The Over expression of the RNA "Goldilocks" and its effects on the bacteria, Escherichia Coli

Laura Taylor

Abstract:

Small non-coding RNAs (sRNAs) are important molecules for translation and gene regulation in bacteria which make them key players in understanding their life cycles and pathogenesis. Furthermore they may lead to potential drug targets and therapeutics in the future. The sRNA Goldilocks, which is produced in Escherichia Coli (*E.coli*) during periods of cell stress and starvation, is one such target. It is hypothesized that Goldilocks interacts with the protein Hu and is involved in the repression of the gal operon. To test this, Goldilocks will be cloned into *E.coli*, so that it is over expressed during normal conditions. A northern blot will be performed to test whether Goldilocks truly is produced. Experiments testing the robustness of the northern blot produced a weak signal. Once the sRNA Goldilocks is properly expressed in *E.Coli* a screen for various phenotypes will be performed. We are particularly interested in the effects that over expressing Goldilocks has on galactose metabolism.

Introduction:

In a world of constant evolution, human beings are fighting a continuous arms race against pathogens. Therefore, it is important to be equipped with as many tools and therapeutics as possible to ensure the ability in the future to ward off these diseases. As is such, in order to develop effective weapons against microorganisms which cause pathogenesis, it is essential to understand them as much as possible. Bacteria in particular, are a prominent vector for diseases, with a countless number of species identified and very few being studied. By piecing together the intricate life cycles of certain pathogenic bacterial strains, it may become possible to develop

unique antibiotics and therapeutics for their relative diseases. The bacteria, Escherichia Coli (*E.Coli*) are widely studied bacteria that can cause severe food borne illness (Mayo Clinic Staff, 2011). Furthermore *E.Coli* makes a great model organism for the study of other related pathogenic bacteria such as Salmonella enterica.

In recent years, the study of small non-coding ribose nucleic acids (sRNA) has increased. Non-coding RNAs can be defined as functional genetic material that does not result in a translated protein. An example of this would be the ribosome, an organelle within the cell that facilitates translation. SRNAs are specific to small non-coding RNAs in bacteria. They play essential roles in the life cycle and replication of bacterial DNA by promoting mechanisms which regulate gene expression (Phillips, 2008). SRNAs are heavily involved in RNA interference which controls gene activity concerning cell defense, differentiation, and protein expression (Phillips, 2008). Currently, scientists are exploring the possibility of using sRNAs as potential drug targets and therapeutics for diseases. Therefore, the more that is understood about their specific role in the cell, the more it will be possible to manipulate them.

In a screen done by B.Tjaden and colleagues (2002) for RNAs in *E.Coli* that are produced in a variety of chemical and environmental conditions, the sRNA C.0293 was identified and seen to only be expressed during periods of cell stress and starvation. Unpublished research done by J.Pellino in recent years has suggested that this RNA, which has been nicknamed Goldilocks due to its ideal size for in vitro RNA studies, interacts with the protein Hu.

Hu is formally known as heat-unstable nucleotide protein and is involved in repressing transcription at the gal operon in *E.coli* (Giuseppe Lia, 2003). The gal operon facilitates the production of the enzyme galactosidase, which is involved in the breakdown of the sugar

galactose. When repressed, the gal gene does not get translated and galactosidase is no longer produced. The repression occurs as a result of the formation of a DNA loop which encompasses the promoter regions of the operon, thereby inactivating it (Giuseppe Lia, 2003). Hu propagates this loop formation (Giuseppe Lia, 2003).

We hypothesize that Goldilocks binds to the protein Hu hereby triggering it to repress the gal operon during periods of cell starvation so that energy is not wasted in the production of galacatosidase in the absence of galactose. To test this, we intend to over express Goldilocks in *E.coli* during normal cell conditions via sub cloning. Through this we hope to identify the effect that it has on galactose metabolism. If our hypothesis is correct, we should see a reduction in the amount of galactose substrate when Goldilocks is produced.

Materials and Methods:

Strain, Plasmid and Primers:

The plasmid, PUC 18, was purified from the E.coli strain K12 using the Promega® Pureyield ™ Plasmid Midiprep System. The oligos for the C.0293over sense and antisense strands were ordered through Integrated DNA Technologies. To create the plasmid insert, the sense and antisense strands were annealed together by combining them in a 1.5mL epindorf tube and heating to 95C, then slow cooling to 40C.

Restriction Digest:

The plasmid was digested using the restriction enzymes, Hind III and Bam HI and buffer E, purchased from Promega. The plasmid was digested for the duration of four hours. It was then treated with phosphotase (CIP) and allowed to incubate at 37C for one hour. The digest was run

through a 1% agarose gel run in 1XTAE at 100mA and stained with Sybr Gold nucleic acid stain for one hour and visualized with UV light. The digested plasmid was cut out and purified using the Promega ®Wizard SV Gel Clean-Up System and resuspended in 50ul of deionized nuclease-free water.

Gene cloning:

The primers were first treated separately with kinase (PNK) and allowed to incubate for an hour at 37C. The sense and antisense strands then were mixed and heated to 95C for 3 minutes and slow cooled down to 40C. A ligation reaction was set up with ratios of insert to plasmids ranging from 2:1 to 4:1 and left at room temperature over night. The ligations were then transformed into competent DHαT5 cells, purchased from Invitrogen, using the heat-shock method of transformation and plated onto LB/AMP agar plates.

In Vitro RNA Transcription:

A transcription reaction was set up for both the sense and antisense strands of C.0293 separately using template DNA purchased through IDT. The transcription was set up using the T7 Mega Short Script kit from Invitrogen. The antisense strand was biotinylated by adding 1ul of Biotin-UTP to the mix. Both strands were incubated at 37C over night. The RNA was purified on a 6% denaturing polyacrylamide gel in 1XTBE at 200V. The RNA was visualized via UV shadowing and eluted into KB buffer. The RNA was ethanol precipitated and resuspended in 80ul of nuclease-free diH_2O.

Northern Blot:

A 6% denaturing polyacrylamide gel with 3ul of sense RNA was run in 1XTBE at 200V. An electroblot was set up for the transfer of RNA to a charged nylon membrane, at 200mA in 0.5XTBE for 30minutes. After the blot, the membrane was cross-linked in a UV Stratalinker® at 1.2mjoules for 1.2minutes while damp. The membrane was then wet in 6XSSC, while a prehybridization solution containing 5mL 6XSSC, 1%PEG,1%BSA, 0.5mL 10%SDS, 0.1mL ssDNA, and 5mL formamide was made. The wet membrane was then incubated with the prehybridization solution in a Hyb oven, rotating overnight, at 42C. The following day, the membrane was washed using the Pierce procedure for the detection of immobilized nucleic acids wash protocol, eliminating the use of the membrane equilibrium buffer, and blocking buffer. Streptavidin-HRP was added to 20mL of 6XSSC and incubated for 30minutes at room temperature. Tetramethylbenzinide (TMB) was used for visualization.

Results and Discussion:

Cloning:

Cloning of the C0293interseted Puc18 plasmid into *E.Coli* was not successful. Ratios of insert to plasmid were tested for the ligations ranging from 1:1 to 1:20. Yet, cell growth was only seen on one of the 1:2 and one of the 1:3 transformation plates. A plasmid miniprep was performed on both colonies to assess whether the plasmid really was incorporated into the cells. For the 2:1 ligation transformation, the miniprep gel yielded one band equivalent to that of the undigested plasmid, thereby suggesting that the digested plasmid was able to close up on itself before the insert could be successfully incorporated. Upon performing the miniprep for the 3:1

colony, no band was present where it was expected to see a band representing a plasmid as can be seen in the image below.

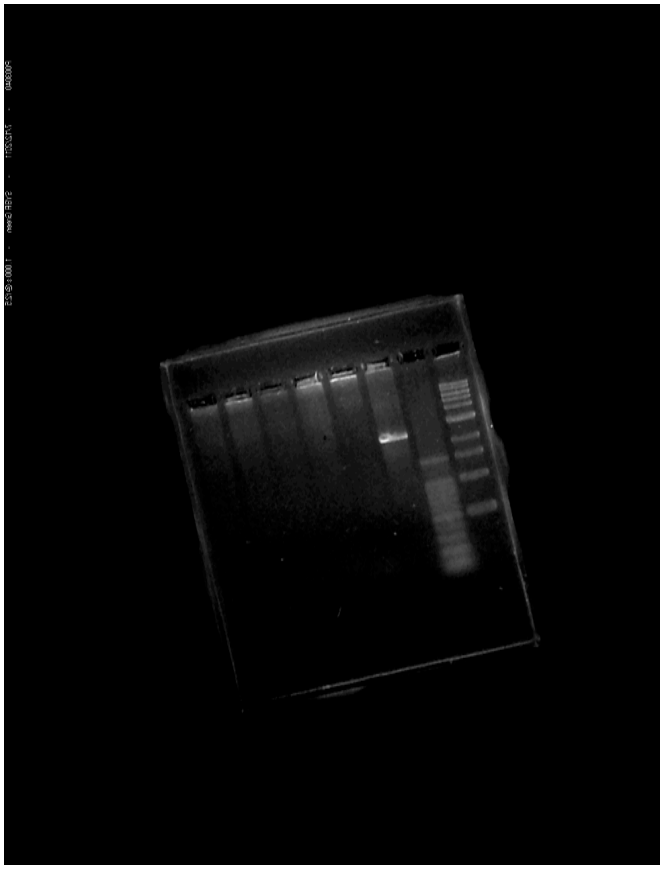

Figure 2: The 3:1 Plasmid Miniprep Gel Results

Lanes 1-5 contained the plasmid minipreps while lane 6 contains the uncut pure Puc18 plasmid. These results suggest that the plates were made improperly and therefore the ampicillin antibiotic used was not present to kill off any bacteria that did not contain the ampicillin-resistant plasmid.

To troubleshoot the cloning process, the insert was re-kinased and annealed and the plasmid was re-digested for a shorter period of time, to prevent enzyme star cutting activity, and spiked with phosphotase again. The ligations were performed using the 2:1 and 3:1 ratios and a

transformation was done, but colonies did not grow. To test the accuracy of the enzymes, the plasmid was single digested by both restriction enzymes separately and run on a gel next to an uncut plasmid control as can be viewed in the image below.

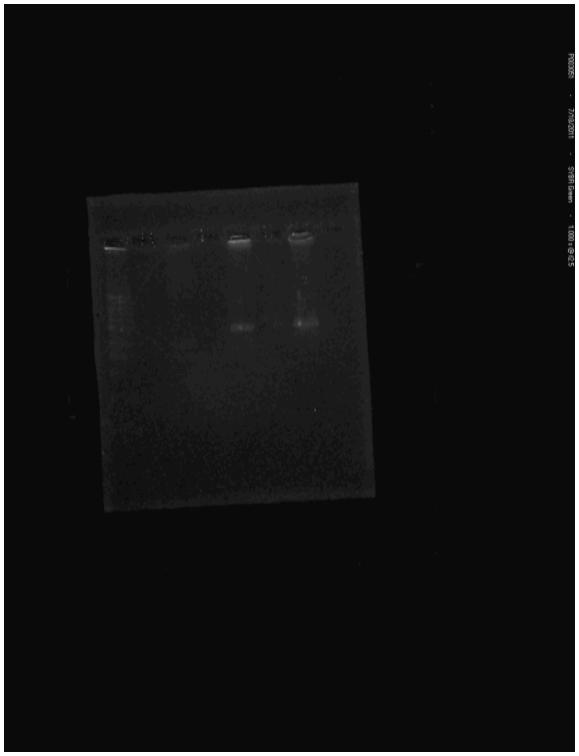

Figure 1: Individual Enzyme Check Gel

In the gel above, lane 2 contains the uncut Puc18 plasmid, lane 4 contains Puc18 digested by HindIII, and lane 6 contains Puc18 digested by Bam HI. It is clear that the bands for both of the enzyme digests are larger than the control plasmid, based on positioning on the gel. Both enzymes appeared to cut efficiently, therefore it was concluded that the enzymes were functional and the plasmid was getting cut only once at the correct site. For the future, a wider scale of ratios will be tried and growing conditions may be altered so that plates may incubate longer or a larger number of cells may be aliquoted per plate.

IN Vitro RNA Transcription:

The first RNA transcription reactions that were performed were successful; however the 2 subsequent reactions produced degraded RNA which could be seen in a long streak on the acrylamide gel rather than a tight band that was produced by the control RNA as seen below.

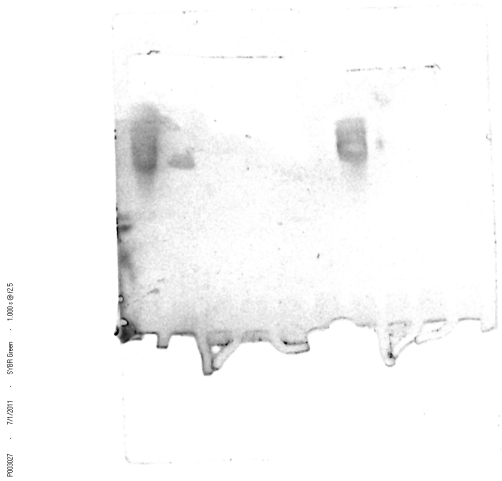

Figure 3: Polyacrylamide RNA#2 Gel

Lanes 1 and 6 contain the transcribed RNA, while lane 2 is a control. It is clear in the image that the transcribed RNA was degraded. The RNA degradation was most likely caused by RNAse contamination.

Northern Blot:

The northern blot was partially successful. At best a slight signal was produced only when just the biotinylated antisense control strand was blotted. The first attempt at implementing a northern blot was done using a formaldehyde-agarose denaturing gel. The RNA was added to

15ml of denaturation mix containing formamide, formaldehyde, and buffer, then heated and dyed before being run at 100V in 1X Buffer E. After staining the gel in sybergold for 30 minutes, there was no evidence of RNA on the gel. It is possible that the RNA had been degraded or the denaturing ingredients may have caused the stain to be less effective.

It decided to switch to using an acrylamide gel, so that the RNA could be visualized after the gel had been run to ensure that no degradation occurred. For the subsequent blot, the sense RNA was run in a 6% acrylamide gel along with the biotinylated antisense strand, which acted as a control. After staining in sybergold, the RNA did not appear degraded so an overnight blot was set up as follows (bottom to top): a nuclease-free container filled with 6XSSC, one whatmann paper, the gel, a nitrocellulose membrane, 2 whatmann papers, and a stack of books for weight. The following day, the membrane was crosslinked in a UV Stratalinker at 1.2mjoules for one minute. Overnight it was soaked in the prehybridization solution at 42C and the following day it was washed using the exact Pierce procedure for detection of immobilized nucleic acids kit. The blot was again unsuccessful.

To troubleshoot, the antisense alone was run to eliminate the possibility of the antisense strand not binding to the sense strand. A range of crosslinking times were also tried and it was decided to try doing an electric transfer instead of a gravity blot. To check whether the electric transfer would be successful, the electroblotted gel was stained for any leftover RNA. No RNA remained on the gel, suggesting either the transfer was indeed successful or the RNA had degraded. However, since the RNA had clearly shown up on the first acrylamide gel run, the second possibility was disregarded as unlikely.

After a slight signal seen on the first electroblotted gel, ways to maximize the signal became the objective. It was concluded that the transfer of the RNA and the crosslinking must have been successful, since the RNA did show a signal; thereby demonstrating that is was effectively blotted and linked onto the membrane. Therefore, the troubleshooting efforts focused on the prehybridization and wash protocol.

A gel was run and washed without the prehybridizing step alongside a gel which had been prehybridized. The prehybridized gel gave off a signal while the other did not. From this it was concluded that the diminished signal was a result of the wash protocol. First, the efficiency of the enzyme, Streptavidin-HRP, was checked by adding 1ul of enzyme to 1ml of TMB. A bright blue change of color was observed within the first 30 seconds, indicating that the enzyme was effective. To check the efficacy of each wash solution used in the Pierce detection kit, the nitrocellulose gel was cut into small squares and a dot of Streptavidin-HRP was placed in the center of each square. Each square was covered in either 1Xwash buffer, substrate equilibration buffer, blocking buffer, or 6XSSC, and then set shaking. Each solution was discarded after 5 minutes of shaking and the membrane squares were covered in TMB. Both the wash buffer and 6XSSC squares showed no depletion in signal, and a dark blue spot was produced. Both the substrate equilibration buffer and blocking buffer showed significant signal degradation and only produced a faint light blue spot. From this test, it was concluded that both the blocking and the substrate equilibration buffers stifled the efficiency Streptavidin-HRP in binding to TMB.

For the future, to try to further maximize the signal, varied lengths of time that the membrane is exposed to the enzyme will be tested. Eventually once the signal strength is more agreeable, the sense strand will be run alongside the antisense strand to detect whether binding of the two strands proves problematic.

To conclude, the insert was not successfully cloned and therefore no over expression of goldilocks occurred. Future directions include continuing to look for an effective method to clone *E.coli* to produce the goldilocks RNA during normal conditions, as well as continued work on optimizing the signal produced by the northern blot, so that when goldilocks is produced in the cell, it can be easily visualized. Once it has been confirmed that Goldilocks is being over expressed, a screen will be performed to assess the difference it makes on the cell with regard to galactose metabolism in comparison to wild-type *E.Coli.*

References:

Giuseppe Lia, D. B.-F. (2003). Supercoiling and denaturation in Gal repressor/heat unstable nucleoid protein (HU)-mediated DNA looping. *PNAS* .

Mayo Clinic Staff. (2011, July 28). *E.Coli*. Retrieved August 6, 2011, from Mayo Clinic: http://www.mayoclinic.com/health/e-coli/DS01007

Phillips, T. (2008) Small non-coding RNA and gene expression. Nature Education 1(1)

Tjaden, B., Saxena, R.M., Stolyar, S., Haynor, D.R., Kolker, E., and Rosenow, C. (2002) Transcriptome analysis of Escherichia coli using high-density oligo nucleotide probe arrays. Nucleic Acids Res, 30, 3732-3738.