Bacterial Two Hybrid Analysis of Chlamydia Trachomatis Inclusion Membrane Protein, CT226

Kayli Nail, Christian Holcomb, and Erika Lutter

Oklahoma State University

Introduction

Chlamydia trachomatis is an obligate intracellular bacterial pathogen which poses severe health problems throughout the world. There are over 90 million new cases annually, making it the most common sexually transmitted disease in the world [1]. *Chlamydia* is treatable with antibiotics, but many of its virulence mechanisms have yet to be fully understood. Specific interactions between *C. trachomatis* and the host cell are important for survival due to its reduced genome [2]. *Chlamydia* is also the leading causes of blindness across the world. Most infections occur in third world countries where the incident rate for sexually transmitted diseases is higher [1]. Evan after a *C. trachomatis* infection has cleared, women can still develop infertility, pelvic inflammatory disease, and are at a higher risk for cervical cancer [3, 4]. *Chlamydia* can pose significant problems during and after infection. *Chlamydia* utilizes two different bodies during its infection process. The elementary body is infectious but not metabolically active, while the reticulate body is metabolically active but noninfectious. It is imperative to understand how *Chlamydia* manipulates the host cell to obtain a better understanding of the pathogen and potentially develop future treatments.

Chlamydia trachomatis contains multiple serovars that cause different diseases in humans. The serovars A-C are one of the leading causes of blindness in the world, serovars D-K cause urogenital infections that are very prevalent in women, and serovars L1-L3 cause lymphogranuloma venereum (LGV) [5, 6]. This research focuses on the L2 serovar, which is primarily known for causing LGV. *Chlamydia trachomatis* infections are often difficult to treat because many of the infections are asymptomatic, but without efficient treatment, these infections can lead to infertility, pelvic inflammatory disease, or even death [6]. Within the host cell *Chlamydia* survive and replicate inside of a parasitic vacuole called an inclusion. When *Chlamydia* first infects a cell, it does so as an elementary body (EB) which converts into a reticulate body (RB). This occurs during its replicative phase and then slowly transitions back to the EB infectious form at the end of the life cycle before existing the cell by lysis or extrusion and further infecting neighboring cells. During its replicative form *Chlamydia* relies heavily on host proteins, nutrients, and lipids for replication and survival [7-9]. It does so by synthesizing proteins that are inserted into the inclusion membrane which can then interact with host cell proteins on the outside of the inclusion. In doing so *Chlamydia* can alter signaling pathways, recruit nutrients, and alter the host's immune response [10-12]. Inclusion membrane proteins are unique to *Chlamydia*, with no other currently known organisms possessing them. These inclusion membrane proteins decorate the inclusion membrane. The genes encoding inclusion membrane proteins are often expressed very early in the developmental cycle and expression is maintained throughout the remainder of the life cycle suggesting a continued role in Chlamydial development [13].

The mechanisms by which *C. trachomatis* alters immune response are not well understood, but recent work done by Lutter lab and other labs [14] have identified that the chlamydial inclusion

membrane protein CT226 could be interacting with host proteins such as Human Flightless homologue II (FLII) and Leucine Rich-Repeat Flightless-Interacting Proteins 1 & 2 (LRRFIP1 and LRRFIP2) (Figure 1). Previous work in the lab has also identified TMOD3 as another potential host interacting partner. This protein does not work within the complex but has been identified as a protein with potential interacting capabilities with CT226. These three proteins (FLII, LRRFIP,1 and LRRFIP2) are known to work as a complex and are also known to interact with each other. In the host this complex regulates the immune response, specifically the inflammasome and is of great interest to understand how *Chlamydia* alters the host immune response. Experiments to date have shown an interaction and localization between CT226 and the proteins in this complex but techniques used so far cannot determine which protein in this complex CT226 is interacting with. The goals of this projects are to test the direct interaction between CT226 and each of the host proteins (LRRFIP1, LRRFIP2, FLII, and TMOD3) by using the bacterial two hybrid system (Figure 2). This system will be used because it is able to individually test each host protein outside of the complex host *Chlamydia* infection. We hypothesized that CT226 will directly interact with at least one of these 4 potential interacting partners (TMOD3, LRRFIP1, LRRFIP2, and FLII) and that this interaction will be detectable using a bacterial two hybrid system.

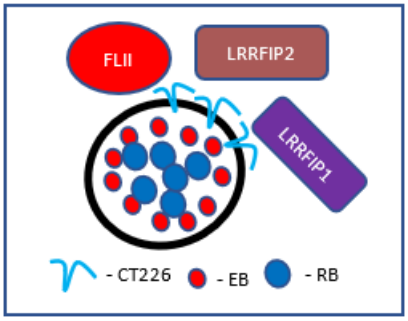

Figure 1. Schematic representation of CT226 interaction with FLII, LRRFIP1, and LRRFIP2

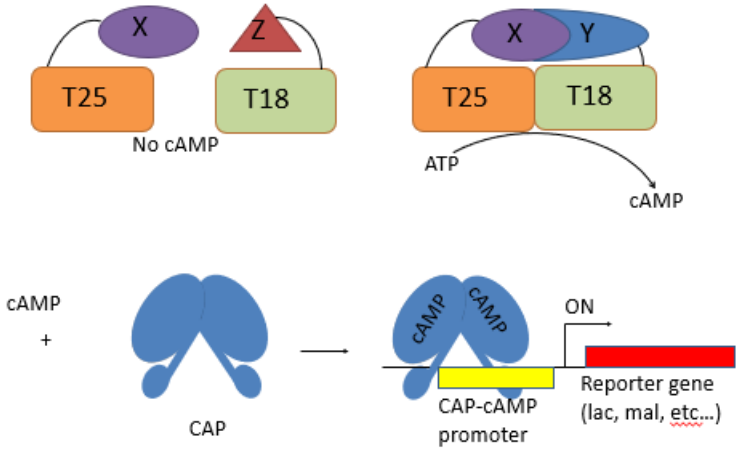

Figure 2: Mechanism of Bacterial-Hybrid Interaction. The bacterial two-hybrid system is being used to measure protein-protein individual interactions between *C. trachomatis* protein CT226 and

LRRFIP1, LRRFIP2, FLII and TMOD3 When two proteins interact, cAMP is generated which interacts with a catabolite activator protein (CAP) complex initiating transcription of target reporter genes including β-galactosidase and ability to utilize maltose for catabolism [15]. The β-galactosidase production will appear as blue colonies on a plate. Previous studies have utilized the bacterial two hybrid system to analyze protein to protein interactions with *Chlamydia* [16].

Materials and Methods

Bacterial Growth

Bacterial stocks for each clone were taken out of cryo storage and grown onto solid media. The solid media consisted of (Laura Bertani Broth) LB plates made with pre-made LB solid, Millipore filtered RO water, and 15g/L of agar. This LB media was then autoclaved on a 20-minute liquid cycle and cooled. Once cooled, ampicillin, kanamycin, or both was added at a 1/1000 ratio to the respective media and then media was poured into 100mm by 15mm petri dishes. After streaking, they were grown overnight at 37◦ Celsius.

Amplification of Insert DNA by PCR

The cloning process began with a PCR amplification of the insert (Primers are listed in Table 1). PCR reaction consisted of: template DNA strand with 1µL of the forward primer, 1 µL of the reverse primer, 1 µL of the DNA template for the desired clone, 9.5µL of sterile water, and 12.5µL of Dreamtaq. Each primer was designed with restriction enzyme digest in mind, so the restriction sites are designed into the primers. The PCR is run under these conditions: 95◦C for 5 minutes, 95◦C for 30 seconds, 52◦C for 30 seconds, 72◦C for 1-5 minutes depending on length of insert, and 72 ◦C for 5 minutes. The cycle is repeated 30 times throughout the cycle.

Table 1: Primers for amplifying genes

Primer Name	Primer Sequence (5'-3')
PKT25-FL2 Forward	AAAGGATCCCATGGAGGCCACCGGGGTGC
PKT25-FL2 Reverse	AAAGGTACCAATGGGCATCTGCGCTCTCCTCC
PUT18C-LRFIP1 Forward	AAAGGATCCCATGACCAGCCCCGCGG
PUT18C-LRFIP1 Reverse	AAAATCGATTTATTAGGACATGGTACAGTCT
PUT18C-FL2 Forward	AAAGTCGACTATGGAGGCCACCGGGGTGC
PUT18C-FL2 Reverse	AAAGGATCCTTAGGGCATCTGCGCTCTCCTCC
PUT18C-TMOD3 Forward	AAAGGATCCCATGGAGCAGAAACTCATCTC
PUT18C-TMOD3 Reverse	AAAATCGATTCACTGGTGATCTCCTTCAAC

DNA Electrophoresis

Once the PCR was completed a gel electrophoresis was made by using 0.48g of agarose and 60mL Tris Acetate EDTA (TAE) buffer. This was heated in the microwave for one minute, or until no solids were left in the liquid, and then poured into a gel castor. Two µL of ethidium bromide was added and mixed into the gel and was left to solidify for 45 minutes. Once the gel

solidified it was placed in the DNA tank and covered in TAE buffer. DNA was mixed with loading dye and loaded into the wells. For every 5µL of product, 3µL of 6X loading dye were mixed with it. The gel was run using a BIO-RAD Power/PAC 300 at 100 V and 400mA for 30 minutes. After completion it was analyzed under UV light to check for product.

Cleaning and Concentrating Insert and Vector DNA

Once the product was confirmed, the insert products were cleaned and concentrated to purify the DNA. This was done by adding a 5:1 ratio of DNA binding buffer to the product. It was then mixed, transferred to a spin column, and centrifuged for 1 minute at 12,000 rpm. The flowthrough was discarded and 200µL of DNA wash buffer was added, mixed, and centrifuged for one minute. The flowthrough was discarded, and the wash step was repeated with 200µL of DNA wash buffer. A new spin tube was received, and the silica column was transferred to the tube. Forty µL of nuclease free water was added to the column and left to sit for a minimum of two minutes. It was then centrifuged for 1 minute at 12,000 rpm. A gel for electrophoresis was made and once the gel solidified it was placed in the TAE buffer mix and the wells were filled with a mixture of product and dye. For every 5µL of product, 3µL of 6X loading dye were mixed with it. The gel was run using a BIO-RAD Power/PAC 300 at 100 V and 400mA for 30 minutes. After completion it was analyzed under UV light to check for product.

Digestion of Insert and Vector

The clean and concentrate product was then digested by 5µL vector, 82µL nuclease free water, 10µL Cutsmart, and 1.5µL each of the two insert specific enzymes.

The vector was made by a plasmid miniprep kit by adding 100µL of 7X lysis buffer to 600µL of *E. coli* culture. It was mixed by inverting the tube and lysed for 1 minute. 350µL of cold neutralization buffer was then added from the 4◦C fridge, and mixed until the sample became yellow, and a precipitate formed. The sample was then centrifuged for 2 minutes at 12,000 rpm, and the supernatant was transferred into a spin column. The spin column was then placed into a collection tube and spun down by the centrifuge for 30 seconds and the flow through was discarded. 200µL of Endo-Wash buffer was then added to the column and centrifuged, the flow through was discarded. Then add 400µL of Wash Buffer, centrifuge at 12,000 rpm for 30 seconds. A new 1.5mL microcentrifuge tube was used and then 30µL of nuclease free water was added to elute. It sat for 1 minute and then centrifuged at 12,000 rpm for 30 seconds to elute the product.

Another digest of the insert was done by 20µL insert, 23µL nuclease free water, 5µL Cutsmart, and 1µL of each insert specific enzyme. The digests were left overnight in a 37◦C water bath. 1.5µL Antarctic phosphatase was added to the vector digest the following morning and continued to digest for another 3 hours. A gel electrophoresis was, and once the gel solidified it was placed in the TAE buffer mix and the wells were filled with a mixture of product and dye. For every 5µL of product, 3µL of 6X loading dye were mixed with it. The gel was run using a BIO-RAD Power/PAC 300 at 100 V and 400mA for 30 minutes. After completion it was analyzed under UV light to check for product.

Cleaning and Concentrating Digested DNA Products

Once the digest products were confirmed, both the insert and vector products were cleaned and concentrated to purify the DNA. This was done by adding a 5:1 ratio of DNA binding buffer to the product. It was then mixed, transferred to a spin column, and centrifuged for 1 minute at 12,000 rpm. The flowthrough was discarded and 200µL of DNA wash buffer was added, mixed, and centrifuged for one minute. The flowthrough was discarded, and the wash step was repeated with 200µL of DNA wash buffer. A new spin tube was received, and the silica column was transferred to the tube. Forty µL of nuclease free water was added to the column and left to sit for a minimum of two minutes. It was then centrifuged for 1 minute at 12,000 rpm. A gel for electrophoresis was made and once the gel solidified it was placed in the TAE buffer mix and the wells were filled with a mixture of product and dye. For every 5µL of product, 3µL of 6X loading dye were mixed with it. The gel was run using a BIO-RAD Power/PAC 300 at 100 V and 400mA for 30 minutes. After completion it was analyzed under UV light to check for product.

Competent Cell Preparation

Competent cells were created by growing an overnight in LB media, dilute 1/100 and grow at 37◦C to an A600 of 0.375-0.6. Once at A600, transfer to a sterile 50mL tube, chill for 5-10 minutes on ice. Spin for 10 minutes in a centrifuge for 5,000-6,000 rpm at 4◦C, discard the supernatant. Resuspend the pellet in 5mL of 0.1M$CaCl_2$ buffer, spin down in the centrifuge for 5 minutes, 4,000 at 4◦C. Resuspend in 5mL of 0.1M $CaCl_2$ buffer, let sit for 30 minutes on ice, spin down in the centrifuge at 4,000 rpm for 5 minutes at 4◦C. Supernatant was discarded and pellet resuspend in 1mL of 0.1M $CaCl_2$ + 15% Glycerol, aliquoted into 100µLand stored at -70◦C.

Ligation and Transformation of Insert and Vector

A ligation was then performed with 5.5µL 2X ligase buffer, 0.5µL quick ligase, 1µL vector, and 5µL insert. It was then left at room temperature for 15 minutes then transformed into one of three possible cell lines: DH5α, DHMI, or BTH101 depending on if it was going to storage or to be used as a reporter cell line. Transformation was performed by letting the competent cell line thaw on ice for 30 minutes. The ligation mixture was added to the competent cells and incubate for 30 minutes on ice. It was the heat shocked for 30 seconds in a 42◦C water bath and then incubated on ice for another 10 minutes. Then 750µL of SOC media was added and incubated shaking for a minimum of 1 hour. After the incubation then 100µL of the transformation mixture was plated onto the correct media plate with antibiotic, ampicillin for any PUT18C clones and kanamycin for any PKT25 clones. The plate is then incubated overnight at 37◦C.

If there was growth on the plate the following day, then a colony PCR was performed on multiple colonies for the same plate. This was done by 1µL forward primer, 1µL reverse primer, 12.5µL TaqMaster 2X, 9.5µL nuclease free water, and a dab of colony. PCR Conditions: 95◦C for 5 minutes, 95◦C for 30 seconds, 52◦C for 30 seconds, 68◦C for 1-5 minutes depending on length of insert, and 68◦C for 5 minutes. A gel electrophoresis was made and once the gel solidified it was placed in the TAE buffer mix and the wells were filled with a mixture of product and dye. For every 5µL of product, 3µL of 6X loading dye were mixed with it. The gel was run using a BIO-RAD Power/PAC 300 at 100 V and 400mA for 30 minutes. After completion it was analyzed under UV light to check for product.

All clones were confirmed via plasmid sequencing.

Liquid β-galactosidase assay

After β-galactosidase production as detected on a plate, a liquid β-galactosidase assay was done. An overnight culture of the positive control, PUT18CZIP + PKT25ZIP was prepared with 25mL of LB broth, a 1 and 1000 concentration of Kanamycin and Ampicillin, and a single blue colony from the plate. It was then incubated overnight in the shaker at 37◦Celsius. After incubation overnight, the OD_{600} was measured using a spectrophotometer by putting 1mL of the overnight culture in a cuvette and using 1mL of LB broth as the blank. After the OD_{600} is measured, 60µL of Chloroform and 30µL of 0.1% SDS is added, vortexed for 10 seconds, then put on ice for approximately 5 minutes. After 5 minutes 20µL of the lysate is added to 980 µL of Buffer Z. Then 200µL of ONPG (4mg/ml) is added to the mix, and the average time for a yellow solution to be seen is 15 minutes. However, once a yellow solution is seen, 500µL Na_2CO_3 stop solution is added. One mL of reaction mixture is then added to a cuvette and OD_{420} and OD_{550} is measured using the spectrophotometer. The β-galactosidase production was measuring this the equation (generating Miller Units):

1000 (OD_{420}-(1.75-OD_{550})/ Time X Volume X OD_{600}

Results

There were two experimental tests, one positive control, and six negative controls that were supposed to be tested. However due to troubleshooting and time constraints, only seven were completed (Table 2). Out of the seven that were tested, only the positive controlled exhibited blue colonies and had β-galactosidase production.

Table 2: Summary of clones

Prey Plasmid	Bait Plasmid	Test Type	Test Result: Beta galactosidase production?
PKT25-CT226	PUT18C-FL2	Experimental 1	No
PKT 25-CT226	PUT18C-TMOD3	Experimental 2	
PKT25-ZIP	PUT18CZIP	Positive Control	Yes
PKT25-CT226	PUT18C	Negative Control	No
PKT25	PUT18C-FL2	Negative Control	No
PKT25-FL2	PUT18C	Negative Control	No
PKT25	PUT18C-TMOD3	Negative Control	
PKT25-ZIP	PUT18C	Negative Control	No
PKT25	PUT18C-ZIP	Negative Control	No

The positive control of PKT25-ZIP + PUT18C-ZIP was transformed to ensure the bacterial two hybrid system was working properly. These genes are both bacterial genes. It was transformed into BTH101competent cells and 100 µL was incubated on LB, containing kanamycin and ampicillin, and the plate was left overnight in the 37◦ C incubator. The double transformation showed colonies that were blue in color (Figure 3). A β-galactosidase production assay was performed after the

culture was grown overnight, and 1.54 was recorded for the positive control. The β-galactosidase production assay for the negative control has yet to be completed.

Figure 3: Double Transformation results streaked out for PKT25-ZIP + PUT18C-ZIP, the positive control. Four colonies were streaked out from the original transformation to be put on a LB plate containing Ampicillin, Kanamycin, IPTG, and Xgal.

The negative controls, PKT25-CT226 + PUT18C (empty), PKT25 (empty) + PUT18C-FL2, PKT25-FL2 + PUT18C (empty), PKT25 (empty) + PUT18C-TMOD3, PKT25ZIP + PUT18C (empty), and PKT25 (empty) + PUT18C-ZIP were then transformed to ensure the bacterial two hybrid system was working properly. Each negative control showed no blue colonies, indicating that there was no β-galactosidase production. However, we have yet to complete the β-galactosidase production assay for the negative controls to confirm that there is no production. We have yet to complete the cloning process for PUT18C-TMOD3, so we were unable to test the double transformation of PUT28C-TMOD3 + PKT25 as a negative control. All tested negative controls grew colonies but did not show any blue color (Figure 4).

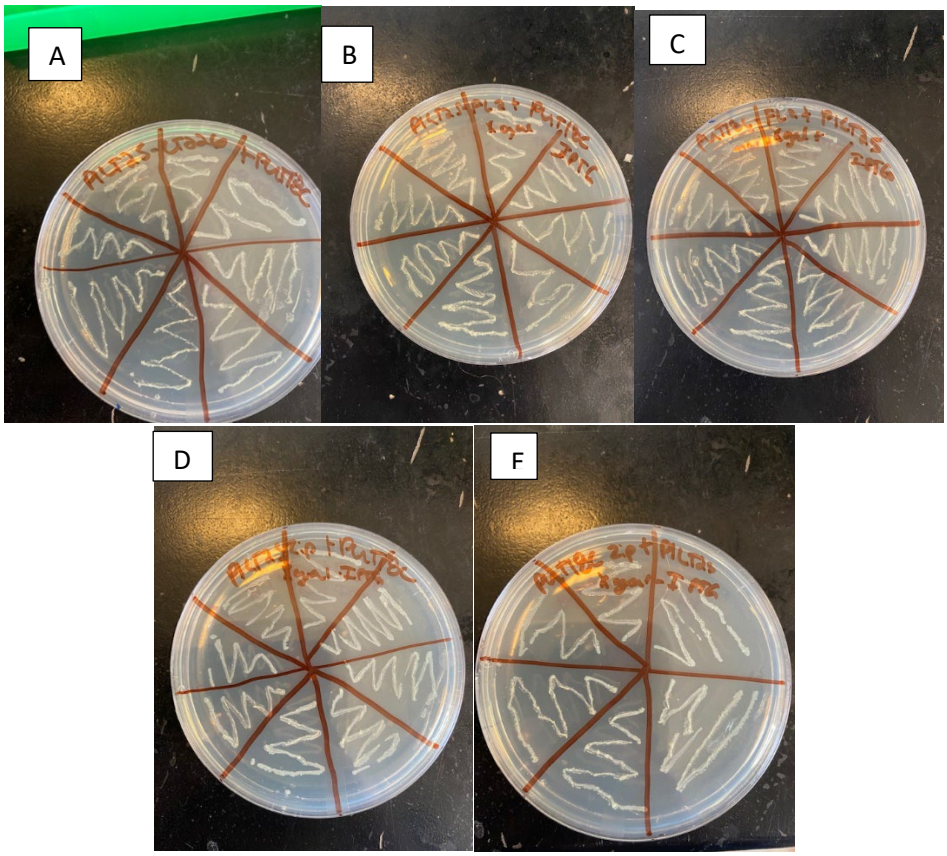

Figure 4: Negative controls for the bacterial two hybrid system. Double Transformation results were struck out based on the number of colonies each transformation had. Each colony was streaked out from the original transformation to be put on a LB plate containing Ampicillin, Kanamycin, IPTG, and Xgal. Plates are ordered by: Plate A: PKT250CT226 + PUT18C, Plate B: PKT25-FL2 + PUT18C, Plate C: PUT18C-FL2 + PKT25, Plate D: PKT25ZIP + PUT18C, Plate E: PUT18CZIP + PKT25.

The test double transformation PKT25-CT226 + PUT18C-FL2 no blue color or any of the colonies, but this result is going to be retested to ensure accuracy (Figure 5). The PKT25-CT226 + PUT18C-TMOD3 double transformation was unable to be performed because of difficulties cloning TMOD3 into PUT18C. However once this has been cloned a double transformation will be done for results.

Figure 5: PKT-25-CT226 + PUT18C-FL2 test double transformation. Double Transformation results were sstruck out based on the number of colonies each transformation had. Each colony was streaked out from the original transformation to be put on a LB plate containing Ampicillin, Kanamycin, IPTG, and Xgal.

All bait and prey systems are going to be retransformed again into their prospective cell lines to ensure that the test results are accurate.

Discussion

A bacterial two hybrid system was used over a yeast two hybrid system due to yeast being eukaryotic and producing actin. Previous efforts using the yeast two hybrid system found TMOD3 to be toxic to yeast cells. The bacterial two hybrid system was used because there is no actin for TMOD3 to interact with in a prokaryotic system. This removes the chance of TMOD3 interacting with proteins other than possible interacting partners. The bacterial two hybrid system removes CT226 out of the context of a Chlamydial infection and into a system that tests for individual interactions. This is advantageous as three of the proteins we are aiming to study are known to act as a complex (FL2, LRFIP1 and LRRFIP2) and need to be assessed individually for an interaction with CT226. For all these reasons: the ease of cloning and individual characterization, the bacterial two hybrid system was chosen.

Using the bacterial two hybrid system I was able to learn a vast number of protocols such as PCR, restriction enzyme digests, clean and concentrate protocols, culturing bacteria, and β-galactosidase assays. It provides a straightforward cloning process that can be easily repeated. The whole process from cloning to β-galactosidase assay can usually be done within two weeks if no troubleshooting is required, which makes it an easy and convenient way to test for individual interactions. One troubleshooting option that we utilized is TOPO cloning technique. It allowed us to skip the PCR step for many of the inserts and go straight to a restriction enzyme digestion from a plasmid prep. This removes a possible mistake being made during the PCR, but it requires a gel extraction from the topo clone before it can be re-cloned into the target vector.

The positive control transformation showed blue colonies, which indicates that there was β-galactosidase production that can be quantified using a β-galactosidase assay. The blue colonies also indicate that there was an interaction that occurred between the bait and prey proteins. This

result was expected. In addition, our negative controls had colonies but no blue color to them, indicating that β-galactosidase was not produced. The lack of blue colonies shows that there was no interaction that occurred between any of the negative control bait and prey proteins This result was also expected. We can conclude that the bacterial two hybrid system is working. Our test plasmids, which contained the inserts CT226 and FL2 showed no blue colonies. This step is being repeated to ensure accuracy as blue colonies were expected. Blue colonies were expected because previously research in Dr. Lutter's lab have indicated that there may an interaction between CT226 and FL2. Based on this knowledge, an interaction, indicated by blue colonies and β-galactosidase production, was expected. However, our results indicated no β-galactosidase production. This experiment is going to be repeated with a new clone of the bait and prey plasmids to ensure the accuracy of this result, but my results indicate there is no interaction between CT226 and FL2.

Future directions for this study are to troubleshoot the cloning of TMOD3 into PUT18C ffor the remaining experimental test. Once this is complete, a transformation will be done and examined for results, and if blue colonies appear, a β-galactosidase assay will be done to quantify the amount of β-galactosidase produced. A β-galactosidase assay will also be done for the negative controls to quantify the interaction level and ensure no β-galactosidase production in all the negative controls. Potential future studies include utilizing the bacterial two hybrid system to test for interactions between CT226 and LRFIP1 and LRFIP2. Previous studies have shown through β-galactosidase assays that LRFIP1 interacts with CT226 [14] and we expect similar results. Potential results for LRFIP2 are currently unknown. *Chlamydia muridarum* is a similar strain to *C. trachomatis*, and has its own CT226, which is a homolog of *C. trachomatis* CT226. The bacterial two hybrid system will be repeated with *C. muridarum* CT226 rather than *C. trachomatis* CT226, but the strategy of cloning will remain the same. Potential results of the bacterial two hybrid system are currently unknown, but since these proteins are homologs, results may be similar.

The bacterial two hybrid system is a convenient way to test for individual protein interactions over complexes. This system provides a great learning tool for many different protocols and requires great patience and critical thinking to complete. The positive control produced β-galactosidase while the negative control did not, indicating that the system works. The test plasmids containing CT226 and FL2 did not yield my expected results and will be repeated to ensure accuracy, but no interaction may be occurring. Potential studies will need to be done utilizing the bacterial two hybrid system to clone LRFIP1, LRFIP2, and TMOD3 to test for potential interacting partners. The entire system will also need to be repeated utilizing the *C. muridarum* CT226 homolog to investigate if the interactions are different. This project has increased my knowledge of molecular techniques, procedures, and problem-solving, and the potential findings for this study and future studies will help scientists have a better understanding of how *C. trachomatis* manipulates the host cell.

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