CHLAMYDIA TRACHOMATIS AND HOST CELL EGRESS

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CHLAMYDIA TRACHOMATIS AND HOST CELL

EGRESS

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Abstract:
Chlamydia trachomatis is the most frequently reported bacterial sexually transmitted infection worldwide. Even after a *C. trachomatis* infection is treated, there is an increased risk for the development of pelvic inflammatory disease and cervical cancer, but the underlying mechanisms are poorly understood. *C. trachomatis* usurps many host cell-signaling pathways from within a membrane bound vacuole, called an inclusion. *C. trachomatis* is also known to synthesize and secrete via the type III secretion system, inclusion membrane proteins (Incs) that are inserted into the inclusion membrane and serve as the interface between *Chlamydia* and the host. *C. trachomatis* is the first bacterial pathogen observed to recruit myosin phosphatase (MYPT1) for means of host cell exit, by extrusion, and does so through the chlamydial Inc protein, CT228. In this study the chlamydial TargeTron™ system was used to genetically inactivate *CT228* in the *C. trachomatis* genome and genetically perform complementation of CT228 *in trans* to successfully restore MYPT1 recruitment in the mutant. Currently, *in vivo* models of infection continue to be instrumental in elucidating the pathogenesis of and immune response to this intracellular bacterium, however significant gaps in our understanding exist when utilizing such. The second objective of this study was to produce a side-by-side comparative analysis in a cervicovaginal murine infection model utilizing the *C. trachomatis* serovars D and L2 and the *C. muridarum* strain, MoPn, by characterizing the time course of infection and *Chlamydia* shed and obtaining gross pathology following clearance of active infection. A key finding herein is the first identification of chlamydial extrusions shed from host cells in an *in vivo* model. Extrusions, a phenotype made possible through a dynamic involving CT228 and MYPT1, had been previously observed solely *in vitro.* The results of this study demonstrate that chlamydial extrusions exist *in vivo*, representing a possible host dissemination method, thus warranting further investigation to determine their role in chlamydial pathogenesis.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Chlamydiae are unique obligate intracellular organisms capable of being human pathogens. Clinically, among human infections*, C. trachomatis* and *C. pneumoniae* are of concern. *C. trachomatis* is separated by clinical manifestation and serovars. Serovars A-C are known to cause infectious blindness in developing countries, most commonly referred to as trachoma, serovars D-K are urogenital in nature and serovars L1, L2 and L3 are known to produce lymphogranuloma venereum (LGV) infections (Baehr et al., 1988; Burton & Mabey, 2009). Most recently, a reclassified member of *C. trachomatis* is *C. muridarum*, a strain known to cause infections in mice, called mouse pneumonitis (MoPn) (Everett et al., 1999).

Chlamydiae require a host cell, for replication, due to their characteristic of being an obligate intracellular pathogen. *Chlamydiae* are unique in that they are biphasic in life cycle; alternating between two forms, Elementary Bodies (EBs) and Reticulate Bodies (RBs). EBs are 300 nm in diameter, metabolically inactive, infectious and extracellular, having the capability to attach to host cells; whereas, RBs are 1000 nm in diameter, intracellularly replicating and modify the inclusion membrane. The chlamydial cell membrane is quite typical of other Gram-negative bacteria, containing a recently identified peptidoglycan layer (Liechti, et al. 2014). After initial attachment to host cells,

Chlamydiae gain entry into the host cell through endocytosis, avoid lysosomal fusion and degradation, form a parasitophorous vacuole, termed an inclusion, and usurp many host cell processes by the recruitment of host proteins.

After forming an inclusion, EBs rapidly differentiate into RBs and after filling the host cell to capacity, the growing numbers of *Chlamydiae* must then exit by one of two mechanisms: lysis, or extrusion. The lytic host cell escape mechanism occurs in a distinct sequence of events: inclusions membrane permeabilization followed by nucleus permeabilization and ultimately, plasma membrane permeabilization (Hybiske & Stephens, 2007). Recently, Yang et al. demonstrated that lytic escape is dictated by the chlamydial plasmid. This single chlamydial plasmid carries eight genes, of which, Pgp4 is a known transcriptional regulator of several chromosomal genes—each required for lytic host cell exit. Furthermore, Yang et al. also show that the type 3 secretion system is required for Pgp4-regulated lytic escape. While the complete mechanism is not yet known, the description of chlamydial lytic escape provides further support of the ability of *Chlamydiae* to manipulate the host cytoskeleton during active infections (Yang et al., 2015).

Alternatively, the chlamydial host cell exit mechanism of extrusion involves a highly dynamic pathway including a series of interactions between host cell proteins and *Chlamydia*-synthesized proteins. The host cell protein MYPT1, a subunit a myosin phosphatase, and the chlamydial inclusion membrane protein, CT228 are known to interact during infection (Lutter et al., 2013). It is known that MYPT1 is recruited to the periphery of the chlamydial inclusion in rich active enzymatic patches, deemed microdomains (Mital et al., 2010). The extrusion mechanism in total is dependent on the

substrate of host protein MYPT1: myosin light chain 2 (MLC2). MYPT1 enzymatically removes phosphate groups from MLC2, dictating overall activity. Phosphorylated MLC2 is in an activated state, as when MYPT1 is interacting with CT228 at the inclusion unable to dephosphorylated the substrate, and MLC2 is able to interact with binding partners myosin IIA and myosin IIB, associating with MLC2 to form an active myosin motor complex. This leads to the overall host cell exit mechanism preference towards extrusion rather than lysis. However, if MYPT1 does not interact with CT228 during infection, lytic events are preferred as MYPT1 does not phosphorylate the substrate MLC2 (Lutter et al., 2013).

The first account of the novel adaptation of the commercially available TargeTron™ system in *Chlamydiae* was published in 2013, when Johnson & Fisher successfully genetically inactivated the chlamydial protein, IncA (Johnson & Fisher, 2013). To continue investigating of the interactions of CT228 and MYPT1, a novel adaptation of the commercially available TargeTron™ system, the second to date in *Chlamydiae*, is described here. Furthermore, the novelty in this investigation if only heightened by the first account of successful complementation *in trans* of *CT228* in the TargeTron™ system.

Additionally, a full comparison of chlamydial strains *in vivo* was utilized to establish a chlamydial murine model for future investigations. The murine model has been used to study chlamydial infections, as there is a close resemblance of genital infections in the mouse resembling human female genital infections (Williams et al., 1981). Following murine genital infection, mice typically resolve infections naturally and develop a subsequent immunity approximately 4 weeks after infection. After the

clearing of infection, more than half of mice are typically resistant to reinfection (Morrison & Caldwell, 2002). It was found that MoPn is more virulent than *C. trachomatis* in the murine genital infection model, causing systemic infections and acute pathology in the genital tract (Nigg, 1942). Within several days following initial infection, neutrophils and monocytes respond at the site of infection (Morrison & Morrison, 2000). Following this neutrophil recruitment, T cells accumulate at the infection site and maintain a critical role during and after infection clearance (Kelly & Rank, 1997; Darville et al., 1997; Ramsey & Rank, 1991; Morrison et al., 1995). CD4+ T cells predominate during the infection time course, in comparison to CD8+ T cells, and in the genital tract mucosa can be observed in small clusters until the resolution of infection (Morrison et al., 1995).

To date, many studies have encompassed topics such as chlamydial infectivity, pathogenicity, infertility and potential vaccine candidates for MoPn and *C. trachomatis* infections in the murine model (Hong et al., 2012, Pal et al., 1999; Schautteet et al., 2011). However, troubles arise in respect to the availability of a wide variety of murine models to choose from. Furthermore, difficulties also lie in the lack of consistency in model utilization. Hence, there lies a broad gap in knowledge, which does not permit for any clean comparisons across the board. A platform can be established for the basis of many investigations to come by characterizing and comparing serovars of *C. trachomatis* serovar D, serovar L2 and *C. muridarum* MoPn *in vivo*, within the same murine infection model. Uniquely, this study is the first to utilize the ocular chlamydial serovar D, the urogenital serovar L2 of *C. trachomatis* and the murine serovar MoPn of *C. muridarum in vivo*. Furthermore, a full comparison of immune response and recoverable infectious

forming progeny is obtained. This study allows for insight into the time course of chlamydial infections and gross pathology following clearance of infection. Unexpectedly, and demonstrated for the first time, is the account of chlamydial extrusions in the *in vivo* murine infection model.

The aim of this thesis was to investigate two distinct, yet related objectives regarding chlamydial pathogenesis:

- Characterize and complement, *in trans*, the chlamydial CT228 mutant created by the adapted TargeTron™ system.
- Perform *in vivo* comparison of *C. trachomatis* serovar L2*, C. trachomatis* serovar D/LC *and C. muridarum* MoPn within the same murine model to establish the timeline of infection and further, to provide evidence of the *in vivo* production of extrusions.

CHAPTER II

REVIEW OF LITERATURE

II. A. Taxonomy

Chlamydiae are capable of infecting a wide array of eukaryotic hosts including humans, ruminants, toads, and birds but typically are observed to replicate within epithelial cells. Based on 16S and 23S rRNA sequences, a phylogenetic analysis of *Chlamydiae* was put forth by Everett *et al*. in which taxonomy of chlamydial species is done through criteria of genetic, morphological and phenotypic means. The only order of *Chlamydiales*, consisting of families: *Chlamydiaceae*, *ParaChlamydiaecae*, *Simkaniaceae* and *Waddliaecae* (Everett, Bust et al., 1999). However, in 2009, Stephens, Myers, et al., proposed to reunite the family *Chlamydiacae* into a single genus: *Chlamydia* (Stephens, Meyers et al., 2009).

II. B. Clinical relevance

Chlamydiaceae are the infectious agents of many human and animal diseases. In humans, two species are of greatest concern: *C. pneumoniae* and *C. trachomatis. C.*

pneumoniae is known to be associated with the formation of pulmonary illness and chronic infections which are associated with an enhanced risk of the development of atherosclerotic, cerebrovascular, and chronic lung disease (Saikku et al.,1988; Martin et al., 1996; Saikku et al., 1992). *C. trachomatis* causes both urogenital and ocular infections. While serovar type is typically separated by which area of the body suffers infection, it has been shown that serovars can infect alternative areas than expected. Typically, urogenital infections are caused by serovars D-K and lymphogranuloma venerum serovars L1-3, whereas ocular based infections serovars also exist. Serovars A-C are the leading cause of infectious blindness worldwide, observed to be mainly located in developing countries (Burton & Mabey, 2009). Human hosts obtain the infection through contact with other infected eye secretions and also contact with flies. Through repetitive blinking, due to the eyelids folding inwards due to the infection, the cornea is scratched beyond repair and blindness often results (Burton & Mabey, 2009).

Chlamydial infections are the most commonly reported sexually transmitted infection (STI) worldwide with an estimated 131 million incidences each year (Newman, et al., 2015). In female hosts, sexually transmitted *C. trachomatis* infections are the major cause of cervicitis, infertility and PID to name a few in female hosts and urethritis and epididymitis in male counterparts (Brunham et al., 1984) (Paavonen & Eggert-Kruse, 1999). In many cases, chlamydial STIs can be present in a silent manner. In fact, asymptomatic female patients are very typical of chlamydial sexually transmitted infections when compared to male counterparts. Nearly 75% of females and 50% of males are asymptomatic in *C. trachomatis* infections (Stamm, 1999; Gonzales et al., 2004). Even after clearing of an infection with antibiotics, such as azithromycin

(Zithromax; 1 g single dose) or doxycycline (100 mg twice daily for seven days), women especially are at high risk for developing pelvic inflammatory disease (PID), infertility, perihepatitis, ectopic pregnancy and tubal scarring and even *Chlamydia*-induced reactive arthritis are also possible (Mishori et al., 2012). It has been suggested that prolonged antimicrobial therapy of up to six months consisting of combinational antibiotics could be effective at treating *Chlamydia*-induced arthritis (Carter et al., 2010). While there are numerous complications after a chlamydial infection, to date the most definitive method of prevention of infection is by practicing abstinence. Also shown to be effective in prevention of infection is a lifestyle of being in a long-term, mutually monogamous relationship (Workowski & Berman, 2011).

C. muridarum, as recently stated, was once defined as a serovar of *C. trachomatis*. However, after phylogenetic 16S analysis and sequencing, it was determined that *C. muridarum* was a separate species. There are currently two isolates of *C. muridarum*: Weiss isolate and Nigg isolate. Differences in the two strains are due to inclusion size, plaque size and single nucleotide polymorphisms (Ramsey et al., 2009). *C. muridarum* is known to cause respiratory infection and pneumonia in mice, mouse pneumonitis (MoPn). Also, MoPn can be used to successfully infect the urogenital tract in mice, serving as the first model for human chlamydial infections (Barron et al., 1981).

II. C. Chlamydial Growth and Development

II. C. i. Entry

The chlamydial entry mechanism has been a bit of a mystery in the past. Previously, it was thought that one of two plausible hypotheses could explain the

mechanism chlamydial host cell entry. The first mechanism of entry involves a sequential, zipper-like microfilament-dependent process of phagocytosis, which requires contact between bacterial adhesins and host cell receptors (Finlay & Cossart, 1997; Wart & Murray, 1984). The second involves uptake into clathrin -coated vesicles by receptor mediated endocytosis (Hodinka et al., 1988). EBs are much larger in size than the normal clathrin-coated vesicles and despite this, they appear capable of exploiting this mechanism of host cell entry. Though strain specific, *Chlamydiae* are capable of entering the host by either route, static or centrifuge-assisted (Prain & Pearce, 1989). However, much has been learned in present times about chlamydial entry.

Currently, the accepted hypothesis of chlamydial host cell entry involves the translocation of an actin-recruiting protein (Tarp) into the host cell (Clifton, et al. 2004). The proposed mechanism is as such: the Tarp protein, also known as chlamydial ORF CT456, is present in EBs in an unphosphorylated state prior to the point of host cell entry. It is suggested that following the attachment of the EB to the host cell, Tarp (CT456) is phosphorylated at its position at the cytoplasmic face of the host plasma membrane where it is then phosphorylated at a tyrosine residue (Fawaz et al., 1997). Following phosphorylation, Tarp, which is a presumed type III secreted effector protein, is observed to recruit actin to the site of initial EB attachment, in which aggregation of actin is observed (Birkelund et al., 1997, Cliffton et al., 2004). The recruitment of actin following chlamydial host cell entry has been well studied in the past. It was found that several proteins, Arp2/3 complex, WAVE2 and various other actin-associated proteins such as Cdc42 and Arf6, colocalize with entering EBs in an ordered and rapid fashion (Carabeo et al, 2004). Also found to be colocalizing at the site of entry was Rac, a GTPase Rho

family member (Carabeo et al., 2002; Carabeo et al, 2004).

After endocytosis, EBs avoid the endocytic pathway and traffic away from lysosomes and rapidly transform into RBs that continue to replicate within the inclusion. Endocytosis is obtained by pinocytosis in clathrin-coated pits or by phagocytosis (Reynolds & Pearce, 1990). Upon entering into eukaryotic cells, F-actin is redistributed and the polymerized actin participates in the redistribution of the microorganisms (Reynolds & Pearce, 1991; Carabeo et al.,2002). Upon *C. trachomatis* attachment to HeLa 229 cells, tyrosine phosphorylation of host cell proteins at the attachment site is induced which consequently is associated with F-actin, aggregation, and Ca^{2+} dependencies, and determines the next set of events in chlamydial infection, in which microfilament network importance is highlighted (Birkelund et al., 1994).

The microfilament networks, as well as dynein and kinesin motor proteins, have distinct roles of internalization and development. Determined through phagocytosis inhibition by the addition of Cytochalasin D and by motor protein blockage, it was observed that unlike *C. trachomatis*, *C. psittaci* can use both microfilament dependent and independent entry pathways. Whereas, internalization and development mainly relied on microfilaments or microtubules, specifically, actin and tubulin networks which were also necessary for growth optimization (Escalante-Ochoa et al., 2000).

II. C. ii. Biphasic Life Cycle

Chlamydiae alternate between two morphological forms, the elementary body (EB) and the reticulate body (RB) (Moulder, 1991). Initially, extracellular EBs attach to susceptible eukaryotic host cells at which point the EBs are internalized and create a membrane bound, parasitophorous vacuole, termed an inclusion. EBs differentiate into

metabolically active forms, RBs, and undergo multiple repeated cycles of binary fission, which leads then into the secondary differentiation back into the metabolically, infectious form of EBs. The host cell fills to capacity due to the growing numbers of *Chlamydia*, at which point they must exit the host cell. Either by lysis, releasing thousands of EBs to infect adjacent neighboring cells, or alternatively by extrusion, a packaged release mechanism in which the inclusion slowly protrudes and detaches from the host cell (Hybiske & Stephens, 2007). Upon treatment with antibiotics, a targeted stressful environment is created in which the developmental cycle is disrupted, causing large aberrant RBs (Hogan et al., 2004). This observation was ultimately attributed to the continued expression of genes controlling DNA replication but was not independent of genes contributing to bacterial cellular division.

II. C. iii. The Elementary Body

EBs are the small (0.3 um), round, electron dense, infectious form of *Chlamydia* (Eb et al., 1976). The EB is highly compacted in nuclear material due to the presence of bacterial histone-like proteins HctA and HctB (Brickman et al., 1993; Barry et al., 1992). The nucleoid location within the cell body suggests for an association with the cell wall or bacterial-inner membrane. EBs were once thought to be unusual in that little to no peptidoglycan is present in the cell membrane, despite *Chlamydia* encoding for peptidoglycan synthesizing genes and also, despite the fact that *Chlamydia* exhibits sensitivity to peptidoglycan targeting antibiotics. However, it was found by a unique cell wall labeling technique involving the use of D- amino acid probes, that in fact, *Chlamydia* does in fact possess a functional peptidoglycan structure (Liechti, et al. 2014). Further structural rigidity is due to highly cross-linked disulfide bond presence amongst

outer membrane proteins rich in cysteine residues. For example: Outer Membrane Protein A (OmpA), Outer Membrane Complex protein B (OmcB), and Outer Membrane Protein Complex A (OmcA) (Hatch, 1999, Liu et al., 2010, Hou et al. 2013). Additionally, hexagonally dispersed protein layer consisting mainly of OmcB at the inner surface of the outer-membrane complex is hypothesized to contribute to cellular stability of the EB.

With the usage of electron microscopy, the EB is presented as a hexagonally organized surface with projections arranged regularly with a center spacing of approximately 50nm (Nichols et al., 1985; Matsumoto, 1982). These supramolecular structures extend 30nm from the EB surface and have rotational symmetry similar to a 9-subunit composition. It has been speculated that these spike-like projections are actually the needle structure of the T3SS. It is now known that all species of the Genus *Chlamydia* within the order *Chlamydiales* contain genomes, which encode for the non-flagellar type III secretion system (NF-T3SS), providing further evidence for this speculation (Büttner, 2012).

II. C. iv. The Reticulate Body

The process of transitioning from a metabolically inactive, infectious form of EB to the metabolically active, non-infectious form of RB can be prevented by addition of antibiotic inhibition preventing transcriptional and translational cellular processes. This suggests that protein expression is required for the beginnings of intracellular growth (Scidmore et al., 1996). The reticulate body (RB), arises quite quickly from the elementary body (EB), following the primary differentiation. RBs are much larger than the 0.3um EB, measuring at approximately 1um. The cytosol of the RB is filled with nucleic acid, which, in comparison to the EB which is highly condensed and filled with

nucleic acid. RBs are non-infectious, metabolically active, and contain ban inner and outer membrane. The RB is covered with projections, which extend from the bacterial surface. Gene expression continues throughout RB development and at mid-development cycle during which, RBs undergo binary fission, exponentially replicate in numbers, eventually going through a secondary differentiation period followed by ultimate host cell egress mechanisms (Rocket & Matsumoto, 2000).

II. D. The Type III Secretion System

Previously, the hypothesized T3SS of *Chlamydiae* was further supported by findings that projections visualized with SEM, were indeed that of the T3SS needle. Based on past models of research in heterologous system in *Yersinia*, inclusion membrane proteins were found to be T3SS substrates (Büttner, 2012). This suggested that EBs must have pre-formed T3SSs, which function rapidly upon the EB-host cell interaction. Further supporting the presence and functioning of the T3SS in the initial stages of host cell attachment and interaction (Clifton et al., 2004).

All species of the Genus *Chlamydia* within the order *Chlamydiales* contain genomes which encode for the non-flagellar type III secretion system (NF-T3SS). More than 20 proteins associate to form an apparatus, the injectisome, to accomplish vector secretion and translocation of anti-host effector proteins. *Chlamydia* is unique in that the NF-T3SS genes are not located on virulence plasmids nor are they arranged into pathogenicity islands. Rather, effector genes are dispersed throughout the chromosome in all chlamydial species. However, apparatus components are grouped into three distinct and major loci. There is no evidence within the genome of transposons, insertion

sequence elements, or horizontal gene transfers that would suggest recent acquisitions of the T3SS capabilities (Stephens et al., 1998). However, *Chlamydia* is consistent with a model described by phylogenetic comparison: the T3SSs have lost the flagellar genes and thus losing motility. Furthermore, the model suggests that additional genes were subsequently acquired and included those required for outer membrane secretin and secreted translocon (Abby et al., 2012). The current evolutionary model suggesting such may serve reason; however, the apparent genetic grouping of NF genes (*cdsD*) along with other genes from the T3S core apparatus suggests that chlamydial T3SS arose after the evolution of a common precursor of the NF-T3SS. However, it is possible that genes were obtained *en bloc* but spontaneously over time, which may serve as evidence of the current chlamydial genomic distribution (Jeffery et al., 2010).

It is at the point of EB contact to a host cell in which the T3SS is activated and actively secreting injectisomes present on RBs. Such secretion through the injectisome is turned off as a result of RBs differentiating back into EBs later during infection. These injectisome serve a key purpose: to mediate the secretion of early developmental cycle effectors. It serves this key purpose to compensate for the fact that synthesis of T3SA genes do not occur until mid-development, correlating with the time in development where *Chlamydiae* start to divide for the first time (Belland et al., 2003). It is also shown that EBs are preloaded with vast amounts of chaperones and effectors; whereas, in late cycles of development, RBs possess little amounts of chaperones and apparatus components (Saka et al., 2011). It is suggested that the NF-T3SS is regulated partially by disulfide bonding from within the T3SA components themselves. The EB envelope is highly covered in disulfide cross-linkages. Such bonds are reduced as the chlamydial

EBs differentiate into RBs (Moulder, 1991). The NF-T3SS needle protein, CdsF, contains many cysteine residues, which is typical amongst secretion system needle proteins (Betts et al., 2008). Furthermore, disulfide bonding within such needle correlates with developmental stages (Betts-Hampikian & Fields, 2011). Hence, the disulfide bonding mechanism could confer structural rigidity and have a role in the control of secretion simultaneously. T3SS chaperones directly bind T3S substrates and facilitate secretion through the injectisome (Fattori et al., 2011). The T3SS chaperones are dimeric, cytosol-localized proteins and function to protect their substrates from degrading, maintaining the partially unfolded state for competence, and prevents substrates from other protein interactions prematurely for the goal of ultimate direct targeting of the substrates to the T3SA components selected for interaction. T3S chaperones are classified and divided into categories based on substrate specificity. Class IA bind single effector substrates while class IB binds multiple effector substrates. Class II binds translocon components and class III binds needle subunit proteins (Parsot et al., 2003). Collectively, T3SS chaperones are proteins, which most likely play critical roles in T3SA assembly, translocon secretion and assembly, and establish the efficient translocation of effector proteins into the host.

II. D. i. Secreted Effector Proteins

During chlamydial infection, protein effectors orchestrate many functions by direct association, enzymatic modification, or mimicry of target host factors to facilitate molecular requirements of virulence in a system (Dean, 2011). *Chlamydiae*, being an obligate intracellular pathogen, possess a wide array of effector proteins. These effector proteins function to direct association, enzymatically modify, mimic target host factors

and facilitate molecular requirements of virulence (Dean, 2011). Detecting chlamydial effector proteins is problematic due to the lack of a predictable T3S substrate secretion signals. Despite effectors from other pathogens containing a consensus domain, all efforts to predict chlamydial effectors in the same way have failed due to short sequence length, error rates in sequence based analyses for prediction verifications, and many false positives in the identification of candidate substrates. Two broad classes of effectors have emerged from recent ongoing studies, and are classified as either invasion-related or inclusion membrane (Inc) class effectors (Dean, 2011; Arnold et al., 2009; Samudrala et al., 2009; Subtil et al., 2005).

II. D. ii. Invasion-related Effector Proteins

Typical of invasion-related, or more commonly known as effector proteins, is the secretion of the protein into the host cell cytosol (Kleba & Stephens, 2008). Past difficulties in the identification of chlamydial host cell cytosolic effector proteins is due to the impossibility to separate the cytosolic compartment of *Chlamydia*-infected cells from the inclusion and inclusion membrane. This is due to the sensitivity observed of the inclusion after lysis to host cells (Heinzen & Hackstadt, 1997). However, Kleba and Stephens describe an important contribution to the *Chlamydial* field with the report of a protocol, in which recovery of a soluble cytosolic fraction from *Chlamydia*-infected cells is indeed possible (Kleba & Stephens, 2008). This work allowed for the identification of more than 20 chlamydial proteins observed to be localized to the cytosolic compartment by the use of cholesterol-dependent cytolysin perfringolysin O (PFO), which permeabilizes the plasma membrane of HeLa 229 cells to obtain a soluble cytosolic protein fraction. However, with this approach there are current limitations: proteins

present in small quantities are not amenable to identification by mass spectrometry, the identified proteins are not representative of the total population of bacterial proteins translocated beyond the inclusion membrane due to limitations of the design, and lastly, it is possible that chlamydial proteins are may be trapped within the cell after PFO treatment due to interaction with other proteins (Kleba & Stephens, 2008). Current efforts in the field are targeted to further identify and characterize chlamydial secreted host cell cytosolic effector proteins (Yang et al., 2015; Markham, et al. 2009).

II. D. iii. Inc-class Effector Proteins

Inc proteins are recognized as T3S substrates fundamental to biogenesis and inclusion modification and maintenance (Subtil et al., 2001; Dehoux et al., 2011). Inc-class effectors are described as proteins localizing to the inclusion membrane, which contain predicted bilobed hydrophobic domains of more than 60 residues (Bannantine et al., 1998). Although a majority of inclusion membrane proteins are hypothesized to have important roles in pathogenesis, current difficulties lie in the prediction of function (Shaw et al., 2000).

First, there is little sequence similarity amongst Incs. Secondly, bioinformatic analysis provides little clues as to function because of this low sequence similarity (Bannantine et al., 2000). Further creating difficulties in prediction and characterization of Incs is an observed poor conservation amongst chlamydial species. For example, IncA was first identified in *C. caviae* (Rockey et al., 1995; Rockey & Rosquist, 1994). Despite this identification, IncA has a function only described in *C. trachomatis* (Hackstadt et al, 1995; Stephens et al., 1998; Hackstadt et al., 1999). However, despite difficulties

mentioned previously mentioned, several Incs have been described in function and characterized. Currently characterized Incs include: CT850, CT229, IncA, IncD, IncG, CrpA and CT228 (CT850, CT229, CT119, CT115, CT118, CT442 and CT228, respectively). CT850 was found to interact with dynein light chain (DYNLT1) which is known to assist in the correct positioning of the inclusion at the MTOC (Mital et al., 2015). The regulation of intracellular trafficking or fusogenicity of the inclusion is in part due to the recruitment of Rab4 to the site of the inclusion membrane during infection by interaction with chlamydial protein, CT229 (Rzomp, et al. 2006). IncA interacts with host endocytic SNAREs at the SNARE motif (Delevoye et al., 2008; Paumet et al., 2009). CT119 IncD interacts with host cell protein, CERT, at an undetermined domain (Derré et al., 2011). Chlamydial protein CT118, IncG, interacts with 14-3-3-beta at the 14-3-3 binding motif (Scidmore & Hackstadt, 2001). CrpA, CT442, elicits a CD8+ T cell response and also partially protects from subsequent infections with *C. trachomatis* (Starnback et al., 2003). Chlamydial protein CT228 was shown to interact with a component of myosin phosphatase, MYPT1. Such interaction controlled the dynamic of host cell exit, to preferably cause the bacteria to exit the cell by extrusion rather than by lysis (Lutter et al., 2013). Other inclusion membrane proteins have yet to be described. This is due in partial to the lack of genetic tools in which to manipulate the bacteria for investigations. But with recently adapted methods, genetic investigations will soon exponentially increase in the chlamydial field.

In terms of inclusion membrane protein conservation, in the past, it was found *in silico* that Incs possess a 40 amino acid hydrophobic domain that was used to predict for approximately 60 Incs (Bannantine et al., 2000; Li et al., 2008; Scidmore-Carlson et al.,

1999). In an investigation performed by Weber et al., it was found that there was a conservation of Incs specifically between *C. trachomatis* serovars L2, A and D. There are at least 7 known orthologs of *C. trachomatis* which possessed N or C- terminal truncations or frameshift mutations: CT036, CT115, CT179, CT192, CT226, CT300 and CT383. In the study performed by Weber et al. it was additionally found that there was a preservation of Chlamydial protein CT228 amongst all three strains tested showing great conservation (Weber et al., 2015; Dehoux, et al., 2011; Shaw et al., 2000).

II. E. Host Cell Exit

For a successful and productive intracellular bacterial infection, two things are necessary: bacterial entry and bacterial exit. While *C. trachomatis* is observed to always be endocytosed into the cell, the mechanism of host cell exit is unique. The bacterium may exit the host cell by one of two mechanisms, lysis or extrusion. The mechanism of lysis involves the systematic permeabilization of membranes whereas extrusion has been described as a packaged release of the chlamydial-filled inclusion, encased in the host cell membrane (Rocket et al., 1996). *C. trachomatis* my exit by either mechanism, but some unique differences in species exist when it comes to exit (Hybiske & Stephens, 2007). *C. trachomatis* serovars L1-L3 and *C. muridarum* strains tend to favor lytic escape whereas the *C. trachomatis* serovars A-K preferably exiting by extrusion (Todd & Caldwell, 1985). More recently, the once unknown release mechanisms of *C. trachomatis* have been investigated and defined, allowing for great insight into chlamydial biology and pathogenicity.

The mechanism of lytic host cell escape consists of an ordered distinct sequence of events: inclusion membrane permeabilization followed by nucleus permeabilization and lastly, plasma membrane permeabilization (Hybiske & Stephens, 2007). In recent times, Yang et al. demonstrated that the chlamydial plasmid dictates lytic escape. The chlamydial plasmid carries eight genes, one of which is Pgp4. Pgp4 is a known transcriptional regulator of several chromosomal genes required for the lytic host cell exit. Furthermore, it was also shown that the T3SS is required for Pgp4 regulated lytic escape. While the mechanism for the process is still unknown, partly due to the lack of identifying the T3S effector protein, the description of chlamydial lytic escape provides further support of the organism to manipulate the cytoskeleton during an active infection (Yang et al., 2015).

The chlamydial host cell exit mechanism of extrusion involves a highly dynamic pathway. Lutter et al. describes that the extrusion host escape is reliant upon an interaction between a subunit of myosin phosphatase and the chlamydial inclusion membrane protein, CT228. Chlamydial gene *CT228* is 588 base pairs in size and is reported to be conserved within *C. trachomatis* serovars A, D and L2 (Weber et al., 2015). *CT228* is translated into a 196 amino acid protein. Based on Kyte & Doolittle hydrophobicity plot analysis, CT228 contains two distinct hydrophobic domains spanning from position 36 to 58; and from 68 to 86 (Appendices: Figure 1). Thus, warranting confidence that CT228 is an inclusion membrane protein fitting current predictions of Incs (Weber et al., 2015). The most closely related species of *C. trachomatis* is known to be the recently reclassified *C. muridarum*, which in the past was known to be a strain of *C. trachomatis* (Ramsey et al.,2009) . In terms of conservation, CT228 amino acids are

conserved when comparing of *C. trachomatis* and *C. muridarum,* by a sequence similarity of 53% (Appendices: Supplemental Figure 2). Upon investigations to determine what host cell protein interacts with CT228 during infection, Lutter et al. reported that CT228 interacts with a component of myosin phosphatase, MYPT1, during infection to regulate the host cell exit mechanism of extrusion (Lutter et al., 2013). Due to CT228 localization to the inclusion membrane, interaction of the two proteins shown by immunofluorescent microscopy imagery of MYPT1 recruitment to be at the periphery of the inclusion in rich active kinase patches, called microdomains (Mital et al., 2010).

The extrusion mechanism is also dependent on not only the function of myosin phosphatase, but also on the ability of the subunit MYPT1 to enzymatically dephosphorylates its substrate, MLC2, myosin light chain 2. MYPT1 enzymatically removes phosphate groups from MLC2, which dictates overall activity of MLC2. When MLC2 is in a phosphorylated active state, myosin IIA and myosin IIB associate with MLC2 to form an active myosin motor complex and the extrusion mechanism of host cell exit is preferred. However, when MLC2 is in a dephosphorylated inactive state, the correlation toward the host cell exit mechanism of lysis is observed. Upon interaction of MYPT1 with CT228, MLC2 is retained in an active phosphorylated state, thus preferring extrusion (Lutter et al., 2013). The protein interaction affecting the extrusion dynamic is best represented with a model (Figure 1).

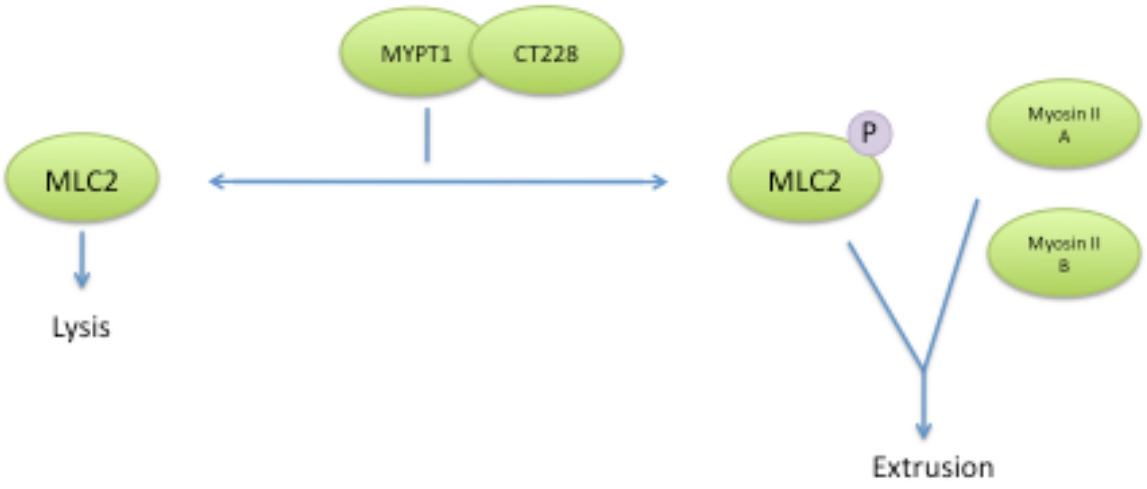

Figure 1. Dynamic of Host-Cell Exit by MYPT1 interaction with CT228. Dephosphorylated MLC2 (inactive) correlates with the host cell exit mechanism of lysis. However, phosphorylated MLC2 may interact with myosin IIA and myosin IIB to form the myosin motor complex which correlates with the host cell exit mechanism of extrusions. The phosphorylation state of MLC2 dictates overall activity which is controlled by MYPT1, a subunit of myosin phosphatase. MYPT1 will enzymatically dephosphorylate the substrate MLC2 under normal conditions. However, *Chlamydia* is known to recruit MYPT1 through interaction with CT228, and deactivate MYPT1. The reasons as to why *Chlamydia* would recruit and deactivate a protein are still an unknown topic for future discussion.

II. F. Development of Genetic Tools

Until recently, *Chlamydia trachomatis* has remained intractable in terms of genetic manipulation. The pathogen is largely hindered by the genetic methods in which to study and manipulate them. Only recently, in 2011, was it described by Wang et al. that these obligate intracellular pathogens could undergo transformation, as demonstrated by *C. trachomatis* serovar L2 transformation with a modified serovars E plasmid (Wang et al., 2011). Further provided from this study was the methods and knowledge of foreign genes being functionally expressed in *C. trachomatis,* which provided the platform for numerous research groups to begin researching chlamydial growth during infection. For example, the approach of chemical mutagenesis was adapted, but as a drawback, this method often resulted in undesired secondary mutation sites (Nguyen & Valdivia, 2014; Demars et al., 2007; Kari et al., 2011). Though this approach is effective, it is quite costly and widely time consuming. Further creating difficulty is that the mutations due to the chemical mutagenesis are often random in nature; making this approach less optimal for many investigations Current mutagenesis methods by Mueller & Fields utilizes a reporter system for assessment of chlamydial protein secretion which allows for an easily adaptable identification of novel secreted proteins (Mueller & Fields, 2015).This expression of recombinant gene products is stable as well as direct, making this new method ideal for more sophisticated studies (Mueller & Fields, 2015). In 2013, Wickstrum et al. described the creation of a tetracycline repressor controlled shuttle vector, shown to be successful for controlled gene expression (Wickstrum et al., 2013). However, this technology failed to localize expressed proteins appropriately in the host cell, as toxicity was noted in several constructs (Wickstrum et al., 2013). This was a limitation until the field was further advanced by the description from Bauler et al. of

recombinant proteins being expressed either conditionally or constitutively, as dictated by promotor control of a uniquely designed shuttle vector system (Bauler et al., 2014). Uniquely, the shuttle vector was not only capable of expressing recombinant inclusion membrane proteins and effector proteins, but was successful in the appropriate localization of such proteins.

Further developing the field, in 2011, Johnson and Fisher describe an alternative method utilizing mobile group II introns to create selectable and targeted chromosomal mutations (Johnson & Fisher, 2013). Mobile group II introns exist in an approximate quarter of bacterial genomes. These mobile group II introns move between genes in a retrotransposition mechanism typically consisting of interactions from an intron encoded protein (IEP) possessing an RNA maturase, endonuclease, and reverse transcriptase (Lambowitz & Zimmerly, 2004). Intron, non-coding regions of an RNA transcript, recognition is based upon sequence recognition between the intron and targeted gene. This results in the insertion of the intron into the resulting target gene in a site dependent manner. Prior to the intron splicing event, the intron is stably inserted into the DNA and in transcriptional events, excised out of the RNA, and results in a wild-type transcript and no change in gene function (Johnson & Fisher, 2013).

This method was provided from the work with the intron L1. LtrB from *Lactococcus lactis* and the targeting of genes of interest by changing the intron recognition sequences, which allows for base pair recognition and insert into newly targeted gene areas (Perutka et al., 2004). The method is made possible through algorithms predicting for optimal base pairing and intron modification and retargeting is performed by PCR-based modifications. *In trans* expression of the gene is subsequent

after removal from the intron, which prevents restoration of the gene function via RNA splicing in post-transcriptional events. To prevent gene restoration via post-transcription RNA splicing events, the gene is removed from the intron and expressed *in trans*. This method is as follows: removal of the IEP, allowing for the intron to carry alternative genes, including a selection cassette. *ltrA* and the intron are provided to the bacterium on a plasmid. Expression of the intron and *ltrA* leads to the subsequent insertion of the plasmid into the desired site of the target gene. After successful insertion, the intron donor plasmid is cured leading to the ultimate absence of LtrA and further prevention of intron splicing, thus insertional gene inactivity.

This method and technology is marketed as TargeTron™ by Sigma. This system was successfully employed in a variety of Gram positive and negative bacteria. Not only Gram negative bacteria, but even an obligate intracellular pathogen, *Ehrlichia chaffeensis* was shown to utilize this technology for directed gene inactivation (Cheng et al., 2013).. With this adopted technology, Johnson and Fisher demonstrated the inactivation of an inclusion membrane protein *incA*. With the investigation, it is now supported that with this technology, adaptation is ideal for the generation of chromosomal mutations in *C. trachomatis*. This is very novel in the chlamydial field, as the *incA:*GII(*bla*) mutant, as established from Johnson and Fisher, was the first site-specific chromosomal gene inactivation in the genus *Chlamydia* (Johnson & Fisher, 2013).

II. G. Murine Model

In respect to infection models, the murine model is the most commonly utilized animal for chlamydial investigations (De Clercq et al., 2013). Despite the utilization of

past animal models in chlamydial infections; such as guinea pigs, non-human primates, swine, rabbit and rats, the murine model possesses numerous benefits for usage (Barron et al., 1981; Tuffrey & Taylor-Robinson, 1981; Mount et al., 1972; Mount et al., 1973; Patton et al., 1983; Patton et al., 1987; Vanrompay et al., 2005; Kaushic et al., 1998; Patton et al., 1982).

Advantages as to utilizing the murine model include: mouse genital tract susceptibility to infection with *C. trachomatis* and *C. muridarum*, small size and ease of handing as well as a low cost (Barron et al., 1981; Tuffrey & Taylor-Robinson, 1981). Following vaginal inoculation, mice typically resolve chlamydial infection within 4 weeks post-infection. However, a few considerations are to be made: the mice strain, the dosage of inoculum, age of the animal and hormone levels. Following bacterial clearing, mice possess adaptive immunity that prevents against reinfection (Barron et al., 1981). C3H murine strains show a severe course of disease and high rate of infertility when compared to other murine strains, due to their lack of innate immune response (de la Maza et al., 1994; Bernstein-Hanley et al., 2006). The inoculating dosage of chlamydial bacterial load affects the time course of infection and the ascension into the higher reproductive tract (Maxion et al., 2004). The utilization of depo-provera prior to murine infection was adapted due to the finding of the various stages of the estrous cycle affecting susceptibility to MoPn vaginal infection (Pal et al., 1998; Kita et al., 1989). This pretreatment alters hormone balance preventing hormonal contributions to the clearing of the infection (Tuffrey & Taylor-Robinson, 1981). Furthermore, older murine strains are not as easily infected as their younger counterparts (Pal et al., 2001). In comparison of *C. muridarum* and *C. trachomatis* infections, *C. trachomatis* is highly dependent on

progesterone pretreatment to establish initial infection compared to *C. muridarum* (Tuffrey & Taylor-Robinson, 1981; Ramsey et al., 1999).

C. trachomatis is capable of murine infection when administered intravaginally. Typical of *C. trachomatis* murine infection is the observation of quickly resolving genital tract infection (Tuffrey et al., 1981). Comparatively, some *C. trachomatis* serovars, both ocular and genital, require a higher bacterial load to establish an infection when compared to *C. muridarum* (Ito et al., 1990; Darville et al., 1997).
Murine *C. trachomatis* direct ovary infections inflict post-infection sequelae: hydrosalpinx and infertility. However, when C3H/HeJ mice are utilized, a naturally ascending infection resulting in salpingitis was observed in some studies (Sturdevant et al., 2010).

Collectively, investigations into the protective immune response brought on by *C. trachomatis* infection within the murine model have been contradictory (De Clercq et al., 2013). The innate immune response is enough alone to eradicate *C. trachomatis* infection, as some studies have shown that in the absence of the adaptive immune system an infection may resolve (Tuffrey et al., 1982; Perry et al., 1999; Brunham & Rey-Ladino, 2005; Hafner et al., 2008; Gondek et al., 2012). In addition to the contradictory findings within the immune response, there are also differing characteristics amongst *C. trachomatis* strains upon murine infection (Ito et al., 1990). These discrepancies assist in portraying that C3H mice are ideal for *C. trachomatis* murine infection, as other murine strains are not as susceptible to infection (Lyons et al., 2005).

C. muridarum, MoPn, was previously classified as a *C. trachomatis* mouse biovar. Originally, *C. muridarum* was isolated from the lungs of mice and upon vaginal

inoculation acute genital tract infection resulted (Nigg, 1942). The observed acute genital tract infection in mice resembled genital infections in female humans and assisted in establishing the murine model as the choice for chlamydial investigations (Barron et al., 1981).

Mice which are infected with MoPn are typically administered the bacterial load in an intravaginal route. However, an alternative approach to infect the upper genital tract to increase genital tract pathology to closer resemble human infection exists. This approach involves infecting the upper genital tract directly, at the ovarian site. However, this route of administration is atypical of the natural route of infection transmission (Swenson et al., 1983; Farris & Morrison, 2011).

Vaginal inoculation of mice with *C. muridarum* results in the recruitment of B cells, CD4+ T cells and CD8+ T cells to the genital tract. CD4+ T cells, responsible for protective adaptive immunity, with respect to the mouse MoPn infection model, are predominantly present through the infection time course (Morrison & Morrison, 2000; Igiesterne et al., 1993). However, CD4+ T cells are not solely responsible for the adaptive immunity observed with MoPn mouse infection. Two additional Th1 cytokines, interleukin-12 and gamma interferon, also contribute to the adaptive protective immune response (Rank et al., 1992; Perry et al., 1997). In regards to primary infection eradication of bacteria, it was found that B cells not only are necessary for clearing but moreover, they also have a role in the resistance of secondary infection (Ramsey et al., 1988; Morrison et al., 2000; Feilzer et al., 1997).

CHAPTER III

METHODOLOGY

III. A. Production of Chemically Competent DH5α and ER2925 *Escherichia coli*

To prepare chemically competent DH5α and ER2925 *Escherichia coli*, glycerol frozen bacterial stocks were struck out with a sterile wooden stick by quadrant streaking onto fresh LB agar plates. DH5α *E. coli* were used to propagate plasmids quickly, mostly for sequence verification following cloning procedures. ER2925 *E. coli* were used for the production of both adenosine and cytosine methylation deficient (*dam*- and *dcm*-, respectively) plasmid products. In the genome of *C. trachomatis*, there is a lack of genes encoding for methyltransferases. The result is a low efficency bacterial transformation of *C. trachomatis* if a methylated plasmid product is used. For these resasons, *C. trachomatis* was transformed solely with plasmids which were *dam*- and *dcm*- deficient (Stephens et al., 1998), Plates were incubated inverted at 37°C over night. A single isolated colony forming unit was used to aseptically inoculate a 5 mL sterile, RNase and DNase-free, polystyrene tube of sterile LB broth. Tubes were incubated over night at 37°C shaking at 220 RPM.

The following morning, 250 mL of sterile LB broth was aliquotted into a sterilized Erlenmeyer glass flask. Two and one half mL of LB broth grown culture was aseptically pipetted into the Erlenmeyer glass flask, filled with 250 mL of LB broth (1:100 dilution). Prepared cultures were returned to the 37°C shaking incubator until the $O.D._{600}$ was approximately 0.5-0.8, at which point, flask filled cultures were placed on ice to slow growth until further downstream processing, all future steps were performed on ice. Cultures were divided equally, checked by weight, into two sterilized, pre-chilled 250 mL centrifugation bottles. Next, centrifugation was performed at 4000 RPM for 10 minutes at 4°C. Supernatant was poured from cell pellets aseptically. The residual cell pellet was resuspended in80 mL of ice cold, pre-chilled TB buffer (80 mL for every 250 mL of culture[10 mM Pipes, 55mM $MnCl_2$, 15mM $CaCl_2$, 250 mM KCl]) and incubated on ice for 10 minutes. Centrifugation was then performed again at 4000 RPM for 10 minutes at 4°C. Once again, supernatant was discarded aseptically and cell pellets were resuspended in 20 mL of ice cold TB buffer (20 mL for every 250 mL of original culture). With a gentle swirl, 1.4 mL of DMSO was added to 20 mL of the TB buffer cell suspension to achieve a final concentration of 7%. Bacterial cultures were incubated on ice for 10 minutes. Pre-chilled, sterilized RNase and DNase-free 1.5 mL tubes were then filled with approximately 200 µL of chemically competent *E. coli* and flash frozen for approximately 1 to 2 minutes by submersion into an approximate 1:1 mixture of absolute ethanol to dry ice. Tubes were then immediately placed at -80°C freezer for long term storage.

III. B. Bacterial Transformation

Five hundred µL aliquots of chemically competent DH5α and ER2925 *E. coli* were stored at -80°C for long term storage until use. Transformation protocol is as follows: cells were thawed on ice for 30 minutes. Plasmid DNA was added to cells. Tubes were gently tapped to mix and incubated on ice for 30 minutes. Tubes were then immediately placed in a 42°C water bath for 30 seconds, and directly then placed back onto ice. Tubes were left on ice for 10 minutes, at minimum, to allow for high stability and recovery by adding 750 µL of SOC (2% w/v tryptone, 0.5% w/v Yeast extract, 8.56 mM NaCl, 2.5 mM KCl, ddH_2O to 1L; autoclaved; 20mM glucose). Tubes were then placed in a shaking incubator at 37°C, 220 RPM to allow for outgrowth. Fifty µL and 250 µL of transformants were then plated onto LB agar supplemented with antibiotics. Plates were inverted and incubated at 37°C overnight.

III. C. Construction of the TargeTron™ *Chlamydia trachomatis* serovar L2, CT228 Mutant

The CT228 mutant was kindly constructed and provided from Dr. Derek Fisher, Southern Illinois University, Carbondale, Illinois. The chlamydial TargeTron™ vector pDFTT3(*bla*) was modified for spectinomycin selection by replacing the *bla* cassette with the *aidA* cassette (Lowden et al., 2015). The Sigma-Aldrich TargeTron™ algorithm was used to predict CT228 insertion sites with lowest E-values to retarget the intron to insert, in an antisense orientation, at 295 base pairs (E-value of 0.200). PCR was utilized to retarget the vector for insertion into *CT228* using Phusion High-Fidelity PCR Master Mix (Thermo-Fisher) and primers 228A, 228B, 228C, and EBS universal, followed by DNA restriction endonuclease digestion as previously described (Johnson & Fisher, 2013) (Table 1). The vector was transformed into *E. coli* DH5α and grown at 37°C in

Luria Bertani (LB) media supplemented with 20 µg/ml chloramphenicol. *C. trachomatis* transformation and antibiotic selection with 500 µg/ml spectinomycin was performed as adapted from Lowden *et al*. Mutants were plaque purified through a series of passages to obtain a passage 4 clonal CT228::GII(*aadA*) final resulting CT228 mutant strain, Δ*CT228*. The isolate was then expanded in L2 mouse fibroblasts and stored in 1X SPG buffer (10 mM sodium phosphate [8 mM Na2HPO4-2 mM NaH2PO4], 220 mM sucrose, 0.50 mM l-glutamic acid) at -80°C.

III. D. PCR validation of the CT228 Mutant

Genomic DNA (gDNA) was extracted from wild type (L2) and CT228 mutant EB (DFC14) stocks using a DNeasy Blood and Tissue Kit (Qiagen). PCR reactions were performed using 50 ng of gDNA or 5 ng of plasmid DNA and Phusion High-Fidelity PCR Master Mix. PCR products were subjected to agarose gel electrophoresis, stained with ethidium bromide and visualized by UV transillumination. To confirm GII insertion, the amplicon produced from PCR reactions using primers CT228F and CT228R (Table 1), was agarose gel purified using the GeneJET gel extraction kit (Thermo-Fisher) and ligated into pJET (Thermo-Fisher) to generate pJET::CT228GII*aadA*. Resulting products were then transformed into *E. coli* DH5α and transformants were selected by antibiotic selection using LB agar supplemented with 100 µg/ml of ampicillin. A single colony was then selected for by overnight growth in LB broth medium supplemented with ampicillin and resulting plasmids were extracted using the GeneJET Plasmid Miniprep kit (Thermo-Fisher). Gene insertion in pJET::CT228GII*aadA* was sequenced with primers JETF and JETR (Table 1) via Sanger sequencing performed by Macrogen USA. Obtained

sequences were analyzed using BioEdit (TA Hall 1999) and Clone Manager (Sci-Ed Software).

III. E. Recombinant DNA Procedures

The complemented CT228 mutant was created by the following procedures. The vector, pBOMB4R (Bauler & Hackstadt, 2014) was kindly provided by Dr. Ted Hackstadt, National Institutes of Health Rocky Mountain Laboratories, Hamilton, Montana. pBOMB4R is a shuttle vector composed of a fragment of the native chlamydial plasmid, an *rpoB* promotor, a multiple cloning site for directional insertion and two origins of replication (Bauler & Hackstadt, 2014). The green fluorescent protein (GFP) contained within pGFP:SW2 was replaced with the mCherry gene, amplified from pmCherry-C1 vector (Clontech). The vector, received on filter paper, was extracted with water. One µL of vector was transformed into DH5alpha *E. coli* and plated onto Luria-Burtani (LB) agar plates containing 100 ug/mL carbenicillin. Clones were picked into 5 mL of broth LB supplemented with 100 ug/mL carbenicillin and grown over night at 37°C and shaking at 220 RPM. Plasmids were extracted with the aid of ZyppyTM Plasmid Miniprep Kit (ZymoResearch) and checked for quality by 0.8% gel agarose electrophoresis. Plasmids were then subjected to recombinant procedures by the following: 30 µL of total pBOMB4R vector was digested with 5 µL of buffer 1.1 (NEB), 1 µL of *KpnI (*NEB) and 1 µL *BamHI*-HF overnight in a 37°C water bath in 600 µL tubes. One µL of Antarctic phosphatase (NEB) was added to the digestion reactions and was allowed to incubate at 37°C for two more hours. Digestions were purified by DNA Clean & Concentrator^{TM} Kit (ZymoResearch) and eluted in 200 µL of DNase RNase free

H_20. Five µL of the purified vector was checked by 0.8% agarose gel electrophoresis. Vector was stored at 4°C until usage.

Full length CT228 constructs were prepared by the following procedures. Polymerase Chain Reactions were set up as follows: 1 µL of primer CT228pBOMBKpnI and 1 µL of primer CT228BOMBBamHI-3 (see in Primer table), 1 µL L2 gDNA from *C. trachomatis* serovar L2 gDNA (Quick-gDNA™ MiniPrep ZymoResearch and 45 µL of Platinum PCR Supermix High Fidelity (Invitrogen) were mixed on ice. Polymerase chain reaction cycles were as follows: 95°C for 2 minutes initial denaturing, 95°C for 30 seconds, 52°C for 30 seconds and 68°C for 1 minutes 30 seconds for 35 cycles along with a 5 minute 68°C final elongation period. Two µL of PCR amplicons were checked by 0.8% agarose gel electrophoresis. The Polymerase Chain Reaction amplicons were subjected to the Clean & Concentrator™ Kit (ZymoResearch) and purified products were digested as follows: 1 µL *BamHI*-HF (NEB) and 1 µL *KpnI* (NEB) and 2.5 µL of buffer 1.1 (NEB) were added and mixed in 600 µL tubes, placed in a water bath set at 37°C overnight. Resulting digests were cleaned once again using the Clean & Concentrator™ Kit (ZymoResearch) and checked for purity by 0.8% agarose gel electrophoresis. Ligation reactions were as follows: 4 µL of amplicon was added with 1 µL of the vector (diluted 1:20 in water) along with 5 µL of 2X Ligation Buffer (NEB) and 1 µL of Quick Ligase (NEB). Reactions were allowed to incubate at room temperature for 30 minutes. The reaction was then transformed into NEB Turbo *E. coli.* Then after sequence confirmation, the plasmid was transformed into a methylation deficient strain of *E. coli* ER2925, (see bacterial transformation section) to serve for bacterial transformation into the TargeTron™ CT228 mutant of *C. trachomatis.* Transformants were then plated onto

LB agar supplemented with 100 ug/mL carbenicillin and grown inverted, overnight at 37°C. Clones were picked and grown in LB liquid broth supplemented with 100 ug/mL carbenicillin overnight at 37°C shaking at 220 RPM. Plasmids were extracted with the aid of ZyppyTM Plasmid Miniprep Kit (ZymoResearch) and eluted in 45 µL. Five µL of the complementation plasmid was then subjected to restriction endonuclease digestion as follows: 5 µL of DNA, 17.5 µL H2O, 2.5 µL buffer 1.1, 0.5 µL *KpnI* and 0.5 µL *BamHI*-HF were added together. Reactions were incubated in a water bath at 37°C to incubate for a total of 4 hours. Entire reactions were assessed by 0.8% agarose gel electrophoresis for appearance of both insert at approximately 600 bp and vector at approximately 10.7 kb. After gel confirmation, 10 µL of the product was sent for sequencing using an Applied Biosystems 3730 DNA analyzer, courtesy of OSU Core Facility. After sequence confirmation and confirmation of correct frame insertion (DNAMAN), the complementation plasmid was then transformed into *C. trachomatis* CT228 mutant strain, DFCT14 (see section chlamydial transformation) producing the complementated CT228 mutant: Δ*CT228*C.

III. F. CT228 Mutant Transformation with the CT228 Complementation Plasmid

After DNA sequence confirmation provided by the OSU Core Facility (DNAMAN), the constructs were transformed into CT228 mutant strain, Δ*CT228* of *C. trachomatis* serovar L2. Plasmid DNA (3µg) was incubated with mutant density gradient purified EBs and 40 µL of $CaCl_2$ buffer (50 mM Tris; 250 mM $CaCl_2$) for 30 minutes at room temperature (Wang et al., 2011; Song et al., 2013; Agaisse & Derré, 2013; Bauler & Hackstadt, 2014). After 30 minutes, cold RPMI 1640 + 10% FBS was added and bacteria were plated into three HeLa 229 cell confluent monolayers seeded in a six-well

plate. Cultures were incubated at 37°C with 5% CO2. To produce passage 1, at 36-40 hours post infection, wells were removed of media and scraped after applying cold sterile distilled water to lyse host cells. The lysed supernatant was centrifuged at 1500 RPM for 5 minutes and supernatant was combined with 1 mL of RPMI 1640 + 10% FBS to infect a fresh monolayer of confluent HeLa 229 cells. Six well plates were centrifuged at 700X g for 1 hour. Media was removed and replaced with 1 mL of RPMI 1640 + 10% FBS supplemented with 1 ug of cyclohexamide/ml and 0.1 U of Penicillin G/ml and incubated for 40 hours. To produce passage two, at 36-40 hours post infection, wells were removed of media and scraped after adding cold sterile distilled water to lyse host cells. Lysate was collected and spun at 1500 RPM for 5 minutes and the supernatant was combined with 2 mL of RPMI 1640 + 10% FBS and used to create a series of dilutions (10^{-17}). Three new 24 well plates of confluent monolayer of HeLa 229 cells were then infected with supernatant from passage two to produce passage three. Plates were centrifuged at 700X g for 1 hour and then lysate was removed and cell monolayers were overlayed with a mixture of 1.1% agarose (sterilized and at 42°C) + 2X RPMI1640 (42°C) + 1ug/mL cyclohexamide. Overlays were allowed to solidify for approximately 10 minutes. RPMI 1640 + 10% FBS + 1:1000 cyclohexamide and 0.1U Penicillin G was then applied to the surface of the agarose overlay and allowed to incubate for 5 days, replacing media every 2 days including cyclohexamide and 0.1 U Penicillin G for consistent selection. For expansion of clones, individual plaques were punched with sterilized, scissor cut P-1000 pipette tip and placed into a 1.5 mL eppendorf tube. One mL of RPMI 1640 + 10% FBS was then added and sample was vortexed for 1 minute. The entire volume was then placed into a T25 flask onto a confluent layer of HeLa 229 cells. At 6 hours post

infection, media was replaced with RPMI 1640 + 10% FBS + 1 mg/mL cyclohexamide and 0.1 U of Penicillin G for selection. Clones were allowed to grow for 72 hours and were then frozen as a backup stock in 1X SPG buffer at -80°C. Frozen plaque purified clones were then used as a crude preparation of EBs to infect confluent HeLa 229 cell monolayers for immunofluorescent microscopy analysis and molecular methods.

III. G. Immunofluorescent Analysis

HeLa 229 cells were seeded onto glass cover slips in 24 well plates and grown to 70% confluency. Cells were infected with *C. trachomatis* serovar L2 mutant CT228 EBs, *C. trachomatis* serovar L2 EBs and *C. trachomatis* serovar L2 CT228 mutant complementation EBs in cold RPMI 1640 + 10% FBS using crude stocks diluted in ice cold RPMI 1640 supplemented with 10% FBS. Infections were allowed to incubate for 24-36 hours post-infection at which point cell monolayers were fixed in methanol, rinsed with 1XPBS three times, 5 minutes each wash, blocked with 2% BSA-PBS for one hour, rinsed 1 time for 5 minutes. Samples were then stained with indicated primary antibodies (See below) for a period of one hour at room temperature, rinsed with 1X PBS 3 times, 5 minutes each and stained with fluorescent secondary antibodies (See below) for a period of 1 hour at room temperature until rinsing, once again 3 times, 5 minutes each rinse. Round glass cover slips over slips were then mounted (Dako Mounting Media) onto glass slides and visualized with a Leica DMI600B inverted microscope with objectives 40X and 63X with oil. Images were edited for only size and color using Adobe Photoshop.

The following antibodies were then prepared in 2% BSA: anti-L2 EB antisera (Generously provided from the Hackstadt lab National Institutes of Health Rocky

Mountain Laboratories)**;** Anti-MYPT1 (US Biologic, Danvers, MA); Anti-phospho (pSer-19)-MLC2 was purchased from Abcam (Cambridge, MA); Anti- Src-pTyr419 (clone 9A6) (Millipore, Billerica, MA) was used to detect active Src-family kinases; Anti-*Chlamydia* LPS (Cell signaling, Danvers, MA); Anti-myosin IIA and anti-myosin IIB were purchased from Thermo Scientific (Thermo Scientific, Rockville, MD); Anti-mouse or anti-rabbit DyLight 594 and DyLight 488 (Jackson ImmunoResearch Laboratories, West Grove, PA) were used as secondaries.

III. H. *CT229-CT224* Operon Expression Assessment

RT-PCR was used to rule out possible polar effects in the *CT228* mutant. HeLa 229 cells were seeded into 24 well plates and grown to 70% confluency. Cells were infected to an MOI of 1 with *C. trachomatis* serovar L2 mutant CT228 EBs or *C. trachomatis* serovar L2 EBs in cold RPMI 1640 + 10% FBS. Infections continued until the point of RNA extraction using Trizol and the Direct-zol RNA kit (ZymoResearch) at 2, 4, 8, 12, 24 and 48 hours post-infection. An in-column digestion was performed using DNase, (Roche) and purified RNA was checked for genomic DNA contamination by the use of PCR, amplifying with internal gene primers of *CT229*, at which point samples were subjected to a second DNase treatment using the DNase I Kit (Roche), and checked once again by PCR before continuing to cDNA synthesis. After confirming an absence genomic DNA contamination, quality by gel electrophoresis and quantitation was performed using UV spectrophotometry. cDNA synthesis was performed with use of Maxima H Minus Reverse Transcriptase (200 U/L) (ThermoFisher). One pg-5pg of cDNA (or 1 μL of H2O, for the negative control) was used for each reaction. One hundred pmol of random hexamer primer and 0.5 mM dNTPs were used as well.

Volumes of each reaction were filled with DNase RNase free water until 15 µL total. Reactions were mixed and then centrifuged for 15 seconds. Following centrifugation, reactions were incubated at 65°C for 5 minutes to remove possible secondary structures and then chilled on ice again for several minutes. Four µL of 5X reverse transcriptase buffer (ThermoFisher) was added to each reaction. Reactions were mixed gently and centrifuged for 15 seconds. Reactions were incubated for 10 minutes at 35°C, 30 minutes at 60°C , and 5 minutes at 85°C . The successful production of cDNA was checked for by gel electrophoresis. A series of PCRs were set up to ensure expression of each gene within the *CT229-CT224* operon with the use of internal gene specific primers (Table 1) and run on a 0.8% agarose gel for evaluation of gene expression.

III. I. Western Blot Analysis

A 24-well plate of confluent HeLa 229 cells was infected at an MOI of approximately 1 with wild type, *C. trachomatis* serovar L2, CT228 mutant and CT228 complementation EBs. At 36 hours post-infection, cell monolayers were removed of supernatant and lysed in 200 µL of Laemmli buffer (Laemmli, 1970) supplemented with 5% beta-mercaptoethanol. Twenty µL of heated (99°C for 4 minutes) protein extracts were separated by SDS-PAGE (10% resolving gel; 85 V for 3 hours) (and transferred to nitrocellulose membrane (125 V for 45 minutes) in transfer buffer (100 mL 10XTris/Gly-SDS Buffer, 200 mL methanol, 700 mL diH_2O). Samples were blocked in 5% skim milk in 2%BSA-PBST buffer (8mM Na_2HPO_4, 150mM NaCl, 2mM KH_2PO_4, 3mM KCl, Tween-20 pH 7.4) and incubated over night with in primary antibodies with constant agitation (anti-CT228) in blocking buffer. Blots were washed 3 times at room temperature with PBST, the first for several seconds and the following 2 for 15 minutes

each. Blots were then incubated in corresponding HRP-conjugated donkey anti-rabbit IgG secondary or HRP-conjugated donkey anti-mouse IgG secondary diluted antibodies (Cell Signaling) for 1 hour at room temperature with constant agitation. Blots were then rinsed 3 more times, 5 minutes each rinse at room temperature, with PBST. Blots were then drained of PBST and developed using equal parts reagent A and reagent B of VisiGlo ECL Western blotting detection reagents (Amresco). Samples were then exposed for 1-3 minutes (ECL Detection machine in NRC).

III. J. Mice Selection

Six week old female inbred C3H/HeJ mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and divided into four groups (n=15 each) and mice were assigned for infection: SHAM, *Chlamydia trachomatis* serovar D/LC, C. *trachomatis* serovar L2, *C. muridarum* mouse pneumonitis (MoPn). C3H/HeJ mice contain a mutation in the TLR4 response locus, making mice susceptible to infection by Gram-negative bacteria due to the innate immune system defect of failure to recognize and bind endotoxin. After arrival, mice were allowed to acclimate to the new environment several days prior to the experiment. Four additional mice were also used strictly for extrusion assessment.

III. K. Cell Strains Used for Murine *in vivo* Experiments

Confluent HeLa 229 cell (ATCC) monolayers were used for assessment of recoverable infectious forming units by dilution and plating techniques in 24 well plates. Cells were overlayed with RPMI 1640 + 10% fetal bovine serum and 1ug/mL cyclohexamide as needed. *C. trachomatis* serovar D/LC (a late clearing, phenotypic strain of serovar D)

was kindly provided from Dr. Harlan D. Caldwell (Rocky Mountain Laboratories, National Institutes of Health, Hamilton, MT).

III. L. Murine Estrous Synchrony

To synchronize estrous of all female inbred 6 week old mice (Jackson Laboratories), mice were sedated individually at a flow rate of 1.5L/minutes O_2 with 3% isoflurane (Henry Schein). Individual mice were subcutaneously administered 150 mg/mL or 20 uL of Depo provera (Pfizer) (29G X ½'' needle and 3/10 mL insulin syringe Covidien Monoject™) at the nape of the neck while the mouse was held flat in a prone position. Mice were then allowed to regain consciousness and returned to cages.

III. M. Infection of Murine Lower Genital Tracts

Normalized EB stocks of *C. trachomatis* serovar L2, and D/LC and *C. muridarum* MoPn were produced to reach a final concentration of $1X10^6$ EBs in 5 µL of 1X SPG buffer (87 mL (0.2 M Na_2HPO_4), 13 mL (0.2 M NaH_2PO_4), 0.72 g L-Glutamic acid, 1 L H2O, pH 7.4 with NaOH, autoclave sterilize). For control mice, 1X SPG was solely administered. Tubes of normalized EBs were kept on ice until infection. All mice were individually sedated with 3% isoflurane at a flow rate of 1.5 L O_2/minute (Figure 15). The three mice groups: D/LC, L2 and MoPn, were vaginally administered 5µL of normalized EBs by gentle pipetting into the opening of the vagina. All strains were appropriated normalized to $1X10^6$ EBs/5 µL in 1X SPG buffer. Control SHAM mice were administered 5 µL of 1XSPG. EBs were normalized as following: 9.2 mL of 1X SPG was added to 5 µL of serovar L2 density gradient purified EBs to produce a final concentration of $1X10^6$ EBs/ 5µL; 8.2 mL of 1X SPG was added to 5µL of concentrated stock serovar D/LC density

gradient purified EBs to produce a final concentration of $1X10^6$ EBs/ 5μL; 156 μL of MoPn density gradient purified EBs were spun down at 10,000 RPM for 5 minutes, supernatant was discarded and pellet was resuspended in 85 μL of 1X SPG to result in a final concentration of $1X10^6$ EBs/ 5μL. Sedation chamber and holding chamber were bleached between mouse groups to prevent cross-infection.

III. N. Swabbing of Murine Vaginal Tracts

At days 3, 7, 10, 14, 17, 21, 14, 28, and 35 post-infection, mice were swabbed for either the assessment of infectious forming units (IFUs) or for extrusion assessment. Mice were individually sedated as previously described and held in the downward supine position. Mice which were used for the assessment of IFUs were swabbed 8 rotations to the right and 8 rotations to the left, and the sample was then placed into 600 μL of 1X SPG buffer and 2 sterile glass beads. Samples were brought to the molecular processing laboratory for further testing. Mice which were used for the extrusion assessment studies were swabbed as follows: the swab was placed into 100 μL of RPMI1640 (10 L H_2O, 10L pack RPMI, 2.9 g L-glutamine, 1.1 g pyruvic acid sodium salt, 20 g sodium bicarbonate, pH 7.0, filter sterilize through 0.2 μm filter) supplemented with 10% fetal bovine serum and the swab handle was cut using sterilized scissors (Further description in extrusion assessment). Samples were brought to the molecular processing laboratory for further testing.

III. O. Assessment of Infectious Forming Units

At days 3, 7, 14, 21, 28 and 35 post-infection, mice were individually sedated with 3% isoflurane at 1.5L O_2/minute until breathing slowed. Mice were then removed

from induction chamber and placed in hand with vaginal opening exposed. Vaginal tracts were swabbed 8 rotations to the right and 8 rotations to the left, for each mouse IFUs (Puritan HydraFlock Sterile Flocked Collection Devices, Puritan Diagnostics, Gullford, Maine). Independent swabs were added to 1.5mL eppendorf tubes with 600 µL1 X SPG and two glass beads, as adopted from previously described studies (Cheng et al., 2008; Chen et al., 2014).

Samples were kept on ice until serial dilution and plating onto HeLa cell monolayers. At days 3, 7, 14, 21, 28 and 35 post-infection, individual samples were vortexed for 30 seconds to assist in shedding infectious EBs from swabs. In each well of a 24 well plate, 900 µL of 1X Hanks Balanced Salt Solution (HBSS) (Thermo Fisher Scientific) was aliquotted. In the first well of the plate, 100 µL of the sample was added creating a 10^{-1} dilution. Mixing was performed by pippetting 10 times into the first well. 100 µL was then taken up and moved to the next well in the same row, producing a 10^{-2} dilution. Contents of well were mixed by pipetting again 10 times. This process was repeated for each well of the row until a 10^{-6} dilution was created. On a separate 24 well plate with confluent HeLa cell monolayers, media was removed and 200 µL of each corresponding sample dilution was placed onto cell monolayers. Plates were spun at 700 X g for 1 hour. 1 mL of RPMI 1640 +10% FBS were added to each well after centrifugation and plates were then incubated for 24-34 hours at 37°C with 5% CO_2.

All wells were then fixed with ice cold 100% methanol for 15 minutes. Wells were washed 3 times for 5-10 minutes each time with 1X PBS. Plates were blocked with 100 µL of 2% BSA made in 1X PBS for 1 hour at room temperature**.** Samples were then stained with 100 µL of 2% BSA-PBS diluted corresponding antibodies for one hour: 1°:

anti-D/LC (mouse monoclonal) anti-Mouse pneumonitis (mouse monoclonal) (courtesy of Dr. Caldwell); anti-L2 (rabbit polyclonal) [1:1000]**;** apply 100 µL of 2°: anti-rb488; anti-ms594 to corresponding wells (mouse with mouse; rabbit in rabbit [Jackson ImmunoResearch Laboratories, Inc.]) [1:1000 and 1:500, respectively]. After samples were allowed to block for a period of 1 hour, all wells were rinsed well with 1X PBS for a minimum of two times, 5 minutes each rinse. Samples were subjected to fluorescent microscopy (10X magnification) for counting**.**

A dilution well with IFUs between 20-200 per field of view was used to perform counts. Twenty fields of view were counted and recorded, (Microsoft Excel) as well as the dilution well for each sample at each sampling time (3, 7, 14, 21, 28 and 35 days post-infection). Enumerations and standard deviations were performed with the aid of Microsoft Excel (average number of IFUs/well X dilution factor X microscope factor X amount plated). Data was then subjected to the box and whisker graph on Prism 7.0 to obtain a comparative analysis of recoverable IFUs verses the days post infection, amongst the *Chlamydia* strains.

III. P. Extrusion Assessment

At days 21 and 24 post-infection, mice were individually sedated with 3% isoflurane at a rate of 1.5L O_2/minute until respiration rate slowed. Mice were then removed from the induction chamber and held during vaginal tract swabbing in which swabs were inserted into the vagina and rotated 8 times to the right and 8 times to the left. Independent swabs were cut and placed into 1.5 mL eppendorf tubes containing 100 µL of RPMI 1640+10% FBS and kept cool until downstream processing for imaging. These

steps were repeated for each mouse until all mice had been sampled. With sterile forceps the swabs were rotated 10 times and gently pressed on the rim of the tube exiting, to remove any residual media and sample. Samples were prepared for Confocal microscopy as follows: 1 µL of NucBlue® Live ReadyProbes® Reagent (Thermo Fisher Scientific) and 1 µL of water solubilized FM®4-64 membrane stain (5µg/mL) (Thermo Fisher Scientific) was added to each sample tube. Tubes were flicked gently approximately 5 times. 10 µL of the sample was then placed on a clean glass microscope slide and a clean glass cover slip was placed on top to create a wet mount for microscopy. Samples were viewed and photographed with 60X magnification with oil on an Olympus Laser Scanning Confocal microscope.

III. Q. Sera Collection

At days 7, 35 and 53 days post infection; mice were euthanized to collect sera for cytokine and immune system activation assessment. Mice were individually sedated with 3% isoflurane at 1.5 L/ minute until respiration rate was approximately 20 respirations/minute. Cervical dislocation was performed and the mouse was then pinned to a dissection plate. Using a 25 gauge needle and 1 cc syringe, whole blood was collected and placed into a 1.5 mL O-ring tube and flicked several times to mix. Whole blood filled tubes were then spun at 600 X g for 10 minutes and stored at -20°C until assessment by ELISA (future experiments not included in this thesis).

III. R. Vaginal Wash for IgA Assessment

To investigate and compare the secretory IgA immune response amongst chlamydial strains, at day 30 days post infection, mice were subjected to a vaginal wash

to determine the presence of IgA by several steps. Briefly, 60 μL of 0.5% BSA-PBS buffer was introduced to individual mice by pipetting. The sample was refluxed five times and transferred, along with any remaining fluid in the vaginal tract, to a corresponding 1.5 mL eppendorf tube. This process was repeated once more and subsequent samples were kept on ice until spinning at 15,000 rpm for 10 minutes at 4°C. Resulting supernatant was removed apart from the pellet and transferred to a new tube and frozen at -20°C until further analysis.

III. S. Gross Physiological Assessment

To assess the anatomical sequelea following the murine infection, at days 64 and 65 post infection, remaining mice were sacrificed. Following cardiac puncture and whole blood collection, mice were dissected for reproductive tracts: ovaries, oviducts, uterine horns, uterus and bladder. Whole reproductive tracts were photographed next to a dissection ruler (VWR) with a 16 megapixel LED flash camera [pixel size 1.12 um, manual focus] and images were adjusted for size and color with Adobe Photoshop. Reproductive tracts were then sent in formalin solution to OSU Veterinary Medicine for histopathological assessment by a trained and licensed Pathologist (results of which are not included in this thesis).

III. T. Murine Cervical Dislocation and Cardiac Puncture

At the end of the time course, mice were individually sedated, as previously described. Mice were cervically dislocated by the following: with the left hand grasp the mouse tail ~1/2" from the base, with the right hand thumb and index finger, place onto neck region; pull the left hand holding the tail the same time as pressing and pulling with

the right hand to dislocate at cervical region. Mouse was pinned at each arm to the dissection plate. With a 25 gauge needle and 1 cc syringe (Sur-Vet Terumo^{R} Syringe), the needles were inserted at a 30° angle directly under the sternum toward the head. The needle was continued in this motion for ~ ½ of the needle length. At the point of the needling approximately ~ ½ the length into the mouse thoracic cavity, the angle of insertion of the needle was adjusted to be as close to 0° as possible. Then, the needle was continued into the thoracic cavity to penetrate the heart. The plunger of the syringe was gently pulled every 3 seconds whilst holding a steady grasp on the syringe to extract as much whole blood as possible. The needle was removed from syringe in disposal container and whole blood was ejected from the syringe into a 1.5 mL O-ring tube, be sure to flick tube to disperse anti-coagulant into the sample. Tubes were labeled with animal number, date, and chlamydial strain of infection. Carcasses were disposed of into biohazard bag (to be bagged up after experiment). Care was taken as to begin with SHAM control mice first. Once all mice were sacrificed and whole blood collected, tubes were spun at 600 X g for 10 minutes. Sera were collected and frozen at-80°C until use.

III. U. Dissection of Murine Reproductive Tracts

At the end of the time course, following cervical dislocation and cardiac puncture, mice reproductive tracts were dissected, photographed and collected for gross and histo-pathological comparisons, taking care to always begin with SHAM control mice. Mice were pinned to dissection trays, and using dissection scissors and forceps, ovaries, uterine horns, uterus and bladder were cut and removed from the mouse. Reproductive tracts were then cleaned of any fatty tissue and photographed.

III. V. Statistical Analysis

Statistical analysis (box and whisker plot and two tailed T-test) was performed in Prism 7 for assessment of IFU recovery.

CHAPTER IV

IN VIVO COMPARISON OF CHLAMYDIAL STRAINS: C. TRACHOMATIS SEROVAR D/LC, C. TRACHOMATIS SEROVAR L2 AND C. MURIDARUM MOPN IN C3H/HEJ MICE

Abstract

Chlamydia trachomatis is the most frequently reported bacterial sexually transmitted infection. Even after a *C. trachomatis* infection is treated, there is an increased risk for the development of pelvic inflammatory disease and cervical cancer, but the mechanisms are poorly understood. As an obligate intracellular pathogen, *C. trachomatis* usurps many host cell-signaling pathways from within a membrane bound vacuole, called an inclusion. *C. trachomatis* is also known to synthesize and secrete via the type III secretion system, inclusion membrane proteins (Incs) that are inserted into the inclusion membrane and serve as the interface between *Chlamydia* and the host. *C. trachomatis* is the first bacterial pathogen observed to recruit myosin phosphatase (MYPT1) for means of host cell exit, and does so through the chlamydial Inc protein, CT228. In this study the chlamydial TargeTron^{TM} system was used to genetically inactivate *CT228* in the *C. trachomatis* genome. TargeTron^{TM} insertion was confirmed by PCR and expression of the *CT229-CT224* operon of the mutant was verified by RT-PCR to rule out polar effects.

The CT228 mutant was verified to be deficient in CT228 production and MYPT1 recruitment by immunofluorescence. Genetic complementation of CT228 *in trans* successfully restored MYPT1 recruitment in the mutant. This study demonstrates successful gene inactivation of the chlamydial protein CT228, successful complementation of the TargeTron™ mutation *in trans*, and confirms the role of CT228 in MYPT1 recruitment. Additionally these studies provide a platform to further investigate the role of CT228 in chlamydial pathogenesis.

Keywords

Need 5 but limit to 8 (*Chlamydia*, TargeTron, Complementation, MYPT1, CT228)

Introduction

Chlamydiae are pathogenic and unique obligate intracellular bacteria that infect a wide variety of animals. *Chlamydia trachomatis* is the causative agent of the number one human sexually transmitted infection in the United States. Serovars A-C are ocular in nature, resulting in infectious blindness; serovars D-K and L1-L3 are urogenital in nature, with L1-L3 resulting in highly invasive LGV (Burton and Mabey, 2009).

Subsequent to clearing of an infection with antibiotics, an associated risk can be observed for the development of numerous sequelea. In developed countries, nearly half the incidences of pelvic inflammatory disease are caused by a chlamydial infection (Khalaf, 2003). Other complications after an infection include fallopian tube scarring, infertility, pelvic inflammatory disease and ectopic pregnancy (Smith et al., 2001).

Chlamydiae are unique in that they possess a biphasic developmental cycle. Upon infection, when the extracellular, non-replicative, infectious elementary body (EB) binds to the surface of a host cell, the EB is endocytosed, avoids the endocytic pathway,

and establishes a parasitophorous vacuole, deemed an inclusion (Moulder, 1991; Hackstadt et al. 1997). The inclusion membrane will serve as the interface between the host and *Chlamydiae*. From within this inclusion, the EB differentiates into the replicative, non-infectious, metabolically active reticulate body (RB). The bacteria synthesize a variety of proteins, which are secreted using a type III secretion system. Some of these proteins, called inclusion membrane proteins (Incs) decorate the inclusion, inserting into the cytosolic face of the inclusion membrane and recruit proteins of the host cell for a variety of functions (Rockey et al., 1997; Scidmore & Hackstadt, 2001; Hackstadt et al., 1999). At approximately 15 hours post infection, RBs begin to convert back to EBs, thus allowing for another cycle of replication. The growing numbers of chlamydia fill the host cell causing the cell to swell, and then exit the host cell by either one of two mechanisms: lysis or extrusion (Hybiske & Stephens, 2007; Todd & Caldwell, 1985).

Most recently, Yang *et al*. describe that the mechanism of lytic exit is dependent on a transcriptional regulator, Pgp4, encoded on a single plasmid possessed by many *Chlamydiae* (Yang et al., 2015). Whereas, the extrusion mechanism of host cell exit is regulated by interactions between host and chlamydial proteins at the site of the inclusion membrane. Lutter et al. were the first to describe that a chlamydial inclusion membrane protein, CT228, recruits a component of myosin phosphatase, MYPT1, to the inclusion during infection. This interaction between CT228 and MYPT1 was observed to regulate the host cell exit mechanism of extrusion (Lutter et al., 2013). Yet, without further characterization of CT228, a greater understanding of how the extrusion method of host cell exit impacts chlamydial pathogenesis could not be established.

Historically, the chlamydial field of research was greatly limited by the availability of genetic tools of which to manipulate the bacterium. The first account of the stable introduction and maintenance of foreign DNA in *Chlamydia spp.* Was reported by Binet and Maurelli (Binet & Maurelli, 2009). Further advancement in the field occurred with the development of the chemical transformation and chemical mutagenesis of *Chlamydia trachomatis* (Wang et al., 2011, Johnson & Fisher, 2013; Gerard et al., 2013; Kari et al., 2011; Nguyen & Valdivia, 2014). These findings allowed for a platform for which numerous research groups would begin to rapidly investigate chlamydial gene function, growth and infectivity. Recent methods allow for the generation of plasmid gene knockouts, chemical mutagenesis and plasmid-based gene knock in approaches, but the field still lacked tools to target selectable chromosomal mutations. (Gerard et al., 2013; Kari et al., 2011; Nguyen & Valdivia, 2014) Johnson and Fisher were the first to report a selectable site-specific chromosomal gene inactivation of *incA* in *C. trachomatis,* by the insertion of a group II intron (Johnson & Fisher, 2013). By utilizing a commercially available TargeTron ™ system (Sigma), the first site-specific gene inactivation in *Chlamydia* was accomplished. Importantly, in the chlamydial-adapted TargeTron™ system, complementation *in trans* has not been published to date.

As TargeTron™ system success with the inactivation of *incA* was observed, we hypothesized that genetic inactivation of *CT228* could be accomplished and that complementation could be utilized to restore MYPT1 recruitment to the inclusion during infection. To test this hypothesis, the TargeTron™ vector platform was modified to allow for spectinomycin selection by replacing the *bla* cassette with the *aadA* cassette and allowed for expression and selection in *C. trachomatis* serovar L2. As predicted, the

CT228 mutant was deficient in CT228 and MYPT1 recruitment to the inclusion during infection. After determining, through RT-PCR, that no polar effects were observed whilst utilizing the CT228 mutant, complementation was performed by incorporation of *CT228* into the vector pBOMB4R, previously shown to successfully express host cell cytosolic chlamydial effector proteins (Bauler & Hackstadt, 2014). Upon complementation, MYPT1 recruitment was restored as demonstrated through immunofluorescent microscopy. These findings demonstrate the second successful chlamydial adaptation of the TargeTron™ system to further characterize the role of CT228 in MYPT1 recruitment during *C. trachomatis* pathogenesis.

Results

Construction of the CT228 Mutant, Δ*CT228*

The chlamydial TargeTron adopted vector, pDFTT3(*bla*) was chosen for modification for use in *C. trachomatis* serovar L2 to genetically inactivate the protein, CT228. Previously described, the vector pDFTT3 contains an *Escherichia coli* origin of replication, a *cat* marker for chloramphenicol selection and necessary intron genes: GII intron, *ltrA* and *bla* genes (Johnson and Fisher, 2013). pDFTT3 was further modified by replacement of the *bla* gene with a spectinomycin cassette (*aadA*), allowing for mutant complementation using the shuttle vector, pBOMB4R. The resulting vector was then retargeted by PCR for antisense insertion into *CT228* at 295 base pairs with an E-value of 0.200 (Figure 2). Colonies carrying a disrupted *CT228* gene were obtained and sequence confirmed to validate intron insertion into the predicted site (data not provided).

Primer name	F/R	Sequence
IntCT224F	Forward	GGAGATAGTGTACCTCTTAG
IntCT224R	Reverse	GAGTGTAGAATAGCCTCTTTC
IntCT225F	Forward	CAACTCCTTTATTCACCG
IntCT225R	Reverse	CCCACCCATGAAATTTAGC
IntCT226F	Forward	GCGAAATAGAGGCGCCATG
IntCT226R	Reverse	CCCACTTTGAAGCTGTTC
IntCT227F	Forward	GTTCCTCTTGCGCTCCAACTC
IntCT227R	Reverse	GAGACACTTATAGTCACG
IntCT228F	Forward	CCATTGCCAACAGCTTCC
IntCT228R	Reverse	GCTTGGTTAGCGTCTATAG
IntCT229F	Forward	CAGGTGCACCTGCTACTC
IntCT229R	Reverse	CGACGGGATGCCTGCACC
BombCT228FBamHI	Forward	AAAGAATCCATGAGTACTACTATTAGCGAA
BombCT228RKpnI	Reverse	AAAGGTACCTTAAGAAGCTTGGTTAGCGTC
incAF	Forward	ACCTTCCTACTCAGCCAATC
incAR	Reverse	AATCGGCGAACTTCTTCTGC
CT228F	Forward	GTACCCGGGATGAGTACTACTATTAGC
CT228R	Reverse	GATCCCCGGGCTAAGAAGCTTGGTTAG
GIIF	Forward	AGCGATGCCGAGAATCTG
GIIR	Reverse	TCTCGGAGTATACGGCTCTG
hyp08F	Forward	CTCGTAATATGCAAGAGCATTGTAAG
hyp08R	Reverse	GGCCGCAGAAGATATTCTGAAG
JETF	Forward	CGACTCACTATAGGGAGAGCGGC
JETR	Reverse	AAGAACATCGATTTTCCATGGCAG
228A		AAAA**AAGCTT**ATAATTATCCTTAAAACACTTTTTGGTGCGCCCAGATAGGGTG
228B		CAGAT**TGTACA**AATGTGGTGATAACAGATAAGTCTTTTTGCTTAACTTACCTTTCTTTGT
228C		TGAACGCAAGTTTCTAATTTCGGTTTGTTTCCGATAGAGGAAAGTGTCT
EBS universal		CGAAATTAGAAACTTGCGTTCAGTAAAC

Table 1. A complete list of primers utilized in this thesis listed from 5' to 3'

A) Wild type locus map **B) CT228::GII(*aadA*)**

Figure 2. Diagram of TargeTron intron insertion.

A) A wild type locus map of *CT228* reveals the predicted intron insertion site at bp 295 with an E-value of 0.200. **B)** The diagram of the *CT228*:GII(*aadA,*) CT228 TargeTron, mutant reveals the incorporation of the GII(*aadA*) intron in an antisense manner into the predicted insertion site. Sanger sequencing established correct insertion, at the predicted site.

CT228 Mutant Analysis

Polymerase Chain Reaction (PCR) was performed using CT228-specific primers flanking the GII(*aadA*) insertion site validated successful intron insertion (Figure 3, Reaction 2). This result was visualized by the increase in mutant product size when compared to wild type. To establish that the *CT228* region was specifically disrupted, a PCR reaction was used with *incA*-gene specific primers. *IncA* is utilized as a control to ensure that the chosen site of insertion was not affecting regions of the chromosome adjacent to *CT228 (*Figure 3, Reaction 1). To justify that the intron had not been lost during passage, PCR validation of the intron was observed in only the mutant (Figure 3, Reaction 3). Primers linking the GII(*aadA*) intron to *CT228* demonstrated the anti-sense orientation of intron insertion relative to CT228 (Figure 3 Reactions 4 and 5). To further provide confidence in intron insertion, Sanger sequencing was utilized to validate intron insertion in an anti-sense manner into the predicted site at 295 bp. Furthermore, PCR was utilized to ensure that the chlamydial cryptic plasmid was not lost during passage or lost upon introduction of the TargeTron plasmid (Figure 3, Reaction 6). Established was the specific site-insertion of the GII(*aadA*) intron into *CT228* in an anti-sense manner. However, with *incA* amplification observed upstream in the 3' direction with respect to

CT228, it was possible downstream polar effects could be observed in the *CT229-CT224* operon, hindering expression of genes downstream.

		Expected sizes, kbp		
	Target Region	**WT**	**Mut**	**pDFTT295**
Rxn1	*incA*	0.561	0.561	
Rxn 2	*CT228*	0.61	2.822	
Rxn 3	intron		1.789	1.789
Rxn 4	*CT228* - intron (CT228F-GIIF)		2.171	
Rxn 5	*CT228* - intron (CT228R-GIIR)		2.44	
Rxn 6	cryptic plasmid	0.477	0.477	

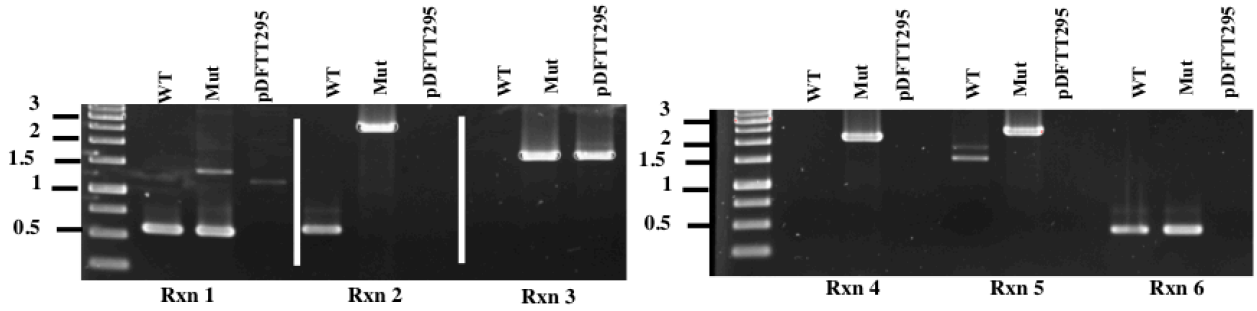

Figure 3. PCR Validation of CT228 Genetic Inactivation

The TargeTron adapted vector, pDFTT(*bla*) was modified for use for the present study by replacing the *bla* gene with the *aadA* cassette to allow for selection using spectinomycin. The vector was retargeted for insertion into the predicted intron insertion site of the *CT228* gene in serovar L2. PCR was utilized using a series of specific primers with template DNA derived from **WT** (wild-type; *C. trachomatis* L2), **Mut** (Δ*CT228*) or from **pDFTT295** (intron) and reactions were assessed with gel electrophoresis. After PCR

validation, Sanger sequencing confirmed that the predicted site (295 bp) contained the intron in an anti-sense manner. Figure provided courtesy of Dr. Derek Fisher.

Expression Validation of the *CT229-CT224* Operon

To establish that polar effects were not observed when using the mutant, reverse Transcriptase PCR (RT-PCR) was utilized with internal gene specific primers to each of the genes within the *CT229-CT224* operon. RNA was extracted from HeLa 229 cell monolayers infected with *C. trachomatis* serovar L2 wild type EBs or mutant CT228 EBs at 24 hours post-infection. RT-PCR was then used to check for gene expression for each gene within the operon (Figure 4). *CT228* is the second gene in the *CT229-CT224* operon and RT-PCR of *CT229* showed amplification from both wild type and mutant cDNA. Thus, indicating that *CT229*, upstream from *CT228* is independent of the mutation and that the cDNA synthesis was successful. RT-PCR amplification of *CT228* showed that amplification was observed from both wild type and mutant RNA. Furthermore, comparison of the product sizes indicates that the intron was retained throughout the time course and was expressed. RT-PCR for each gene downstream of *CT228* (*CT227*, *CT226*, *CT225* and *CT224*) from both wild type and mutant show amplification, thus providing that polar effects due to *CT228* GII(*aadA*) insertion are not hindering expression of genes downstream of *CT228*. Furthermore, this ensured that future complementation of the mutant could be performed by the addition of the *CT228* gene solely to the shuttle vector, pBOMB4R.

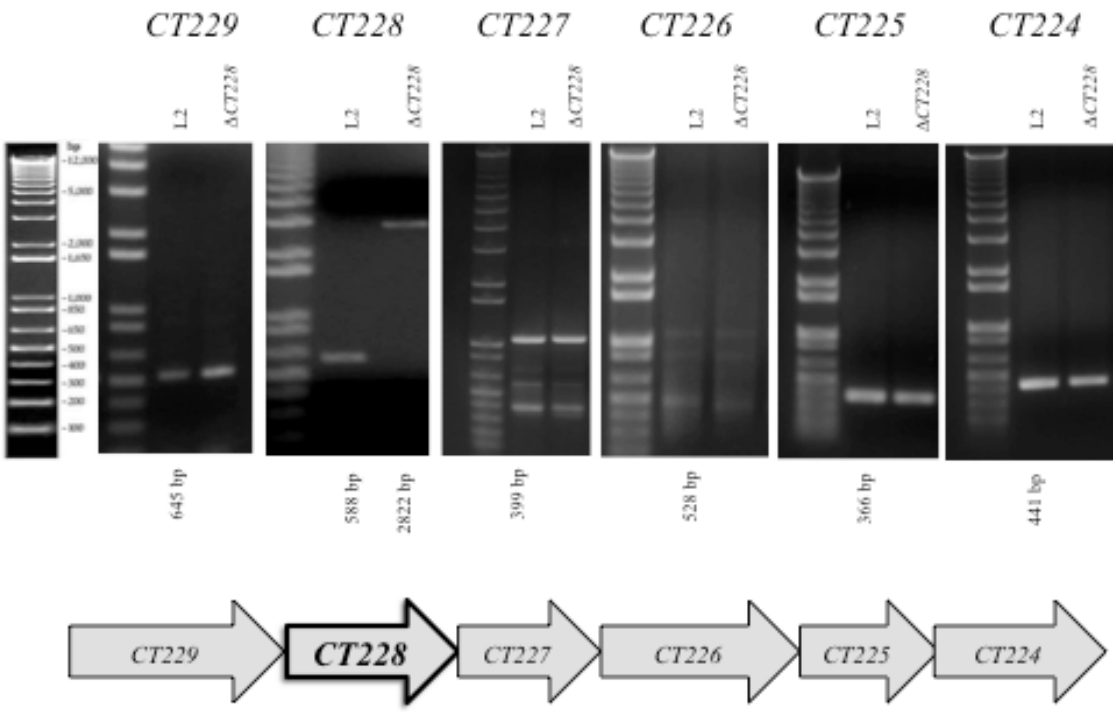

Figure 4. The *CT229-CT224* operon of the CT228 mutant, Δ*CT228,* is free of polar effects

To validate that the downstream genes of *CT228* (*CT227*, *CT226*, *CT225* and *CT224*) were not affected by the insertion of GII(*aadA*), RT-PCR was utilized. RT-PCR was performed using internal gene-specific primers for each gene within the operon. Panels display expression of all genes within the *CT229-CT224* operon from both *C. trachomatis* L2 wild type *and C. trachomatis* CT228 mutant, Δ*CT228.*

C. trachomatis CT228 Mutant Validation Protein Recruitment to the Inclusion Membrane

The inclusion membrane protein CT228 is localized to the inclusion membrane periphery during infection. To verify mutant loss of CT228 recruitment to the inclusion membrane, *C. trachomatis* L2 and CT228 mutant, Δ*CT228,* EBs were used to infect confluent HeLa 229 cell monolayers. At 36 hours post infection, when cells are infected and have observable large inclusions, cell monolayers were fixed and stained with anti-CT228 and anti-total MYPT1 and corresponding secondary antibodies. Immunofluorescent microscopy was used to validate that in the mutant, Δ*CT228* was deficient in CT228 production. Furthermore, CT228 was not localized to the periphery of the inclusion in contrast to wild type localization of CT228 at the inclusion membrane (Figure 5).

A component of myosin phosphatase, MYPT1, is known to interact with CT228 during infection, affecting the dynamic of the host cell exit method of extrusion (Lutter et al., 2013). Myosin Light Chain 2 (MLC2) is a known substrate of MYPT1. MYPT1 will enzymatically dephosphorylated MLC2, when active. MYPT1, when inactive and interacting with CT228, is prevented from the enzymatic function on the substrate MLC2. This leaves MLC2 in an active, phosphorylated state (Lutter et al., 2013). Active MLC2 is known to interact with binding partners, Myosin IIA and Myosin IIB to form a myosin motor complex, which in turn correlates with the host cell exit mechanism of extrusion.

The loss of MYPT1 to the site of the inclusion periphery during infection with the CT228 mutant, Δ*CT228* was observed in comparison to the presence of MYPT1 upon infection with *C. trachomatis* L2 (Figure 6). Thus, providing that in the mutant, MYPT1 is not to the periphery during infection. When assessing recruitment of MLC2, Myosin IIA and Myosin IIB to the site of the inclusion during infection, phospho-specific antibodies were used as to detect the presence of phosphorylation states of the protein, which indicate activity of the overall protein. As observed, MLC2, Myosin IIA and Myosin IIB are all recruited to discrete areas of kinase activity around the inclusion, called microdomains (Figures 6-9). Adapted from previous investigations, staining with anti-Src (Src family kinases) is used as a marker for microdomains (Mital and Hackstadt, 2011). Thus, providing evidence that these proteins are recruited to these areas in both wild type and mutant.

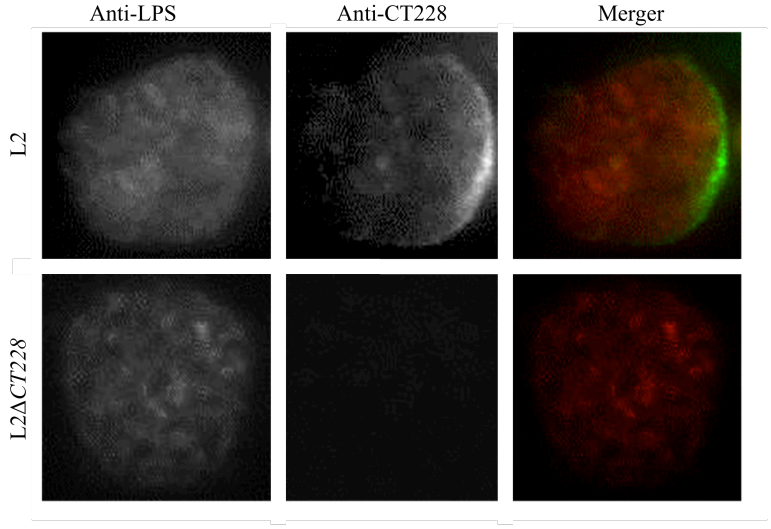

Figure 5. Immunofluorescent microscopy verifies the loss of CT228 production in the CT228 mutant

HeLa 229 cell confluent monolayers were infected with *C. trachomatis* L2 wild type or CT228 mutant, Δ*CT228*. Infections were fixed with methanol and cells were stained with anti-LPS to visualize the inclusions within each cell and α-CT228 to observe the localization and production of CT228. Observed above is the loss of CT228 production upon infection with CT228 mutant, Δ*CT228*.

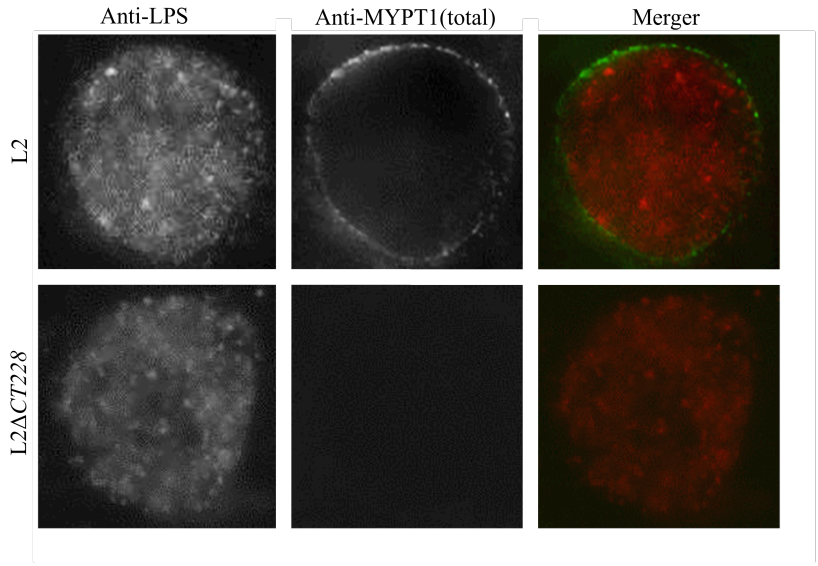

Figure 6. Immunofluorescent microscopy validates the loss MYPT1 recruitment in the CT228 mutant

HeLa 229 cell confluent monolayers were infected with *C. trachomatis* L2, wild type, or CT228 mutant, Δ*CT228,* EBs. Infections were fixed with methanol and cells were stained with α-LPS to visualize the inclusions within each cell and α-MYPT1 (total; both phosphorylated and non-phosphorylated) to observe the localization to the inclusion membrane. Observed above is the loss of MYPT1 recruitment to the inclusion membrane periphery upon infection with the CT228 mutant, Δ*CT228*.

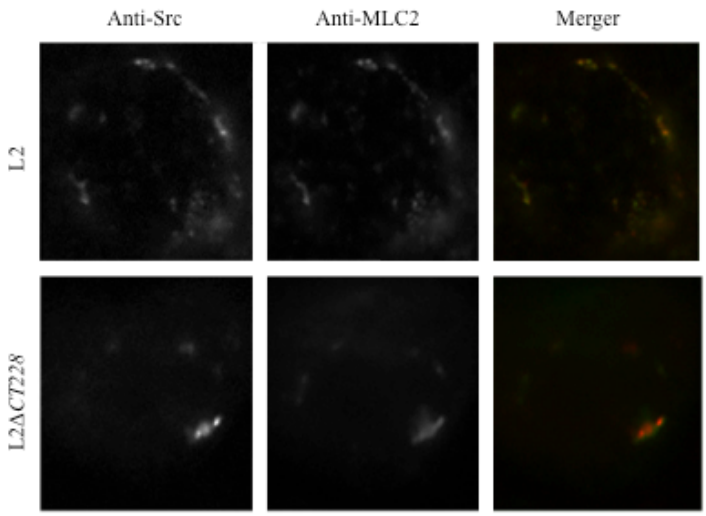

Figure 7. Immunofluorescent microscopy verifies the recruitment of MLC2 in the CT228 mutant

HeLa 229 cell confluent monolayers were infected with *C. trachomatis* L2 Wild Type or CT228 mutant, Δ*CT228,* EBs. Following infections, monolayers were fixed with methanol and cells were stained with phospho-specific antibodies: anti-Src (pTyr419) and anti-MLC2 (pSer19) to visualize the recruitment of phosphorylated proteins to the inclusion membrane during infection. Observed above is the recruitment of MLC2 to microdomains of the inclusion membrane. This recruitment of MLC2 to the inclusion membrane during infection with mutant, Δ*CT228,* is similar to the recruitment observed upon infection with *C. trachomatis* L2.

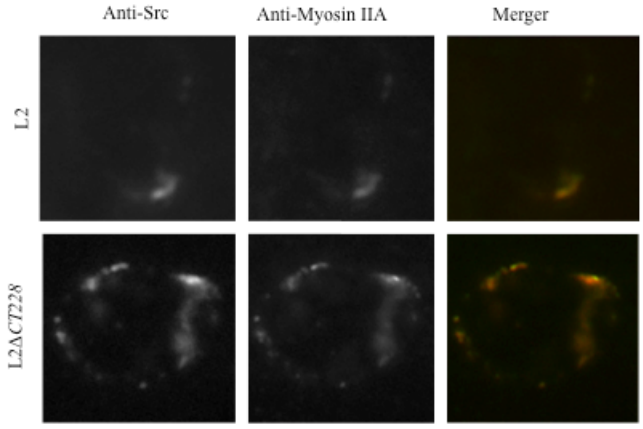

Figure 8. Immunofluorescent microscopy reveals the recruitment of Myosin IIA in the mutant

HeLa 229 cell confluent monolayers were infected with *C. trachomatis* L2 wild type, or CT228 mutant, Δ*CT228,* EBs. Infections were fixed with methanol and cells were stained with phospho-specific antibodies: anti-Src (pTyr419) and anti-Myosin IIA, to visualize the recruitment of phosphorylated proteins to the inclusion membrane during infection. Observed above is the recruitment of Myosin IIA to microdomains of the inclusion membrane. This recruitment of Myosin IIA to the inclusion membrane during infection with Δ*CT228* is similar to the recruitment observed upon infection with *C. trachomatis* L2.

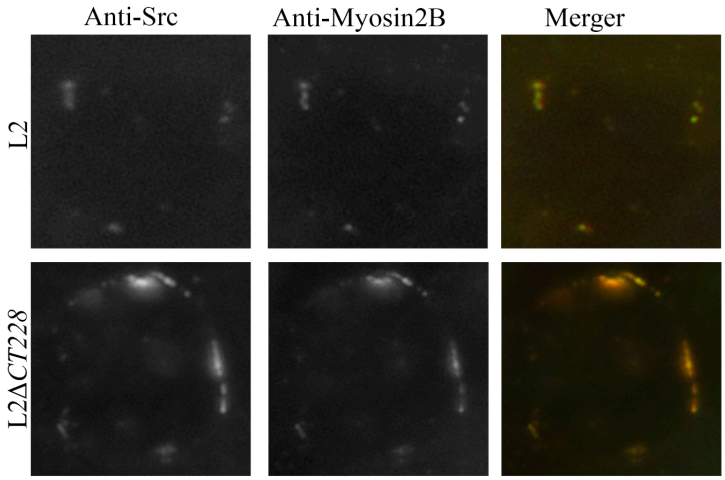

Figure 9. Immunofluorescent microscopy establishes the presence of Myosin IIB in the mutant

HeLa 229 cell confluent monolayers were infected with *C. trachomatis* L2 wild type, or CT228 mutant, Δ*CT228*, EBs. Infections were fixed with methanol and cells were stained with phospho-specific antibodies: anti-Src (pTyr419) and anti-Myosin IIB, to visualize the recruitment of phosphorylated proteins to the inclusion membrane during infection. Observed above is the recruitment of Myosin IIB to microdomains of the inclusion membrane. This recruitment of Myosin IIB to the inclusion membrane during infection with Δ*CT228* is similar to the recruitment observed upon infection with *C. trachomatis* L2.

Immunofluorescent Validation of the CT228 Mutant Complementation

A chlamydial adapted TargeTron mutation has yet to be complemented. As such, the goal was to complement the *CT228* TargeTron mutant and verify the recruitment of proteins necessary for the host cell exit mechanism of extrusion, to the inclusion during infection. The shuttle vector, pBOMB4R was utilized. The pBOMB4R vector has been used previously to investigate inclusion membrane proteins during infection (Weber et al., 2015). Complementation of the *CT228* mutant was possible as pBOMB4R contains a β-lactamase resistance cassette, allowing for selection by the addition of Penicillin G (Bauler & Hackstadt, 2014). The adaptation of the TargeTron plasmid, replacement the *bla* cassette with the *aadA* spectinomycin cassette, was instrumental for successful complementation of the mutant strain.

CT228 was amplified from *C. trachomatis* serovar L2 434/Bu and was cloned into the *BamHI* and *KpnI* sites of pBOMB4R. Following sequence confirmation, the construct was transformed in CT228 mutant, Δ*CT228*, EBs. After several selective passages using Penicillin G, a clonal plaque was obtained and expanded (see Methods and Materials). The *CT228* mutant complementation plasmid was used to infect confluent HeLa 229 cell monolayers, along with wild-type *C. trachomatis* serovar L2, and CT228 mutant, Δ*CT228*; allowing for a direct comparison of wild-type, mutant and complemented strains. Infected cell monolayers were fixed and stained with corresponding primary and secondary antibodies and subjected to immunofluorescent microscopy.

Immunofluorescent microscopy analysis showed consistent results for *C. trachomatis* serovar L2. Both CT228 and MYPT1 are shown to be present at the site of

the inclusion, surrounding the periphery of the inclusion during infection (Figures 9 and 10, respectively). Also consistent with previous findings is the loss of CT228 and MYPT1 at the site of the inclusion during infection with Δ*CT228* (Figures 10 and 11). However, upon infection by the mutant complement, Δ*CT228*C, CT228 is again present at the site of the inclusion in a similar manner to wild-type (Figure 10). However, the amount of protein recruitment appears to be at lower levels. This could be partially in fact by the construct being under control of the *rpoB* promoter, allowing for low levels of constitutively expressed proteins. Similarly, upon infection by the CT228 mutant complement, Δ*CT228*C, MYPT1 is also observed at the periphery of inclusion membrane during infection (Figure 11). It is shown that complementation of the CT228 TargeTron mutant was successful, as MYPT1 recruitment to the inclusion during infection was restored. To further ensure that complementation of the mutant was indeed successful, Western blot analysis was utilized to confirm expression of CT228 in the complemented strain.

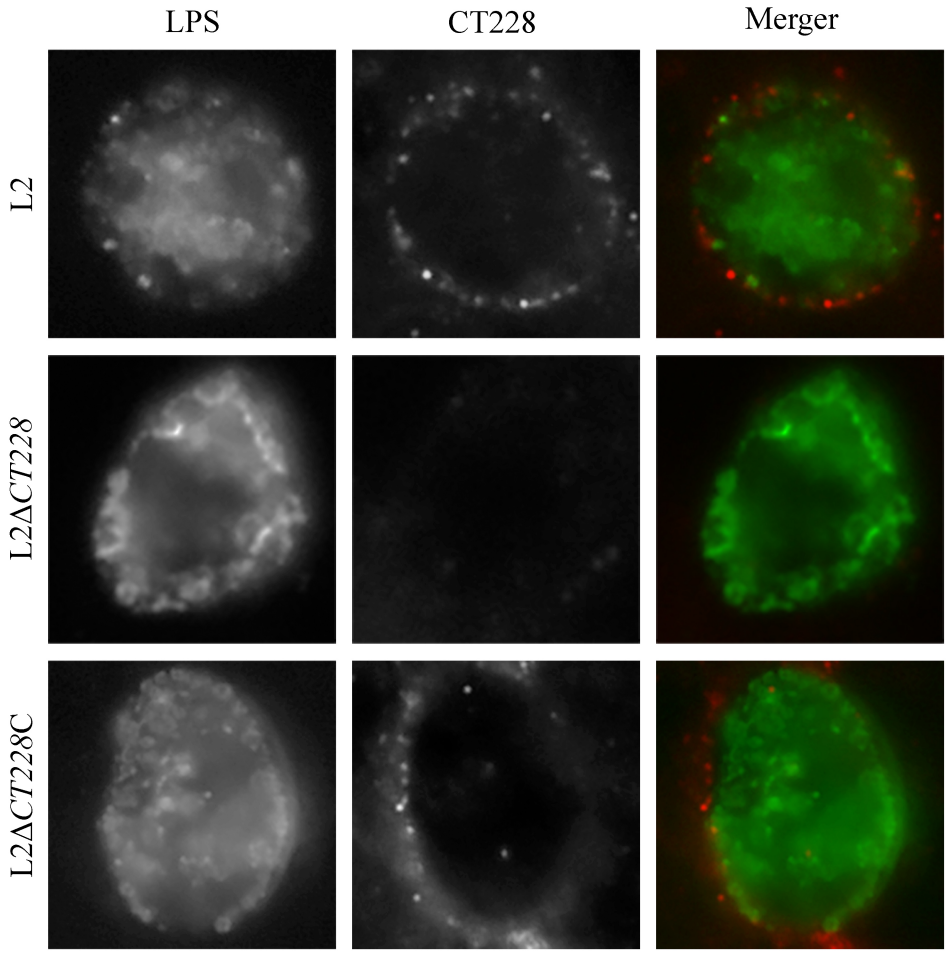

Figure 10. Immunofluorescent microscopy reveals restoration and localization of CT228 to the inclusion membrane when infecting with the complemented CT228 mutant

HeLa 229 cell confluent monolayers were infected with *C. trachomatis* L2 (wild type strain), Δ*CT228* (CT228 mutant) or Δ*CT228*C (CT228 complemented mutant). Infections were fixed with methanol and cells were stained with anti-LPS to visualize the inclusions

within each HeLa 229 cell and α-CT228 to observe the localization and production of CT228. Observed above is the loss of CT228 production upon infection with the mutant, Δ*CT228* and restoration of protein production with the CT228 complemented mutant, Δ*CT228*C.

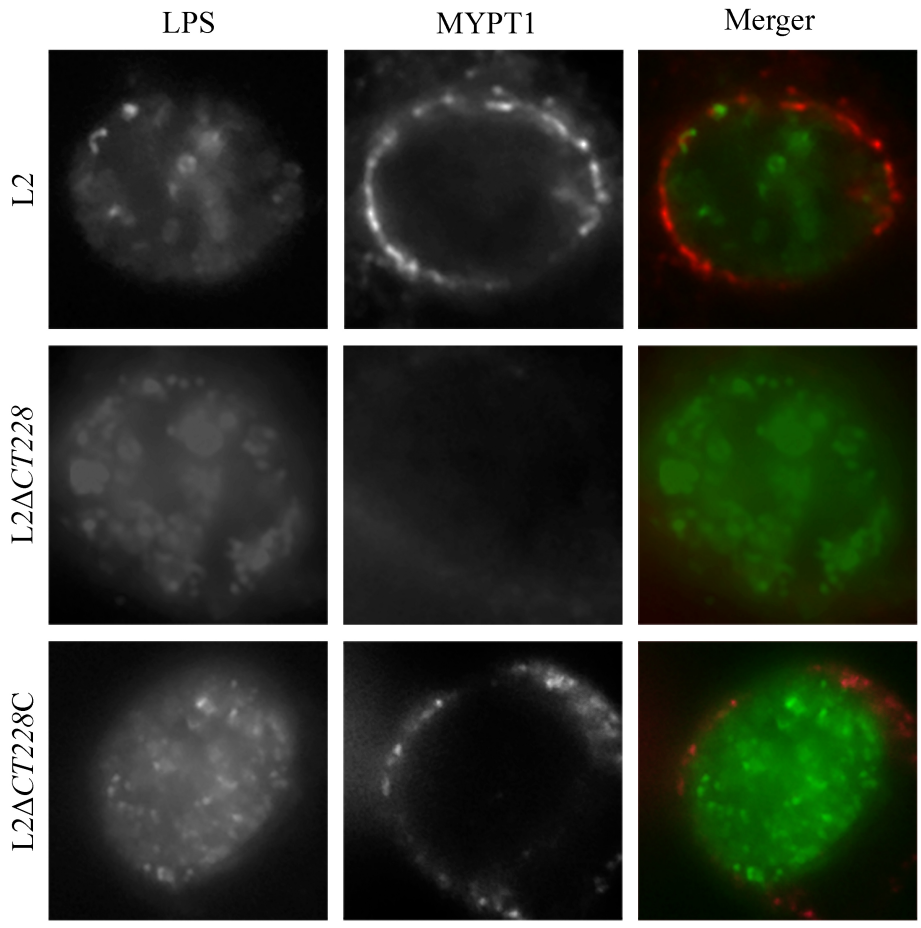

Figure 11. Immunofluorescent microscopy verifies that CT228 complemented mutant restores MYPT1 recruitment upon infection

HeLa 229 cell confluent monolayers were infected with *C. trachomatis* L2 (wild type strain), Δ*CT228* (mutant) or Δ*CT228*C (complemented mutant). Infections were fixed with methanol and cells were stained with anti-LPS to visualize the inclusions within each HeLa 229 cell and anti-MYPT1 (total; phosphorylated and non-phosphorylated) to observe the localization and production of CT228. Observed above is the loss of MYPT1 recruitment upon infection with the CT228 mutant, Δ*CT228*, and restoration of protein recruitment with the complemented mutant, Δ*CT228*C, similar to recruitment of wild type.

Western Blot Analysis of Whole Cell Lysates Infected with *C. trachomatis* L2, Δ*CT228* and Δ*CT228*C

To establish that the mutant was indeed deficient in CT228 production and that the CT228 mutant complementation had restored the CT228 protein deficit, HeLa 229 cell confluent monolayers were infected with *C. trachomatis* L2 wild type*, C. trachomatis* serovar L2 CT228 mutant (Δ*CT228)* and *C. trachomatis* serovar L2 CT228 mutant complement (Δ*CT228*C) at an MOI of approximately 1. At 36, 42 and 48 hours post infection, monolayers were lysed and collected with Laemmli buffer and subjected to SDS-PAGE gel electrophoresis. Samples were transferred to nitrocellulose membrane and resulting blots were developed using anti-MOMP and anti-CT228 primary antibodies and HRP-conjugated secondary antibodies serving for ECL detection (Figure 12).

Figure 12. Western Blot Analysis verifies production of CT228 in Wild Type, CT228 Mutant and CT228 Mutant Complement

Western blot analysis was used to assess whole cell lysates separated by SDS-PAGE and stained with α-CT228 at 36 hours post-infection. Consecutive bands at approximately 21 kDa are apparent in lanes corresponding to wild type, **L2** (left lane) CT228 mutant, **Δ*CT228*** (middle lane) and the CT228 complemented mutant, **Δ*CT228*C** (right lane).

Extrusion Assessment of *C. trachomatis* L2 Wild Type, CT228 Mutant and Complemented CT228 Mutant

To assess the differences of extrusion numbers between *C. trachomatis* L2 wild type, Δ*CT228* mutant and Δ*CT228*C complemented mutant. HeLa 229 cell confluent monolayers were infected at an MOI of approximately 1 for each strain. At 36, 42 and 48 hours post-infection, supernatants were collected, spun by centrifugation, stained with 1µL NucBlue® Live ReadyProbes® nucleic acid stain (Thermo Fisher Scientific) and enumerated for *Chlamydia*-filled host cell membrane encased extrusions, devoid of host cell nuclei. Statistical analysis was provided by Prism 7.0 using a one-way ANOVA multiple comparisons (Figure 13) However, it is plausible that contradictory results could be established if the use of a *dam-dcm-* methylation deficient plasmid was not used. This

could have decreased transformation efficiency producing such potential contradictory results. Furthermore, it is possible that differences in cell culturing procedures or extrusion collection and enumeration could also account for differences in contrasting phenotype (Key & Fisher, 2016). In addition, Hayes et al. report that the *C. trachomats* serovar B/Jali-20/OT Chlamydial major outer membrane protein (MOMP) sequence was found to contain 12 point mutations within the genome in the region encoding for the major outer membrane protein (MOMP) (Hayes et al., 1990). This yields the production of five amino acid changes of MOMP, along with a known truncation event in the *CT228* gene (Seth-Smith et al., 2009; Zuck et al., 2016; Hayes et al., 1990). The *C. trachomatis* serovar L2 CT228 mutant, Δ*CT228* possesses only the singe GII intron insertion mutation event in comparison to the *C. trachomatis* serovar B strain, which naturally harbors additional mutation types. These differences of strain type utilized in investigations, as well as mutations harbored within the strains can explain differences observed in the results of extrusions, as it is quite possible the B/Jali strain is mutated in other locations that could possibly have a role in the extrusion process.

Collectively, extrusion numbers at every time point of collection, 36, 42 and 48 hours post-infection, were found to be significant between groups L2 and mutant, Δ*CT228,* as well as CT228 mutant complement, Δ*CT228*C (*=P<0.01). However, statistical significance at every time point of collection, 36, 42 and 48 hours post-infection, was not found to be between groups L2 and Δ*CT228*C (P>0.05). These findings suggest that extrusions were produced in significantly larger numbers by the CT228 mutant, Δ*CT228,* at each time point when compared to the wild-type or complemented strains. Furthermore, there was an observable lack of statistical significance amongst wild-type and complemented strain produced extrusions. This

suggests that the complemented strain partially restored the extrusions phenotype, decreasing the numbers of overall produced extrusions, similar to the wild-type phenotype. While restoration to wild-type is not exact, this suggests a similar conclusion in comparison to immunofluorescent analysis: while restoration of the deficit is observable, it is indeed observable at low levels. Again, this could be due to a couple factors. First, the transformation efficiency could be lower than anticipated. Or, alternatively, expression under the control of the *rpoB* promoter in the pBOMB4R vector could be allowing for a lower level of expression, not high enough for full restoration of the deficit. In conclusion, complementation of the CT228 mutant was performed *in trans* by reintroduction of the CT228 as mediated by the shuttle vector, pBOMB4R. Complementation was observed to restore not only protein deficits, but additionally, recruitment to the inclusion membrane. Moreover, upon complementation of the mutant, the phenotypic extrusion host cell exit mechanism was restored. Novel to the success of this investigation was this result, for the first time, the complementation of a chlamydial-adapted use of the TargeTron™ system. As such, it is evident that the TargeTron™ based gene inactivation system proves again to be a valid platform for which to continue to genetically investigate a once intractable obligate intracellular bacterium.

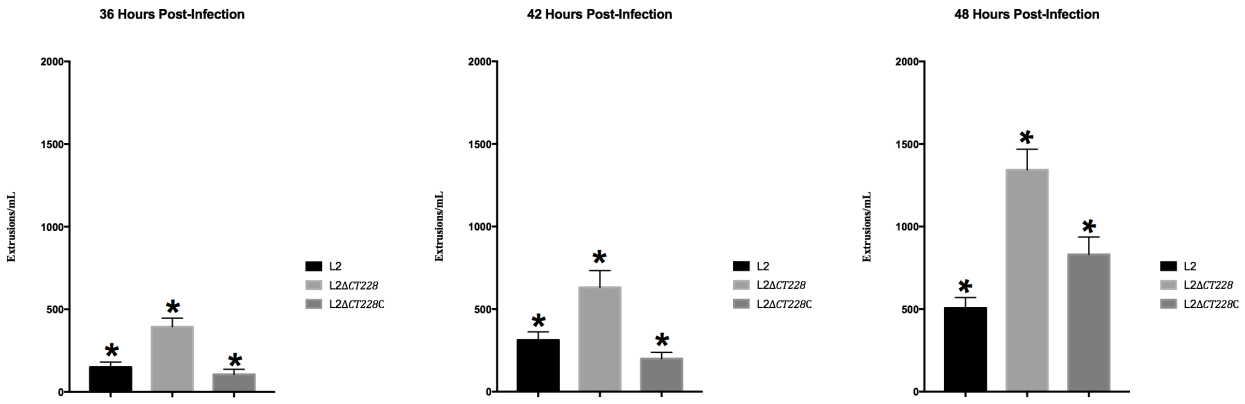

Figure 13. Comparison of L2, Δ*CT228* and Δ*CT228*C extrusion production at 36, 42 and 48 hours post-infection.

At 36, 42 and 48 hours post-infection, supernatant was removed from *Chlamydia*-infected HeLa 229 cell monolayers. Supernatant was stained to view live cell nucleic acid centrifuged to pellet extrusions. Samples were loaded onto glass slides with hemocytometer cover slips and 8 grids counted for each sample. Results were analyzed (one way ANOVA) and graphed using Prism 7.0 (XY graph).

Upon Western blot analysis, it was anticipated result that the mutant would yield a higher band, as there was previous evidence supporting such, as provided by RT-PCR. However, upon translation of the sequence of the mutant (provided by Dr. Derek Fisher in validation of the mutant), it was found that in Frame 3 of the results of translation there is a near 170 amino acid stretch which is translated immediately followed by two stop codons (Figure 14). Upon Western blot analysis, the expected size of the wild type protein is 196 amino acids as well as the complemented CT228 mutant. However, in comparison, when taking the truncation event into consideration of the CT228 mutant, only a difference of 2 kDa would be observable on a Western Blot, a difference not observable by eye.

Figure 14. SIB ExPASy *in silico* Frame 3 translation results of the sequenced

Δ*CT228* mutant shown in a 5'3' manner.

DNA sequencing of the Δ*CT228* mutant were entered into the ExPASy translate tool provided by Swiss Institute of Bioinformatics. Observable is the start codon Met followed by a near 175 amino acid protein followed by two Stop codons.

Discussion

Historically, obligate intracellular pathogen, *Chlamydia*, was considered to be genetically intractable until 2001, when Wang *et al.* described the first transformation method. From there, the genetic tools of which to investigate *Chlamydia* have advanced to chemical mutagenesis, chromosomal gene inactivation's and even plasmid-based gene knock outs (Johnson & Fisher, 2013; Gerard et al., 2013; Kari et al., 2011; Nguyen & Valdivia, 2014). Represented here, is the second adaptation of the chlamydial-based TargeTron™ system of which, genetic chromosomal inactivation of the chlamydial Inc, *CT228*, was accomplished.

In 2013, Johnson & Fisher were the first to report the successful utilization of the TargeTron™ system to genetically inactivate *incA*. The TargeTron™ system works by the incorporation of a non-coding region of DNA, the group II intron. Insertion into a chosen site of a target gene is accomplished by the mode of an RNA protein complex, which is easy mutable with PCR. The utilization of the TargeTron™ system has also been used to investigate various other bacteria including Gram positive and other Gram negative, as well. However, to date, in the Chlamydial host, restoration of mutant deficit was not performed by complementation. Importantly, this study is the first to complement a mutant TargeTron™ strain *in trans* to restore near wild-type phenotype.

Adaptation, yet again, of the GII(*bla*) mutant, replacing the *bla*, beta-lactamase resistance cassette with the *aadA*, spectinomycin resistance gene, was essential to the success of this investigation. This modification allowed for the selection of stable mutants possessing the mutation. Furthermore, the modification allowed for complementation of the mutant by using the pBOMB4R shuttle vector, shown to work previously with inclusion membrane proteins (Bauler & Hackstadt, 2014). Adaptation of the TargeTron™ system was successful in the Chlamydial system once again, based on the non-essential function of CT228. Furthermore, validating that polar effects, when using the *CT228* mutant, did not compromise operon expression was key in the successful complementation of the mutant. It was anticipated result that the mutant would yield a higher band by Western blot analysis as previous evidence was provided by RT-PCR. However, it was found that there is a truncation event, resulting in a near 170 amino acid stretch, which is translated. Upon Western blot analysis, the expected size of the wild type protein is 196 amino acids as similarly observed in the complemented CT228 mutant. However, in comparison, when taking the truncation event into consideration of the CT228 mutant, only a difference of 2 kDa would be observable on a Western Blot, a difference not observable by eye with use of this method. This discrepancy and difference could explain the Western Blot analysis results observed in this work. However, as a result, validity is provided to future use of this chromosomal inactivation system as it produces chlamydial proteins of which functionality is compromised are a result. The TargeTron^{TM} system is thus warranted to serve again as a chlamydial investigational system to gain further future insight into protein function.

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CHAPTER V

IN VIVO COMPARISON OF CHLAMYDIAL STRAINS: C. TRACHOMATIS SEROVAR D/LC, C. TRACHOMATIS SEROVAR L2 AND C. MURIDARUM MOPN IN C3H/HEJ MICE

Abstract

Chlamydia trachomatis is the leading cause of bacterial sexually transmitted infections, preventable blindness, and is a risk factor for the development cervical cancer. Untreated, asymptomatic infection as well as frequent re-infection are common and drive pelvic inflammatory disease, ectopic pregnancy, and infertility. *In vivo* models of infection continue to be instrumental in elucidating the pathogenesis of and immune response to this intracellular bacterium, however significant gaps in our understanding exist. The objective of this study was to produce a side-by-side comparative analysis in a cervicovaginal murine infection model utilizing the *C. trachomatis* serovars D and L2 and the *C. muridarum* strain MoPn by characterizing the (i) time course of infection and *Chlamydia* shed, (ii) early, mid and late immune response to infection, and (iii) gross pathology following clearance of active infection. A key finding herein is the first identification of chlamydial extrusions shed from host cells in an *in vivo* model.

Extrusions, a recently appreciated mode of host-cell exit and potential means of dissemination, had been previously observed solely *in vitro.* The results of this study demonstrate that chlamydial extrusions exist *in vivo* thus warrant further investigation to determine their role in chlamydial pathogenesis.

Keywords

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Chlamydia, cervicovaginal, extrusions

Introduction

There are five recognized species of *Chlamydia*: *C. trachomatis, C. pecorum, C. psittaci, C. pneumoniae* and the most recently reclassified, *C. muridarum*, *C. pecorum* and *C. psittacti* are pathogens of mainly avian and lower animals, however human infections may occur. *C. pneumonaie* is known for inflicting respiratory infections and can lead to the harmful development of arthrosclerosis and cardiovascular disease (Grayston, 2000). *C. trachomatis* is a pathogen of mucosal areas, causing ocular trachoma and urogenital infections. There are 15 serovariants: serovars A-C cause ocular trachoma, serovars D-K and L1-L3 are associated with urogenital sexually transmitted infections. Serovars D-K are less severe than serovars L1-L3 and may cause cervicitis and urethritis or may be asymptomatic. Pathologically distinct LGV infections caused by serovars L1-L3 are more invasive in nature and are characterized in infection through dissemination into distant lymph nodes near the site of infection by mediation of macrophages (Schachter, 1999; Burton and Mabey, 2009; Herweg & Rudel, 2016). Although an infection can be cleared with the use of antibiotics, ongoing complications

may arise after; such as pelvic inflammatory disease (PID), endometriosis, tubal scarring, ectopic pregnancy and even cervical cancer (Smith et al., 2001). Due to the similarities to *C. trachomatis*, *C. muridarum*, a mouse pneumonitis (MoPn) murine strain has used as a model to study *C. trachomatis* human infections (Ramsey et al., 2009).

Chlamydiae are a group of obligate intracellular bacteria that possess a unique biphasic life cycle consisting of two alternating forms: infectious, non-replicative, extracellular elementary bodies (EBs) and non-infectious, metabolically active, replicative reticulate bodies (RBs) (Hackstadt, 1999). Upon binding of the EB to the host cell, the EB is endocytized and contained within a membrane bound vacuole, deemed an inclusion, and avoids lysosomal fusion (Moulder, 1991; Hackstadt et al. 1997). From within the protection of the inclusion, EBs quickly differentiate into metabolically active RBs, which replicate by mode of binary fission. Through a process of genomic DNA condensation and cross-linking of outer membranous proteins, the RBs then reorganize into infectious EBs. Upon the host cell swelling to fit the growing numbers of chlamydia, the *Chlamydia* will exit the cell by one of two mechanisms: lysis or extrusion (Todd & Caldwell, 1985; Hybiske & Stephens, 2007).

Most recently, the mechanism of lytic escape was found to be dependent on a virulence-associated gene transcriptional regulator, Pgp4, encoded on a single plasmid possessed by many *Chlamydia* (Yang et al., 2015; Song et al. 2013). Alternatively, the extrusion mechanism of host cell exit, in which the inclusion exits the cell by budding out and pinching off, encased in host cell membrane, is regulated by interactions between host and chlamydial proteins at the site of the inclusion membrane. The chlamydial inclusion membrane protein, CT228, recruits a component of myosin phosphatase,

MYPT1, to the inclusion during infection to regulate the host cell exit mechanism of extrusion (Lutter et al., 2013). However, current investigations have yet to identify existence of extrusions *in vivo* (Lutter et al., 2013; Hybiske & Stephens, 2007; Herweg & Rudel, 2016; Zuck et al. 2016; Chen et al., 2014). Such findings could heighten the knowledge of chlamydial pathogenesis and provide validity into extrusion dissemination of infection to surrounding tissues.

The use of the murine model to investigate chlamydial infections has been used widely due to close resemblance of acute genital tract infections in human females. After infection, mice typically naturally resolve infections in approximately 4 weeks and develop subsequent immunity and after such, more than half of mice are typically resistant to reinfection (Morrison & Caldwell, 2002). Differences in mouse immune response to infection occur between *C. trachomatis* strains and MoPn strains (Williams et al., 1981). MoPn has been described to be more virulent than *C. trachomatis* in mice, causing systemic infections and acute pathology in the genital tract (Nigg, 1942). Within days of primary infection, neutrophils and monocytes respond to the site of infection (Morrison & Morrison, 2000). After neutrophil recruitment, T cell accumulation can be observed at the infection and maintain a critical role during and after infection clearance (Kelly & Rank, 1997; Darville et al., 1997; Ramsey & Rank, 1991; Morrison et al., 1995). Both CD4+ and CD8+ T cells can be observed during the course of infection, however CD4+ T cells predominate, and in small clusters can be observed in the genital tract mucosa until infection resolution.

To date, much knowledge has been gained in infectivity, pathogenicity, fertility and potential vaccine candidates for MoPn and *C. trachomatis* infections in the murine

model (Hong et al., 2012, Pal et al., 1999; Schautteet et al., 2011). However, with numerous murine models used and due to the lack in consistency in strain usage, there is a wide gap in knowledge. By characterizing and comparing strains of *C. trachomatis* serovar D/LC, serovar L2 and *C. muridarum* MoPn *in vivo*, a platform can be provided of which to form the basis of many investigations to come. Here, we demonstrate the first comparison of the ocular serovar D, the urogenital infecting serovar L2 of *C. trachomatis* and murine specific, MoPn of *C. muridarum in vivo*. This comparison allows for insight into the time course of chlamydial infections and gross pathology following clearance of infection. Furthermore, demonstrated is the complete first account of chlamydial extrusions in an *in vivo* murine model.

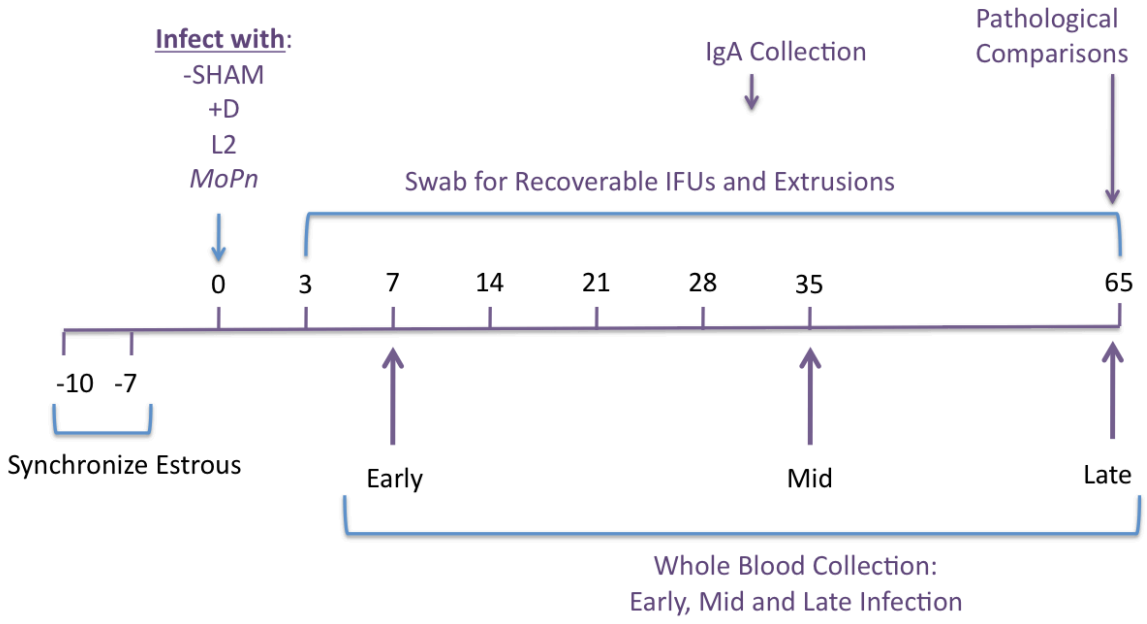


Figure 15. Time course of murine infection.

Six week old C3H-HeJ female mice were ordered from Jackson Laboratories and at 10 and 7 days prior to infection, mice were subcutaneously injected with Depo-provera

to synchronize estrous (Days -10 and -7, respectively). Infection (Day 0) was performed by pipetting infectious *Chlamydia* serovars diluted in 1X SPG into the lower genital tract. Lower genital tracts were swabbed for infectious EBs at days 3, 7, 14, 21, 28, 35 and 59 post-infection. At days 10, 17, 24 and 31 post-infection, lower genital tracts were swabbed for the presence of extrusions. At days 7, 35 and 65 post-infection, sera was obtained by whole blood collection. Between days 28 and 35, lower vaginal tracts were flushed with bovine serum albumin to collect IgA. At day 65, remaining mice were euthanized and whole reproductive tracts were dissected, photographed for gross pathological comparisons, and sent for histopathology.

Results

Lower vaginal infection results in increased recoverable Infectious Forming Units of *C. trachomatis* serovar L2.

Ten and 7 days prior to infection, mice were subcutaneously administered Depo-provera (Pfizer, NY, NY) to synchronize estrous. Mice were then separated into groups and infected in the lower genital tract with $1X10^6$ EBs of a chlamydial strain: *C. trachomatis* serovar D/LC, *C. trachomatis* serovar L2 or *C. muridarum* MoPn. At days 3, 7, 14, 21, 28, 35 and 59 post infection, mice were individually sedated and vaginally swabbed 8 rotations to the right and 8 rotations to the left for recoverable (Figure 15). Swabs were emerged in 1.5 mL sterile tubes with 600 μL of SPG buffer with two sterile glass beads. Samples were vortexed and serially diluted in HBSS and plated onto confluent HeLa 229 cell monolayers. Approximately 36-40 hours post-infection, infected cell monolayers were fixed with methanol and stained with appropriate primary and

secondary antibodies to visualize inclusions. Recoverable IFUs, means and standard deviations were calculated and graphed as a box and whisker plot (Microsoft Office Excel; PRISM 7) (Figure 16).

At the beginning of the time course, the amounts of shedding of live organisms were within the same log scale, suggesting that all mice were infected equally. However, at day 7 post-infection, differences in live organism shedding between groups are evident. At day 14 post infection, group *C. trachomatis* serovar L2 mice exhibit a 3-log fold increase in recoverable live organisms compared to serovar *C. trachomatis* D/LC and *C. muridarum* MoPn. After day 21, all strain recoverable IFUs continue to decline. The presence of recoverable organisms within each group at day 35 post infection, suggested that a subsequent sampling needed to be performed at day 59 to ensure that the immune system had cleared the infection. It was evident that there is a near 2-log fold difference between the maximum produced extrusion levels of *C. trachomatis* serovar L2 compared to the recovery of both *C. trachomatis* serovar D/LC and *C. muridarum* MoPn.

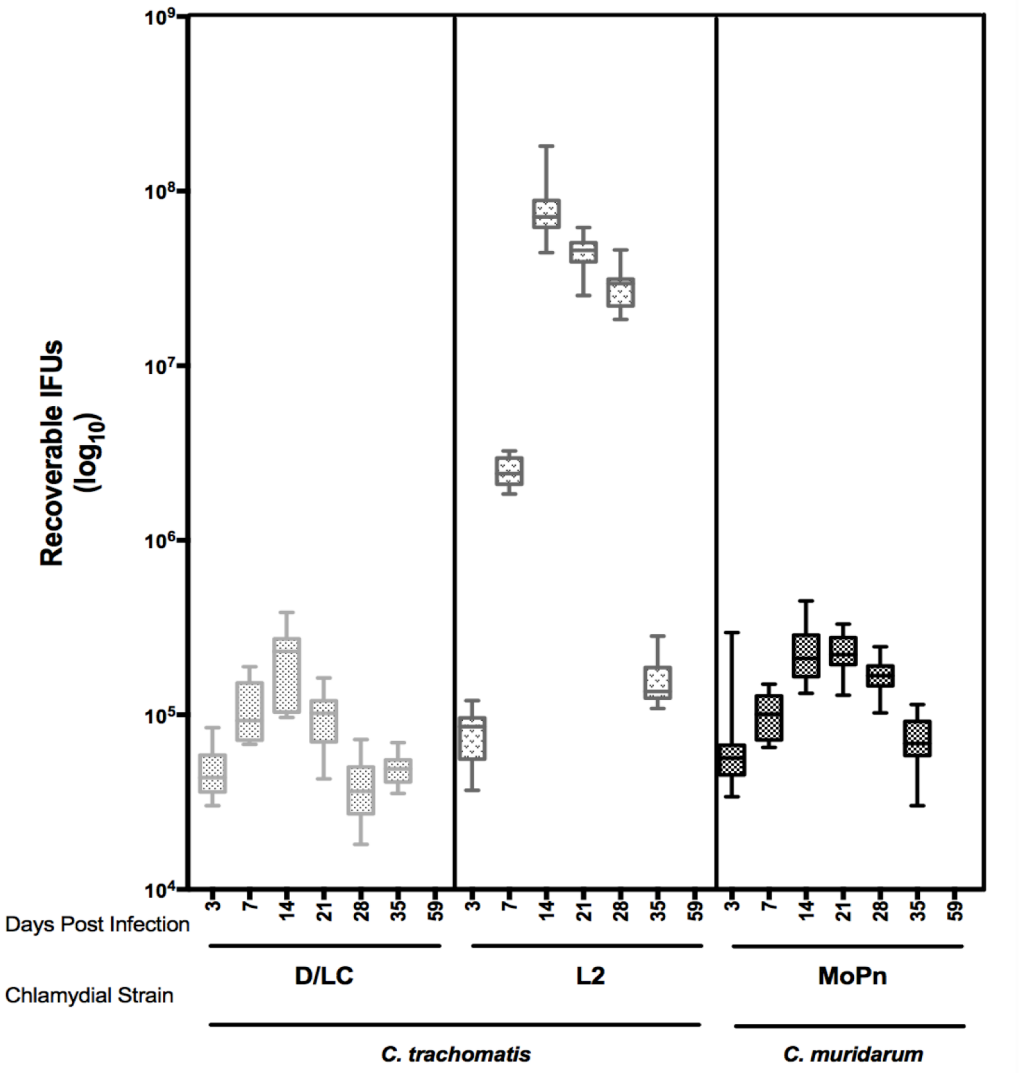


Figure 16. Comparison of recoverable IFUs obtained from lower vaginal swabs in C3H/HeJ mice infected with Chlamydial serovars D, L2 and MoPn.

Mice were Depo-Provera treated and vaginally infected with 1X 10^6 elementary bodies of corresponding serovar. Comparisons of recoverable IFUs were obtained by swabbing vaginal tracts and enumerating on HeLa 229 cell monolayers. Data is separated by chlamydial serovar and presented in logarithmic scale (log_{10}) and separated by day. Group means are displayed as well as standard deviations by error bars.

Swabbing of vaginal tracts during Chlamydial infection reveals recoverable extrusions *in vivo*

It is known that *Chlamydiae* may exit the host cell by one of two mechanisms: lysis or extrusion. Extrusions are defined to be chlamydial-filled, host membrane enveloped bodies devoid of host nuclei. The chlamydial extrusion process is defined in several steps involving, first, the protuberance of the inclusion out of the host cell, second, pinching of inclusion into distinct compartments and lastly, the separation of the pinched inclusion out of the cell to the periphery (Hybiske & Stephens, 2007). However, it was once assumed that extrusions were an artifact of tissue culture, with the majority of published extrusion images being derived *in vitro* or *in situ* (Neeper et al., 1990; Wyrick et al., 1993).

A surprising finding within this study was establishing that extrusions were recoverable *in vivo*, showing that extrusions were not an artifact of tissue culture methods. Mice were individually swabbed at days 21 and 24 post infection. Swabs were placed into RPMI1640 media supplemented with 10% fetal bovine serum and subjected to staining with FM®4-64 plasma membrane dye and NucBlue® Live ReadyProbes® nucleic acid stain. Samples were mixed gently and mounted onto glass slides for Confocal imaging (Olympus). Images were obtained at 63iX magnification with immersion oil. Images were obtained for differential interference contrast (DIC), FM®4-64 (excitation 506 nm, emission 750nm) and NucBlue® Live ReadyProbes® (360 nm, emission 460 nm) (Figure 17). DIC images show that extrusions recovered in vivo were consistent with extrusion derived from tissue culture in terms of shape. While not all extrusions are perfectly spherical, evident through FM®4-64 staining, each extrusion is

in fact enveloped in host cell membrane (Zuck, et al. 2016). Furthermore, as demonstrated by NucBlue® Live ReadyProbes® staining, each extrusion was found to be devoid of host cell nuclei. As a conclusion, extrusions were recovered from live murine lower genital tracts from *C. trachomatis* serovar D/LC, *C. trachomatis* L2 and *C. muridarum* MoPn during the course of infection.

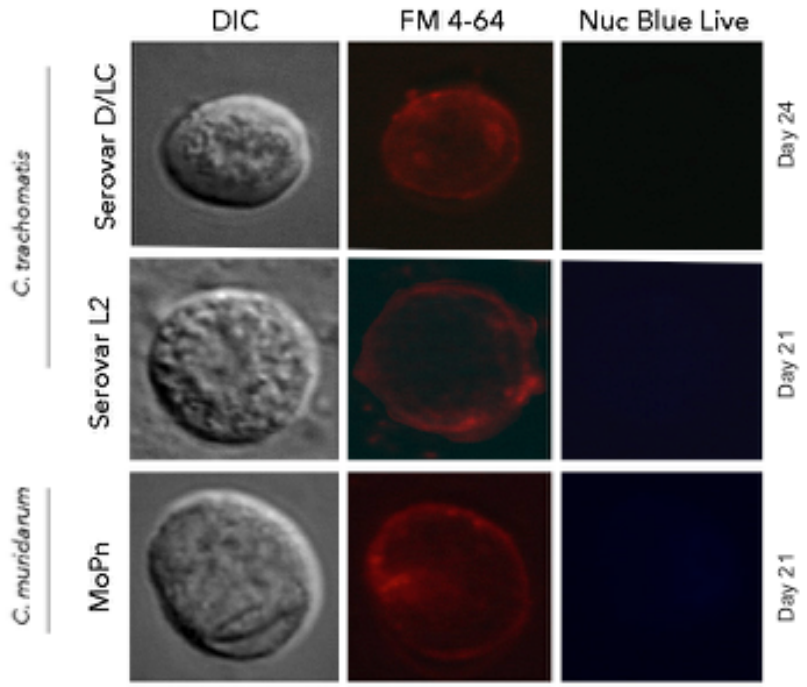


Figure 17. Presence of extrusions *in vivo* after infection with Chlamydial serovars D, L2 and MoPn.

Extrusions were found to be present from all Chlamydial serovars during infection in C3H/HeJ mice. Mice were Depo-provera treated and vaginally infected with $1X10^6$ elementary bodies of corresponding serovar or strain. Samples were obtained by swabbing the vaginal tract and collecting in RPMI 1640 medium supplemented with 10%

FBS. Samples were stained with of NucBlue® Live ReadyProbes® Reagent and FM®4-64 plasma membrane dye and mounted onto glass slides with glass coverslips. Olympus Laser Scanning Confocal microscopy was used to obtain images above. Images were cropped, sized, aligned and adjusted for brightness using Adobe Photoshop.

Observation of gross reproductive tracts reveals distinct differences in anatomical pathology following chlamydial infection

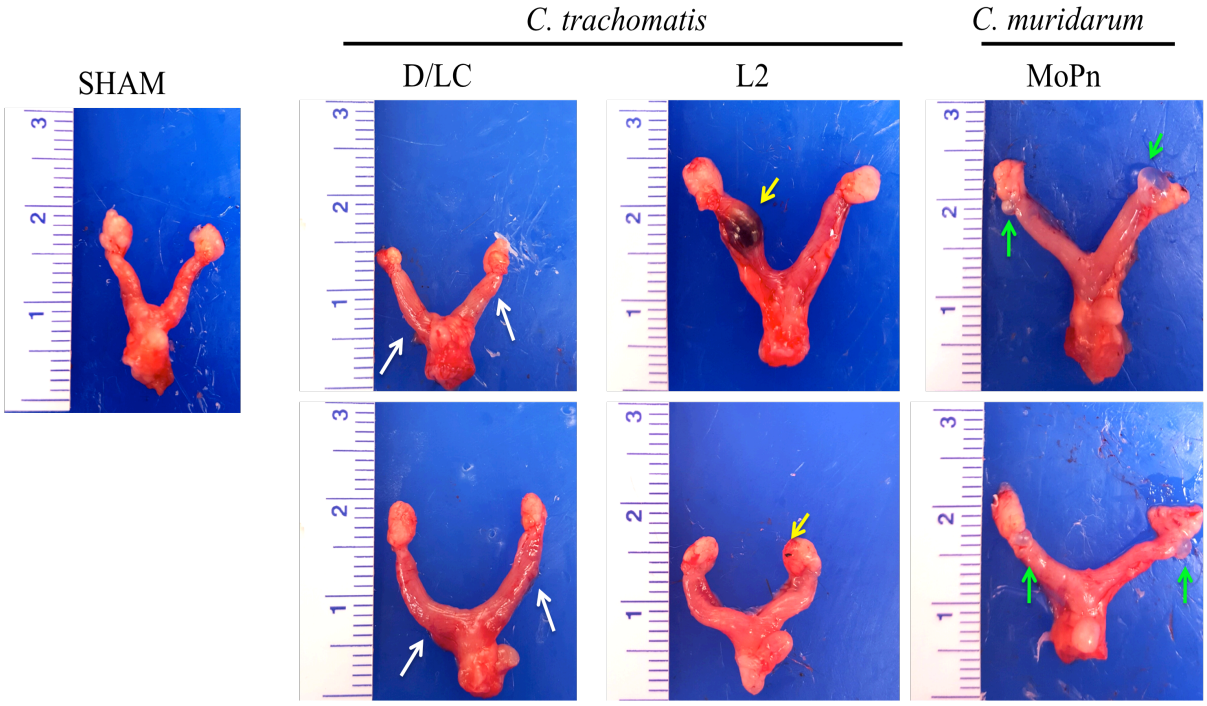
To further assess upper reproductive tracts and to bridge a comparison between the recoverable live organisms to the pathological severity of infection, mice were subjected to gross pathological assessment. It has been described that C3H/HeJ mice are susceptible to the development of hydrosalpinx following lower genital infection with *C. muridarum* (Chen et al., 2014; Lei et al., 2014). The murine pathology very much mimics the tubal pathology observed in humans after a *C. trachomatis* infection (Lei et al., 2014).

Upon imaging, SHAM mice, as a control, were found to possess healthy reproductive tracts: ovaries, uterine horns, and uterus. *C. muridarum* MoPn infected C3H/HeJ mice were found to have distinct observable pathology, bilateral hydrosalpix, consistent with previous findings within the same murine model (Chen et al., 2014). To establish a platform for further chlamydial investigations, it was necessary to perform a comparison of reproductive tracts with human *C. trachomatis* serovars typical of both ocular and genital infections. Comparison of anatomical sequelae after infection with *C. trachomatis* serovar D/LC and L2 revealed notable differences. Upon observation of reproductive tracts from D/LC mice was uterine horn inflammation, relatable to PID. In contrast, L2 mice had observable uterine horn occlusion and the presence of dark tissue

areas on the ovary. Comparison of mice reproductive tracts after infection demonstrates the differences in anatomical pathology following a lower genital tract infection. Due to the differences observed in whole reproductive tracts, histopathological assessment was required to validate further establish a distinct pathology associated with infection of independent chlamydial strains.

Figure 18. Anatomical assessment of murine reproductive tracts.

At days 63 and 64 post infection, mice were euthanized and reproductive tracts were imaged and sent for histopathological assessment. Individual images captured above demonstrate anatomical sequelea due to infection. (**SHAM)** Sample depicts a



healthy reproductive tract. (**D/LC)** Top and bottom images depict observable right and left oviduct inflammation (white arrows). (**L2**) Top image depicts a black occlusion in the top portion of the left uterine horn (yellow arrow top). Bottom image depicts the presence

of dark tissue on ovary (bottom yellow arrow). (**MoPn**) Top and bottom images depict observable hydrosalpinx near both right and left oviducts (green arrows top and bottom).

Discussion

The murine model has been used widely in past to investigate chlamydial bacteriology and pathology of the host after infection. However, with numerous murine strains available for use, and numerous pathogenic chlamydial strains, a platform establishing the clear differences in pathology, recoverable live organisms and immunological response was lacking. This investigation proves to be sufficient for establishing such a platform for which to base future investigations. The data from the present study taken together with previous findings suggest that the pathology of host reproductive tissues and recoverable shed live organisms do not necessarily correlate to the severity of infection. Evident from the assessment of live recoverable organisms is that *C. trachomatis* serovar L2, yielded nearly 3-log fold more recoverable infectious forming progeny compared to *C. trachomatis* serovar D/LC and *C. muridarum* MoPn (Figure 15). Unique of this investigation, was the use of *C. trachomatis* serovar D/LC for comparisons. The usage of this strain was relevant in that in human hosts, the infection may present for long periods of time until subsequent treatment. In past literature, Sturdevant et al. identified that when using C3H/HeJ mice were used as a model for *C. trachomatis* serovar D infection, there are two clearance phenotypes observed: early and late (Sturdevant et al., 2010). For this study, the serovar D late clearing phenotype was used to infect mice, to produce an overall, more virulent infection. Consistent with findings is that serovar D/LC infection produces a long lasting infection, clearing after day 35 post-infection. Furthermore, all mice were observed to clear infections by day

59 post-infection. This result was consistent with findings from research performed in both *in vivo* and *in situ* approaches (Lyons et al. 2005; Ito et al., 1990).

When comparing the results of recoverable IFU studies to the anatomical sequelae pathology, it was evident that despite a lower level of shed live organisms, *C. muridarum* MoPn infections had a long lasting effect on reproductive tracts, causing the development of hydrosalpinx in all mice, consistent with previous findings (Chen et al., 2014). However, in comparison to *C. trachomatis* serovar D/LC infected mice, a difference was noted in terms of gross observation of reproductive tracts. Serovar D/LC infected mice had less distinct differences in reproductive tracts, but rather the consistent presence of inflammation within each individual mouse, suggestive of the development of PID. Unique of *C. trachomatis* serovar L2 infected mice was the presence of a dark colored, uterine horn occlusion as well as dark growths on the ovary (Figure 18). As a conclusion of anatomical sequelae after the infection had resolved, notable differences, unique to serovar of infection were noted.

Human infection of *C. trachomatis* is linked to the development of serious sequelae amongst women, such as pelvic inflammatory disease (PID). (Brunham et al., 1988). PID is associated with inflammation of the uterus, fallopian tubes and ovaries in the human and is thought to be a development following a viral or bacterial infection (Paavonen et al., 2008). Gynecological consequences of PID in the human often result in ectopic pregnancy, infertility, and damage to the fallopian tubes (Chow et al., 1990; Ness et al., 2008; Robertson et al., 1987; Stamm 2008; Westrom et al., 1992). These consequences are a result from damage to the cilia lining of the fallopian tube or due to

occlusions or adhesion formation (Safrin et al., 1993; Stacey et al., 1992; Buchan et al., 1993).

Upon infection of the host cell by Chlamydia, there resides a process of which irreparable damage is produced in host tissue. Specifically, it is the non-immune host epithelial cells, which produce the most inflammatory response, driving the inflammatory response in which the result is the release of tissue damaging molecules (Rasmussen, et al. 1997). The production of matrix metalloproteases (MMPs) by the host cell and the release of cytokines and chemokines from adjacent macrophages, it is observable that proteolysis and remodeling of infected and adjacent tissue occurs. Continued release of chemokines from *Chlamydia*-infected cells drives adaptive T cells and B cells to be recruited, following the initial recruitment of neutrophils and monocytes (Morrison & Morrison, 2000; Rank & Barron, 1983). Specifically, the Interleukin 1 (IL-1) release has been shown to have a novel role in the pathogenesis of reproductive tract pathology following and infection (Hvid, et al. 2007). Furthermore, the production of tumor necrosis factor (TNF) leads to increased expression of adhesion molecules, which then leads to activation of the innate, then, adaptive immune system to target the Chlamydial infection (Ault et al., 1996; Kelly et al., 2001). It is well characterized that after these immune responses, damage to the genital tract is observed in the form of scarring. However, the scarring is observed to be in the area of infection, despite the resolve of inflammatory processes (Darville & Hiltke, 2010). As taken as a result collectively from gross pathological assessment of murine reproductive tracts following a long term infection, it was presumable that upper genital tract pathology was present in the form of inflammation. This agrees with past findings of reproductive pathology in C3H mice

following a Chlamydial infection , as it is more often observed to be more severe than other mouse strains (Darville et al. 1997; de la Maza et al., 1994).

Due to the lack of a comparative assessment of *C. trachomatis* infections within the same murine strain, both ocular and genital strains, D/LC and L2, respectively were introduced into the C3H/HeJ murine model to allow for direct comparison. This allowed for the evaluation of the development of PID within the murine model when infecting with *C. trachomatis* serovars D/LC and L2. A goal of this study was to assess gross pathological differences amongst mice groups following infection (gross pathology images provided in Figure 17).

An aim of this study was to identify extrusions derived from a live *in vivo* murine model. Consistent with the definition of an extrusion, being chlamydial-filled, host membrane enveloped bodies devoid of host nuclei, images were obtained from mice infected with each chlamydial strain demonstrating the existence of extrusions retrieved from live C3H/HeJ mice. The chlamydial extrusion process is separated in a series of steps: (i) protuberance of the inclusion out of the host cell, (ii) pinching of inclusion into distinct compartments (iii) separation of the pinched inclusion out of the cell to the periphery (Hybiske & Stephens, 2007). With this finding, we demonstrate that previously accepted knowledge of extrusions being derived by *in vitro* and *in situ* methods solely is no longer the case, thus leading to new questions of chlamydial pathogenesis (Neeper et al., 1990; Wyrick et al., 1993). As a conclusion on the existence of extrusions retrieved from live C3H/HeJ mice infected with *C. trachomatis* and *C. muridarum*, it was found that extrusions are in fact being shed from the vaginal tract during infection with all three strains, which was provided by Confocal microscopy analysis. Following infection with

each strain of *Chlamydia*, extrusions were found to be encased in host cell plasma membrane, as provided by staining with FM4-64 that is specific for plasma membrane. Furthermore, NucBlue Live DNA stain provided that these extrusions were devoid of host cell nuclei, currently fitting the description of extrusions as being host cell membrane encased, *Chlamydia*-filled spherical sacs, which are devoid of host cell nuclei. This was essential in differentiating that it was not a possibility that the inclusion was expelled from host cells lysed by the swabbing process. However, enumeration was not a possible result of this experiment. There lies a possibility that the enumeration of extrusions was not a possibility due to the physiological nature of the vaginal tract from which samples were taken. There lie a few possibilities of explaining this enumeration difficulty. First, it is possible that the vaginal acidity could be responsible for decreased extrusion events being retrieved from the vaginal tract. The pH of the vagina is known to be moderately acidic, fluctuating along with hormonal changes throughout the estrous cycle (Kelly, 1990). Also affected by hormonal cycles in the vagina is mucosal secretion (Sharif & Olufowobi, 2006). It is possible that extrusions were retained within the cervical mucosal secretions during the infection timeline. Observed in this study is that the fluidity of cervical secretions varied amongst strains used for infection. There lies a real possibility that the cervical mucous retained the extrusions. In conclusion, due to possibility that the careful experimental design of retrieving the extrusions without agitation or centrifugation, extrusions could either be lost during or prior to sampling due to pH acidity in the vagina or, quite possibly extrusions could be retained in mucosal secretions in the sample following processing and might not located in the media of which was used for wet mounting methods.

Considering that the host cell exit method of extrusion is indeed occurring *in vivo*, future research should be focused on further characterizing chlamydial-host interactions involving chlamydial protein, CT228 as well as identifying the advantage to which *Chlamydiae* obtain by utilizing this mechanism of host cell exit. It was been suggested by *in vitro* methodological approaches that *Chlamydiae* utilize the host cell exit mechanism of extrusion to subsequently promote engulfment by macrophages (Zuck et al., 2016; Herweg & Rudel, 2016). Aside from the novel identification of recoverable extrusions derived after a chlamydial infection *in vivo*, in this present study, we have demonstrated the comparable levels of recoverable IFUs derived from C3H/HeJ vaginal tracts infected with chlamydial strains *C. trachomatis* serovar D/LC, *C. trachomatis* serovar L2 and *C. muridarum* MoPn. Furthermore, gross anatomical reproductive tract assessment and histopathological analysis at days 64 and 65 post-infection revealed distinct characteristics specific to chlamydial strain infection. Thus, we demonstrate that a platform for future investigations is established; C3H/HeJ mice are ideal for chlamydial infections of the murine model. This work provides a basis for further investigations into chlamydial-host pathogenesis.

SUMMARY OF CHLAMYDIAL-HOST INTERACTIONS

Chlamydia trachomatis is the bacterial causative agent of the most commonly reported urogenital sexually transmitted infection (STI) worldwide (Newman et al., 2015). While main public health concerns are focused on *C. trachomatis* urogenital infection, *C. trachomatis* is also known to infect other mucosal sites such as the eye, causing infectious blindness or more commonly known as, trachoma. Chlamydial STIs are often presented asymptomatically, however females have a risk for the development

of tubal infertility and scarring and PID as a sequelae after infection and male counterparts exhibit the risk for development of epididymitis and urethritis (Brunham et al., 1984; Mishori et al., 2012). Recently, it has been suggested that antimicrobial therapies continued for up to 6 months following infection hold the possibility treating some of these sequelae (Carter et al., 2010). However, the best plan of prevention against the transmission of chlamydial infections is by practicing abstinence.

Chlamydiae are obligate intracellular biphasic pathogens which reside and replicate within a parasitophoros vacuole, deemed an inclusion (Moulder, 1991). Upon endocytosis into the host cell, the infectious elementary body (EB) will congregate to form a single inclusion where the EBs will rapidly differentiate into metabolically active, non-infectious reticulate bodies (RBs) (Hybiske & Stephens, 2007). From within the inclusion, *Chlamydiae* must highjack and redirect cellular traffic for growth and development. The bacterium is capable of doing such through the T3SSin which *Chlamydia* synthesized proteins are secreted to interact with host cell proteins at either the site of the inclusion membrane, or alternatively, will be secreted into the host cell cytosol (Dean, 2011; Kleba & Stephens, 2008).

As a result of growth and replication, the large numbers of *Chlamydia* will cause the host cell to swell, making escape necessary for a productive infection to result. *Chlamydia* may exit the cell by one of two mechanisms: lysis or extrusion. The lytic host cell escape mechanism is reliant upon a systematic procedure of permeabilization events regulated by the Chlamydial transcriptional regulator, Pgp4 (Hybiske & Stephens, 2007; Yang et al., 2015). Whereas, the chlamydial host cell exit mechanism of extrusion is regulated by a highly dynamic, enzymatic pathway involving interaction of chlamydial

inclusion membrane protein CT228 with a host cell protein and component of myosin phosphatase, MYPT1 (Lutter et al., 2013).

The chlamydial host cell exit mechanism of extrusion was once thought to be an artifact of tissue culture, as no *in vivo* investigations resulted in the recovery of viable extrusions. During investigations of the *in vivo* comparison of chlamydial infections, it was a question of if extrusions were being produced. Surprisingly, extrusions were detected from all strains used for the study, roughly about 3 weeks post-infection. Extrusions were identified as being host cell membrane encased, *Chlamydia*-filled inclusions, devoid of host cell nuclei, separate from the host cell. As a conclusion, for the first time to date, it is observed that extrusions are recoverable from a live animal model following urogenital *Chlamydia* infection. This adds significant knowledge to the Chlamydial field, as investigations can now focus more on host-pathogen interactions involving host cell exit and pathogenesis.

In the near future, for the first time *in vivo*, a Chlamydial TargeTron™ CT228 mutant, Δ*CT228* will be used for live murine infection studies. It will be interesting to observe if extrusion levels are heightened when sampling from the infected live animal model as predicted, based on *in vitro* preliminary date presented in this study. Furthermore, consistent with past findings, when using the murine infection model, there is a distinct pathological sequelae: tubal occlusion, PID, inflammation and hydrosalpinx development. Identification of pathological sequelae following infection with the first Chlamydial mutant should yield strong insight into differences between wild-type and mutant, as this has not be yet performed. The merging of the two projects at hand, into one complete *in vivo* analysis of the CT228 mutant will yield great insight into

Chlamydial pathogenesis as there are current limitations of *in vitro* and *in situ* based approaches.

Benefits of the TargeTron™ system adaptation include higher insertion efficiencies when compared to homologous recombination methods, minimal host system requirements and a relatively short amount of time to produce a mutant strain (Perutka et al., 2003; Chen et al., 2005). However, when utilizing the TargeTron™ Chlamydial adapted system, it was a possibility that polar effects would result as the incorporation of the Group II intron could affect expression of downstream genes, as *CT228* is second in line of the *CT229-CT224* operon. Due to the fact that promoter studies have not been performed within the operon, there was a chance that polar effects resided after genetic mutation. By performing RT-PCR we ruled out that polar effects were observed from the operon when using the *CT228* TargeTron™ mutant.

Newer genetic tools are already being developed within the Chlamydial field as a result of the original adaptation, such as by gene deletion by fluorescence reported allelic exchange mutagenesis (FRAEM), which would allow for gene deletion rather than mutation. This adaptation allows for further investigational studies without compromising bacterial growth, essential when utilizing intracellular bacteria (Mueller et al., 2016). The Chlamydial field has a long road ahead in terms of genetic methodologies; but the future appears promising with such newly developed methods.

REFERENCES

1. Abby SS, Roch EPC. (2012) The non-flagellar type III secretion system evolved from the bacterial flagellum and diversified into host-cell adapted systems. PLoS Genet. 8:e1002983. http://dx.doi.org/10.1371/journal.pgen.1002983.
2. Arnold R, Brandmaier S, Kleine F, Tischler P, Heinz E, Behrens S, Niinikoski A, Mewes HW, Horn M, Rattei T. (2009) Sequence-based prediction of type III secreted proteins. PLoS Pathog. 5:e10000376. http://dx.doi.org/10.1371.journal.ppat.1000376.
3. Ault KA, Kelly KA, Ruther PE. *Chlamydia trachomatis* enhances the expression of matrix metalloproteinase's in an in vitro model of the human fallopian tube infection. Am J Obstet Gynecol 2002;187(5):1377-83.
4. Baehr W, Zhang YX, Joseph T, Su H, Nano FE, Everett KD & Caldwell HD. (1988) Mapping antigenic domains expressed by *Chlamydia trachomatis* major outer membrane protein genes. Proc Natl Acad Sci U S A 85(11):4000-4.
5. Balin BJ, Gerard HC, Arking EJ, Appelt DM, Branigan PF, Abrams JT Whittum-

6. Hudson JA and Hudson AP. (1998) Identification and localization of *Chlamydia pneumoniae* in the Alzheimer's brain. Med. Microbiol. Immunol. (Berl.) 187, 23-42.
7. Bannantine JP1, Griffiths RS, Viratyosin W, Brown WJ, Rockey DD. (2000) A secondary structure motif predictive of protein localization to the chlamydial inclusion membrane. Cell Microbiol. 2(1):35-47.
8. Bannantine J, Rockey D, Hackstadt T. (1998) Tandem genes of *Chlamydia psittaci* that encode proteins localized to the inclusion membrane. Mol. Microbio. 28:1017-1026. http://dx.doi.org/10.1046/j.1365-2958.1998.00867.x.
9. Barron AL, White HJ, Rank RG, Soloff BL, Moses EB. (1981) A new animal model for the study of Chlamydia trachomatis genital infections: infection of mice with the agent of mouse pneumonitis. J Infect Dis. 143(1):63-6.
10. Barry III CE Hayes SF and Hackstadt, T. (1992) Nucleoid condensation in Escherichia coli that expresses a Chlamydial histone homolog. Science 256, 377-379.
11. Bashour M, Robert CM, Dagher HM. (2013) Chlamydial Keratitis and Conjunctivitis. Copeland Robert A Jr, Afshari Natalie A. Copeland and Afshari's Principles and Practice of Cornea (Volume 1 and Volume 2). Jaypee; 1: 346-364/27.
12. Bauler LD and Hackstadt T. (2014) Expression and targeting of secreted proteins from *Chlamydia trachomatis*. J Bacteriol 196(7)1325-34.
13. Belland R, Zhong G, Crane D, Hogan D, Sturdevant D, Sharma J, Beatty WL, Caldwell HD. (2003) Genomic transcriptional profiling of the developmental

cycle of *Chlamydia trachomatis*. Proc. Natl. Acad. Sci. U. S. A. 100:8478-8483. http://dx.doi.org/10.1073/pnas.1331135100.

14. Bernstein-Hanley I, Balsara ZR, Ulmer W, Coers J, Starnbach MN, Dietrich WF (2006) Genetic analysis of susceptibility to *Chlamydia trachomatis* in mouse. Genes Immun. 7(2):122-9.
15. Betts-Hampikian HJ, Fields KA. (2011) Disulfide bonding within components of the *Chlamydia* type III secretion apparatus correlates with development. J. Bacteriol. 193:6950-6959. http://dx.doi. Org/10.1128/JB.05163-11.
16. Betts HJ, Twiggs LE, Sal MS, Wyrick PB, Fields KA. (2008) Bioinformatic and biochemical evidence for the identification of the type III secretion system needle protein of *Chlamydia trachomatis*. J. Bacteriol. 190: 1680-1690.
17. Birkelund S, Johnsen H, Christiansen G. (1994) *Chlamydia trachomatis* serovar L2 induces protein tyrosine phosphorylation during uptake by HeLa cells. Infect Immun. 62(11): 4900–4908. PMCID: PMC303205]
18. Birkelund S, Bni L, Pallini V, Sanchez-Campillo M, Liberatori S, Ostergaard S, Holm A & Christiansen G. (1997) Electrophoresis 18, 563-567.
19. Brickman TJ, Barry ICE and Hackstadt T. (1993) Molecular cloning and expression of *hctB* encoding a strain-variant Chlamydial histone-like protein with DNA-binding activity. J. Bacteriol. 175, 4272-4281.
20. Brunham RC, Binns B, Guijon F, et al. (1988) Etiology and outcome of acute pelvic inflammatory disease. J Infect Dis 158((3)):510-517.
21. Brunham RC, Rey-Ladino J. (2005) Immunology of Chlamydia infection: implications for a *Chlamydia trachomatis* vaccine. Nat Rev Immunol.5(2):149-

61.

22. Buchan H, Vessey M, Goldacre M, Fairweather J. (1993) Morbidity following pelvic inflammatory disease. Br J Obstet Gynaecol 100((6)):558-562.
23. Burton MJ and Mabey DC (2009) The global burden of trachoma: a review. PLoS Negl Trop Dis. 3(10):e460.
24. Büttner D. (2012) Protein export according to schedule: architecture, assembly, and regulation of type III secretion systems from plant- and animal-pathogenic bacteria. Microbiol. Mol. Biol. Rev. 76:262-310. http://dx.doi.org/10.1128/MMBR.05017-11.
25. Carabeo RA, Grieshaber SS, Fischer E, Hackstadt T. (2002) *Chlamydia* trachomatis induces remodeling of the actin cytoskeleton during attachment and entry into HeLa cells. Infect Immun. 70(7):3793-803.
26. Carabeo RA, Grieshaber SS, Hasenkrug A, Dooley C & Hackstadt T. (2004) Requirement for the Rac GTPase in *Chlamydia trachomatis* invasion of non-phagocytic cells. Traffic 5, 418-425.
27. Carter JD, Espinoza LR, Inman RD, et al. (2010) Combination antibiotics as a treatment for chronic chlamydia-induced reactive arthritis: a double-blind, placebo-controlled, prospective trial. Arthritis Rheum. 62(5)1298-1307.
28. Carter JD, Hudson AP. (2010) The evolving story of chlamydia-induced reactive arthritis. Curr Opin Rheumatol. 22(4):424-430.
29. Chen J, Hongbo Z, Zhou Z, Zhangsheng Y, Ding Y, Zhou Z, Zhong E, Arulanandam B, Baseman J, and Zhong G. (2014) Chlamydial Induction of Hydrosalpinx in 11 Strains of Mice Reveals Multiple Host Mechanisms from

Preventing Upper Genital Tract Pathology. PLoS One 9(4): e95076.

30. Chen, Y, McClane, BA, Fisher, DJ, Rood JI and Gupta P. (2005) Construction of an Alpha Toxin Gene Knockout Mutant of *Clostridium perfringens* Type A by Use of a Mobile Group II Intron. Appl Environ Microbiol 71(11):7542-7547.
31. Cheng C, Nair AD, Indukuri VV, Gong S, Felsheim RF, et al. (2013) Targeted and random mutagenesis of *Ehrlichia chaffeensis* for the identification of genes required for in vivo infection. PLoS Pathog 9: e1003171.
32. Cheng W, Shivshanker P, Li Z, Chen L, Yeh IT, Zhong G. (2008) Caspase-1 contributes to *Chlamydia trachomatis*-induced upper urogenital tract inflammatory pathologies without affecting the course of infection. PLoS One 76(2): 515-522.
33. Chow JM, Yonekura ML, Richwald GA, Greenland S, Sweet RL, Schachter. (1990) The association between *Chlamydia trachomatis* and ectopic pregnancy. JAMA. 263(23):3164-3167.
34. Clifton, DR Fields, KA, Grieshaber, SS Dooley, CA, Fischer, ER, Mead DJ, Carabeo, RA and Hackstadt T. (2004) From the Cover: A Chlamydial type III translocated protein is tyrosine-phosphorylated at the site of entry and associated with recruitment of actin. Proc. Natl. Acad. Sci. USA 101, 10166-10171.
35. Darville T, Andrews CW, Laffoon KK, Shymasani W, Kishen LR, Rank RG (1997) Mouse strain-dependent variation in the course and outcome of chlamydial genital tract infection is associated with differences in host response. Infect Immun 65(8): 3065-3073.
36. Darville T and Hiltke T (2010) Pathogenesis of Genital Tract Disease Due to

Chlamydia trachomatis. J Infect Dis 201(Supplement 2):S114-S125.

37. De Clercq E, Kalmar I and Vanrompay D. (2013) Animal Models for Studying Female Genital Tract Infection with *Chlamydia trachomatis.* Infect Immun 81(9): 3060-3067.
38. de la Maza LM, Pal S, Khamesipour A, Peterson EM. (1994) Intravaginal inoculation of mice with the C*hlamydia trachomatis* mouse pneumonitis biovar results in infertility Infect Immun. 62(5):2094-7.
39. Dean P. (2011) Functional domains and motifs of bacterial type III effector proteins and their roles in infection. FEMS Microbiology Reviews 35: 1100-1125.
40. Dehoux P, Flores R, Dauga C, Zhong G, Subtil A. (2011) Multi-genome indentification and characterization of chlamydiae-specific type III secretion substrates: the Inc proteins. BMC Genomics 12:109. http://dx.doi.org/10.1186/1471-2164-12-109.
41. Delevoye C, Nilges M, Dehoux P, Paumet F, Perrinet S, Dautry-Varsat A, Subtil A. (2008) SNARE protein mimicry by an intracellular bacterium. PLoS Pathog. 4:e1000022. http://dx.doi.org/10.1371/journal.ppat.1000022.
42. Demars R, Weinfurter J, Guex E, Lin J, Potucek Y. (2007) Lateral gene transfer in vitro in the intracellular pathogen *Chlamydia trachomatis*. J Bacteriol. 189(3):991-1003.
43. Derré I, Swiss R, Agaissse H. (2011) The lipid transfer protein CERT interacts with the *Chlamydia* inclusion protein IncD and participates to ER-*Chlamydia* inclusion membrane contact sites. PLoS Pathog. 7:e1002092.
44. Eb F, Orfila J and Lefebvre JF. (1976) Ultrastructural study of the development of

the agent of ewe's abortion. J. Ultrastruct. Res. 56, 177-185.

45. Escalante-Ochoa C, Ducatelle, R and Haesebrouck, F. (2000) Optimal development of *Chlamydophila psittaci* in L929 fibroblast and BGM epithelial cells requires the participation of microfilaments and microtubule-motor proteins. Microbial Pathogenesis 28, 321 – 333.
46. Everett KDE. (2000) *Chlamydia* and *Chlamydiales*: more than meets the eye. Veterinary Microbiology 75(2):109-126.
47. Everett, KD, Bush RM and Andersen, AA. (1999) Emended description of the order *Chlamydiales*, proposal of *Parachlamyd*iaceae fam. nov. and *Simkaniaceae* fam. nov., each containing one monotypic genus, revised taxonomy of the family *Chlamydiaceae,* including a new genus and five new species, and standards for the identification of organisms. Int J Syst Bacteriol 49 Pt 2: 415-440.
48. Farris CM, Morrison RP. (2011) Vaccination against Chlamydia genital infection utilizing the murine *C. muridarum* model. Infect Immun. 79(3):986-96
49. Fattori J, Prando A, Martins AM, Rodrigues FH, Tasic L. (2011) Bacterial secretion chaperones. Protein Pept. Lett. 18:158-166. http://dx.doi.org/10.2174/092986611794475048
50. Fawaz F, van Ooij, C, Mutka S & Engle J. (1997) Infect Immun 65, 5301-5308.
51. Finlay BB & Cossart P. (1997) Exploitation of mammalian host cell functions by bacterial pathogens. Science 276, 718 – 725.
52. Gaede W, Reckling C, Dresenkamp B, Kenklies S, Schubert E, Noack U, Irmscher M, Ludwig C, Hotzel H, Sachse K. (2008) *Chlamydophila psittaci*

Infections in Humans during an Outbreak of Psittacosis from Poultry in Germany. Zoonoses Public Health 55(4):184-188.

53. Godek DC, Olive AJ, Stary G, Starnbach MN. (2012) CD4+ T cells are necessary and sufficient to confer protection against *Chlamydia trachomatis* infection in the murine upper genital tract J Immunol. 189(5):2441-9.
54. Gonzles GF, Munoz G, Sanchez R, Henkel R, Gallegos-Avila G, Diaz-Gutierrez O, et al. (2004) Update on the impact of *Chlamydia trachomatis* infection on male fertility. Andrologia 36:1-23. Doi:10.1046/j.0303-4569.2003.00593.x
55. Grayston JT, Aldous MB, Easton A, Wang S, Kuo C, Campbell LA, and Altman J. (1993) Evidence that *Chlamydia pneumoniae* Causes Pneumonia and Bronchitis. J Infect Dis. 168 (5): 1231-1235 doi:10.1093/infdis/168.5.1231
56. Grayston, J. T. (2000) Background and current knowledge of *Chlamydia pneumoniae* and atherosclerosis. J. Infect. Dis. 181: S402-S410.
57. Hackstadt, T. (1999) *Chlamydia*: intracellular biology, pathogenesis, and immunity. Cell biology: 101-138.
58. Hackstadt T, Fischer ER, Scidmore MA, Rockey DD & Heizen RA. (1997) Origins and functions of the chlamydial inclusion. Trends Microbiol. 5(7):288-93.
59. Hackstadt T, Scidmore MA, Rockey DD (1995) Lipid metabolism in *Chlamydia trachomatis*-infected cells: directed trafficking of Golgi-derived sphingolipids to the chlamydial inclusion. Proc Natl Acad Sci U S A 92:4877-4881. Doi:10.1073/pnas.92.11.4877

60. Hackstadt T, Scidmore-Carlson MA, Shaw EI, Fisher ER (1999) The *Chlamydia trachomatis* IncA protein is required for homotypic vesicle fusion. Cell Microbiol 1:199-130. Doi:10.1046/j.1462-5822.1999.00012.x

61. Hafner L, Beagley K, Timms P. (2008) *Chlamydia trachomatis* infection: host immune responses and potential vaccines. Mucosal Immunol. 1(2):116-30.

62. Hahn, D.L., Dodge, R.W. and Golubjatnikov, R. (1991) Association of *Chlamydia pneumoniae* (strain TWAR) infection with wheezing, asthmatic bronchitis, and adult-onset asthma. Jama 266, 225-230.

63. Hayes, LJ, Pickett MA, Conlan JW, Ferris S, Everson JS, Ward ME & Clarke IN (1990). The major outer-membrane proteins of *Chlamydia trachomatis* serovars A and B: intra-serovar amino acid changes do not alter specificities of serovar- and C subspecies-reactive antibody-binding domains. J Gen Microbiol. 136:1559-66.

64. Heinzen RA, and Hackstadt T. (1997) The *Chlamydia trachomatis* parasitophorous vacuolar membrane is not passively permeable to low-molecular-weight compounds. Infect. Immun. 65:1088-1094

65. Herweg JA, Rudel T. (2016) Interaction of Chlamydiae with human macrophages. FEBS J. 283(4):608-18.

66. Hodinka RL, Davis CH, Choong J & Wyrick P. (1988) Ultrastructural study of endocytosis of *C. trachomatis* by McCoy cells. Infection and Immunity 56, 1456 – 1463.

67. Hogan RJ, Mathews SA, Mukhopadhyay S, Summersgill JT. and Timms P. (2004) Chlamydial persistence: beyond the biphasic paradigm. Infect. Immun. 72,

1843-1855.

68. Hong Y, Karunakara KP, Jiang X, Shen C, Anderson P, Brunham RC (2012) *Chlamydia muridarum* T cell Antigens and Adjuvants That Induce Protective Immunity in Mice. Infect Immun 80(4): 510-1518.
69. Hou S, Lei L, Yang Z, Qi M, Liu Q, Zhong G. (2013) *Chlamydia trachomatis* outer membrane complex protein B is processed by the protease CPAF. J Bacteriol. 195(5):951-957.
70. Hvid M, Baczynska A, Delerun B, et al. Interleukin-1 is the initiator of fallopian tube descruction during *Chlamydia trachomatis* infection. Cell Microbiol 2007:9:2795-03.
71. Hybiske K and Stephens RS (2007) Mechanisms of host cell exit by the intracellular bacterium *Chlamydia*. PNAS. 104 (11430-11435).
72. Igietseme JU, Ramsey KH, Magee DM, Williams DM, Kincy TJ, Rank RG. (1993) Resolution of murine chlamydial genital infection by the adoptive transfer of a biovar-specific, Th1 lymphocyte clone.Reg Immunol. 5(6):317-24.
73. Ito JI Jr, Lyons JM, Airo-Brown LP. (1990) Variation in virulence among oculogenital serovars of Chlamydia trachomatis in experimental genital tract infection. Infect Immun. 58(6):2021-3.
74. Jeffery BM, Suchland RJ, Quinn KL, Davidson JR, Stamm WE, Rockey DD. (2010) Genome sequencing of recent clinical *Chlamydia trachomatis* strains identifies loci associated with tissue tropism and regions of apparent recombination. Infect. Immun. 78:2544-2553. http://dx.doi.org10.1128/IAI.01324-09

75. Johnson CM and Fisher DJ (2013) Site-Specific, Insertional Inactivation of IncA in *Chlamydia trachomatis* Using a Group II Intron. PLoS ONE 8(12): e83989. Doi:10.1371/journal.pone.0083989 .

76. Kari L, Goheen MM, Randall LB, Taylor LD, Carlson JH, Whitmire WM, Virok D, Rajaram K, Endresz V, McClarty G, Nelson DE, Caldwell HD. (2011) Generation of targeted *Chlamydia trachomatis* null mutants. Proc Natl Acad Sci U S A. 108(17):7189-93.

77. Kazdan JJ, Schachter J, Okumoto M. (1967) Inclusion conjunctivitis in the guinea pig. American Journal of Ophthalmology 64: 116–124.

78. Kleba, B. and Stephens RS. (2008) Chlamydial effector proteins localized to the host cell cytoplasmic compartment. Infect. Immun. 76(11):4842-4850.

79. Kelly KA, Rank R. (1997) Identification of homing receptors that mediate the recruitment of CD4 T cells to the genital tract following intravaginal infection with *Chlamydia trachomatis*. Infect Immun 65(12): 5198-5208.

80. Key, CE and Fisher DJ. (2016) Use of Group II Intron Technology for Targeted Mutagenesis in *Chlamydia trachomatis*. Methods in Molecular Biology. 1498:163-177.

81. Kita E, Yagyu Y, Nishikawa F, Hamuro A, Oku D, Emoto M, Katsui N, Kashiba S. (1989) Alterations of host resistance to mouse typhoid infection by sex hormones. J Leukoc Biol. 46(6):538-46.

82. Kaushic C, Murdin AD, Underdown BJ, Wira CR. (1998) *Chlamydia trachomatis* infection in the female reproductive tract of the rat: influence of progesterone on infectivity and immune response. Infect. Immun. 66:893–898

83. Kelly KG. (1990) Tests on vaginal discharge. Clinical Methods: The History, Physical, and Laboratory Examinations. (3rd edition): 833-835.

84. Kuo CC, Jackson LA, Campbell LA, and Grayston JT. (1995) *Chlamydia pneumonaie* (TWAR). Clin. Microbiol. Rev. 8(4): 451-61.

85. Kuo, C.C., Shor, A., Campbell, L.A., Fukushi, H., Patton, D.L. and Grayston, J.T. (1993) Demonstration of *Chlamydia pneumon*iae in atherosclerotic lesions of coronary arteries. J. Infect. Dis. 167, 841-849.

86. Laemmli, UK. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227(5259):680-685.

87. Lagae S, Kalmar I, Laroucau K, Vorimore F, Vanrompay D. (2014) Emerging *Chlamydia psittaci* infections in chickens and examination of transmission to humans. Journal of Medical Microbiology. 63 (Pt_3): 399

88. Lambowitz AM, Zimmerly S (2004) Mobile group II introns. Annu Rev Genet 38:1-35.

89. Leichti GW, Hall KE, Kalinda A, Brun YV, VanNieuwenhze M & Maurelli AT. (2014) A new metabolic cell-wall labeling method reveals peptidoglycan in *Chlamydia trachomatis*. Nature 506, 057-510.

90. Li Z, Chen C, Chen D, Wu Y, Zhong Y, Zhong G. (2008) Characterization of fifty putative inclusion membrane proteins encoded in the *Chlamydia trachomatis* genome. Infect Immun 76:2746-57.

91. Liu X, Afrane M & Clemmer D. (2010) Identification of *Chlamydia trachomatis* Outer Membrane Complex Proteins by Differential Proteomics. J Bacteriol. 192 (11): 2852-2860.

92. Lowden NM., Yeruva L, Johnson CM, Bowlin AK, & Fisher, DJ. (2015) Use of aminoglycoside 3′ adenyltransferase as a selection marker for *Chlamydia trachomatis* intron-mutagenesis and in vivo intron stability. BMC Research Notes, 8, 570. http://doi.org/10.1186/s13104-015-1542-9.

93. Lutter EI, Barger AC, Nair V, Hackstadt T. (2013) *Chlamydia trachomatis* inclusion membrane protein CT228 recruits elements of the myosin phosphatase pathway to regulate release mechanisms. Cell reports. 3(6):1921-1931. doi:10.1016/j.celrep.2013.04.027.

94. Lyons JM, Morré SA, Airo-Brown LP, Peña AS, Ito JI (2005) Acquired homotypic and heterotypic immunity against oculogenital *Chlamydia trachomatis* serovars following female genital tract infection in mice. BMC Infect Dis. 5:105.

95. Markham, AP, Jaafar, ZA, Kemege, KE, et al. (2009) Biophysical Characterization of *Chlamydia trachomatis* CT584 Supports Its Potential Role as a Type III Secretion Needle Tip Protein. 48(43)10353-10361.

96. Matsumoto A. (1973) Fine structures of cell envelopes of *Chlamydia* organisms as revealed by freeze-etching and negative staining techniques. J. Bacteriol. 116, 1355-1363.

97. Matsumoto, A. (1982) Electron microscopic observations of surface projections on *Chlamydia psittaci* reticulate bodies. J. Bacteriol. 150, 358-364.

98. Matsumoto, A. (1982) Surface projections of *Chlamydia psittaci* elementary bodies as revealed by freeze-deep-etching. J. Bacteriol. 151, 1040-1042.

99. Maxion HK, Liu W, Chang MH, Kelly KA. (2004) The infecting dose of *Chlamydia muridarum* modulates the innate immune response and ascending

infection Infect Immun. 72(11):6330-40.

100. Mishori, R, McClaskey E, Winklerprins, V. (2012) *Chlamydia trachomatis* infections: screening, diagnosis, and management. Am Fam Physician. 86(12)1127-1132.

101. Mital J, Lutter EI, Barger AC, Dooley CA and Hackstadt T. (2015) *Chalmydia trachomatis* inclusion membrane protein CT850 interacts with dynein light chain DYNLT1 (Tctex1). Biochem Biophys Res Commun. 462(2):165-170.

102. Mital J, Miller NJ, Dorward DW, Dooley CA, Hackstadt T. (2013) Role for chlamydial Inclusion Membrane Proteins in Inclusion Membrane Structure and Biogenesis. PLoS ONE 8(5):e63426. Doi: 10.1371/journal.pone.0063426.

103. Mital J, Miller NJ, Fischer ER, Hackstadt T. (2010) Specific chlamydial inclusion membrane proteins associate with active Src family kinases in microdomains that interact with the host microtubule network. Cell Microbiol. 12(9):1235-49.

104. Morrison RP, Feilzer K, Tumas DB. (1995) Gene knockout mice establish a primary protective role for major histocompatability complex class II-restricted responses in Chlamydia trachomatis genital tract infection. Infect Immun 63(12): 4661-4668.

105. Morrison RP& Caldwell HD. (2002) Immunity to Murine Chlmaydial Genital Infection. Infect Immun 70(6): 2741-2751. Differences in mouse immune response to infection occur between C. trachomatis strains and MoPn strains

106. Morrison SG, Morrison RP. (2000) *In situ* analysis of the evolution of the primary immune response in murine *Chlamydia trachomatis* genital tract infection. Infect Immun. 68(5):2870-9.

107. Morrison SG, Su H, Caldwell HD, Morrison RP. (2000) Immunity to murine *Chlamydia trachomatis* genital tract reinfection involves B cells and CD4(+) T cells but not CD8(+) T cells (2000) Infect Immun. 68(12):6979-87.

108. Moulder JW. (1991) Interaction of *Chlamydiae* and host cells *in vitro*. Microbiol. Rev. 55, 143-190.

109. Mount DT, Bigazzi PE, Barron AL. (1972) Infection of genital tract and transmission of ocular infection to newborns by the agent of guinea pig inclusion conjunctivitis. Infect. Immun. 5:921–926

110. Mount DT, Bigazzi PE, Barron AL. (1973). Experimental genital infection of male guinea pigs with the agent of guinea pig inclusion conjunctivitis and transmission to females. Infect. Immun. 8:925–930

111. Mueller KE, Wolf K, Fields KA. (2016) Gene Deletion by Fluorescence-Reported Allelic Exchange Mutagenesis in *Chlamydia trachomatis*. mBio 7(1):e01817-15.

112. Neeper ID, Patton DL and Kuo CC. (1990) Ciinematographic observations of growth cycles of Chlamydia trachomatis in primary cultures of human amniotic cells. Infect Immu. 58(7):2042-47.

113. Ness RB, Soper DE, Richter HE, Randall H, Peipert JF, Nelson DB, Schubeck D, McNeeley SG, Trout W, Bass DF, Hutchinson K, Kip K, Brunham RC. Chlamydia antibodies, Chlamydia heat shock protein, and adverse sequelae after pelvic

inflammatory disease: the PID evaluation and Clinical Health (PEACH) study. Sex Transm Dis. 35(2)129-35.

114. Newman, L. et al. (2015) Global estimates of the prevalence and incidence of four curable sexually transmitted infections in 2012 based on systematic review and global reporting. PLoS ONE. (10):12

115. Nguyen B, Valdivia R.(2014) A chemical mutagenesis approach to identify virulence determinants in the obligate intracellular pathogen *Chlamydia trachomatis.* Methods Mol Biol. 1197:347-58. doi: 10.1007/978-1-4939-1261-2_20.

116. Nichols BA., Setzer PY, Pang, F and Dawson CR (1985) New view of the surface projections of *Chlamydia trachomatis*. J. Bacteriol. 164, 344-349.

117. Nigg, C. (1942) Unidentified virus which produces pneumonaie and systemic infection in mice. Science 95: 49.

118. Paavonen J, Westrom L, Eschenbach DA. (2008) Pelvic inflammatory disease. In: Holmes KK, Sparling PF, Stamm WE, editors. Sexually transmitted diseases. New York: McGraw Hill Medical. 1017-1050.

119. Pal S, Hui W, Peterson EM, de la Maza LM. (1998) Factors influencing the induction of infertility in a mouse model of *Chlamydia trachomatis* ascending genital tract infection J Med Microbiol. 47(7):599-605.

120. Pal S, Peterson EM, de la Maza LM. (1999) A Murine Model for the Study of *Chlamydia trachomatis* Genital Infections during Pregnancy. Kaufmann SHE, ed. Infection and Immunity. 67(5):2607-2610.

121. Pal S, Peterson EM, de la Maza LM. (2001) Susceptibility of mice to vaginal infection with *Chlamydia trachomatis* mouse pneumonitis is dependent on the age of

the animal. Infect Immun.69(8):5203-6/

122. Parsot C, Hamiaux C, Page A. (2003) The various and varying roles of specific chaperones in type III secretion systems. Curr. Opin. Microbiol. 6:7-14. http://dx.doi.org/10.1016/S1369-5274(02)00002-4

123. Patton DL, Halbert SA, Wang SP. (1982) Experimental salpingitis in rabbits provoked by *Chlamydia trachomatis*. Fertil. Steril. 37:691–700

124. Patton DL, Halbert SA, Kuo CC, Wang SP, Holmes KK. (1983) Host response to primary *Chlamydia trachomatis* infection of the fallopian tube in pig-tailed monkeys. Fertil. Steril. 40:829–840

125. Patton DL, Kuo CC, Wang SP, Brenner RM, Sternfeld MD, Morse SA, Barnes RC. (1987) Chlamydial infection of subcutaneous fimbrial transplants in cynomolgus and rhesus monkeys. J. Infect. Dis. 155:229–235 [PubMed]

126. Paumet F, Wesolowski J, Garcia-Diaz A, Delevoye C Aulner N, Shuman HA, Subtil A, Rothman JE. (2009) Intracellular bacteria encode inhibitory SNARE-like proteins. PLoS One 4:e7375. http://dx.doi.org/10.1371/journal.pone.0007375.

127. Perry LL, Feilzer K, Caldwell HD. (1997) Immunity to *Chlamydia trachomatis* is mediated by T helper 1 cells through IFN-gamma-dependent and -independent pathways. J Immunol. 158(7):3344-52.

128. Perry LL, Su H, Feilzer K, Messer R, Hughes S, Whitmire W, Caldwell HD. (1999) Differential sensitivity of distinct *Chlamydia trachomatis* isolates to IFN-gamma-mediated inhibition. J Immunol. 162(6):3541-8.

129. Perutka J, Wang W, Goerlitz D, Lambowitz AM (2004) Use of computer designed group II introns to disrupt *Escherichia coli* DExH/D-box protein and DNA Helicase genes. J Mol Biol 336: 421-439.

130. Prain CJ & Pearce JH. (1989) Ultrastructural studies on the intracellular fate of *Chlamydia psittaci* (strain guinea pig inclusion conjunctivitis) and *C. trachomatis* (strain lymphogranuloma venereum 434): modulation of intracellular events and relationship with endocytic mechanism. Journal of General Microbiology (135)2107 – 2123.

131. Rackstraw S, Viswalingam ND, Goh BT. (1998) Can chlamydial conjunctivitis result from direct ejaculation into the eye? Int J STD AIDS. 2006 Sep. 17(9):639-41.

132. Ramsey, K. H., Rank, R.G. (1991) Resolution of chlamydial genital infection with antigen-specific T-lymphocyte lines. Infect Immun 59(3): 925-931.

133. Ramsey KH, Soderberg LS, Rank RG. (1988) Resolution of chlamydial genital infection in B-cell-deficient mice and immunity to reinfection. Infect Immun. 56(5):1320-5.

134. Ramsey KH, Sigar IM, Schrimpsema JH, Denman CJ, Bowlin AK, Myers GAS, Rank RG. (2009) Strain and Virulence Diversity in the Mouse Pathogen *Chlamydia muridarum.* Infect Immun 77(8): 3284-3293.

135. Rank RG, Barron AL. Effect of antithymocyte serum on the course of chlamydial genital infection in female guinea pigs. Infect Immun 1983;41(2):876-9.

136. Rank RG, Ramsey KH, Pack EA, Williams DM. Effect of gamma interferon on resolution of murine chlamydial genital infection (1992) Infect Immun. 60(10):4427-9.

137. Reinhold P, Kirschvink N, Theegarten D & Berndt A. (2008) An experimentally induced *Chlamydia suis* infection in pigs results in severe lung function disorders and pulmonary inflammation. Vet Res 39:35.

138. Reynolds DJ & Pearce JH. (1990) Characterization of the cytochalasin D-resistant (pinocytic) mechanisms of endocytosis utilized by chlamydiae. Infect Immun. 58(10):3208-16.

139. Reynolds DJ & Pearce JH. (1991) Endocytic mechanisms utilized by *Chlamydiae* and their influence on induction of productive infection. Infect Immun. 59(9):3033-9.

140. Robertson JN, Ward ME, Conway D, Caul EO. (1987) Chlamydial and gonococcal antibodies in sera of intertile women with tubal obstruction. J Clin Pathol 40:377-8

141. Rockey DD, Fischer ER, Hackstadt T. (1996) Temporal analysis of the developing chlamydia psittaci inclusion by use of fluorescence and electron microscopy. Infect Immun. 64(10):4269-78.

142. Rockey DD, Heinzen RA, Hackstadt T (1995) Cloning and characterization of a *Chlmaydia psittaci* gene coding for a protein localized to the inclusion membrane of infected cells. Mol Microbiol 15: 617-626. Doi: 10.1111/j.1365-2958.1995.tb02371.

143. Rockey DD, Rosquist JL (1994) Protein antigens of *Chlmaydia psittaci* present in infected cells but no detected in the infectious elementary body. Infect Immun 62:106-112.

144. Rzomp KA, Moorhead AR and Scidmore MA. (2006) The GTPase Rab4 Interacts with *Chlamydia trachomatis* Inclusion Membrane Protein CT229. Infect Immun. 74(90:5362-5373.

145. Safrin S, Schachter J, Dahrouge D, Sweet RL. (1992) Long-term sequelae of acute pelvic inflammatory disease: a retrospective cohort study. Am J Obstet Gynecol 166((4)):1300-1305.

146. Saikku P, Leinonen M, Tenkanen L, Linnanmäki E, Ekman MR, Manninen V, Mänttäri M, Frick MH & Huttunen JK. (1992) Chronic *Chlamydia pneumoniae* infection as a risk factor for coronary heart disease in the Helsinki Heart Study. Ann Intern Med 116(4):373-8.

147. Saikku P, Leinonen M, Mattila K, Ekman MR, Nieminen MS, Makela PH, Huttunen JK and Valtonen V. (1988) Serological evidence of an association of a novel *Chlamydia*, TWAR, with chronic coronary heart disease and acute myocardial infarction. Lancet 2, 983-986.

148. Saka HA, Thompson JW, Chen Y-S, Kumar Y, Dubois LG, Moseley AM, Valdvia RH. (2011) Quantitative proteomics reveals metabolic and pathogenic properties of *Chlamydia trachomatis* developmental forms. Mol. Microbiol. 82:1185-1203. http://dx.doi.org/10.1111/j.1365-2958.2011.07877.x.

149. Samudrala R, Heffron F, McDermott J. (2009) Accurate prediction of secreted substrates and identification of a conserved putative secretion signal for type III secretion systems. PLoS Pathog. 5:e10000375.

150. Schachter J. (1978) Chlamydial infections (second of three parts). N. Engl. J. Med. 298, 490-495.

151. Schachter, J. (1999) *Chlamydia*: intracellular biology, pathogenesis, and immunity. Infection and disease epidemiology (R.S. Stephens (ed.)): 139-169.

152. Schautteet K, De Clercq E, and Vanrompay D. (2011) *Chlamydia trachomatis* vaccine research through the years. Infectious Diseases in Obstetrics and Gynecology 2011: 9.

153. Scidmore M, Hackstadt T. (2001) Mammalian 14-3-3-beta associates with the *Chlamydia trachomatis* inclusion membrane via its interaction with IncG. Mol. Microbiol. 39:1638-1650. http://dx.doi.org/10.1046/j.1365-2958.2001.02355.x

154. Scidmore MA, Rockey DD, Fischer ER, Heinzen RA and Hackstadt T. (1996) Vesicular interactions of the *Chlamydia trachomatis* inclusion are determined by Chlamydial early protein synthesis rather than route of entry. Infect. Immun. 64, 5366-5372.

155. Scidmore-Carlson MA, Shaw EI, Dooley CA, Fisher ER & Hackstadt T. (1999) Identification and characterization of a *Chlamydia trachomatis* early operon encoding four novel inclusion membrane proteins. Mol Microbiol 33:753-65.

156. Sharif K, Olufowobi I. (2006) The structure, chemistry and physics of human cervical mucus. The Cervix. (2nd edition):157-68.

157. Shaw EI, Dooley, CA, Fisher ER, Scidmore MA, Fields, KA, et al. (2000) Three temporal classes of gene expression during the *Chlamydia trachomatis* developmental cycle. Mol Microbiol 37:913-925. Doi: 10.1046/j.1365-2958.2000.02057.

158. Smith JS, Muñoz N, Franceschi S, Eluf-Neto J, Herrero R & Peeling RW (2001) *Chlamydia trachomatis* and Cervical Squamous Cell Carcinoma. JAMA. 285(13):1703-1706. doi:10.1001/jama.285.13.1703

159. Seth-Smith HM, Harris SR, Persson K, Marsh P, Barron A, Bignell A, Bjartling C,

Clark L, Cutcliffe LT, Lambden PR, Lennard N, Lockey SJ, Quail MA, Salim O, Skilton RJ, Wang Y, Holland MJ, Parkhill J, Thomson NR, Clarke IN. (2009) Co-evolution of genomes and plasmids within *Chlamydia trachomatis* and the emergence in Sweden of a new variant strain. Genomics 10:239.

160. Song L, Carlson JH, Whitmire WM, Kari L, Virtaneva K, Sturdevant, DE, Watkins H, Zhou B, Sturdevant GL, Porcella SF, McClarty G, Caldwell HD (2013) *Chlamydia trachomatis* plasmid-encoded Pgp4 is a transcriptional regulator of virulence-associated genes. Infect Immun 81(3): 636-644.
161. Spiliopoulou D., et al. (1992) Protection of mice infected with *C. trachomatis* from abortion by passive immunization. Proceedings of the European Society for Chlamydia Research. Uppsala University Center for STD Research, Uppsala, Sweden 103.
162. Stacey CM, Munday PE, Taylor-Robinson D, et al. (1992) A longitudinal study of pelvic inflammatory disease. Br J Obstet Gynaecol 99((12)):994-999.
163. Stamm WE. (1999) *Chlamydia trachomatis* infections: progress and problems. J Innfect Dis 179 (suppl 2): S2380-3. Doi:10.1086/513844
164. Stamm WE. (2008) *Chlamydia trachomatis* infections in the adult. Sexually transmitted diseases. New York: McGraw Hill Medical. 575-594.
165. Starnback, MN, Loomis WP, Ovendale P, Regan D, Hess B, Alderson MR, and Fling SP. (2003) An inclusion membrane protein from *Chlamydia trachomatis* enters the MCH Class I Pathway and Stimulates a CD8+ T Cell Response. Journal of Immunology (171);4742-4749.

166. Stephens RS. (1999) reviewed in Developmental biology In: *Chlamydia*: intracellular Biology, Pathogenesis, and Immunity ASM Press, 29-67.

167. Stephens RS, Kalman S, Lammel C. Fan J, Marathe R, Aravind L, Mitchell W, Olinger L, Tatusov RL, Zhao Q, Koonin EV, Davis RW. (1998) Genome sequence of an obligate intracellular pathogen of humans: *Chlamydia trachomatis.* Science 282: 754-759. http://dx.doi.org/10.1126/science.282.5389.754.

168. Stephens RS, Myers G, Eppinger M., et al. (2009) Divergence without difference: phylogenetics and taxonomy of *Chlamydia* resolved. FEMS immunology and medical microbiology 55, 115-119.

169. Sturdevant GL, Kari L, Gardner DJ, Olivares-Zavaleta N, Randall LB, Whitmire WM, Carlson JH, Goheen MM, Selleck EM, Martens C, Caldwell HD. (2010) Frameshift mutations in a single novel virulence factor alter the in vivo pathogenicity of *Chlamydia trachomatis* for the female murine genital tract. Infect Immun. 78(9):3660-8.

170. Su H, Feilzer K, Caldwell HD, Morrison RP. (1997) *Chlamydia trachomatis* genital tract infection of antibody-deficient gene knockout mice Infect Immun. 65(6):1993-9

171. Subtil A, Delevoye C, Balana M, Tastevin L, Perrinet S, Dautry-Varsat A. (2005) A directed screen for chlamydial proteins secreted by a type III mechanism identifies a translocated protein and numerous other new candidates. Mol. Microbiol. 56:1636-1647. http://dx.doi.org/10.1111/j.1365-2958.2005.04647.x.

172. Subtil A, Parscot C, Dautry-Varsat A. (2001) Secretion of predicted Inc proteins of *Chlamydia pneumoniae* by a heterologous type III machinery. Mol. Microbiol. 39:792-800. http://dx.doi.org10.1046/j.1365-2958.2001.02272.2.

173. Swenson CE, Donegan E, Schachter J. C (1983) *Chlamydia trachomatis*-induced salpingitis in mice. J Infect Dis. 148(6):1101-7.

174. Sykes J. (2001) Feline Upper Respiratory Tract Pathogens: *Chlamydophila felis*. Compendium on Continuing Education for the Practicing Veterinarian, 23(3): 231-240.

175. Todd, W.J. and Caldwell, H.D. (1985) The interaction of *Chlamydia trachomatis* with host cells: ultrastructural studies of the mechanism of release of a biovar II strain from HeLa 229 cells. J Infect Dis. 151(6):1037-44.

176. Tuffrey M, Falder P, Taylor-Robinson D. (1982) Genital-tract infection and disease in nude and immunologically competent mice after inoculation of a human strain of *Chlamydia trachomatis*. Br J Exp Pathol. 63(5):539-46.

177. Tuffrey M, Taylor-Robinson D. (1981) Progesterone as a key factor in the development of a mouse model for genital-tract infection with *Chlamydia trachomatis*. FEMS Microbiol. Lett. 12:111–115

178. Vanrompay D, Hoang TQ, De Vos L, Verminnen K, Harkinezhad T, Chiers K, Morre SA, Cox E. (2005) Specific-pathogen-free pigs as an animal model for studying *Chlamydia trachomatis* genital infection. Infect. Immun. 73:8317–8321

179. Vanrompay D, Lyons JM, Morre SA. (2006) Animal models for the study of *Chlamydia trachomatis* infections in the female genital infection. Drugs Today (Barc) 42(Suppl A):55–63

180. Wang Y, Kahane S, Cutcliffe LT, Skilton RJ, Lambden PR, et al. (2011) Development of a transformation system for *Chlamydia trachomatis*: restoration of

glycogen biosynthesis by acquisition of a plasmid shuttle vector. PLoS Pathog 7:e1002258.

181. Ward, M. E. & Murray, A. (1984) Control mechanisms governing the infectivity of C. trachomatis for HeLa cells: mechanisms of endocytosis. Journal of General Microbiology 130, 1765 – 1780.
182. Weber MM, Bauler LD, Lam J & Hackstadt T (2015): Expression and Localization of Predicted Inclusion Membrane Proteins in *Chlamydia trachomatis.* J Infect Immun. 83(12):4710-15.
183. Westrom L, Joesoef R, Reynolds G, Hagdu A, Thompson SE. (1992) Pelvic inflammatory disease and fertility: a cohort study of 1,844 women with laparoscopically verified disease and 657 control women with normal laparoscopic results. Sex Transm Dis 19((4)):185-192.
184. Williams DM, S J, Drutz, DJ, Sumaya, CV (1981) Pneumonia due to *Chlamydia trachomatis* in the immunocompromised (nude) mouse. J Infect Dis 143: 238-241.
185. Williams D & Sullivan A. (2010) Ocular disease in the guinea pig (*Cavia procellus*): a survey of 1000 animals. Veterinary Ophthalmology Supplement 1:54-62.
186. Wimmer MLJ, Sandmann-Strupp R, Saikku P & Haberl RL. (1996) Association of Chlamydial Infection with Cerebrovascular Disease. Stroke 27(12):2207-10.
187. Workowski KA, Berman S (2011) Centers for Disease Control and Prevention. Sexually transmitted diseases treatment guidelines. MMWR Recomm Rep. 59(RR-12):1-110.

188. Wyrick PB, Davis CH, Knight ST, Choong J, Raulston JE, Schramm N. (1993) An *in vitro* human epithelial cell culture system for studying the pathogenesis of *Chlamydia trachomatis*. Sex.Transm.Dis. 20:248–256

189. Yang C, Starr T, Song L, Carlson JH, Sturdevant GL, Beare PA, Whitmire WM1, Caldwell HD (2015) Chlamydial Lytic Exit from Host Cells Is Plasmid Regulated. MBio.6(6):e01648-15. doi: 10.1128/mBio.01648-15.

190. Yang, ZS, Tang , L Sun, X, Chai, JJ & Zhong, GM. (2015) Characterization of CPAF Critical Residues and Secretion during *Chlamydia trachomatis* Infection. 83(6):2234-2241. Doi:10.1128/IAI.00275-15.

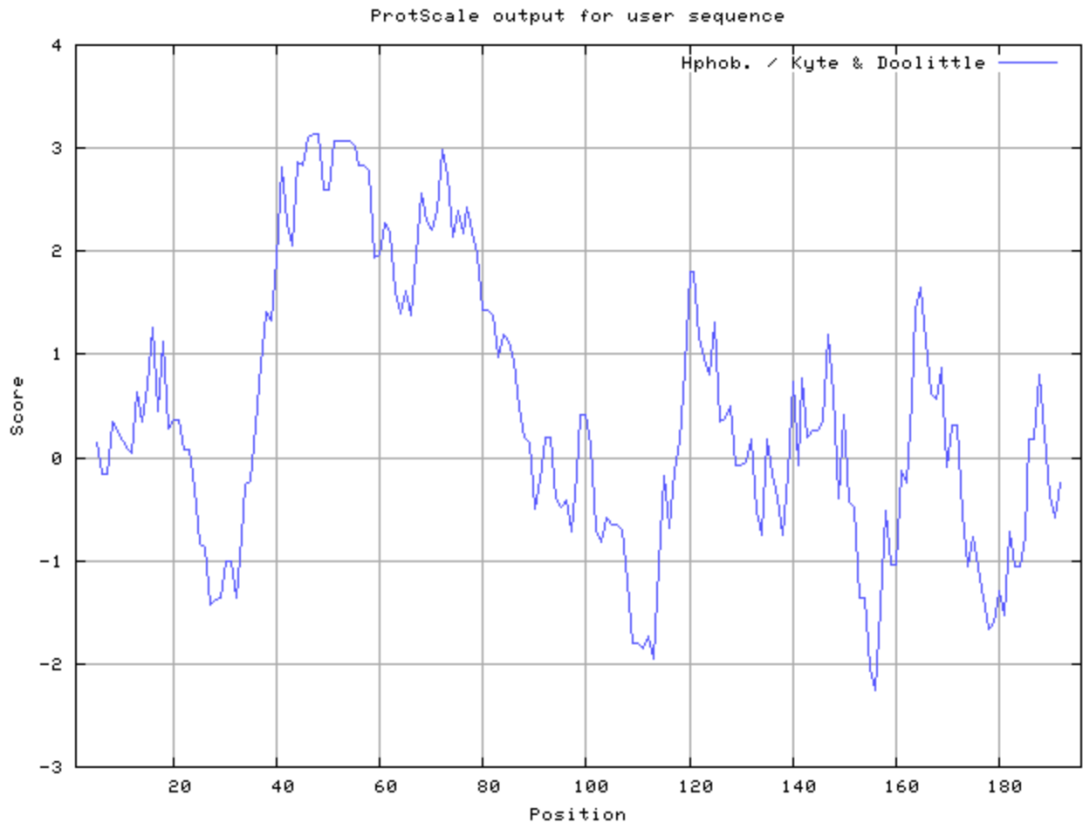
191. Zuck M, Ellis, T, Venida A, Hybiske K. (2016) Extrusions promote engulfment and C*hlamydia* survival within macrophages. bioRxiv: 1-13.

192. Zuck M, Sherrid A, Suchland R, Ellis T & Hybiske K. (2016) Conservation of extrusion as an exit mechanism for *Chlamydia*. Pathog Dis. 74(7). Doi:10.1093/femspd/ftw093

APPENDICES

Figure A1. Kyte and Doolittle hydrophobicity plot of the Inc CT228.

Hydrophobicity analysis of translated amino acids provided of *CT228* sequence provide that two hydrophobic domains are observed from positions 36 to 86 of the 196 amino acid protein.



hypothetical protein [Chlamydia muridarum]

Sequence ID: ref|WP_010230619.1| **Length:** 196 **Number of Matches:** 1

See 10 more title(s)

Range 1: 4 to 191 GenPept Graphics Next Match Previous Match

Score	Expect	Method	Identities	Positives	Gaps
190 bits(482)	7e-57	Compositional matrix adjust.	102/192(53%)	140/192(72%)	4/192(2%)

```
Query  5    ISGDASSLPLPTASCVEIKSTSSSTKGNTCSKILDIALAIVGALVVVAGVLALVLCASNV  64
            ISG  SSL +   S  +I+S ++  K + CS+ILD+AL +VGA++VVAGVL LV+CAS+
Sbjct  4    ISGSGSSLRIAPQS--KIESVTTEAKSSRCSRILDVALLVVGAMIVVAGVLTLVVCASS-  60

Query  65   IFTAIGIAALIIGSACVGAGISRLMCRSSYASLEAKNVLAEQRLRNLSEEKDALVSVSFI  124
             F A+G+AAL++GSAC+GAG+SR+ CRS  ++LEAKN+   +RL  L E +DAL S+  I
Sbjct  61   -FNALGVAALVLGSACLGAGLSRIACRSFCSNLEAKNIANTRRLSGLIEARDALTSIVSI  119

Query  125  NKMFLRGLTDDLQALEAKAIEVEIDCLDRLEKNEQALLSDVRLVLSSYTRWLDSAEKEKA  184
            NK  L+ L  ++Q LE K IE E+D L +L++NE+ LL DVRLV SSYTRWL ++E+EK
Sbjct  120  NKALLQKLAKEIQILENKVIEAELDLLAKLDENEKRLLEDVRLVFSSYTRWLKASEQEKL  179

Query  185  ALKASIDANQAS  196
             LK+SI    AS
Sbjct  180  VLKSSIRQTGAS  191
```

Figure A2: *C. trachomatis* and *C. muridarum* CT228 homology.

CT228 of *C. trachomatis* and of *C. muridarum* was found to be more than 50% conserved.

VITA

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