5-10-2017

The Search for Binding Partners of the Chlamydia trachomatis Phosphatase Cpp1

Lea Matschke
Southern Illinois University Carbondale, lea.matschke@siu.edu

THE SEARCH FOR BINDING PARTNERS OF THE CHLAMYDIA TRACHOMATIS PHOSPHATASE CPP1

Lea Matschke

A thesis submitted to the University Honors Program in partial fulfillment of the requirements for the Honors Diploma

Southern Illinois University

May 10, 2017

Abstract

Chlamydia trachomatis is a Gram negative, obligate intracellular bacterium responsible for the most common reportable bacterial sexually transmitted infection in the United States and the leading infectious cause of preventable blindness worldwide. This pathogen undergoes a unique biphasic developmental cycle where it alternates between its infectious form, termed the elementary body (EB), and its replicative form, termed the reticulate body (RB). We hypothesize that reversible phosphorylation regulates the differentiation of the bacterium between these two forms. In order to study phosphorylation in *Chlamydia*, it is necessary to examine the role of individual kinases and phosphatases in the organism. My project focuses on using the Bacteriomatch II Two-Hybrid System to search for binding partners for the chlamydial phosphatase protein Cpp1. In particular, my project will test for interactions with a cysteine desulfurase and the elongation factor EF-Tu. Binding will be determined by streptomycin resistance and histidine production using the Bacteriomatch system. Successful identification of binding partners for Cpp1 could further our understanding of essential regulatory mechanisms in *C. trachomatis* and could lead to the identification of potential drug targets in the future.

Introduction

Chlamydia are obligate intracellular bacterial pathogens responsible for numerous human diseases, including the most common reportable bacterial sexually transmitted infection in the United States (Vasilevsky *et al.*, 2014). In particular, *Chlamydia trachomatis* in humans is divided into three biovars: serovars A-C, serovars D-K, and serovars L1-L3. Serovars A-C cause trachoma (Vasilevsky *et al.*, 2014), the leading infectious cause of preventable blindness worldwide (Centers for Disease Control and Prevention, 2009). Serovars D-K cause urethritis, pelvic inflammatory disease, and neonatal conjunctivitis. Lastly, serovars L1-L3 cause lymphogranuloma venereum (Vasilevsky *et al.*, 2014). *C. trachomatis* undergoes a unique biphasic developmental cycle where it alternates between its infectious form, termed the elementary body (EB), and the replicative form, termed the reticulate body (RB) (Claywell *et al.*, 2016). Upon infection of a host, the elementary body utilizes a type III secretion system to facilitate its entry into an epithelial host cell (Moore and Ouellette, 2014). Once inside the host cell, the bacteria differentiate from EBs into RBs and replicate inside a host-derived inclusion until RBs asynchronously differentiate back into EBs (Claywell *et al.*, 2016). However, little is known about how the bacterium regulates the differentiation between its two forms.

Reversible phosphorylation on Ser/Thr/Tyr residues has been gaining recognition as an important global regulatory mechanism in many pathogens (Mijakovic, Macek, 2012). Phosphoproteomic analysis of *C. caviae*, a close relative of *C.* trachomatis, showed a threefold increase in phosphoproteins in the EB form when compared to the RB form, and only three phosphoproteins were shared (Fisher *et al.*, 2015). 80% of the genes in *C. caviae* have homologs in *C. trachomatis*, and 41 of the 42 phosphoproteins identified have homologues in all chlamydial species. Additionally, phosphoproteins in the EB form were found to have functions

in central and secondary metabolism, amino acid metabolism, and virulence, whereas phosphoproteins in the RB form functioned in protein synthesis and folding (Fisher *et al.*, 2015). This supports the hypothesis that the differentiation of *C. trachomatis* between its two forms is regulated by reversible phosphorylation.

Reversible phosphorylation typically involves the addition and removal of phosphate groups by kinases and phosphatases, respectively. *C. trachomatis* has two characterized Hank's type kinases, Pkn1 and PknD, which have been shown to autophosphorylate Ser/Thr residues and to interact with each other (Verma, Maurelli, 2003). It also encodes a putative pseudokinase Pkn5, which lacks conserved catalytic residues, and a putative arginine kinase, McsB (Verma, Maurelli, 2003). The bacterium also produces three phosphatases: Cpp1, RsbU, and CT_589 (Hua *et al*., 2006). The latter two phosphatases are assigned to the partner-switching mechanism in *C. trachomatis* (Hua *et al*., 2006), which is hypothesized to regulate sigma 66 availability (Thompson *et al.*, 2015). This makes Cpp1 a likely candidate as the cognate phosphatase of Pkn1 and PknD and implies that it might have a significant role in regulating the EB to RB transition. For an overview of phosphorylation in *Chlamydia*, see Claywell *et al.*, 2016.

Cpp1 is a characterized protein phosphatase type 2C that has been shown to dephosphorylate Ser/Thr/Tyr phosphopeptides, suggesting that it may be able to dephosphorylate multiple substrates in *C. trachomatis* (Claywell, Fisher, 2016). However, the substrates of Cpp1 *in vivo* have not been identified, and it is necessary to investigate this topic in order to understand the role of the enzyme. My research will use a bacterial two-hybrid system to identify binding partners of Cpp1. However, before using a two-hybrid system, it is essential to first determine if the system-required protein tags interfere with the activity of the enzyme. The first portion of my research used pNPP hydrolysis assays to look for increased phosphatase activity in strains

containing Cpp1 compared to empty vector strains. Increased phosphatase activity would indicate that the protein tags did not interfere with enzyme activity and that the system would be a suitable system to use in the search for binding partners. The Bacterial Adenylate Cyclase-based Two-Hybrid (BACTH) system by Euromedex and the Bacteriomatch II Two-Hybrid system by Stratagene were both tested, and the latter was identified as a suitable two-hybrid system. After this experiment, I will move forward with the Bacteriomatch system to identify binding partners of Cpp1. If successful, this would help further our understanding of the role of reversible phosphorylation in chlamydial regulation and could lead to the identification of drug targets for use in treating chlamydial infections.

The BACTH system utilizes the unique ability of an adenylate cyclase protein from *Bordetella pertussis* to become active if two fragments of its catalytic domain are brought together. These two fragments, T18 and T25, can be fused to an enzyme and another protein and will produce cAMP if the two proteins interact. Once enough cAMP is produced, the cAMP will bind to CAP proteins and activate transcription of the *lac* operon downstream from the cAMP-CAP promoter (Figure 1). The reporter gene is *lacZ*, which encodes for β-galactosidase. β-galactosidase assays can be used to detect increased production of β-galactosidase in *Escherichia coli* strains containing the constructs. Growth on X-gal media can also be used to detect β-galactosidase production by blue-white screening (Karimova *et al*., 1998).

In the Bacteriomatch system, the enzyme is fused to the λCI repressor, and the potential binding partner is fused to the α subunit of the RNA polymerase. When the λCI operator site is moved upstream of a promoter, interaction between the enzyme and its binding partner allows for the recruitment of the RNAP to the promoter, and transcription of reporter genes occurs. In this system, histidine biosynthesis genes and streptomycin resistance genes are the reporter genes

(Figure 2). After cotransformation of plasmid constructs containing the enzyme and the potential binding partner into a strain auxotrophic for histidine, interaction between the two proteins would result in the ability of the bacterium to grow on media containing streptomycin and lacking histidine supplementation (Agilent Technologies, 2008). My research involved making plasmid constructs for each system containing Cpp1, putting these plasmids in *E. coli*, and comparing phosphatase activity of these strains with strains containing only empty vector plasmids.

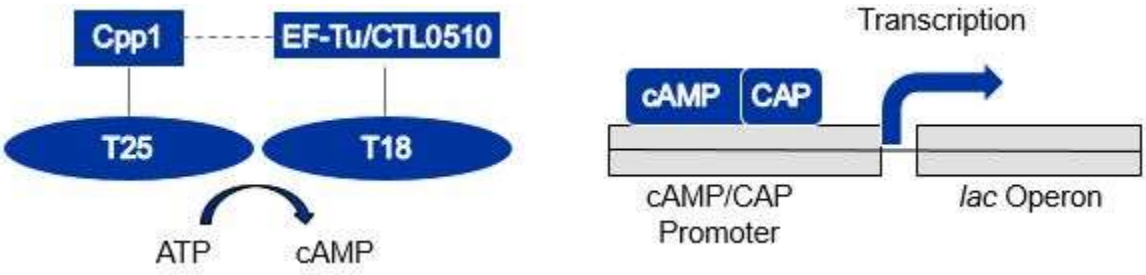

Figure 1. Principle of the BACTH System.

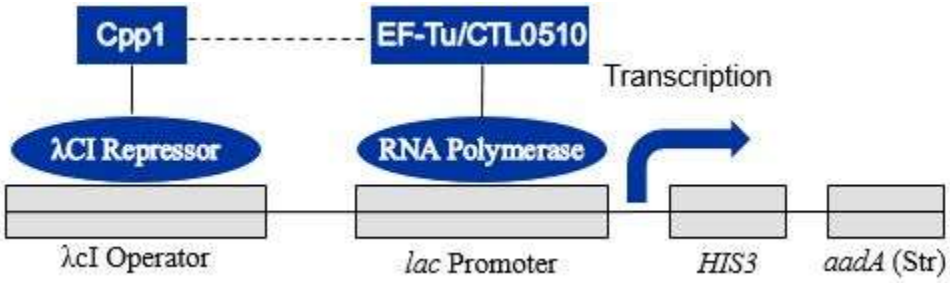

Figure 2. Principle of the Bacteriomatch II Two-Hybrid System.

Procedure

In order to test for Cpp1 activity when fused to tags required by the BACTH system, we inserted *cpp1* into the system-supplied plasmids. We were able to obtain plasmids that would produce Cpp1 fused to both fragments at the N-terminus or the C-terminus (Figure 3). Plasmids were transformed into BTH101 and DHM1 strains of *E. coli*, and cultures were grown to mid-logarithmic phase at 37^{O}C before induction with varying levels of IPTG. OD600 readings were taken at least every half hour until values were between 0.7 and 0.9. Protein production was initially induced with 0.5 mM IPTG at 18^{O}C for 24 hours. IPTG concentrations of 0 mM and 1 mM were also tested with the DHM1 strains After induction, OD600 values for each individual culture were taken, and cultures were pelleted before storage at -20^{O}C.

In order to test for Cpp1 activity when fused to tags required by the Bacteriomatch system, *cpp1* was inserted into the system-supplied plasmid. This construct and an empty vector plasmid were transformed separately into *E. coli* (Figure 4), and cultures were again grown to mid-logarithmic phase at 37^{O}C before induction. Protein production was initially induced with 30μM of IPTG at 18^{O}C for 16-20 hours and 37^{O}C for 4-5 hours. Induction was also performed at room temperature for 16-20 hours and 30^{O}C for 5-6 hours. OD600 readings for each culture were taken before pelleting and storage at -20^{O}C. Cells were lysed by sonication, and pNPP hydrolysis assays were performed on crude whole-cell lysates using $MnCl_2$, pH 8 assay buffer, and p-nitrophenyl phosphate (pNPP) (Claywell, Fisher, 2016). Purified Cpp1 protein was used as a positive control, and assay buffer alone along with empty vector strains were used as negative controls. Absorbance values were taken using a plate reader, and these values were divided by corresponding OD600 readings to correct for growth differences between strains.

After analysis of phosphatase activity using each system, the Bacteriomatch system was identified as a suitable bacterial two-hybrid system. *cpp1* along with the genes for potential binding partners *tufA*, which encodes for elongation factor EF-TU, and *CTL0510*, which is a cysteine desulfurase found in an operon with *cpp1* in the chlamydial genome, were amplified by PCR from *C. trachomatis* L2 434 genomic DNA. After amplification, PCR products were inserted into pJET1.2/blunt cloning vector. These plasmids were digested using restriction enzymes, and digested inserts were purified using a gel clean-up kit. pBT bait plasmid and pTRG target plasmids were also digested before inserts were ligated into the plasmids at the MCS (Figure 5). Plasmids were transformed into the XL1 blue MRF' kan strain of *E. coli*, and colonies containing the inserts were identified using colony PCR. Sequence analysis was done to confirm the presence of the inserts in the plasmids before continuing. pBT plasmid containing *cpp1* and pTRG plasmid containing either *tufA* or *CTL0510* were cotransformed into the reporter strain. This is where we currently are in the project, but we will be plating cotransformants on media lacking histidine and containing streptomycin in the future to test for interactions between our potential binding partners and Cpp1. We are hoping that either TufA or CTL0510 interacts with Cpp1 and allows the cotransformants to grow on the selective media.

Figure 3. Tags in strains used for testing the BACTH system.

Figure 4. Tags in strains used for testing the Bacteriomatch II Two-Hybrid System.

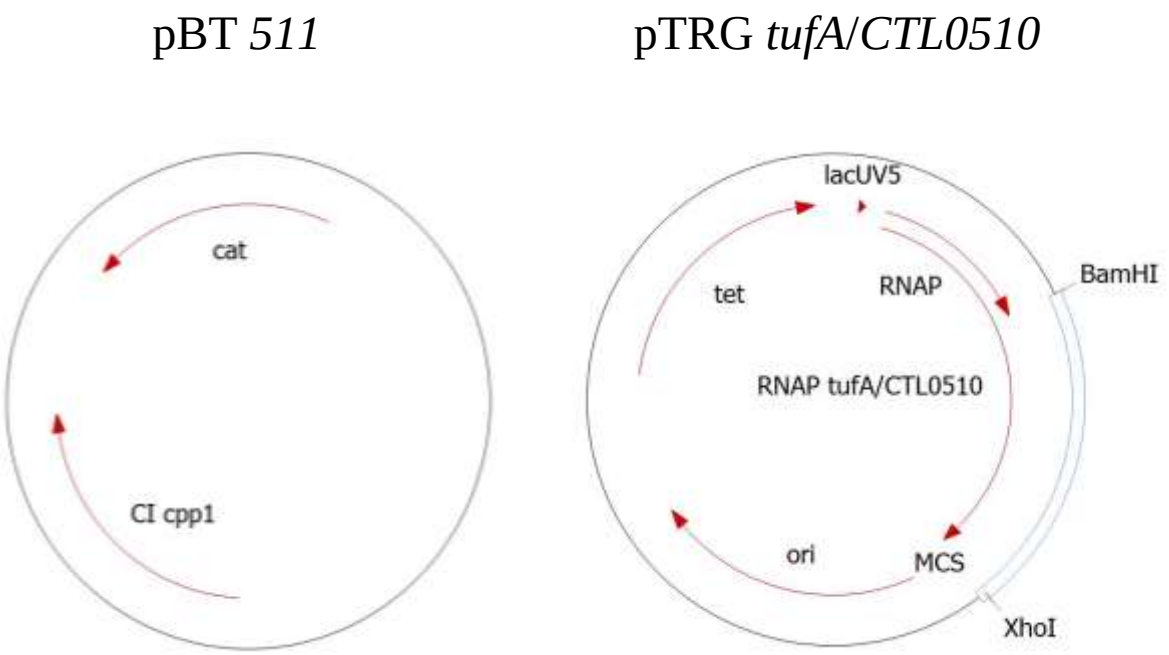

Figure 5. Vector maps of constructs used to test for interactions between Cpp1 and potential binding partners.

Results

For the BACTH system, we saw no increases in phosphatase activity in strains containing Cpp1 when compared to strains not containing Cpp1 even after varying induction temperatures, inducer concentrations, and the bacterial strain (Figure 6, Figure 7). There was no significant difference or pattern when comparing A405 values for strains labelled with “511” in the legends

of the figures. It is important to note that the positive control typically had values too high for the plate reader to read within the first few minutes of the assay. Therefore, the values used in the figures only represent the first couple of readings taken, and these values appear much lesser than the others because the other values were adjusted for culture absorbance. The values for the positive control were not adjusted for culture absorbance since purified protein was used in these wells. Although it is not represented clearly in the graphs, the positive control values were much higher than the values for any other wells in the experiment.

In contrast, when testing the Bacteriomatch system, we saw significant increases in phosphatase activity in strains containing Cpp1 (labelled with "511" in the legends) when compared to strains not containing Cpp1 (Figure 8, Figure 9). However, this increase in activity seems to be more significant when protein production is induced at lower temperatures for longer times. At 18^{O}C, we saw the largest increase in phosphatase activity in the DF204 strain, but there was little difference at 37^{O}C (Figure 8). Because it would take a very long time to grow cultures at 18^{O}C when testing for interactions, we wanted to find a warmer temperature where functional Cpp1 production was still significant. We ran pNPP hydrolysis assays at room temperature and 30^{O}C, and we saw the same pattern of increased phosphatase activity in Cpp1-containing strains. Induction at room temperature seemed to produce enough Cpp1 to allow for significant increases in phosphatase activity, and growth at room temperature would take a more reasonable amount of time when using this system to search for binding partners.

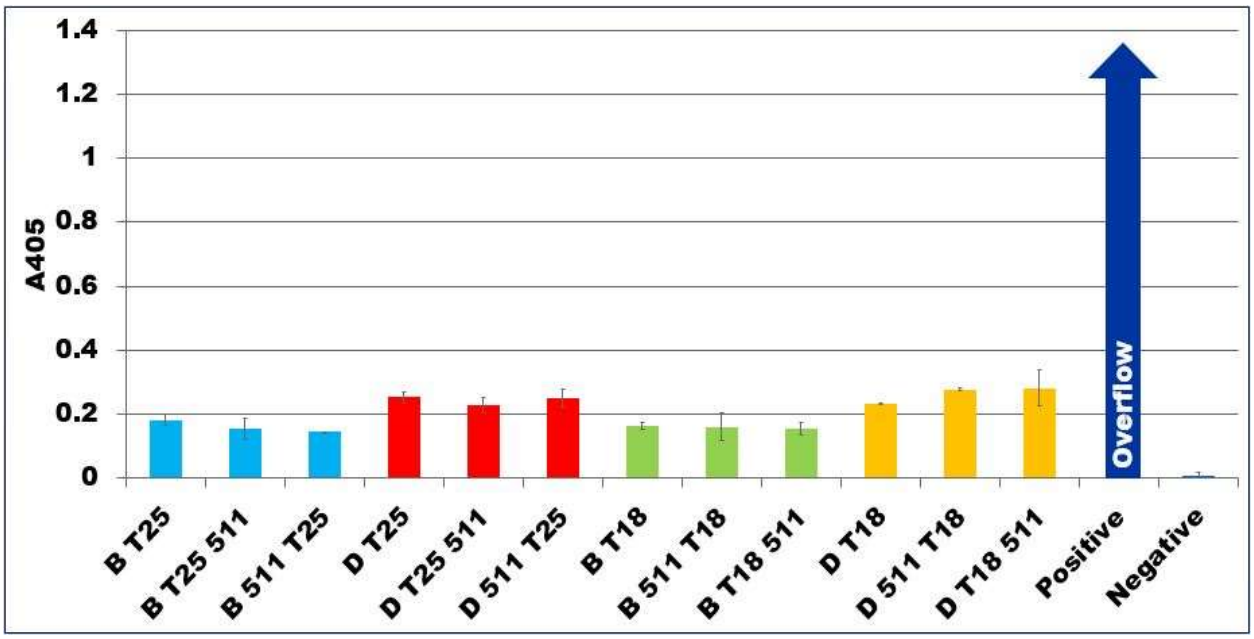

Figure 6. Absorbance values for pNPP hydrolysis assays after induction with 0.5 mM IPTG at 18ºC for 24 hours. Strains labelled B were BTH101 *E. coli* strains, and strains labelled D were DHM1 *E. coli* strains.

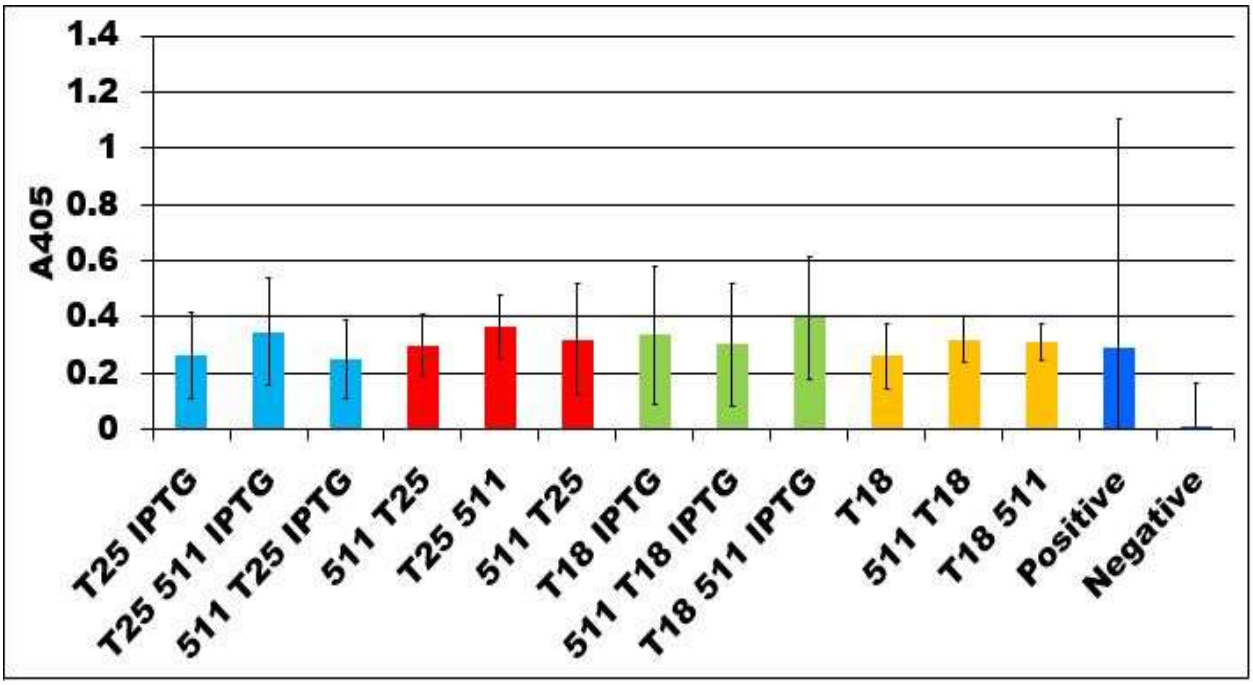

Figure 7. Absorbance values for pNPP hydrolysis assays after induction at 18ºC for 24 hours. The DHM1 strains labelled with IPTG were induced with 1 mM IPTG. The other DHM1 strains were not supplemented with IPTG.

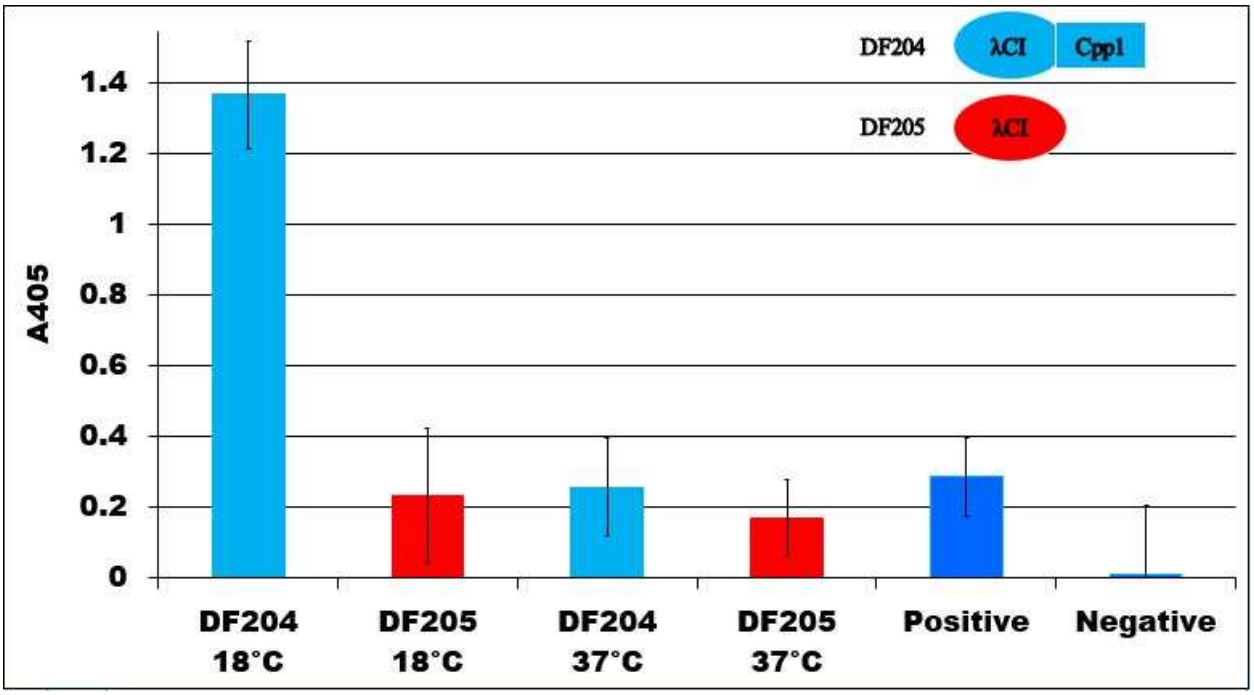

Figure 8. Absorbance values for pNPP hydrolysis assays after induction at 18ºC for 16-20 hours or at 37ºC for 4-5 hours as indicated. IPTG concentration for induction was 30 µM for all strains.

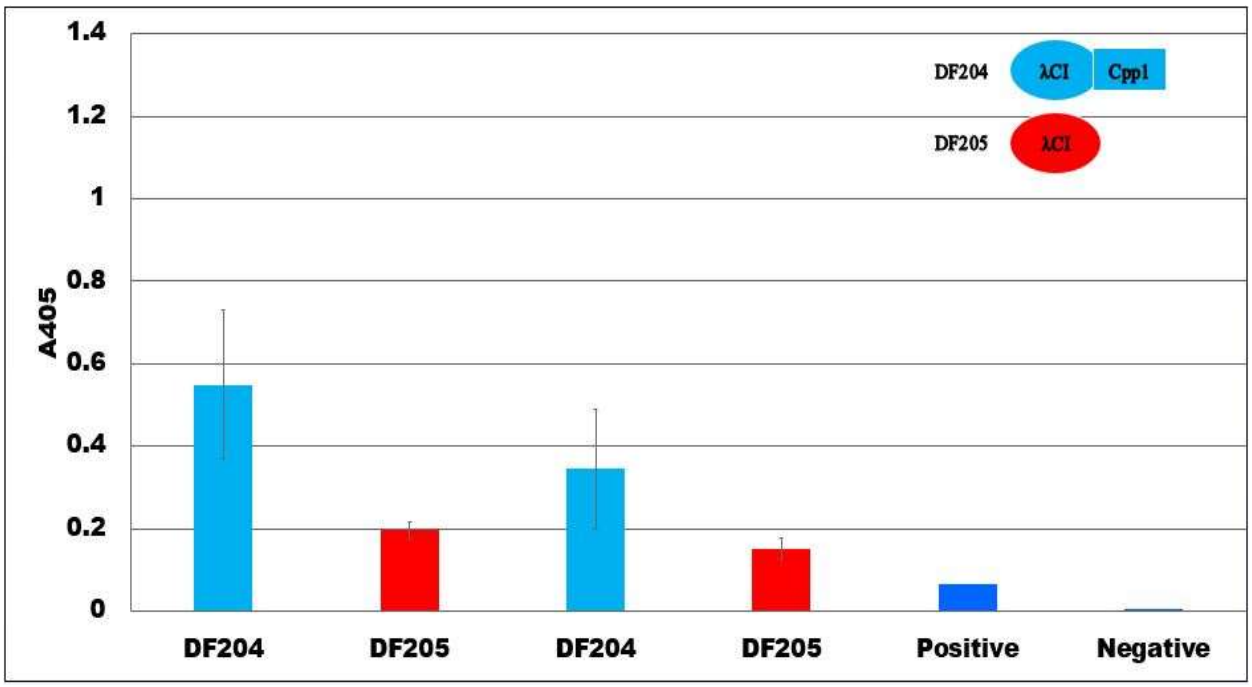

Figure 9. Absorbance values for pNPP hydrolysis assay after induction with 30 µM IPTG for 16-20 hours at RT or 5-6 hours at 30^{O}C.

Discussion

After looking for increases in phosphatase activity in strains containing Cpp1 when compared to empty vector strains, we concluded that the Bacteriomatch system is a suitable bacterial two-hybrid system to use to test for binding partners of Cpp1, whereas the BACTH system is not. Even after changing induction conditions and the strains used, we saw no significant differences or patterns in phosphatase activity between the strains used to test the BACTH system (Figure 6, Figure 7). The system-required protein tags likely interfered with folding or catalysis of Cpp1. However, when Cpp1 was fused to the CI repressor as in the Bacteriomatch system, we saw increases in phosphatase activity in the DF204 strain when compared to the empty vector strain (Figure 8, Figure 9). We also saw that the difference in

phosphatase activity was larger when induction was performed at lower temperatures. This may have been due to the enzyme having an optimal temperature for folding or due to the buildup of toxic products in the cells. Because growth at 18^{O}C would take a long time, we will likely grow the bacteria at room temperature when using the system in the future. This slightly warmer temperature still produced a significant difference in phosphatase activity.

In the future, we will be using the Bacteriomatch system to test the elongation factor TufA and the cysteine desulfurase CTL0510 for interactions with Cpp1. *CTL0510* is of interest because it is found in an operon with *cpp1* in the genome of *C. trachomatis*. In addition, TufA was identified as a phosphoprotein in the phosphoproteomic analysis of *C. caviae* discussed earlier (Fisher *et al.*, 2015). This may indicate that *C. trachomatis* regulates the differentiation between its two developmental forms with a mechanism similar to that of *Bacillus subtilis*, which uses phosphorylation of TufA to regulate sporulation of the bacterium (Pereira *et al.*, 2015). We have already been successful in creating constructs with *cpp1* inserted into the pBT plasmid and *tufA* and *CTL0510* inserted into pTRG plasmids. We will be cotransforming these plasmids into the Bacteriomatch reporter strain and plating on media containing streptomycin and lacking histidine. Now that we have identified a suitable system, we are hoping that we will be able to identify at least one binding partner for Cpp1. Identification of a binding partner for this phosphatase could lead to identification of drug targets and could help elucidate the role of reversible phosphorylation in developmental regulation of *C. trachomatis.*

Acknowledgements

Thank you to Dr. Fisher for his guidance, instruction, and assistance throughout this project. In addition, thank you to Jae Claywell for his time and involvement throughout the first portion of the project and to the Fisher laboratory for the encouragement and valuable input. This research was funded by the Center for Undergraduate Research and Creative Activities at Southern Illinois University Carbondale and the grant NIAID R15AI109566 issued to Derek J. Fisher through the National Institutes of Health.

References

1. BacterioMatch II Two-Hybrid System Vector Kit. (2008). Retrieved May 8, 2017.
2. Centers for Disease Control and Prevention, United States Department of Health and Human Services. (2009, December 28). *Hygiene-Related Diseases*. Retrieved May 9, 2017, from https://www.cdc.gov/healthywater/hygiene/disease/trachoma.html.
3. Claywell J. E., Fisher D. J. (2016). CTL0511 from *Chlamydia trachomatis* is a type 2C protein phosphatase with broad substrate specificity. J. Bacteriol. 198, 1827–1836. 10.1128/JB.00025-16.
4. Claywell, J. E., Matschke, L. M., & Fisher, D. J. (2016). The Impact of Protein Phosphorylation on Chlamydial Physiology. *Frontiers in Cellular and Infection Microbiology*, *6*, 197. http://doi.org/10.3389/fcimb.2016.00197.
5. Fisher D. J., Adams N. E., Maurelli A. T. (2015). Phosphoproteomic analysis of the *Chlamydia caviae* elementary body and reticulate body forms. Microbiology 161, 1648–1658. 10.1099/mic.0.000116.
6. Hua, L., Hefty, P. S., Lee, Y. J., Lee, Y. M., Stephens, R. S., and Price, C. W. (2006). Core of the partner switching signaling mechanism is conserved in the obligate intracellular pathogen *Chlamydia trachomatis*. Mol. Microbiol. 59, 623–636. doi: 10.1111/j.1365-2958.2005.04962.x.
7. Karimova, G., Pidoux, J., Ullmann, A., & Ladant, D. (1998). A bacterial two-hybrid system based on a reconstituted signal transduction pathway. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(10), 5752–5756.
8. Mijakovic, I., Macek, B. (2012). Impact of phosphoproteomics on studies of bacterial physiology. FEMS microbiology reviews 36, 877-892.

9. Moore, E. R., and Ouellette, S. P. (2014). Reconceptualizing the chlamydial inclusion as a pathogen-specified parasitic organelle: an expanded role for Inc proteins. Front. Cell. Infect. Microbiol. 4:157. doi: 10.3389/fcimb.2014. 00157.
10. Pereira, S. F. F., Gonzalez, R. L., and Dworkin, J. (2015). Protein synthesis during cellular quiescence is inhibited by phosphorylation of a translational elongation factor. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(25), E3274–E3281. http://doi.org/10.1073/pnas.1505297112.
11. Thompson, C. C., Griffiths, C., Nicod, S. S., Lowden, N. M., Wigneshweraraj, S., Fisher, D. J., *et al*. (2015). The Rsb phosphoregulatory network controls availability of the primary sigma factor in Chlamydia trachomatis and influences the kinetics of growth and development. PLoS Pathog. 11:e1005125. doi: 10.1371/journal.ppat.1005125.
12. Vasilevsky, S., Greub, G., Nardelli-Haefliger, D., & Baud, D. (2014). Genital Chlamydia trachomatis: Understanding the Roles of Innate and Adaptive Immunity in Vaccine Research. *Clinical Microbiology Reviews*, *27*(2), 346–370. http://doi.org/10.1128/CMR.00105-13.
13. Verma, A., Maurelli, A. T. (2003). Identification of Two Eukaryote-Like Serine/Threonine Kinases Encoded by *Chlamydia trachomatis* Serovar L2 and Characterization of Interacting Partners of Pkn1. *Infection and Immunity*, *71*(10), 5772–5784. http://doi.org/10.1128/IAI.71.10.5772-5784.2003.