 17. Schematic of full crRNA reporter construct. The crRNA-GFPLVA-tt composite part is ligated downstream of a promoter (either pBad or pLac). The dark green represents the BioBrick scar that occurs when two parts are ligated together.

The crRNA-GVPLVA-tt did not cut out of the plasmid even though it has verified XbaI and PstI sites and was able to be cut out with EcoRI and PstI (Figure 18). This is most likely caused by the base pairs TC being the first two bases after the XbaI site. The *dam* methylase recognizes the

GATC sequence formed when TC follows the XbaI site and methylates the adenine residue. When the site is methylated, the enzyme XbaI cannot cut the site (NEB Dam, 2007). This problem can be circumvented by transforming the plasmid into *dam⁻* cells such as NEB's C2925 *E. coli* competent cells.
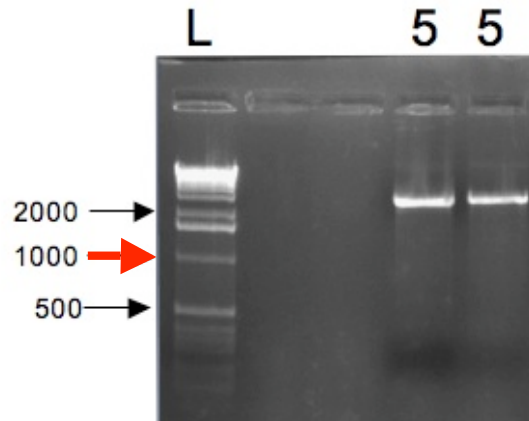
Figure 18. Gel analysis of crRNA-GFPLVA-tt XbaI digestion. The miniprep of crRNA-GFPLVA-tt from colony 5 was digested with XbaI and Pst1. No insert appeared at ~975 bp (red arrow) although the insert does appear when digested with EcoRI and PstI as seen in figure 14.
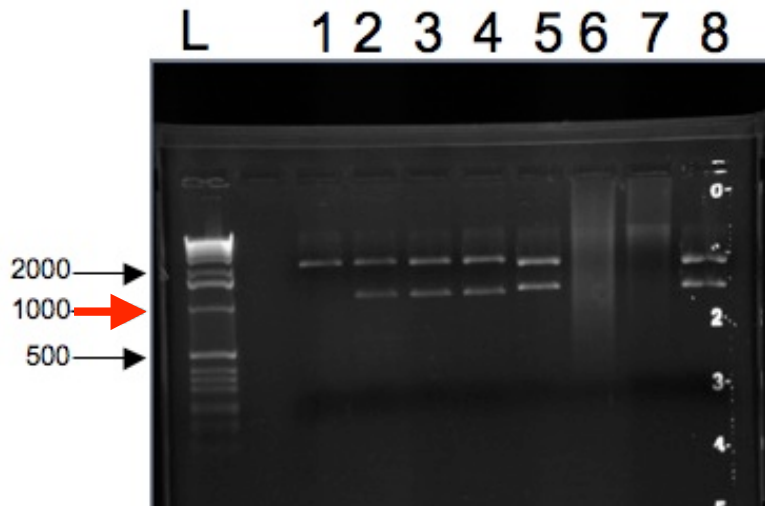
The DNA from crRNA-GFPLVA-tt colony 2 was transformed into *dam-/dcm-* competent *E. coli* cells. Two colonies from the transformation were picked and their plasmid DNA was cut with XbaI and PstI (Figure 19). Both plasmid digestions produced bands at approximately 975 bp, the size of crRNA-GFPLVA-tt; therefore, the methylation of the adenine by *dam* had previously prevented the insert from being cut.
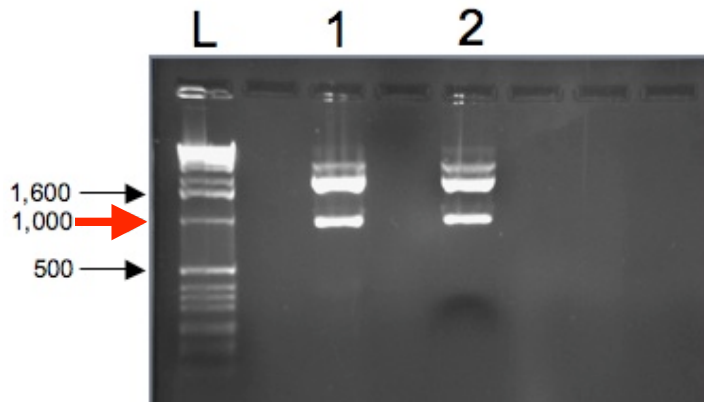
Figure 19. XbaI and PstI restriction analysis of crRNA-GFPLVA-tt from *dam-* cells. The miniprep of crRNA-GVPLVA-tt from colony 2 was digested with XbaI and Pst1. Insert appeared at ~975 bp (red arrow), the correct length of crRNA-GFPLVA-tt.

To make the crRNA construct functional, a promoter needed to be inserted upstream of crRNA-GFPLVA-tt. As different promoters have different strengths, two different promoter-crRNA constructs were made (Figure 20). One used pLac while the other used pBad. The

current primers for the taRNA include the pBad promoter; therefore, the pBad-crRNA construct should always produce GFP when the taRNA gene is in the cell.

Figure 20. Gel analysis of potential pBad-crRNA-GFPLVA-tt and pLac-crRNA-GFPLVA-tt colonies. Lane L is 1 Kb Ladder. DNA in lanes 1-4 are plasmid DNA from individual colonies of the pBad-crRNA-GFPLVA-tt transformation and were digested with EcoRI and PstI. DNA in the lanes 5-8 are plasmid DNA from individual colonies of the pLac-crRNA-GFPLVA-tt transformation and were digested with EcoRI and PstI. Both constructs should be slightly larger than 1 KB (red arrow).

Once the promoter-crRNA-GFPLVA-tt construct was made, the taRNA construct and promoter-RBS-GFPLVA-tt construct needed to be generated before testing the functionality of the crRNA.

taRNA construction:

Once the PCR technique was shown to generate crRNA, six primers were designed that split the taRNA sequence into three segments. The three segments would then be used as primers to generate the full taRNA (Figure 21). All of the primers had been designed with four extra bases added on instead of just the end primers that contain restriction enzyme sites; therefore, the middle primers had to be redesigned. While the middle primers were being reordered, the bad primers were used to test PCR conditions and protocols (Figure 22).


Figure 21. Schematic of taRNA construct. The full taRNA sequence (black, red, and grey) is placed directly downstream of its promoter pBad (purple). The whole construct is flanked by BioBrick ends (green). The transcribed product is shown on the right.
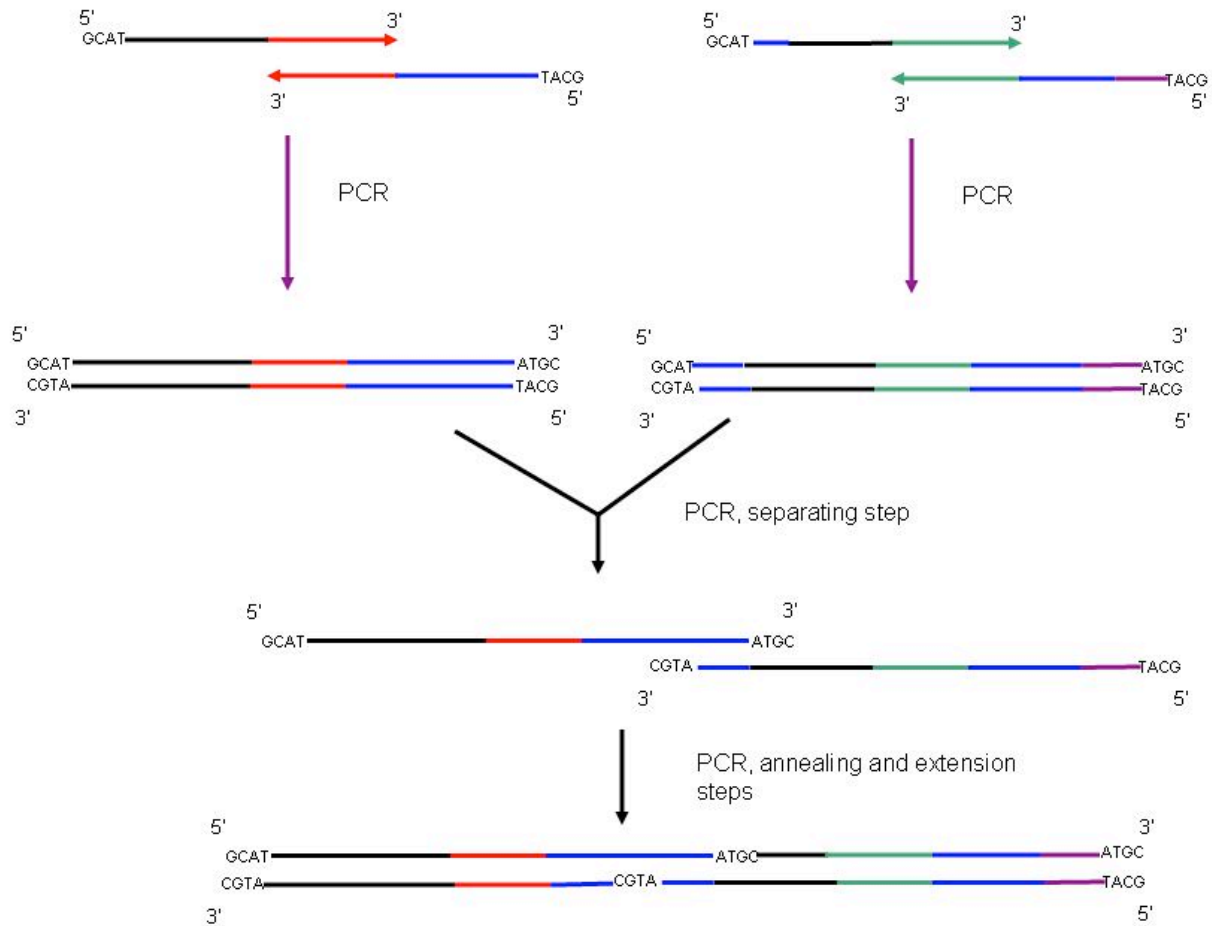
Figure 22. taRNA PCR protocol with bad primers. As in the protocol used to generate crRNA, primers without template were used to produce segments of the taRNA. Each segment had sections of overlap (shared colored sections) the segments that flank it (black). The segments were then used as primers in the next PCR reaction. The four extra bases on each internal primer would not allow for the desired product to be formed.

First a shotgun PCR was attempted. All of the primers were added to the PCR at a concentration of 1 μM each. This attempt did not result in the desired ~340 bp product (Figure 23).
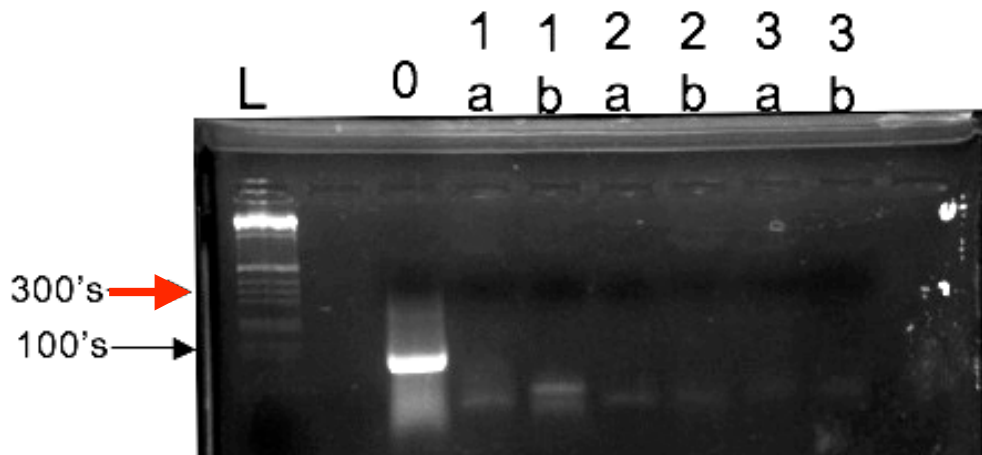
Figure 23. Gel analysis of shotgun PCR reaction for taRNA. 2% agarose gel of a shotgun PCR approach to generating the taRNA. Lane L contains a 1 Kb ladder. 0 is the product of a PCR using the primers for all three segments of the taRNA. The product in 0 should be in the 300's (red arrow). 1a (74 bp) is the PCR with only the first segment's forward primer. 1b (74 bp) is the PCR with only the first segment's reverse primer. 2a (74 bp) is the PCR with only the second segment's forward primer. 2b (75 bp) is the PCR with only the second segment's reverse primer. 3a (82 bp) is the PCR with only the third segment's forward primer. 3b (77 bp) is the PCR with only the third segment's reverse primer. The middle primers (1b, 2a, 2b, 3a) had four extra bases each that would not base pair with the other segments. The controls showed that primer dimer amplification was not occurring.

A piecewise protocol, where each individual segment was generated and then combined with the following segment, was then attempted. The individual segments were successfully generated, but the full construct was unable to be generated (Figure 24). The inability of the full construct to be formed most likely results from the extra bases on the ends of each strand not base pairing; thus, the polymerase was unable to add more bases on to the end of the strands (Figure 25). If PCR with the proper primers does not work, the problem could stem from self-complementation in the longer segments.
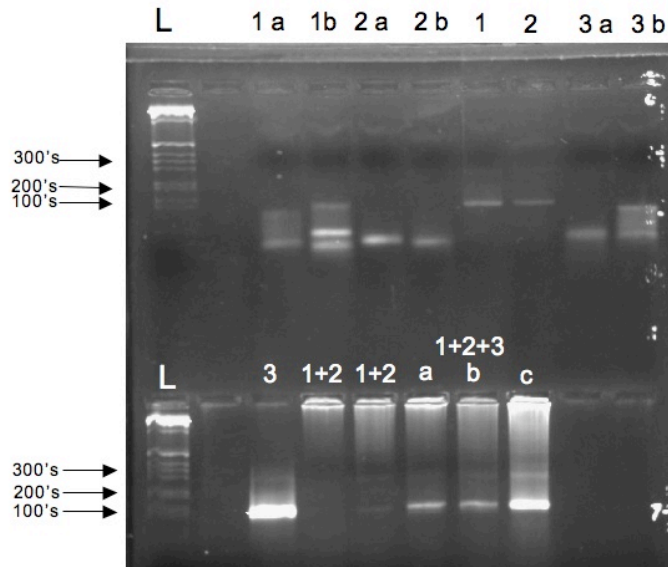
Figure 24. Gel analysis of piecewise PCR reaction for taRNA. The lanes marked L contain 1 Kb ladder. Lanes 1a, 1b, 2a, 2b, 3a, and 3b are single primer control PCR. Lanes 1, 2, and 3 are the actual PCR reactions for the three segments of taRNA that are later used as overlapping primers to generate the full 340 bp taRNA. The size of these segments ranges from 125 bp (1 and 2) to 139 bp (3). The first lane labeled 1+2 is the PCR using 100 ng each of 1 and 2 as primers. The second lane labeled 1+2 is the PCR using 535 ng each of 1 and 2 as primers. These two lanes should have had a band at about 200, but did not. 1+2+3 a is the PCR using 100 ng each of 3 and the product of the first lane of 1+2. 1+2+3 b is the PCR using 100 ng each of 3 and the product of the second lane of 1+2. 1+2+3 c is the PCR using 535 ng each of 3 and the product of the second lane of 1+2. These three lanes should have had a band at 340 bp. There may be a faint band. These primers also had the four extra bases that do not pair with the other segments.
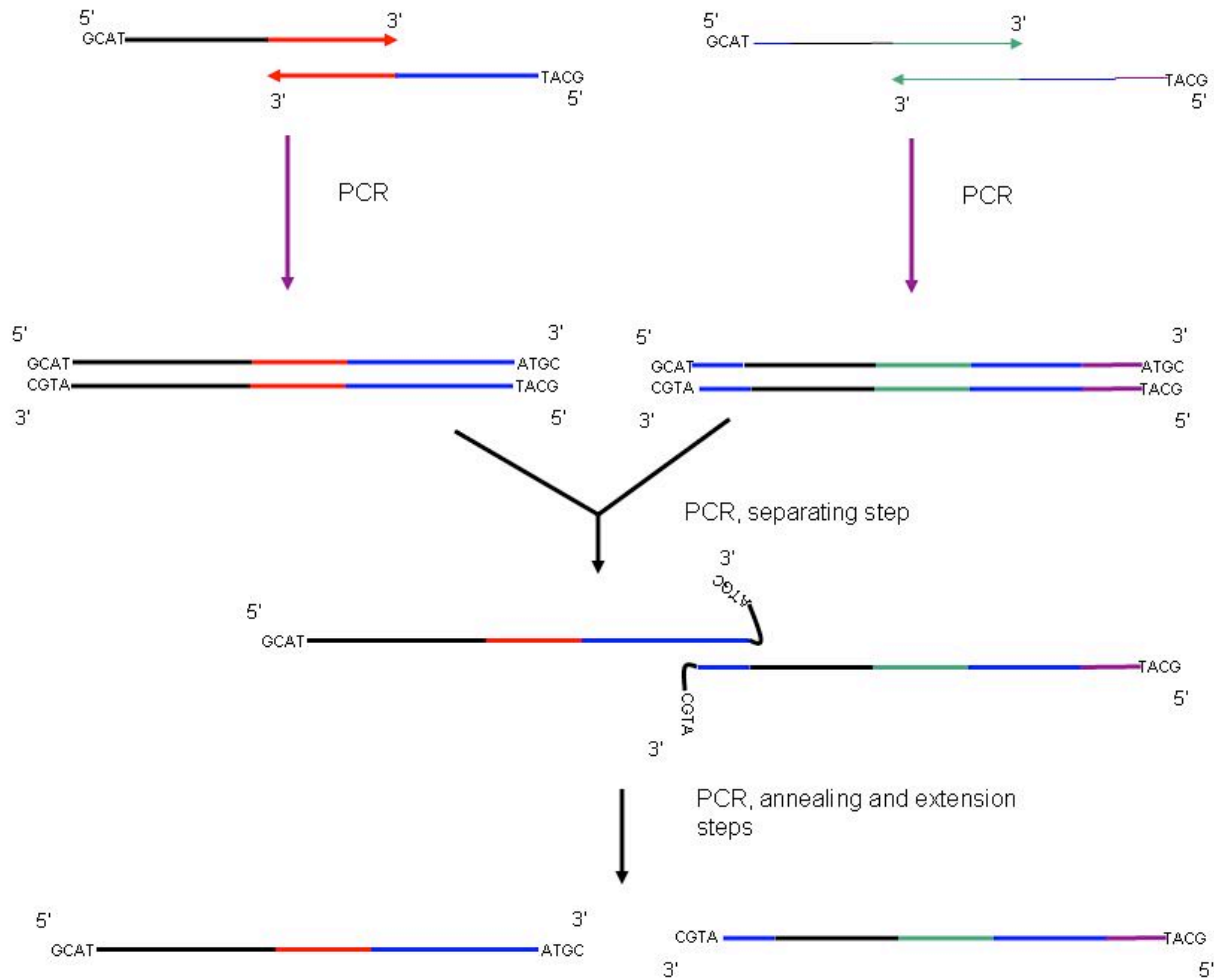
Figure 25. Problem with primers. Each segment has the sections of overlap (shared colored sections) and the segments that flanked it (black). The segments were then used as primers in the next PCR reaction. The four extra bases on each internal primer would not allow for the desired product to be formed as they did not pair up with the other primer.

The piecewise protocol as well as the all primers in one reaction PCR was retried with the correct primers. The single reaction PCR most likely did not work because the primers possibly had too much self-complementarity and bound easily to primers other than the correct overlap. While the single reaction did not work, the piecewise was able to generate the segments (Figure 26a). Those segments successfully generated the next segments. When segments one and two were combined, there was a faint band in the 200's, the approximate length of the new desired segment (Figure 26 b). To confirm that segments generated by using previously generated segments as primers where correct, either the forward primer of segment 1 and the reverse primer of segment 2 or the forward primer of segment 2 and the reverse primer of segment 3 were used to amplify these segments by PCR. Using these primers at least 1+2 and 2+3s seem to have been amplified above the template only controls (Figure 26 c).   The DNA from these

bands were purified and used as the next primers.
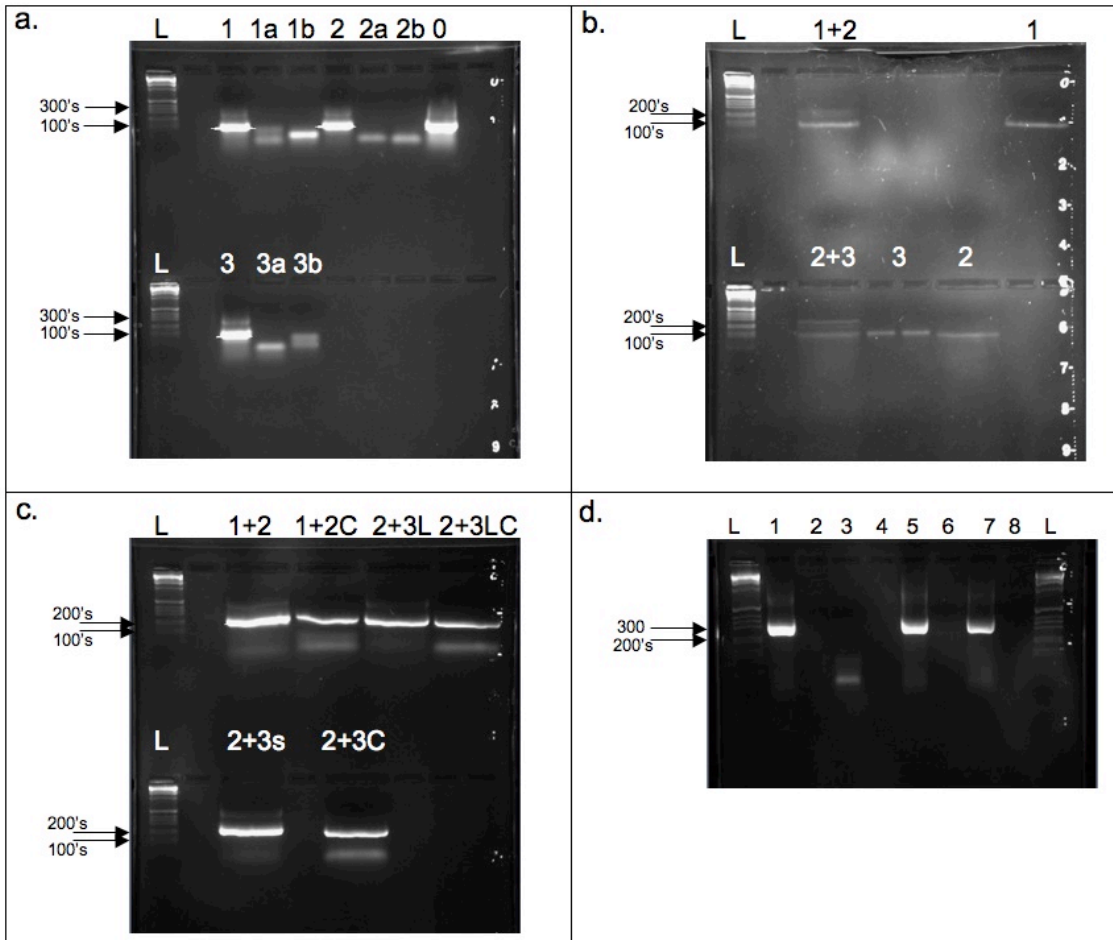
Figure 26. Gel Analysis of piecewise PCR reactions. a. Lane 1 is the PCR reaction for the first segment. Lane 1a is the reaction with forward primer only. Lane 1b is the reaction with reverse primer only. Lane 2 is the second segment. Lanes 2a and 2b are its single-primer controls. Lane 0 is the reaction with all of the primers. Lane 3 is the third segment. 3a and 3b are its controls. Lanes 1, 2, and 3 all have bands in the 100's, the approximate size of the desired segments. **b.** Lane 1+2 is the PCR reaction using segments 1 and 2 from the previous reactions. A band in the 200's was generated. Lane 2+3 is the reaction using segments 2+3 from the previous reactions. In the 2+3 lane, there are two bands in the 200's (one of the bands was only visible on a gel box). Lanes 1, 2, and 3 are single segment reaction controls. **c.** Lanes 1+2, 2+3L, and 2+3s are PCR reactions using either the forward primer of segment 1 and the reverse primer of segment 2 or the forward primer of segment 2 and the reverse primer of segment 3. Using these primers at least 1+2 and 2+3s seem to have been amplified above the template only controls. **d.** The following reactions used 1 ul of a previous reaction that should have generated (1+2)+3, 1+(2+3L), or 1+(2+3s) as template. The forward primer of segment 1 and the reverse primer of segment 3 were used as template. Lanes 1, 5, and 7 used (1+2)+3, 1+(2+3L), and 1+(2+3s) respectively as template. Lane 3 is the primer control. Lanes 2, 6, and 8 are template controls. Lanes 1, 5, and 7 show bands of approximately 300 bp in length. The wells are overloaded and most likely ran fast.

As the gel of the final taRNA reactions (Figure 26 d) had bands slightly lower than expected but the lanes were overloaded, I decided to transform the DNA into cells and sequence the parts. The transformation was successful for all forms of the full taRNA and the band size seemed to match better except for the taRNA 1+(2+3L), which seemed to be slightly larger than the expected 340 bp (Figure 27). The sequence data showed that 1+(2+3L) actually had an extra section, which accounts for its larger size (Figure 28). taRNA 1+(2+3s) from colony 3 was a perfect match to the designed sequence (Figure 29 a). Only one base was ambiguous, but the chromatogram suggested that it really was an adenine (Figure 29 b).
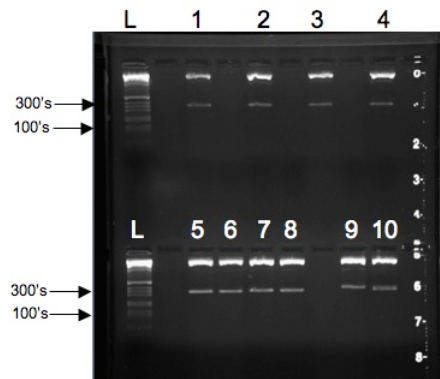

Figure 27. Gel analysis of potential taRNA colonies. Lanes 1, 2, 3, and 4 are digested plasmid minipreps of colonies from the transformation with 1+2+3 taRNA in pSB1A2. Lanes 5, 6, 7, and 8 are digested plasmid minipreps of colonies from the transformation with 1+(2+3s) in pSB1A2. These lanes have bands of approximately 340 bp in length (middle band of the 300's in ladder), which is the size of a full taRNA. Lanes 9 and 10 are digested plasmid minipreps of colonies from the transformation with 1+(2+3L) taRNA in pSB1A2. Their bands seem to be slightly higher than 340 bp.
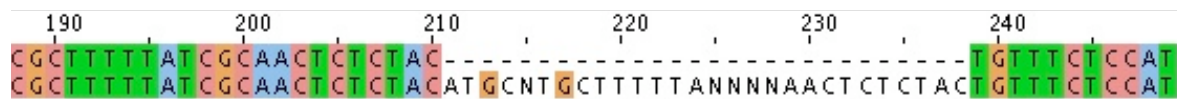
Figure 28. Sequence analysis of taRNA generated from the 1+(2+3L) PCR reaction. The taRNA produced from the segments 1+(2+3L) (bottom line) contains a 29 bp stretch as well as other mutations not originally part of the designed sequence of the taRNA (top line).

a.
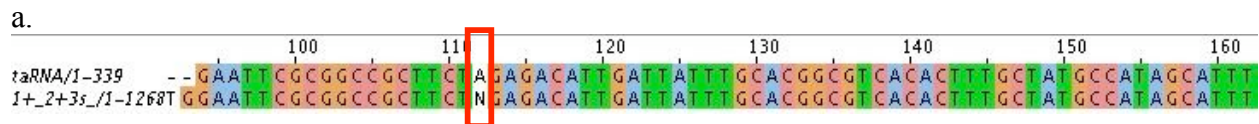
b.
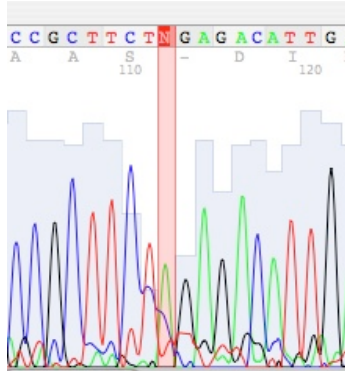
Figure 29. Sequence analysis of taRNA generated by PCR reaction 1+(2+3s). a. The taRNA reference sequence (top line) matches the taRNA DNA from colony 3 of the transformation of plasmid containing the product of the 1+(2+3s) PCR reaction into pSB1A2 except for a single A → N at base 112 (red box). **b.** The chromatogram of colony three's taRNA sequence shows a distinct A peak with some slight background C signal that could confuse the reading.

Once the taRNA was generated and sequence verified, the taRNA needed to be placed into the same plasmid as the crRNA construct. By placing them in the same plasmid instead of in the same cell on two different types of plasmid, the potential for extreme disproportions in the number of taRNA transcripts compared to crRNA transcripts decreases. If the taRNA was inserted into a cell with crRNA on a separate plasmid, it would need to be on a plasmid with a different origin of replication. Otherwise, either the crRNA plasmid or the taRNA plasmid would be kicked out of the cell. The problem with using a plasmid with a different origin of replication is that the plasmids would have different copy numbers. Therefore, the taRNA might be working but not have enough to produce any noticeable effect because the crRNA was on a high copy plasmid (200-500 per cell).

Construction of RBS:

To properly understand how effective the taRNA is at returning GFP translation, a positive control that only has the promoter, RBS, GFPLVA, and terminator is needed. This construct would show the maximum fluorescence that cells could have if no crRNA were interfering or if the taRNA worked perfectly. Because every RBS has a different strength, I needed to build this construct with the exact same RBS that was used in the crRNA construct.

For the first attempt at generating the RBS (59 bp with BioBrick ends and extra bases to allow restriction enzymes to cut, Figure 30), I employed the primer dimer PCR. I predicted the reaction would produce enough product to efficiently cut and ligate. The method did generate what might have been the desired RBS (Figure 31), but retrieving the DNA was difficult. I could not use any kits as they do not catch DNA that size. While the freeze and squeeze method may have retrieved the DNA, the transformations were highly inefficient. No colonies with the RBS were found.

Figure 30. Schematic of RBS. Above is the construct for the positive control's RBS. The RBS is the same as in the crRNA construct. The BioBrick ends flank the part.
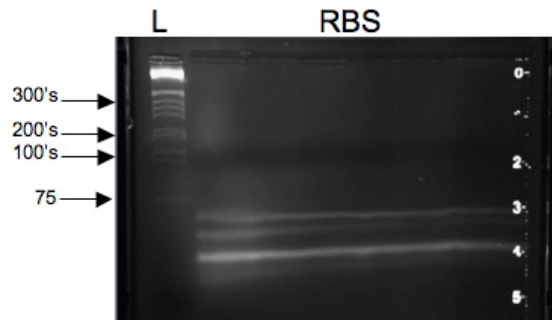
Figure 31. Gel analysis of RBS generated by PCR protocol. Two bands appear that could possibly be the 59 bp RBS.

As cleaning and retrieving the RBS after using the PCR method was unsuccessful, I had the RBS synthesized as single stranded oligos that had EcoRI and PstI sticky ends (Figure 32).

5'   3'
  aattcgcggccgcttctagagAGAGGAGAtactagtagcggccgctgca
    gcgccggcgaagatctcTCTCCTCTatgatcatcgccggcg
3'   5'

Figure 32. RBS oligos synthesized for annealing protocol. The top and bottom strands of the RBS were synthesized as if they had been cut with EcoRI and PstI.

While very little RBS DNA is needed in a transformation, single stranded RBS could block double stranded RBS from being ligated into the vector. The vector also showed high background in control transformations. Because the RBS DNA was synthesized, the 5' end does not have a phosphate; therefore, the phosphate cannot be removed from the vector by alkaline phosphatase to increase efficiency. Currently the two oligo assembly method has also failed to yield a useable RBS. I will try to complete this after submitting my thesis.

Building and testing taRNA with crRNA construct:

As mentioned previously, the taRNA and crRNA parts would work most efficiently in the same plasmid (Figure 33). Although the crRNA constructs were originally built with two different promoters, only the construct using the pLac promoter was combined with the taRNA part as digestion of the pBad construct continually exhibited bands from enzyme star activity and time constraints did not allow for further attempts (Figure 34). Transformations using plasmid

with taRNA as either an upstream and downstream receiving vector were inefficient; therefore, the phosphate was removed from the receiving vectors with alkaline phosphatase. I proceeded with the downstream transformation and successfully generated the construct taRNA-pLac-crRNA-GFPLVA-tt (Figure 35).
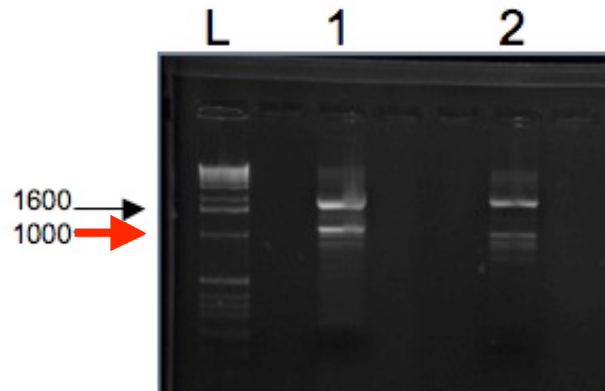

Figure 33. Schematic of taRNA and crRNA constructs in a single plasmid. A. The full crRNA reporter construct has been inserted downstream of the taRNA construct. Dark green represents the SpeI/XbaI scar. B. The cull crRNA reporter construct has been inserted upstream of the taRNA construct. Dark green represents the SpeI/XbaI scar.

Figure 34. Gel purification of pLac-crRNA-GFPLVA-tt and pBad-crRNA-GFPLVA-tt cut with EcoRI and SpeI. Lane 1 is the pLac-crRNA-GFPLVA-tt construct. It has a prominent band at just above 1000 bp, which is most likely the desired construct. There does seem to be some star activity. Lane 2 is the pBad-crRNA-GFPLVA-tt construct. The band at about 1000 bp is about as intense as the bands generated through star activity.
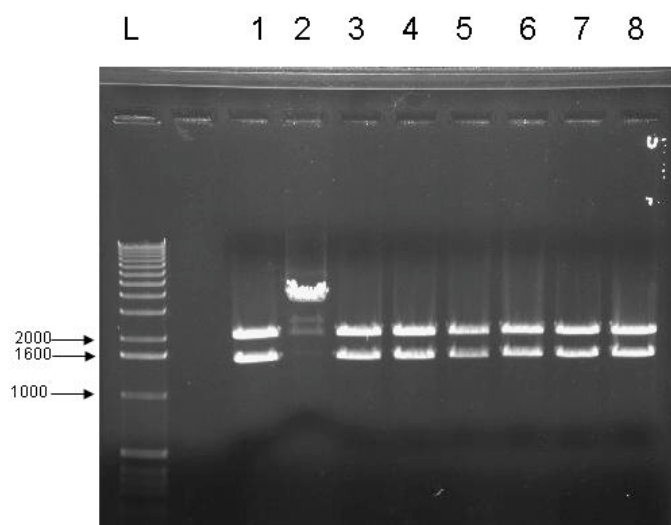
Figure 35. Gel analysis of potential taRNA-pLac-crRNA-GFPLVA-tt colonies. Lanes 1, 3, 4, 5, 6, 7, and 8 have bands slightly lower than 1,600 bp. The taRNA-pLac-crRNA-GFPLVA-tt construct is 1,515 bp; thus, these bands are most likely the desired construct.

Now that the full device has been built, flow cytometry will be performed to test functionality after the thesis has been submitted.

Discussion:
Biosensor technologies will benefit greatly from the development of ligand-controlled riboregulators in bacteria. Currently, antiswitches allow for ligand-controlled regulation of translation in eukaryotes while riboregulators permit for transcription factor-controlled regulation of translation in bacteria (Bayer, 2005; Isaacs, 2004). The goal of my research was to develop a device that combined antiswitches and riboregulators and thus performed ligand-controlled regulation of translation in bacteria. While time has not allowed for the final ligand-controlled taRNA to be built, advances made with this research suggest the potential for future building of the ligand-controlled taRNA and has provided several tools for making the process doable without relying on full gene synthesis.

While the actual structure of folded RNA can only be seen through X-ray crystallography, the algorithms used by mFold and RNAstructure can predict two-dimensional folding. By combining antiswitch and taRNA sequences, I designed a sequence that should be both ligand-controlled and targeting crRNA. Using RNAstructure, one potential folding for the sequence was shown to be similar to an antiswitch but with different sequences (Figures 6 and 8).

The construction of new BioBrick standardized parts has been difficult for many synthetic laboratories. A technique such as oligo annealing may efficiently generate a promoter or other basic part but fail to generate effectively a different part like an RBS. Full gene synthesis remains expensive and depending on what other contracts the company has may take months before the DNA reaches the lab. The PCR technique that I have developed to generate self-complementary DNA parts adds to our toolbox. My own previous attempts to use oligo annealing to generate crRNA demonstrated a need for a new technique. The PCR method allows

for oligos to be made into the full construct relatively quickly as shown by the building of the crRNA and taRNA with this technique. The PCR method worked because each individual primer was designed to have minimum self-complementarity. Once all segments were combined, the overlap was strong enough to ensure that at least a few molecules would anneal properly. In PCR, one template molecule can be amplified a billion fold in thirty cycles. Since the original end primers would only amplify the constructs that had the beginning of the first segment and end of the third, the researcher sees a clear difference between the desired band and any other bands. Because the full construct has areas of self-complementarity, the DNA would be more likely to fold onto itself in the last stage than bind with the second strand of DNA. When folded over onto itself, polymerases would not amplify the folded form; thus, the primers would only amplify the desired construct (Figure 22 d). Hopefully in the future, programmers will write code to analyze and design the primers more effectively than hand analysis used in conjunction with sequence alignment programs. This method will prove useful when work continues forward with building ligand-controlled riboregulators in bacteria.

While I was unable to build the ligand-controlled taRNA, the work done with JM109 cells containing the GFP gene that have been exposed to theophylline has provided necessary information for future testing of the ligand-controlled riboregulator system. Future testing should use theophylline concentrations lower than 10 mM. If higher concentrations must be used, the growth time must be longer. The slower growth rate produced with high concentrations of theophylline could actually be beneficial to generating more accurate readings of GFP when using flow cytometry as GFP has a high dilution rate when cells divide rapidly (Leveau and Lindow, 2001); however, the cells may grow slower because the higher concentrations negatively affect normal cell function. To achieve the slower growth rate while keeping cells healthier, the later experiments with the actual ligand-controlled riboregulator might grow cells at a lower temperature in a lower concentration of theophylline.

While I completed the necessary beginning work in the process of developing ligand-controlled riboregulators in bacteria, I need to test the functionality of the device. To truly understand how well the device is functioning, I need to build the positive control construct using the same RBS as the crRNA construct. Previous research shows that small differences can change the strength of an RBS and thus the amount of translation that occurs for a RNA (Yokobayashi, 2002). Once the positive control has been built, functionality data can be understood fully for both the transcription factor-controlled and the ligand-controlled riboregulator.

Before building the ligand-controlled riboregulator, I need to test the functionality of the transcription factor-controlled riboregulator that I generated. The results of those functionality tests will indicate whether the sequence of the ligand-controlled riboregulator needs tweaking to allow it to accommodate the BioBrick ends.

While I have yet to build the ligand-controlled riboregulator, I have almost concluded the necessary background work. The crRNA target reporter construct has already been built; therefore, only a ligand-controlled taRNA must be built to have a fully functioning device. Future work to physically produce the ligand-controlled taRNA could potentially result in a prototype biosensor. The aptamer could then be changed. Such a switch would allow for the detection of any chemical that has a corresponding aptamer. Biosensors would truly benefit from further work in this field.

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