Characterization of the chlamydial T3S translocator proteins

Characterization of the chlamydial type III secretion translocator proteins CopB and CopD

By

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LAY ABSTRACT

Chlamydia species are gram-negative bacteria that cause a wide range of human disease, including pneumonia, blinding trachoma, and sexually transmitted infections. However, our current understanding of the mechanisms by which *Chlamydiae* infects its host is relatively unknown. Using various biochemical techniques, the function of two proteins that are believed to be important for chlamydial pathogenesis are explored. Using an animal model for chlamydial genital infections, a novel vaccine was developed to evaluate its ability to protect against a live *Chlamydia* challenge and reduce the prevalence of sequelae.

ABSTRACT

Chlamydiae spp. are a significant cause of disease worldwide. *Chlamydia* pneumoniae is a respiratory pathogen which is believed to cause approximately 10% of community-acquired pneumoniae. Furthermore, *C. pneumoniae* infections have been associated with the development of atherosclerosis, exacerbation of asthma, multiple sclerosis and Alzheimer's. *Chlamydia trachomatis*, the causative agent of trachoma, is responsible for over 6 million cases of preventable blindness each year. Furthermore, *C. trachomatis* is the leading bacterial sexually transmitted infection in North America. Yet, given how prevalent chlamydial infections are, there is still limited information regarding the pathogenic mechanisms by which *Chlamydia* infects its host.

Type III secretion is a conserved pathogenic virulence factor used by gram-negative bacteria. Type III secretion has been extensively studied in other bacterial genera, but given the difficulty of working with *Chlamydia*, there have been few advances in our understanding of the role of type III secretion in chlamydial pathogenesis. A set of proteins, called translocators, are believed to be indispensable for infection; however, a comprehensive biochemical characterization of them has not been undertaken. Using GST-pulldown assays, interactions between *Chlamydia* outer protein (Cop) B and D from *C. pneumoniae* and other components of the type III secretion system are characterized. Furthermore, site-directed mutagenesis was used to elucidate the critical amino acids involved in interaction between the putative chaperone, LcrH_1, and both

CopB and CopD. We also demonstrate that CopD is capable of forming higher ordered structures, and that CopD and LcrH_1 interact in a 1:1 ratio. Using polyclonal antibodies directed towards the translocator proteins, we demonstrate that CopB and CopD may be surface exposed prior to host cell contact and critical to infection. A synthetic peptide consisting of the chaperone binding domain was capable of blocking the interaction between LcrH_1 and the translocator proteins, as well as inhibiting infection in vitro. Lastly, an animal model of *Chlamydia* infection was developed to explore whether the use of type III secretion system proteins could be used to protect against *Chlamydia* infection and *Chlamydia* induced pathology.

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book big enough, or thesis long enough to describe what you mean to me. All I can say is six words: Thank you, and I love you.

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LIST OF ALL ABBREVIATION AND SYMBOLS

°C	Degrees Celsius
AC	Cytoplasmic ancillary proteins
BA	Basal apparatus
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
BSA	Bovine Serum Albumin
Cds	Contact-dependent secretion
Cop	*Chlamydia* outer protein
CpG	Unmethylated DNA
C-terminal	Carboxy terminal
Da	Dalton
DNA	Deoxyribonucleic acid
EB	Elementary body
ELISA	Enzyme-linked Immunosorbent Assay
GAGs	Glycosaminoglycans
GST	Glutathione-S-Transferase
GST-tag	Glutathione-S-Transferase tag
HCl	Hydrochloric acid
His-tag	Polyhistidine-tag
HM	Host membrane
hpi	Hours post infect
IB	Intermediate body
IFUs	Inclusion forming units
IM	Inner membrane
IN	Intranasal
IPTG	Isopropyl β-D-1-thiogalactopyranoside
KCl	Potassium chloride
kDa	Kilodalton
L	Litre
LDAO	Lauryldimethylamine oxide
M	Molar
mL	Millilitre
mM	Millimolar
NaCl	Sodium chloride
NC	Needle complex
ng	nanogram
N-terminal	Amino terminal

OM	Outer membrane
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDI	protein disulfide isomerase
PID	Pelvic inflammatory disease
PP	Periplasm
qRT-PCR	Quantitative Real-time Polymerase Chain Reaction
RB	Reticulate body
RNA	Ribonucleic acid
RNase	Ribonuclease
RPM	Revolutions per minute
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
Spp.	Species
T3S	Type III Secretion
T3SS	Type III Secretion System
TC	Tip complex
TCA	Trichloroacetic acid
TR	Translocators
TRIS	Tris(hydroxymethyl)aminomethane
UGT	Upper genital tract
x *g*	times gravity
µg	Micrograms
µL	Microliter
µM	Micromolar

CHAPTER ONE - INTRODUCTION

Chlamydiae

In 1909, Halberstaedter and von Prowazek took infected conjunctival scrapings from humans and infected orang-utans and observed cytoplasmic inclusions via light microscopy in epithelial cells. The causative agent identified at the time was *Chlamydia trachomatis*, but was classified as a protozoan named *Chlamydozoa*. From 1929-1930, there was an outbreak of psittacosis, a zoonotic respiratory infection, and the organism responsible *Chlamydia psittaci*, was misclassified as a "large virus" (Schachter & Caldwell, 1980). It wasn't until the mid-1960's that James Moulder provided evidence that *Chlamydiae* were not viruses, but in fact bacteria, by the presence of a cell envelope, RNA and DNA, and the existence of ribosomes via electron microscopy(Moulder, 1965; Moulder, Grisso, & Cho, 1965; Schechter, Tribby, & Moulder, 1964). The first specimen of *Chlamydia pneumoniae* (TWAR) was collected from a control subject in a trachoma vaccine study, but was identified as *C. psittaci* until 1989 when Grayston *et. al.* identified it as *C. pneumoniae* (Grayston, 1989; Grayston, Wang, Kuo, & Campbell, 1989).

Taxonomy

Based on a sequence comparison of the 16s ribosomal subunit, bacteria of the order *Chlamydiales* have a sequence identify of > 80%, which include: *Chalmydiaceae*, *Parachlamydiaceae*, *Simkaniaceae*, and *Waddliaceae (Corsaro, Valassina, & Venditti, 2003)*. The *Chlamydiaceae* family can be further divided

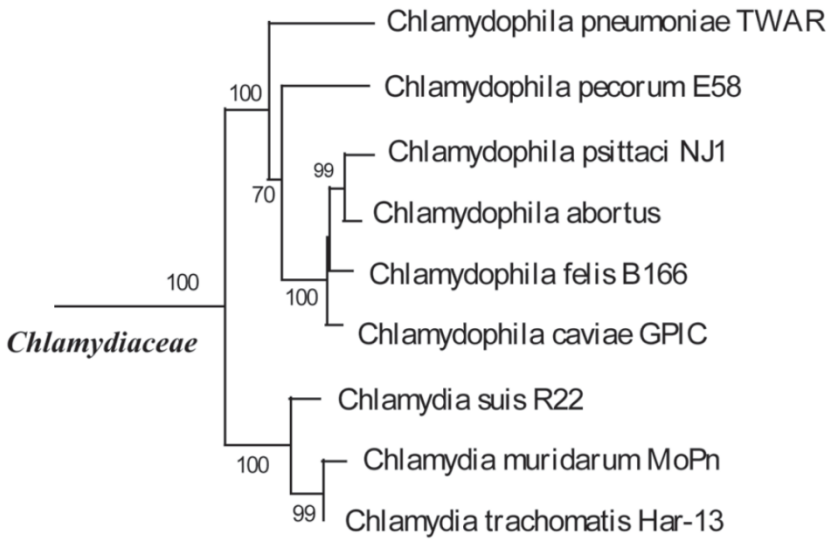

Figure 1: Adapted from Corsaro *et. al* (2003). Phylogenetic tree based on a 1200bp segment of the 16S RNA sequence

into two genera: *Chlamydia* and *Chlamydophila*. The two genera, *Chlamydia* and *Chlamydophila* were created based on a genetic analysis of the ribosomal operon, outer membrane proteins (Omp1 and Omp2) and the RNase P genes (Corsaro et al., 2003). Furthermore, the genera are also characterized by unique phenotypic properties. Members of the *Chlamydia* genus have the ability to synthesize glycogen, contain two 16s rRNA genes, are sensitive to sulfadiazine antibiotics, and possess non-fusogenic inclusion bodies (Corsaro et al., 2003). Alternatively, members of the *Chlamydophila* genus do not produce any detectable amounts of glycogen, are resistant to sulfadiazines, have fusogenic inclusion bodies, and one 16s rRNA gene (Corsaro et al., 2003). The *Chlamydia* genome is approximately 1.05 Mbp, whereas members of the *Chlamydophila* genus have a genome size of 1.2 Mbp (Corsaro et al., 2003). Additionally, both genera contain an extrachromosal plasmid (Corsaro et al., 2003). Currently, there are three members of the

Chlamydia genus, *C. trachomatis*, *C. suis*, and *C. muridarum*; and six members in the *Chlamydophila* genus which are, *C. pneumoniae*, *C. psittaci, C. pecorum, C. abortus, C. caviae*, and *C. felis(Corsaro et al., 2003)*. A 2001 publication discussed the issues with the original classification of *Chlamydia* and *Chlamydophila* into two separate genera based on very limited differences among the two(Schachter et al., 2001). Thus, I will use *Chlamydophila* or *Chlamydia* interchangeably to describe *C. pneumoniae* (Schachter et al., 2001). Figure 1 shows a phylogenetic tree of *Chlamydiaceae*.

Life cycle and Pathogenesis of *Chlamydia*

Chlamydiae have a unique biphasic lifecycle consisting of an infectious EB and a metabolically active RB. The EB is much smaller in size (~0.3um) as compared to the RB (~1.0um) (Chi, Kuo, & Grayston, 1987; Miyashita, Kanamoto, & Matsumoto, 1993), is electron dense, round, and often described as being pear shaped with a

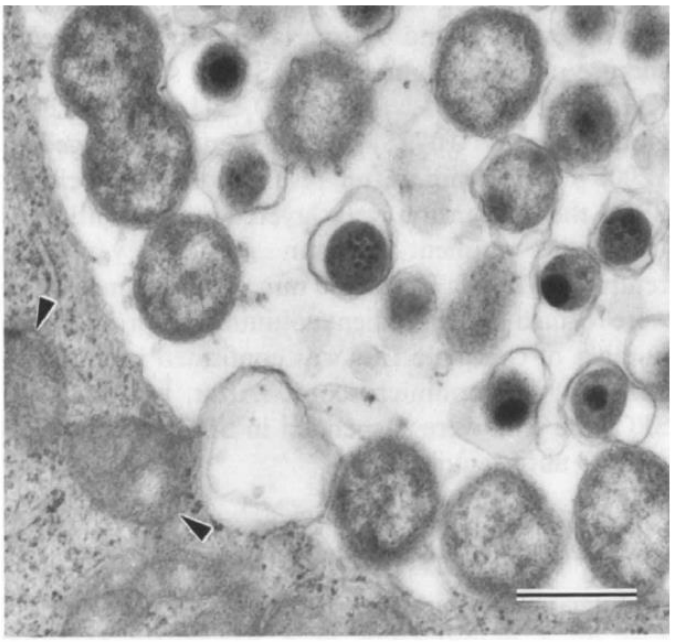

Figure 2: Electron micrograph of *C. pneumoniae* infected HeLa cells. Arrows indicate mitochondria, Bar is 1 µm. Adapted from Miyashita et. al (1993).

relatively large periplasmic region (Chi et al., 1987; Miyashita et al., 1993). Furthermore, nucleic acids in the EB exists in a condensed state, with genetic material bound to histone-like proteins (Barry, Hayes, & Hackstadt, 1992). Lastly, the outer membrane of most Gram-negative bacteria is rigid because of the presence of peptidoglycans in the outer membrane (OM). Though *Chlamydia* is believed to have a full complement of genes required for peptidoglycan synthesis, the rigidity of the outer membrane in the EB is derived from extensive disulfide bonds formed between cysteine rich proteins that are located in the OM (Hatch, 1996; Moulder, 1993). As for the RB, it appears electron diffuse, as opposed to the EB, because of a relative homogenous distribution of cytoplasmic contents, and an uncondensed nucleoid (Chi et al., 1987; Miyashita et al., 1993). Figure 2 above is an electron micrograph of an inclusion body containing *C. pneumoniae* EBs and RBs.

The mechanism for attachment and invasion of *Chlamydia* into the host remains relatively obscure (Dautry-Varsat, Subtil, & Hackstadt, 2005). However, it is believed that for most species of *Chlamydia*, attachment to the host cell may occur through reversible, electrostatic interactions with heparan-sulfate containing glycosaminoglycans (GAGs), either on the bacterium or host cell (Dautry-Varsat et al., 2005). These heparan-sulfate containing GAGs may act to bring together the bacterium and the host cell (Dautry-Varsat et al., 2005). An eukaryotic membrane protein called protein disulfide isomerase (PDI) may be involved in attachment as a CHO mutant cell line deficient in PDI is not

permissive to *C. pneumoniae* infection (Dautry-Varsat et al., 2005). *Chlamydiae* may target to areas on the eukaryotic membrane that are rich in cholesterol and sphingomylein, within lipid rafts (Dautry-Varsat et al., 2005). Furthermore, pre-treating cells with a cholesterol depleting agent (HMG-CoA reductase inhibitors) reduces infectivity of *Chlamydia* (Dautry-Varsat et al., 2005). Once *Chlamydia* is attached to its' host, Coombes and Mahony (2002) showed the formation of actin pedestal-like structures at the point of contact between the bacterium and the host cell (Dautry-Varsat et al., 2005). An effector protein, named TARP, which is secreted by the type III secretion system may be responsible for triggering the formation of the actin pedestal-like structures because it has been shown to influence actin polymerization (Dautry-Varsat et al., 2005). Upon host cell binding certain pathways that have been shown to be involved in actin reorganization, such as MEK-ERK and PI-3, are known to be activated (Coombes & Mahony, 2002). Ultimately, this process leads to internalization of *Chlamydia* into a vacuole termed an inclusion.

Being an obligate, intracellular bacterium, *Chlamydia* spends all of its time replicating within the inclusion. Due to its reduced genome size, *Chlamydia* is unable to produce all the nutrients needed for replication, thus it must scavenge nutrients from the host, such as cholesterol, ATP, and sphingomyelin (Carabeo, Mead, & Hackstadt, 2003; Hackstadt, Scidmore, & Rockey, 1995). To accomplish this, the inclusion (containing *Chlamydia*) translocates to a peri-Golgi location to intercept exocytic vacuoles containing sphingomyelin and other

nutrients from the exocytic pathway (Al-Younes, Rudel, & Meyer, 1999; Grieshaber, Grieshaber, & Hackstadt, 2003). The EBs differentiate into RBs, following an unknown signal, then replicate by binary fission. In *C. pneumoniae*, after approximately 40 hours, the RBs begin to differentiate back into EBs through an unidentified signal (Hybiske & Stephens, 2007). It has been suggested that the expanding inclusion, combined with anchoring of the RBs to the inclusion membrane by means of the T3S injectisome and subsequent detachment through T3S inactivation, is involved in this RB to EB conversion (Peters, Wilson, Myers, Timms, & Bavoil, 2007). Occasionally, an intermediate body (IB) will form, which occurs during the RB to EB differentiation. Finally, the process of replication is completed when *Chlamydiae* exit the cell following host cell lysis, killing the host cell, or through a process called extrusion, where the bacteria can directly enter a nearby cell, leaving the original host intact (Hybiske & Stephens, 2007). Figure 3 below is a simplistic representation of the *Chlamydia* lifecycle.

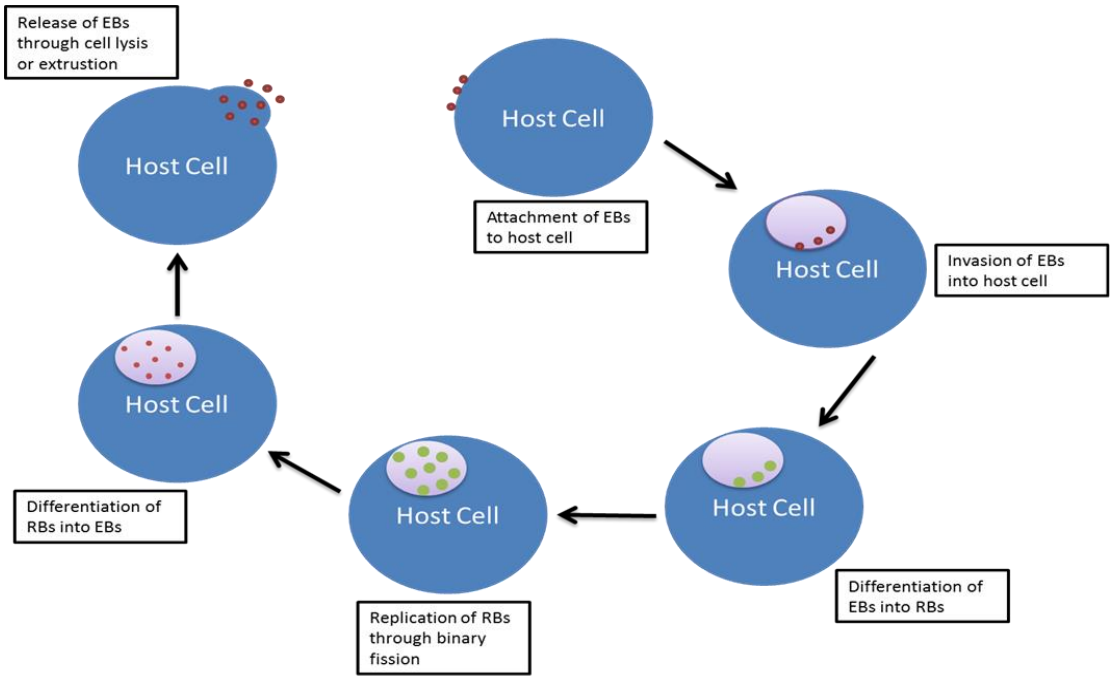

Figure 3: Simplistic overview of the *Chlamydia* developmental cycle.

Type III secretion system

Secretion systems are used by numerous eukaryotes and prokaryotes to secrete molecules from the cytoplasm to the extracellular space, or directly to another cell. Prokaryotes have developed seven different secretion systems to facilitate essential functions for survival. Type III secretion (T3S) is used by numerous gram-negative

To date, type III secretion (T3S) is believed to be one of the most complex secretion systems (Beeckman & Vanrompay, 2010). The type III secretion system (T3SS) is a highly conserved system among pathogenic and commensal Gram-negative bacteria (Beeckman & Vanrompay, 2010; Hueck, 1998). The T3SS is composed of 20-30 proteins, many of which are conserved across bacteria that use T3S, such as, *Salmonella spp., Shigella spp., Yersinia spp.,*

Escherichia spp., and of course, *Chlamydia spp.* (Beeckman & Vanrompay, 2010; Hueck, 1998). This system allows for the direct injection of effector proteins into host cells (Beeckman & Vanrompay, 2010). The system as a whole can be referred to as the injectisome, which can be broken down to smaller functional components (Cornelis, 2006).

In 1998, a needle complex was visualized on the outside of *Salmonella spp*., but more than 25 years before that, in the late 1970's and early 1980's Matsumoto identified projections on the surface of *C. psittaci (Kubori et al., 1998)*. Matsumoto identified these structures which became known as Matsumoto projections, which are believed to be injectisomes (Matsumoto, 1981, 1982a, 1982b; Matsumoto, Fujiwara, & Higashi, 1976). However, it was not until 1997 that Hsia *et. al.* identified genes within *C. psittaci* that encode putative T3S proteins (Hsia, Pannekoek, Ingerowski, & Bavoil, 1997) To date, putative T3S genes have been identified in ten distinct operons containing 37 different genes (Hefty & Stephens, 2007). Typically, Gram-negative bacteria that utilize T3S have their genes clustered into specific pathogenicity islands, which may be encoded chromosomally or on a plasmid rather than being scattered over numerous operons, as is the case for *Chlamydia spp* (Hefty & Stephens, 2007). Although bioinformatics have shown that *Chlamydia spp*. may contain all the genes required to form a functional T3S apparatus, there is still limited data about its functionality. See Figure 4 for a proposed structure of the T3SS of *Chlamydia.*

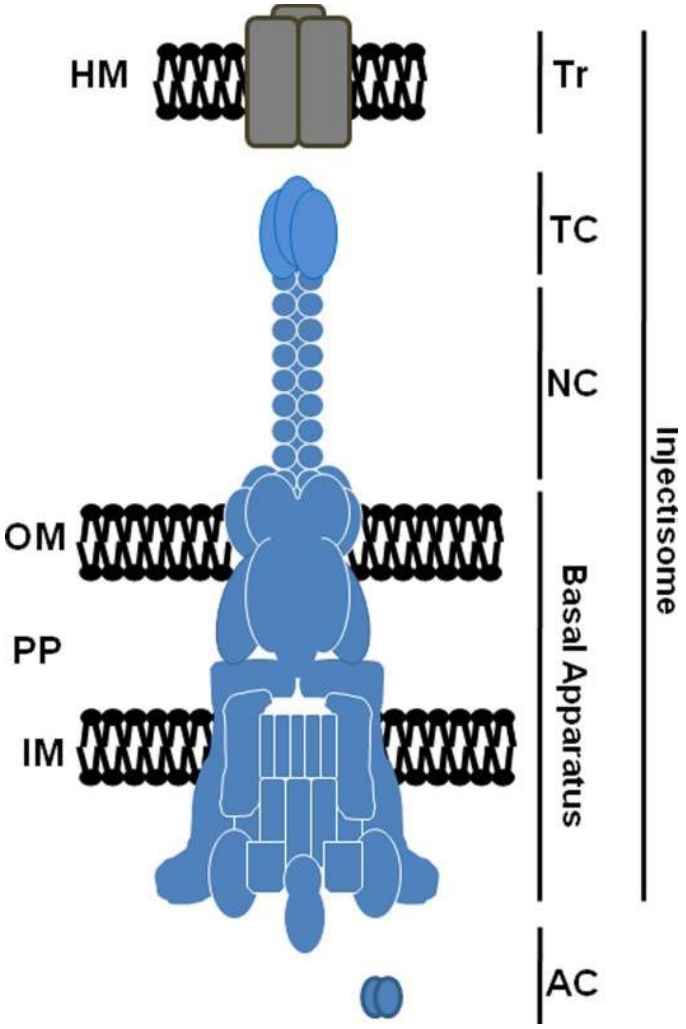

Figure 4: A schematic diagram of the T3S apparatus from *Chlamydia* as described by Betts-Hampikian & Fields (2010). (TC = Tip Complex; NC = Needle Complex; BA =Basal Apparatus; AC = Cytoplasmic Ancillary Proteins; HM = Host Membrane; TR = Translocator; OM = Outer membrane; IM = Inner membrane; PP = Periplasm).

Studying the T3SS in *Chlamydia spp*. is difficult, due in part to its genetic intractability and its obligate, intracellular nature (Stephens, 1992). However, much of the information regarding T3S has been elucidated from other well characterized systems in *Yersinia spp*., *Salmonella spp*., *Escherichia spp*., and *Shigella spp* (Beeckman & Vanrompay, 2010). In *Chlamydia*, proteins involved in T3S are abbreviated as either Cds (Contact-dependent secretion), or Cop (*Chlamydia* outer protein) (Beeckman & Vanrompay, 2010). Based on predicted models in other species, CdsC, also called secretin, is believed to be an integral,

pore-forming outer membrane protein. The inner membrane components of the T3S apparatus are believed to be composed of CdsD,J,R,S,T,U,V (Beeckman & Vanrompay, 2010). CdsD, an inner membrane protein, may contain a C-terminal domain that spans the periplasm to interact with the outer membrane secretin ring, CdsC (Diepold et al., 2010). CdsJ, an inner membrane protein, along with CdsC and CdsD, may be the first structural proteins inserted into the membranes during the formation of the T3S apparatus (Diepold et al., 2010). Furthermore, CdsU a protein with four transmembrane domains is believed to play a role in substrate-switching between early, middle, and late effector proteins (Deane et al., 2008; Zarivach et al., 2008). The needle complex is believed to be formed from approximately 140 CdsF monomers, and is anchored to a protein in the inner membrane (Broz et al., 2007). The length of the needle complex is thought to be regulated by the putative cytoplasmic ruler protein, CdsP (Journet, Agrain, Broz, & Cornelis, 2003; Wagner et al., 2009). There is mounting evidence in other species that CdsP orthologues may regulate needle length, as mutations in CdsP orthologues increase or decrease the alpha helical content of ruler protein, increasing or decreasing the length of the needle, respectively (Wagner et al., 2009). CdsQ, which forms the putative cytoplasmic ring (C-ring), is thought to act as a hub for proteins, shuttling them to the base of the T3S apparatus for secretion (Spaeth, Chen, & Valdivia, 2009). *Chlamydia spp*. encode a putative ATPase called CdsN (Hueck, 1998). The ATPase is believed to provide the energy required to dissociate an effector protein from its chaperone, and play a

role in protein unfolding, as only partially folded proteins are small enough to fit through the needle (Beeckman & Vanrompay, 2010; Stone, Johnson, Bulir, Gilchrist, & Mahony, 2008). The activity of CdsN is regulated by a protein called CdsL (Stone CB, 2011). On the tip of the needle, outside the OM, there is the needle-tip complex hypothesized to consist of the sensor protein, putatively Cpn0803, and a translocator, CopB (Espina et al., 2006; Markham, Jaafar, Kemege, Middaugh, & Hefty, 2009; Veenendaal et al., 2007). Cpn0803 is believed to detect the host cell, and then trigger an event that transduces a signal through the injectisome, triggering secretion of effector proteins. Bioinformatic analysis has led to the prediction that *Chlamydia* may contain two sets of hydrophobic translocator proteins, CopB (*cpn0809*), CopD (*cpn0808*), CopB2 (*cpn1019*), and CopD2 (*cpn1020*), while most other species have one set of translocators (Ouellette, Abdelrahman, Belland, & Byrne, 2005)

Chlamydia translocator proteins

The translocators are an essential group of three proteins that play a role in sensing the host cell and forming a pore in the host cell membrane to allow for translocation of effector proteins (Mattei et al., 2011). In other bacterial species, there are three proteins that are considered translocators, a hydrophilic host-cell sensing protein, also called the needle tip protein, and two hydrophobic pore-forming proteins (Mattei et al., 2011). The hydrophilic translocator is referred to as the V-antigen because immunization with LcrV from *Yersinia spp*. provides protective immunity (Anderson et al., 1996). The crystal structure of the V-antigen

in other bacterial species has been solved and the protein has been well characterized. The needle tip protein is indispensable for translocation of effector proteins into the host cell. Knockouts of the needle tip protein in other bacterial species prevent proper insertion of the pore-forming translocators into the host, and thus block translocation of effector proteins (Broz et al., 2007; Goure et al., 2004). Furthermore, the needle tip protein may be in a hexameric or pentameric state (Gebus, Faudry, Bohn, Elsen, & Attree, 2008). Also, there is conflicting evidence to suggest that one of the hydrophobic translocators may be present on the outside of the cell in a complex with the needle-tip protein (Espina et al., 2006; Veenendaal et al., 2007).

As with the needle-tip protein, the hydrophobic translocators are indispensable for the infection process, as knockouts with either or both hydrophobic translocator missing are unable to secrete effector proteins into the host cell (Mattei et al., 2011). Studies have shown that PopB from *Pseudomonas* is able to spontaneously insert into lipid membranes (Faudry, Vernier, Neumann, Forge, & Attree, 2006; Goure et al., 2004). Furthermore, a knockout of PopB prevented PopD insertion into the host cell membrane as well as preventing secretion of effector proteins into the host (Faudry et al., 2006; Goure et al., 2004). Taken together, this data suggests that PopB may act as a scaffold for PopD membrane insertion, and that PopB and PopD are both required for effector secretion (Faudry et al., 2006). YopB, IpaB, PopB and other orthologues, sometimes referred to as the major hydrophobic translocator due to their size, are

known to have two predicted transmembrane domains, an N-terminal coiled-coil domain, and an N-terminal chaperone binding domain (Mattei et al., 2011). Furthermore, analysis of SipB from *Salmonella spp*. indicates that the major hydrophobic translocator may form a hexamer (Hayward, McGhie, & Koronakis, 2000). Furthermore, it has been shown that the C-terminus of IpaB is not only responsible for binding to the needle protein, but is also involved in regulating secretion (Shen, Saurya, Wagner, Nishioka, & Blocker, 2010). Prior to secretion, there is conflicting evidence as to the location of the major hydrophobic translocator. There is evidence to suggest that IpaB is located outside the bacteria in complex with the needle-tip protein, or possibly located within the bacterial cytosol (Espina et al., 2006; Veenendaal et al., 2007).

The other hydrophobic translocator can be referred to as the minor hydrophobic translocator because of its relative size. YopD, PopD, IpaC and their orthologues are relatively conserved between different species (approx. 30% similarity) (Mattei et al., 2011). Structural analysis of the minor hydrophobic translocator shows that it has one larger transmembrane domain near the middle or C-terminus of the protein, an N-terminal chaperone binding domain, and coiled-coil domain or amphiphatic helix in the C-terminus which may mediate interactions with the needle-tip protein (Mattei et al., 2011). In addition to aiding in pore formation, there is evidence to suggest that the minor hydrophobic translocator has effector function. SipC, from *Salmonella*, triggers actin nucleation; whereas as the C-terminus of IpaC recruits and activates Src kinase

to stimulate actin rearrangement during invasion (Chang et al., 2007; Mounier et al., 2009). When both the major and minor hydrophobic translocators form a pore in the host cell, the diameter of the pore corresponds to the diameter of the needle complex (Mattei et al., 2011). As mentioned previously, *Chlamydia* is predicted to have two sets of hydrophobic translocators. Two sets of hydrophobic translocators have been observed in other bacterial species that use two type III secretion apparatuses. For example in *Salmonella*, the two sets of hydrophobic translocators are encoded on separate pathogenicity islands, SPI-1 and SPI-2 (Holzer & Hensel, 2010). In SPI-1 the hydrophobic translocators are SipB and SipC; where as in SPI-2, the hydrophobic translocators are SseB and SseC (Holzer & Hensel, 2010; Miki, Okada, Shimada, & Danbara, 2004). These two sets of hydrophobic translocators are used at different time points in the life cycle of *Salmonella*. SipB and SipC are critical during the invasion process whereas SseB and SseC are important for replication (Holzer & Hensel, 2010; Miki et al., 2004). To date, there is very little evidence to suggest that *Chlamydia spp*. have two T3SSs. Besides the two aforementioned papers by Ouellette *et. al*. and Fields *et. al*., there is very little data characterizing the hydrophobic translocators in *Chlamydia* spp. (Ouellette et al., 2005) Spaeth et. al. (2009) confirmed the interaction between Scc2 and CopB, but also identified an interaction between Scc2 and CopD (Spaeth et al., 2009). More recent work on CopB and CopB2 indicates that when ectopically expressed in eukaryotic cells, CopB can be found associated with the cytoplasmic membrane, and CopB2 associates with the

inclusion membrane; suggesting a role in invasion, and replication, respectively. Due to the high homology between the translocator proteins of different T3SS, significant insight can be made regarding the function of chlamydial translocator proteins.

Prior to bacterial secretion, the translocator proteins are located in the bacterial cytosol bound to their cognate type II chaperone. Type II chaperones, which are specific for the translocator proteins, are meant to prevent premature homo- and hetero-oligomerization between the major and minor hydrophobic translocator proteins before secretion. Crystal structures of the chaperone have been resolved with a peptide encompassing a conserved PxLxxP motif, which is located within the N-terminus of the translocator proteins (Lunelli, Lokareddy, Zychlinsky, & Kolbe, 2009). Controversy remains as to whether the translocator proteins are located on the needle tip-complex prior to host cell contact, or are recruited to the tip upon activation of secretion. However, the translocators are first uncoupled from their chaperone via the T3S ATPase prior to passing through the needle apparatus. Once on the tip of the needle apparatus, the translocator proteins insert into the host cell membrane, forming a pore and allowing for the translocation of effector proteins. It has been demonstrated in other bacterial T3SS that the translocator proteins may have effector function within the host cell, but there has been no evidence to date regarding the effector function of the translocator proteins from *Chlamydia spp.*

Further research is needed to elucidate the role of the two sets of hydrophobic translocators in the *Chlamydia* developmental cycle, and their importance in type III secretion.

Type III secretion proteins as vaccine candidates

After 50 years of research into *Chlamydia* vaccines, there are currently no approved vaccines for the prevention of *Chlamydia* infection and its subsequent sequelae. Early work into *Chlamydia* vaccines utilized inactivated EBs in attempts to prevent trachoma; however, when compared to the unvaccinated group, the children vaccinated with formalin-fixed EBs were at risk for developing worsened pathology upon exposure to *Chlamydia* (Bell, Nichols, & Haddad, 1963). In 1981, Caldwell et. al. demonstrated the purification and immunogenicity of the major outer membrane protein (MOMP) from *C. trachomatis (Caldwell, Kromhout, & Schachter, 1981)*. MOMP, which is an integral membrane protein accounting for approximately 60% of the total protein in the cells, has been the subject of extensive vaccine work since the 1980s. Despite years of research, MOMP has failed to provide significant protection against *Chlamydia* induced pathology, and has limited cross-serovar protection. In other bacteria that utilize the type III secretion system, including *Yersinia spp., Pseudomonas spp*., and *Shigella spp*., there has been significant advances in vaccine development for these species by utilizing T3S proteins as immunogens (Gebus et al., 2008; Heine et al., 2014; Heine et al., 2013; Martinez-Becerra, Chen, et al., 2013; Martinez-Becerra,

Scobey, et al., 2013). In fact, early work in *Yersinia spp*. identified anti-LcrV antibodies as being able to block *Yersinia* infection (Zauberman et al., 2008). Furthermore, YopB and YopD, which are orthologous to CopB and CopD from *Chlamydia*, are able to protect against Yersinia infection when used as an antigen (Ivanov et al., 2008). The tip protein has been has been successfully used in numerous gram-negative bacteria to prevent against infection. However, the tip protein has yet to be identified in any *Chlamydia spp*.. In *Shigella spp*, researchers have started to use a translocator protein, IpaB, in combination with the tip protein, IpaD, as antigens to protect against lethal Shigella infection in mice. In attempts to identify dominant antigens that could be used in *Chlamydia* vaccines, researchers, in independent experiments, have identified CopB and CopD as possible dominant antigens during a *Chlamydia* infection (Goodall, Yeo, Huang, Raggiaschi, & Gaston, 2001). Furthermore, unlike MOMP which has a significant degree of amino acid sequence variability between each of the serovars which would afford minimal levels of protection, CopB and CopD are conserved across *Chlamydia* serovars, and highly conserved between *Chlamydia spp*. Together, the translocator proteins from *Chlamydia spp*. may represent a novel group of antigens for a *Chlamydia* vaccine.

Significance of *Chlamydia* research

Chlamydia cause a significant burden of disease worldwide (Centers for Disease & Prevention, 2011). The Gram-negative pathogen *C. trachomatis* is reported to be responsible for over 6 million cases of blindness in developing

countries each year, and is the number one sexually transmitted bacterial infection in North America. *C. trachomatis* infection can lead to PID (pelvic inflammatory disease), salpingitis, and infertility in women and epididymitis and infertility among men (Peipert, 2003). However, one of the most salient features of a *Chlamydia trachomatis* infection in women is that in upwards of 70% infected individuals are completely asymptomatic (Peipert, 2003). *C. pneumoniae* infections are usually limited to the respiratory tract, and has been noted to cause up to 10% of community-acquired pneumonia (Marrie, Peeling, Reid, De Carolis, & Canadian Community-Acquired Pneumonia, 2003). Epidemiological studies of serum samples have placed the seroprevalance of *C. pneumoniae* between 40-85% (Miyashita, Fukano, Yoshida, Niki, & Matsushima, 2002). Lastly, *C. pneumoniae* has been implicated in other diseases, such as atherosclerosis, Alzheimer's disease, Multiple Sclerosis, and exacerbation of asthma (Contini et al., 2008; Gerard et al., 2006; Grayston, 2005; Sutherland & Martin, 2007). Taken together, infections from *Chlamydia* species represent a major public health concern. Further research focusing on the pathogenesis of *Chlamydia* is therefore required to develop novel therapies to treat infection reduce morbidity associated with chlamydial infections.

CHAPTER TWO

Preface to Chapter Two

In this chapter, a comprehensive biochemical characterization of one of the translocator proteins from *Chlamydia pneumoniae* is described. Various biochemical techniques were utilized to characterize CopD, including GST-pulldown assays for protein interaction studies. Furthermore, site-directed mutagenesis was used to examine the necessity of specific amino acids for the interaction between LcrH_1 and CopD. Using size-exclusion chromatography (SEC), the oligomeric state of CopD was assessed in the presence and absence of its chaperone, LcrH_1. Lastly, antibodies generated towards an N-terminal epitope of CopD were incubated with *C. pneumoniae* to examine their ability to neutralize infection.

The publication presented in Chapter 2 is published in PLoS one. Most of the experiments were performed by myself. Daniel Waltho, Christopher Stone, Kenneth Mwawasi, and Jordon Nelson assisted with cloning and protein purification. Dr. James Mahony and I were responsible for the experimental design and generation of the manuscript.

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Chlamydia pneumoniae CopD translocator protein plays a critical role in type III secretion (T3S) and infection

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ABSTRACT

Pathogenic Gram-negative bacteria use type III secretion (T3S) to inject effector proteins into the host cell to create appropriate conditions for infection and intracellular replication. *Chlamydia spp.* are believed to use T3S to infect their host cell, and the translocator proteins are an essential component of this system. *Chlamydia pneumoniae* contains genes encoding two sets of translocator proteins; CopB and CopD, and CopB2 and CopD2. In this study, we identified novel interactions between CopD and three type III secretion proteins; namely, CopN, CdsN, and CdsF. We identified a CopD putative chaperone binding motif, PxLxxP, within the N-terminal region (CopD amino acids 120-125), which was necessary for interaction with its putative chaperone LcrH_1. Using size exclusion chromatography, we showed that CopD and LcrH_1 formed higher order structures in solution with CopD and LcrH_1 binding in a ratio of 1:1, which is unique for T3SS translocator proteins. Lastly, we showed that antibodies to CopD reduced *C. pneumoniae* infectivity by >95%. Collectively, this data suggests that CopD plays a critical role in pathogenesis and likely functions as a hydrophobic translocator of the type III secretion system in *Chlamydia pneumoniae*.

INTRODUCTION

Chlamydia species are obligate intracellular pathogens with a unique biphasic lifecycle initiated by the attachment of the metabolically quiescent elementary body (EB) to the host cell and subsequent invasion into a plasma-membrane derived vacuole termed an inclusion body [1]. Inside the inclusion, EB transform into metabolically active reticulate bodies (RB) that remain associated with the inclusion membrane [1]. *Chlamydia* RB are thought to interact with the host cell cytoplasm across the inclusion membrane using the type III secretion (T3S) injectisome. *Chlamydiae* are capable of commandeering host cell pathways to acquire lipids, cholesterol, and other nutrients crucial for growth and replication and some of these functions may be mediated by T3S. RB continue to replicate until an unknown signal triggers differentiation into EB, which temporally coincides with detachment of the RB and the T3S injectisome from the inclusion membrane [2]. *Chlamydiae* then exit the cell through either lysis or a packaged release mechanism termed extrusion [1]. The complete replication cycle takes approximately 48-72 hours depending on the species.

T3S is a virulence mechanism used by several Gram-negative bacteria, including *Yersinia, E. coli,* and *Salmonella* to inject effector proteins from the bacterial cytoplasm directly into the host cell [3, 4]. The T3SS consists of 20 to 25 components, all of which form a functional T3S injectisome [3, 4]. The needle filament protein (YscF in *Yersnia*) extends from the bacterial outer membrane into the extracellular matrix, and houses a distal needle-tip complex. This needle-tip

complex contains the needle-tip protein (LcrV orthologs) and the translocators (YopB and YopB orthologs), which are involved in sensing the host cell and initiating secretion [5]. Upon host cell contact, a signal is transmitted to the inner membrane effector recognition complex, which consists of several membrane proteins including an ATPase [6]. The ATPase binds effector-chaperone complexes, dissociating the effector from their cognate chaperone followed by unfolding to facilitate their passage through the injectisome [6]. The translocators present at the tip of the complex initiate pore formation in the host cell in preparation for effector secretion.

In contrast to other chaperones that play a role in protein folding and assembly of macro-molecular structures, T3S chaperones maintain proteins in a secretion competent state. Type I chaperones are subdivided into two categories; type IA and type IB [7]. Type IA chaperones are known to bind to one specific effector protein, whereas type IB chaperones are capable of binding to more than one effector protein, such as Slc1 [7]. Type I chaperones share some similar biochemical properties, including a low molecular weight (~20 kDa), an acidic isoelectric point (pI), and interacting with cognate effectors as a homo-dimer [7]. Furthermore, these chaperones bind conserved chaperone binding domains (CBD) that encompass hydrophobic regions on their binding partner, usually located at the N-terminus of their cognate effector. Type II chaperones are chaperones for hydrophobic, oligomeric translocator proteins, and include SycD (*Y. enterocolitica*), LcrH (*Y. pestis*), SicA (*Salmonella spp*.), IpgC (*Shigella spp*.),

Scc2/Scc3 (Specific chlamydia chaperone) (*Chlamydia trachomatis*), and Cpn0811 (LcrH_1), and Cpn1021 (LcrH_2) (*C. pneumoniae*) [4, 7, 8]. Amino acid sequence analysis has shown the presence of tetratricopeptide repeat (TPR) domains present in all type II chaperones, including *Chlamydia* [9]. TPR domains are a common structural motif for protein-protein interactions in chaperones, which are present in eukaryotic chaperones such as Hsp70 and Hsp90 [9, 10]. Furthermore, type II chaperones maintain the hydrophobic translocators in a secretion competent state, and prevent premature hetero- or homo-oligomerization of the translocators in the bacterial cytosol by masking the oligomerization domain [10].

The translocator proteins form a translocon pore in the host cell membrane and are broadly divided into hydrophobic and hydrophilic translocators [10]. The hydrophilic translocator is the sensor protein, which detects the host cell and triggers secretion of effectors through an as yet unknown mechanism [10]. This sensing mechanism has been linked with cholesterol-rich lipid rafts, and other small molecules in the host cell membrane. The hydrophobic translocators are believed to be early effector proteins, consisting of the major hydrophobic translocator (YopB in *Yersinia*) and the minor hydrophobic translocator (YopD in *Yersinia*). In *Yersinia,* YopB and YopD form the translocon pore in the host cell membrane, which is instrumental in docking the injectisome to the membrane via the needle filament protein and allowing translocation of effector proteins into the host cell cytoplasm. The hydrophobic translocators in *Chlamydia* have been poorly

characterized. Genetic analysis of *C. pneumoniae* has identified two putative pairs of translocators; namely, *cpn0809* and *cpn0808* (CopB and CopD, respectively) and *cpn1020* and *cpn1019* (CopB2 and CopD2, respectively) [8]. Cpn0808, annotated as CopD, is believed to be the minor hydrophobic translocator [2, 11]. The minor hydrophobic translocator (YopD orthologs) in other bacteria interact with other key components of the T3SS, including the filament protein, the major hydrophobic translocator, the ATPase, and the plug protein [10, 12, 13]. Initial work on the translocators by Fields *et. al.* indicated that the translocator proteins, CopB and CopD, from *Chlamydia trachomatis* may be type III secreted, as evidenced by a heterologous secretion system. Furthermore, they suggest that the SycD orthologous proteins, Scc2 and Scc3, may function as translocator chaperones [11]. More recent work by Chellas-Gery *et. al.* has expanded our knowledge of CopB*,* and implicated it as a potential translocator protein from *Chlamydia trachomatis* [14]*.* These translocator proteins have dedicated chaperones (LcrH_1 and LcrH_2) that maintain them in a partially unfolded, secretion-competent state until they are translocated to the needle-tip complex.

In this report, we characterize *cpn0808* (CopD), a putative translocator protein of *C. pneumoniae* to assess its role in chlamydial T3S*.* We identified novel interactions between CopD and the type III secretion proteins CopN, CdsN, and CdsF. We identified a specific N-terminal region, $CopD_{1\text{-}157,}$ containing a putative chaperone binding motif, PxLxxP, which is required for interaction with the LcrH_1

chaperone. Collectively, this data supports a role for CopD as a hydrophobic translocator of the T3SS in *Chlamydia pneumoniae*.

METHODS & MATERIALS

Cloning

All T3S genes were cloned from *C. pneumoniae* CWL029 (VR1310, ATCC) using genomic DNA as template. Since full-length CopD was toxic to *E. coli,* fragments excluding transmembrane domains based on TMpred software were cloned. The following genes were cloned into the pDONR201 vector with *attB*-containing primers via the Gateway cloning system (Invitrogen) (subscript denotes amino acid number): *cdsN*, *lcrH_1, copN, cdsF, copD*$_{1-137}$*, copD*$_{158-206}$*, copD*$_{227-444}$*, copD*$_{1-157}$*,* ^{P120A}*copD*$_{1-157}$*,* ^{L122A}*copD*$_{1-157}$*, and* ^{P125A}*copD*$_{1-157}$. The pDONR201 vector containing the respective gene were then cloned into pDEST17 (N-Terminal 6xHis-tag), pDEST15 (N-Terminal GST-tag) and pDEST-HisMBP (N-Terminal 6xHis-Maltose Binding Protein-tag) for protein expression. Full length *copD* and *lcrH_1* was amplified with appropriate restriction sites for cloning into MCS1 (N-Terminal 6xHis-tag) and MCS2 (C-Terminal S-tag), respectively, of the pET-DUET vector (Novagen). All constructs were verified by sequencing at the MOBIX Laboratory at McMaster University.

Protein Expression and Purification

All expression constructs were transformed into *E. coli* Rosetta pLysS strains to minimize protein expression prior to induction with isopropyl β-D

galactosidase (IPTG). Briefly, 6 L of LB containing 100 µg/mL ampicillin was inoculated with 1:50 dilution of an overnight culture. The culture was then grown at 37°C with shaking at 250 RPM until an absorbance at 600 nm of 0.600 was reached. Prior to induction with 0.2 mM IPTG, the cultures were cooled to 16°C on ice. After induction, the cultures were left incubating at room temperature with shaking at 250 RPM for 3 hours. After induction, the bacteria were pelleted at 8000 x *g* in a Sorval RC-5B centrifuge at 4°C. The bacterial pellets were washed once with cold phosphate-buffered saline (PBS) and then resuspended in either Nickel A (20 mM TRIS-HCl pH 7.0, 500 mM KCl, 0.03% LDAO, 10 mM imidazole, 10% glycerol) or PBS, depending on the downstream application. The bacteria were then subjected to sonication and pelleted at 50000 x *g* to isolate the soluble protein. Polyhistidine-tagged proteins were purified using fast protein liquid chromatography (FPLC) using a Ni-NTA His-Trap HP column (GE Healthcare), and washed with 5%,10%, and 15% Nickel B before elution in 100% Nickel B (20 mM TRIS-HCl pH 7.0, 500 mM KCl, 0.03% LDAO, 300 mM imidazole, 10% glycerol). Prior to size exclusion chromatography, proteins were buffer exchanged into phosphate buffered saline with 0.03% LDAO.

Glutathione-S-transferase (GST) pull-down assay

Glutathione-agarose beads (Sigma) were swollen in distilled water for 2 hours at room temperature, and then washed with PBS. GST-tagged proteins were bound to 1 mL of glutathione-agarose beads for two hours at 4ºC while rocking. The GST-bound agarose beads were centrifuged at 3000 x *g* for 5 minutes to

remove the supernatant and then blocked with 5% bovine serum albumin (BSA) in PBS + 0.1% TWEEN-20 overnight at 4ºC while on a rocking platform. Blocked beads (50 µL) were mixed with 1 mL of *E. coli* lysates containing our overexpressed His-tagged protein, and left rocking at 4ºC for 2 hours. The glutathione-agarose beads were then centrifuged at 16000 x *g* for 10 seconds and the supernatant was removed, and the pellet washed with high salt wash buffer (500 mM KCl, 20 mM TRIS-HCl pH 7.0, 0.1% Triton X-100). Washing was repeated seven times. The last supernatant wash was collected, trichloroacetic acid (TCA) precipitated, and analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot to confirm that His-tagged protein was removed. The glutathione-agarose beads were then resuspended in 30 µL of Laemmli buffer and heated at 90ºC for 20 minutes. The samples were then analysed by SDS-PAGE and Western blot analysis using a mouse anti-His antibody (Sigma).

Bioinformatics

To identify orthologous sequences, CopD was subjected to BLASTP (Basic Local Alignment Search Tool Protein) and PSI-BLAST using the default parameters, and excluding *Chlamydia* spp. from the search. Structural characteristics within CopD were analyzed using the TMpred tool to identify transmembrane domains, using a minimum transmembrane window of 17 and maximum of 33. COILS online software was used to predict the presence of coiled-coil domains within CopD, using MTIDK scoring matrices, and weighting for positions a & d [14].

Size exclusion chromatography

Full length His-CopD and LcrH_1-S-tag (300 µL, 4.5 mg/mL) mixture was subjected to size exclusion chromatography in PBS + 0.03% LDAO (Sigma) on a Superdex S200 10/300 GL gel filtration column (Amersham Biosciences, Piscataway, New Jersey) at 0.5 mL/min [15]. Elution fractions of 1 mL were collected at a flow rate of 0.5 mL/min, with each fraction analyzed via an anti-His and anti-S-tag Western blot. Size prediction of elution complexes was performed after the column was standardized using a LMW and HMW gel filtration standard kit (GE Life Sciences) [15].

Inhibition of *C. pneumoniae* infection

A polyclonal antibody to a 15 amino acid peptide (SSKGEKSEKSGKSKC) was produced and obtained from GenScript (New Jersey). *C. pneumoniae* was pre-incubated for 2 hours at 37°C with various dilutions of affinity purified CopD antibody, control antibody, or pre-immune sera. Infection was performed as previously described by Johnson *et. al.* [16]. After 72 hours, chlamydial inclusions were stained with the Pathfinder *Chlamydia* detection reagent (BioRad). Multiple fields of view were visualized and percent reduction of infection was calculated compared to control infection. Statistical significance was calculated using a Student's t-test.

RESULTS

Bioinformatic analysis of CopD

Translocator proteins are highly conserved across bacterial species because of their critical role in T3S [3, 4]. We used a bioinformatic approach to examine similarities between translocators from *Chlamydia* and other bacterial species. Despite the inability to identify an orthologous protein through PSI-BLAST and BLASTP, CopD contains many structural and sequence characteristics consistent with a translocator protein. Cpn0808 is a 444 amino acid protein consisting of two *in silico* predicted transmembrane domains and one coiled-coil domain. The TMpred server was applied for transmembrane domain prediction with a minimum and maximum hydrophobic window of 17 and 33 amino acids, respectively. Consistent with orthologous minor hydrophobic translocators, *viz.* YopD or IpaC, Cpn0808 contains two transmembrane domains consisting of amino acids 138-157 and 207-226 [10, 14]. Furthermore, the coiled-coil domain prediction program (COILS) identified a 21 amino acid coiled-coil domain spanning amino acids 278-298 (Figure 1). It is known that translocator chaperones bind within the N-terminus of typical translocator proteins [17]. Previous studies on bacterial T3SS translocators have identified a conserved chaperone binding motif; namely, PxLxxP [17]. We identified that same motif within Cpn0808 (PSLPTP) at amino acid 120, which could function as the chaperone binding site. Collectively, the bioinformatic analysis of CopD suggests that CopD is a hydrophobic translocator protein of the type III secretion system.

Interaction of CopD with Type III Components

To investigate protein interactions of CopD with other T3SS proteins we performed *in vitro* interaction studies using glutathione-S-transferase (GST) pull-down assays. We identified novel interactions between Cpn0808 and CopN, CdsF, CdsN. These assays were performed under high salt conditions (500 mM NaCl) in the presence of 0.1% Triton X-100 to eliminate non-specific interactions. Due to the difficulty of working with full-length CopD in *E. coli*, fragments of CopD were cloned and used for pull down assays. We identified an interaction between $CopD_{1-157}$, $CopD_{158-206}$ and the needle protein, CdsF (Figure 2A). In orthologous systems, the minor hydrophobic translocator is known to interact with the plug protein, and we found that amino acids 158-206 of CopD interacts with CopN (Figure 2B) [18]. Furthermore, the N-terminal fragment of $CopD_{1-137}$ interacted with the T3SS ATPase, CdsN (Figure 2C). The observation that CopD interacts with three key proteins of the T3S apparatus suggests that CopD plays an important role in chlamydial T3S.

Interaction of LcrH_1 and CopD

Analysis of YopD orthologs indicates the presence of a conserved CBD containing a PxLxxP motif [17]. Bioinformatic analysis of CopD revealed a PxLxxP motif extending from amino acid 120 to 125. Before determining the essential amino acids in this PxLxxP motif, we showed that LcrH_1 interacted with full-length CopD using a co-expression and purification in the presence of high salt (500 mM KCl) and detergent (0.03% LDAO) (data not shown). To identify the specific binding region of LcrH_1 on CopD, fragments of CopD were made to identify the interacting

domains. GST-CopD$_{1-157}$ interacted with His-LcrH_1 in the presence of high salt (500 mM KCl) and detergent (0.1% Triton X-100), but did not interact with GST-CopD$_{1-137}$, or GST-CopD$_{227-444}$ (Figure 3A). Together, these results suggest that amino acids 1-157 of CopD contains a required domain for interaction with the putative chaperone LcrH_1.

To identify the necessity of the chaperone binding motif, an alanine walkthrough of the conserved amino acids in the CopD PxLxxP motif was performed to examine the requirement of the three conserved amino acids for binding LcrH_1. To ensure that the CBD variants retained the ability to interact with previously identified binding partners, we performed a GST pull down assay of GST-$^{\Delta P120A}$CopD$_{1-157}$ and CdsF. Figure 3C demonstrates that the variants retained the ability to interact with CdsF. His-LcrH_1 interacted with GST-CopD$_{1-157}$, but not with GST-$^{\Delta P120A}$CopD$_{1-157}$, GST-$^{\Delta L122A}$CopD$_{1-157}$, and GST-$^{\Delta P125A}$CopD$_{1-157}$ (Figure 3B). These results suggest that amino acids 120,122 and 125 of CopD are required for interaction with LcrH_1. However, in the absence of being able to show that each variant does not result in a major conformational change in the molecule thereby precluding interaction with CdsF, we cannot say with certainty that these amino acids are essential for CopD binding with its partners.

Oligomerization of CopD

Since translocators of other bacterial secretion systems are capable of forming higher order structures including homo- or hetero-oligomers, we explored

whether CopD could form homo-oligomers. To determine whether CopD forms higher-order structures, His-CopD was co-expressed with LcrH_1-S-tag and subjected to size-exclusion chromatography in the presence of 0.03% LDAO. His-CopD eluted from the column with peaks corresponding to a decamer, tetramer and monomer, as determined by Western Blot using antibodies to the poly-histidine tag. When the membrane was probed with an anti-S-tag antibody, LcrH_1 was detected only in the fraction containing monomeric CopD (Figure 4). Similar results were obtained when His-CopD and LcrH_1-S-tag were subjected to size exclusion chromatography in the presence of 0.1% Triton X-100 (data not shown). This data indicates that CopD is capable of forming higher order structures in solution and that LcrH_1 binds only to monomeric CopD [19, 20].

Neutralization of *C. pneumoniae* infection with antibodies to CopB and CopD

Since T3S translocators are believed to be surface exposed and antibodies generated against these antigens could potentially block invasion of host cells, we investigated the possibility that antibodies to CopD might inhibit *C. pneumoniae* infection. We used affinity purified polyclonal antibodies raised against an N-terminal CopD peptide (SSKGEKSEKSGKSKC) to investigate whether these antibodies could block infection. The CopD 16-mer within the N-terminal region was predicted to be immunogenic since it is hydrophilic with a net positive charge. Based on these characteristics, this sequence of amino acids is most likely solvent exposed and capable of interacting with an antibody. We first showed that the anti-CopD antibodies reacted with both recombinant and native CopD from *C.*

pneumoniae by Western blot analysis prior to performing infection inhibition assays (Figure 5, Panel F). Pre-incubation of *C. pneumoniae* EB with affinity purified anti-CopD antibody inhibited infection by 98%, as compared to control antibody, as determined by IF staining of inclusion bodies (Figure 5). This suggested that CopD may be either surface exposed or secreted and that it plays a critical role in infection of cells.

DISCUSSION

T3S systems have been well-characterized for several bacterial systems, but chlamydial T3S remains poorly characterized, and little is known about the chlamydial translocator proteins. In this study, we characterized the putative minor hydrophobic translocator CopD (Cpn0808) of *C. pneumoniae*. We showed that CopD interacted with other *Chlamydia* type III secretion proteins including, CopN, CdsF, and CdsN. Bioinformatic analysis revealed that CopD contains structural features consistent with other minor hydrophobic translocators, including two transmembrane domains and a coiled-coil domain. In addition, CopD interacted with its putative chaperone, LcrH_1, first, by an essential chaperone binding motif of PxLxxP at amino acids 120-125, and second, via a predicted transmembrane domain at amino acids 138-157. We also showed that LcrH_1 interacts with only monomeric CopD in a 1:1 ratio and not tetrameric or decameric CopD, as evidenced by size exclusion chromatography. Finally, we show that polyclonal antibodies directed against an N-terminal epitope of CopD inhibited chlamydial

infection. Collectively, these findings are consistent with CopD functioning as a hydrophobic translocator of the *C. pneumoniae* T3S apparatus.

Since T3S knock-outs cannot be made in *Chlamydia* it is not possible to unequivocally demonstrate the role of individual T3S components. We have used *in vitro* protein interactions to demonstrate interactions between CopD and three other T3S proteins. CdsN, the ATPase of the *Chlamydia* T3SS, plays a key role in substrate selection and mediates effector-chaperone disassociation prior to secretion, allowing effectors to be translocated through the injectisome. We demonstrated that the N-terminal fragment of CopD interacted with CdsN. This suggests that CopD is delivered to the base of the needle apparatus, possibly associated with its putative chaperone, allowing CdsN to dissociate the effector-chaperone complex to initiate secretion. The filament protein, CdsF, and its orthologs in other bacteria, form the needle of the injectisome and is believed to play a role in facilitating the insertion of translocators into the host cell membrane [21]. We have demonstrated that CopD interacts with CdsF, and have identified two specific regions of CopD, *viz.* $CopD_{1\text{-}157}$ and $CopD_{158\text{-}206}$ that facilitate this interaction. In *Yersinia spp.*, the plug protein, YopN, has been shown to interact with YopD [18]. We explored this interaction in *C. pneumoniae* using a GST pull down assay and confirmed the interaction between $CopD_{158\text{-}206}$ and CopN (Supplemental Figure 1). A summary of the interactions identified in this study appear in Figure 6. It is interesting to note that all of the protein interactions that we have identified occur within the N-terminus of CopD, and we have not identified

any proteins that interact within the C-terminus of CopD. Based on orthologous T3S systems, we believe that this C-terminal domain of CopD is involved in homo-oligomerization, membrane interaction, or other effector functions consistent with orthologs as suggested by other T3S systems [22-27].

A bioinformatic analysis of CopD demonstrated predicted structural and sequence motifs found in other hydrophobic translocator proteins [22, 28, 29]. PSI-BLAST or BLASTP failed to identify a CopD orthologous protein outside of the *Chlamydiales* order, but revealed that CopD contains two predicted transmembrane domains (amino acids 138-157 and 207-226) and a predicted coiled-coil domain (amino acids 207-226), characteristic structures of other hydrophobic translocators. The toxicity of full-length CopD in *E. coli* could be due to the presence of two transmembrane domains of CopD, which may insert into the bacterial membrane, forming homo-oligomers and thus disrupting normal membrane function and causing cell death. This phenomenon has been observed with other translocator proteins when they are not co-expressed with their putative chaperone [30]. Since expression of full-length CopD alone yielded insoluble protein, CopD was co-expressed with its putative chaperone, LcrH_1. In principle SEC allows for the estimation of molecular weight from the elution volume. Since Western blot results indicate the presence of both CopD and LcrH_1 in the third elution peak, it is most likely that CopD (44.5 kDa) and LcrH_1 (25.9 kDa) are present as a hetero-dimer with a combined molecular weight of 70 kDa. If CopD or LcrH_1 formed homo-dimers they would be expected to elute in a different

volume. In addition, any LcrH_1 in the mixture must be complexed with His-CopD since the complex was purified on Ni-NTA agarose. Our SEC data suggesting that CopD and LcrH_1 interact in a 1:1 molar ratio, is consistent with a 1:1 molar ratio shown for IpaB and IpgC in *Shigella* [30, 31]. Another difference found in *Chlamydia* T3SS is that they possess two sets of predicted translocators, despite only having a single T3SS [2, 8, 14]. Furthermore, we found that CopD formed higher order structures in the absence of LcrH_1, corresponding to decamers and tetramers. Together, this data suggests that the role of LcrH_1 is to maintain CopD in a monomeric state, preventing homo-oligomerization of CopD prior to secretion. The presence of a conserved chaperone binding motif, PxLxxP, within the N-terminal region supports the possibility of CopD being a translocator protein. Furthermore, the coiled-coil domain located near the C-terminus may function to bring the C-terminal domain of the protein in contact with the membrane to mediate its effector function, such as actin polymerization. Further studies are required to elucidate the function of this C-terminal region. Collectively, the bioinformatics data supports that CopD may serve as a hydrophobic translocator for chlamydial T3S.

It is well known that LcrH_1 orthologs interact within the N-terminal region of translocator proteins. In this report, we show that LcrH_1 interacts with the N-terminus of CopD (amino acids 1-157). A hydrophobic sequence of amino acids, 138-157, of CopD appear to play a critical role in the interaction with LcrH_1, as GST-CopD$_{1\text{-}157}$ but not CopD$_{1\text{-}138}$ interacted with His-LcrH_1. In other T3S systems, LcrH_1 is believed to function by masking hydrophobic amino acids and

preventing premature homo- and hetero-oligomerization prior to secretion, corroborating the importance of amino acids 138-157 for interaction.

In addition to amino acids 138-157, the interaction is dependent on amino acids 120-125 within the PxLxxP motif, suggestive of two possible binding domains. A mutational analysis of the conserved CBD motif, PxLxxP (P120→A, L122→A, P125→A), of CopD was undertaken to examine the role of these conserved amino acids. An alanine walk through of the conserved amino acids of the PxLxxP motif was performed. Substitution of each of the three amino acids in the PxLxxP motif (P120→A, L122→A, P125→A) abolished the interaction with LcrH_1, indicating the essential role of these amino acids in the CopD/LcrH_1 interaction. To our knowledge, this is the first demonstration of conserved amino acids within the predicted CBD motif of a chlamydial translocator protein. In *C. pneumoniae,* two proteins, CopB and CopD, are believed to form the translocon pore in the host cell. Like CopD, CopB (Cpn0809) is in a contiguous operon with its predicted chaperone, LcrH_1. It is not surprising that starting at amino acid 166, CopB contains a putative chaperone binding domain motif of PxLxxP. However, further studies are required to identify the functionality of this motif in CopB. Examining the predicted orthologous proteins to CopD in the *Chlamydiaceae* family reveals similarities in the predicted chaperone binding domain (Table 1). Of the members of the *Chlamydiaceae, C. pneumoniae, C. psittaci, C. pecorum, and C. abortus* all contain the same PxLxxP, but interestingly, *C. trachomatis* and *C. muridarum* contain an AxLxxP motif. The variability in the motif in *C. trachomatis* is

not surprising since the orthologs in *C. trachomatis* and *C. muridarum* have only 56% and 54% sequence identity, respectively, which is the lowest sequence identity among the sequences examined from the *Chlamydiaceae* family. The high sequence identity (>70%) between LcrH_1 orthologs within the *Chlamydiaceae* family, and the conserved CBD domain, or variant thereof, located within the N-terminal region of the CopD orthologs suggests that the CopD-LcrH_1 interaction may be conserved within the *Chlamydiaceae* family.

We have shown that antibodies to CopD inhibit the ability of *C. pneumoniae* to infect cells, which is consistent with observations seen with antibodies to orthologous translocator proteins in other bacteria including *Shigella* (IpaB and IpaD) and *Yersinia* (YopD) [32-34]. Since CopD antibodies are unlikely to enter EBs, neutralization with CopD antibodies suggests that that CopD is either surface exposed or secreted during the infection cycle. This observation is consistent with *Chlamydia* being an obligate intracellular pathogen that is dependent on T3S to infect cells. The production of CopD late during the replication cycle when RB differentiate into infectious EB is also consistent with a role for CopD in T3S and infection [8]. Given the essential nature of the translocator proteins in T3S, these proteins may represent an excellent target for drug development and a vaccine candidate.

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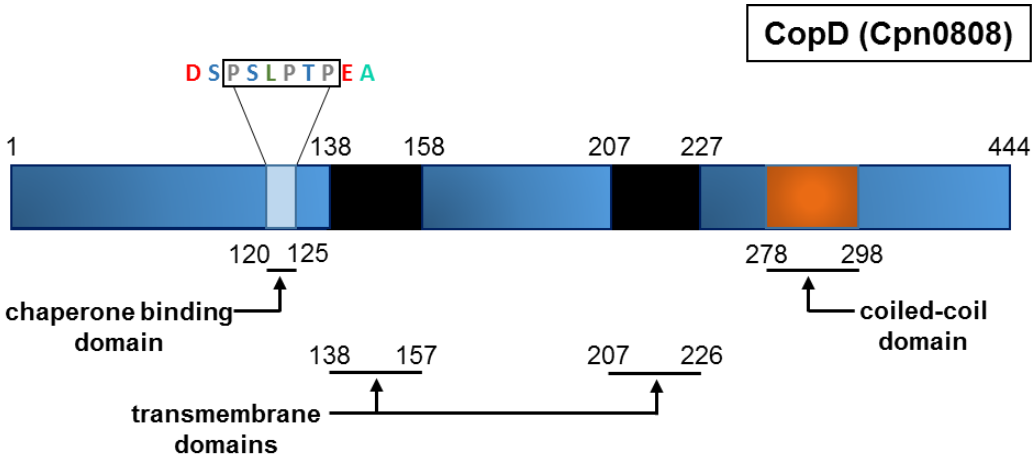


Figure 1: Topographic overview of structural prediction of CopD. Solid black regions represent transmembrane domains. Orange block represents a predicted coiled-coil domain in the C-terminus of the protein. Light blue depicts predicted Chaperone Binding Domain (CBD) located from amino acids 120-125.

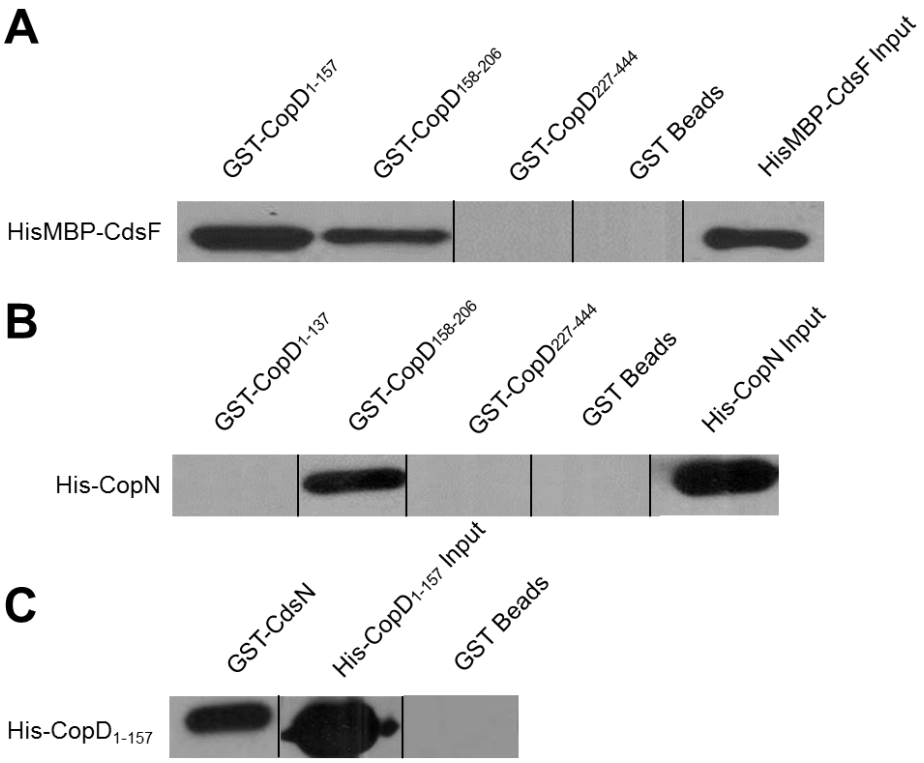


Figure 2: *Chlamydia* Outer Protein (Cop) D Interacts with T3S proteins CdsF, CopN, and CdsN. GST-CopD$_{1-157}$ or GST-CopD$_{158-206}$ bound to glutathione-agarose beads (bait) pulled HisMBP-CdsF (prey) out of an *E. coli* lysate in the presence of a high salt wash buffer (500mM NaCl). Furthermore, GST-CopD$_{158-206}$ pulled His-CopN out of an *E. coli* lysate in the presence of a high salt wash buffer. Lastly, GST-CdsN pulled His-CopD$_{1-157}$ out of an *E. coli* lysate in the presence of a high salt wash buffer.

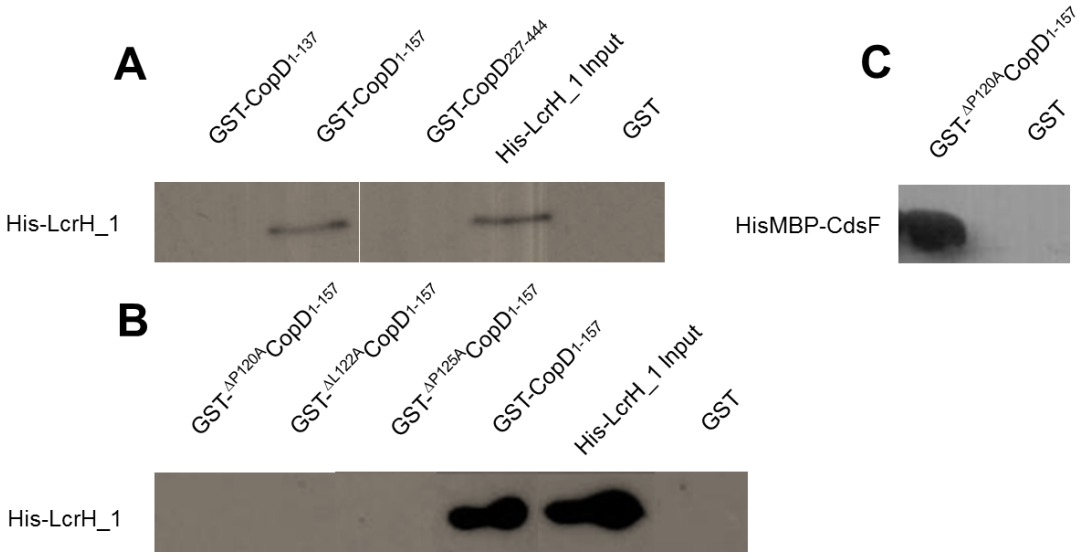
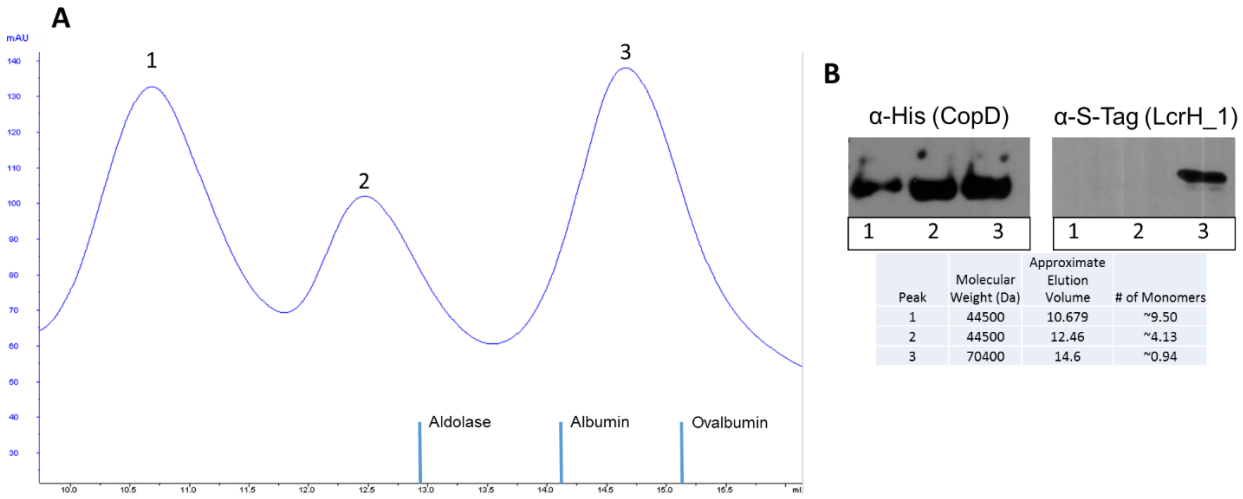


Figure 3: LcrH_1 (*Cpn0811)* interacts with CopD at amino acids 120-125. Recombinant LcrH_1 interacted with amino acids 1-157 of CopD. CopD mutants were created through overlapping PCR to create $^{P120A}CopD_{1\text{-}157}$, $^{L122A}CopD_{1\text{-}157}$, and $^{P125A}CopD_{1\text{-}157}$. Mutations at the conserved amino acids within the predicted chaperone binding domain disrupted the interaction between $CopD_{1\text{-}157}$ and the chaperone LcrH_1, but not other identified interactions.



Peak	Molecular Weight (Da)	Approximate Elution Volume	# of Monomers
1	44500	10.679	~9.50
2	44500	12.46	~4.13
3	70400	14.6	~0.94

Figure 4: Interaction of CopD and LcrH_1 occurs in a 1:1 ratio assessed by size exclusion chromatography of co-expressed proteins. CopD and LcrH_1 were co-expressed, purified, and subjected to size exclusion chromatography. Elution peaks were analyzed by Western Blot using anti-His antibody for CopD and anti-S-tag for LcrH_1. Panel A shows size exclusion chromatography elution peaks. Panel B shows the Western Blot result for the three peaks, and the corresponding the number of CopD monomers present in peaks 1, 2, and 3.

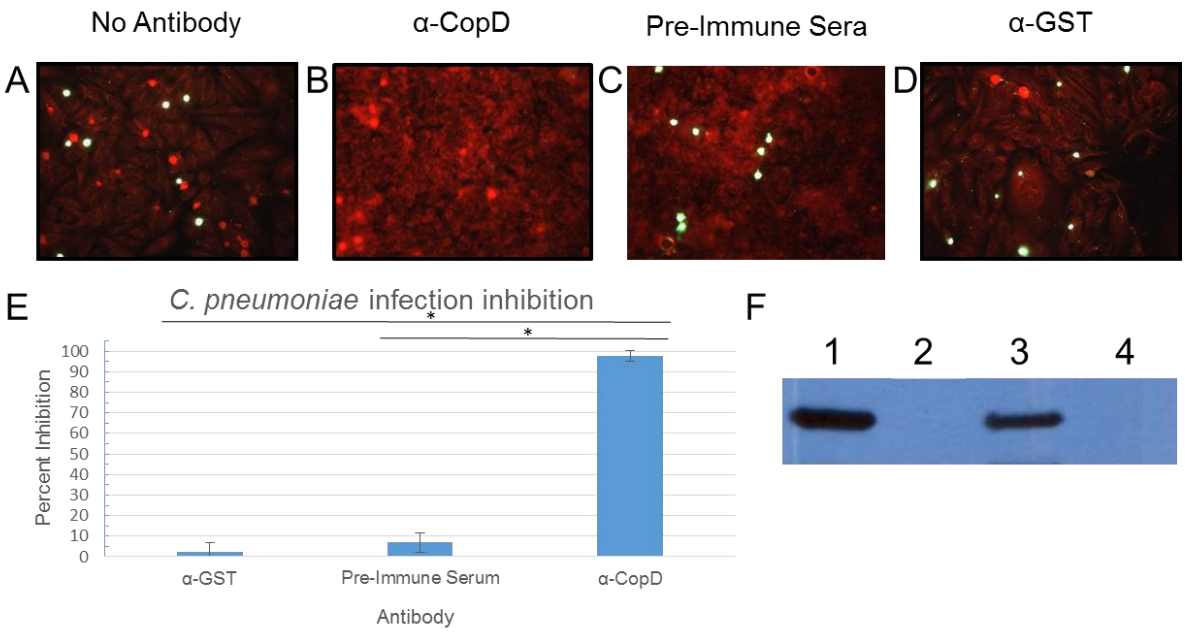


Figure 5: Inhibition of *Chlamydia pneumoniae* with CopD antibodies.

Panels A-D show inhibition assay results performed with either no antibody (A), CopD antibody (B), pre-immune sera (C), or control antibody (α-GST) (D). Panel E shows the degree of inhibition by of CopD antibodies compared to control antibodies. Chlamydial inclusions are stained green, while HeLa cells are stained red by Evan's blue counterstain. Panel F demonstrates reactivity of anti-CopD with (1) *C. pneumonia* infected HeLa cell lysate, (2) uninfected HeLa cell lysate, (3) recombinant GST-$CopD_{1\text{-}157}$ produced in *E. coli*, and (4) recombinant GST produced in *E. coli.* Experiments were performed in triplicate. Error bars represent 2 standard deviations. Images represent random fields of view. * = $P < 0.0001$

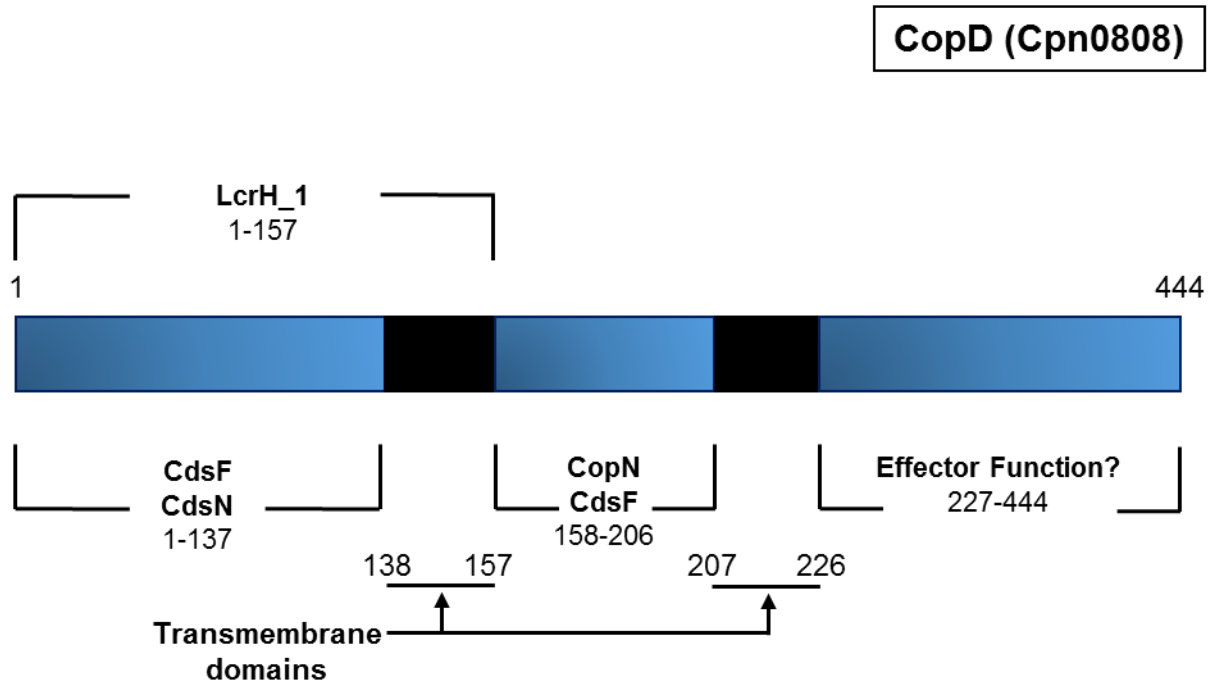
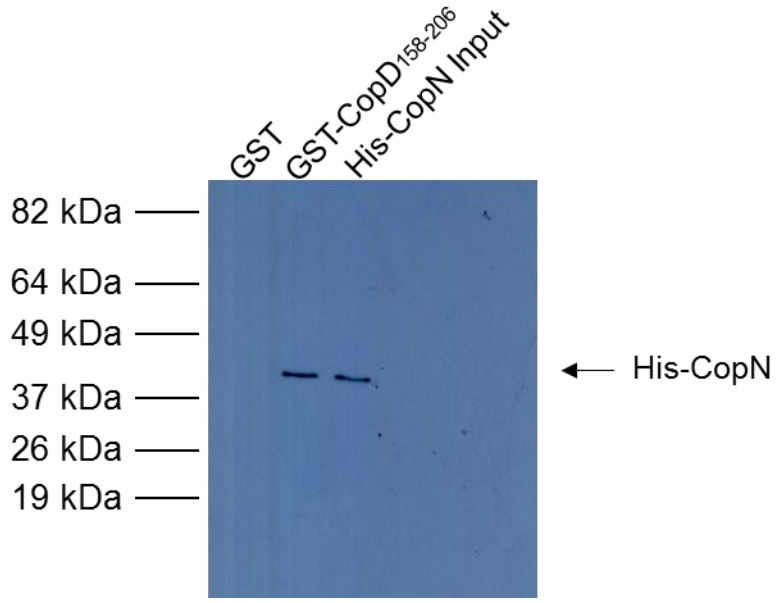


Figure 6: Summary of proposed interactions between CopD and type III secretion proteins. Interactions identified through Glutathione-S-Transferase pull down assays mapped to a topographic overview of CopD.

	P1		P3			P6	Percent Identity
CopD (*C. pneumoniae*)	P	S	L	P	T	P	100%
CT579 (*C. trachomatis*)	A	T	L	P	S	P	54%
CopD1 (*C. psittaci CP3*)	P	Q	L	P	T	P	63%
TC_0868 (*C. muridarum*)	A	S	L	P	S	P	56%
CPE3_0915 (*C. pecorum P787*)	P	Q	L	P	S	P	58%
CAB924 (*C. abortus S26/3*)	P	Q	L	P	T	P	63%
YopD (*Y. enterocolitica*)	P	E	L	I	K	P	15%
SipC (*S. enterica*)	P	T	L	S	A	P	12%

Table 1: Comparison of putative chaperone binding domains between *Chlamydiaceae* and other T3SS containing Gram-negative bacteria. Putative chaperone binding domains were identified within the N-terminal regions of orthologous proteins to CopD from *C. pneumoniae.* P1, P3, P6, represent positions 1, 3, and 6, respectively of the PxLxxP motif. Percent identity refers to amino acid sequence identity comparing CopD to full length sequences of orthologous proteins.



Supplemental Figure 1: *Chlamydia* Outer Protein (Cop) D Interacts with CopN in a pull down assay. GST-CopD$_{158-206}$ bound to agarose beads reacted with an *E. coli* lysate over-expressing His-CopN in the presence of a high salt wash buffer (500 mM KCl) (middle lane). His-CopN did not interact with Glutathione-S-Transferase (GST) beads in the absence of CopD (left lane). His-CopN input is shown in right lane. The anti-His antibody was specific and did not react with other proteins.

CHAPTER THREE

Preface to Chapter 3

In this chapter, a comprehensive biochemical characterization of one of the translocator proteins from *Chlamydia* pneumoniae is described. Various biochemical techniques were utilized to characterize CopB, including GST-pulldown assays for protein interaction studies. Furthermore, site-directed mutagenesis was used to examine the necessity of specific amino acids for the interaction between LcrH_1 and CopB. Using a synthetic peptide derived from the chaperone binding domain of CopB, the peptide was pre-incubated with both the translocator proteins to examine its ability to block interaction with their chaperone, LcrH_1. Furthermore, monolayers of HeLa cells were infected with C. pneumoniae and then treated with the synthetic peptide to explore its ability to inhibit *Chlamydia* infection. Lastly, antibodies generated towards an N-terminal epitope of CopB were incubated with C. pneumoniae to examine their ability to neutralize infection.

The publication presented in Chapter 3 is published BMC Microbiology. Most of the experiments were performed by myself. Daniel Waltho, Christopher Stone, Steven Liang, Christopher Chiang, Kenneth Mwawasi, Jordon Nelson, Steven Zhang, Samantha Mihalco, and Zachariah Scinocca assisted with cloning and protein purification. Dr. James Mahony and I were responsible for the experimental design and generation of the manuscript.

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Chlamydia Outer Protein (Cop) B from *Chlamydia pneumoniae* possesses characteristic features of a type III secretion (T3S) translocator protein

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ABSTRACT

BACKGROUND: Chlamydia *spp*. are believed to use a conserved virulence factor called type III secretion (T3S) to facilitate the delivery of effector proteins from the bacterial pathogen to the host cell. Important early effector proteins of the type III secretion system (T3SS) are a class of proteins called the translocators. The translocator proteins insert into the host cell membrane to form a pore, allowing the injectisome to dock onto the host cell to facilitate translocation of effectors. CopB is a predicted hydrophobic translocator protein within the chlamydial T3SS.

RESULTS: In this study, we identified a novel interaction between the hydrophobic translocator, CopB, and the putative filament protein, CdsF. Furthermore, we identified a conserved PxLxxP motif in CopB (amino acid residues 166-171), which is required for interaction with its cognate chaperone, LcrH_1. Using a synthetic peptide derived from the chaperone binding motif of CopB, we were able to block the LcrH_1 interaction with either CopB or CopD; this CopB peptide was capable of inhibiting *C. pneumoniae* infection of HeLa cells at micromolar concentrations. An antibody raised against the N-terminus of CopB was able to inhibit *C. pneumoniae* infection of HeLa cells.

CONCLUSION: The inhibition of the LcrH_1:CopB interaction with a cognate peptide and subsequent inhibition of host cell infection provides strong evidence that T3S is an essential virulence factor for chlamydial infection and pathogenesis. Together, these results support that CopB plays the role of a hydrophobic translocator.

KEYWORDS: *Chlamydia,* type III secretion, antibody, antigen, translocator

COMPETING INTERESTS: The author(s) declare that they have no competing Interests.

INTRODUCTION

Chlamydia infections represent a significant disease burden worldwide. *C. trachomatis* infection can lead to pelvic inflammatory disease (PID), salpingitis, and infertility in women and epididymitis and infertility among men [1]. Furthermore, *Chlamydia pneumoniae* is a respiratory pathogen causing approximately 10% of community acquired pneumonia [2]. Additionally, *C. pneumoniae* infections have been associated with Asthma exacerbations, cardiovascular disease, Multiple Sclerosis, and Alzheimer's [3-7]. Combined, *Chlamydia pneumoniae* and *Chlamydia trachomatis* represent a significant disease burden. An essential component of *Chlamydia*'s survival is creating an environmental niche that exhibits the necessary requirements for replication and survival. Type III secretion (T3S) is a complex mechanism utilized by important Gram-negative bacterial pathogens. *Salmonella*, *Shigella*, *Yersinia*, *Pseudomonas*, and *Chlamydia* all contain the highly conserved type III secretion system (T3SS) of approximately 20-30 proteins [8-11]. To manipulate its host's environment, these bacteria secrete toxic effector proteins directly into their target cell. Functionally, the whole apparatus can be referred to as an injectisome; however, it consists of smaller functional components, which include the cytoplasmic C-ring, the inner and outer membrane rings, the needle complex, and needle-tip complex [8-10, 12]. Each of these components display numerous essential protein-protein interactions. Despite the identification and characterization of many putative T3S proteins, it remains unclear whether

Chlamydia truly has a functional T3SS, and whether it plays a role in replication and survival given the absence of a robust genetic manipulation system for gene knockouts [13].

Chlamydia spp. undergo a unique biphasic life-cycle starting with an infectious, non-metabolically active elementary body (EB) [14-16]. Upon attachment of the EB to the host cell, there is a conformational change within the host membrane that allows for invasion of the EB into a membrane-derived vacuole termed an inclusion [16]. Once inside the inclusion, an as yet unknown signal triggers differentiation of the EB into a metabolically-active, non-infectious reticulate body (RB) that divides through binary fission until late in the infection cycle [16]. The infectious EB will then leave either through a packaged release mechanism, called extrusion, or through cell lysis, to repeat the infection cycle [17-19]. Throughout this process the T3SS to play an essential role in pathogenicity [12].

The translocator proteins of the T3S system are believed to be critical to the survival of *Chlamydia,* by forming a pore in the host cell membrane to allow for translocation of effector proteins from the bacterial cytosol to the host cell cytoplasm [8-10]. Analysis of the chlamydial genome suggests that there may be two sets of translocator proteins, CopB and B2 and CopD and D2, both of which are located in the same operon as a predicted class II chaperone [20]. To date, there has been limited characterization of the translocator proteins from *Chlamydia spp..* Early work on the translocator proteins in *Chlamydia* indicated

that both CopB and CopB2 can be secreted from *Yersinia spp.* in a T3S-dependent manner and that Scc2 co-precipitated with CopB from a *C. trachomatis* infected monolayer [21]. More recently, localization experiments have shown that CopB and CopB2, when ectopically expressed in HeLa cells, associate with the cytoplasmic and inclusion membrane, respectively [22]. Our laboratory has previously characterized the minor hydrophobic translocator (CopD) from *Chlamydia pneumoniae*. We have shown that it associates with T3S components and contains an essential PxLxxP motif for interaction with its class II chaperone, LcrH_1 [23]. Although there is limited information on the biochemical characterization of chlamydial translocator proteins owing to the inherent difficulties of working *Chlamydia spp.*, many hypotheses can be made regarding the possible function of the translocator proteins based on orthologous T3SS translocator proteins. However, Chlamydia spp. are unique among T3SS containing Gram-negative bacteria; thus, further studies are required to fully understand the function of the translocator proteins.

In this report, we characterize the putative T3SS translocator protein CopB of *C. pneumonia,* explore interactions between CopB and other T3SS proteins, and characterize the chaperone binding domain of CopB. In addition, we generated a novel peptide mimetic that blocks the interaction between the translocators, CopB and CopD, and their chaperone, LcrH_1, and showed that the peptide mimetic prevents infection. We also identify a CopB epitope which is

immunogenic and elicits neutralizing antibodies that block *C. pneumoniae* infection supporting an essential role for CopB in the infection of host cells

METHODS & MATERIALS

Cloning

T3SS genes were cloned via PCR using genomic DNA from *C. pneumoniae* CWL029 [23]. Fragments of CopB, excluding the transmembrane domains, were cloned due to toxicity of full length CopB in *E. coli.* Using the Gateway cloning system (Invitrogen) the following genes were cloned into the pDONR201 vector with *attB*-containing primers (note: subscript denotes amino acid number): *lcrH_1, copN, copD*$_{1\text{-}157}$*, cpn0803, copB*$_{1\text{-}180}$*, copB*$_{1\text{-}200}$*, copB*$_{1\text{-}255}$*, copB*$_{275\text{-}382}$*, copB*$_{407\text{-}493}$*,* ^{P166A}*copB*$_{1\text{-}200}$*,* ^{L168A}*copB*$_{1\text{-}200}$*, and* ^{P168A}*copB*$_{1\text{-}200}$. Each of the pDONR201 vectors were then used in an LR reaction to transfer the gene into expression vectors, pDEST17 (N-Terminal 6xHis-tag), pDEST15 (N-Terminal GST-tag) and pDEST-HisMBP (N-Terminal 6xHis-Maltose Binding Protein-tag). Prior to protein expression, all constructs were verified by Sanger sequencing at the MOBIX laboratory (McMaster University).

Protein Expression and Purification

All constructs were transformed into *E. coli* BL21 and recombinant protein was expressed following induction with Isopropyl β-D-thiogalactopyranoside (IPTG). Protein expression and purification were performed as described by Bulir *et. al.* (2014), with the following modifications [23]. Briefly, 6 L of LB containing 100

µg/mL ampicillin was inoculated with 1:100 dilution of an overnight culture and split equally into 6x 2 L flasks. The cultures were then grown at 37°C with shaking at 250 RPM until an optical density of 0.500 at 600 nm was reached. Cultures were induced with 0.2 mM IPTG and were left incubating at room temperature, shaking at 250 RPM for 3 hours.

Glutathione-S-transferase (GST) pull-down assay

Glutathione-S-transferase pull-down assays were performed as described by Bulir *et. al.* (2014) [23]. Briefly, GST-tagged proteins were bound to 1 mL GST beads for one hour at 4^{o}C on a mixing platform. GST beads were centrifuged at 3000 x *g* for 5 minutes to remove the supernatant and then blocked with blocking solution (5% BSA in PBS + 0.1% TWEEN-20) overnight at 4^{o}C. Blocked beads (50-100 µL) were mixed with *E. coli* lysates containing overexpressed His-tagged protein for one hour. For experiments involving the blockade of interaction between GST- and His-tagged constructs, the chemically synthesized peptide was incubated with the bait construct for 1 hour at 4^{o}C prior to the addition of the overexpressed His-tagged *E. coli* lysate. The beads were then centrifuged at 16000 x *g* for 10 seconds, the supernatant was removed, and the pellet was washed with high salt wash buffer (500 mM KCl, 20 mM TRIS-HCl pH 7.0, 0.1% Triton X-100). The washing procedure was repeated seven times to ensure complete removal of adventitiously bound protein. For GST pull-downs involving synthetically produced peptide, the peptides were used at a concentration of 500 uM. The glutathione-agarose beads were then resuspended in 75 µL of SDS-

PAGE loading dye. The samples were analysed by SDS-PAGE and Western blot analysis using a mouse anti-His antibody (GenScript, New Jersey).

Bioinformatics

Orthologous proteins to CopB were identified using BLASTP (Basic Local Alignment Search Tool Protein) and PSI-BLAST, excluding *Chlamydiaceae* family from the search. CopB was analyzed using the TMpred software tool to predict transmembrane domains, using a minimum transmembrane window of 17 and maximum of 33. Coiled-coil prediction software, COILS, was used to predict the presence of coiled-coil domains within CopB, using the MTIDK scoring matrices, and weighting for positions a & d.

Antibody and Peptide Inhibition of *C. pneumoniae* infection in HeLa cells

Infection was performed as previously described by Johnson *et. al.* [15]. At approximately 72 hours post infection, chlamydial inclusions were stained with the Pathfinder *Chlamydia* detection reagent (BioRad) and visualized with multiple, random fields of view visualized. Percent reduction of infection was calculated compared to a control infection, and statistical significance was calculated using a Student's t-test. A polyclonal antibody raised against a 15 amino acid peptide (SGKDKTSSTTKTETC) from CopB was obtained from GenScript (New Jersey). *C. pneumoniae* was pre-incubated for 2 hours at 37°C with dilutions of affinity purified CopB antibody, control antibody (anti-GST), or pre-immunization sera. Additionally, chlamydial infection inhibition was performed using a synthetic

peptide (500 uM), vehicle alone (PBS), or control peptide (anti-RSV peptide). Briefly, $5x10^5$ IFUs were incubated with the peptide or vehicle alone (PBS) for 2 hours at 37°C prior to performing a standard infection and inclusions were visualized as previously described.

RESULTS

Bioinformatic analysis of *Chlamydia* outer protein (Cop) B

Translocator proteins have a conserved function across numerous bacterial species, facilitating the translocation of effector proteins from the bacterial cytosol to the host cell cytoplasm through formation of pores within the host cell membrane. However, there is limited sequence orthology between *Chlamydia spp*. translocators and other well-characterized bacterial translocator proteins. BLAST-P analysis identified potentially orthologous sequences in the recently sequenced genome of *Bacteroides fragilis* with an expect value of $6e^{-141}$ and percent identity of 54%. CopB is a 493 amino acid protein with a predicted molecular weight of 50.5 kDa. Potential transmembrane domains were identified using online prediction software, TMpred, which suggests the presence of two transmembrane domains, spanning amino acids 256-274 and 383-406, respectively, and a hydrophobic stretch of amino acids from 180-200. COILS software identified three potential coiled-coil domains located at amino acids 117-140, 234-347, and 410-437. Sequence analysis of the N-terminal region of CopB identified a conserved

chaperone binding motif of PxLxxP at amino acids 166-171 with the sequence of PELPKP (Figure 1). Together, these results are consistent with features characteristically found in T3S translocator proteins [22, 23].

CopB interacts with the putative needle filament protein, CdsF

CopB is believed to be a T3S protein, and thus it should interact with other proteins within the T3SS [10]. Cloning fragments of CopB lacking the transmembrane domains allowed us to identify specific domains of CopB that are responsible for interactions with other type III secretion components. GST pulldowns were performed between CopB and Cpn0803, CdsF, and CopN. No interactions were observed between any fragments of CopB and Cpn0803 or CopN (Figure 2A,B). There was a positive interaction between the N-terminal (amino acids 1-255) and middle fragment of CopB and CdsF, but not the C-terminus of CopB (Figure 2C). These observations are consistent with a role in the T3S apparatus of *Chlamydia pneumonia*, since translocator proteins from orthologous systems have been shown to interact with the needle filament protein.

LcrH_1 Interacts within the N-terminus of CopB

Cpn0811 (LcrH_1) is a small, basic isoelectric protein located upstream in the same operon as CopB (Cpn0809) [20]. We explored the possible interaction between LcrH_1 and CopB and found that His-LcrH_1 interacts within the N-terminus of CopB (Figure 3A). Both $CopB_{1-200}$ and $CopB_{1-255}$ interacted with His-

LcrH_1, but $CopB_{1-180}$ did not, suggesting the hydrophobic stretch of amino acids spanning residues 180-200 plays an important role in this interaction. Since $CopB_{1-200}$ was the smallest truncation construct that maintained an interaction with His-LcrH_1, we examined the amino acid sequence for the presence of a conserved chaperone binding motif, PxLxxP, which begins at amino acid 166. To elucidate the importance of the conserved motif, we performed an alanine walkthrough of the conserved amino acids in the PxLxxP motif starting at amino acid 166($^{P166A}CopB_{1-200}$, $^{L168A}CopB_{1-200}$, $^{P171A}CopB_{1-200}$). Mutation of the PxLxxP motif abrogated the interaction between His-LcrH_1 and CopB (Figure 3B). To ensure that the absence of interaction was the result of the specific amino acid substitution, as opposed to gross misfolding of the mutant protein, $^{L168A}CopB_{1-200}$ was subjected to a GST pulldown against CdsF. As expected, $^{L168A}CopB_{1-200}$ maintained the interaction with HisMBP-CdsF (Figure 3C), suggesting that the PxLxxP is a critical interaction domain between the chaperone and CopB.

A CopB peptide mimetic blocks the LcrH_1 and CopB interaction

Given the necessity of the PxLxxP motif for the interaction between translocator proteins and their class II chaperones, a synthetic peptide containing the chaperone binding motif was synthesized and tested for its ability to block the interaction between LcrH_1 and both CopB and CopD. To determine whether a synthetic peptide consisting of a cell penetrating peptide sequence (YGRKKRRQRRR) and the 10 amino acids (ETPELPKPGV) encompassing the chaperone binding motif of CopB is capable of preventing the

chaperone:translocator interaction, the peptide was incubated with GST-CopB$_{1-200}$ or GST-CopD$_{1-157}$ prior to the addition of His-LcrH_1. In the presence of the peptide, no interaction was observed between the putative translocators, CopB and CopD, and LcrH_1 under high salt conditions (Figure 4A). To explore the hypothesis that the CopB:LcrH_1 and CopD:LcrH_1 interaction are essential for infection, we then tested the ability of the peptide to block *C. pneumoniae* infection. We pre-incubated *C. pneumoniae* with the peptide or vehicle alone and then infected host cells. The peptide inhibited infection by 90% compared to the control infection with vehicle alone (Figure 4B).

Anti-CopB antibody inhibits *C. pneumoniae*

Since T3S translocators are believed to be surface exposed proteins in other T3SS, we hypothesized that antibodies to CopB would inhibit infection [24-26]. We generated an antibody to a peptide (15-mer) in the N-terminal region of CopB and tested its ability to inhibit *C. pneumoniae* infection. To test whether this antibody could inhibit infection, we pre-incubated *C. pneumoniae* with the polyclonal antibody for 1 hour at 37°C prior to infection. *C. pneumoniae* infection was inhibited by the CopB antibody. (Figure 5A-D), resulting in a 98% reduction in inclusion forming units, as compared to control antibody (Figure 5E). Using a Western blot, polyclonal antibodies were able to detect both recombinant and native CopB (Figure 5F). The ability of the CopB antibody to block infection suggests that CopB is surfaced exposed, and plays a critical role in the infection process.

DISCUSSION

Despite our increasing understanding of the T3SS in *Chlamydia spp.*, there is limited or no evidence for a direct role for the translocator proteins during infection. Our laboratory has previously characterized the putative minor hydrophobic translocator, CopD, showing that it plays an essential role during chlamydial infection [23]. In this report, we provide an initial characterization of the major hydrophobic translocator, CopB. The interaction of CopB with the filament protein CdsF suggests that it plays an essential role in T3S. As seen with other translocator proteins, the putative chaperone located immediately upstream of CopB interacted with the first N-terminal 200 amino acids of CopB. Using an alanine walkthrough of the conserved PxLxxP motif, we show that amino acids P166, L168, and P171, in addition to amino acids 180-200, are required for the interaction between CopB and its' cognate chaperone LcrH_1. We demonstrated that a cognate CopB peptide encompassing the chaperone binding motif can block the interaction between LcrH_1 and both CopB and CopD, suggesting that the CBD is a critical binding domain. Furthermore, we show that this peptide when pre-incubated with *C. pneumoniae*, blocked infection. Together, these results strongly suggest that the PxLxxP motif is required for the translocator-chaperone interaction, and for infection. We also show that a polyclonal antibody raised against an N-terminal epitope within CopB significantly reduced infection. Collectively, these results are consistent with CopB's role as a translocator within the C*hlamydia* T3SS.

Initial bioinformatic studies were performed to gain insight into the role of CopB in *C. pneumonia* [21-23]. Chellas-Géry *et. al.* identified potential hydrophobic and coiled-coil domains within CopB from *C. trachomatis* [22]. Given the moderate level of sequence identity between *C. trachomatis* and *C. pneumonia* CopB (approximately 52% amino acid identity), a thorough bioinformatics analysis of CopB was performed. BLASTP analysis of CopB yielded one significant result from *Bacteroides fragilis*, typically a commensal bacterium found in the gastrointestinal tract, but no matches were found for other T3S systems, suggesting that the *C. pneumoniae* T3SS may be quite unique among orthologous systems, which is in keeping with *Chlamydiae* containing an ancient T3SS. Although no orthologous sequences of the chlamydial translocator proteins were identified in the archetypal secretion systems using our bioinformatics approach, the proteins are predicted to have similar structure and function. Since CopB is likely anchored within the host-cell cytoplasm to facilitate translocation of effector proteins, we utilized TMpred online software to identify potential transmembrane regions. Our analysis identified two potential transmembrane domains spanning amino acids 256-274 and 383-406, respectively. This is consistent with other translocator proteins possessing two transmembrane domains to anchor themselves within the host cell membrane [27, 28]. Using the COILS online prediction software, we identified three potential coiled-coil domains, which may be important for mediating protein-protein or protein-membrane interactions. The N-terminus of CopB contains a conserved PxLxxP motif, followed by a sequence of hydrophobic amino acids, which is seen

in other translocator proteins from *C. pneumonia,* and has been shown to be important for mediating the essential translcoator:chaperone interaction in other bacterial systems (*Shigella, Yersinia, Salmonella*) [23].

Due to the difficulty in genetically manipulating *Chlamydia* and the inherent challenges of establishing structure-function relationships for T3S proteins of obligate intracellular pathogens dependent on T3S for infection, it is difficult to ascertain the role of chlamydial T3SS proteins. We therefore explored the possible interaction between CopB and other proteins within the chlamydial T3SS. The needle filament protein, CdsF in *Chlamydia*, is believed to polymerize forming needle structure for the translocation of effector proteins. The translocator proteins are believed to be docked on the tip of the injectisome to form the needle-tip complex, prior to host cell contact. Two domains of CopB, amino acids 1-255 and 275-382, interacted with CdsF using a GST-pulldown assay. CopN, the putative plug protein, is believed to be localized to the base of the needle apparatus and prevents premature secretion of effector proteins. No apparent interaction between CopN and CopB was observed using a GST-pulldown assay. Although an interaction was observed between CopD and CopN, the lack of interaction between CopB and CopN suggests that CopB may be secreted through the apparatus and docked on the end of the needle complex before CopN plugs the needle apparatus. Considering the fact that recent work has suggested that Cpn0803 may be a chaperone protein given its biophysical properties and putative interactions, it is not surprising that CopB failed to interact with Cpn0803 [29, 30]. The interaction

between CopB and the needle filament protein, CdsF, in this report is a novel observation not previously reported in the literature. It has been reported that the translocators are recruited to the tip of the needle complex either upon detecting host cell contact or under secretion conditions. Once the translocators are inserted into the host cell membrane, the filament protein must anchor to the host cell via the translocator proteins, which are now imbedded in the host membrane. This result is consistent with the role of the hydrophobic translocator proteins in other T3SS since the needle protein must interact with the translocator proteins on the host cell to facilitate translocation of effector proteins [31, 32].

Interactions between class II chaperones and translocator proteins have been documented in *Chlamydiae spp.* previously. Initial identification of the LcrH_1 and CopB interaction, from *C. trachomatis*, was performed by Fields *et. al*. (2005) [10, 21]. Using a GST pulldown, we demonstrated that the N-terminus of $CopB_{1-255}$ interacts with LcrH_1, which is in keeping with LcrH_1 orthologs interacting within the N-terminus of translocator proteins [28, 33]. An additional truncation series showed that the removal of the hydrophobic amino acids from 180-200 eliminated the interaction of LcrH_1 and CopB despite the presence of the PxLxxP motif within the $CopB_{1-180}$ construct. The PxLxxP motif is conserved in members of the *Chlamydiaceae* family, despite the low amino acid sequence identity, suggestive of an important role for the chaperone binding motif (Table 1). An alanine walkthrough of the conserved amino acids in the PxLxxP motif disrupted the interaction between $CopB_{1-200}$ and LcrH_1. Our data indicates that the interaction

between CopB and LcrH_1 is dependent on both the PxLxxP motif and the $CopB_{180-200}$ domains.

The co-crystal structure of class II chaperones (LcrH_1 orthologs) with the translocator PxLxxP domain confirms the interaction between the two proteins [28]. Based on this interaction we hypothesized that a cognate peptide of CopB containing the PxLxxP peptide sequence could disrupt the translocator-chaperone interaction. We therefore tested a peptide containing the 10 amino acids encompassing the PxLxxP domain plus a cell penetrating peptide sequence to test this hypothesis. Since the PxLxxP motif is conserved between CopB and CopD, we pre-incubated LcrH_1 with the cognate peptide then added either CopB or CopD fragments to the GST pull down and showed that the peptide blocked the interaction between LcrH_1, and CopB and CopD. Since the cell penetrating peptide sequence allows proteins to enter cells, we hypothesized that this cognate peptide would block chlamydial infection and intracellular replication [34, 35]. After pre-treating *C. pneumoniae* with this peptide, we observed a significant reduction of 90%, compared to control infection, with vehicle alone. Since it is currently not possible to create genetic knockouts in *Chlamydia*, peptide mimetics could be used to create functional knockouts to study the resultant phenotype. The peptides ability to significantly reduce infection reinforces the importance of the chaperone binding motif for the chaperone-translocator interaction and may represent a novel target for therapeutic intervention using peptide mimetics.

CONCLUSIONS

Antibodies to the translocator proteins in orthologous secretion systems have been shown to inhibit infection, suggesting that the translocator proteins play an essential role during infection [36-40]. Using antibodies raised to an N-terminal epitope of CopB, we demonstrated that anti-CopB antibodies inhibited *C. pneumoniae* infection by 98%. Inhibition of infection by anti-CopB antibodies indicates that CopB is surface exposed at some time during infection and play an essential role in infection. Given the fact that CopB is surface exposed during the initial phase of chlamydial infection, the translocator proteins may represent a novel class of antigens for use in vaccination strategies to prevent chlamydial infections.

AUTHOR CONTRIBUTIONS: DCB performed most of the experimental work. DAW, CBS, SL, CKWC, KAW, JCN, SWZ, SPM, and ZCS aided in preparation of reagents, and cloned constructs. JBM coordinated the experiments, and edited the manuscript. All authors read, and approved of, the final manuscript.

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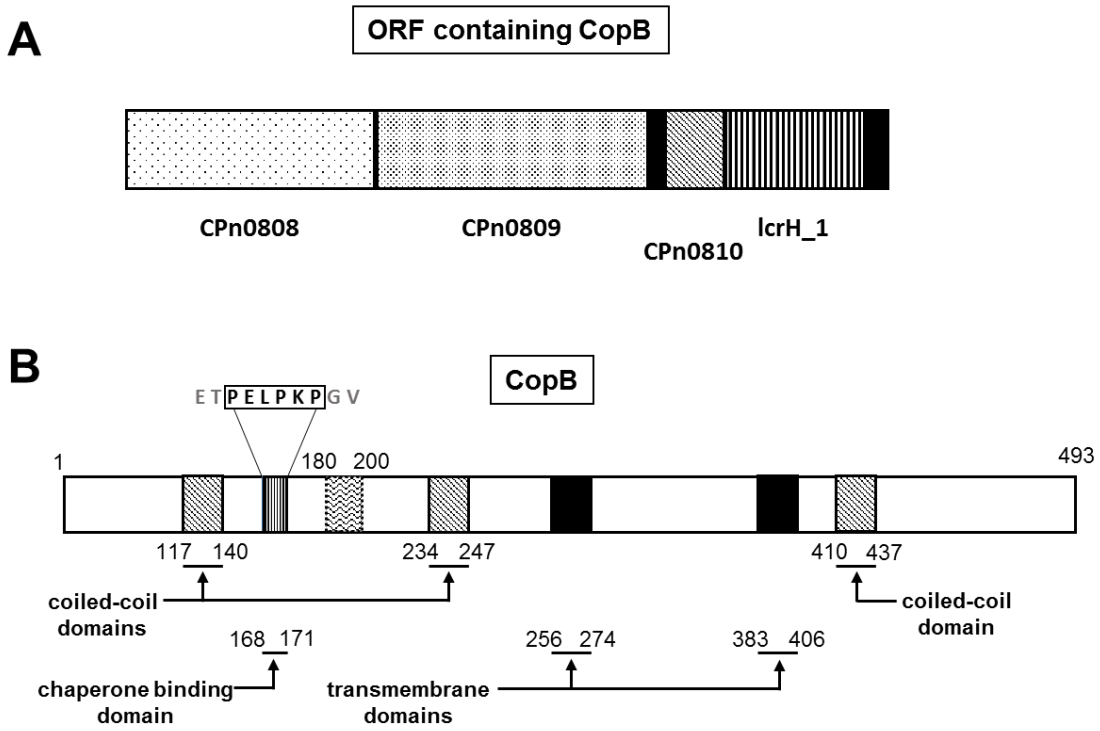

Figure 1: Genetic organization and topographic overview of structural prediction of CopB. Solid black regions represent transmembrane domains. A) Genetic organization of the ORF containing the putative translocator, CopB and CopD, and the TPR-domain containing chaperone, LcrH_1. B) Diagonal stripes represent predicted coiled-coil domain in the C-terminus of the protein. Vertical stripes depict predicted Chaperone Binding Domain spanning amino acid residues 168-171. The hydrophobic region is shown from amino acids 180-200.

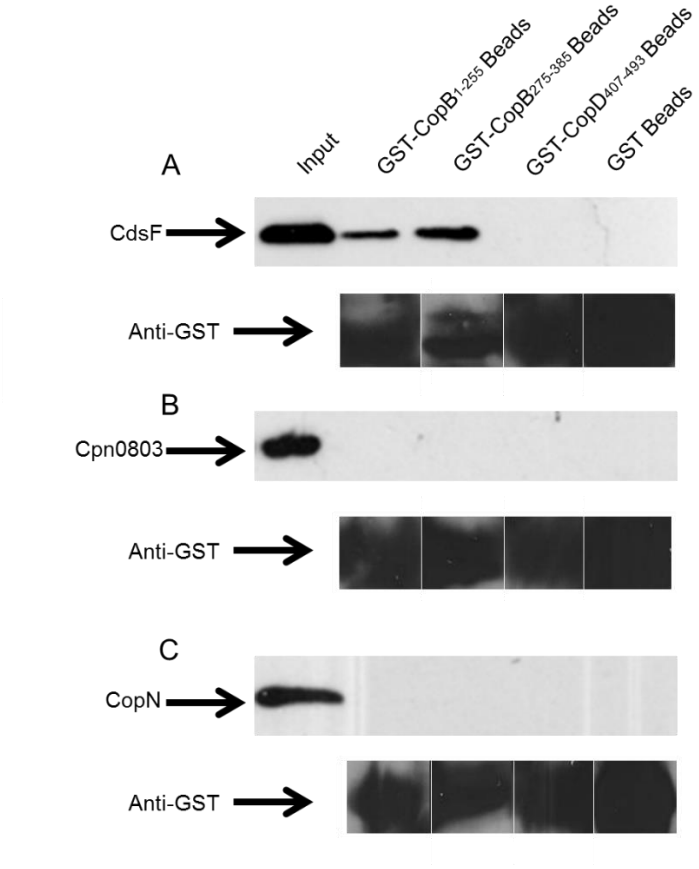

Figure 2: *Chlamydia* Outer Protein (Cop) B Interacts with T3S proteins. GST-CopB$_{1-255}$ or GST-CopB$_{275-385}$ bound to glutathione-agarose beads (bait) pulled HisMBP-CdsF (prey) out of an *E. coli* lysate in the presence of a high salt wash buffer (500mM NaCl). Furthermore, GST-CopB fragments did not pull His-CopN or Cpn0803 out of an *E. coli* lysate in the presence of a high salt wash buffer.

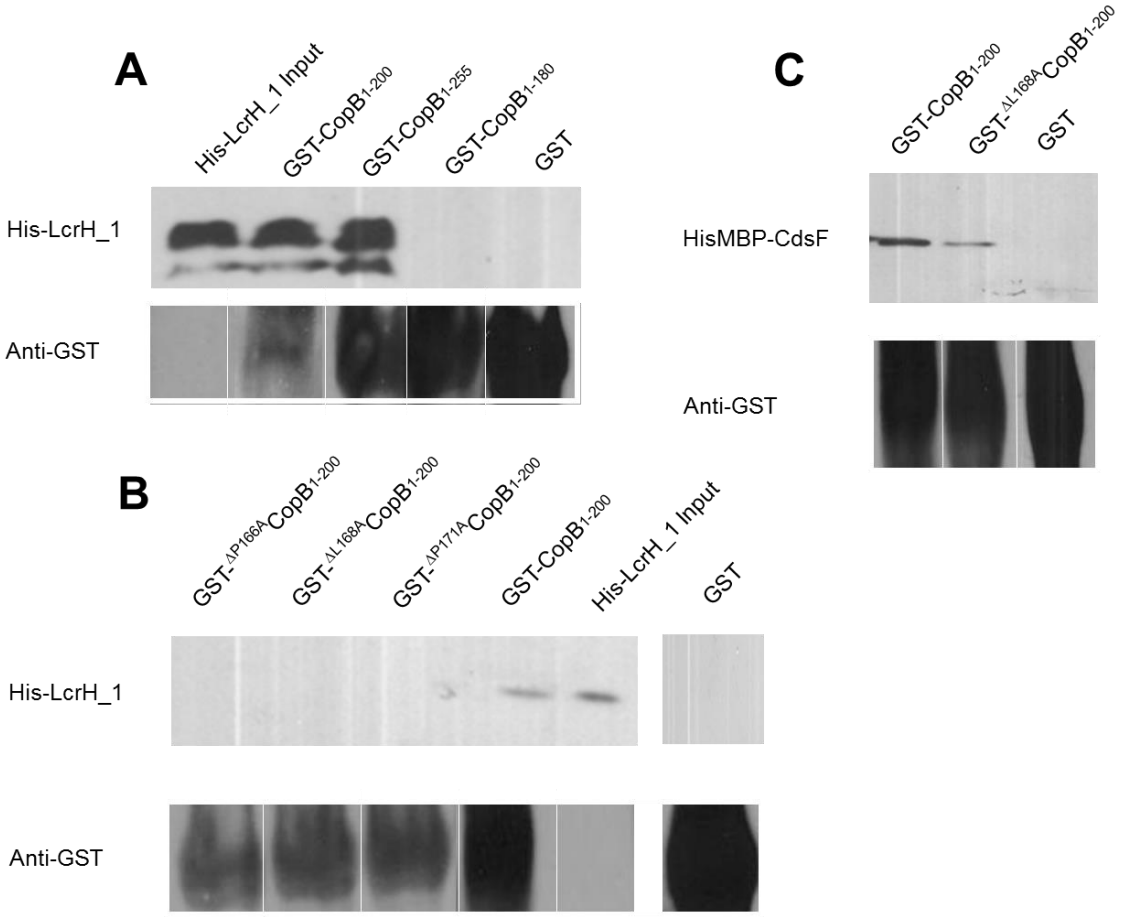

Figure 3: LcrH_1 (*Cpn0811)* interacts with CopB. Recombinant LcrH_1 interacted with amino acids 1-200 of CopB. CopB mutants were created using Gblock synthesis: $^{P166A}CopB_{1-200}$, $^{L168A}CopB_{1-200}$, and $^{P171A}CopD_{1-200.}$ Mutations at the conserved amino acids within the predicted chaperone binding domain disrupted the interaction between $CopB_{1-200}$ and the chaperone LcrH_1, but not other identified interactions.

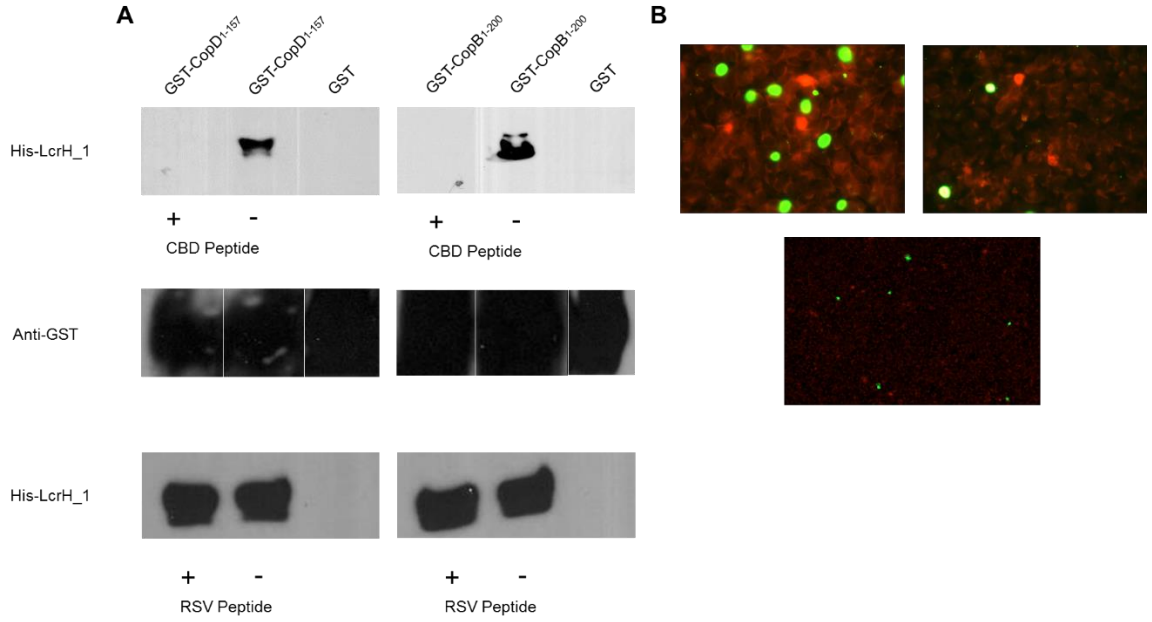

Figure 4: Peptide inhibition of the translocator:chaperone interaction. Panel A: Recombinant GST-CopD$_{1\text{-}157}$ or GST-CopB$_{1\text{-}200}$ was pre-incubated with 500 µM of the chaperone binding domain peptide mimetic (+) or vehicle alone (-). CopD$_{1\text{-}157}$ and GST-CopB$_{1\text{-}200}$ did not interact with its putative chaperone in the presence of the CBD peptide, but did so in the presence of a control peptide or vehicle alone. Panel B: Left image is *C. pneumoniae* incubated with vehicle alone (PBS), right image is *C. pneumoniae* incubated with 500 µM CBD Peptide, bottom image is *C. pneumonia* incubated with 50 uM anti-RSV peptide. Chlamydial inclusions are stained green, while HeLa cells are stained red by Evan's blue counterstain.

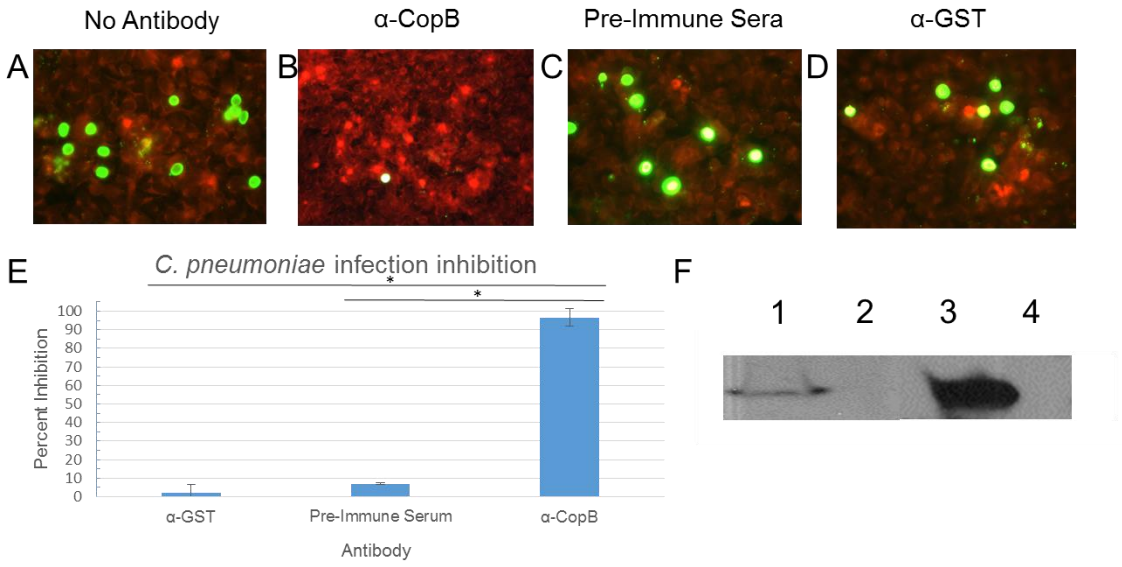

Figure 5: Inhibition of *Chlamydia pneumoniae* with CopB antibodies.

Panels A-D show inhibition assay results performed with either no antibody (A), CopB antibody (B), pre-immune sera (C), or control antibody (α-GST) (D). Panel E shows the degree of inhibition by of CopB antibodies compared to control antibodies. Chlamydial inclusions are stained green, while HeLa cells are stained red by Evan's blue counterstain. Panel F demonstrates reactivity of anti-CopB with (1) *C. pneumoniae* infected HeLa cell lysate, (2) uninfected HeLa cell lysate, (3) recombinant GST-CopB$_{1\text{-}255}$ produced in *E. coli*, and (4) recombinant GST produced in *E. coli.* Experiments were performed in triplicate. Error bars represent 2 standard deviations. Images represent random fields of view. * = P < 0.0001

	P1		P3			P6	Percent Identity
CopB (*C. pneumoniae*)	P	E	L	P	K	P	100%
CT578 (*C. trachomatis* serovar D)	P	G	L	P	K	P	52%
SseC like family protein (*C. psittaci*)	P	D	L	P	K	P	53%
TC_0867 (*C. muridarum*)	P	G	L	P	K	P	50%
CPE1_0913 (*C. pecorum*)	P	E	L	T	P	P	53%
CAB923 (*C. abortus S26/3*)	P	D	L	P	K	P	54%
YopB (*Y. enterocolitica*)	P	A	L	G	R	P	18%
IpaB (*S. dysenteriae*)	P	E	L	K	A	P	17%

Table 1: Comparison of putative chaperone binding domains between *Chlamydiaceae* family members and other T3SS containing Gram-negative bacteria. Putative chaperone binding domains were identified within the N-terminal regions of orthologous proteins to CopB from *C. pneumoniae.* P1, P3, P6, represent positions 1, 3, and 6, respectively of the PxLxxP motif. Percent identity refers to amino acid sequence identity comparing full length CopB to full length sequences of orthologous proteins.

CHAPTER FOUR

Preface to Chapter 4

In this chapter, the design and testing of a novel chlamydial antigen to prevent *Chlamydia* infections is described. Using traditional cloning methods, a fusion protein consisting of CopB, CopD and CT584 was generated and purified via FPLC. Mice were vaccinated with the antigen, and then subsequently challenged with C. muridarum. The mice were analyzed using various outcome measures, including bacterial shedding, gross pathology, generation of neutralizing antibodies, and serum titres.

The manuscript presented in Chapter 4 is submitted to Vaccine. The production and purification of BD584 was performed by myself. The animal work was performed by Steven Liang, Amanda Lee and myself. In vitro experiments examining antibody titres, and neutralization were performed by Steven Liang and myself. Sylvia Chong tested the vaginal swabs for bacterial load using qPCR. Dr. James Mahony, Steven Liang and I were responsible for generating the manuscript. All authors on the manuscript were responsible for the experimental design.

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Immunization with chlamydial type III secretion antigens reduces vaginal shedding and prevents fallopian tube pathology following live *C. muridarum* challenge

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ABSTRACT

Chlamydia trachomatis infections in women are often asymptomatic and if left untreated can lead to significant late sequelae including pelvic inflammatory disease and tubal factor infertility. Vaccine development efforts over the past three decades have been unproductive and there is no vaccine approved for use in humans. The existence of serologically distinct strains or serovars of *C. trachomatis* mandates a vaccine that will provide protection against multiple serovars. *Chlamydiae spp.* use a highly conserved type III secretion system (T3SS) composed of both structural and effector proteins which is an essential virulence factor for infection and intracellular replication. In this study we evaluated a novel fusion protein antigen (BD584) which consists of three T3SS proteins from *C. trachomatis* (CopB, CopD, and CT584) as a potential chlamydial vaccine candidate. Intranasal immunization with BD584 elicited serum neutralizing antibodies that inhibited *C. trachomatis* infection *in vitro*. Following intravaginal challenge with *C. muridarum*, immunized mice had a 95% reduction in chlamydial shedding from the vagina at the peak of infection and cleared the infection sooner than control mice. Immunization with BD584 also reduced the rate of hydrosalpinx by 87.5% compared to control mice. Together, these results suggest that highly conserved proteins of the chlamydial T3SS may represent good candidates for a *Chlamydia* vaccine.

INTRODUCTION

Chlamydia trachomatis is the most common sexually transmitted bacterial infection in North America and in many developed countries around the world [1]. The World Health Organization has estimated that there are 92 million new chlamydial infections globally every year, with the highest incidence occurring in under developed regions of the world where adequate control programs are significantly lacking [2]. Chlamydial infection can lead to acute inflammation characterized by redness, edema, and discharge, presenting as mucopurulent cervicitis in women and non-gonococcal urethritis in men [3]. It has been estimated that more than 70% of women and 50% of men experience subclinical, asymptomatic infections. Since individuals with asymptomatic infections do not seek medical treatment, untreated infections in women can lead to upper genital tract (UGT) infection, pelvic inflammatory disease (PID), hydrosalpinx, and tubal infertility [3]. The high rate of asymptomatic infections, together with the severity of the infection-induced pathology, suggests that controlling *C. trachomatis* infections would benefit from a vaccine.

Early vaccine studies were initiated after the isolation of the etiologic agent of trachoma in 1957 [4]. Children were vaccinated with formalin-fixed chlamydial elementary bodies (EBs), which conferred only partial, serovar-specific, and short-lived immunity. Furthermore, when compared to their unvaccinated counterparts, a small number of vaccinated children had a greater risk of developing exacerbated disease following exposure to *Chlamydia* [4, 5]. The

same observations were made when similar experiments were carried out in non-human primates [6]. The development of undesirable immune responses following vaccination with intact *Chlamydia* suggests the presence of deleterious antigen in EBs, highlighting the need for an effective and broadly protective subunit vaccine.

Chlamydial vaccine research efforts over the past three decades have led to the discovery of a large number of protective antigens [5, 7, 8]. Efforts have largely been focused on the major outer membrane protein (MOMP), and more recently on polymorphic membrane proteins (Pmps), both of which elicit antibody and cell-mediated protective immune responses. However, the use of MOMP as an antigen has been hampered by its complex structure and its allelic variation among different serovars of *C. trachomatis*. Antigenic variation seen with MOMP and Pmps suggests that a single antigen may not be sufficient in a *Chlamydia* vaccine, and that immunization with a multi-subunit vaccine consisting of novel antigens may represent a better strategy.

As a highly evolved obligate intracellular pathogen with a small genome size, chlamydia likely employs many virulence factors to manipulate the host cell environment ensuring successful infection. One essential virulence factor is the T3SS, which is required for cell invasion. Many of the T3SS proteins are surface exposed and as such can be targeted by neutralizing antibodies. For this reason the use of T3SS components as antigens in the development of vaccines against other pathogenic bacteria has recently garnered attention. Antibodies to the T3S

tip proteins LcrV in *Yersinia spp.* and PcrV in *Pseudomonas aeruginosa* have been shown to block infection [9, 10]. LcrV is a well-characterized *Yersinia* antigen and is currently being tested as a subunit vaccine against plague. A broadly protective vaccine consisting of T3S translocator proteins IpaB and IpaD has been shown to be effective against *Shigella* [11, 12]. There is much interest in IpaB and IpaD as vaccine candidates, because of their ability to induce cross-protective immunity in mouse models of *Shigella* infection. Since the T3S system proteins are highly conserved across chlamydial species, these proteins may represent good candidates for use in a *Chlamydia* vaccine.

We have previously shown that rabbits immunized with T3S proteins CopB, CopD and Cpn0803 from *C. pneumoniae* were able to produce neutralizing antibodies that blocked infection *in vitro* by 98% [13]. We now show that immunization with a novel multi-component vaccine (BD584) consisting of the N-terminal 100 amino acids of *C. trachomatis* CopB, CopD, and full length *CT584* reduced both bacterial shedding from the vagina and upper genital tract pathology following intra-vaginal challenge in mice.

METHODS & MATERIALS

Hydrophilicity prediction

Amino acid sequences of CopB, CopD and CT584 were analyzed for transmembrane domains using an online prediction software, TMpred, which predicts transmembrane domains, using a minimum transmembrane window of 17

and maximum of 33. Proteins containing predicted transmembrane domains were subjected to Kyte-Doolittle hydrophobicity prediction using a window size of 9.

Cloning

Genomic DNA from *C. trachomatis* serovar D was used as the source for all genes cloned in this study. Using Platinum *pfx* DNA amplification enzymes (Life Technologies), the following PCR products were generated (note: subscript denotes amino acid number): $CopB_{1-100}$ with a 5' BamHI restriction site and 3' EcoRI restriction, $CopD_{1-100}$ with a 5' EcoRI restriction site and a 3' SalI restriction site, full length CT584 with a 5' SalI restriction site and 3' HindIII restriction site. PCR products were digested with their respective endonuclease (New England Biolabs) (Figure 1). The multiple cloning site (MCS) 1 of pET-DUET was restriction digested with BamHI and HindIII (New England Biolabs). Restriction digested $CopB_{1-100}$, $CopD_{1-100}$, CT584, and pET-DUET were ligated in a 3:3:3:1 ratio using T4 DNA Ligase (Invitrogen) and transformed into NEB Turbo Cells (New England Biolabs). Prior to protein expression, all constructs were verified by Sanger sequencing at the MOBIX laboratory (McMaster University).

Protein Expression and Purification

The cloned construct in pET-DUET was transformed into *E. coli* BL21(DE3) cells for protein production and purification. For protein expression, an overnight culture of bacteria was sub-cultured 1:50 into 6L of pre-warmed LB in the presence of 100 ug/mL ampicillin. The bacteria were incubated at 37°C while shaking at 250

RPM until an optical density of 0.600 at 600 nm was reached. Protein expression was induced by the addition of 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and remained shaking for 3 hours at 37°C. The bacteria were harvested via centrifugation at 10,000x g for 5 minutes using a Sorvall 5B centrifuge. The bacterial pellets were washed once with phosphate buffered saline (PBS) and then pelleted again before resuspending it in Nickel A buffer for protein purification (20 mM TRIS-HCl pH 7.4, 500 mM KCl, 0.03% LDAO, 10% glycerol, 10 mM imidazole). To release soluble protein from the bacterial cytoplasm, the bacteria were sonicated and then pelleted at 40,000x g for 45 minutes. The supernatant was loaded onto a 1 mL HisTRAP column (GE Life Sciences) using an AKTA FPLC (GE Life Sciences). The bacteria were washed with approximately 20 column volumes of 100% Nickel A, 5% Nickel B (20 mM TRIS-HCl pH 7.4, 500 mM KCl, 0.03% LDAO, 10% glycerol, 300 mM imidazole), 10% Nickel B, and 15% Nickel B before eluting in 100% Nickel B. The protein was buffer exchanged into PBS using a desalting column and an AKTA FPLC. Purified protein was concentrated to 250 uL using a centrifugal filter (EMD-Millipore) and protein concentration was determined using a BioRad Protein DC Assay with protein standards.

Enzyme-linked Immunosorbant Assay (ELISA) for Immunoglobulin Detection

An indirect ELISA was used to detect the presence of antibodies against immunizing antigens. Briefly, cheek bleeds or vaginal washes were collected from the mice two weeks following the second immunization. The samples were pelleted in a microcentrifuge at 16,000 rpm, and the supernatant was collected. Serial

dilutions of the supernatants, from either the vaginal washes or serum, were made from 1:25-1:3200. Approximately 250 ng of antigen were immobilized on nickel coated plates (Pierce) for 1 hour at room temperature with shaking. The 96 well plate was then washed 1 time with PBS+0.1%Tween 20 (PBS-T). 200 uL of blocking solution (5% BSA in PBS-T) was added to each well and incubated at room temperature for 1 hour. After removal of the blocking solution, 100 uL of the serial dilutions (serum or vaginal washes) were added to the wells and incubated for 1 hour at room temperature while shaking. The serial dilutions were removed, and wells were washed with 200 uL of PBS-T and incubated for 5 minutes at room temperature with shaking, and repeated three times. After the final wash was removed, a 1:1000 dilution of goat anti-mouse HRP-conjugated secondary antibody (Sigma) was in made in 5% BSA + PBS-T, and 100 uL was added to each well and incubated at room temperature with shaking for 1 hour. After repeating the previously described washing procedure, Ultra TMB (Pierce) was used to develop the ELISA as per the manufacture specifications. Briefly, 100 uL of Ultra TMB was added to each well, after a 30 minute incubation at room temperature, 100 uL of stop solution (1 M H_2SO_4) was added to each well. The absorbance of each well was measured at 450 nm.

Intranasal Immunization

Mice (n = 5 per group) were sedated with 5% Isoflurane in 4 L/min oxygen. The mouse was held at a slight angle to position the nose towards the ground and then using a micropipette, the vaccine was delivered to the right nares while the

mouse was waking from anesthesia. Vaccinated mice received 20 ug of antigen and 10 ug of CpG in 15 uL of PBS. Control mice received vehicle (PBS) alone. Immunizations were administered twice, at 6 weeks and 3 weeks prior to infection.

Chlamydia muridarum vaginal challenge

C. muridarum was grown in McCoy cells and harvested 48 hours post infection. *C. muridarum* was aliquoted and stored in a sucrose-phosphate-glutamate (SPG) buffer at -80 °C. Eight week old female C57/BL6 mice (Charles River) were used for all animal experiments. Mice were given 2.5 mg of medroxyprogesterone acetate (Depo-Provera, Pfizer) subcutaneously one week prior to infection to force the mice into the diestrus phase of the estrous cycle, thereby increasing the susceptibility to *C. muridarum* infection. The mice received $1x10^5$ inclusion forming units (IFUs) of *C. muridarum* intravaginally. Non-infected controls received PBS only. Each group consisted of five mice.

Quantitation of vaginal shedding

Quantitative real-time PCR was used to quantitate chlamydial shedding. Vaginal swabs (REF 516CS01, Copan, Italy) were collected from each mouse on days 1, 3, 5, 7, 10, 14 days post infection. Mouse vaginal swabs were placed in SK38 tubes containing ceramic beads (Berkin Technologies, Montigny, France) and stored at -20C until testing. Before nucleic acid extraction, 1 mL of lysis buffer (bioMerieux) was added to the swab. The tubes were vortexed for 5 minutes and left stand at room temperature for 15 minutes. The tubes were then spun at 14000

rpm for 2 minutes. The supernatant was collected and then subsequently extracted on an easyMAG extractor using generic 2.01 protocol. Quantitative PCR was performed using a RotorGene Q instrument (Qiagen). A pair of primers and a FAM-BHQ probe were used to target the MOMP gene from *C. muridarum*. qRT-PCR was performed in a 20 uL reaction containing 1 x SensiFAST Probe Mix (Bioline), 0.4 uM forward primer, 0.4 uM reverse primer, 0.2 uM prove and 5 uL of template. The amplification profile consisted of 95°C for 3 minutes, followed by 45 cycles of 95°C for 10 seconds, and 55ºC for 40 seconds. Standards used for quantitation were from the MOMP gene, cloned into the pGEM-T easy vector system.

Serum neutralization of *C. muridarum* Infection

C. muridarum was propagated in McCoy cells using DMEM supplemented with 5% BSA, 1 ug/mL cyclohexamide, and 50 ug/mL gentamicin. Briefly, confluent shell vials of McCoy cells were infected by removing growth media and replacing it with supplemented DMEM containing *C. muridarum* at an MOI of 1.0 and serum from either vaccinated or control mice diluted 1:10. The shell vial was centrifuged for 1 hour at 3000x g at room temperature. After centrifugation, shell vials were incubated at 37°C with 5% CO2 for 48 hours. Chlamydial inclusions were stained with the Pathfinder *Chlamydia* detection reagent (BioRad) and visualized with multiple, random fields of view visualized. Percent reduction of infection was calculated compared to a control infection, and statistical significance was calculated using a Student's t-test.

RESULTS

Type III secretion proteins are highly conserved between *C. trachomatis* and *C. muridarum.*

To determine whether the three components of the trivalent BD584 antigen are genetically conserved between *Chlamydiae spp.*, a bioinformatic analysis was performed to examine the amino acid identity of $CopB_{1-100}$, $CopD_{1-100}$, and $CT584_{FL}$ between *C. trachomatis* and *C. muridarum.* BLAST-P analysis revealed that $CopB_{1-100}$, $CopD_{1-100}$, and $CT584_{FL}$, were highly conserved between *C. trachomatis* and *C. muridarum*, with 86%, 78%, and 97% amino acid identity, respectively (Figure 2). We have previously shown that antibodies generated against N-terminal peptides of CopB and CopD were capable of neutralizing Chlamydiae *spp*. Cloning of $CopB_{1-100}$, $CopD_{1-100}$, and $CT584_{FL}$ into pET-DUET1 generated an N-terminal polyhistidine-tagged fusion protein hereafter referred to as BD584 with a predicted molecular weight of approximately 47 kDa. Following purification via FPLC, BD584 was greater than 95% pure as analyzed by SDS-PAGE (Figure 1).

Chlamydial T3S proteins elicit neutralizing antibodies

Two groups of mice (N=5) were immunized twice intranasally with both BD584 and CpG adjuvant, or with PBS alone (control group). Following immunization, sera were tested for antibodies to BD584 by ELISA (Figure 3). Serum from immunized mice had anti BD584 antibodies with an end point titer of

1:3200 while serum from control mice had no detectable BD584 antibodies. To determine whether BD584 immunization elicited neutralizing antibodies, sera were pooled and tested using a neutralization assay. Pooled serum from the immunizaed mice reduced the number of inclusions by 77% while pooled control sera failed to neutralize *C. muridarum*. (Figure 4)

Immunization with BD584 reduces bacterial shedding of *C. muridarum*.

Mice were immunized then challenged vaginally to determine whether BD584 immunization reduced chlamydial shedding. Vaginal swabs were collected at various time points during the course of infection (days 1, 3, 5, 7, 10, 14, 32) and tested for the presence of *C. muridarum* by qPCR. On day 3 post-infection, vaccinated mice had an 85% reduction in bacterial shedding as compared to control mice (mean of 256 versus 1504 copies, respectively) At the peak of infection, days 5 and 7, a 95% reduction in bacterial shedding was observed in the vaccinated group (mean of 90 and 13 copies, on day 5 and 7) compared to the control group (mean of 1555 and 121 copies, on day 5 and 7) (Figure 5). Reductions in chlamydial shedding were significantly different between the immunized and control groups on days 3 ($p = 0.028$), 5 ($p = 0.0023$) and 7 ($p = 0.0022$). Vaccinated mice also cleared the infection sooner than control mice as no *Chlamydia* was detected 32 days post infection in the vaccinated mice (Figure 5).

Immunization with BD584 reduces *Chlamydia*-induced immunopathology

One of the primary goals for an effective Chlamydial vaccine is to reduce or prevent upper genital tract pathology following lower tract infection. To assess the ability of BD584 immunization to reduce *Chlamydia*-induced immunopathology of the ovarian ducts, mice were vaccinated then challenged as previously described. Animals were sacrificed 35 days post infection to assess the development of hydrosalpinx. In the vaccinated group of five mice, only 1 out of 10 ovaries had hydrosalpinx, which was significantly lower ($p = 0.0055$) than that seen in the control group where 8 out of 10 ovaries had hydrosalpinx (Figure 6).

DISCUSSION

Despite decades of research on *Chlamydia* immunobiology, limited success has been achieved in vaccine development [6, 14]. Given the success of vaccines derived from T3SS proteins in other bacterial species, we developed a novel trivalent antigen consisting of three T3S chlamydia proteins and evaluated this antigen in a *C. muridarum* mouse model of infection [9-11, 15-18]. The highly conserved nature of T3SS proteins across different bacterial species highlights the essential nature of this virulence factor and suggests that immunization with T3S antigens could produce an effective pan-serovar *Chlamydia* vaccine [19-21]. We show here for the first time that T3SS antigens from *Chlamydia* represent a good vaccine candidate. Intranasal immunization of mice with BD584 antigen plus CpG adjuvant elicited serum antibodies against BD584 which were capable of

neutralizing *Chlamydia* infection *in vitro*. Mice vaccinated with BD584 and challenged with *C. muridarum* had a reduction in both bacterial shedding ($p < 0.05$) and *Chlamydia*-induced fallopian tube pathology ($p < 0.05$). Together, this data suggests that BD584 represents a good chlamydial vaccine candidate.

Since there are fifteen serovars of *C. trachomatis*, an ideal vaccine should provide cross-serovar protection. The T3SS is structurally conserved across many gram-negative bacteria and is an essential virulence factor. *Chlamydia spp.* are obligate intracellular pathogens and use a T3SS to infect host cells. Despite the amino acid sequence differences seen between orthologous T3S proteins of other bacteria, the amino acid sequence of T3S components is highly conserved between chlamydial species, and between serovars of *C. trachomatis* [13, 22, 23]. Our BD584 antigen was selected because it is highly conserved between *C. trachomatis* serovars and *C. muridarum*. MOMP has been considered the archetype for immunization against *Chlamydia* infections for the past 30 years [6, 24]. However, vaccination with one *C. trachomatis* serovar of MOMP provides protection against the same serovar but only limited cross-protection across other serovars [24]. We immunized mice with T3SS proteins derived from *C. trachomatis* and challenged with live *C. muridarum* to demonstrate that these antigens can provide protection across chlamydial species and serovars. To date, few studies have examined the use of T3S proteins as antigens to vaccinate against *Chlamydia*. In other gram-negative bacteria, there has been considerable success in vaccination strategies using components of the T3SS; specifically, it has been

demonstrated that when orthologs of CopB from *Shigella spp.* are used as antigens, in combination with other T3S proteins, there is significant protection against *in vivo* challenge with *Shigella* [11, 15-17, 25]. Immunization with BD584 represents a novel approach for prevention of chlamydial infection and/or *Chlamydia*-induced pathology. Experiments are in progress to determine the level of protection afforded by BD584 compared with other chlamydial components such as MOMP.

It has been well documented in the literature that a cell-mediated immune response is critical to protecting and clearing a *Chlamydia* infection. Using CpG adjuvant with BD584, we intranasally vaccinated mice in attempts to generate a mucosal Th1 polarized immune response [6]. To ensure that our immunization strategy was successful, sera from immunized mice were tested for the presence of antigen specific serum IgG. Previously, we have shown that antibodies raised against the translocators, CopB and CopD, can inhibit infection suggesting that antibodies directed towards these proteins block an essential aspect of T3S during infection [13, 26]. Serum collected from mice immunized with BD584 reduced infectivity by 77% when compared to control serum. This reduction in infectivity by serum antibodies directed against the translocators suggests that these antibodies are directed against surface exposed epitopes on CopB and CopD. The mechanism of neutralization remains to be elucidated, but is presumably due to the antibodies rendering the T3SS inactive and preventing host cell infection. Our vaccine preparation contained CpG adjuvant, a TLR9 agonist, known to polarize

the immune response towards a Th1 immune response. It has been shown previously that a Th1 immune response is an essential component for the induction of protective immunity against chlamydial infection. Experiments to characterize the mechanism of protection are currently underway.

An important characteristic of a *Chlamydia* vaccine would be the ability to decrease bacterial shedding to reduce transmission and to produce an immune response to prevent *Chlamydia*-induced immunopathology. Recently, it has been shown that approximately 300 IFUs are required to establish an active *Chlamydia* infection in mice [27]. During the course of a *Chlamydia* infection in mice, bacterial shedding occurs for approximately 14-35 days before being cleared from the lower genital tract [6]. In the mouse model of infection, it is believed that pathology in the upper genital tract occurs as a result of an ascending infection from the lower genital tract. To assess the ability of BD584 immunization to reduce bacterial shedding, vaginal swabs were collected and analyzed by qPCR. During the peak of infection, 3-7 days post infection, vaccination with BD584 reduced bacterial shedding by 95% compared to control vaccinated mice. In our study immunized mice shed approximately 90 IFUs compared to more than 1500 IFUs in the control vaccinated mice on day 5 at the peak of infection. Furthermore, *C. muridarum* was not detectable in vaginal swabs on day 32 post-infection in the vaccinated mice; whereas, it was detected in control vaccinated mice. Vaccination with BD584 significantly reduced bacterial shedding to less than 300 IFUs at all time points, suggesting that BD584 may decrease transmission of *Chlamydia* infections. Since

Chlamydia infections causes significant UGT pathology we examined the UGT following *Chlamydia* challenge to determine whether vaccination could prevent *Chlamydia*-induced immunopathology. Vaccination with BD584 decreased the rate of hydrosalpinx from 80% to 10%. The reduction in bacterial shedding, coupled with protection against *Chlamydia*-induced pathology suggests that BD584 affords a significant degree of protection and could be an effective vaccine for human use. Since a Th1 immune response is required to clear a chlamydial infection and elicit antibodies to prevent infection, the BD584 vaccine likely produced a Th1 polarized immune response, and anti-chlamydial antibodies targeted against the chlamydial T3SS proteins. The immune mechanism of protection afforded by BD584 is not known but the combined neutralizing antibodies and cell-mediated immune response could be instrumental in reducing bacterial shedding and pathology. We are currently investigating the immune mechanism of protection.

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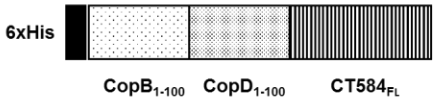
(a)

Primer	Sequence
$CopB_{1-100}$(+)	GTTGTTAA**GGATCC**TATGTCCCTTTCATCTTCTTCG
$CopB_{1-100}$(-)	GTTGTTAA**GAATTC**GGATGTCGCATCGGCAGAT
$CopD_{1-100}$(+)	GTTGTTAA**GAATTC**ATGACAACAGGAGTACGTGG
$CopD_{1-100}$(-)	GTTGTTAA**GTCGAC**TGCAGTTGCTGTTTGGCTAG
$CT584_{FL}$(+)	GTTGTTAA**GTCGAC**ATGACGACGAAACCCAAAACT
$CT584_{FL}$(-)	GTTGTTAA**AAGCTT**TTACACAGATTTCGTTAATTCTT

(b)



(c)

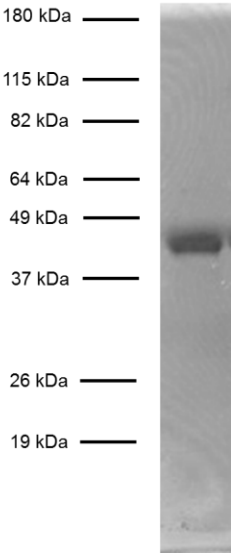


Figure 1: Oligonucleotide primers used to generate recombinant protein BD584. (a) BD584 was constructed by amplifying the genes for CopB, CopD and CT584 using genomic DNA from *C. trachomatis* serovar D using the primers indicated. PCR products were digested with their respective endonuclease (in bold), and then ligated into the MCS-1 of the pET-DUET1 vector. (b) Organization of the final trivalent antigen, termed BD584. (c) BD584 was expressed from pET-DUET in *E. coli* BL21 and purified by FPLC on a Ni-NTA agarose column, buffer exchanged into PBS and analyzed by SDS-PAGE. The positions of molecular weight markers are shown on the left. Note: (+) denotes forward primer, (-) denotes reverse primer, subscripts denotes amino acid number, FL = full length.

Figure 2: Conserved amino acid sequences of CopB$_{1\text{-}100}$, CopD$_{1\text{-}100}$, and CT584$_{FL}$ orthologs. BLAST-P analysis was performed using the amino acid sequences of CopB$_{1\text{-}100}$, CopD$_{1\text{-}100}$, and CT584$_{FL}$ from *C. trachomatis*. Alignment between *C. trachomatis* and *C. muridarum* orthologs revealed 86%, 78%, and 97% amino acid identity between CopB$_{1\text{-}100}$, CopD$_{1\text{-}100}$, and CT584$_{FL}$, respectively.

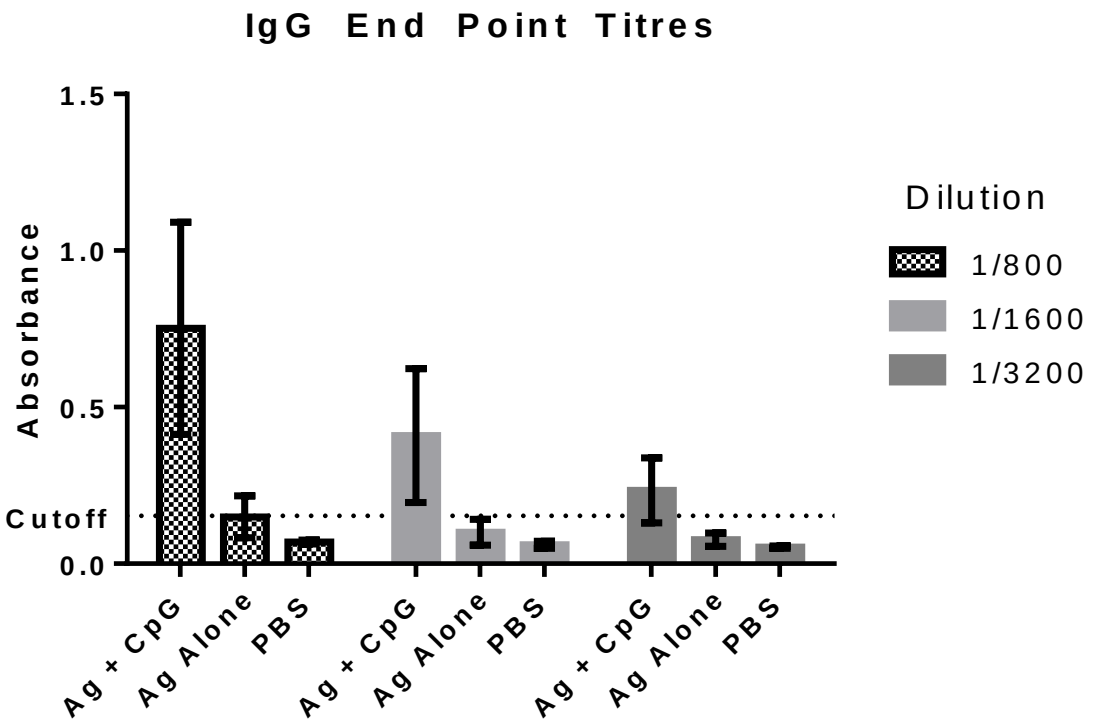


Figure 3: Measurement of serum antibodies to BD584 in immunized mice. Serum from vaccinated and control mice were tested by ELISA at serial two-fold dilutions from 1:25 to 1:3200 for specific anti-BD584 antibodies as described in the Methods section. Absorbance for the last three dilutions 1:800, 1:1600, and 1:3200 is shown. Histogram bars represent the mean absorbance for BD584+CpG, BD584 alone, and PBS + SEMs.

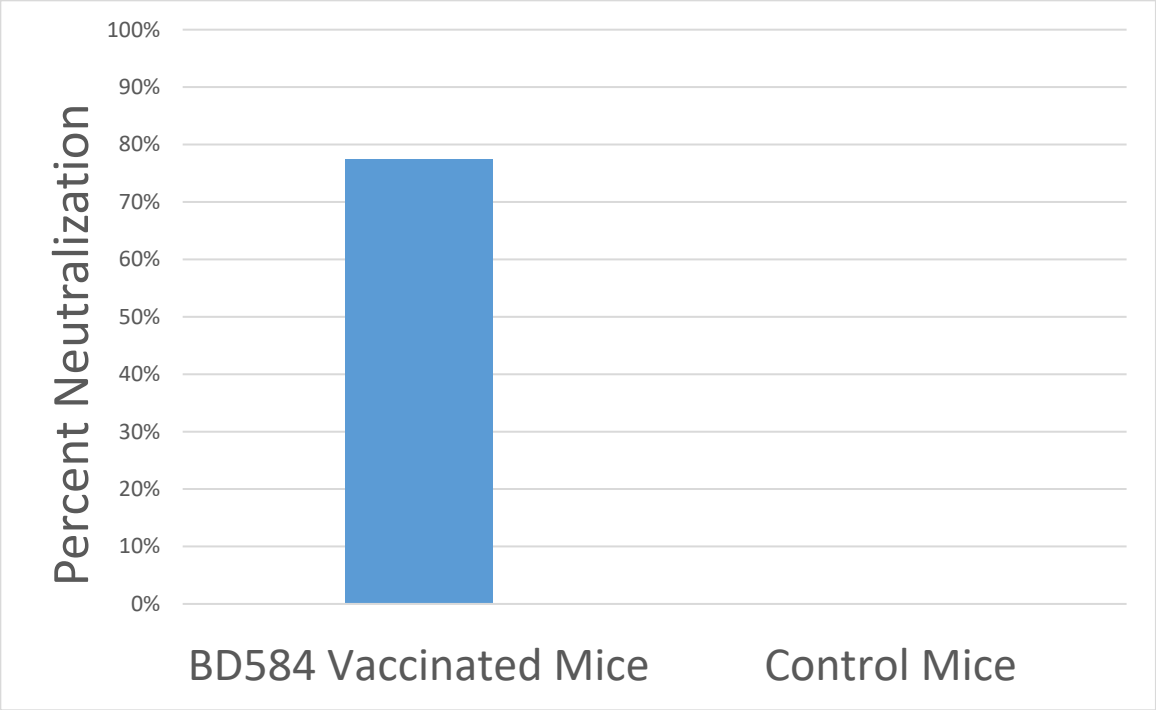


Figure 4: Presence of serum neutralizing antibodies in mice. BD584 vaccinated mice (N=5) and control mice (N=5) were bled, and tested for neutralizing antibodies to *C. muridarum*. Serum (diluted 1:10) was pre-incubated with *C. muridarum* for 1 hour at 37°C and the mixture put on a cell monolayer as described in Materials and Methods. Inclusions were counted at 48 hr. A mean reduction of approximately 77% was observed with serum from the vaccinated group and no reduction was seen with the control group.

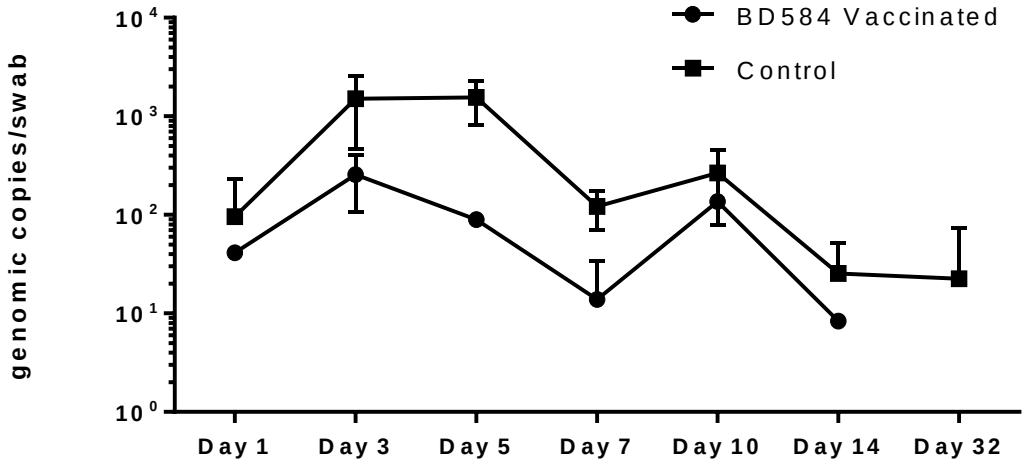


Figure 5: Vaginal shedding of *C. muridarum* in BD584 vaccinated and control mice determined qPCR. Vaginal swabs were collected on days 1, 3, 5, 7, 10, and 14 post infection and *C. muridarum* shedding was determined using qPCR. A 95% reduction in bacterial shedding was observed in the vaccinated group compared to the control group at peak of infection on days 5 and 7.

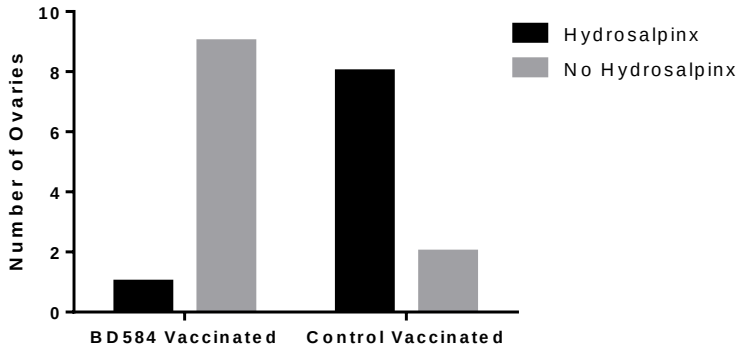


Figure 6: Presence of oviduct pathology (hydrosalpinx) at 35 days post infection. Mice were sacrificed 35 days after intravaginal challenge with *C. muridarum*, and upper genital tracts harvested and analyzed for the presence of hydrosalpinx using a pathology grading scores as described in Materials and Methods. Hydrosalpinx was present in 8/10 ovaries collected from the five unvaccinated mice, compared with only 1/10 ovaries in the vaccinated mice. Fisher's exact test; $p = 0.0055$.

CHAPTER FIVE - DISCUSSION

Chlamydia's T3SS is believed to be an essential virulence factor required for invasion of host cells and intracellular survival; however, as an obligate intracellular pathogen with limited systems for genetic manipulation, studying the T3SS within *Chlamydia spp.* is an arduous process. The translocator proteins are believed to be essential components of this secretion system, facilitating the translocation of bacterial effector proteins in to the host cell. Early genetic studies identified putative chlamydial genes for T3SS components, including translocator proteins; yet, even after their identification, there have been limited studies to characterize these proteins. Most of the data we have regarding the function of the translocator proteins within *Chlamydia* is modeled from orthologous secretion systems. However, understanding how the translocator proteins function within *Chlamydia* may unlock further information regarding translocator proteins in other bacterial species which utilize T3SS since *Chlamydia spp.* are believed to be one of the first archetypal bacteria to possess a T3SS (Betts-Hampikian & Fields, 2010; Kim, 2001).

Genetic Studies of *Chlamydia spp.* indicate that *Chlamydia* may contain two sets of translocator proteins, with both sets encoded downstream of a type II chaperone protein (Ouellette et al., 2005). Though functionally the translocator proteins are believed to be highly conserved between species, there is a significant variation in amino acid sequence. CopD, which is believed to be the minor hydrophilic translocator, is located in the same operon as CopB and LcrH_1. Working with translocator proteins is exceptionally difficult as they are

membrane bound proteins, thus to functionally characterize them it is easier to work with truncated version excluding the transmembrane domains. It was previously noted in *C. trachomatis* that Scc2 (LcrH_1) interacts with CopD; however, this interaction has not been characterized (Fields, Fischer, Mead, & Hackstadt, 2005). Similarly, no data exists except for the initial identification of the interaction between CopB and Scc2 (Fields et al., 2005). Using this data as the foundation for my graduate work, I expanded what is known regarding the translocator proteins from *Chlamydia spp*., and the following section outlines this contribution to the *Chlamydia* field, and the implications of the research to the understand of *Chlamydia* biology.

Chlamydia translocator proteins: Does *Chlamydia* have them, and what do they do?

Bioinformatic analysis is an excellent starting point in the initial characterization of a previously uncharacterized protein. Genetic analysis of *Chlamydia spp*. indicates that there are potentially two sets of translocator proteins, yet is believed to have only one T3S apparatus. Transcriptional analysis of the operons encoding CopB/CopD and CopB2/CopD2 indicates that these operons are transcribed at different times in the *Chlamydia* developmental cycle. However, the literature has failed to describe any potential implications from this observation. One set of translocator proteins, CopB/D, are upregulated late in the chlamydial lifecycle, during RB to EB transition. Presumably, this increased production of CopB and CopD late in the infection cycle is to prepare

CopB and CopD in elementary bodies for the next infection cycle. Alternatively, CopB2 and CopD2 are upregulated in mid-lifecycle of *Chlamydia*, during the replication of RBs. Why would *Chlamydia* have two sets of translocators, yet have only one T3S apparatus, which is unlike other bacteria that have two T3SS, such as *Salmonella*'s SPI-1 and SPI-2 system (Jones, Hulme, Barrow, & Wigley, 2007). During the infectious cycle, *Chlamydia* is intimately associated with the inclusion membrane to maintain a constant efflux of effector proteins into the host cell to maintain its intracellular niche. This data suggests that the first set of translocator proteins, CopB and CopD, are important for invasion of the EB into the host cell, whereas CopB2 and CopD2 are critical for intracellular RB replication. However, the most interesting fact is that there is only one annotated T3SS in *Chlamydia* (Hsia et al., 1997). *Chlamydia* has a significantly reduced genome size in comparison to other bacteria. Unlike other bacteria that have multiple secretion systems, such as *Salmonella*, it does not seem to be an evolutionary fit idea to encode multiple copies of structural components of T3S proteins when only the proteins that are important for invasion and replication can be interchanged with one another depending on the expression of such protein. Thus, it is possible that *Chlamydia* may have a T3SS that is composed of a core of structural proteins that are maintained during the life-cycle, with proteins that can be swapped in-and-out during the lifecycle to meet the different needs of the infectious EB and replicating RB. The translocator proteins are not the only evidence for this observation. Work by Stone *et. al.* indicates that *Chlamydia*

possesses a flagellar ATPase, yet *Chlamydia* is a non-motile bacteria (Stone, Bulir, Gilchrist, Toor, & Mahony, 2010). These experiments demonstrated that these flagellar components interacted with T3S proteins, further reinforcing the fact that *Chlamydia* could form a hybrid T3S apparatus consisting of proteins that are swapped in and out depending on the stage of the chlamydial life cycle. This simple observation that *Chlamydia* possesses two sets of translocator proteins, yet one T3S apparatus, has significant implications as it could be the first bacteria with a “mix-and-match” or “hybrid” T3S apparatus.

Structural bioinformatics studies of both CopB and CopD suggest that they contain features that are characteristic of translocator proteins such as numerous transmembrane domains to mediate membrane insertion, coiled-coil domains for protein and membrane interaction, and a putative chaperone binding domain (Chellas-Gery, Wolf, Tisoncik, Hackstadt, & Fields, 2011; Lunelli et al., 2009). Using this data to guide protein interaction studies, it was no surprise when an N-terminal fragment of both CopB and CopD (both of which contain the chaperone binding motif) interacted with the chaperone, LcrH_1. The chaperone binding motif (PxLxxP) appears to be an essential motif for interaction between type II chaperones and the translocator proteins, as this motif is highly conserved across many bacterial species, and site-directed mutagenesis of the conserved amino acids ablates interaction between the two proteins.

Given the necessity of this small CBD motif for an essential interaction to *Chlamydia* survival, this interaction is amenable to disruption using a peptide

mimetic, and may represent a novel therapeutic target for *Chlamydia* infections. LcrH_1 is believed to keep the translocator proteins in a secretion competent state while also preventing homo- and hetero-oligomerization. As effector-chaperone interactions have been well-elucidated in other bacterial T3SSs, I explored whether CopB or CopD interact with CdsN, and showed that both are capable of interaction with CdsN. Whether the translocator proteins are pre-docked on the tip of the injectisome needle apparatus prior to host cell contact, or recruited to the tip after host cell contact, it is reasonable to assume that than upon activation of secretion, the translocator proteins must be uncoupled from their putative chaperone via the T3SS ATPase, CdsN.

Further protein interaction studies were performed using CopB and CopD to examine other possible interactions within the chlamydial T3SS. As it is believed that the translocator proteins are docked on the tip of the needle apparatus, it is logical to assume that there should be an interaction between the translocator proteins and filament protein, CdsF, which makes up the T3S needle. I demonstrated an interaction between an N-terminal and middle fragment of both CopB and CopD were observed, suggesting that at some point during infection that the translocator proteins do interact with the filament protein. However, further experiments will be required to elucidate whether that translocator proteins are organized on the needle-tip prior to host cell contact, as this would have important implications for novel vaccine targets. Initial work with Cpn0803 described it as the needle-tip protein in *C. pneumoniae*. However, new

information regarding Cpn0803 appears to suggest that it may be a chaperone protein, and therefore it was not surprising when it failed to interact with CopB (Pais, Milho, Almeida, & Mota, 2013). The interactions observed between CopB and CopD and other components of the T3SS are in keeping with interactions observed in other bacterial T3SSs, providing evidence that CopB and CopD, are in fact, translocator proteins.

One common feature of the translocator proteins is that they are able to oligomerize by forming hetero- and homo-oligomers. It has been well documented in the literature that the translocator proteins can oligomerize (Dickenson et al., 2013; Gebus et al., 2008; Montagner, Arquint, & Cornelis, 2011). We initially tried to express full-length CopB and CopD, however, over-expression in *E. coli* was toxic and any protein that was produced was insoluble. However, soluble protein was able to be produced when the translocator proteins were co-expressed with LcrH_1, further evidence that LcrH_1 is the chaperone for CopB and CopD. When CopD was subjected to size exclusion chromatography, it migrated through the column as either an aggregate of approximately 10 monomers, a biologically plausible tetramer, or in a monomeric state bound to LcrH_1. I was unable to determine whether CopB formed multimers even though soluble CopB protein was produced, as it was consistently contaminated with other bacterial proteins. Despite the inability to show oligomerization of CopB, these findings reinforce the fact that the

chaperone protein, LcrH_1, is most likely a class II chaperone as it interacts with the translocator proteins.

Whether the translocator proteins are pre-loaded onto the tip of the needle apparatus prior to host cell contact is a topic of much debate, as there has been conflicting information in other secretion systems. Furthermore, even less is known about the cellular localization of the translocator proteins within *Chlamydia* (Chellas-Gery et al., 2011). Using polyclonal antibodies generated against an N-terminal epitope of both CopB and CopD, it is possible to discern the potential cellular location of the translocator proteins. Pre-incubation of *C. pneumoniae* with either of these antibodies reduced the number of inclusion by upwards of 98% compared to control antibodies, suggesting that the translocator proteins may be surface exposed prior to host cell contact. However, future experiments should aim to further localize the translocator proteins as we are only capable of stating that the translocator proteins are surface localized at some point during infection, and it is unclear if host cell contact is required for this recruitment to occur. Regardless, antibodies directed towards the translocator proteins are able to inhibit *Chlamydia* infection *in vitro*, which suggests that these proteins are surface exposed and may be excellent candidates for vaccine studies.

Limitations and future directions on the biochemical characterization of proteins within *Chlamydia spp.*: At the tipping point.

As mentioned previously, studying *Chlamydia* is an arduous task, and attempting to biochemically characterize a protein has required extensive use of *in vitro* methods, at times making interpretation of the data difficult. However, as my graduate work is winding down, we are on the precipice of significant advances in the understanding of *Chlamydia* biology. Researchers have started to produce non-lethal mutants through both random, and site directed mutagenesis (Kokes et al., 2015; Nguyen & Valdivia, 2013). Consequently, the *Chlamydia* field is now able to take a reverse genetics approach to characterize chlamydial proteins by ascribing phenotypic changes to specific amino acid substitutions. However, at the moment, reverse genetics has little utility in elucidating proteins involved in pathogenesis (i.e. T3S proteins) as it is believed that T3S proteins are critical to *Chlamydia's* survival since it is an obligate intracellular protein and we presently do not have an axenic media for *Chlamydia* spp.. With that said, thanks to the pioneering work of Dr. Ian Clarke in *Chlamydia* genetics, it is now possible to transform *Chlamydia spp*. (Wang et al., 2013). Using this new transformation system, it is now possible to insert exogenous genes into *Chlamydia*. The genes, which can encode fusion proteins, make it easier to study protein-protein interactions in a system which more readily mimics natural *Chlamydia* biology. A logical progression from this present work could explore the transformation of C*hlamydia* with fusion proteins of LcrH_1, CopB,

and CopD to study protein-protein interactions, and cellular localization. However, for the time being, the necessity of the translocator proteins to the intracellular survival and replication of Chlamydia is still speculative and requires significant advances in *Chlamydia* genetics and biology to conclusively ascribe an essential role in chlamydia pathogenesis.

Type III secretion proteins – novel antigens for protection against *Chlamydia*-induced immunopathology

As discussed previously, Chlamydia infections are the leading bacterial sexually transmitted infection in North America. Pelvic inflammatory disease (PID) is a potential consequence of Chlamydia infection and can lead to hydrosalpinix and subsequently infertility, even after being treated with antibiotics. Furthermore, upwards of 75% of females, and 50% of males are asymptomatic and unknowingly infected with Chlamydia. Given this information, a *Chlamydia* vaccine could have a significant impact on preventing PID and infertility. However, over 50 years of research into a *Chlamydia* vaccine has yielded limited success. We initially generated antibodies towards the translocator proteins in attempts to better understand the interactions ad localization of the translocator proteins throughout the lifecycle of *Chlamydia*. However, after observing the ability of these antibodies to neutralize infection, this suggested that CopB and CopD may be excellent immunogens to prevent against chlamydial infection. Previous experiments with collaborators had identified Cpn0803 (or CT584 in *C. trachomatis*) as an antigen with potential to protect against *Chlamydia*-induced

pathology. Using this knowledge, we combined the predicted antigenic regions of CopB and CopD (the first 100 amino acids of each), with full length CT584, all from *C. trachomatis*, into one chimeric fusion protein. After setting up a mouse model of *Chlamydia* infection using *C. muridarum*, which has a highly reproducible endpoint of hydrosalpinix, mice were vaccinated intranasally with 20 ug of BD584 with 10 ug of CpG. Using a Th1 polarizing adjuvant allows the immune response to be polarized towards cellular-mediated immunity (CMI), as CMI has been demonstrated to be critical for preventing and clearing *Chlamydia* infections. Mice were vaccinated twice, three weeks apart, prior to being challenged with *C. muridarum*. To study the effects on bacterial shedding, vaginal swabs were collected on Days 1, 3, 5, 7, 10, 14 post infection, and analyzed via qPCR for the presence of *C. muridarum*. A significant reductions of >90% in bacterial shedding were observed during peak of infection in the vaccinated group as compared to the control group on Days 5 and 7. It has been demonstrated that 300 IFUs are required to establish a chlamydial infection in mice, and given the fact that vaccinated mice had < 300 IFUs in their vaginal swabs at peak of infection suggest that this vaccination strategy may prevent transmission of *Chlamydia* infection (Carey, Cunningham, Hafner, Timms, & Beagley, 2009). Furthermore, 32 days post infection, the mice were sacrificed and the fallopian tubes were examined for the presence of hydrosalpinx. In the vaccinated group, hydrosalpinx was present in only 1/10 fallopian tubes, as compared to 8/10 in the unvaccinated group. Taken together, this data suggests

that vaccination with BD584 could represent a novel class of antigens used to protect against *Chlamydia* infection and associated pathology. However, to further understand how this vaccination strategy is able to afford such protection, it would be prudent to begin to characterize the immune response produced via vaccination with these T3S proteins. This work is presently ongoing by other members of the Mahony laboratory.

Closing Remarks

The name *Chlamydia* has Greek origins, and is derived from the word khlamus, which means “cloak” (Byrne, 2003). Though some may believe that the naming of *Chlamydia* is incorrect since it was not known at the time of its initial identification that the “cloak-like mantle” was actually cytoplasmic inclusions filled with the bacteria, not the bacteria itself. From the initial identification of *Chlamydia* in the 1910s as a protozoan, to it being classified as a “large virus” in the 1930’s, and finally being identified as a bacteria in the mid-1960’s, *Chlamydia* has always been incredibly difficult to study - it concealed its identity for over 50 years. The presence of T3S genes in *Chlamydia* were identified in 1997, and almost two decades later, we are just on the cusp of understanding T3S within *Chlamydia*. However, to me, the name could not be any more fitting. After almost 100 years after its initial identification, and the technological advances that have revolutionized microbiology, we are just beginning to remove the “cloak” shrouding *Chlamydia’s* biology.

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