Expression of an Epitope Tagged Tarp Effector in Chlamydia Trachomatis

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EXPRESSION OF AN EPITOPE TAGGED TARP EFFECTOR IN CHLAMYDIA TRACHOMATIS

by

BRENDA N. NGUYEN

A thesis submitted in partial fulfillment of the requirements for the Honors in the Major Program in Molecular Biology and Microbiology in the College of Medicine and in the Burnett Honors College at the University of Central Florida Orlando, FL

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ABSTRACT

Previous studies performed on *Chlamydia trachomatis* have demonstrated how these obligate intracellular microbes invade host cells through the utilization of secreted effector proteins. One secreted effector called Tarp (translocated actin recruiting protein) is implicated in cytoskeleton rearrangements that promote bacterial entry into the host cell. The focus of our study is to create a plasmid that carries the *tarP* gene that when transcribed and translated from within *Chlamydia trachomatis* will generate a c-Myc epitope tagged Tarp. The tag will be used in future studies to track the progression of the protein through the infectious process and will allow us to distinguish this protein from the Tarp effector expressed from the endogenous wild type gene. The epitope-tagged Tarp expression plasmid will be used as a template to construct Tarp deletion mutants. The mutant forms will be created in regions that have been biochemically characterized and predicted to be important to the invasion process of the pathogen. Observations on the potential phenotypes of these mutants and the possibility of allelic exchange will also be pursued in the future.
DEDICATION

I dedicate this work to my loving parents, Donald and Day Thi Nguyen, as well as my caring siblings.

I also dedicate this to all of my friends who have supported me and encouraged me throughout this entire time.
ACKNOWLEDGEMENTS

I would like to express sincere gratitude to my mentor and head of thesis chair, Dr. Travis Jewett because without his teaching and guidance, I will not have the confidence and opportunity to take this big leap into research.

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LIST OF MEDIA/ABBREVIATIONS/NOMENCLATURE/ACRONYMS

AB/α: antibodies

ABD: actin binding domain

Ag: antigen

CAT: chloramphenicol acetyl transferase

DsRed: Discosoma red fluorescent protein

EB: elementary body

HRP: horseradish peroxidase

IFU: infectious units

L2: serovar LGV 2

LGV: lymphogranuloma venereum

Ori: origin of replication

pCtSV.1: plasmid-C. trachomatis shuttle vector #1

PhosD: phosphorylation domain

PID: pelvic inflammatory disease

PRD: proline domain
RB: reticulate body

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

STI: sexually transmitted infections

TARP: translocated actin recruiting protein

WT: wild-type
INTRODUCTION

*Chlamydia trachomatis* LGV 2

*Chlamydia trachomatis* is a gram-negative, intracellular bacterium which replicates exclusively within mammalian host cells [10]. This microorganism falls under the genus of *Chlamydia* and is among one of the three major chlamydial species recognized within the scientific community to cause human diseases. The other 2 species are *Chlamyphila pneumonia* and *Chlamyphila psittaci* which have their pathogenicity geared towards the respiratory systems, whereas *C. trachomatis* infections are more commonly found in the reproductive and ocular systems [7, 8]. The infection may be contracted through the mucosal membranes, smooth muscles, and epithelial cells. The severity and persistence of the infection is partially determined by the location of the infection [9]. Details of the infectious process will be covered later in the “Developmental Cycle and Infectious Process” section. The bacterium of interest within this study is further broken down into biovars and serovars, which are trachoma and LGV 2 respectively [7]. Another distinguishing characteristic of *Chlamydia* is that it harbors a plasmid of 7.5 kilobase pairs and one complete developmental cycle takes approximately 48 hours *in vitro*. This plasmid will be the main focus of my study [6, 11].

Epidemiology

*Chlamydia trachomatis* is the leading cause of preventable blindness worldwide and infertility in both males and females in the nation [1, 2]. The infection may result in severe inflammation or remain dormant for an extended period of time. A non-discriminatory pathogen, populations of all gender and race has an equal chance of contracting the disease. The disease is
most prominent among sexually active adults and among populations in less developed communities that have a poorer standard of hygiene. Sexually active patients with chlamydial infections are often asymptomatic, and are at risk for infertility due to infections in the genitourinary tract. Repeated infections or chronic infection in woman can lead to pelvic inflammatory disease (PID) and eventually infertility [1]. In the year 2010, about 1 million cases of chlamydial infections were reported nationwide but may have been grossly underestimated due to the absence of symptoms in people who are infected. The greatest occurrence of infection is observed in the African American and Native American populations. *Chlamydia* is the most common type of bacterial sexually transmitted infections (STI) within the United States and Japan [13].

**Developmental Cycle and Infectious Process**

Figure 1. The Developmental Life Cycle of *Chlamydia trachomatis*.

The bacterium first makes contact with the host cell in their infectious form, called elementary bodies (EB). Once these pathogens enter through the cell membrane with the help of effector proteins, such as TARP, they
are enclosed within a vesicle called an inclusion body. Within the inclusion body, the EBs differentiate into their metabolically active form called reticulate bodies (RB) and begin to replicate. After several rounds, the RBs revert back into EBs and leave the host cell through either exocytosis or cell lysis and will go on to infect the nearby host cells.

As shown in Figure 1, the obligate intracellular prokaryote *C. trachomatis* is present in one of two developmental forms. During the infectious phase, the bacteria are called the elementary bodies (EB) and during the noninfectious phase they are called the reticulate bodies (RB) [5]. Based on past *in vitro* studies, it was shown that the effector protein Tarp (translocated actin recruiting protein) played an important role in allowing *Chlamydia trachomatis* to enter host cells [3]. Tarp has 3 characterized protein domains which consist of an actin binding domain (ABD), phosphorylation domain (PhosD), and a proline rich domain (PRD), as depicted in Figure 2 [3, 4].

![Figure 2. TARP’s ABD is specialized for G-actin and F-actin binding.](image)

In a recent *Chlamydia* publication, of which I was a co-author, our lab provided evidence that TARP has a very diverse ABD. In further TARP protein assays, it was shown that TARP caused nucleation and bundling of different forms of actin, which includes globular and fibrous actin.
The ABD and PRD are critical for Tarp mediated actin nucleation which is believed to be required for invading host cells. Actin polymerization results in the formation of pseudopods which extend around the bacterium and engulf it into the cytoplasm. Intracellular *C. trachomatis* is enclosed within an endosome, called an inclusion body. Within this protective niche, the bacterium transforms from an EB to an RB to replicate within the host cell [3]. At the end of the developmental cycle, RBs convert back to EBs and are liberated from the host cell either through exocytosis or cell lysis to infect new cells [5].

*Chlamydia trachomatis* appears to have evolved over time through a close relationship with the eukaryotic host and consequently has acquired the ability to manipulate the host signal transduction cascades to their benefit. Pathogenicity of the bacterium lies in the fact that they are often shielded from the adaptive immune system of humans because of the sanctuary that they have found in inclusion bodies. These structures allow them to evade opsonization, neutralization, and T cell recognition. Moreover, after *C. trachomatis* has invaded the host cell, they express proteins that elicit transcription factors, such as NF-κB, which signals the recruitment of cellular proinflammatory chemokines and inhibits apoptosis of the infected cell [9]. These elicited chemokines will cause inflammation of the infected tissues within the host which will eventually lead to diseases previously mentioned. Since the bacterium lacks muramic acid within the peptidoglycan cell wall, treatment with penicillin is theoretically futile. However, when penicillin is administered, the developmental cycle comes to a halt even though the bacteria are still viable. Stronger antibiotics, such as azithromycin, are more effective at eliminating the infection [10].
Oftentimes the infection can be cleared out on its own because *Chlamydia* within epithelial cells is more prone to be removed from the body when the cell undergoes the transition phase of shedding off in order for new cells to rise to the surface. Another possibility is clearance through the body’s adaptive immune response which has been shown to be tied to CD8+ cytotoxic T cells, IgA, IFN-γ, and T_{H1} CD4+ T cells. However, *C. trachomatis* has been shown to manifest an immune evasion strategy which is through the expression of serovar specific antigens within the genus and species. This diversity limits our body’s ability to develop immunity to all *Chlamydia* serovars/biovars resulting in new infections or reinfections of *C. trachomatis* [9].

**DNA technology**

Studies performed on *C. trachomatis* have been limited due to the absence of DNA technology available to manipulate and experiment with the bacterial genome. Only recently, a new protocol has been devised by the lab of Dr. Ian Clark that allows for the transformation of DNA into *Chlamydia* [12]. This new breakthrough is a major stepping stone towards understanding the functionality of different genes that may play an important role in pathogenicity. The transformation system also allows for new genes to be introduced into the bacteria for the first time [7].

**Hypothesis:** Expression of epitope tagged Tarp effector mutants from within *C. trachomatis* will identify those protein domains required for bacterial entry and development.

Genetic manipulation of *C. trachomatis* has proven difficult, but recently a transformation system has been developed by Dr. Ian Clark’s lab to transform foreign DNA into
the bacteria. The goal of this study is focused on using this new technology to express epitope tagged genes in *C. trachomatis* [12].

In addition, shuttle vectors have been created in my past experiments which will enable for chlamydial and foreign genes to be expressed in both *E. coli* and *C. trachomatis* with ease. The genes transformed into these bacteria allows for it to be selected through resistance to a specific antibiotic. The application of this method will be further elaborated in the sections to come.
PURPOSE

The primary focus of my study is on the creation of a plasmid that will carry the genetic sequence that expresses the *C. trachomatis* Tarp effector. In order to distinguish between the endogenous Tarp expressed on the bacterial chromosome and the Tarp from the plasmid, Tarp will be engineered with an epitope tag that can be detected with monoclonal antibodies. The significance of this study lies in the fact that the expression of epitope tagged *C. trachomatis* effectors has never been pursued before because a transformation system for this prokaryote was not present until recently. If shown to be successful, this project will potentially lead to a better understanding of how chlamydial effectors mediate bacterial entry into host cells. The clone will then be used for creating Tarp mutants in *Chlamydia trachomatis* in order to determine if a phenotype can be observed from introducing a second copy of Tarp into the bacterium. Therefore, this study can be perceived as a “tool-building” step on the road heading towards revealing the ways to knockout genes within *Chlamydia trachomatis*.

Another objective of this study is to highlight the importance of the unpublished work completed in the past within my lab and how these results have played a great contributive factor towards accomplishing my desired tasks and the projects to be pursued in the future. In this paper, various significant experiments would be discussed, such as the impact of the creation of truncated wild-type chlamydial plasmids, the developmental cycle of *Chlamydia trachomatis* observed over time under the persistent exposure of antibiotics, and how actin nucleation changes with respect to the interaction between actin and various forms of mutant TARP present. These various projects have subsequently provided the stepping stones towards the success of my study.
METHODS

The shuttle vector used in our studies was comprised of an *Escherichia coli* origin of replication and the β-lactamase gene from pBR328, the entire *C. trachomatis* plasmid sequence, and an Inc-D promoter that was originally used in past experiments to express DsRed and CAT proteins. In this study, the Inc-D promoter, DsRed, and CAT genetic sequence were removed from the chlamydial vector (Figure 3).

![Diagram](image)

**Figure 3. Shuttle Vector and Potential New Mutations Induced.**

The *C. trachomatis* shuttle vector pCtSV.1 was adapted to allow for the expression of c-Myc tagged Tarp by the tarP promoter (tarP). In frame deletions were incorporated into the tarP gene to encode for g-actin
binding domain (pCtSV.TarpΔABD) and f-actin binding domain (pCtSV.Tarp ΔFAB1&2) deletions respectively.

The TARP promoter and TARP genetic sequence were amplified from pGEM-3Z using primers carrying the sequence for the c-Myc tag epitope (Figure 4). The PCR product was used as the insert and ligated to the vector afore mentioned. The new constructed plasmid was first transformed into *E. coli* (DH5α cells) and selected through ampicillin resistance. A miniprep was performed on the viable colonies and the plasmids were retrieved, confirmed, and sequenced.

**Figure 4. Plasmid for Tarp promoter + Tarp protein Amplification.**

The *C. trachomatis* Tarp effector genetic sequence along with the Tarp promoter will be amplified from a pre-designed pGEM-3Z+9kbTARP plasmid. The insert will be incorporated into the pCtSV.1 shuttle vector and it will replace the DsRed and CAT genes along with the IncD promoter.
This newly, constructed TARP plasmid with an epitope tag was used as the template for creating TARP mutants. Specific sequences were deleted in major TARP domains, in which past

Figure 5. TARP molecular structure and actin protein interactions of WT vs. mutants.

A. The wild-type TARP effector protein is divided into 3 major domains called the phosD, PRD, and ABD, which is further separated into fibrous and globular actin binding domains. The molecular structure of TARP mutants are shown in comparison to the WT sequence with specific domains removed. B. The TARP protein expressed from each mutant are run on an SDS-PAGE and their size corresponds to the amount of sequence that was removed from the original encoding sequence. Confirmation of the specific deletions of each domain is done through the use of monoclonal AB that have an affinity for the particular domain that is removed. C. The effects of WT TARP and mutant forms on the nucleation of actin. D. A shuttle vector was created to coexpress the GFP and Tarp protein, along with other Tarp mutants as shown in image A. This vector when transfected into Hela cells did indeed express both proteins. GFP fused Tarp appeared more
clustered inside the cell. When actin AB are placed onto these cells (as shown in second column), the fibers turned red and under the merged view it showed that even when the ABD was absent, actin was still nucleated. This led to the discovery of G-actin and F-actin binding domains [4].

research has shown to possibly play a key role in host cell invasion. The domains that have been noted for importance were the phosphorylation domain (phosD), proline domain (PRD), G-actin binding domain (ABD), and the F-actin binding domains (FAB 1 and 2) (Figure 2, 5). As shown in Figure 5, through extensive protein biochemical assays, TARP was implicated for playing an important role in host cell actin nucleation. Most importantly, the coexpression of both GFP and mutant TARP protein enabled for the presence of different actin binding domains to be determined. Of the various domains, previously discussed, the first mutant TARP pursued was the deletion within the phosphorylation domain. The mutant TARP insert was digested from a previously created plasmid called the GSTRecA + His6 pGEX-6P-1 and was ligated to the epitope tagged vector (Figure 6). Other TARP mutants could potentially be created through the same process. The vector was transformed into DH5α cells in order to obtain a larger quantity of the plasmids.

The plasmids retrieved from DH5α cells were then transformed into ER2925 cells in order de-methylate the DNA and made them compatible in C. trachomatis. The retrieved and confirmed plasmids were transformed into C. trachomatis for expression. An intriguing aspect of the application of the new transformation system was that in our study we were able to select for our specific transformant through the use of the penicillin resistance gene as the selectable marker. Since the resistant gene was only present in the wild-type epitope-tagged and mutant TARP plasmids, the only chlamydial pathogen that should survive were the bacteria that have been successfully transformed and carried our vector.
Figure 6. Plasmid carrying the TARP mutant sequence in the phosphorylation domain.

A segment of the TARP sequence that carries the complete deletion of the phosphorylation domain was taken from this plasmid. The digested insert later replaced the Phos domain sequence in the epitope-tagged plasmid.

Another technology that has allowed for detection of the newly introduced genes within the chlamydial system was antibodies that acted in ligand-protein interactions. An organized protocol was laid out to distinguish the expressed protein of interest with great sensitivity. In order to identify the protein, a primary antibody was used that carried strong affinity for the protein and a secondary antibody was applied to detect the presence of the bound primary. In
order to visualize these interactions, a chemiluminescent substrate was added that has an affinity for the secondary antibody. When exposed to UV radiation, the X-ray film would capture any trace of ligand-protein interactions present.

This new protocol was applied to detect the expression of the epitope tagged Tarp protein through the use of monoclonal antibodies that specifically recognized the c-Myc tag. In order to determine the presence of epitope tagged Tarp effector, the collected proteins from lysed *Chlamydia* were purified and separated on an SDS-PAGE protein gel. The protein gel was transferred onto a membrane in order to perform a Western Blot and from that membrane the c-Myc tag was detected with specialized antibodies that recognized the epitope. The initial primary antibody used was a c-Myc 9E10 monoclonal antibody that bound to the epitope. Afterwards, a secondary HRP conjugated antibody was applied that recognized the primary 9E10. A chemiluminescent substrate placed over the membrane produced light that was detected by X-ray film that localized the presence of the tagged Tarp on the transferred membrane.
RESULTS

Creation of Epitope-Tagged Wild-type and Mutant TARP

The c-Myc tag epitope and phosphorylation domain deletion were successfully introduced into the TARP shuttle vector. The presence of the epitope and absence of the PhosD were confirmed through sequencing, digestion, and PCR verification, as shown in Figure 7 and Figure 8 respectively.

Figure 7. Digestion and PCR confirmation of wild-type tagged TARP in retrieved plasmid.

Clones were retrieved from possible positive E. coli transformants that may carry the constructed vector with the full length, tagged TARP sequence. The presence of the genetic sequence was confirmed through the digestion of the mini-prep retrieved plasmid and PCR amplification using specific primers that recognized the TARP promoter and c-Myc tag sequence.
In Figure 7, the second and third lane are bands representing the exact size of the chlamydial vector backbone and tagged-TARP insert that should be present if epitope-tagged TARP was indeed successfully transformed into \textit{Escherichia coli}. A closer look at Figure 7, the fourth and fifth lanes show the relative size of two digested plasmids, labeled 5a and 5b, which were retrieved from transformed ampicillin resistant \textit{E. coli} clones. These two digestions were each separated into two bands that lined up perfectly with the vector and insert in the previous two lanes. Therefore, there is great possibility that these bands represent the segments in the desired plasmid. The last 2 lanes display the PCR verification reactions in which the TARP protein is amplified from the two clones. From this figure, it can be observed that TARP and the epitope tag were successfully introduced with great certainty; sequencing confirmed their presence and the reading frame was correct.

Figure 8. Digestion and PCR confirmation of mutant TARP from retrieved plasmids.

Clones were retrieved from possible, successful \textit{E. coli} transformants that may carry the mutant-TARP sequence with the phosphorylation domain removed. The plasmids purified from the clones through
miniprep were digested with restriction enzymes specific for the beginning of the TARP promoter and directly after the c-Myc tag. PCR amplification of the plasmids were also performed using primers that recognized the promoter and epitope sequence.

In Figure 8, the second, third, and fourth lanes are the digestions of 3 plasmids retrieved from potential *E. coli* transformants that may carry the PhosD TARP mutant sequence. In all 3 plasmids, the two bands that dropped out appeared to line up with one another which could possibly mean that the 3 clones all carried the same plasmid. In the fifth lane is the digestion of the original epitope-tagged plasmid from the former experiment with the two bands showing the size of the wild-type tagged-TARP sequence and the chlamydial vector backbone. In the 3 former lanes, the larger band lined up with the fifth lane’s vector backbone but the inserts were different in size as expected. The 3 former lanes showed a much smaller insert compared to the fifth lane because the TARP sequence had the phosphorylation domain removed. As a result, the insert in the wild-type epitope TARP is larger in size and appears higher on the DNA ladder. The 6th-8th lanes, on the other hand, are PCR reactions performed on the plasmids of the 3 clones. In the image, for all 3 lanes, mutant TARP was amplified which matched the size of the digested insert in lanes 2-4. In the last lane is the PCR amplification of wild-type TARP and the size of the band is the same as the digested insert in lane 5.

The expression of the mutant TARP from the shuttle vector inside *Chlamydia trachomatis* was determined through antibody-protein interactions. PhosD mutant TARP expression was detected from the host-bacterial lysate. The result of the protein assay is found in Figure 9. In the immunoblot image captured, the 4th lane shows anti-TARP antibody interaction against the TARP protein expressed from the shuttle vector labeled L2+ pCTSV.TarpΔphos. However, in this lane 2 bands were visible on the immunoblot because the Tarp antibody not
only detected the wild-type TARP expressed on *Chlamydia*’s genomic DNA but also the mutant TARP expressed off of the engineered plasmid. This is most apparent when comparing the tagged-mutant protein against the expression of the original genomic TARP (L2) and wild-type shuttle vector TARP (L2+pCtSV.1) which shows only one band of Ag-AB interaction. Also, when these latter samples and mutant TARP were exposed to anti-c-Myc AB, no bands appeared for L2 and L2+pCtSV.1 because these proteins lacked the epitope tag. The mutant phosD TARP (L2+ pCTSV. TarpΔphos) clone, however, did show expression as expected.

**Figure 9. Detection of Tarp and c-Myc protein expression with Tarp and c-Myc AB.**

The protein lysate retrieved from *Chlamydia*-host infections were purified and separated by SDS-PAGE. The gel was transferred over to a membrane to conduct ligand-protein binding assay with TARP and c-Myc epitope acting as the ligand for detection with their respective monoclonal antibodies.
Truncating the Wild-type Chlamydial Plasmid

One of the previous chlamydial projects that I have performed was devoted towards creating deletions within the wild-type chlamydial plasmid found within the pathogen itself. The purpose of the project was to truncate the bacterial plasmid to determine if the transformation efficiency into *E. coli* and *C. trachomatis* could be improved. With such a large plasmid, it posed as a restriction on the size of the foreign gene that can be introduced into the prokaryote. Moreover, the results of transformation into the pathogen could reveal which segment within the plasmid carried an important factor in the developmental cycle of the bacteria. Potentially, these truncated plasmids could be used as the templates within chlamydial transformations instead of employing the complete WT plasmid sequence as the vector backbone which is very large and can hinder transformation efficiency.

The project was carried out by first starting out with a plasmid our lab has engineered called pCtSV.1 (*Figure 10*). The shuttle vector possessed an IncD promoter that controlled the expression of DsRed and CAT proteins and ligated to it was the complete chlamydial plasmid sequence. The shuttle vector, therefore, served as the template and parts of the complete bacterial sequence of different sizes were slowly taken away. As depicted in *Figure 10*, when the truncated constructs were digested with the same restriction enzymes, the same vector backbone is observed in all samples but the size of the insert that drops out varied depending on the amount of DNA bases that have been removed from the chlamydial sequence.
The digestion observed above is the result of the truncations of the original chlamydial plasmid. The original template (found in Figure 5) carried the complete bacterial sequence, an *E. coli* origin of replication, β-lactamase gene, Inc D promoter, and genes encoding DsRed and CAT proteins. The *E. coli* origin of replication (Ori), as well as the inherent chlamydial Ori, allows for the plasmid to shuttle between two different transformation systems. In this experiment, segments of the original chlamydial sequence were slowly removed to form different size of plasmids to determine if transformation efficiency can be increased if the size of the shuttle vector is reduced.

The 4000 base pair band observed in all five digestion reactions was the original backbone, which carried the genetic sequence for the *E. coli*’s origin of replication, β-lactamase gene, IncD promoter, as well as the DsRed and CAT genes. In the first plasmid, P1, retrieved from a transformed clone, about 6,000 base pairs were removed from the chlamydial plasmid sequence. Therefore, P1 carried an insert close in size to 1,000 base pairs. For clones P2 and P3, about 5,000 and 3,000 base pairs were removed respectively from the original 7,500 base pairs. Taking
a closer look at the P3 digestion reaction, the DNA gel shows a slight overlap to two DNA bands which was expected because the vector backbone was similar in size to the chlamydial insert. In clones P4 and P5, about 2,000 and 1,000 base pairs were removed respectively from the template.

**Creation of Antibiotic Resistant *Chlamydia trachomatis***

The plasmid pCtSV.1, a crucial shuttle vector employed within this study, was created from a previous unpublished project which allowed for the DNA to have the ability to replicate within *Chlamydia trachomatis* and *Escherichia coli*. Moreover, this plasmid provided the antibiotic selectable marker, which was crucial in this project, through the organism’s growth and resistance to ampicillin treatment. When this shuttle vector was shown to be successfully replicated and expressed within *E. coli*, the plasmid was transformed into *C. trachomatis* and an observable phenotype was recorded. The phenotype studied was progeny counts, based on the presence of inclusion bodies. The assumption made in the study was that only one elementary body (also called infectious units), with proper dilutions, would enter only one host cell and create one inclusion body. Therefore, when cells lyse and new infections occur, the number of EBs (IFUs) were determined indirectly through the number inclusion bodies that results after the lysis.

In this project, two types of plasmids were used to transform into *C. trachomatis* which included the shuttle vector pCtSV.1 and the wild-type plasmid originally found within the pathogen. The developmental cycles of the two transformations were observed over a 102-hour period under two separate conditions. As shown in **Figure 11**, the WT *Chlamydia* and transformed pCtSV.1 clone were both treated with and without penicillin after they have infected
host cells. The effects on progeny counts were then determined through inclusion bodies count. When the quantity of IFUs were recorded and plotted onto a logarithmic graph, the data displayed that there was no significant changes in progeny counts between the pCtSV.1 clones whether it was grown in the presence or absence of penicillin. The wild-type clone, however, was not able to thrive and grow in the presence of penicillin because these clones did not possess the β-lactamase gene. When the WT was grown in the absence of penicillin, on the other hand, they grew rapidly in the same manner that was observed with the pCtSV.1 transformants.

![Graph showing IFUs/mL over time](image)

**Figure 11.** *C. trachomatis* transformed with the chlamydial shuttle vector pCtSV.1 confers penicillin resistance.

*C. trachomatis* LGV L2 (wild type: WT) was transformed with the chlamydial shuttle vector and a transformed clone was isolated after multiple developmental cycles in the presence of penicillin (pCtSV.1). The presence of the shuttle vector was confirmed following DNA isolation and recovery of the plasmid in *E. coli*. The growth curve shown represents the infectious EBs (IFUs) recovered from McCoy cells infected with our wild type (WT) and transformed clone (pCtSV.1) in the presence or absence of penicillin. EBs were recovered every 6 hours from 24 to 102 hours post infection. The number of infectious EBs harvested was determined by the introduction of recovered material to new McCoy cells and inclusions were counted after 24 hours.
The presence of the inclusion bodies was visualized through the application of an immunofluorescent assay (IFA). In this assay an anti-L2 EB antibody was used to bind to EBs present within the inclusion bodies. A secondary antibody was then used to detect this primary AB and the one used was Alexa 488 which allowed for visualization of EB’s at a particular wavelength. The image in Figure 12 supports the idea that, under penicillin treatment, WT cannot grow whereas the pCtSV.1 clone would not have any problems.

![Figure 12. IFA of penicillin resistant C. trachomatis transformants.](image)

*C. trachomatis* EBs were transformed with shuttle vector pCtSV.1 (+ DNA) or mock treated (- DNA) according to the protocol developed by Dr. Ian Clarke [42]. One developmental cycle (one cycle equals approximately 2 days) occurred in the absence of antibiotics followed with 4 developmental cycles (4 passages) in the presence of penicillin. For each passage, approximately 10% of the preparation was added to a 24 well plate containing fresh McCoy cells and chlamydial inclusions were detected after 24 hours with an anti-L2 EB antibody and Alexa 488 conjugated secondary antibody. No *C. trachomatis* inclusions were detected from the mock transformed bacteria after two passages with penicillin.
DISCUSSION

From the data gathered, it is evident that a model is now present with the potential of introducing mutations into the chlamydial genomic DNA. Never before has a copy of an endogenous gene been successfully tagged and expressed within the chlamydial system on a separate plasmid. This leads the way towards enabling the Chlamydia scientific community to gain one step closer towards creating a gene knockout within the pathogen and determining the function of each gene. Not only can C. trachomatis now be tagged with a selectable marker bound to a protein of interest but also selected through ampicillin resistance because it carries the gene necessary to survive the antibiotic treatment.

The transformation with the delta Phos Tarp, in addition, has shown to have a greater turnover rate in entering the bacterial pathogen which may implicate that this domain is not vital to the invasion process of C. trachomatis. The deletion of this domain, on the contrary, may enable the prokaryote to become more pathogenic. These speculations, however, need to be furthered analyzed in future assays in contrast to the wild type Tarp to test its validity.

Another major contribution that this study has provided is the characterization of the Tarp promoter sequence. In chlamydial literature the Tarp promoter has never been characterized; therefore, the scientific community is still unaware of the size of the gene and how much of it is necessary for initiation of transcription. With the success of having expression of Tarp and c-myc epitope off of the plasmid that I have created, implicates that 200 base pairs of the Tarp promoter is sufficient for proper functioning. This promoter can now be employed in future studies to express other mutant forms of Tarp as well as other desired foreign genes. Although
my study focused primarily on the “tool-building” process, the success of this experiment is a major stepping stone towards gaining a better understanding of the chlamydial genome. This opens the path towards numerous possibilities to the future studies that may lie ahead.
FUTURE STUDIES

My future endeavors are aimed at creating more mutant Tarp constructs, using the wild-type tagged Tarp clone as the template. For instance, after the success with deletion of the phosphorylation domain, the other domains were left untouched. Therefore, mutations can still be made in the PRD, ABD, and FAB as well as combinations of the different deletions (Figure 5). The mutant TARP will potentially be introduced into the genomic DNA through recombination and the effects of a mutant gene can be observed through its phenotype. When a gene is either removed or introduced into a genome, there may be unprecedented effects on the organism’s pathogenicity, development, and viability. All of these various changes can be tracked and studied through the use of invasion assays, developmental assays, and progeny counts respectively. Since the tagged-effector TARP has been mutated, once it undergoes allelic exchange with the genomic TARP, it is very likely that *Chlamydia trachomatis* may have a diminished infectiousness, a retarded growth rate, and decreased amount of progeny. However, these are only mere speculations that will require further experimentations to confirm their accuracy.

Moreover, the truncated plasmids that were previously created in a past experiment could be used for transforming the mutant Tarp with greater efficiency because a smaller plasmid enters the pathogen at a faster rate. In addition, in a recent study performed by the Wayne State University has provided some evidence that the transformation of *Chlamydia trachomatis* compounded with dendromeres have the potential of increasing the success of introducing foreign genes into the bacteria by 80%. This new method will be implemented in the near future with my studies on transforming *C. trachomatis* with my new Tarp mutant genes.
REFERENCES


