Evaluating the Potential Use of a Cellulose Binding Domain to Protect Oysters from *Perkinsus marinus* in the Chesapeake Bay

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The Eastern Oyster (*Crassostrea virginica*) population of the Chesapeake Bay has been declining steadily over the past century due to a disease called Dermo. Dermo is a disease caused by the *Perkinsus marinus* parasite, which attaches to the galectin receptors on the outer membrane of oyster cells. The cells of the oyster are destroyed by the replication of this protist within the host's cells. When the host dies, these new spores are released into the water and infect other oysters. Since the Bay is close to us, we understand the significant contribution oysters have to Maryland's economy. The purpose of this project is to create a synthetic seaweed that will capture the protist, *P. marinus*. We have cultivated the cellulose through *Gluconacetobacter xylinus*, a bacteria, in a specific liquid media. In order to confirm we can attach a protein to the cellulose, we have constructed a DNA sequence containing the genes for a Cellulose Binding Domain (CBD) and a Blue Fluorescent Protein, replicated it via Pollymerase Chain Reaction, and plan to transform it into *Escherichia coli*. This protein will be extracted from the *E. Coli*, spread over the cellulose, and observed under ultraviolet light to see if the cellulose glows blue. If so, then the protein will have successfully adhered to the cellulose and will provide the foundation for splicing the galectin receptor to the CBD. The galectin receptor binds as a tetramer and is located on the outer membrane of the oyster cells. This is how the *P. marinus* enters and infects the oysters.

Keywords: Oysters, Chesapeake Bay, Cellulose, *Perkinsus marinus*, Galectin

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Watch a video introduction by the authors at
https://youtu.be/2PqSPFt27AQ

The issue we would like to address is the infection of the Eastern Oyster (*Crassostrea virginica*) that results in the disease Dermo. The group collectively decided to attempt to fix this issue since it is very close to home. To begin, the group did extensive research on the oysters in the bay and the conditions that were causing their population to decrease. In regards to the conditions that were harming the oysters, we came to the conclusion that parasites were the threat that we would like to confront first.

The parasite, *Perkinsus marinus*, infects its host by entering its bloodstream, facilitated by galectin receptors on the oyster cells (Bower 2013). Galectin is a type of protein located on the outside of oyster cells. Some of its functions include mediation of cell-to-cell interactions, cell-to-matrix adhesion, and transmembrane signalling. The parasite manages to replicate within the body of the host, namely within the host's cells, causing the oyster's health to deteriorate as the parasite replicates. The oyster's death shortly follows, releasing once again another set of spores that will continue to infect more oysters.

We plan to create cellulose sheets that the protist will bind to so we can create a parasite-specific "flypaper." We plan on conducting a series of experiments to show that only the *P. marinus* protist will be targeted, leaving other microbes that are beneficial to the Chesapeake unharmed. The first steps toward being able to launch our project were accomplished with the help of Imperial College. They sent us a sample of the cellulose-forming bacteria, *G. xylinus* iGEM, which the Imperial College team discovered. The team also supplied us with the BioBrick parts they created in order to bind green fluorescent protein and other proteins to the bacterial cellulose. We conclude that the cellulose that we would be creating would be environmentally safe, as no harsh chemicals are being added in producing this cellulose, and the bacteria would not be introduced to the Chesapeake Bay.

We have also reached out to the 2014 University of Maryland iGEM team, who introduced us to the concept of using galectin to trap *P. marinus*. We will be building on their research when we combine galectin with the Cellulose Binding Domain (CBD) to make a cellulose-anchored protist trap.

Materials and Methods

General Lab Safety. During our lab procedures, some general lab safety guidelines must be in place. First, due to the many different kinds of bacteria present on our hands and possibly cross contamination, gloves must always be worn throughout the experiment. Second, safety goggles must be worn as a precautionary step, just in case media or bacteria gets into the eyes. Regular school protocol is assumed in case of a fire, and a chemical shower is in place in case of a chemical spill. Hazardous waste is placed in a safe, well-labeled biohazard container until it is picked up by a waste management company for proper disposal. Apparel for students is casual with no open toed shoes and no long sleeves when dealing with fire. Lab safety also includes sterilizing the inoculating loop whenever transferring bacteria from one test tube into different test tubes. Lastly, at the end of every experiment, materials should be thoroughly washed and dried. Likewise, hands should also be thoroughly washed with warm water and soap. Throughout the year, we have carefully labeled and inventoried our cultures and reagents so that everyone remains aware of what we are working with and storing in the lab.

Culturing *Gluconacetobacter xylinus*. The culturing of the *G. xylinus* bacteria began with the receipt of the *G. xylinus* from the Imperial College of London. This bacteria has the ability to produce a natural cellulose that would be ideal for the housing our synthetic galectin. After receiving the bacteria, we attempted to find the most efficient method for culturing the bacteria and producing the cellulose. In order to do this, we used conical tubes and liquid medium. We made a large amount of liquid media at the start of last year and have been using it to culture the bacteria ever since. To make the media, we first combined 250 mL of distilled water to 10 g of dextrose in one bottle. In a second bottle, we combined 210 mL of distilled water with the following reagents: 40 mL 0.1 M citric acid, 2.5 g yeast extract (0.5% w/v), 2.5 g trypticase peptone (0.5% w/v), 1.35 g Na_2HPO_4 (0.27% w/v), and 7.5 g agar if making Hestrin Schramm (HS) agar plates. HS agar is a media that allows the bacteria to produce the cellulose. We then autoclaved both bottles and combined them. After we had the liquid media, we began to subculture the bacteria and grow the cellulose in conical tubes. To prevent contamination, we began by heating up metal forceps using a bunsen burner flame. To rapidly cool down the forceps, we then immersed them in sterile media. Using these sterilized forceps, we removed a cellulose sheet from the conical tube and ripped off a piece of the sheet. This piece of cellulose was then placed in a new conical tube containing 5 mL of HS media. These new conical tubes were then incubated at room temperature for two weeks. This results in a successful subculture of bacteria and a newly formed cellulose sheet floating at the top of the conical tube. Reference our methods paper (Amin et al. 2016) to see detailed steps for how to make the media and subculture the bacteria.

Transforming *Escherichia coli*. After several attempts to transform the *E. coli*, we finally came across an efficient and effective procedure. Our original plan for transformation began by adding 5 mL of nutrient broth to a 50 mL conical tube, inoculating the media with *E. coli*, and incubating the culture overnight at 37°C. The following day, we actually began the transformation procedures using the Mix & Go E. coli Transformation Kit (Zymo Research Corp, Irvine CA). In the morning, we added 6 mL of ZymoBroth to a 50 mL conical tube and inoculated the media with 120 µL of the overnight *E. coli* culture. The subcultured bacteria had to incubate for approximately 5-6 h at room temperature. Towards the end of the incubation period, we combined 210 µL 2X Wash Buffer and 210 µL of Dilution Buffer in a microcentrifuge tube. We also combined 55 µL 2X Competent Buffer and 55 µL of Dilution Buffer in another microcentrifuge tube. These were both stored on ice. We added an aliquot 1.4 mL of the 5-6 h *E. coli* culture samples into four microcentrifuge tubes. After being placed on ice for 10 min., we pelleted the cells by centrifuging the tubes at 8,000 RPM for 10 min. We then removed the supernatant from each of the tubes and re-suspended the pelleted cells in 100 µL 1X Wash Buffer by gently pipetting. We combined the four 100 µL cell suspensions into a single microcentrifuge tube and then re-pelleted the cells by centrifuging the tube again at 8,000 RPM for 10 min. We completely removed the supernatant from the tube by pipetting, being careful to not disturb the cell pellet. We then re-suspended the cells in 100 µL 1X Competent Buffer and placed them on ice. We added 1-5 µL of plasmid DNA to the re-suspended cells and mixed by gently vortexing. After allowing the cells to sit on ice for 2 min., we added 900 µL of super optimal broth with catabolite repression (SOC) medium

to the cell suspension and incubated at 37°C for 1 h. Simultaneously, we prepared an antibiotic-treated agar plate and stored the plate at 37°C until needed. This was made by adding the following to form a single drop in the center of the plate: 100 µL 100 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), 100 µL 10% (w/v) Arabinose, 20 µL 20 mg/mL X-Gal, 20 µL 100 mg/mL ampicillin, 20 µL 50 mg/mL Kanamycin, 20 µL 50 mg/mL Tetracycline, and 20 µL 50 mg/mL chloramphenicol. We then used a disposable spreader to spread the added reagents evenly over the entire plate. After the 1 h incubation period, we spread 200 µL of the SOC cell culture on the pre-warmed antibiotic-treated agar plate. We then incubated the plate at 37°C overnight and looked for the presence of antibiotic-resistant transformants. After an unsuccessful transformation, we made some adaptations to these procedures in order to increase efficiency. We inserted a step in which we incubated the cells for 1 h in SOC prior to plating on chloramphenicol-treated plates. We also subcultured the *E. coli* at room temperature. These two changes helped increase the effectiveness of the transformations.

Performing PCR Analysis. In order to test for the presence of the Cellular Binding Domain-Green Fluorescent Protein (CBD-GFP) plasmid, we utilized Middletown High School team's Polymerase Chain Reaction (PCR) machine. We gave them four different subcultures, each taken from a separate colony and each hopefully containing the BioBrick BBa_K1321356. They combined 12.5 µL Q5® High-Fidelity 2X Master Mix (New England Biolabs, Ipswich MA), 1.25 µL forward primer, 1.25 µL of reverse primer, 1.0 µL of genomic DNA, and 9 µL of nuclease free water in a PCR tube. They then thermal cycled using the following program: 30 s at 98°C, 10 s at 98°C, 20 s at a temperature determined by the online NEB T_m Calculator, 20 s at 72°C, repeat 35 times from step 2, 120 s at 72°C, indefinitely 10°C, and then store the completed reaction at -20°C until needed. In order to test for the plasmid, they added a 1 µL aliquot of each culture to a PCR instead of the template, essentially as described in the procedure above. However, this time the initial PCR phase was held at 95°C for 10 min. to disrupt the cells and release the DNA. They used the universal BioBrick primers VF2 and VR with an annealing temperature of 66°C and viewed the PCR products on a FlashGel (Lonza Group, Basel, Switzerland) per the manufacturer's protocol. The BBa_K1321356 insert is 1252 bp and the positioning of the primers around the insert add another 333 bp. Therefore, we expected to get a PCR product of around 1,585 bp.

Testing Cellulose Binding. Unfortunately, we once again failed to produce transformants using the Zymo Research kit. As we continued to trouble shoot the assay, we inserted a step in which we incubate the cells for 1 h in SOC prior to plating on chloramphenicol-treated plates. We also subcultured the *E. coli* at room temperature, a technique that is known to increase transformation efficiencies. We only transformed the *E. coli* with two separate plasmids in this experiment to ensure the assay works before wasting further samples. Specifically, we inoculated one chloramphenicol-treated plate with *E. coli* that had been transformed with 2 µL BBa_K1321356 CBD-GFP plasmid. Similarly, a chloramphenicol-treated plate was transformed with 2 uL BBa_K1321357. Finally, a third untreated plate was spread with *E. coli* that had been transformed without a plasmid. We also transferred 1 µL of the Imperial College-supplied CBD and BBa_K1321359 into fresh microcentrifuge tubes and passed the samples to the Middletown High School Bioengineering team so that they can test for the presence of the CBD-GFP insert using PCR. This experiment was a partial success with colonies appearing on the first of the chloramphenicol-treated plates. We confirmed that the bacteria on this plate actually contains GFP-encoding genes by subculturing them onto a plate treated with chloramphenicol and IPTG.We accomplished this by treating a nutrient broth agar plate with chloramphenicol and IPTG. We then used inoculating loops to transfer each of the colonies from plated BBa_K1321356 transformants onto the subcultured BBa_K1321356 CBD-GFP transformants.The plates will be incubated overnight at 37°C and examined for fluorescence under a ultraviolet (UV) light.

Evaluating IPTG Effects on Protein Expression. The first step in evaluating the effect of IPTG on protein expression is to create a 0.1 M solution of IPTG. In order to create this solution, we added 119 mg IPTG to 5 mL distilled water. This solution created will be added to our plates with the amount of media consistent throughout each plate. On Plate 1, 4 µL of the 0.1 M IPTG solution is added to 2 µL of 50 mg/mL chloramphenicol. Since the plasmid is antibiotic resistant, the chloramphenicol added is used to confirm the protein expression is due to the IPTG. The following 4 plates contain an additional 4 µL of 0.1 M IPTG (4 µL, 8 µL, 12 µL, etc.) with the 2 µL of 50 mg/mL chloramphenicol the same in each tube as well. After preparing the plates, we re-suspended a single colony from plate 003-016-01 that had been transformed with the CBD-GFP plasmid in 1 mL nutrient broth and used an inoculating loop to spread some of it on each of the plates. When doing so, we streaked for isolation using a four-quadrant streak technique. The plates were then placed in the incubator overnight at 37°C and then we moved the plates to the fridge where they will be stored until we could observe them the following week.

Preparing of New Operons. In order to prepare a new operon, we researched the base pairs of the parts needed such as oyster galectin (BBa_K1489005), a CBD (BBa_K1321340), and a Blue Fluorescent Protein (BFP) (BBa_K592100). We also had to make sure to add restriction enzymes and termination sequences, but we had to keep the sequence under 2,000 bp, so we were unable to include the oyster galectin sequence due to its size. After checking to make sure there weren't any restriction enzyme sites in the middle of the sequence, we then submitted our sequences to Integrated DNA Technologies (IDT; Coralville, IA) for synthesis as gBlocks. Once received, we amplified the sequence through PCR using a thermocycler and then purified our new samples with a purification kit. Once the sequence was purified, we ran the sequences through a Lonza FlashGel to observe the size of the base pairs in the sequence and check for the presence of parts like the BFP and CBD.

Results

Culturing *G. xylinus* Bacteria to Produce Cellulose. After receiving the *G. xylinus* sample from Imperial College on December 3, 2013, we streaked some of the bacteria onto a plate and let it grow for a week. Two colonies were growing after a week; one was contaminated, and one was small but viable. Since this method of growth seemed unsuccessful, we tried four different cultures to decide the best method. We made two petri dishes and two conical tubes. One of the petri dishes and one of the conical tubes were contaminated but the other two were what was expected. The samples that didn't appear to have any

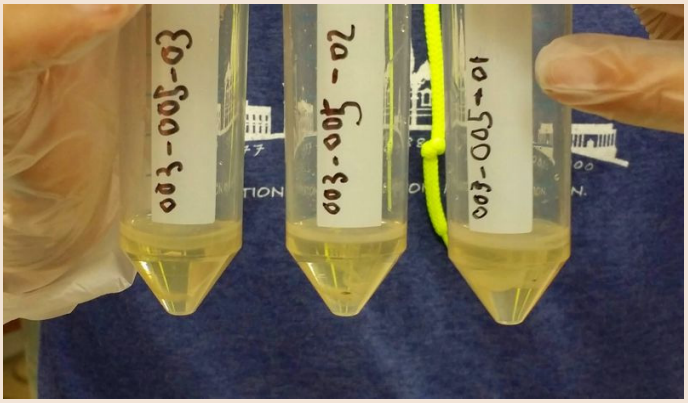

Figure 1. *Using three cultures of Gluconacetobacter xylinus, the Tuscarora Bioengineering Team was able to produce multiple cellulose sheets. The faint cellulose sheet, which floats at the top of the media, is clearly visible in all three test tubes. This is a positive result, as the team will now be able to use these cultures in future experiments.*

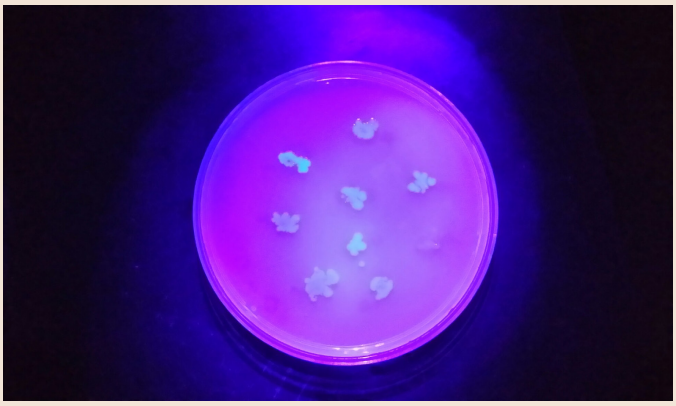

Figure 2. *Tuscarora's Bioengineering Team inoculated chloramphenicol treated plates with Escherichia coli transformed with the Cellular Binding Domain-Green Fluorescent Protein (CBD-GFP) plasmid. After performing a CBD-GFP bacterial transformation, these colonies were subcultured on plates with isopropyl β-D-1-thiogalactopyranoside (IPTG). The IPTG acts as an inducer of the GFP and caused the colonies on the plate to grow.*

contaminants had a thick sheet of cellulose floating at the top. To reproduce more cellulose, we separated pieces of the one successful conical tube into three separate tubes to subculture. Using the same process, we successfully subcultured nine pieces of cellulose in each of the three cultures. The cellulose sheets were seen floating on top of the media in the conical tubes and were stored for later use (Figure 1). This technique for subculturing *G. xylinus* worked well, giving us three fresh cultures for use in future experiments.

Transforming Cells with Plasmids for the CBD. After producing unsuccessful transformants using the Zymo Research Kit, we continued to troubleshoot for a solution. In order to increase transformation efficiencies, we inserted a step where we incubated the cells for 1 h in SOC media prior to plating on chloramphenicol treated plates and also subcultured the *E. coli* at room temperature. We inoculated one chloramphenicol-treated plate with *E. coli* that was transformed with the CBD-GFP plasmid (BBa_K1321356). Similarly, we transformed a second chloramphenicol-treated plate with a second CBD-GFP plasmid (BBa_K1321357). Finally, a third untreated plate was spread with *E. coli* that had been transformed without a plasmid (control). We examined the plates and found small colonies on BBa_K1321356, which was one of the plates with the CBD-GFP-transformed bacteria. The second plate with the transformed bacteria did not contain any colonies. The negative control, as anticipated, had a dense lawn of bacteria growth. Our changes to the procedure seemed to increase the transformation efficiency. To confirm these results, we subcultured these colonies on plates with IPTG. This IPTG would induce the production of the GFP protein and cause the colonies to glow. We began by mixing 0.119 g IPTG with 5 mL nuclease-free water to obtain the 100 mM IPTG required for our experiment. After supplementing the plate (BBa_K1321356) with chloramphenicol and IPTG, we transferred nine colonies from the earlier plate to numbered regions on a plate and placed the plate in the incubator. The next day, BBa_K1321356 was removed from the incubator and stored at 4°C. When the lights were turned off and the plate was placed near the black lights, the colonies were fluorescent. A photograph of the plate, taken under a UV light on 3 June, showed that many, if not all, of the subcultured colonies were fluorescing (Figure 2).

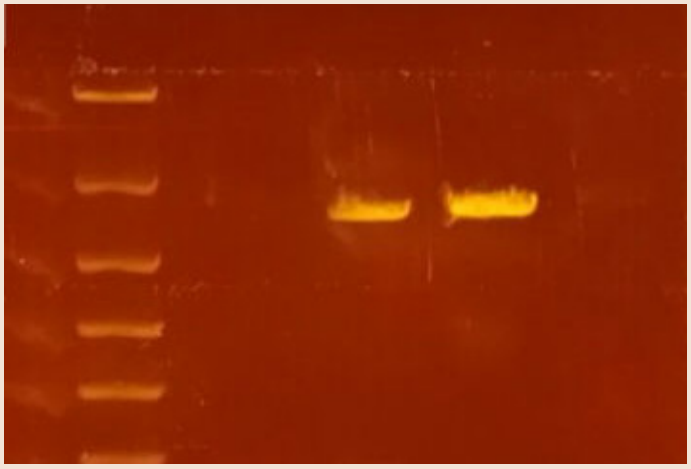

Figure 3. *Running the two Polymerase Chain Reaction (PCR) products on a FlashGel reveals that both samples contain a DNA sample of the expected size.*

In retrospect, we realized that the weak fluorescence in some strains likely resulted from the fact that we added only 2 µL 100 mM IPTG to the plate when we probably should have added as much as 100 µL of the inducer. We were able to successfully conclude that we transformed colonies with the CBD-GFP expressing plasmid.

Confirming the Presence of the Plasmid by PCR. Middletown High School obtained a thermocycler, which they generously offered to use to test for the presence of our plasmid. We had recently transformed *E. coli* with the CBD-GFP containing BioBrick, BBa_K1321356 and gave them subcultures, each taken from a separate colony and hopefully each containing the BioBrick. In

order to test this they added a 1 µL aliquot of each culture to a PCR instead of the template. However, this time their initial PCR phase will hold at 95°C for 10 min. to disrupt the cells and release the DNA. They placed the PCR products on a FlashGel. The BBa_K1321356 insert is 1,252 bp, and the positioning of the primers around the insert add another 333 bp. Therefore, we expect to get a PCR product of around 1,585 bp.The bands observed for samples 2 and 3 correspond with the anticipated 1,585 bp PCR products (Figure 3). Our transformation experiment was successful because each reagent contains bacteria harboring the BBa_K1321356 plasmid.

Testing the CBD. By testing the CBD, we would be able to positively confirm that the binding domain was attached to the cellulose we made. In order to test this, the cells had to be lysed in order to place the CBD gene into the plasmid. Theoretically, this should've worked, but the testing solution was the same color

Figure 4. *Cellular Binding Domain-Green Fluorescent Protein (CBD-GFP) and GFP cultures under UV light after lysing.*

as the control with the UV light shined on it (Figure 4). Therefore, we were unable to differentiate if the "glow" was due to the CBD binding to the cellulose. In order to resolve this problem, multiple solutions were proposed by the group. One solution was to change the UV light that we were using to a blue light instead of a green light due to the wavelength. Another solution was to increase the IPTG concentration to produce more of the CDB-GFP gene. Both the blue UV light and the increased IPTG concentrations were tested, used, and led to our first experiment for the new school year.

In order to increase the protein expression of the CBD gene, GFP proteins were tagged to the CBD gene. Therefore, by using UV light, the GFP would glow, resulting in the CBD gene attached and knowing transformation was occurring. By having GFP-CBD genes and GFP genes in separate plates, we could induce the fluorescent protein. Sadly, the plate with the GFP-CBD genes did not glow, but the plate with only the GFP protein did. Our results raised questions on the errors involved in our experiment. With discussion among our team, we speculated that the source of the absence of glowing was caused by the IPTG concentration. Therefore, in the following experiment, we tested different concentrations of IPTG at 0.2 nM, 0.4 nM, 0.6 nM, 0.8 nM, and 1 nM to determine if IPTG would increase the expression. We did this by preparing five plates with the concentrations previously listed and we suspended a single colony transformed with the CBD-GFP plasmid. Due to the fact that none of the test tubes glowed, we concluded the increased concentrations of IPTG was not the source.

Selecting a Different Fluorescent Protein. Due to the lack of success identifying and culturing previous samples using transformations, we chose to create our own sequences to send to IDT: one containing CBD and BFP and one containing just BFP. We decided to use BFP rather than GFP because the excitation wavelength of the former protein more closely matched the wavelength of the lights that we were using. The sequence with just BFP will be used as a control to compare to the sequence with the CBD and BFP. Using the iGEMS part registry, we found gene sequences for oyster galectin (BBa_K1489005), a CBD (BBa_K1321340), and a BFP (BBa_K592100) that matched the wavelength of the pen lights we use to analyze samples. We had to identify a fluorescent protein with minimal base pairs, and that worked under a light with a specific wavelength (400-500 nm). Because IDT can't produce gBlocks over 2,000 bp in length, we weren't able to include a gene for the oyster galectin. However, we concluded that we could add the galectin protein later using the 3A assembly process. Constructing the specific DNA sequence that we needed required parts in addition to the CBD and BFP. To start off the gene construction, we used a specific sequence that was recognized by the restriction enzyme XbaI. We then added an IPTG sensitive promoter sequence to allow the operon to be inducible. A ribosome binding sequence was needed to start gene transcription, which was followed by the BFP sequence and linked to the CBD sequence with a flexible linker sequence. The sequence was ended with a stop codon, two terminators, and another restriction enzyme site for the enzyme SpeI. We also checked to make sure no other areas included the sequences for the BioBrick restriction enzymes to prevent the genes from being accidentally cut during the assembly process. The second sequence was constructed in the same order but without the CBD. After constructing both sequences on the computer, we checked the base pairs in an online program to verify the protein sequence created. Once received, the sequences were amplified using PCR to use for further testing.

Amplifying and Purifying New Constructs. Using a thermocycler, we amplified both of the gBlock constructs we received from IDT and looked for appropriately-sized bands on the Flash

Gel. These results are shown in Figure 5. The first well is the base pair ladder in which we identify how large a sample is. In the second well we placed the BFP DNA, which had a band size of 1,104 bp. In the gel, it is shown under the sixth band, meaning it is around 1,250 bp. We concluded the BFP was present. In the third band, we placed the BFP + CBD which is 1,476 bands. Its band was between the sixth and seventh bands, which means it is 1,250-2,000 bp. We concluded the BFP + CBD are present. The constructs were also purified.

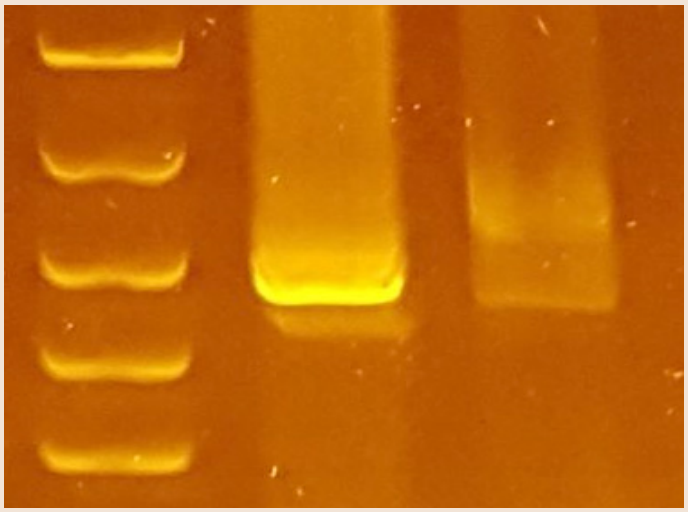

Figure 5. *Polymerase Chain Reaction products were run on a gel to confirm the presence of Blue Fluorescent Protein (BFP) and Cellulose Binding Domain (CBD) using the length of each band and the number of base pairs. The first band on the left was the ladder, the middle band was BFP, and the band on the right side was BFP + CBD.*

Discussions

We have learned so much while attempting to create a cellulose sheet with the infused galectin to attract the *P. marinus* parasite. We first learned how to work with bacteria and culture it. We then got into the process of getting the bacteria to take a plasmid so it would create the cellulose sheet with the galectin. Within that, we learned how to micropipette and how to do a gel electrophoresis. With some failures, we went on to create our own DNA by writing a base pair code and then sending it to a company to be made. We really learned what goes into making DNA and what is required to have a complete gene. We also built our own thermocycler, which was a great experience in which we learned how such an essential machine works. Overall, the group came much closer to science and learned a whole lot from the different experiments and projects we carried out throughout the year.

With the DNA we created, we will now put it into a plasmid that will then be put into the *G. xylinus* bacteria. We will then take the cellulose produced from the *G. xylinus* and test it with actual *P. marinus* to see if it works at collecting and trapping the parasite. Once confirmed, we will follow up with the Chesapeake Bay Foundation, who we have come into contact with about our project. We were talking about possible testing with oysters, which would prove if the cellulose sheet actually works. Through live testing, we will determine the functionality of the cellulose created and then move on to finding a way to mass produce the cellulose to hopefully create a working trap for *P. marinus*.

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