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Characterization of the CIP Protease System in the Growth and Development of Chlamydia trachomatis

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Characterization of the Clp Protease System in the Growth and Development of *Chlamydia trachomatis*

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ABSTRACT

Characterization of the Clp Protease System in the Growth and Development of *Chlamydia trachomatis*

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Chlamydia is an obligate intracellular bacterium that differentiates between two distinct forms during its developmental cycle: elementary bodies (EBs) and reticulate bodies (RBs). The EB is the small, electron dense form that mediates host cell infection. Within the cell, the EB differentiates into the RB, which is the replicative form that develops within a host membrane derived vesicle, termed an inclusion. RBs replicate within this inclusion and eventually differentiate back into EBs. Upon accumulation of EBs at the end of the developmental cycle, the host cell lyses, releasing the EBs for infection of proximal cells. The EB and RB have distinct proteomic profiles, and, given the unique functional and morphological forms, the role of proteomic turnover through protein degradation is understudied in *Chlamydia*. We hypothesize that the Clp protease system plays an integral role in protein turnover by targeting specific proteins from one developmental form or the other for degradation. *Chlamydia* contains five genes encoding five *clp* genes: *clpX*, *clpC*, two *clpP* paralogs, and *clpB*. Homotypic oligomerization of the Clp proteins was determined with bacterial two-hybrid assays and native-PAGE gels. Transcriptional analysis via RT-qPCR determined these genes are expressed mid-cycle.

Antibiotics that non-specifically activated the ClpPs negatively affected chlamydial development. Additionally, inducible, poly-histidine tagged inactive *clp* mutants were used to determine the effect of overexpression on *Chlamydia*. Taken together, these data suggest that i) the Clp system of *Chlamydia* functions comparably to other bacteria and ii) Clp proteins are important for chlamydial growth and development.

Keywords: *Chlamydia*, differentiation, protein turnover, protein quality control, Clp protease

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CHAPTER ONE

Introduction

C. trachomatis (Ctr) is one of the most prevalent human sexually transmitted infections (STIs) and the leading cause of infectious preventable blindness worldwide [5]. As many as 60-80% of cases are asymptomatic [6], which, if left untreated, infection can lead to complications such as pelvic inflammatory disease, possibly resulting in infertility and ectopic pregnancies in women [4]. An infant may also acquire the infection during vaginal delivery should the mother be infected, which can result in conjunctivitis and irreversible blindness if left untreated [2]. Multiple different serotypes presenting several clinical manifestations exist. Serovars A-C are commonly associated with trachoma, while D-K are common agents of sexually transmitted infection. The L1-L3 variants can also cause STIs but can present as more invasive diseases such as systemic infection and lymphogranuloma venereum. Important to chlamydial pathogenesis is their completion of a developmental cycle and evasion the host immune system in chronic infections. *Chlamydiae* have significantly reduced their genome size, suggesting that most preserved genes likely serve an important function to bacterial fitness [3].

Chlamydia trachomatis is an obligate intracellular human pathogen with a complex developmental cycle (see Figure 1 for an overview and [1] for detailed review). *Chlamydia* differentiate between two distinct functional and morphological forms over the course of 48 to 72 hours (*in vitro*) depending on the strain. The electron dense elementary body (EB) is the infectious, metabolically quiescent form, and the reticulate body (RB) is the non-

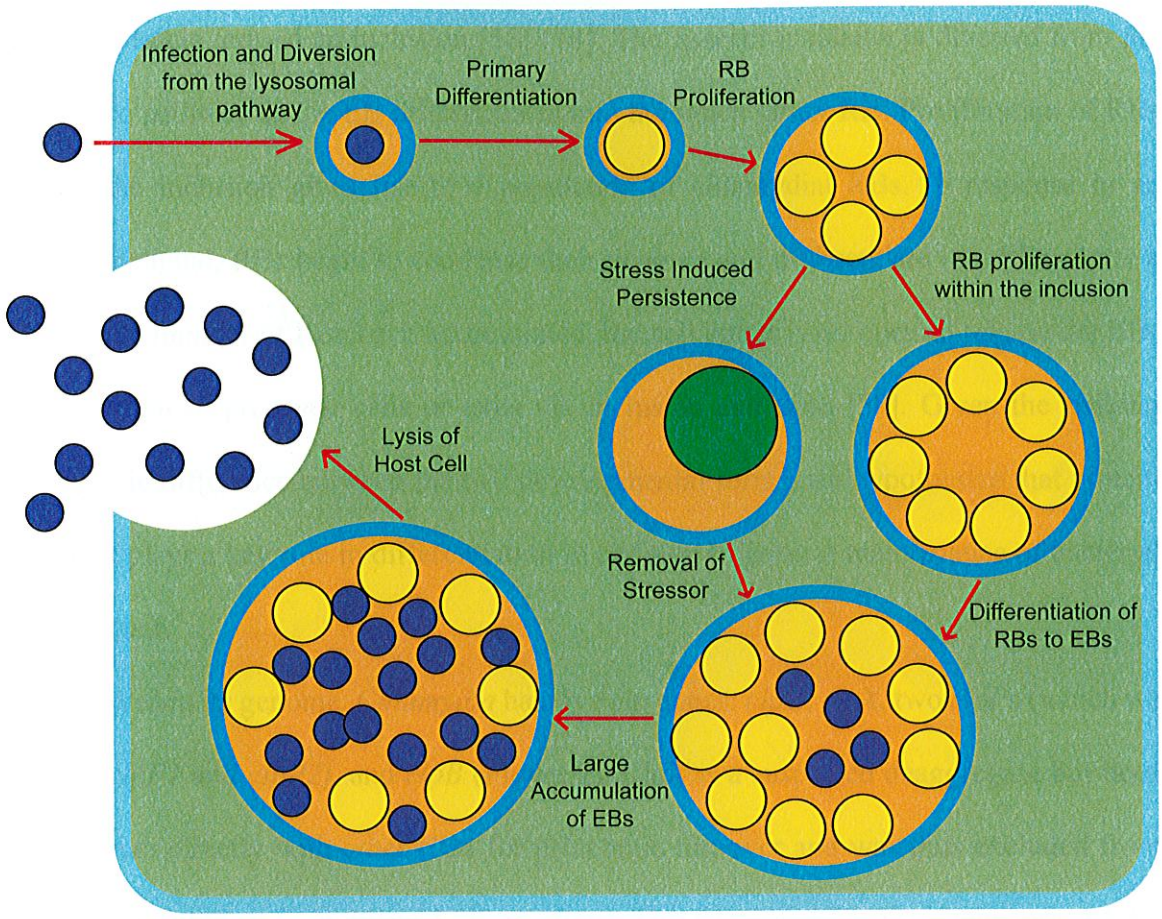

Figure 1: The chlamydial developmental cycle. Not illustrated to scale. It should be noted that the correct terminology is developmental cycle rather than life cycle, as the latter suggests a terminal differentiation or death of the bacterium.

infectious, metabolically active, replicative form. The typical size of each form is 0.3μm and ~1μm, respectively [1]. A single EB facilitates infection of a host cell by attaching to the plasma membrane and inducing endocytosis into the cell within a host membrane derived vesicle termed an inclusion [58] [14]. The nascent inclusion is diverted from the endocytic pathway as the EB differentiates into the much larger RB. Proliferation of RBs within the inclusion gives rise to a population of chlamydial RBs. In response to an unknown signal, RBs begin to condense their genome and differentiate into EBs. After a significant number of EBs have accumulated, the cell either lyses open to release the EBs for infection of proximal cells or exits via inclusion extrusion [59]. Given the striking phenotypic difference between the two developmental forms, we hypothesize that protein turnover plays a key role in differentiation in addition to general maintenance of bacterial homeostasis.

Within its genome, *Chlamydia* has five *clp* genes: *clpC*, *clpX*, two *clpP*s (which we termed *clpP1* and *clpP2*), and *clpB* (Figure 2). ClpB is a suspected deaggregase but does not interact directly with other Clps for proteomic turnover and was thus excluded from our primary studies (unpublished observation). The ClpC and ClpX proteins are AAA+ (ATPases Associated with various cellular Activities) unfoldases that serve as adapter proteins that utilize ATP hydrolysis to linearize target proteins [7] [8]. ATP binding, but not necessarily hydrolysis, allows for a homo-hexamer of these proteins to interact with a ClpP complex in other organisms [9] [10]. ClpP is a serine protease that gives peptidase function to the resultant complex [11]. ClpP proteins oligomerize into a tetradecameric complex of a stack of two heptamers that can then perform proteolytic function upon interaction with the adaptor protein oligomer [16] [12]. Given the reductive nature of the

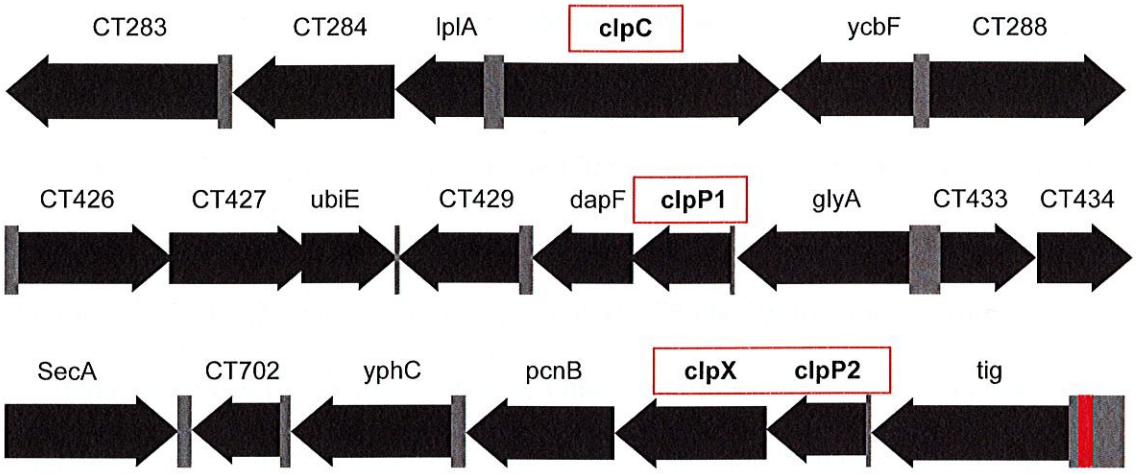

Figure 2: Genomic map of the chlamydial *clp* genes. Numbers are as follows: *clpC* (CT286), *clpP1* (CT431), *clpP2* (CT705), and *clpX* (CT706). Of note is the juxtaposition of *clpP2* and *clpX* in tandem within the same operon. Not shown is *clpB* (CT113).

chlamydial genome (~1mbp vs ~5mbp of *E. coli*), that *Chlamydia* encodes two proteolytic *clpP* paralogs leads us to hypothesize that each serves a distinct function.

As seen in multiple sequence alignments (Figures 3-5), the Clp proteins contain a remarkable level of evolutionary conservation. ClpP1 and ClpP2 align to the more studied *E. coli* ClpP in both secondary and tertiary structure. The serine active sites, in addition to the hydrophobic pocket, are well conserved with the most variability limited to the oligomer interface (Figure 3). The two isoforms share only 39% sequence similarity (data not shown), which suggests a potential for a dual function of this protease system. ClpX and ClpC both demonstrate retention of the IGF/L loop and Walker A/B ATP binding and hydrolysis motifs, further strengthening our hypothesis that these proteins can serve as adaptors to feed substrates into the proteolytic ClpPs. While these proteins possess functional structures that suggest conserved molecular mechanisms of action, the apparent differences in interaction interfaces and recognition motifs may result in differential interaction of the two ClpPs with the respective chaperone proteins.

Our current hypothesis is that ClpC interacts with ClpP1 and that ClpX interacts with ClpP2 (Figure 6). We base our prediction of ClpP2X interaction due to the juxtaposition of the two genes within the same operon (Figure 2). We then reasoned that ClpP1 would serve as the proteolytic subunit that would interact with ClpC, as both are independent of each other (Figure 2). The Baker lab at MIT characterized the dual Clp system of *P. aeruginosa* and showed that system could potentially have a dual function contributing to virulence and fitness of the bacteria [13]. The two *P. aeruginosa* ClpP proteins share a low amount of similarity via protein alignment, as do the chlamydial ClpPs, strengthening the idea of independent functions for each. Based on our initial

bioinformatic studies and knowledge of the chlamydial developmental cycle, we hypothesize that the chlamydial Clp protease system plays a unique role in differentiation and bacterial physiology. We thus initiated studies to establish an initial characterization of this protein system in *C. trachomatis*.

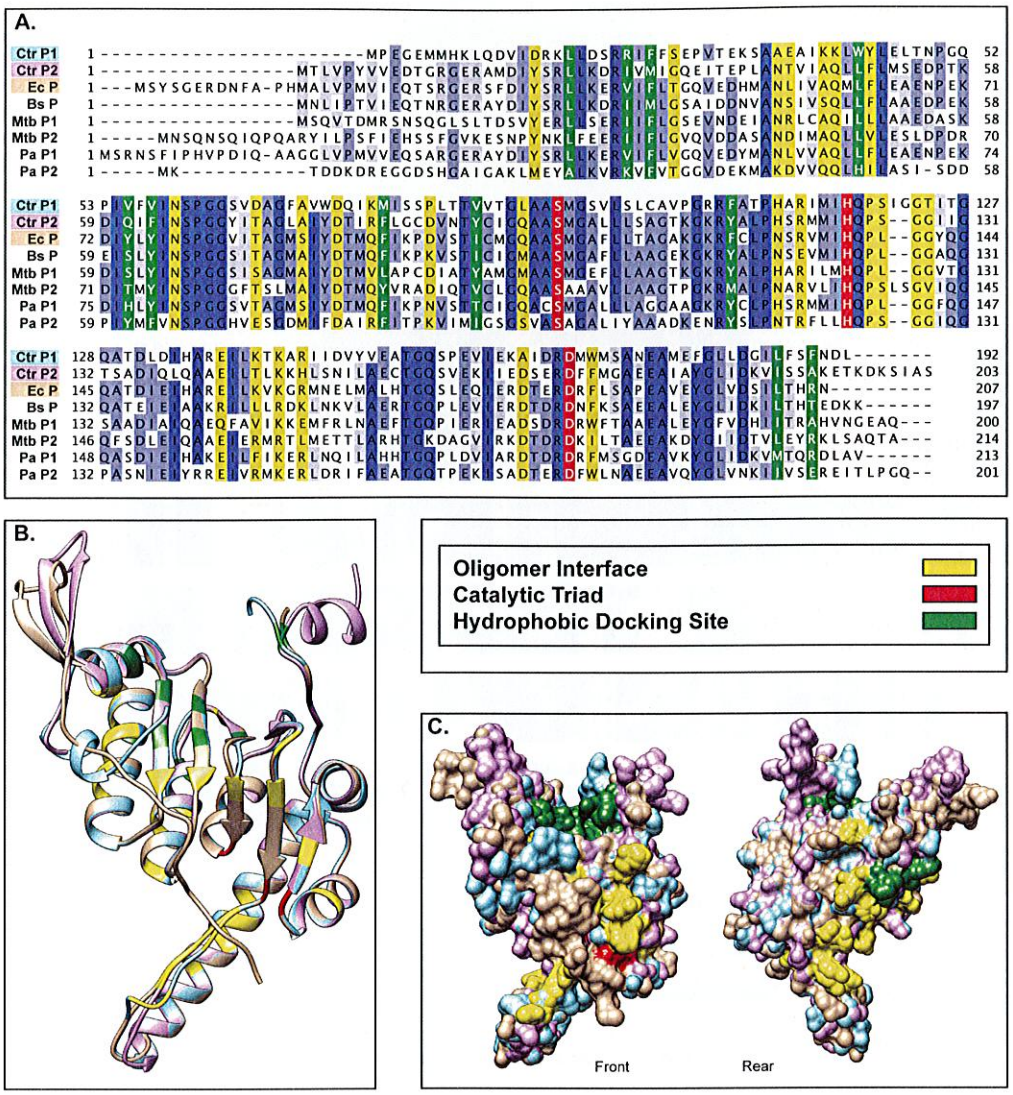

Figure 3: ClpP multiple sequence alignment. (A) Alignment performed using Clustal Omega default settings and presented using Jalview Version 2. Organisms presented are *C. trachomatis* (Ctr), *E. coli* (Ec), *B. subtilis* (Bs), *M. tuberculosis* (Mtb), and *P. aeruginosa* (Pa). **(B) Predicted 3D** structural alignment of Ctr ClpP1 (light blue), Ctr ClpP2 (pink), and Ec ClpP (tan). **(C) Surface model** of the 3D alignment presented in (B), rotated 180° around the y axis to show "front" and "rear" of the proteins.

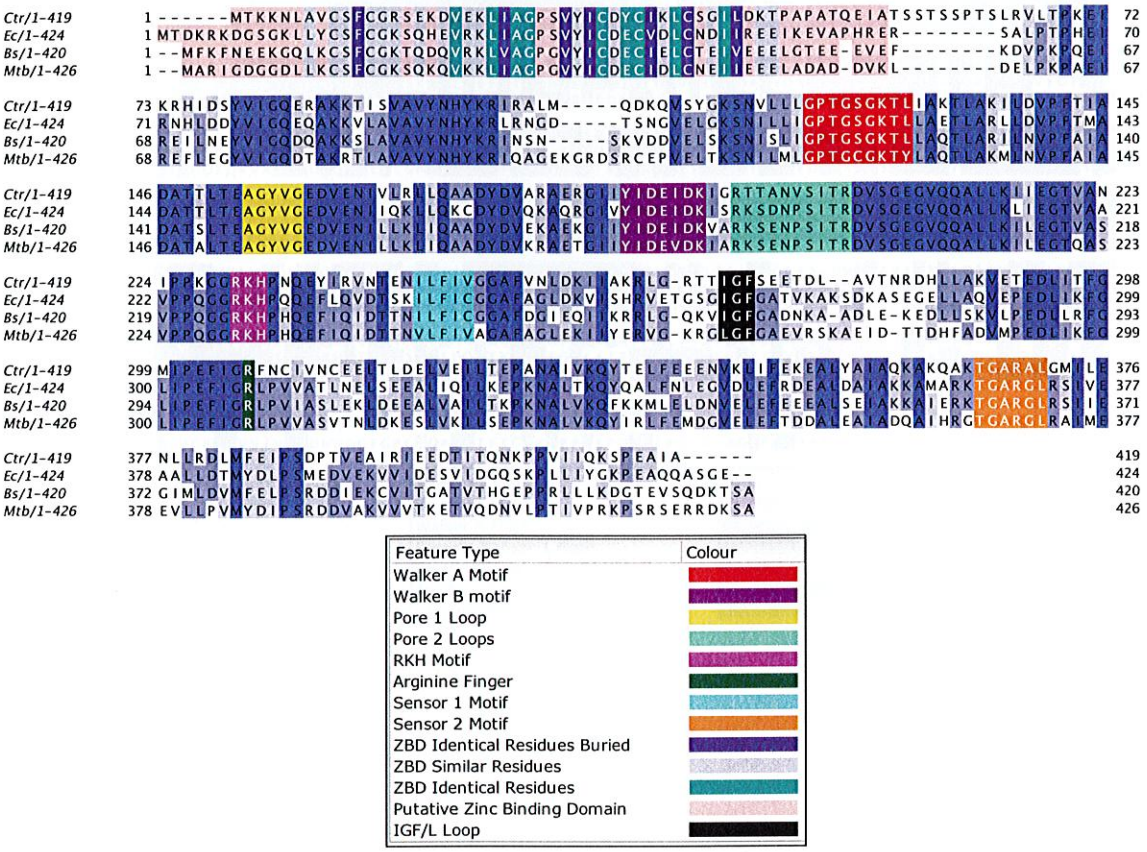

Figure 4: ClpX multiple sequence alignment. Alignment parameters and organism abbreviations are the same as discussed in figure X. ZBD=zinc-binding domain.

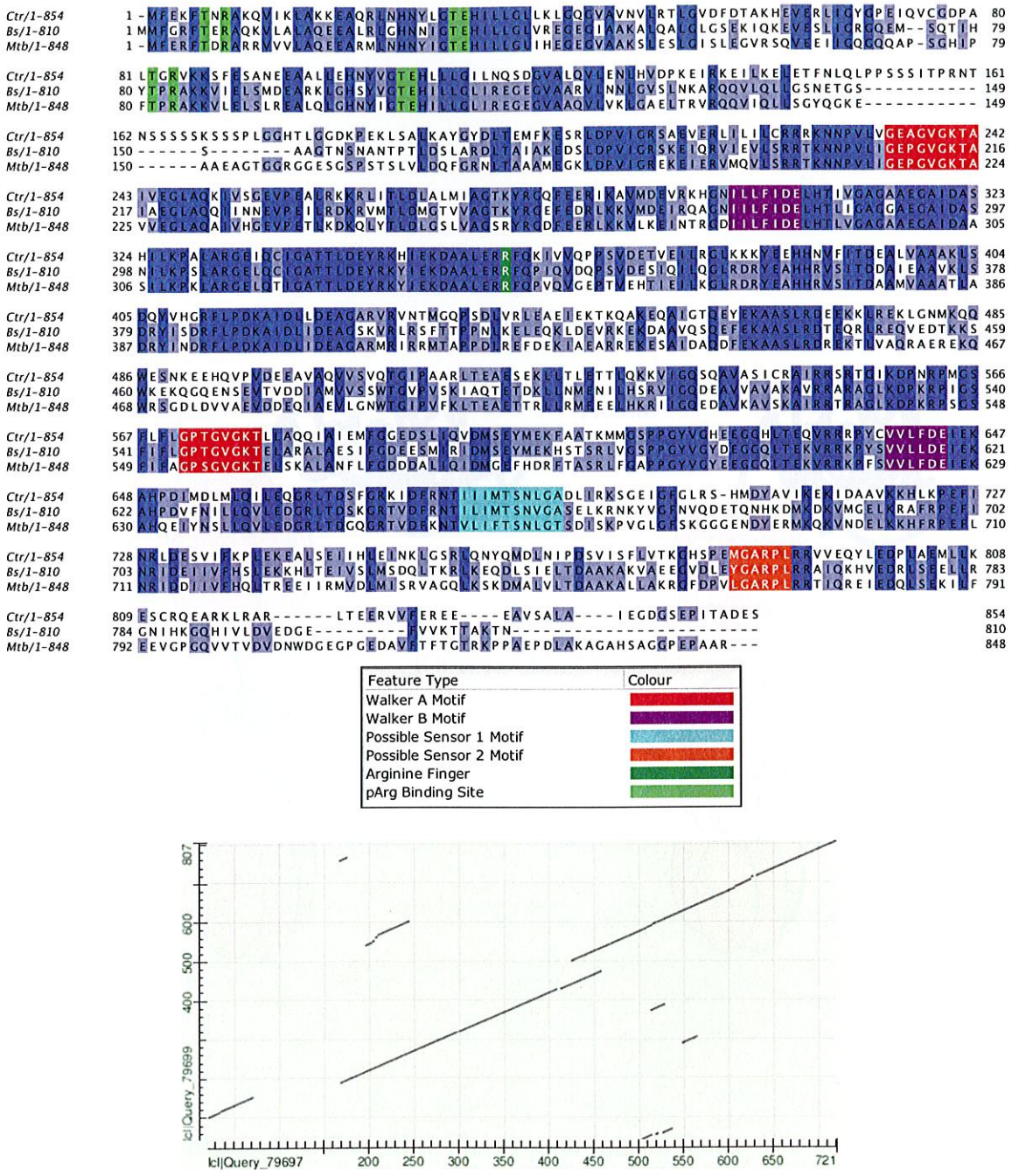

Figure 5: (A) ClpC Multiple Sequence Alignment. Alignment parameters and organism abbreviations are the same as discussed in figure X. **(B) Alignment of Ctr ClpC (Y axis) and Ec ClpA (X axis).** Shown to demonstrate similarity but highlight variation from ClpA, another Type II AAA+ protease.

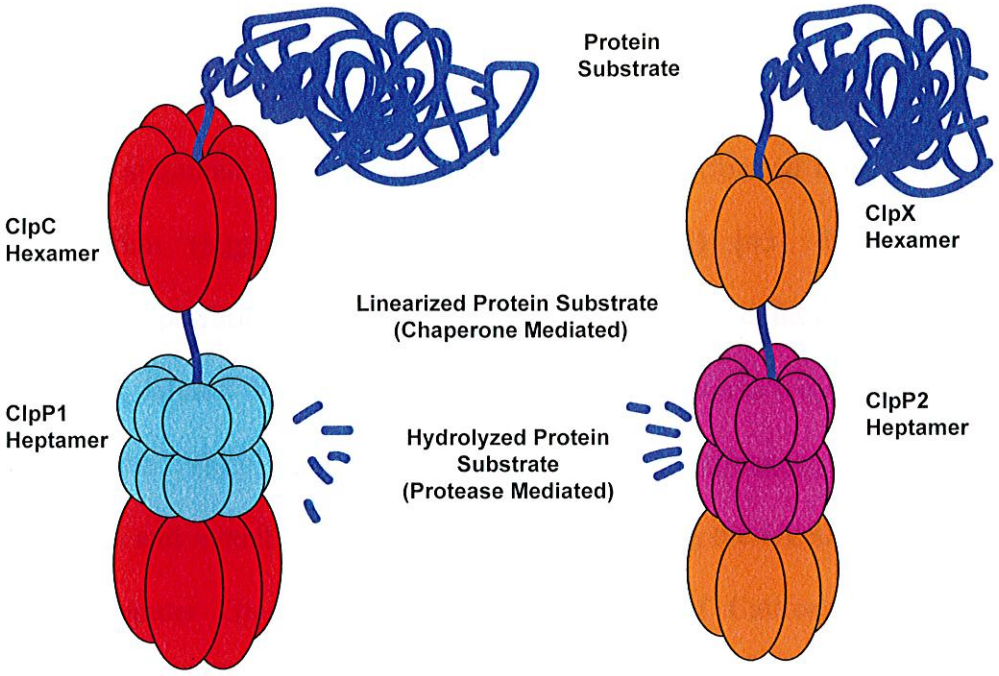

Figure 6: Hypothesized chlamydial Clp protein interactions. Our current model for oligomerization is ClpC interacting with ClpP1 and ClpX interacting with ClpP2.

CHAPTER TWO

Materials and Methods

Bioinformatics Analysis: Gene maps and sequences of genes of *Chlamydia trachomatis* used were obtained from STDGen database (http://stdgen.northwestern.edu). Protein sequences from *E. coli, B. subtilis, M. tuberculosis,* and *P. aeruginosa* were acquired from the NCBI protein database (https://www.ncbi.nlm.nih.gov/guide/proteins/). The ClpP1 vs. ClpP2 protein alignment to find sequence identity was performed using NCBI Protein BLAST function (https://blast.ncbi.nlm.nih.gov/Blast.cgi) [43]. Multiple sequence alignments were performed using Clustal Omega [31] with default settings and were presented using Jalview Version 2 [30]. Predicted 3D structures were acquired from the Phyre2 website (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) [47]. Protein models and model alignments were rendered using the UCSF Chimera package from the Computer Graphics Laboratory, University of California, San Francisco (supported by NIH P41 RR-01081) [46].

Strains and Cell Culture: The human epithelial cell line HEp2 was utilized in the antibiotic studies and was routinely cultivated and passaged in Iscove's Modified Dulbecco's Medium (IMDM, Gibco/ThermoFisher; Waltham, MA), and 10% FBS (Sigma; St. Louis, MO). For the purpose of chlamydial transformation, McCoy mouse fibroblasts were used, and human epithelial HeLa cells were used for attempted plaque purification of transformations. Both of these cell lines were passaged routinely in Dulbecco's Modified

Eagle's Medium (DMEM, Gibco/ThermoFisher). Density gradient purified *Chlamydia trachomatis* L2/434/Bu (ATCC VR902B) EBs were used for the antibiotic studies. The antibiotic ACP1b (a generous gift from Dr. Walid Houry, University of Toronto [32]) used for preliminary studies prior to synthesis was resuspended in DMSO in 20mM stocks and frozen at -80° C. Frozen stocks of this antibiotic were used only once to avoid freeze-thawing. Antibiotic stocks of ACP1, ACP1a, and ACP1b were synthesized, resuspended at 25 mg/mL in DMSO, and frozen at -20°C.

Plasmid Construction: A full list of the primers and plasmids used is included in Appendices 1&2. Plasmids for the Bacterial Adenylate Cyclase Two-Hybrid (BACTH) system were cloned using the Gateway® recombination system. The genes were amplified from *Chlamydia trachomatis* L2 genomic DNA with primers designed to add an *attB* recombination site on either side of the gene. The PCR products were then incubated with a pDONR^{TM}221 entry vector (containing *attP* recombination sites) in the presence of BP Clonase II (Invitrogen) that inserts the gene via the flanking *attB* sites and removes *ccdB* endotoxin flanked by the *attP* sites encoded on the plasmid, allowing for positive selection. The result of the BP reaction was an entry vector containing the gene of interest flanked by *attL* sites. 2μL were transformed into DH5α chemically competent *E. coli* and plated onto an LB agar plate containing 50μg/mL kanamycin. Plasmid from an individual colony was purified and used for the LR reaction into one of three destination vectors (pST25-DEST, pSNT25-DEST, or pUT18C-DEST). The same entry vector for any given gene was used for all three LR reactions to insert into the destination vector with LR Clonase II. 150ng of the entry vector was incubated with 150ng of destination vector for 1 hour at room

temperature. 2μL were used to transform XL1 *E. coli*, which were plated on the appropriate selection plate. Purified plasmid from an individual colony was sequence verified prior to use the in BACTH assay (see below).

Constructs for Chlamydial transformation were created using the Ligation Independent Cloning (LIC) (New England Biolabs) protocol. To add the C-terminal 6xHis tag to the Clp proteins, the genes were cloned out of the genome with a primer to add the poly-histidine tag. These products served as the template for PCR reactions to add the necessary overlap for the LIC reaction. Primers were generated using the NEBuilder® assembly tool available from New England BioLabs (http://nebuilder.neb.com). The backbone used was the pTLR2 derivative of the pASK plasmid [35] The pTLR2 backbone was digested using FastDigest BshTI and Eco52I restriction enzymes (ThermoFisher), and then 50ng of digested plasmid was incubated with a 2:1 ratio of insert copy number to backbone [2*(insert size/backbone size)*50] and 2x LIC master mix (NEB). Following a 15 minute incubation of the reaction mix at 50° C, 2μL of the reaction was transformed into DH5α chemically competent *E. coli* (NEB) and plated on the appropriate antibiotic selection plate. Positive clone sequences were verified by Eurofins Genomics. Sequence verified plasmids were transformed into *-dam/-dcm E. coli* (New England BioLabs) in order to produce demethylated plasmid, which was sequence verified prior to transformation into *C. trachomatis* (see below). The previously described procedure was also utilized in the construction of ClpP2X wild type and mutant operons. A FLAG tag was added to the *clpP2* gene and a 6xHis tag was added to the *clpX* gene by PCR. The intergenic region between the two genes was added after the addition of the FLAG tag to *clpP2*. The

PCR to add the overlap for both constructs to be used in the ligation system was performed using these templates.

Strains created or used in this study are listed in Appendix 2. Bacteria were maintained on LB agar plates and grown in LB medium or on LB agar plates supplemented with 100 μg/ml ampicillin as needed. Chlamydial genomic DNA for cloning was obtained from EBs using the DNeasy Blood & Tissue Kit from Qiagen and E. coli genomic DNA was isolated using sodium hydroxide lysis of colonies. The chlamydial clpP1 and clpP2 along with clpP from *E. coli* were amplified via PCR using the primers listed in Appendix 1 and Phusion polymerase (Thermo Scientific). PCR products were cloned into the pLATE31 expression vector from Thermo Scientific as directed by the manufacturer to create fusion proteins with a C-terminal 6x His-tag. Plasmids were initially transformed into E. coli NEB10 and selected on LB agar ampicillin plates. Transformants were screened for inserts using colony PCR with Fermentas Master Mix (Thermo Scientific) and positive clones were grown for plasmid isolation (GeneJet Plasmid Miniprep Kit, Thermo Scientific). DNA inserts were sequenced by Macrogen USA and sequence-verified plasmids were then transformed into *E. coli* BL21(DE3) bacteria for protein production.

Determining Protein-Protein Interactions with the BACTH System: To test interaction of the Clp proteins, the (BACTH) assay was utilized. This assay relies on reconstitution of adenylate cyclase activity in adenylate cyclase deficient (Δ*cya*) DHT1 *E. coli*. The genes of interest are translationally fused to one of either subunit, denoted as T18 and T25, of the *B. pertussis* adenylate cyclase toxin catalytic region. Each *clp* gene (sequences acquired from STDGen gene database) cloned into one of the pST25, pSNT25, or pUT18C

Gateway® vectors was tested for both homotypic and heterotypic interactions [45]. One plasmid from the T25 background and one from the T18 background were co-transformed into chemically competent DHT1 *E. coli* and were plated on a double antibiotic minimal M63 medium selection plate supplemented with 0.5mM IPTG for induction of the construct, 40μg/mL Xgal to give a visual readout upon cleavage, and 0.2% maltose as a unique carbon source. These plates also contain casein hydrolysate to supplement the bacteria with the branched chain amino acids because Δ*cya* DHT1 *E. coli* cannot synthesize these amino acids. Blue colonies were indicative of positive interaction between proteins, as both the *lac* and *mal* operons require reconstituted cAMP production from interacting T25 and T18 fragments. Leucine zipper motifs were used for controls in pKT25 and pUT18C backgrounds on the appropriate antibiotic selection plates because these have been previously shown to interact [29]. Blue colonies were then picked for use in a β-galactosidase assay to quantify protein interactions. Random positive colonies were used to inoculate individual wells and grown 24 hours at 30° in M63 with 0.2% maltose and appropriate antibiotics. These bacteria were permeabilized with 0.1% SDS and chloroform prior to addition of 0.1% o-nitrophenol-β-galactoside (ONPG). The reaction was stopped using 1M $NaHCO_3$ after precisely 20 minutes of incubation at room temperature. Absorbance at the 405 wavelength was recorded and normalized to bacterial growth (OD_{600}), dilution factor, and time (in minutes) of incubation prior to stopping the reaction. Enzyme activity was reported in relative units (RU) of β-galactosidase activity.

Purification of Recombinant ClpP1 and ClpP2: His-tagged Ctr ClpP1, Ctr ClpP2, and *E. coli* (Ec) ClpP were purified using 500mL cultures of BL21(DE3) *E. coli* transformed with

the respective plasmid. Samples were induced with 0.5mM IPTG and incubated with shaking for 20 hours at 18°C. Cultures were pelleted and frozen at -20°C prior to purifications. Buffers used are listed in Appendix 3. Samples were suspended and sonicated, and lysates were run through a HisPur Cobalt Resin (Thermo Scientific) and washed in buffer A. Proteins were eluted from the resin using buffer B. Buffer exchange for buffer C was performed using a Millipore Amicon Ultra 15 filtration units (3 kDa cut-off). ClpP proteins were quantified using the Bio-Rad Protein assay, assessed for purity on 12% SDS-PAGE gels with Coomassie staining, and identified using anti-His-tag Western blot. Blotting was performed using a mouse monoclonal anti-6x His antibody (1:1000; Millipore HIS.H8) and a goat anti-mouse IgG HRP conjugated secondary antibody (1:2000; Millipore AP124P). Protein samples were aliquoted and stored at -80°C.

In Vitro *Analysis of ClpP1 and ClpP2 Homo-Oligomerization:* 5μg of purified protein was incubated in buffer D at 37°C for 1 hour before being run on a BioRad MiniProtean 4-20% gradient gel for Native-PAGE. Samples were run for 90 minutes at 200V. Gels were assessed using Coomassie staining.

***Assessment of Clp Activity* In Vitro: Analysis of ClpP protease activity fluorometric peptide assay:** The ClpPs (at 1 μM) or papain (positive control, 0.1 μM) were added to 500 μM of Suc-Luc-Tyr-AMC (Boston Biochem) dissolved in buffer E (50 mM Tris-HCl [pH 8], 200 mM KCl, and 1 mM DTT). Final reaction volumes were 50 μl. Reactions were monitored over six hours at 37 °C using a BioTek Synergy HT plate reader set at an excitation of 340/360 and an emission of 440/460 with readings taken at five-minute

intervals. **Casein degradation assays:** Casein (Sigma-Aldrich) was dissolved in buffer E and 1 μg was used per assay. Samples containing casein and 1 μM of the respective ClpP or papain (positive control) were incubated at 37 °C for various time periods with or without the compounds. Reactions were halted by mixing with 2x Laemmli buffer containing β-mercaptoethanol and heating at 90-100 °C for 5 minutes. Samples were analyzed for digestion of casein using 12% SDS-PAGE gels followed by staining with Coomassie Brilliant Blue.

Transcript Analysis Using RT-qPCR:

Assays performed by Dr. Scot Ouellette. They have proven to be pertinent to this research project and were thus included in this text.

C. trachomatis ***propagation and detection of ClpP2:*** DFCT28, a GFP-expressing C. trachomatis 434/Bu clone (PMID 28396349), was routinely grown in and titered (using the IFU assay) on L2 cells as described in (PMID: 18770550). Briefly, cells are maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and grown at 37 °C with 5% CO_2. For chlamydial infection experiments, HeLa cells were grown until confluent in 6 well tissue culture dishes and then infected with DFCT28 at an MOI of ~3 using centrifugation at 545 g for one hour. The infected cells were then incubated at 37 °C with 5% CO_2 with DMEM/FBS supplemented with 0.2 μg/ml cycloheximide and 1X non-essential amino acids. At various times post infection, the medium was removed, cells were washed twice with 2 ml of PBS, and cells were lysed via addition of 200 μl Laemmli buffer with β-mercaptoethanol followed by heating at 90-100

°C for 5 minutes. Chlamydial protein samples or purified, recombinant ClpP samples were run on 12% SDS-PAGE gels and either stained for total protein with Coomassie Brilliant Blue or transferred to nitrocellulose for Western blotting. Blots were probed with a rabbit polyclonal anti-ClpP1 (*Pseudomonas aeruginosa* designation, similar to ClpP2 from *C. trachomatis*) diluted 1:10,000 in 5% milk Tris-buffered saline (mTBS) or anti-Clp2 (*Pseudomonas aeruginosa* designation, similar to ClpP1 from *C. trachomatis*) at 1:2,500. The antibodies were kindly provided by Dr. Tania Baker (Massachusetts Institute of Technology) (PMID: 27849175). After incubating with primary antibodies, blots were washed with Tween (0.5%)-TBS (TTBS) and then probed with a goat, anti-rabbit IgG poly-HRP conjugated secondary antibody (Thermo Scientific 32260) diluted 1:1000 in mTBS. As a control for chlamydial protein, blots were also probed for the major outer membrane protein (MOMP) using a mouse monoclonal anti-MOMP antibody (1:1000; Abcam, ab41193) and a goat anti-mouse IgG HRP conjugated secondary antibody (1:2000). After incubation with the secondary antibodies, blots were washed with TTBS followed by TBS and then incubated with chemiluminescent substrate (EMD Millipore Immobilon ECL) followed by imaging on a Bio-Rad Chemidoc MP.

Chlamydial Transformation: Protocol followed was a modification of the protocol developed by Mueller and Fields [57]. For transformation, 10^6 *Chlamydia trachomatis* serovar L2 EBs that have been cured of the endogenous plasmid were incubated with 2μg of unmethylated plasmid in a volume of 50μL $CaCl_2$ at room temperature for 30 minutes. Each reaction was sufficient for a confluent monolayer of McCoy cells that had been plated a day prior. The transformants were added to a 1mL overlay of room temperature HBSS

per well, and an additional 1mL of HBSS was then added to each well. The plate was centrifuged at 400xg for 15 minutes at room temperature, where the beginning of this step was recorded as the time of infection. Following the spin, the plate was incubated for 15 minutes at 37° C. This infection was recorded as T_0. The inoculum was aspirated at the end of the incubation and replaced with antibiotic-free DMEM+10% FBS. 8 hours post-infection, the medium was replaced with DMEM containing 1μg/mL cycloheximide, 10μg/mL gentamicin, and 1U/mL penicillin. Cells infected with transformants were passaged every 48 hours until a population of penicillin resistant bacteria was established. These EBs were then harvested and frozen in 2SP solution at -80° C prior to titration.

Determining the Effect of Overexpression of Clp Mutant Proteins via Immunofluorescence Analysis: Transformed *Chlamydia trachomatis* containing a mutant *clp* gene under control of an anhydrotetracycline (aTc) inducible promoter was used to infect a monolayer of HEp2 cells on coverslips with penicillin as a selection agent. Samples were induced with varying amounts of aTc at 10 hours post-infection and were methanol fixed after either a 6 hour or 14 hour pulse. Fixed cells were incubated with an anti-L2 guinea pig primary antibody to stain for the organism and a goat anti-guinea pig Alexa488 conjugated secondary antibody for visualization. Additionally, a mouse anti-6xHis tag was used, followed by a goat anti-mouse Alexa594 secondary antibody for visualization. Finally, the samples were stained with DAPI for insight into the host cell and bacterial DNA. Representative images were taken on a Zeiss Fluoview confocal microscope with a 60x2x objective and were equally color corrected using Adobe Photoshop CC.

Analysis of the Effect of Overexpression of Wild Type and Mutant ClpP2X Operons: Chlamydial transformants containing either the ClpP2X wild type or the ClpP2X mutant operon under the aTc inducible promoter were used to infect a confluent monolayer of HEp2 cells plated on coverslips. Penicillin was added at 1U/mL to maintain selective pressure. Samples were induced 10 hours post-infection and methanol fixed after either a 6 or 14 hour pulse. Coverslips were stained using a guinea pig anti-L2 primary antibody and a goat anti-guinea pig Alexa405 conjugated secondary antibody for visualization of the bacteria. To assess the location of the ClpP2 proteins, a mouse anti-FLAG primary and goat anti-mouse Alexa594 conjugated secondary antibody were used. For ClpX protein analysis, a rabbit anti-6xHis primary antibody (a generous gift from Dr. Michael Chaussee, USD) and goat anti-rabbit Alexa488 secondary antibody were used. Representative images were taken on a Zeiss Fluoview confocal microscope using both 60x2x and 60x10x objectives. Images were equally color corrected using Adobe Photoshop CS4.

Testing of the ClpP specific antibiotics on **Chlamydia trachomatis:** Following synthesis of the ACP1, ACP1a, and ACP1b chemicals (drugs synthesized by Dr. Martin Conda-Sheridan of UNMC), discovered and characterized by Dr. Walid Houry and colleagues [32], initial studies to assess the effect on Ctr in addition to effects on host cell growth by this antibiotic were conducted. For assessment of cell viability upon treatment, four wells of a 96 well plate with a confluent monolayer of HEp2 cells either was or was not infected with density gradient purified wild type *Chlamydia trachomatis* L2 with an MOI of 1. These wells were either treated or not with 25 or 50μg/mL of ACP1, ACP1a, or ACP1b, with a set of DMSO only samples used as a control. Antibiotics were added eight hours

post-infection (hpi). At 24 hpi, 100μL of 2x Resazurin (Abcam) was added to three wells of each treatment condition, adding only DMEM to the fourth as a background control. Following a four hour incubation at 37° C, absorbance at the 570nM wavelength was recorded using a Tecan plate reader. The wells were averaged, subtracting background absorbance from samples without dye. Absorbance was reported as percentage of the untreated samples. To quantify the effect of the drug on *Chlamydia*, variable treatments of each drug (25μg and 50μg) were added eight hours post-infection. IFUs were collected in 2SP and frozen at -80° C prior to titration. Calculations took the average number of inclusions over 15 fields of view multiplied by the number of fields of view possible by a 20x objective and corrected for the dilution factor and volume of inoculum to yield IFUs/mL.

CHAPTER THREE

Results

The Clp proteins demonstrate homotypic interaction *in vitro*. We sought to determine whether these Clp protein intermolecular interactions were functionally similar to that of other bacterial Clp systems. Due to the extensive homology of the Clp proteins to other bacteria as seen in the multiple sequence alignments, we predicted that interactions of the Clp proteins are conserved across bacterial species. To perform this analysis, we first utilized the BACTH system. We detected and quantified homotypic interactions for all four proteins (Figure 7A&B). We tested heterotypic interactions as well but failed to detect any positive interactions. Such issues were likely due to one of several reasons. First, full complex formation is required for interaction between the AAA+ proteins and the ClpP proteins [9]. Because the adenylate cyclase toxin fragments are translationally fused to the target, the bulk of the fragment (T18 and T25 are 21.8 kDa and 25.6 kDa, respectively, including linkages added) may hinder proper oligomer formation and block heterotypic complex formation. Second, positive results in this assay require the interaction of only two proteins, so we cannot assume complete hexamer or heptamer formation from these data. Third, another potential issue is the presence of the endogenous Clp system in *E. coli*. These components may be interacting with the recombinant proteins and result in a chimeric oligomer with structural differences that block larger complex formation. Finally,

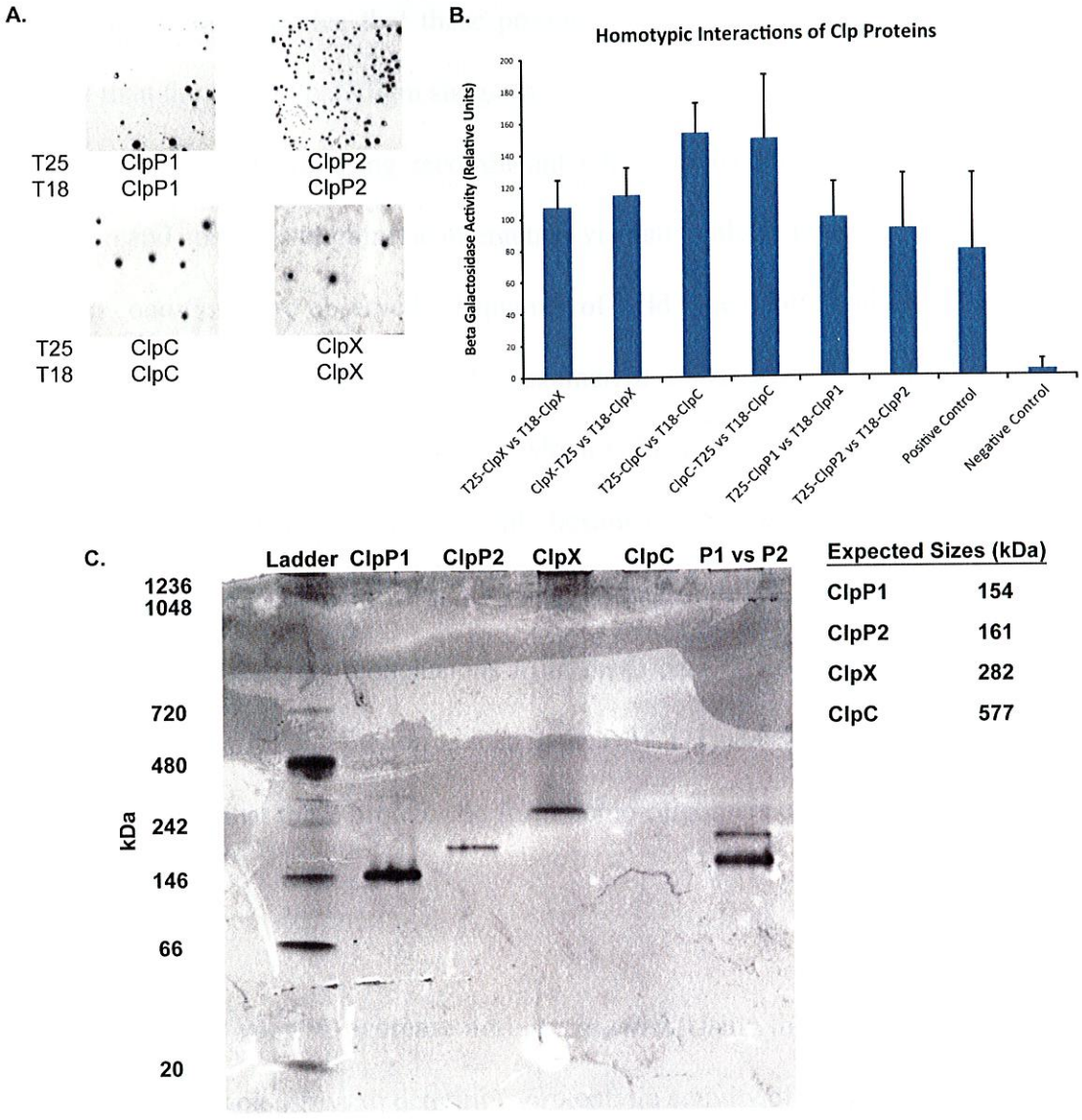

Figure 7: (A) Clp positive homotypic BACTH assays. Colonies were color corrected from blue to grayscale. **(B) β-Galactosidase assay results for homotypic Clp interactions.** Results are considered positive when at least five times the negative control. **(C) Native PAGE Coomassie blot.** Samples incubated in buffer D at 30° C for 15 minutes prior to run. 4-20% gradient gel. Run at 200V for 90 minutes. Expect heptamer/hexamer sizes in table to the right.

we cannot rule out the idea that these proteins do not interact and function markedly different than the current paradigm suggests.

Our *in vitro* work using recombinant Clp proteins further validates homotypic interaction and confirms oligomeric interaction via native blot. Figure 7C shows that, with the given conditions, we observed a heptamer of wild type ClpP1 and ClpP2 at 154 and 166 kDa, respectively. ClpX appears to hexamerize at 282 kDa, as well. Seemingly no protein was present in the ClpC samples, which is likely a technical error of loading too little protein. The expected size of a ClpC hexamer is 577 kDa. Shown in the text are samples done in the denoted buffer at 30° C, so other testable conditions should be assessed prior to drawing substantial conclusions from these data. These assays also confirm the lack of heterotypic ClpP interaction as seen in the BACTH assays. The observed homotypic interactions prove that these proteins do form homo-oligomers consistent with our current model.

ClpP1 and ClpP2 vary in protease activity *in vitro*. Using the recombinant ClpP1 and ClpP2 for degradation assays to determine proteolytic activity of the chlamydial ClpPs, we noted an apparent difference in protease activity. Our first assays used the small fluorogenic substrate, Suc-LY-AMC, which should be able to enter the ClpP complex without any additional activation. These data demonstrated that ClpP2 but not ClpP1 is able to allow small peptides into the complex to be degraded. While the *E. coli* ClpP exhibits several orders of magnitude more activity than those of *Chlamydia*, we conclude that at least ClpP2 is also able to degrade smaller substrates (Figure 8A&B). Turning to larger substrate degradation in the presence of the Clp activators, we observed that ClpP2

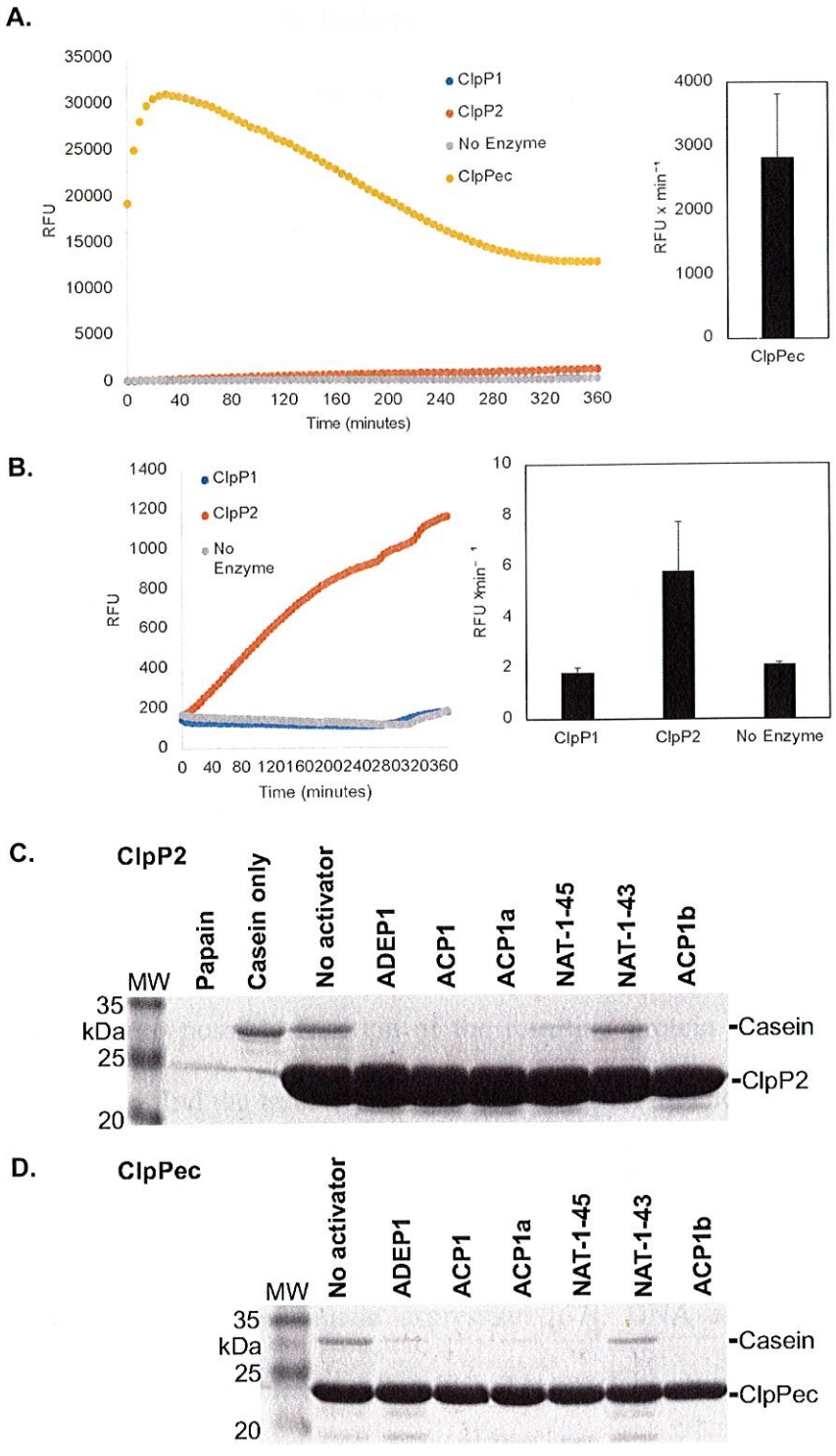

Figure 8: (A) RFU change in Suc-LY-AMC assay. Ec ClpP included, average reported. **(B) Ec ClpP excluded**. Same assay as (A), highlights only Ctr. **(C &D) Casein degradation assays with the ACP antibiotics.** Amounts reported in the text.

is also able to be activated to hydrolyze larger peptides with disordered regions that typically have no defined structure (i.e. casein). Taken together, we reason that at least ClpP2 exerts proteolytic activity, although these assays need to be optimized prior ruling out the absence of ClpP1 activity.

The *clp* genes are expressed as RB specific genes. In our initial characterization, we determined transcription patterns of each of the four *clp* genes of interest. Because chlamydiae are developmentally regulated bacteria, transcription patterns differ as the organism differentiates from one developmental form to the other. Genetic expression in *Chlamydia* can be roughly categorized into three different categories: early-, mid-, and late-developmental cycle genes. Early genes are involved in initial differentiation from an EB to an RB or evasion of the host immune system. Mid genes are RB specific and play a role in division and development of the RBs. Late genes function in differentiation from an RB to an EB. The specificity of a transcript to a particular point of the developmental cycle can give an insight into possible function of the resulting protein. Using this premise, we initiated studies to find the transcription profiles using RT-qPCR. We used the data from extensively studied genes as a basis to compare our data. Chlamydial early upstream operon (*euo*) served as the model for an early cycle gene [65, 66] while *omcB* represented transcript levels of late cycle gene expression [67]. DNA and RNA were analyzed following an infection of epithelial cells with wild type *C. trachomatis* L2. Our data (Figure 9A) suggest that all four *clp* genes are mid cycle, RB specific genes since the level of transcripts peaks mid-developmental cycle.

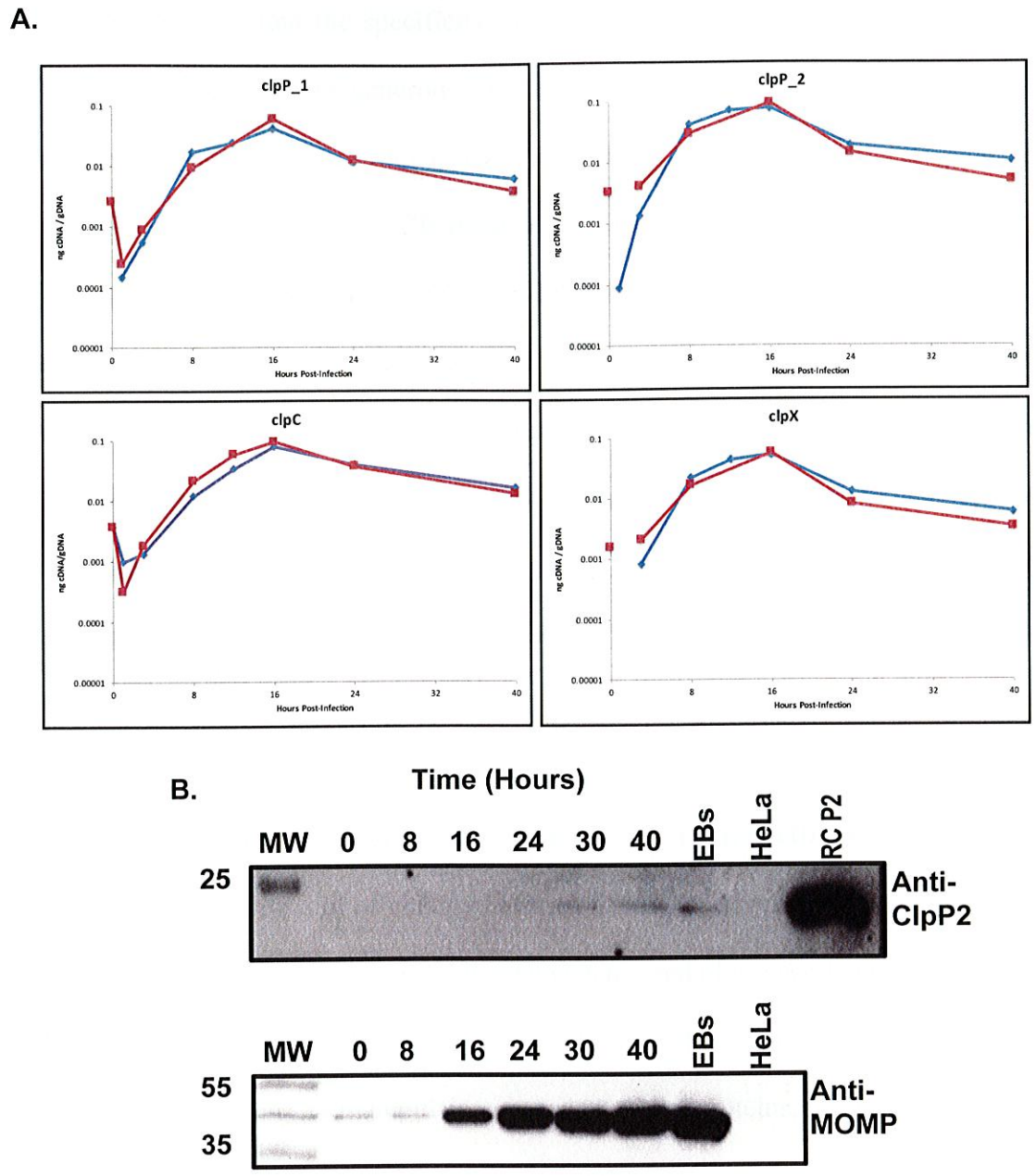

Figure 9: (A) RT-qPCR data for all four *clp* genes. Of note is the peak levels of transcript expression at approximately 16hpi. The abundance and maintenance of transcription suggests that these transcripts are RB specific. Courtesy of Dr. Scot Ouellette. **(B) Western blotting of chlamydial lysates for ClpP2.** Targeting of ClpP2 using a ClpP specific antibody in *P. aeruginosa* (of Dr. Tania Baker, MIT). Courtesy of Dr. Derek Fisher

Further supporting the specificity of the Clps to RBs is the western blot using ClpP2-specific antibodies (a generous gift from Dr. Tania Baker, MIT). That ClpP2 translation becomes apparent from roughly 8 to 16 hours post-infection (Figure 9B), correlating with an increase of an RB population. Given the transcriptional profile of the other *clp* genes, we extrapolate that we would see this pattern of translation of the other three Clps. This model is consistent with our hypothesis of possible function of protein quality control or chaperone activity of division proteins as characterized in other bacteria, as the bulk of protein production and division occurs during this phase of development. Additionally, because these transcripts increase around the time when RB to EB differentiation begins, the idea that the Clp proteins play a role in this process is a strong possibility as well.

Overexpression of inactive Clp proteins exerts differential effects on *Chlamydia*. Using the relatively new tool of chlamydial transformation developed by the Clarke lab [34], we transformed *C. trachomatis* L2 that had been cured of its cryptic plasmid (denoted as –pL2). We utilized an anhydrotetracycline (aTc) inducible shuttle vector developed by the lab of Dr. Scott Hefty to manipulate expression of our proteins, where we are able to induce increasing levels of recombinant protein expression with increasing amounts of aTc [35]. Due to the apparent sensitivity of *Chlamydia* to the expression of these proteins (demonstrated by a complete loss of viability with induction at time of infection), we induced expression 10 hours post-infection to allow a primary infection to be established. Both 6 and 14 hour pulses of aTc induction were utilized to determine inclusion morphology at 16 and 24 hours post infection (hpi). Overexpression of ClpP1mut and

ClpCmut appeared to have a negative impact on inclusion size (Figures 10 & 11). This is not unexpected, as these recombinant proteins may be forming complexes with the endogenous Clp subunits, resulting in reduced or blocked function. Of particular note is the graded response by ClpC in which the highest level of overexpression appeared to induce a state of persistence. That an aberrant inclusion stains for ClpCmut suggests that this effect is resultant of overexpression rather than plasmid rejection. ClpP1mut overexpression appears to reduce inclusion size (Figure 10), which fits with a model where ClpP1 and ClpC are important to maintain bacterial homeostasis by reduction of misfolded proteins via the aforementioned pArg tagging system; consequently, disruption of this system negatively impacts the organisms. Wild type transformations of recombinant ClpP1 and ClpC in our shuttle plasmid failed. As we have observed leaky expression in all of our Clp transformants with our inducible plasmid (data not shown), even minute amounts of additional wild type Clp proteins could reduce transformation efficiency as the bacteria reject the plasmid to eliminate extra expression.

Evaluating the effects ClpXmut and ClpP2mut overexpression, we observed little to no effect on inclusion morphology. The explanation for these phenomena is harder to predict, as we would expect that disruption of systems where ClpX and ClpP2 contribute to ribosomal rescue [27] and ClpX acts as a chaperone to division proteins [53] would demonstrate a significant negative impact on organism growth. These overexpression data suggest little to no effect on the inclusion, even by metabolic burden of expression of more protein (Figures 12 & 13). We speculate that the robust growth indicates that normal levels of wild type proteins are maintained and that ClpP2 and ClpX are able to interact immediately following translation, thus preventing the disruption of the system by the

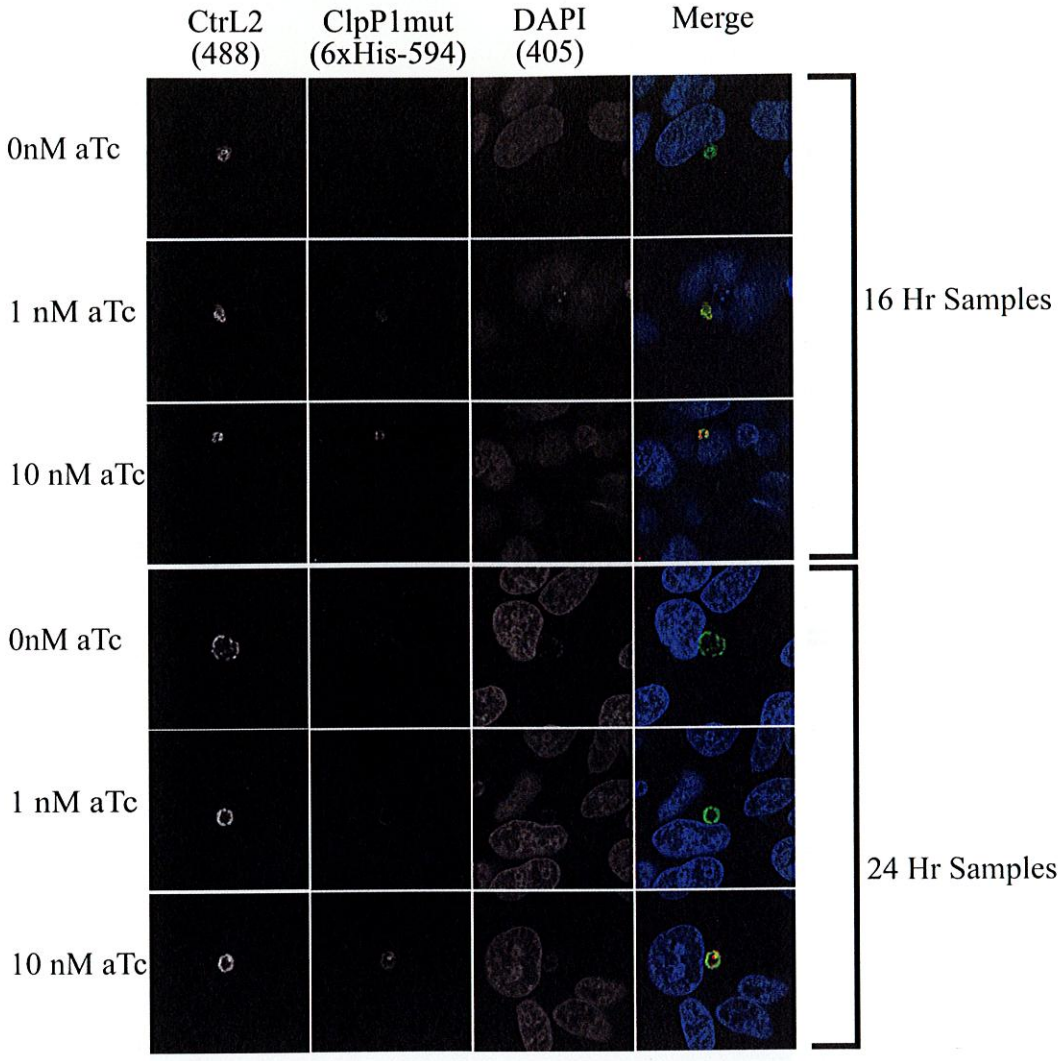

Figure 10: ClpP1(S92A) mutant overexpression immunofluorescence assay. Organism stain targets the MOMP protein of Ctr. The ClpP1mut protein was stained with a mouse anti-6xHis tag primary antibody. DAPI was used for visualization of DNA. Images are representative of observed transformants in the infection. Hours on the right indicate times fixed post-infection. Mutant ClpP1 overexpression from these studies appears to reduce inclusion diameter even in lower levels of overexpression.

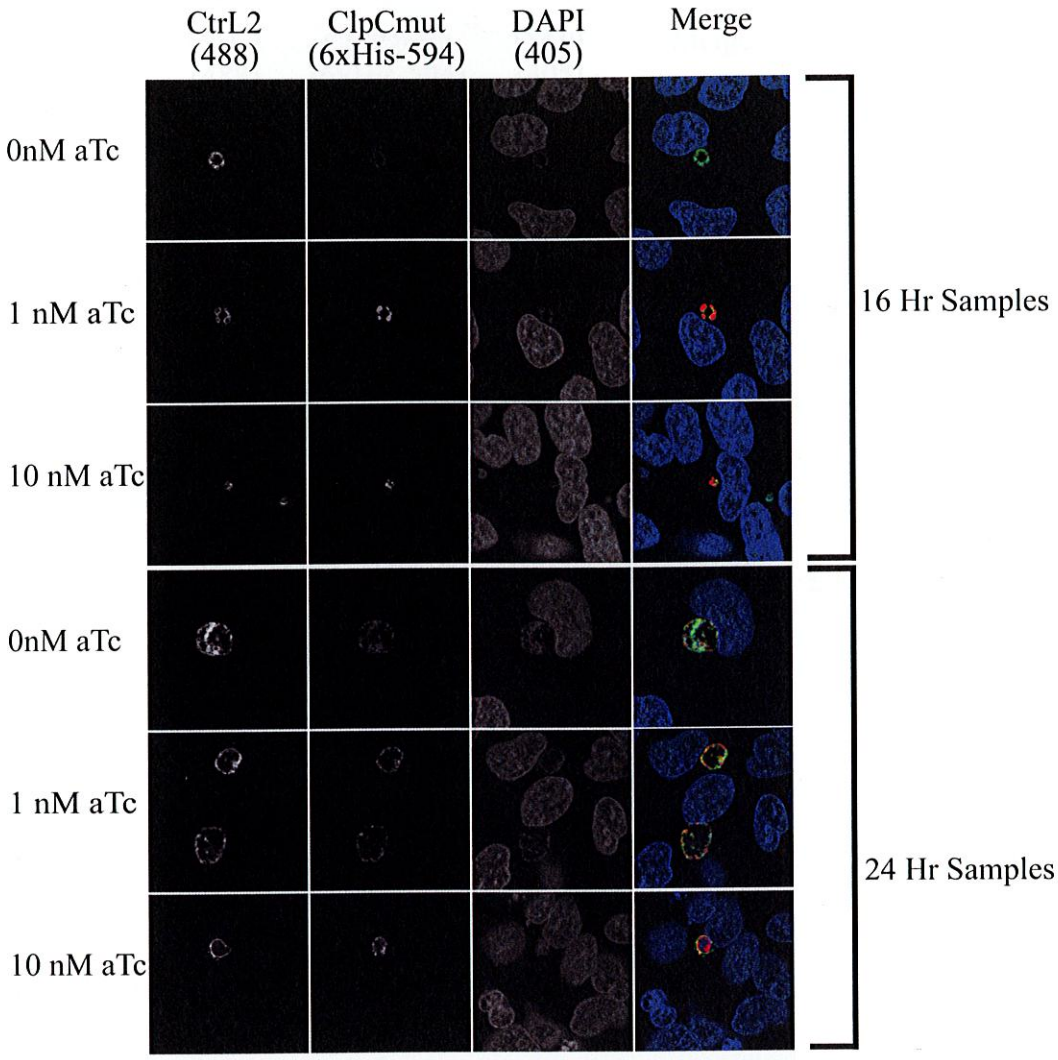

Figure 11: ClpC (E306A, E644A) mutant overexpression immunofluorescence assay. Staining parameters and test conditions are the same as described in the legend to Figure 7. Some leaky expression is noted in the 0nM aTc samples. Overexpression clearly reduces inclusion diameter in both 16 and 24 hour samples and appears to induce a persistent form in the highest level of overexpression in the 24 hour samples.

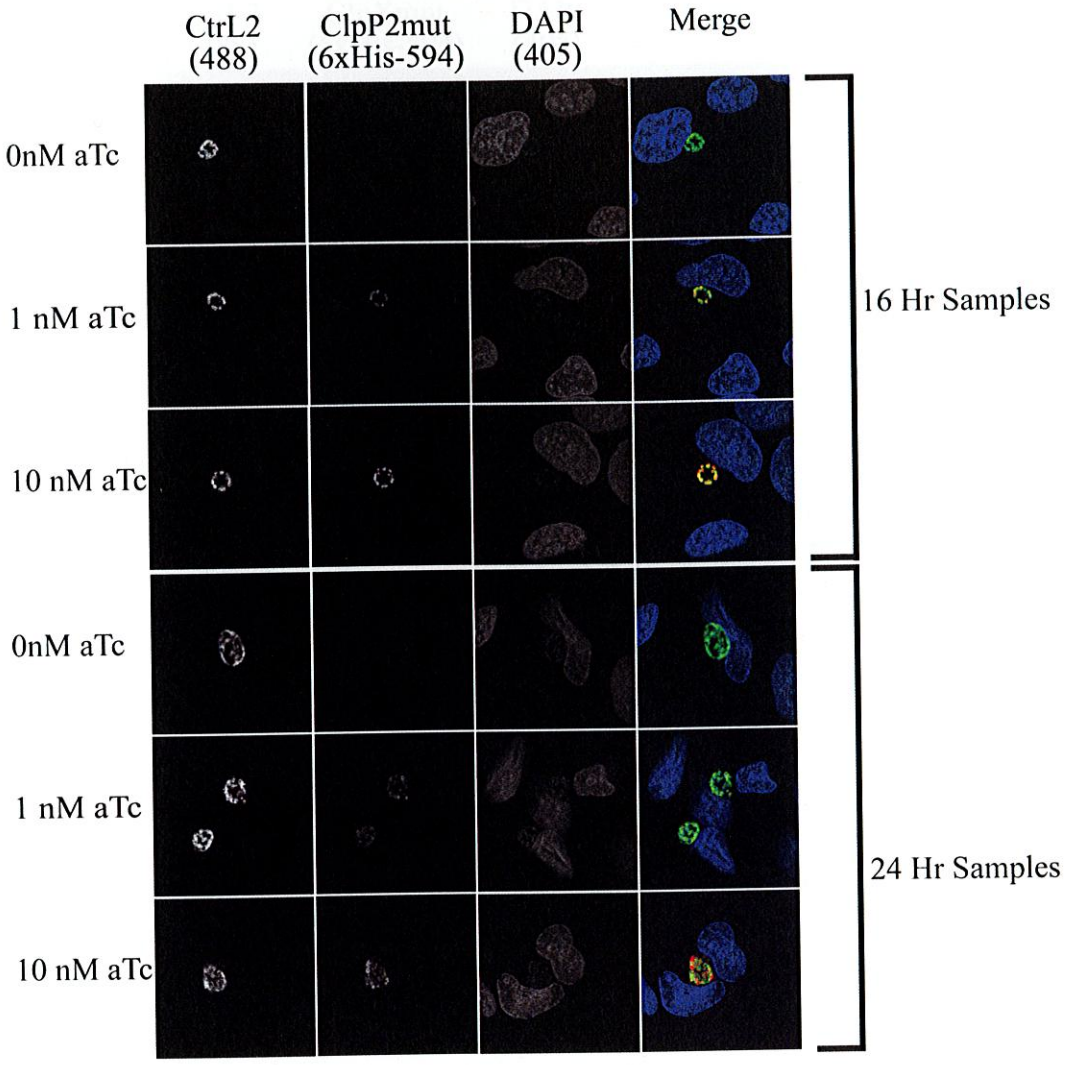

Figure 12: ClpP2(S98A) mutant overexpression immunofluorescence assay. Staining parameters and test conditions as described previously. Overexpression of inactive ClpP2 displays no obvious effect on inclusion morphology. The robust expression with localization within individual organisms confirms successful transformants, but function still remains hard to predict from this study.

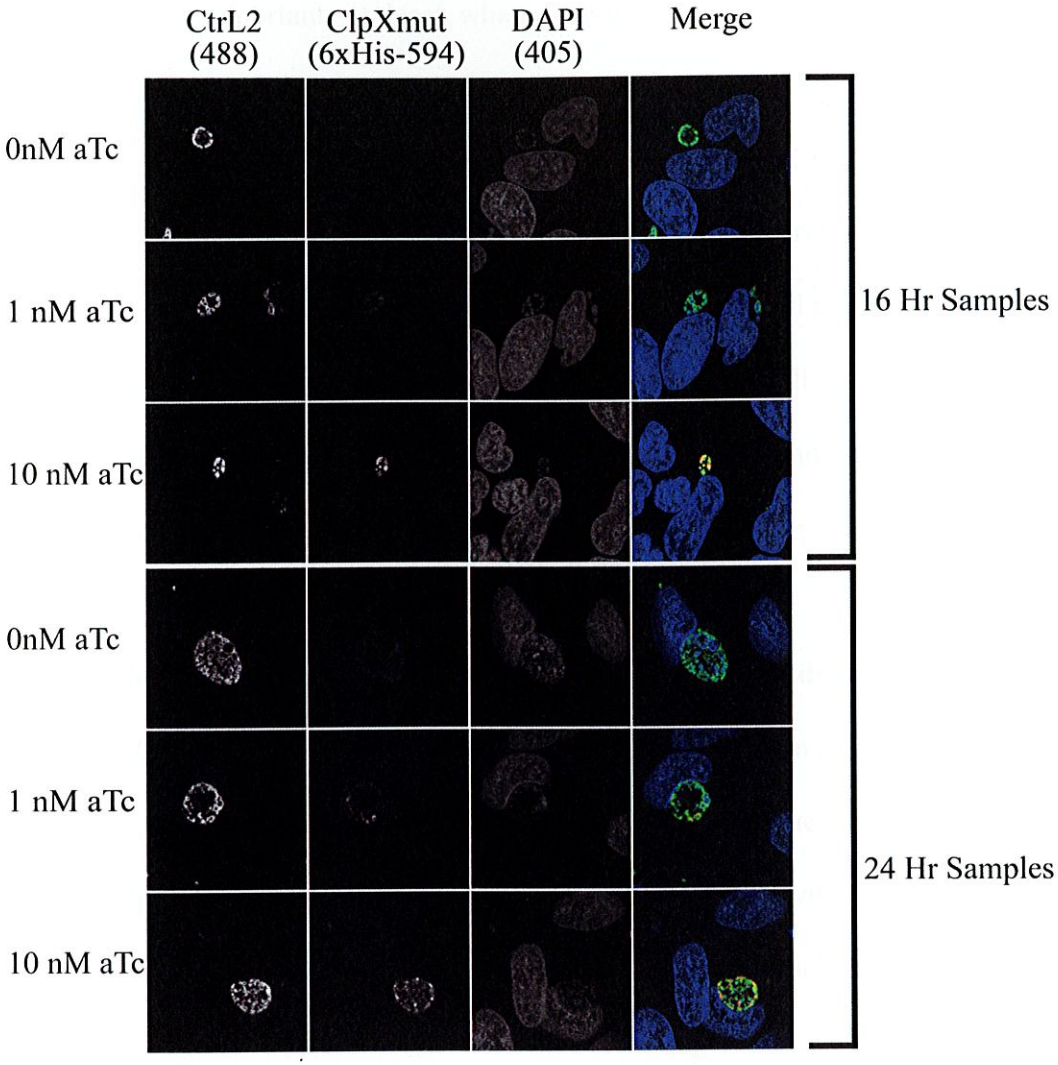

Figure 13: ClpX(E187A) mutant overexpression immunofluorescence assay. Staining parameters and test conditions as described previously. The discrepancy between the apparent graded response of the 16 hour samples and the seemingly unaffected 24 hour samples is of interest. A disconnect suggests importance to ClpX earlier in the developmental cycle, but more testing is required to validate such a conjecture.

inactive mutants. Transformations of *C. trachomatis* with wild type ClpX and ClpP2 (individually) failed, which strengthens the hypothesis that the ratio of these two proteins to one another is important, at least when functional. Given that negative results are typically not overly informative, more testing is necessary to determine the meaning of these data. As discussed previously pertaining to ClpP1 and ClpC mutant proteins, basal levels of transcription due to leaky repression may allow enough protein expression to shift the balance of these proteins out of homeostasis, stressing the cell and leading to plasmid rejection. Since wild type proteins expressed on a plasmid can still oligomerize and target their native substrate(s), over activity due to elevated protein abundance could explain the rejection of the wild type encoding plasmid.

Overexpression of wild type and mutant ClpP2X operons yields distinct phenotypes. Because the *clpP2* and *clpX* genes are positioned in tandem within the same operon in the chlamydial genome as shown in Figure 2, we replicated this in the pTLR2 plasmid while tagging the ClpP2 protein with a FLAG tag and the ClpX protein with a 6xHis tag. We also maintained the intergenic region between these genes to ensure stoichiometric conservation of protein abundances. Transformants were induced using variable concentrations of aTc for different pulse lengths (as described above) and then stained for visualization via immunofluorescence. While we could not isolate a clonal population of the mutant ClpP2X operon, we were able to plaque purify the transformants containing the wild type ClpP2X. Upon observation, we noticed that the mutant ClpP2 and mutant ClpX appeared to localize together in punctate dots (Figure 14). Inclusion size appeared to be negatively impacted as well. While we were unable to achieve successful transformation of individual wild type

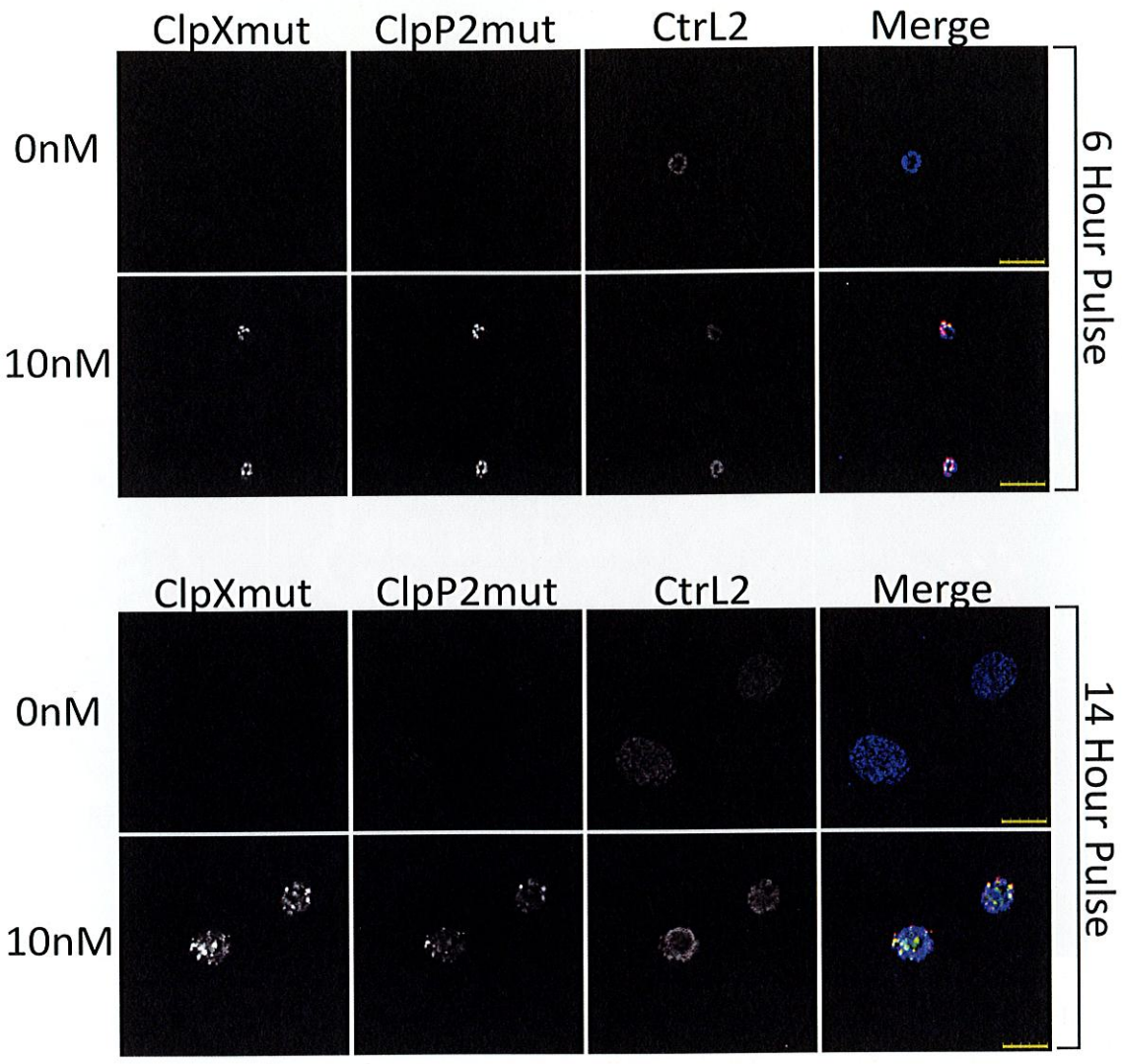

Figure 14: ClpP2(S98A)ClpX(E187A) overexpression immunofluorescence assay. Parameters same as described above, where 6 hour pulse refers to samples fixed 16 hours post-infection and 14 hour pulse refers to samples fixed 24 hours post-infection. Scale bars are 10μm. Co-expression at high levels of both inactive ClpP2 and ClpX appears to reduce inclusion size, with both recombinant proteins seeming to localize together within the organisms.

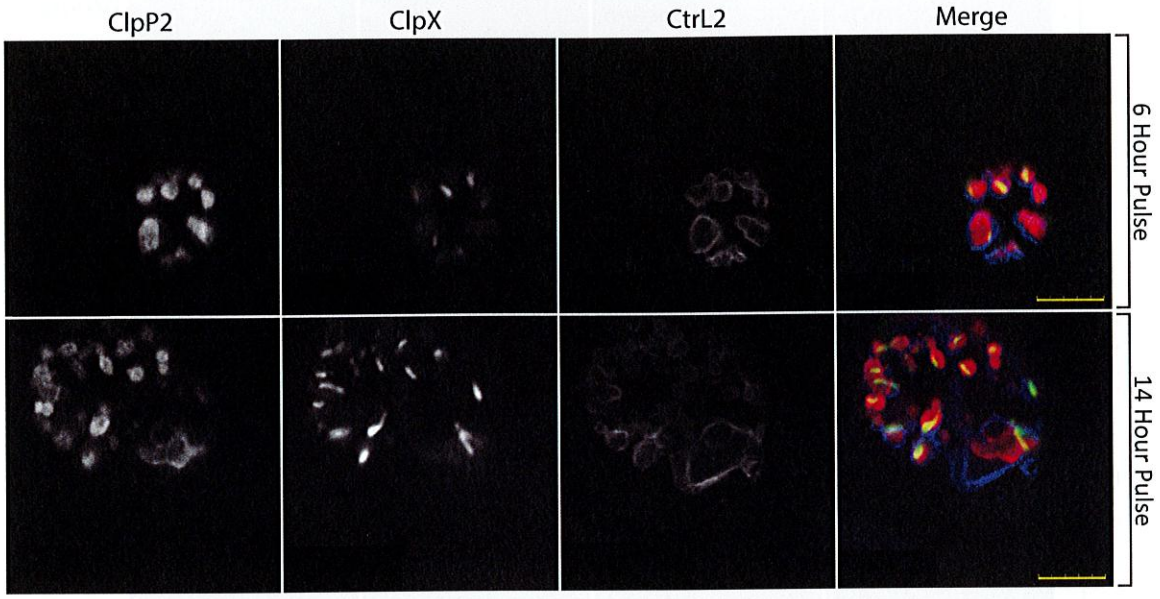

Figure 16: ClpP2X Overexpression. Same parameters as above, (10nM treatment) but at 60x2x magnification to better visualize the distinct localization of ClpX. Scale bars are 5μm.

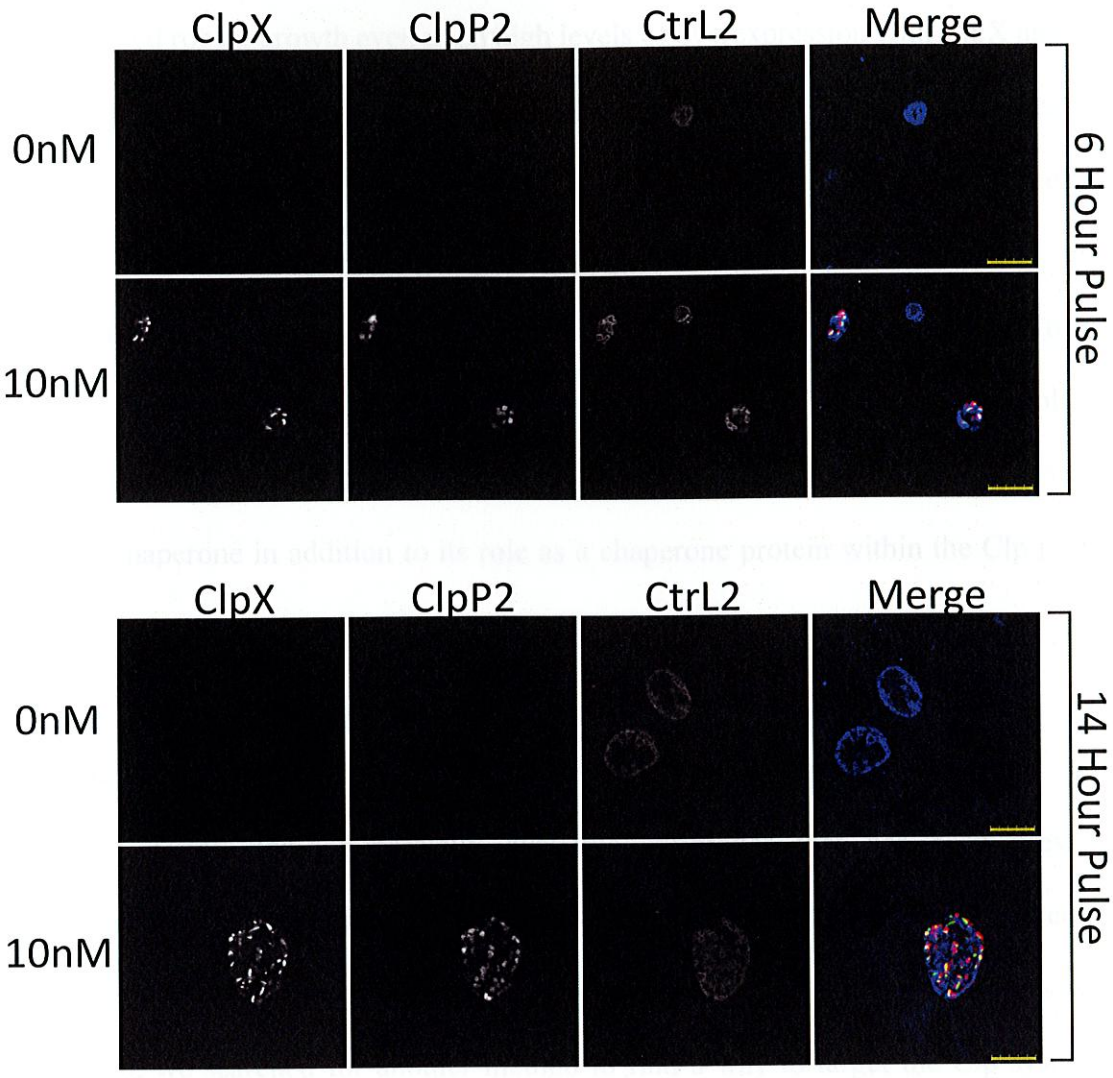

Figure 15: ClpP2X operon construct overexpression immunofluorescence assay. Parameters as described above. Scale bars are 10μm. Overexpression seems to have no negative effect on inclusion morphology. While ClpP2 (red) is entirely cytosolic within the organisms, ClpX (green) appears to show distinct localization. Organism stain is blue.

Clp components, the wild type P2X operon was successful; moreover, the inclusions demonstrated robust growth even upon high levels of overexpression with ClpX appearing to localize in an almost linear fashion (Figures 15 & 16). These data suggest that ratios of ClpP2 to ClpX are also important to bacterial homeostasis. While the ClpP2 was entirely cytosolic within the RBs (as anticipated), ClpX showed specific localization within individual RBs along what could potentially be a plane of division in addition to being cytosolic. This suggests potential involvement with proteins known to be involved in division in other bacteria [26] [44]. Future studies will investigate the possibility of ClpX to act as chaperone in addition to its role as a chaperone protein within the Clp protease system.

Antibiotic targeting of ClpP suggests a vital function of regulation by AAA+ proteases. Using non-commercially available antibiotics that has been previously characterized to be functionally analogous to ADEP compounds [32], we explored their effect on *Chlamydia*. Since we are currently unable to knock out the Clp genes within *Chlamydia*, we searched for another method to find a way to target the Clp system for characterization. To study our system, we first attempted to use acyldepsipeptide (ADEP) ClpP protease activators that uncouple the need for the AAA+ chaperones to target proteins to the ClpP complex [33]. These studies relied heavily on whether or not the compound affected the host cells as well, as human mitochondria also contain a ClpP homolog. We sought an alternative to the ADEP compounds because we had previously found excessive toxicity to host epithelial cells when used at bacteriocidal concentrations (data not shown), which could skew our IFU secondary infection data. We synthesized and used a drug that

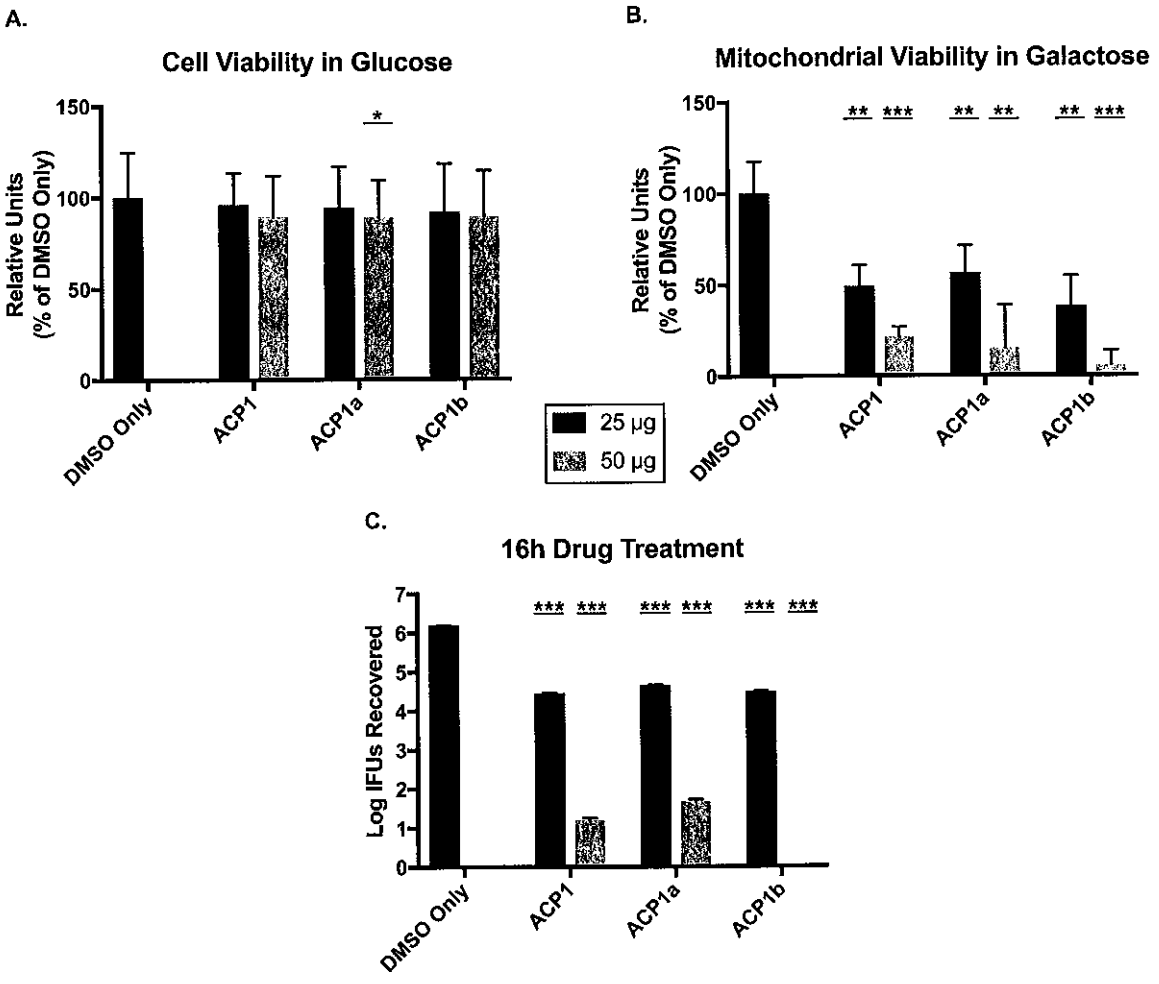

Figure 17: Activators of cylindrical proteases (ACP) studies. * P<0.05, ** P<0.01, ***P<0.0001 with all experimental values being compared to the DMSO only control via student's two-tailed T test. **(A) Cell Viability** in DMEM with glucose as the carbon source. **(B) Mitochondrial viability** in DMEM with galactose as the carbon source. **(C) IFU titration** of *Chlamydia* infected, ACP1/a/b treated or not cells that were lysed and used to reinfect a new monolayer. Numbers reported as $\log_{10}$ of IFUs recovered.

a lab had characterized, termed Activator of Cylindrical Proteases (ACP). *In vitro* assays (Figure 8) showed an increase in protease activity when ClpP2 (as well as Ec ClpP) was incubated with a more "difficult" substrate (casein) in the presence of ACP1 cassette of drugs, suggesting functionality of the drug on the Clp proteins as previously described. The Resazurin cell viability studies indicate that inhibition of *Chlamydia* as seen in the IFU secondary infection models (Figure 17A&B) is specific to the bacteria rather than a result of host cell death. The limited reduction of host cell viability in glucose containing DMEM as opposed to the significant drop in mitochondrial viability in galactose containing DMEM suggests that, while the drugs may be acting on the mitochondria, the ACP chemicals do not exert a strong enough effect on host epithelial cells to account for the reduction in inclusion forming units recovered following treatment and reinfection. Moreover, the ACP drugs remarkably inhibited *Chlamydia* in cell culture (Figure 17C). With such drastic effects reported, we conclude that regulation of the chlamydial ClpPs is absolutely vital to bacterial fitness.

CHAPTER FOUR

Discussion

The Clp protease system has been extensively studied primarily in *E. coli* but also in a multitude of different organisms (see [11] for review). To our knowledge, our studies are the first of their kind in *Chlamydia* and could potentially provide insight into chlamydial physiology in addition to yielding more information about the complex developmental cycle of this organism. We were able to utilize bioinformatic techniques to provide insight into the potential conserved function of the Clp proteins. Indeed, we found significant similarity of the chlamydial proteins to those of more studied organisms such as *E. coli*, with most of the hallmark conserved residues present.

Since the discovery of the ClpP protease [79], interaction studies showed that the ClpP proteins typically interact as a tetradecameric stack of two heptamers [70]. The serine active sites are buried within these barrel-like structures [71]. ClpP hydrolysis of large protein substrates is increasingly stimulated in the presence of AAA+ chaperone binding [77], although our primary studies focused on ATPase-independent proteolysis. Complexed ClpP alone processes small substrates of up to 30 amino acids regardless of the presence of ATP, suggesting that active site availability and complex accessibility are the two main regulatory mechanisms of ClpP [73]. Substrates that enter this complex by either chaperone-mediated or chaperone-independent are hydrolyzed into short peptides of 6-8 amino acids with some degree of amino acid specificity [80] [81]. While not unprecedented, the presence of two encoded ClpP homologs is unusual. In P. aeruginosa,

another bacterium encoding dual ClpP proteases, the two isoforms have been shown to not interact in with the given test conditions, based off the different transcriptional profiles and differential effects on virulence factor production [13]. Conversely, the two ClpP proteins of M. tuberculosis and L. monocytogenes heterologously interact with two homotypic heptamers, which modulates an increase in function [28, 37, 75]. The activation of one homotypic ClpP heptamer activates the heptamer of the other isoform in M. tuberculosis, providing a novel mechanism for activation [18]. Of note is that L. monocytogenes may also form a completely homotypic tetradecamer of one ClpP paralog [76]. Again, the Clp proteolytic subunits played an integral role in virulence factor production and bacterial survival during intracellular growth [72]. Taken together, these studies demonstrate the diverse function of dual ClpP peptidase systems in the developmental cycles of various bacteria.

Both ClpP1 and ClpP2 have hydrophobic residues aligned with those of other bacterial ClpPs, suggesting the presence of AAA+ adaptor protein docking sites that allow recognition of the IGF/L motifs of these proteins (Figures 3-5) [19]. Both ClpP1 and ClpP2 in addition to the other bacterial ClpPs presented all show an extremely high conservation of catalytic triad alignment (in multiple sequence alignment and 3D predicted structures (Figure 3B)). These results are not unanticipated given the evolutionary conservation of these proteins from prokaryotes to human mitochondria. Perhaps the largest discrepancy among the ClpP proteolytic individual subunits is the residues present within the interaction interface. While we have not yet tested these distinct differences to determine their function in protein interactions, they have not escaped our interest. The striking difference of ClpP1 interaction residues from those of ClpP2 coupled with our data

suggesting no interaction between ClpP1 and ClpP2 strengthens our hypothesis that these proteins do serve functions independent of one another

ClpC and ClpX contain the evolutionarily retained IGF/L loop motifs that facilitate interaction with the ClpP tetradecameric complex [18]. Chlamydial ClpX and ClpC also retained the ATP binding and hydrolysis sites known as the Walker A and Walker B motifs, respectively [20] [24]. The presence of only one set of these motifs classifies ClpX as a type I AAA+ protease while ClpC contains two sets, classifying it as a type II AAA+ protease. Chlamydial ClpX also has conserved pore loops for recognition of ssrA tagged substrates associated with stalled ribosome rescue, showing potential as a player in that system as well [21]. Interestingly, the authors demonstrated that the RKH motif and pore 1&2 loops aid in recognition of the ssrA tag in addition to a protein known as SspB, which is a stress protein present in other bacteria. While *Chlamydia* retain those motifs within their encoded ClpX protein, no SspB homolog has been identified to date (unpublished observation). *Chlamydia* also encodes both the tmRNA gene and a SmpB homolog, which has been shown in other bacteria to assist in tmRNA recognition of stalled ribosomes [56] [55]. Because ClpX shows preservation of these three conserved pore loops that have been implicated in substrate recognition by the hexameric complex, a ClpXP complex existing *in vivo* and playing a role in tagged protein degradation seems highly likely [19]. The retention of the N-terminal zinc binding domain (ZBD) strengthens the idea of the function of chlamydial ClpX as a proteolytic chaperone, as such a structure has been implicated in ClpX dimerization in addition to hexamerization for proteolytic activity [51] [52]. The conservation of the cysteine residues that function in chelating zinc suggests that *Chlamydia* is able to utilize the N-terminal domain of its ClpX for oligomerization, thereby

allowing for functionality as an unfoldase. Interestingly, the zinc binding domain is not necessary for ssrA recognition but implicates ClpX in differential recognition of substrates where the unfoldase function may be more important for reorganization of protein complexes rather than unfoldase activity [53]. Future investigations will dissect the particular role of ClpX to chlamydial biology in terms of independent chaperone function in addition to its role in protein turnover.

Our hypothesis that ClpC is involved in proteomic turnover is strengthened by the presence of conserved N-terminal phosphorylated arginine (pArg) docking sites. The targeting of proteins containing phosphorylated arginines in addition to the ClpC protein itself have been shown in heat shock responses in other bacteria [22] [25]; however, heat shock studies performed on *Chlamydia* have failed to implicate ClpC in these responses [23]. While the function of pArg recognition has yet to be elucidated in *Chlamydia*, these residues provide substantial evidence for the role of ClpC in misfolded, pArg-tagged protein degradation. We have not ruled out that arginine phosphorylation may also occur at particular time points during the developmental cycle, which could serve as a signal for differentiation. Although the presence of a ClpC homolog in *C. trachomatis* is not unprecedented, ClpC is primarily found in Gram positive bacteria, while ClpA is the primary type II AAA+ protease in Gram negative bacteria (unpublished observation). A pairwise alignment of Ctr ClpC and Ec ClpA (Figure 5B) shows how these two type II AAA+ orthologs can play vastly different roles while still utilizing a similar underlying mechanism of action. Our mutant overexpression studies also revealed that disruption of the ClpC system yields a strikingly negative phenotype. Such an effect fits with a model where ClpC may be involved in protein quality control through recognition of

phosphorylated arginines (pArg), as observed in *B. subtilis* [22]. Disruption of this system with inactive ClpC proteins may contribute to an accumulation of misfolded proteins, which could be an underlying cause of induced persistence via cytoxicity. Another possibility explaining the effect of ClpCmut overexpression could be disruption from a delicate homeostasis of some sort of transcriptional regulator [54], the caveat being that these previous studies report competence genes and not persistence genes. *Chlamydia* contains a suspected arginine kinase and adaptor protein similar to those found in *B. subtilis* [22] [40-42]; future studies will be conducted to determine the role of these proteins with the Clp system. One interesting finding pertaining to the chlamydial ClpC is the absence of a MecA ortholog. Direct interaction between ClpC and MecA has been documented in other organisms, particularly in the function of degradation of ComK as a competence factor in *B. subtilis* [20] [54]. Since *Chlamydia* encodes no proteins showing significant similarity to either MecA or ComK, chlamydial ClpC may potentially act as a regulator of a virulence factor. Nevertheless, the function of ClpC appears to be important to *Chlamydia*, as overexpression of inactive ClpC appears to induce a persistent form (Figure 8). From a purely bioinformatic standpoint, we reasonably assume that the chlamydial Clp proteins should at least demonstrate conserved interactions, though differences in function will be the target of future studies.

We demonstrated that the Clp proteins of *Chlamydia* interact homotypically, as observed in other bacteria. Although we were unable to show heterologous interaction with our assay, we reason that this may be due to complications of the assay rather than negative protein complex interactions. Extrapolating from our current BACTH data and the previously discussed homology, we still predict that complexes form *in vivo*. Studies to

prove this experimentally both *in vivo* and *in vitro* will be conducted to test this hypothesis as well as to validate the homotypic oligomerization of these proteins. The successful *clpP2X* operon transformed into Ctr strengthens our prediction via microscopy and IFA of ClpP2 and ClpX intermolecular interaction. While ClpX also appears to be localizing in a distinct pattern that could suggest division protein chaperone function [26] [44], we also saw cytosolic staining that could be ClpX complexing with the cytosolic ClpP2. In *M. tuberculosis* expressing a dual ClpP system, the researchers demonstrated that the ClpPs form heterologous, non-canonical complexes [28] [37]. While we have considered the notion, our data support a model of dual functionality via separate homotypic ClpP complex formation based on the lack of homology between ClpP1 and ClpP2.

Immunofluorescence assays of the ClpP1 and ClpP2 inactive mutant transformed Ctr clones suggest that some fundamental difference between ClpP1 and ClpP2 exists. The graded response of ClpP1 to increasing amounts of overexpression reveals that disruption of the ClpP1 homeostasis impacts the organisms more so than that of a disruption of ClpP2 homeostasis. Inactive ClpP2 does not appear to reduce inclusion size upon higher levels of overexpression. Given the distinct differences of mutant protein overexpression, we reasoned that each ClpP must serve an independent function. That ClpP1 plays a role in misfolded protein proteolysis seems likely, as inhibited function via the serine active site mutation could allow for aggregation of misfolded proteins that leads to cytotoxicity. The *in vivo* function of ClpP2 is harder to predict, as overall impact on the organisms appears to be minimal. We were unable to isolate clonal populations of ClpP1, as leaky expression from our shuttle vector potentially allowed for low levels of protein expression that led to subsequent occasional rejection of the plasmid, thus inducing persistence and confounding

the isolation of a uniform population. We also attempted the transformation of wild type constructs but failed multiple times. The inability to transform individual wild type Clp encoding plasmids into *Chlamydia* could also be a result of the leaky expression and suggests that ratios of wild type ClpP1 and ClpP2 to their respective cofactors may play a vital role in homeostasis of the bacteria. The repression offered by the tetracycline-inducible pTLR2 is effective but not perfect and allows for leaky expression of our recombinant proteins. We have so far been unable to prove experimentally that the basal level of expression is contributing to the lack of transformants, but multiple unsuccessful attempts lead us to believe that ratios of functional Clp enzymes are important to bacterial homeostasis. As aforementioned, overexpression the ClpP2X wild type operon yielded robust inclusion growth. This observation solidifies the hypothesis of the importance of ratios and also suggests ClpP2 and ClpX interaction. Inclusion diameter does not seem to be negatively impacted despite the additional metabolic burden of overexpression of two proteins; rather, the inclusions appear to be comparable in size if not larger than the uninduced samples. The significance of this phenomenon will be the subject of future investigations.

The transcriptional patterns of our proteins of interest all fit with the mid-developmental cycle, RB specific profile. These data suggest that the Clp proteins are not involved in inclusion organization, as would be the case with early genes. Rather, that these proteases are primarily expressed during RB proliferation and remain high throughout the remainder of the developmental cycle provides evidence for participation in control of protein quality and differentiation. A high level of protein production by the RBs increases the risk of misfolded proteins and stalled ribosomes, so the Clps could provide a rescue

mechanism to prevent cytotoxicity. We also are interested in identifying substrates using the Clp^{Trap} method [36], which shows promise as we have already engineered these inactive proteins and transformed them into *Chlamydia* (as described above in mutant overexpression studies). Successful affinity purification may prove the involvement of the Clp system in phosphorylated arginine recognition via mass spectrometry, thereby validating the observed genomic evidence that this system exists in *Chlamydia*. This trap method may also yield a Clp target that modulates differentiation, which would provide a mechanistic insight into the transition from RB to EB.

The binding of the ClpC or ClpX chaperones potentiates ClpP activity; accordingly, removing the need for this interaction to undergo proteolysis of larger substrates shows a severely negative impact on the organisms [17]. We have demonstrated this reduction in chlamydial viability in our initial studies using the ACP1 antibiotics. Our *in vitro* purified protein data further shows that, in the presence of the ClpP activators, chlamydial ClpP can hydrolyze more "difficult" substrates. These data suggest that the significant reduction of bacterial viability following treatment is on account of drug induced dysregulation of chlamydial ClpPs rather than some other mechanism. We see that, with our cell viability studies, the drugs directly affect the bacteria rather than merely reduce host cell viability, which confirms that the reduction of inclusion forming units upon drug treatment is due to the negative effects of the drugs on the organisms and is not due to the loss of host cells. Not surprisingly, our data show that drug treatment significantly reduces chlamydial viability. Removal of the need for an ATPase chaperone and stabilization of the N termini results in highly unregulated proteolysis. While we do not know what proteins are targeted during dysregulation of chlamydial ClpPs or whether the targeting still retains specificity,

our data reinforce the idea that regulation of the destructive ClpP proteases is vital to the survival of the organism. Because the hypothesized activity stems from drug binding in the hydrophobic pockets of the ClpP complex to relax the N terminal regions of the oligomer, we plan to mutate these residues to polar amino acids, thereby removing the interaction and abolishing ACP1-induced activity. An important caveat to these assays is that we showed a definite effect on the mitochondria. Given that *Chlamydia* are parasitic and intercept nutrients from the host cells, we cannot rule out that the negative effect on the mitochondria may reduce chlamydial viability by reducing production of some unknown, mitochondrial-produce factors upon which *Chlamydia* is reliant. Nevertheless, we feel confident that our data are indicative of a direct effect on the bacteria despite any potential off-target effects.

We have shown here an initial characterization of the chlamydial Clp protease system. The data acquired and presented provide a foundation on which we intend to build future studies. Overall, the sensitivity of the organisms to this system suggests a vital function in maintenance of bacterial physiology and a possible role in differentiation of *Chlamydia*. We will pursue all aspects of this system in the future to determine the essentiality and function of the Clp protease system in the growth and development of *Chlamydia*.

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Appendix I

Mutagenesis Cloning		
Forward Sequence	Reverse Sequence	Description
TTTAGCAGCAGCAATGGGATCTG	CCTGTAACAACTGTAGTC	Mutation of the ClpP1 serine active site
ACAAGCCGCTGCAATGGGAGCGC	CCAATGCAGTACGTATTTACATCACAG	Mutation of the ClpP2 serine active site
TTACATTGATGCAATCGATAAAATTGGTCGC	ATAATGCCTCGCTCTGCTC	Mutation of ClpX Walker motif
ATTTATTGATGCACTTCACACGATTGTTG	AAGAGGATGTTCCCATGTTTAC	Mutation of ClpC Walker motif (1 of 2)
GTTGTTTGATGCTATTGAAAAAGCACATCC	ACAACACAGTAAGGGCGG	Mutation of ClpC Walker motif (2 of 2)

Genomic Cloning		
Forward Sequence	Reverse Sequence	Description
ATCCCACCGGTATGCCTGAAGGGGAAATGATGCA	ATATTCGGCCGTTAGTGATGATGATGGTGATGCA AGTCGTTAAAAGAGAAGAGA	Addition of 6xHis tag to ClpP1
ACCCCACCGGTATGACGTTGGTACCATACGTTGT	ATATTCGGCCGTTAGTGATGATGATGGTGATGA GACGCAATACTCTTATCTT	Addition of 6xHis tag to ClpP2
CCCCCCCCCACCGGTATGACAAAAAA AAATCTTGCGGTCTGTTCT	ATATTCGGCCGTTAGTGATGGTGATGGTGATGA GCAATCGCCTCTGGTGATTTCTGAAT	Addition of 6xHis tag to ClpX
ATATTCGGCCGTTAGTGATGGTGATGGTGATGTG ATTCATCAGCTGTAATAGGCT	ATCCCACCGGTATGTTTGAGAAGTTTACCAATCG CGCAAAGCAA	Addition of 6xHis tag to ClpC
ACCCCACCGGTATGACGTTGGTACCATACGTTGT	ACTTATCGTCGTCATCCTTGTAGTCAGACGCAAT ACTCTTATCTTTTG	Addition of FLAG tag to ClpP2
AATTAACAAGTTTGTACAAAAAAGCAGGCTTTA TGCCTGAAGGGGAAATGATGCATAAGTTGCAAG	AATTACCACTTTGTACAAGAAAGCTGGGTTCAA GTCGTTAAAAGAGAAGAGAATCCCATCTAACA	Addition of attB sites to ClpP1
AATTAACAAGTTTGTACAAAAAAGCAGGCTTTA TGACGTTGGTACCATACGTTGTTGAAGACACGG	AATTACCACTTTGTACAAGAAAGCTGGGTTAGA CGCAATACTCTTATCTTTTGTCTCTTTAGCAGA	Addition of attB sites to ClpP2
AATTAACAAGTTTGTACAAAAAAGCAGGCTTTA TGACAAAAAAAAATCTTGCGGTCTGTTCTTTTTG	AATTACCACTTTGTACAAGAAAGCTGGGTTAGC AATCGCCTCTGGTGATTTCTGAATAATGACCG	Addition of attB sites to ClpX
AGAAGGAGATATAACTATGCCTGAAGGGGAAA TGATGCATAAG	GTGGTGGTGATGGTGATGGCCCAAGTCGTTAAA AGAGAAGAGAATCCC	Cloning of ClpP1 into pLATE31
AGAAGGAGATATAACTATGACGTTGGTACCATA CGTTGTTGAAG	GTGGTGGTGATGGTGATGGCCAGACGCAATACT CTTATCTTTTGTC	Cloning of ClpP2 into pLATE31
AGAAGGAGATATAACTATGACAAAAAAAAATC TTGCGGTCTGTTC	GTGGTGGTGATGGTGATGGCCAGCAATCGCCTC TGGTGATTTC	Cloning of ClpX into pLATE31
AGAAGGAGATATAACTATGTTTGAGAAGTTTAC CAATCGCGCAAAGCAAG	GTGGTGGTGATGGTGATGGCCTGATTCATCAGCT GTAATAG	Cloning of ClpC into pLATE31

Ligation Independent Cloning		
Forward Sequence	Reverse Sequence	Description
TTTGTTTAAGAAGGAGATAATGCCTGAAGGGGA AATG	CCATTTTTCACTTCACAGGTCAACCTTAGTGATG GTGATGGTGATG	Addition of overlap for pTLR2-ClpP1construction
TTTGTTTAACTTTAAGAAGGAGATAATGACGTTG GTACCATA	CCATTTTTCACTTCACAGGTCAACCTTAGTGATG GTGATGGTGATG	Addition of overlap for pTLR2-ClpP2 construction
TTTGTTTAACTTTAAGAAGGAGATAATGTTTGAG AAGTTTACCAATC	CCATTTTTCACTTCACAGGTCAACCTTAGTGATG GTGATGGTGATG	Addition of overlap for pTLR2-ClpC construction
TTTGTTTAACTTTAAGAAGGAGATAATGACAAA AAAAAATCTTGCG	CCATTTTTCACTTCACAGGTCAACCTTAGTGATG GTGATGGTGATG	Addition of overlap for pTLR2-ClpX construction
TAACAATTCTCTACTTATCGTCGTCATCCTTG	TGACGACGATAAGTAGAGAATTGTTATGACAAA AAAAAATC	Addition of overlap to ClpX (Fwd) and ClpP2 (Rev) for pTLR2-ClpP2X

Appendix II

Strains			
Strain	Organism	Genotype	Purpose
DH5α	E. coli	fhuA2, Δ(argF-lacZ)U169, phoA, glnV44, Φ80, Δ(lacZ)M15, gyrA96, recA1, relA1, endA1, thi-1, hsdR17	Plasmid Cloning
DHT1	E. coli	F-, cya-854 , recA1, endA1, gyrA96 (Nal r) , thi1, hsdR17 , spoT1 , rfbD1, glnV44(AS), tetR	BACTH Assay
dam-/dcm-	E. coli	ara-14, leuB6, fhuA31, lacY1, tsx78, glnV44, galK2, galT22, mcrA, dcm-6, hisG4, rfbD1, R(zgb210::Tn10), TetS, endA1, rspL136, (StrR)dam13::Tn9, (CamR), xylA-5, mtl-1, thi-1, mcrB1, hsdR2	Unmethylated Plasmid Cloning
DH10β	E. coli	Δ(ara-leu), 7697 araD139, fhuA, ΔlacX74, galK16, galE15, ϕ80dlacZΔM15, (e14-), recA1, relA1, endA1, nupG, rpsL, (StrR), rph, spoT1, Δ(mrr-hsdRMS-mcrBC)	Plasmid Cloning
BL21(DE3)	E. coli	fhuA2, [lon], ompT, gal, [dcm], ΔhsdS, λ DE3 (λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5)	Protein Purification
Ctr L2	C. trachomatis	Chlamydia trachomatis L2/434/Bu (ATCC VR902B)	Drug Testing (inhibition and cell viability)
Ctr -pL2	C. trachomatis	Chlamydia trachomatis L2 (25667R)	Chlamydial Transformation

Plasmids		
Name	Features	Description
pST25-ClpP1	aadA, lacO, lacI, p15A ORI, CAP Binding Site, attR1, attR2, T25 (*B. pertussis* toxin fragment), ClpP1	BACTH C-terminal T25-ClpP1 Fusion Vector
pST25-ClpP2	aadA, lacO, lacI, p15A ORI, CAP Binding Site, attR1, attR2, T25 (*B. pertussis* toxin fragment), ClpP2	BACTH C-terminal T25-ClpP2 Fusion Vector
pST25-ClpX	aadA, lacO, lacI, p15A ORI, CAP Binding Site, attR1, attR2, T25 (*B. pertussis* toxin fragment), ClpX	BACTH C-terminal T25-ClpX Fusion Vector
pST25-ClpC	aadA, lacO, lacI, p15A ORI, CAP Binding Site, attR1, attR2, T25 (*B. pertussis* toxin fragment), ClpC	BACTH C-terminal T25-ClpC Fusion Vector
pSNT25-ClpP1	aadA, lacO, lacI, p15A ORI, CAP Binding Site, attR1, attR2, T25 (*B. pertussis* toxin fragment), ClpP1	BACTH N-terminal ClpP1-T25 Fusion Vector
pSNT25-ClpP2	aadA, lacO, lacI, p15A ORI, CAP Binding Site, attR1, attR2, T25 (*B. pertussis* toxin fragment), ClpP2	BACTH N-terminal ClpP2-T25 Fusion Vector
pSNT25-ClpX	aadA, lacO, lacI, p15A ORI, CAP Binding Site, attR1, attR2, T25 (*B. pertussis* toxin fragment), ClpX	BACTH N-terminal ClpX-T25 Fusion Vector
pSNT25-ClpC	aadA, lacO, lacI, p15A ORI, CAP Binding Site, attR1, attR2, T25 (*B. pertussis* toxin fragment), ClpC	BACTH N-terminal ClpC-T25 Fusion Vector
pUT18C-ClpP1	bla, lacO, lacI, ColE1 ORI, CAP Binding Site, attR1, attR2, T25 (*B. pertussis* toxin fragment), ClpP1	BACTH C-terminal T18-ClpP1 Fusion Vector
pUT18C-ClpP2	bla, lacO, lacI, ColE1 ORI, CAP Binding Site, attR1, attR2, T25 (*B. pertussis* toxin fragment), ClpP1	BACTH C-terminal T18-ClpP2 Fusion Vector
pUT18C-ClpX	bla, lacO, lacI, ColE1 ORI, CAP Binding Site, attR1, attR2, T25 (*B. pertussis* toxin fragment), ClpP1	BACTH C-terminal T18-ClpX Fusion Vector
pUT18C-ClpC	bla, lacO, lacI, ColE1 ORI, CAP Binding Site, attR1, attR2, T25 (*B. pertussis* toxin fragment), ClpP1	BACTH C-terminal T18-ClpC Fusion Vector
pLATE31-ClpP1	bla, lacO, lacI, pT7, 6xHis, pTet, rop, pMB1 ORI, ClpP1	Protein Expression of 6xHis tagged ClpP1
pLATE31-ClpP2	bla, lacO, lacI, pT7, 6xHis, pTet, rop, pMB1 ORI, ClpP2	Protein Expression of 6xHis tagged ClpP2
pLATE31-ClpX	bla, lacO, lacI, pT7, 6xHis, pTet, rop, pMB1 ORI, ClpX	Protein Expression of 6xHis tagged ClpX
pLATE31-ClpC	bla, lacO, lacI, pT7, 6xHis, pTet, rop, pMB1 ORI, ClpC	Protein Expression of 6xHis tagged ClpC
pTLR2-ClpP1mut::L2	tetR, tetO, pTet (of pASK), bla, ColE1 ORI, ClpP1mut (6xHis tagged)	Chlamydial transformation and overexpresion ClpP1mut
pTLR2-ClpP2mut::L2	tetR, tetO, pTet (of pASK), bla, ColE1 ORI, ClpP2mut (6xHis tagged)	Chlamydial transformation and overexpresion of ClpP2mut
pTLR2-ClpXmut::L2	tetR, tetO, pTet (of pASK), bla, ColE1 ORI, ClpXmut (6xHis tagged)	Chlamydial transformation and overexpresion of ClpXmut
pTLR2-ClpCmut::L2	tetR, tetO, pTet (of pASK), bla, ColE1 ORI, ClpCmut (6xHis tagged)	Chlamydial transformation and overexpresion ClpCmut
pTLR2-ClpP2Xmut::L2	tetR, tetO, pTet (of pASK), bla, ColE1 ORI, ClpP2mut (FLAG tagged), ClpXmut (6xHis tagged)	Chlamydial transformation and expresion ClpP2mut & ClpXmut
pTLR2-ClpP2X::L2	tetR, tetO, pTet (of pASK), bla, ColE1 ORI, ClpP2 (FLAG tagged), ClpX (6xHis tagged)	Chlamydial transformation and overexpresion ClpP2 & ClpX

Appendix III

Buffers		
Buffer	Components	Description
Buffer A	25mM Tris (pH 7.5), 150mM NaCl, 10mM Imidazole, 10% glycerol	Wash buffer for ClpP(1&2)
Buffer B	25mM Tris (pH 7.5), 150mM NaCl, 300mM Imidazole, 10% glycerol	Elution buffer for ClpP(1&2)
Buffer C	25mM Tris (pH 7.5), 150mM NaCl, 10% glycerol	Storage buffer for ClpP(1&2)
Buffer D	25 mM Tris-HCl [pH 7.5], 5 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 1 mM DTT	Native-PAGE Assay Buffer
Buffer E	50 mM Tris-HCl [pH 8], 200 mM KCl, and 1 mM DTT	Fluorometric and casein degradation assay buffer
Buffer F	25 mM Tris-HCL [pH 7.5], 300 mM NaCl, 10 mM Imidazole	Wash buffer for ClpX&C
Buffer G	25 mM Tris-HCL [pH 7.5], 300 mM NaCl, 300 mM Imidazole	Elution buffer for ClpX&C
Buffer H	25 mM Tris-HCL [pH 7.5], 300 mM NaCl	Storage buffer for ClpPX&C