in vivo Assay: Bacterial Small RNAs May Regulate Eukaryotic Gene Expression

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ABSTRACT

Wolbachia is present in 70% of insects because of this it is widely studied. Research suggests that one or more small RNAs, made by the *Wolbachia* bacteria is responsible for initiating and maintaining the ability to manipulate its host's reproduction which is a phenomenon called cytoplasmic incompatibility (CI). Early evidence suggests that some *Wolbachia*- derived small RNAs hybridize to *Drosophila* protein-coding genes, which regulate protein expression. If the small RNAs are produced in enough volume to bind to *Drosophila* targets with enough strength they could regulate protein expression. The bacterial small RNA sequences have been derived from RNA library pools sequenced using RNA-Seq. A plasmid was designed from two existing plasmids. The pHd-DsRed plasmid and the act5c plasmid, which each contain specific pieces that were needed to complete the designed plasmid. For the pHd-DsRed plasmid, restriction enzyme digestion was used to cut the section of the plasmid needed and a gel electrophoresis was used to separate the piece from the rest of the plasmid. For the act5c plasmid, PCR was used to amplify and isolate a section of the plasmid. The pac5-DsRed plasmid, containing the ACT5C promoter, a fluorescence gene, and an ampicillin resistance gene, is transformed into cells and selected for using ampicillin plates.

1. Introduction

Gene expression can be affected by several different factors, external and internal. For example, you might have the gene that Usain Bolt has that indicates you run fast, however, you may run really slow simply because that gene is not activated. In recent years, microRNAs (miRNAs) have become a topic of research for geneticists and other scientists, as well. They are not a well-researched topic, therefore, there are many questions floating around about these small RNAs. The question I will be focusing on in my research will pertain to small RNA interactions

with DNA. I will be using Drosophila cell culture and *Wolbachia* bacteria to determine if small RNAs from bacteria affect the host's gene expression.

Small noncoding RNAs, 18-30 nucleotides in length, are one of those factors that affect gene expression. MiRNAs are a class of small RNAs that are around 20 nucleotides in length. MiRNAs only exist in Eukaryotic organisms and are known to affect gene expression. They are a relatively new discovery in the genetics field so there has not been much research done on the subject. Their basic structure and a little about how they work is known, however, not much is known about how they affect their surroundings. They are involved in almost every cellular process and are a critical part of animal development, cell differentiation, and homeostasis (Gerbert, 2019). These factors and their abundance make them a great resource for research and show a need for more studies on the subject. A 'hairpin' structure, which is where the miRNA sequences are embedded, develops as mRNAs are transcribed by RNA polymerase II (Ha, 2014). This hairpin is cut by enzymes making the miRNAs, which then float around in the cytoplasm until they bind to a target sequence at the 3' untranslated region. We are proposing that the bacterial small noncoding RNA mechanism is similar to the miRNA mechanism.

There is also evidence that small RNAs produced by viruses and the host are involved with each other (Kabekkodu, 2018). This intercellular communication opens up the question which began my project: 'Can these small RNAs from bacteria and viruses affect host gene expression?' If this is the case it would have to change the way antibiotics are used and designed. If the bacteria that live in us naturally affect gene expression in the body, then antibiotics that kill all bacteria in the area are in turn affecting which genes are expressed or inhibited.

Drosophila melanogaster is often used to model genetic research because of its rapid life cycle, short life span, small size, easy generation of mutant animals, and they share about 40% of the nucleotide and protein sequence in their homologs with mammalian species (Baena, 2019). This project will be using Drosophila cell cultures instead of a live model. Cell cultures was chosen because in previous projects conducted by former students there were complications with live models. I, also, have previous experience working with cell cultures, which opened the opportunity for this project. Cell cultures are a very straightforward way to determine the results of this project. Drosophila were chosen for this project because it has 466 known miRNAs (Ha, 2014) and usually approval for experimental settings from animal welfare ethical review boards is not needed (Baena, 2019). All of these factors have led to Drosophila being well researched which leaves few unknown variables that could affect this research outside of what is being tested.

This project will use two types of Drosophila cultures, one infected with *Wolbachia* and one uninfected. *Wolbachia* is a part of the order Rickettsiales, which is a group of intracellular bacteria that is made up of species with parasitic, mutualistic and commensal relationships with their hosts (Werren, 2008). There were several factors that made *Wolbachia* the choice bacteria for this project. The first reason, *Wolbachia* have a unique feature called cytoplasmic incompatibility (CI) which is when one or more small RNAs made by the bacteria is responsible for initiating and maintaining the ability to manipulate its host's reproduction which is a

phenomenon. The second reason, Wolbachia usually do not infect vertebrates (Werren, 2008). The third reason, Dr. Hagen had a contact that could provide Drosophila cultures infected and uninfected with *Wolbachia.*

Once cultures are grown plasmids will be transformed into them. Plasmids are rings that have three key features: an antibiotic resistance gene, an origin of replication site, and multiple cloning sites. The plasmid used in this project is the pHD-DsRed plasmid (Figure 1). It contains the DsRed fluorescent gene. The pHD-DsRed plasmid is designed for rapid generation of gene-specific donor templates and are custom synthesized (Gratz, 2014). There is evidence that this plasmid is very compatible with Drosophila. It has also been used in Dr. Hagen's lab previously, which provided an advantage in jump starting this research project. The pHD-DsRed plasmid's multiple cloning sites were designed to conserve as many restriction sites as possible to make the vector compatible with homology arms (Gratz, 2014). This plasmid will have to be manipulated slightly to be completely applicable to this research project. The promoter will have to be replaced and a target sequence will need to be added as well.

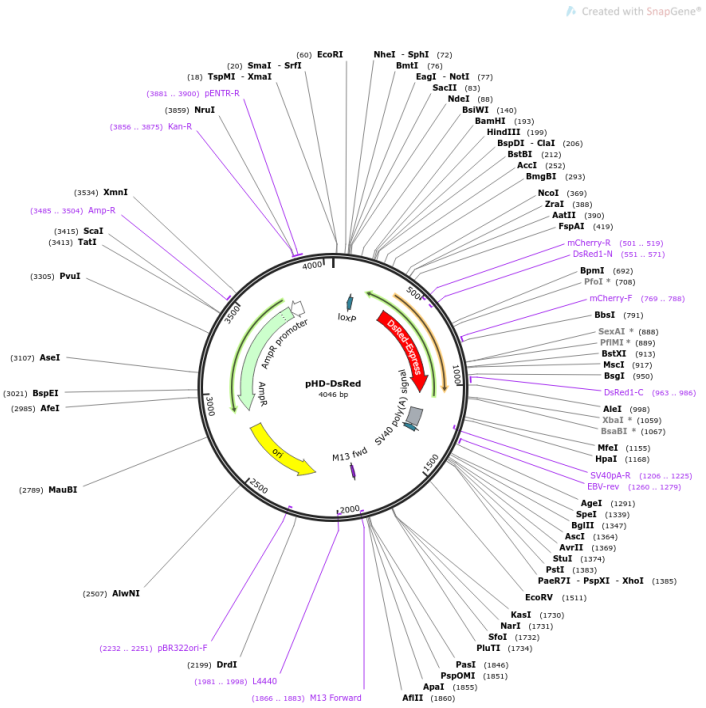

Figure 1: pHd-DsRed plasmid

The promoter on the plasmids have been changed to a promoter that is more applicable to Drosophila systems. The decision was between two promoters, copia transposon promoter (COPIA) and actin 5C promoter (ACT5C). When comparing the two in both price and quality of the promoter the decision was made to get the ACT5C promoter. Both produce similar fluorescence signals in Drosophila (Qin, 2010), but when comparing prices, the ACT5C promoter made the most sense for this project. Promoters act as on and off switches. For this project, a promoter that is on at all times was required. This allows us to limit the influences of other cellular factors on our results. The complete plasmid will consist of several pieces. The piece will include: the ACT5C promoter, the body of the pHD-DsRed, and the 3' UTR sequences will be stitched together using this kit. The full plasmid will then be transformed into the cell culture and go through an electrical shock process to trigger the absorption of the plasmid into the cells.

The goal of the project I am proposing is to identify interactions between small noncoding RNAs from *Wolbachia* bacteria and protein-coding genes in *Drosophila* JW cells.

This project will allow continued research of molecular relations between bacteria and host interactions. I will generate a DNA plasmid that contains the promoter, ACT5C, which acts as an on or off switch that is driving the DsRed gene, a fluorescent gene, and a 3' UTR target sequence, which the small noncoding RNAs will attach to. I will then insert the plasmid into the lung cells, one uninfected culture and one infected with *Wolbachia*. If the small noncoding RNAs generated by *Wolbachia* are complementary to the target sequences in the plasmids, are produced and hybridized with sufficient strength, there is expected to be a visual reduction or inactivity of fluorescence expression. Picture it like a flashlight that is always turned on and shining. If a piece of paper blocks the light, then it will shine less or not at all. If the small RNAs are not produced in sufficient quantity or strongly connected, the small RNAs will be able to be identified as non-targeting. The correctly assembled transgenic plasmid will lead to CRISPR being able to identify small RNAs influenced by *Wolbachia* within lung cell lines.

This project will lead to a better understanding of the relationship between *Wolbachia* bacteria and their Drosophila hosts. This project will help determine whether the bacteria in our bodies, either helpful or harmful, affects the expression of our genes. Which in turn will lead to a greater understanding of how antibiotics and bacteria affect us at the genetic level. Antibiotics currently kill all bacteria in an area, not just the good or the bad. Therefore, my project could bring that characteristic into question and allow us to consider ways to limit the bacteria affected by antibiotics, specifically to infectious or harmful bacteria. It also has the potentially to limit the expression of genetic cancers or stop them altogether. Cancer researchers and antibiotic developers will have new questions to research, and it will show other scientists valuable information about how outside forces can affect the genome.

This project will combine the use of Drosophila cultures, one group infected with *Wolbachia* and one group uninfected, to determine if small noncoding RNAs from bacteria affect gene expression in the host. The DNA plasmid will consist of a pHD-DsRed with an added ACT5C promoter replacing the original and added 3'UTR sequences that was found in data from RNA library pools that were created. The DNA plasmid will be used to insert the fluorescent gene, DsRed, and the 3'UTR sequence the small RNA will attach to. This project will lead to a better understanding of interactions between bacteria and hosts at the molecular level and genetics as a whole.

2. Experimental Details

Two groups of *Drosophila* cells were purchased, one infected with *Wolbachia* and the other uninfected. Eight small RNA library pools were created from the two groups of *Drosophila* cells, four from the uninfected and four from the *Wolbachia* infected, using the NEBNext Small RNA Library Prep Set. The first step is to ligate the 3' SR adaptor, to do this use 1 µg of total RNA from each sample, 1 µl of 3'SR adaptor for Illumina, and nuclease-free water to bring the total volume to 7 µl were combined. The samples are incubated in a preheated thermal cycler for 2 minutes at 70℃ and then is transferred to ice. The following components were added to the samples: 10 µl of 3' Ligation Reaction Buffer (2X) and 3 µl of 3' Ligation Enzyme Mix. The samples were vortexed and incubated for one hour at 25℃ in a thermal cycler. The second step

is to hybridize the reverse transcription primer, to do this add 4.5 µl of nuclease-free water and 1 µl of SR RT primer for Illumina and then mix the solution. Place the tube in a thermocycler with a heated lid and run it for 5 minutes at 75℃ then 15 minutes at 37℃ then 15 minutes at 25℃ then hold at 4℃. The third step is to ligate the 5' SR adaptor, which was briefly denatured at 70℃ for two minutes. Add 1 µl of the denatured 5' SR adapter for Illumina, 1 µl 5' ligation reaction buffer (10x), and 2.5 µl 5' ligation enzyme mix to bring the total volume to 30 µl for each sample. Mix the solution and incubate for one hour at 25℃ in a thermal cycler. The fourth step is to perform reverse transcription to do this add 8 µl of the first strand synthesis reaction buffer, 1 µl of the murine RNase inhibitor, and 1 µl of the ProtoScript II reverse transcriptase and mix the solution. Next, incubate the solution for 60 minutes at 50℃ and immediately after start step five. The fifth step is to perform PCR amplification. Add and mix well 50 µl of LongAmp Taq 2X master mix, 2.5 µl SR primer for Illumina, 2,5 µl Index (X) primer, and 5 µl nuclease-free water. Use the recommended cycling conditions for the PCR program. The final step is to perform a quality control check and size selection, in this experiment, a bioanalyzer high-sensitivity DNA chip was used to evaluate quality.

To obtain plasmids cells needed to be grown. Plasmid-containing bacteria were grown in glycerol and quadrant streaked on ampicillin-coated LB Agar plates. To make the LB agar selection plates add 12.5g of LB Broth to 500 mL of deionized distilled water and stir until the clumps are gone. Then add 7.5g of agar, it will not dissolve, and autoclave for a 30-minute liquid cycle. Once the agar has cooled to the touch, use a serological pipet to transfer 25 µl to each plate and let them solidify. After the plates have solidified add 25 µl of ampicillin as you use them. The plates growing plasmid-containing bacteria are incubated overnight at 37℃ and several colonies are selected from those plates to be grown in 3 ml of LB broth with 3 µl of ampicillin overnight. LB broth is made by adding 12.5g of LB broth to 500 mL of deionized distilled water and stirring until the clumps are dissolved. Once the plasmid-containing bacteria has been grown in liquid culture use the Monarch Plasmid DNA Miniprep Kit to purify plasmid DNA. Using the Monarch Plasmid DNA Miniprep Kit pellet 1 ml of the bacterial culture by centrifuging for 30 seconds at 13,000 RPM and discard the supernatant after. Then mix the pellet with 200 µl of the plasmid resuspension buffer by vortexing. To lyse the cells, add 200 µl of the plasmid lysis buffer, invert the tubes until the solution changes to dark pink, and incubate for one minute. Add 400 µl of the plasmid neutralization buffer to neutralize the lysate. Invert the tubes until the color of the solution is yellow and a precipitate forms and incubate for two minutes. Centrifuge the solution for five minutes and transfer the supernatant to a spin column. Centrifuge the spin column for one minute and discard the flow-through. Add 200 µl of plasmid wash buffer 1 to the spin column and centrifuge for one minute. Then add 400 µl of plasmid wash buffer 2 and centrifuge for one minute. Transfer the column to a new tub and add 30 µl of elution buffer, let the elution buffer saturate the filter for one minute then centrifuge for one minute.

In order to isolate the DsRed fluorescence gene, the ampicillin resistance gene, and the origin of repliation pieces from the pHd-DsRed plasmid a double restriction enzyme digest was used. A double digest was conducted using 2 mg of plasmid DNA, 1 µl of NCOI, 1 µl of BamHI,

2 µl 10x tango buffer, and then water is added to reach a total volume of 20 µl. Incubate the solution at 37℃ for one hour and heat kill the enzymes at 70℃ for 20 minutes. The next step is to purify using agarose gel electrophoresis. The agarose gel should be a 0.8% gel with 60 ml of TBE that runs for an hour and 45 minutes. The final step to obtain the cut pHd-DsRed plasmid is to extract the isolated piece of the plasmid by cutting the band out of the gel and extracting the DNA using the QIAquick Gel Extraction Kit. The first step when using the QIAquick Gel Extraction Kit is to weigh the gel slice and add three volumes of that amount in QG buffer. Once the QG buffer is added incubate the solution at 50℃ for 10 minutes, shaking occasionally. After the gel slice is melted add the weight of the gel slice in isopropanol to the solution and mix. Add the entire solution into the spin column and centrifuge – all centrifuge steps are one minute, then add 500 µl of QG buffer to the spin column and centrifuge, then add 750 µl of PE buffer to the spin column and incubate at room temperature for four minutes then centrifuge. Spin briefly in a tabletop centrifuge before transferring the spin column to a new tube and adding 30 µl of buffer EB. Once buffer EB is added let it incubate at room temperature for one minute then centrifuge.

To obtain the promoter from the ACT5C plasmid two PCR amplification reactions were used. The promoter was too large for one PCR amplification reaction to work successfully. Multiple smaller amplifications with overlapping primers allowed for successful amplification. The first reaction has 2 µl of template DNA in it with a concentration of 87.3 ng/ µl, 5 µl of 5x Green GoTaq Flexi buffer, 2 µl of dNTPs, 1.5 µl of $MgCl_2$, 0.5 µl of the AC5_DsRed_1F primer, 0.5 µl of the AC5_DsRed_1R primer, 0.5 µl of Taq, and enough deionized distilled water to reach a total of 25 µl in the reaction. The second reaction has 2 µl of template DNA in it with a concentration of 87.3 ng/ µl, 5 µl of 5x Green GoTaq Flexi buffer, 2 µl of dNTPs, 1.5 µl of $MgCl_2$, 0.5 µl of the AC5_DsRed_2F primer, 0.5 µl of the AC5_DsRed_2R primer, 0.5 µl of Taq, and enough deionized distilled water to reach a total of 25 µl in the reaction. Run the reactions through a PCR program with the following parameters: initial denaturation at 95℃ for one minute; for 35 cycles have denaturation at 95℃ for 30 seconds, annealing at 47℃ for 30 seconds, and extension at 72℃ for one minute and 30 seconds; final extension at 72℃ for 10 minutes; and to finish out the program set it to 4℃ for infinity. After the PCR program finishes, run the reaction through agarose gel electrophoresis to check for the proper size of the promoter and to purify the DNA from the gel using the QIAquick Gel Extraction Kit.

The next step is ligating the pieces from each plasmid, the pHd-DsRed and ACT5C, together to build the final plasmid, the pAc5 plasmid. In order to ligate the pieces together the NEBuilder HiFi DNA Assembly Kit is used. To reach a total of 0.06 pmols of DNA when using 2 fragments, 0.02 pmols of the vector, the pHd-DsRed plasmid, and 0.04 pmols of the insert, the ACT5C plasmid, according to the ratio and recommended pmols of DNA fragments as described in the reaction protocol. The vector needs to have a concentration of 60 ng added to the tube. The insert needs to have a concentration of 75 ng added to the tube. The amount of µl needed for the vector and insert is determined on an individual basis by the concentration of the DNA sample you have at the time. Add 10 µl of Assembly Master Mix to the tube and add the amount of

water required to make the total volume reach 20 µl. Incubate the sample(s) in a thermocycler at 50℃ for 60 minutes then store on ice.

The next step is continuing to use the NEBuilder HiFi DNA Assembly Kit to transform the cells. First, thaw the competent cell tube from New England Biolabs on ice and add 2 µl of the assembly mix to the cells. Pipet gently to mix and place the mixture on ice for 30 minutes, then heat shock at 42℃ for 30 seconds. After heat shock, transfer the tubes to ice for 2 minutes and add 950 µl of room temperature SOC medium to the tube. Incubate the tube at 37℃ for 60 minutes while shaking at 250 rpm. While the tube is incubating, warm the selection plates to 37℃ and add 25 µl of ampicillin to the plates. After the selection plates are warmed, light a Bunsen burner to sterilize the air and the spread bar then spread 100 µl of the cells onto the selection plates and incubate overnight at 37℃. The next day, warm the selection plates to 37℃ and add 25 µl of ampicillin to the plates. Once the plates are warmed draw 3 lines horizontally and 3 lines vertically on the bottom of the plate to make a 12-space grid. Label the spaces and label 12 PCR tubes 1-12. Remove the spread plates from the incubator from the day before and select 12 random colonies. Use an autoclaved toothpick to pick up one of the selected colonies and swap the PCR tube with the toothpick. Next, stab the toothpick into the agar plate in the coordinating space and repeat this process for the other 11 colonies with different toothpicks. Once all 12 colonies have been selected and stab cultured, incubate the plate overnight at 37℃. The next day, remove the plate from the incubator and wrap the plate in parafilm to store at 4℃.

To check the proper assembly and ligation of the plasmid pieces we run a specific PCR program to check the binding sites. Using the PCR tubes that have the stab culture samples in them add a master mix containing 5 µl of 5x Green GoTaq Flexi buffer, 2 µl of dNTPs, 1.5 µl of $MgCl_2$, 0.5 µl of the red_ac5_junction_ F primer, 0.5 µl of the red_ac5_junction_R primer, 0.5 µl of Taq, and 15 µl deionized distilled water for each of the samples you are running, in this case, 12. Place the 12 samples in the thermocycler and start the program. The program consists of three stages. The first stage, initial denaturation, runs at 95℃ for two minutes. The second stage, consists of denaturation which runs at 95℃ for 30 seconds, annealing which runs at 50℃ for 30 seconds, and extension which runs at 72℃ for 30 seconds. The third stage, final extension, runs at 72℃ for five minutes and then drops to 4℃ and lasts forever. After the PCR program finishes gel electrophoresis needs to be set up and run to check for the proper sizing, 218 bps, and that the pieces are properly assembled in the pAc5 plasmid.

3. Results

Successful restriction enzyme digestion was conducted on the pHd-DsRed. The NCOI enzyme cut at 1552 bp mark. The BamHI enzyme cut at the 1376 mark. When run on a gel the expected band size was seen (Figure 2) and cut from the band for purification using the QIAquick Gel Extraction Kit.

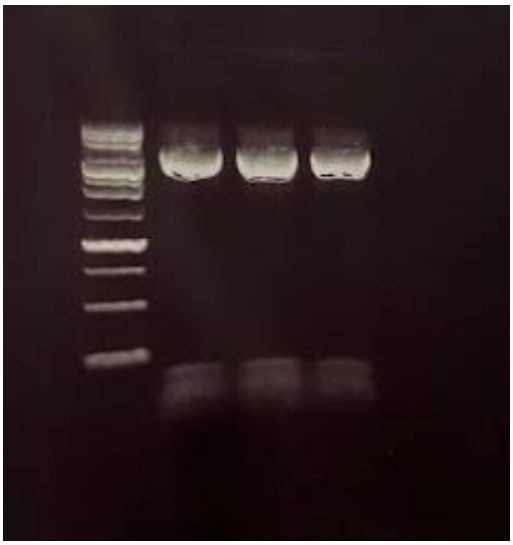

Figure 2: Restriction Enzyme Digest Results from NCOI and BamHI double digest.

Successful amplification of the promoter from ACT5C was conducted. These are gel images from the AC5 promoter amplification. From figure 3 you can see that there was amplification and that it was the size we expected to see. The reaction run with the 1f and 1R primers is on the right of the gel image. The reaction run with the 2F and 2R primers is on the left of the gel image. The primers amplified the correct sequence and expected sizes were seen when run on a gel. The correctly sized bands were cut from the gel and DNA was extracted using the QIAquick Gel Extraction Kit.

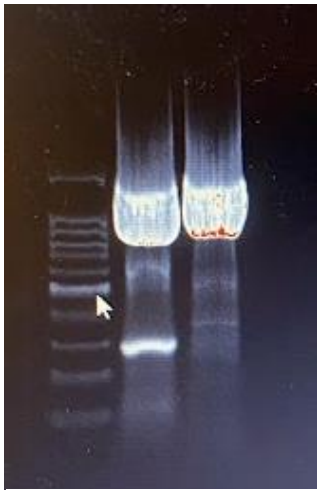

Figure 3: AC5 Promoter Amplification Result

These gel images (Figure 4 and Figure 5) depict the successful ligation and transformation of the pAc5 plasmid. We know the ligation was successful because the primers used in this PCR reaction straddle one of the spots where the plasmid pieces connected, and the product is the expected 218 bps in length. We know the transformation was successful because

the PCR was run using the cells we transfected with the ligated plasmid. These images prove the pAc5 plasmid was built correctly and is performing its intended role thus far.

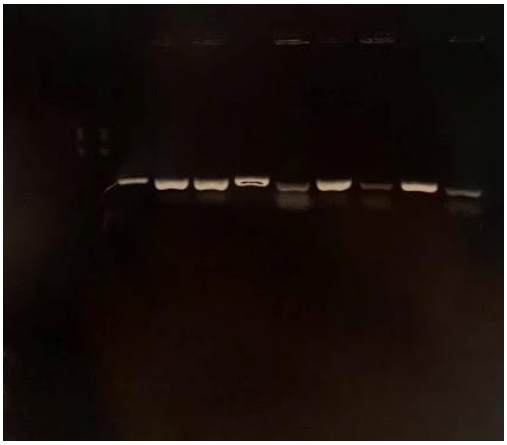

Figure 4: Ligation and Transformation Results Samples 1-9

Figure 5: Ligation and Transformation Results Samples 10-12

4. Discussion and Conclusions

The pAc5 plasmid was successfully assembled to contain the constituently active promoter, AC5, and the fluorescence gene and the ampicillin resistance gene, pHd-DsRed. This plasmid can now be used in the next phase of the *Drosophila* project, which includes adding 3' UTR target sponges to the plasmid and transforming the plasmids into infected and uninfected *Drosophila* cells. The building of the pAc5 plasmid had several complications. For example, the PCR product to check for the proper assembly was never amplified in gels, with many changes. (insert gel images) It was assumed that the assembly was not happening properly because the pHd-DsRed plasmid was not digested well. This was assumed because cells were growing on ampicillin-coated plates and the ampicillin resistance gene came from the pHd-DsRed plasmid. However, it was not realized until later in the project that both the plasmids used to construct the pAc5 contained ampicillin resistance genes. This was discovered when DNA from cells that were run through PCR to check for the assembly was sequenced. It became very apparent that the pHd-DsRed plasmid was nowhere in the DNA sequence of those cells. It was quickly realized the primers used to amplify the promoter from the ACT5C plasmid were amplifying much more than the promoter alone. New primers were designed to target the promoter more specifically through the use of the DNA sequences we obtained.

This project will lead to a better understanding of the relationship between *Wolbachia* bacteria and their *Drosophila* hosts. The results of this project will help determine whether the bacteria in our bodies, either helpful or harmful, is affecting the expression of our genes. This will lead to a greater understanding of how antibiotics and bacteria affect us at the genetic level. Antibiotics currently kill all bacteria in an area, not just the good or the bad. Therefore, my project could bring that characteristic into question and allow us to consider ways we can limit

the bacteria affected by antibiotics, specifically to infectious or harmful bacteria. It also can potentially limit the expression of genetic cancers or stop them altogether. Cancer researchers and antibiotic developers will have new research questions, showing other scientists valuable information about how outside forces can affect the genome.

5. Summary

In conclusion, gene expression can be affected by various factors, possibly including bacterial small non-coding RNAs. *Drosophila* cell culture and *Wolbachia* bacteria were used to investigate whether small RNAs from bacteria affect gene expression in the host. MiRNAs are involved in almost every cellular process, and the bacterial small non-coding RNA mechanism is believed to be similar to the miRNA mechanism. The project used two existing types of plasmids to build a new plasmid that will be transformed into *Drosophila* calls after the addition of a target sequence. There is a need for further research on small RNAs and the potential impact on antibiotics if bacteria naturally affect gene expression in the body.

6. Appendices

6a. Papers Published

6b. Acknowledgments

I would like to acknowledge and thank Dr. and Mrs. Niblack for their generous contribution. I would also like to thank Dr. Darren Hagen for his support and assistance, in and out of the laboratory over the past two years. I would also like to thank Anna Goldkamp for her help, enthusiasm, and support over the last two years.

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