CT226 and TMOD3 Interaction in *Chlamydia trachomatis* Infection

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ABSTRACT

Chlamydia trachomatis is the most commonly reported sexually transmitted infection in the United States. Infection, even after successful treatment, can lead to pelvic inflammatory disease, ectopic pregnancy, tubal infertility and increased risk of cervical cancer. The reasons for these medical complications are not very well understood, and neither is the mechanism of immune response to *C. trachomatis*. Insights into how our cells respond to infection by *C. trachomatis* could provide future therapeutic targets for the lasting medical complications. One of the Chlamydial proteins, CT226, has been found to interact with the host protein TMOD3. We used a yeast two-hybrid strategy to confirm the interaction between the Chlamydial protein, CT226, and the host protein TMOD3. TMOD3 is involved in actin recruitment within the cell, and is responsible for the structural integrity of both the cell and the inclusion. This interaction between CT226 and TMOD3 is a new *Chlamydia*-host interaction that could provide significant insights into how *Chlamydia* manipulates host proteins during infection. By studying this interaction, our goal was to be able to better understand the impacts that *C. trachomatis* has on the host cell, and this could allow for a better understanding of how we could treat the ongoing symptoms of infection. While confirming the interaction between these two proteins through mating experiments, we found that there appears to be a weak interaction between the two proteins.

1. Introduction

Chlamydia trachomatis is the most commonly reported sexually transmitted infection in the United States, and it is a leading factor in female infertility (CDC, 2014). It affects an estimated 3 million people annually but does not frequently produce recognizable symptoms, which can lead to further spread of the infection. *Chlamydia* symptoms can include pain when urinating and pelvic pain. Due to the rare occurrence of noticeable symptoms upon infection, most cases are often diagnosed after severe damage has occurred to the reproductive tract. Even after successful treatment, damage caused by Chlamydial infection can lead to pelvic inflammatory disease, ectopic pregnancy, tubal infertility and increased risk of cervical cancer (Becker, 1996). Pelvic inflammatory disease affects the female reproductive tract, and can be the cause of abdominal pain and other symptoms, and left untreated can cause the formation of scar tissue in and around the fallopian tubes that can lead to infertility, ectopic pregnancy, and long-term abdominal pain. Ectopic pregnancy occurs as the fertilized egg implants outside of the uterus, most commonly in a fallopian tube. If ectopic pregnancies are not caught early enough, this could cause the fallopian tube to rupture, and in most cases the fetus does not survive, and the mother's life is put at risk. Chlamydial infection originates at the cervix, which also puts the woman at a higher risk for cervical cancer.

The reasons behind these medical complications even after treatment of *Chlamydia* are not very well understood, and neither is the mechanism of immune response to *C. trachomatis*. *C. trachomatis* is an obligate intracellular pathogen that is unable to create its own energy, and therefor relies on the host cell to provide the necessary nutrients for cell metabolism (Becker, 1996). *C. trachomatis* replicates inside of the host cell, in an inclusion—a membrane-bound compartment within the cell—and releases inclusion membrane proteins, or Incs, which control host-pathogen interactions across the inclusion (Mirrashidi, 2015). Once the Chlamydial cells have replicated inside the inclusion, they are released from the host cell, and can now infect nearby cells within in the host organism (Elwell, 2016). CT226 is an Inc that recruits certain host proteins to a specific region on the inclusion membrane (Olson, 2019). The previously mentioned study by Mirrashidi (2015) explores the Inc-human interactome of *Chlamydia* infection, and suggests that there is an interaction between CT226 and four host proteins, including TMOD3. TMOD3, when present in an uninfected cell, typically blocks the elongation of actin filaments at the pointed end (Fisher, 2006).

The present study uses the yeast two-hybrid protocol to study the interaction between a Chlamydial Inc and a host protein. The yeast two-hybrid system consists of separately transforming two separate sets of yeast cells. The first is transformed with a plasmid of

interest, in this case encoding the Chlamydial protein CT226, and the second is transformed to contain the other protein of interest, TMOD3. The two separate yeast strains are then mated through a set of experiments that fuses bait and prey to other protein fragments containing transcription factors that allow us to visualize whether the proteins have interacted by plating the strains on selective media so that the system is indicative of the strength of interaction of our bait and prey. We then determine the strength of the interaction using techniques such as a Western Blot (Makuch, 2014).

2. Experimental Details

Preparation of Yeast Strains: To visualize the interaction between CT226 and TMOD3, we used the yeast two-hybrid protocol from Clontech. First, we grew the yeast on YPAD plates. This was accomplished by quadrant streaking the yeast strains AH109 and Y187 onto freshly made YPAD plates that were incubated overnight at 30^{o}C. The yeast strains were then each individually transformed, using the Frozen-EZ Yeast Transformation II Kit (Zymo Research), to contain their respective plasmid DNA- AH109 contains the bait, pGBKT7-CT226, and Y187 contains the prey, pGADT7-TMOD3. After transformation, each was grown for 2-3 days on dropout plates (plates lacking specific amino acids) at 30^{o}C. AH109 transformations were grown on SD-Trp plates, meaning that they lack the amino acid Tryptophan. Y187 transformations were grown on SD- Leu plates that lack the amino acid Leucine. This was done to ensure that the cells were properly transformed, because only cells that contain the plasmid DNA would have the ability to grow on plates without the specific amino acid. This process was also completed with several controls with known growth expectations later in the experiments, AH109- pGBKT7-53 and AH109-pGBKT7-Lam, Y187-pGADT7-T and Y187-pGADT7-AD. These transformed cells were re-plated on their respective dropout plates and used for mating experiments.

Yeast Mating Experiments: To perform the mating experiments, a few colonies from each plate were incubated in their respective liquid dropout medias overnight at 30^{o}C. These overnight incubations were then used to start the mating experiments. To do this we placed 500uL of YPAD+, 100uL of bait (AH109), and 100uL of prey (Y187) were added into a 1ml tube and incubated at 30^{o}C overnight. These overnight incubations were then plated onto SD-TL plates (plates lacking both Tryptophan or Leucine, which selects for yeast diploids), SD-TLH (plates lacking Tryptophan, Leucine, and Histidine, which selects for mutants that contain an activated transcription factor that allows for growth on further selective media), and SD-TLHA (plates lacking Tryptophan, Leucine, Histidine, and Alanine, which selects for mutants that contain two activated transcription factors that allow for growth on even further selective media).

Preparation of Cell Extracts For protein collection, we grew overnight cultures using one isolated colony from each intended strain. This overnight culture was used to inoculate fresh media with the entire overnight culture. This inoculation was incubated until the OD_{600} reached 0.4-0.6, which takes about 4-6 hours. After the ideal OD_{600} was obtained, the culture was chilled and centrifuged for 5 minutes at 1000xg. The supernatant was poured off, and the pellet was re-suspended in ice-cold H_2O followed by centrifugation at 1000xg for 5 minutes. The resulting pellet was quickly frozen using liquid nitrogen, and stored at -80^oC overnight. A cracking buffer was freshly prepared and warmed to 60^oC. This prewarmed cracking buffer was used to resuspend the pellet and quickly thaw the cells. Next, we add tiny glass beads to the cell suspension and 80 uL per 7.5 OD_{600} units in a 1.5 mL tube. This tube was heated at 70^oC for 10 minutes and then vigorously vortexed for 1 minute. The tube was centrifuged at 14,000 rpm for 5 minutes and the supernatant was removed and transferred into a fresh 1.5 mL tube on ice. The pellet was placed in a boiling water bath for 3-5 minutes, vortexed for 1 minute, and centrifuged again at 14,000 rpm for 5 minutes. The supernatant was poured off into the tube containing the previous supernatant and then boiled briefly. The samples were ready to be loaded onto a freshly prepared SDS-PAGE.

SDS-PAGE and Western Blot: After casting the SDS-PAGE gel, the protein samples were thawed. Ten uL of protein ladder and 20 uL of each sample was loaded into individual wells on the gel. The gel was electrophoresed at 125 V for 1.5 hours. The resulting protein gel was transferred to a nitrocellulose membrane through electro-blotting by running at 100 V for 1 hour. The resulting blot was blocked with a 5% milk solution in TBST and incubated at room temperature for 1 hour. After rocking for 1 hour, the milk was poured off and the membrane rinsed with 1x TBST buffer three times for 5 min each. Primary antibody to Gal4AD (1:1000 dilution) and Myc tag (1:100 dilution)) for detection of prey and bait fusion proteins respectively, was added to the blots and incubated for 1 hour. This was followed by another 3 washes of 1x TBST. Five mL of milk and 5uL of the anti-rabbit/mouse secondary antibody (conjugated to Horse radish peroxidase) was added to the membrane, incubated for 1 hour followed by 3 more washes in 1x TBST. The TBST was poured off and the substrates, Signalfire ECL reagents, were added to the membrane and left to sit for 5 minutes and the Western Blot images were acquired using Fluorchem E FE0622 system (ProteinSimple).

3. Results

All yeast matings showed growth on SD-TL, the growth control. However, only the positive control, pGBKT7-53 with pGADT7-T presented growth on any of the other two plates, SD-TLH and SD-TLHA. These experiments were repeated several times, but the results remained the same, all matings showed growth on the SD-TL plates, but there was

no growth on either the SD-TLH or SD-TLHA plates, which indicates that the bait and prey were not interacting to active the transcription factors that allow for growth at higher stringency. However, through the project we found that strain Y187 transformed with pGADT7-TMOD3 grew very slowly, and we suspected TMOD3 may be interacting with the yeast actin complexes which in turn was inhibiting growth. The lagging growth of strain Y187 harboring the pGADT7-TMOD3 construct can be seen in Figure 1.

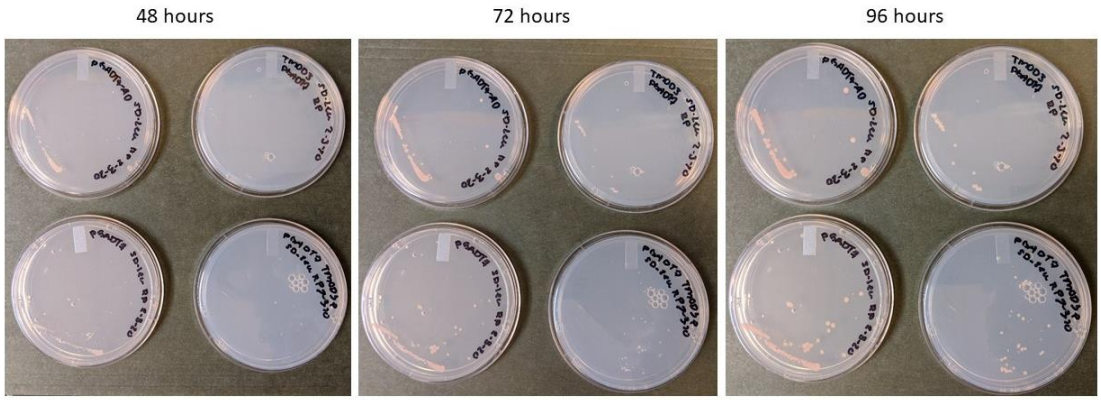

Figure 1: Growth comparison of yeast constructs. Shown is the relative slow growth of Y187 pGADT7-TMOD3 in comparison with Y187 pGADT7-T and Y187 pGADT7-AD. Even after incubation for 96 hours, Y187 pGADT7-TMOD3 (the two plates on the right in all 3 pictures) shows very little growth, while the other transformations showed growth after 48 hours.

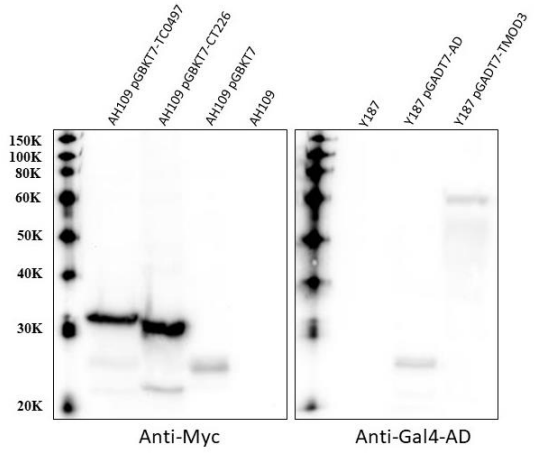

Figure 2: Western blot analysis of protein expression in yeast. Shown is the Western Blot of AH109 pGBKT7-C0497, AH109 pGBKT7-CT226, AH109 pGBKT7, and AH109 and similarly, Y187 pGADT7-AD and Y187 pGADT7-TMOD3.

We checked the expression of TMOD3, using a Western Blot, to verify that the proteins were being expressed the yeast strains. The slow growth of the Y187 transformed with TMOD3 raised concerns about whether there might be expression issues. We checked the expression of the other proteins to ensure that the transformations were effective, and to verify that these strains would be effective controls (the baits are tagged with Myc and the preys are tagged with Gal4-AD). Shown in Figure 2 is the Western Blot of AH109, AH109 pGBKT7-CT226, AH109 pGBKT7, and AH109 pGBKT7-TC0497 and similarly, Y187, Y187 pGADT7-AD and

Y187 pGADT7-TMOD3. This Western Blot shows the relative abundance of each expressed protein, which is very close to our expectations. Although Y187 pGADT7-TMOD3 is present, it is a relatively very low abundance as compared to the other strains.

To troubleshoot the slow growth of yeast cells transformed to contain TMOD3, we had to transform yeast to contain a truncated version of TMOD3. We used the truncated versions of TMOD3 to attempt to minimize the interference that TMOD3 had on cell growth. In this approach, we cloned TMOD3 into the pGADT7-AD plasmid in two separate halves, pGADT7-TMOD3 TM cap (containing the first 181 amino acids), and the pGADT7-TMOD3 LRR cap (containing the last 178 amino acids), depicted in Figure 3. The resulting plasmids were transformed into the Y187 strain.

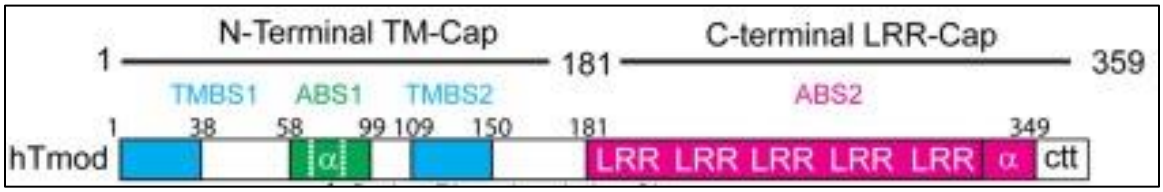

Figure 3: Schematic Representation of TMOD3. Shown above is an illustration of the TM and LRR caps of TMOD3 that were transformed into the Y187 yeast strain. The TM cap contains the first 181 amino acids of the TMOD3 protein, including the transmembrane domain, and the LRR cap contains the last 187 amino acids, the Leucine-rich-repeat. Figure adapted from Parreno *et al*., 2018.

We hoped that this would allow us to minimize the possible toxicity of TMOD3 to the cells, but still test the interaction of this specific protein. We attempted to use a Western Blot to verify that we could detect the proper expression in the protein samples in the TMOD3-TM and TMOD3-LRR caps, but unfortunately, the western blot was not successful, due to technical error, and we were not able to verify this. Due to this complication, those specific protein interactions would need to be verified in future studies.

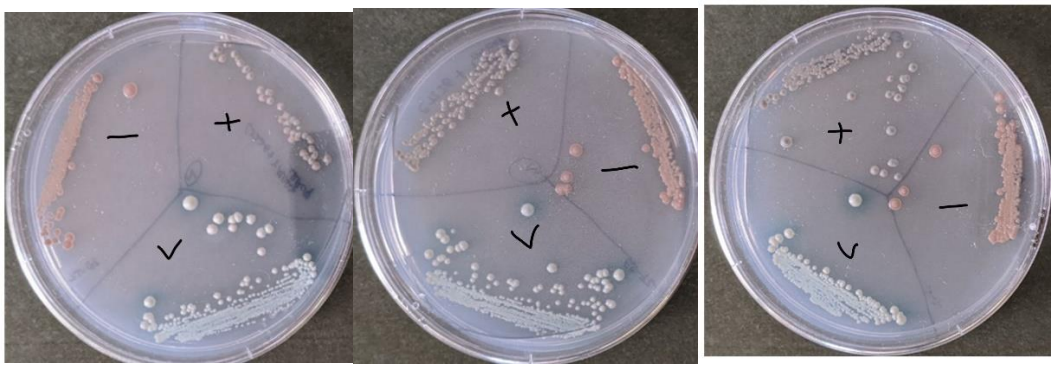

Figure 4: Growth comparison of mating experiment. Shown are the yeast matings plated on SD-TL plates containing X-Gal. Each plate contains both pGADT7-T + pGBKT7-Lam as a negative control (presents as pink colonies), and pGADT7-T + pGBKT7-53 as a positive control (presents as blue colonies). On the left, the variable strain is pGBKT7-CT226 + pGADT7-TMOD3 LRR cap (presents as blueish-grey colonies). In the middle, the variable strain is the pGBKT7-CT226 + pGADT7-TMOD3 TM cap (presents as blueish-grey colonies). On the right, the variable strain is pGBKT7-CT226 + pGADT7-TMOD3 (presents as blueish-grey colonies).

On each SD-TL plate shown above, the positive control- pGADT7-T + pGBKT7-53 presented blue colonies, showing that it does metabolize X-Gal, a substrate that, when added to these plates, turns colonies blue if they contain Beta-Galactosidase, and are therefore able to metabolize the substrate. Likewise, on each SD-TL plate shown above, the negative control- pGADT7-T + pGBKT7-Lam presented pink colonies, showing that it cannot metabolize X-Gal. In the picture on the left, the variable strain is pGBKT7-CT226 + pGADT7-TMOD3 LRR cap, and when left for 2-3 days, it presents blueish-grey colonies, which indicates that there is a slight interaction that allows these cells to metabolize X-Gal. The middle picture displays the pGBKT7-CT226 + pGADT7-TMOD3 TM cap strain, and also presents blueish-grey colonies, which indicates that there is a slight interaction that allows these cells to metabolize X-Gal. Last, the plate on the right displays pGBKT7-CT226 + pGADT7-TMOD3 as the variable, which also presents blueish-grey colonies, which indicates that there is a slight interaction that allows these cells to metabolize X-Gal. Each variant of TMOD3 presents these blueish-grey colonies that indicate that there is a weak interaction between the proteins CT226 and TMOD3.

As mentioned before, when mated and then plated on SD-TL, SD-TLH, and SD-TLHA plates, the strains that contained TMOD3, TMOD3-TM cap, and TMOD3-LRR cap did not grow on SD-TLH or SD-TLHA plates. This result indicates that there is not an interaction between CT226 and TMOD3, however, the SD-TL plates contained X-Gal and when left for 2-3 days, the growth on the SD-TL plates began to show slight blue coloration (pictured above, in Figure 4). The blue coloration of these cells indicates a slight reaction, because otherwise the cells would be unable to metabolize X-Gal.

4. Discussion

Our findings showed that CT226 and TMOD3 produce a weak interaction. This was shown through the addition of X-Gal to the plates that were used to visualize the mating experiments, SD-TL, SD-TLH, and SD-TLHA. At first glance, the mated cells did not grow on the triple dropout plates, indicating that the two proteins were not interacting, as this would produce the ability for the cells to grow in the absence of all three growth mediums. However, after left for a few days, the colonies on the plates began to turn

blue. This coloration indicates that the cells were able to utilize X-Gal, and shows that the proteins did have some interaction, although it was weak. The cells only showed this blue tint after being left for several days, which indicates the weak interaction. Although growth of TMOD3 was never obtained on SD-TLH or SD-TLHA, which would have indicated a stronger reaction, the growth that occurred on the SD-TL plates was able to metabolize X-Gal. This ability to metabolize X-Gal indicates an interaction because it is indicative of a recombinant protein. These cells would not be able to metabolize the substrate X-Gal if there was no interaction, so this is a good indication that our hypothesis was not completely incorrect. This study will be replicated in a bacterial two-hybrid to eradicate the error that was brought in by the use of yeast cells. Our hope is that in the bacterial two-hybrid system, these proteins will show a stronger interaction that does not interfere with cell function, such as growth, as we have seen in yeast cells.

5. Conclusions

Our results suggest that there is a weak interaction between CT226 and TMOD3, but unfortunately did not provide evidence of a strong/measurable interaction. This may be due to the TMOD3 protein interfering with the yeast actin machinery and that may have resulted in the slowed growth of the yeast. To verify the interaction between these two proteins, further work will be done in the lab. In the future, this study will be reproduced in a bacterial two-hybrid system, which uses bacterial cells instead of yeast cells, to better visualize the interaction. Our hope is that this system would eliminate the slow growth effect of TMOD3 because there is no longer any influence from other eukaryotic proteins. Furthermore, because yeast cells are eukaryotic, their cytoskeletons contain actin, which TMOD3 is known to bind (Parreno, 2018). We hypothesize that the probable interaction between TMOD3 and the yeast actin proteins is what resulted in the growth defect observed with pGADT7-TMOD3 in the yeast cells. This actin interaction is another reason why the project will be repeated in a bacterial two-hybrid system.

6. Summary

The interaction between CT226 and TMOD3 is a *Chlamydia*-host interaction that could provide significant insights into how *C. trachomatis* are manipulating host proteins during infection. Our goal was to be able to better understand the interactions that *C. trachomatis* has on the host cell and this could allow for a better understanding of how we could treat the ongoing symptoms of infection. While confirming the interaction between these two proteins through mating experiments, we verified that there is a weak interaction between CT226 and TMOD3 through the metabolization of X-Gal on the SD-TL plates. Although further experimentation will need to be done to verify these results,

the indication of a weak interaction supports our hypothesis that there is a direct interaction between CT226 and TMOD3 when infection occurs.

7. Appendices

7a. Papers Published

Planned to present research at the following (all canceled due to COVID-19):

- Annual Department of Microbiology and Molecular Genetics Symposium
- American Society of Microbiology, Missouri Valley Branch Meeting
- Oklahoma Research Day

7b. Acknowledgements

I would like to thank Dr. and Mrs. Niblack for providing the funds, and ultimately making this experience possible for me. I gained a deeper insight into the field of scientific research and even had the opportunity to work on a project that I believe will be beneficial to my future career as an OB/GYN. Over the past year I was able to obtain a greater appreciation for all of the work, and complications, that come along with scientific research. This study provided me with hands on experience in the field of microbiology, but it also allowed me to experience the reality that scientific discoveries do not happen overnight. We had several complications throughout this past year, but we were able to overcome them and complete our project. I learned endurance and patience from an academic standpoint and I would like to thank Dr. and Mrs. Niblack for the opportunity to have these experiences in such an exciting research field.

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