Genetic Knockdown System for *Chlamydia trachomatis*

Undergraduate Honors Thesis

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Abstract:

Chlamydia trachomatis is an obligate intracellular pathogen that is responsible for the highest number of reported cases of sexually transmitted infections. Until recently *Chlamydia* has been genetically intractable, thereby limiting genetic approaches. Unfortunately, gene inactivation by TargeTron or antibiotic cassette insertion can result in polar effects of neighboring genes making it difficult to study the genes within operons. This study focuses on developing a novel knockdown strategy by expressing the reverse complement specific *Chlamydia* genes on a shuttle plasmid. Once cloned, the plasmids are transformed back into *Chlamydia* and the genes expressed *in trans* will be transcribed and bind the RNA of the corresponding gene producing double stranded RNA which is degraded. This method will allow us to look at individual genes in an operon without the polar effects of mutations. This strategy is being used on an operon containing 6 genes. After the reverse complement of each gene is expressed, the decreased expression of the target gene will be assessed by reverse transcription PCR. These experiments are the first to utilize a gene specific knockdown strategy in *Chlamydia*.

Introduction:

Chlamydia trachomatis Background

Chlamydia trachomatis is an obligate intracellular pathogen that is commonly sexually transmitted among humans (Elwell et al., 2016). In fact, it is the most commonly reported sexually transmitted disease in the United States with an estimated three million *Chlamydia* infections each year (CDC). The vast majority of these infections, almost two-thirds of them, are found in the age group of 15-24 years with an estimated one in every 20 women in this group being infected (CDC). With the local population being a significant percentage of this highly infected age group, the effects of this easily transmittable disease are seen in this community.

Of the approximately three million infected people, only about one half have been found to seek treatment because *Chlamydia* infections are often asymptomatic (Newman et al. 2015). Due to the lack of symptoms, the statistics regarding *Chlamydia* infections are much lower than the actual incidence number of infections that occur. The lack of education on sexually transmitted infections and safe sex practices contribute to the high rate of infection, especially among young adult women (Newman et al. 2015). The stigma that surrounds sexually transmitted infections as well as the lack of symptoms in a large number of *Chlamydia* infections often leads individuals to neglect seeking treatment for their infections for long periods of time. Many who are unaware of an infection due to it being asymptomatic will not ever receive treatment unless they regularly get tested for sexually transmitted diseases. Many males are carriers of asymptomatic *Chlamydia* and pass their infections along to their sexual partners without knowing they are infected and transmitting a

disease (CDC). Untreated infections can have short and long-term negative consequences. However, even after antibiotic treatment and clearance of the infection, long term health problems such as pelvic inflammatory disease, scarring of the fallopian tubes, tubal factor infertility and ectopic pregnancies (Bakken et al. 2007) are still of great concern (Low et al. 2006). It is currently unknown what the cause of these long-term health effects are which is why research must continue to be done on the topic. With the high rate of infectivity and the lack of education on the spreading of sexually transmitted infections, it is pertinent that research continue to be done to find the cause of these long-term effects in order to find options to treat and potentially prevent them.

For decades *Chlamydia trachomatis* was genetically intractable which made deciphering the role of specific genes in pathogenesis difficult (Heuer et al. 2007). Within the last five years, new advancements have led to multiple mechanisms to introduce gene mutations by allelic exchange or TargeTron insertion (Johnson and Fisher 2013). The problem with such mutations is that they insert a large piece of DNA into the gene of interest to inactivate it and this can cause polar effects on the expression of neighboring genes (Mueller 2016). This is highly problematic with *Chlamydia* since it has a highly reduced genome and most genes are found in operons. As such, the goal of this project was to develop a genetic knockdown system that could be transformed into *Chlamydia* to block transcription of specific genes without creating a mutant strain.

The initial goal of this project was to clone six *Chlamydial* genes from a specific operon that is only found in human pathogenic strains of *Chlamydia* into a shuttle vector (Lutter et al. 2012), a plasmid that is a fusion of a *Chlamydia* plasmid and an *E. coli* plasmid (Bauler et al. 2014, Witkin et al. 2017). Each gene is cloned in the reverse complement in

order to produce an mRNA transcript that will bind to the mRNA produced in the bacterial genome (Ji et al. 2014, Tierney& Lamour, 2005). The result would be that the newly introduced reverse complement mRNA would bind to the genomic mRNA resulting in double stranded mRNA that would be degraded. This would decrease expression of the gene of interest. This method will allow us to look at individual genes in an operon without the polar effects of mutations. These experiments will be the first to utilize a gene specific knockdown strategy in *Chlamydia*.

Methods:

PCR primers were designed to amplify each gene in the reverse complement. This means that the forward primer was designed to the complementary strand (- strand) at the end of the gene and the reverse primer was designed to the reading strand (+ strand) at the start of the gene. Each PCR product generated the reverse complement of each gene that would be cloned into the shuttle vector pBOMB-Tet-mCherry (Bauler and Hackstadt 2014). This plasmid allows us to clone constructs in *E. coli* and then transfer the final plasmid into *Chlamydia* for expression studies. The vector map and multiple cloning site for pBOMB-Tet-mCherry is shown in Figure 1 and the primers used are seen in Table 1.

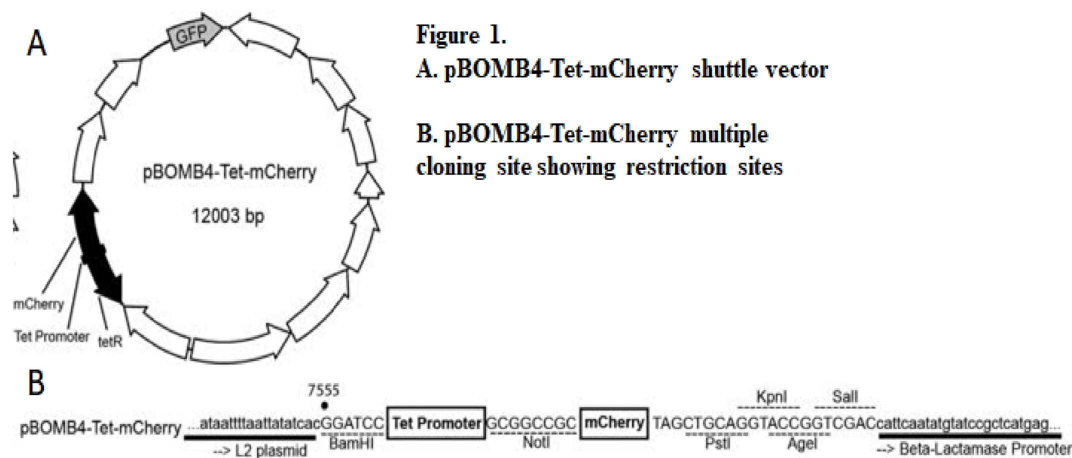

Figure 1.
A. pBOMB4-Tet-mCherry shuttle vector

B. pBOMB4-Tet-mCherry multiple cloning site showing restriction sites

Table 1: Primers used in this study

Gene	Primer sequence 5'→3'	Restriction site
CT226	AAAGCGGCCGCCCTACAGACTTTCTTCCAATACAC	Not 1
CT226	AAACTGCAGATGTTTAATATTTCTTTTTGTTG	Pst1
CT228	AAAGCGGCCGCCTAAGAAGCTTGGTTAGCGTC	Not 1
CT228	AAACTGCAGATGAGTACTACTATTAGCGG	Pst1
CT229	TTTGCGGCCGCTTATTTTTTACGACGGGATGC	Not1
CT229	AAACTGCAGATGAGCTGTTCTAATGTTAATTCAG	Pst1

Of the 6 genes in the *CT229-CT224* operon, we focused on *CT226*, *CT227* and *CT229* for amplification and cloning. Primers to the other genes were designed and experiments on those genes would continue once the first 3 genes were done. The reason these genes were chosen is that they either have a function identified in lab or have functions published. In our lab, *CT226* protein interacts with flightless homologue 1 (FLII), however, the overall function has not yet been determined (Sah and Lutter, unpublished). *CT228* protein interacts with MYPT1 to regulate the mechanism of *Chlamydial* host cell exit (Lutter et al. 2013) and *CT229* protein interacts with RAB4A (Rzomp et al. 2006). A schematic representation of the genes in the *CT229-CT224* operon with their primer location is shown in Figure 2, below.

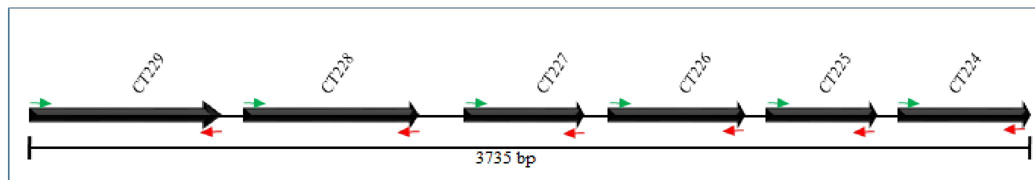

Figure 2. Schematic representation of the *CT229-CT224* operon and primer locations. Gene are shown approximate to actual size with the location of forward primers (green) and reverse primers (red) shown.

For PCR the following protocol was used for each gene. The PCR mix contains a combination of 25 microliters of DreamTaq Master Mix 2X, 1 microliter of the forward primer as described above, 1 microliter of the reverse primer as described above, 1 microliter of the template *Chlamydia* DNA, and 22 microliters of purified water to create a total of 50 microliters of solution. The solution was then amplified by PCR under the following conditions: initial denaturation was performed for 1 cycle at 95° C for 3 minutes, denaturation was performed for 40 cycles at 95° C for 30 seconds per cycle, annealing was performed for 40 cycles at 52° C for 30 seconds per cycle, extension was performed for 40 cycles at 72° C for 1 minute per cycle, and final extension was performed for 1 cycle at 72°

C for 15 minutes. To ensure correct amplification, each of the PCR products were run on an agarose gel at 115V for 30 minutes. The images were then exposed to an ultraviolet light on a low setting in order to visualize the DNA fragments. This allowed the identification of the genes by ensuring their location on the agarose gel was consistent with the proper length of the intended gene. Once the PCR products were identified as being in the correct position on the gel, each gene proceeded to the restriction digestion and purification steps.

Initially, we used the restriction sites and enzymes Kpn1 and Not1. The pBOMB-Tet-mCherry vector was cut with the same primer specific restriction digest at restriction sites Kpn1 and Not1. The restriction digest procedure is as follows: 10 microliters of the plasmid DNA, 10 microliters of the CutSmart buffer, 1 microliter of the restriction enzymes Not1 and Kpn1, all combined with 78 microliters of water put into a 37°C water bath overnight. The vector was treated with Antarctic Phosphatase to remove any phosphate groups from the ends of the DNA in order to prevent any religation of the vector to itself if any of it was only cut with one restriction site instead of both. Each of the genes were also purified by the Zymo Research DNA Clean & Concentrator kit and eluted in 25 microliters of water. The cells were then ligated and transformed into *Escherichia coli* DH5α cells. The ligation process began by combining 5.5 microliters of the quick ligase buffer, 1 microliter of the vector, 4 microliters of each of the PCR products, individually, and 0.5 microliters of the quick ligase enzyme. They were then mixed and left sitting at room temperature for 30 minutes. To transform these ligation products, 250 microliters of DH5α cells were thawed on ice and then combined with 5.5 microliters of the ligation products. This mixture then sat on ice for 30 minutes. They were then heat shocked at 42°C for 30 seconds and then returned to sitting on ice for 10 minutes. After this, the products were combined with 750 microliters of SOC and

were shaken in the 37°C incubator for 2 hours. The transformed cells were then grown on Ampicillin resistant plates to select for the cells that contained the *Chlamydia* gene of interest. After many unsuccessful attempts at cloning, new primers were designed that replaced the restriction sites to see if an increased compatibility with the vector resulted. The restriction site of Kpn1 was replaced with a restriction digest specific to restriction site Pst1 with all other conditions remaining the same. A new vector was also generated with matching restriction sites for subsequent cloning reactions. Multiple cloning reactions were attempted with no success.

The strategy for the cloning procedure was altered from the initial attempt at directional cloning and was changed to using procedures of TOPO cloning. TOPO cloning allows us to sub clone a PCR amplicon directly into a plasmid, pCRTOPO 2.1. For the TOPO cloning reaction freshly amplified PCR product for each gene was mixed with a salt solution and pCRTOPO2.1 vector for 5 min. The mixture was transformed into *E. coli* DH5α and selected for on LB plates containing Ampicillin and X-gal. White colonies were repicked and each plasmid was isolated and digested with Not1 and Pst1 to verify amplicon insertion. The inserts were gel excised, cleaned with Zymo Research Gel Extraction kit, verified by gel electrophoresis and ligated into linearized pBOMB-Tet-mCherry vector. Ligations and transformations were performed as described above.

Once clones were achieved, the plasmids were then prepared for sequencing through the Zymo Research Zippy Plasmid Midi Prep kit. The plasmids were then separated on an agarose gel to verify that the inserts were present in the plasmids. After confirmation of plasmids, they were then sent to the Biochemistry department for sequencing. After the constructs were cloned we needed to get the plasmids into the proper methylation state for

transformation into *Chlamydia. Chlamydia* will update plasmid DNA if it is not methylated thus the need for methylating the plasmids. Most cloning strains produce methylated DNA, including our *E. coli* strain DH5α, so we had to transform the final plasmid products into *E. coli* ER2508 which is mutated for *Dam*, *Dcm* and *Mcr* methylation systems.

Results:

The reverse complement of the genes *CT226*, *CT228*, and *CT229* were all successfully PCR amplified as described above. The results of the agarose gel electrophoresis of each of the PCR products of the 3 genes are visualized in Figure 3. This figure confirms that each of the three genes were properly amplified because the location of the band on the agarose gel in comparison with the DNA Ladder in lane 1 was the expected size.

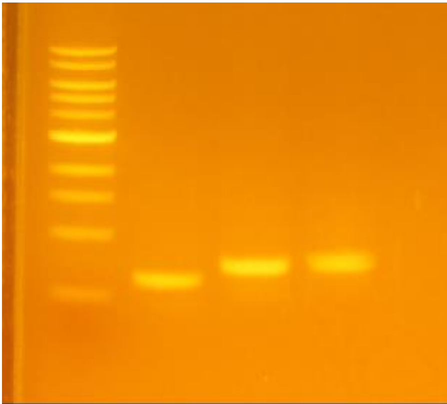

Figure 3. PCR amplification of *CT226, CT228* and *CT229*. The first lane of this figure shows a 1Kb ladder followed by the initial PCR products of the reverse complement of the genes *CT226*, *CT228*, and *CT229* in lanes 2, 3, and 4.

Once the PCR products were confirmed, the vector as well as each gene underwent restriction digestion through the above mentioned procedure. The vector was then cleaned using a gel purification procedure to remove the nucleotides, enzymes, and salts from the solution which could interfere with subsequent reactions. Each of the genes were cleaned with the Zippy DNA Clean Up Kit as previously mentioned. The digested and cleaned vector and genes were then visualized by agarose gel electrophoresis. This provided evidence that the initially circular vector was properly cut and linearized due to its appearance as a single band in Figure 4. This gel also provided proof of each of the genes being recovered after the cleaning procedures by showing they are in the same position on the gel in Figure 4 as they were in Figure 3. Once the vector was seen to be linearized and it was confirmed that the genes were successfully recovered after the cleaning procedure, the genes were then TOPO cloned into the vector using the method described above. To differentiate between the cells

that successfully inserted the *Chlamydia* DNA and those that did not, a blue and white selection procedure was used. The white colonies represented in Figure 5 below are the successfully cloned cells and were subsequently picked from the plates and grown in a broth culture. The blue colonies show the cells in which *Chlamydia* genes did not successfully insert and therefore were not picked from the plate. The image in Figure 5 is an example of what a blue white selection test looks like as all of the white colonies on the plates used in this experiment were picked for culture before an image could be taken.

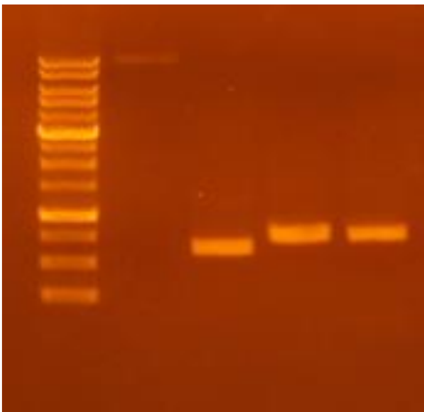

Figure 4. Electrophoresis visualization of linearized vector and purified inserts for cloning reactions. The first lane of this figure shows a 1 Kb ladder followed by the cut, linearized, and purified vector pBOMB-Tet-mCherry in lane 2. Lanes 3, 4, and 5 show the restriction digested and cleaned genes *CT226*, *CT228*, and *CT229*, respectively.

Figure 5. Blue white screening of TOPO Cloning reactions. This image is an example of how blue white selection differentiate between the cells containing plasmids with inserts (white) and those that lack inserts (blue).

The white colonies that were picked from the TOPO cloning reactions were struck onto LB plates containing Ampicillin and incubated for about 18 hours. The cells that grew on these plates were then used to set up overnight broth cultures for plasmid extraction. Plasmids were purified, restriction digested with the enzymes Not1 and Pst1 through the procedure described above. All of these restriction digests were separated by agarose gel electrophoresis to confirm the TOPO cloning was successful. Multiple clones for each gene were tested and we were able to visualize which constructs were successfully TOPO cloned due to the presence of three distinct bands. The genes *CT226, CT228, and CT229* were all successfully TOPO cloned and visualized in Figure 6 in lanes 5, 9, and 12, respectively as seen by the presence of three distinct bands in each of the before mentioned lanes. The TOPO vector also contains a Not1 site outside of the multiple cloning site which cuts the TOPO plasmid into 2 pieces which is why we look for 3 distinct bands. The third DNA fragment is our gene of interest in each clone. The genes were gel extracted, directionally cloned into the shuttle plasmid, and constructs for each of them were successfully made.

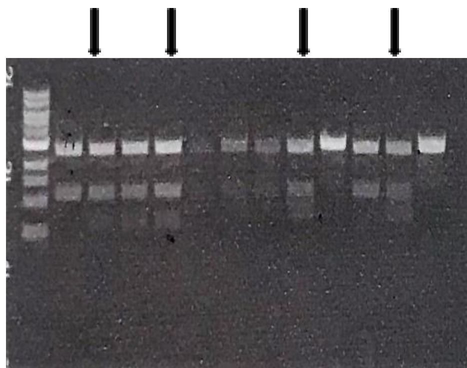

Figure 6. Restriction digest of TOPO Cloning reactions. Lane 1 contains a 1Kb ladder. Lane 2 contains an example of an empty TOPO cloning. Lanes 5, 9, and 12 contain the successfully TOPO cloned genes *CT226, CT228,* and *CT229*, respectively.

Discussion and Conclusion:

Genetic manipulation of obligate intracellular pathogens is very challenging and each obligate intracellular pathogen comes with its own unique obstacles to overcome (Wood et al 2014). Genetic work with the obligate intracellular pathogen *Chlamydia* is a very new and therefore a very difficult process. Only recently (within the last 5 years) have tools and techniques been developed to help researchers genetically alter *Chlamydia.* One tool in particular was the development of 3 shuttle vectors, one of which pBOMB-Tet-mCherry was used in this project (Bauler and Hackstadt 2014). The shuttle plasmids consist of a fusion of an *E. coli* plasmid and *Chlamydia* plasmid into a single plasmid. Because these plasmids contain *E. coli* and *Chlamydia* origins of replication they allow for us to clone genes in an *E. coli* background and then transform the resulting clones into *Chlamydia*. Before the ability to genetically transform *Chlamydia* and the creation of these shuttle plasmids, it was impossible to express any genes *in trans* in *Chlamydia* (Bauler and Hackstadt 2014). Even with the creation of these shuttle plasmids, the process of genetic mutation in *Chlamydia* is long and difficult. In addition, due to its small genome size and the arrangement of a majority of its genes in operons, genetic mutations cause polar effects on the genes surrounding the mutated gene making it impossible to tell the function of the individual gene (Mueller 2016). Multiple techniques have focused on making mutation using the TargeTron system (Johnson and Fisher, 2013), allelic exchange with the insertion of an antibiotic cassette to inactivate the target gene (Mueller et al. 2016), and even the development of CRISPR technologies that can be adapted to *Chlamydia* (Oullette 2018). However, with the development of these techniques, some problems regarding polar effects have arisen.

This was the basis for the development of a novel knockdown strategy. The design for this project was chosen based on these new advancements and in order to avoid issues found in other experiments. The knockdown strategy was also chosen because it will allow for the ability to understand the function of a single gene without any polar effects from neighboring genes which is the ultimate goal of this project. There are, however, many challenges with cloning obligate intracellular pathogens, especially those with a reduced genome like *Chlamydia trachomatis* (Elwell et al. 2016).

To summarize the results from this study, each of the three target *Chlamydial* genes *CT226, CT228*, and *CT229* were successfully amplified in the reverse complement and confirmed through agarose gel electrophoresis. The next steps of verifying if the knockdown strategy works well would be exciting as it would represent a very new strategy to manipulate *Chlamydia* genetically. We do, however, believe these next steps will be successful as similar techniques have successfully been used in other pathogens. A recent study in another obligate intracellular pathogen, *Rickettsia rickettsi* used single-stranded synthetic DNA that was complementary to the Shine-Dalgarno (start codon regions of the bacterial mRNA) to reduce protein expression from their target genes of interest (Pelc wt al 2015). An additional study in *E. coli*, looking at genes within a highly coordinated operon (similar to the genetic arrangement of genes in the *Chlamydia* genome) used the same approach as we did in this study to assess the function of each gene in the operon separately (Goh et al 2015).

Most of the previous research on *Chlamydia trachomatis* dealt with *Chlamydia* while it was in the host cell. These new methods previously discussed have only recently allowed

for work with *Chlamydia* independently from its host cell. That is why the genetic knockdown strategy designed for this study is novel because it only recently became a possibility. There are many practical applications for a genetic knockdown system in *Chlamydia trachomatis*. Since the genome is quite small (has a reduced genome) and most of its genes are arranged in operons, there is a good chance that a majority of the genes in the *Chlamydia* genome may be essential (Elwell et al. 2016). This knockdown strategy will allow for the function of each gene to individually be reduced or removed during different time intervals of the infection process without the need to generate a mutant. This would be very useful to assess genes that may be essential at different stages of the infection process. This will also allow for any gene in an entire operon to be removed from expression without having any polar effects in neighboring genes which is a huge concern when making *Chlamydial* mutants (Heuer et al. 2007). Since the expression of the genes cloned can be controlled via the tetracycline inducible promoter throughout the entire infectious process, we will be able to understand the function of each gene at any point in time.

The ultimate goal of this study is to find which gene or genes is responsible for the long-term health effects that *Chlamydia trachomatis* infections can cause. By identifying the function of each gene in the *CT229-CT224* operon, we will be able to see exactly how each gene contributes to the cause of these symptoms. This will allow for more targeted treatment of symptoms because the source of them will be made available.

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