CHARACTERIZATION OF COPN (*CPN0324*) THE PUTATIVE TYPE III SECRETION SYSTEM PLUG PROTEIN OF *CHLAMYDIA PNEUMONIAE*

CHARACTERIZATION OF COPN (*CPN0324*) THE PUTATIVE TYPE III SECRETION SYSTEM PLUG PROTEIN OF *CHLAMYDIA PNEUMONIAE*

By TIFFANY LEE LEIGHTON, H.B.Sc.

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Descriptive Note

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TITLE: Characterization of CopN (*Cpn0324*) the Putative Type III Secretion System
       Plug Protein of *Chlamydia pneumoniae*

AUTHOR: Tiffany L. Leighton, H.B.Sc. (Trent University)

SUPERVISOR: Dr. James B. Mahony

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To Mom, Dad, Darrell & Tristan
With all my love

ABSTRACT

Chlamydia pneumoniae is a Gram-negative, intracellular bacterium which utilizes a type III secretion system for virulence. This system injects virulence associated proteins into a host cell and ultimately hijacks host intracellular machinery required for the bacteria to propagate and reproduce. Chlamydia outer protein N or CopN (*Cpn0324*), is a member of a family of proteins found in pathogenic bacteria, which inhibits premature secretion of effector proteins by plugging the base of the injectisome. The lack of a genetic system to manipulate the bacteria hampers the identification of proteins within the T3S field. The work presented in this thesis establishes the role of CopN as the plug protein of *Chlamydia pneumoniae*, and examines protein interactions within the individual CopN domains.

The structure of CopN was first explored by limited proteolysis to establish the domain boundaries. We found three domains, an N-terminal, central domain, and C-terminal domain. Next, we used the full length protein as well as a series of truncations to examine the interactions within each domain. Using a subset of the known protein interactions between CopN and other T3S proteins, we found that the proposed chaperones Scc1 and Scc4 bind in the N-terminal region. There were no apparent interactions in the central domain, whereas FliI, FliF, CopD1$_{158-206}$ and Scc3 all bound within the C-terminal region of CopN. Finally the secretion of CopN in a HeLa cell model was addressed throughout the course of an infection. CopN was detected in the host cell immediately after infection, and then was not detectable again until late

infection. Overall, I have characterized the individual domains of CopN and present data

to support the role of CopN as the plug protein in the T3SS of *Chlamydia pneumoniae*.

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LIST OF ABBREVIATIONS AND SYMBOLS

aa   -amino acids
ABC   -ATP binding casette
AMP   -ampicillin; antibiotic
ATCC   -American Type Culture Collection
ATP   -adenosine triphosphate
β-ME   -beta-mercaptoethanol
BSA   -bovine serum albumin
CAD   -coronary artery disease
CBD   -chaperone binding domain
Cds   -contact dependent secretion
COMP   -Chlamydial outer membrane complex
COPD   -chronic obstructive pulmonary disease
CopN   -Chlamydial out protein N
Cpn   -*Chlamydia pneumoniae*
Cpsi   -*Chlamydia psittaci*
CT   -*Chlamydia trachomatis*
C-terminus   -carboxyl terminus
CWL029   -respiratory isolate of *C. pneumoniae*
EB(s)   -elementary body (bodies)
ECL   -enhanced chemiluminescence
EDTA   -ethylenediaminetetra-acetic acid
ELISA   -enzyme-linked immunosorbent assay
DNA   -deoxyribonucleic acid
FBS   -fetal bovine serum
GSP   -general secretory pathway
GST   -glutathione-s-transferase
HeLa cells   -immortal human cell line; for *C. pneumoniae* propagation
His_6 -common tag formed of 6 Histidine residues
hpi   -hours post infection
HRP   -horseradish peroxidase
IB   -intermediate body
IM   -inner membrane
IPTG   -isopropyl-β-D-thiogalactopyranoside
KAN   -kanamycin; antibiotic
kDa   -kilo Daltons
LB   -Luria-Bertani broth
LDAO   -lauryldimethylamine-oxide
MEM   -minimum essential medium
MOI   -multiplicity of infection
MOMP   -major outer membrane protein
N-terminus   -amino terminus

NC	-needle complex
OD	-optical density
OM	-outer membrane
ORF	-open reading frame
PAGE	-polyacrylamide gel electrophoresis
PBS	-phosphate buffered saline
PCR	-polymerase chain reaction
RB(s)	-reticulate body (bodies)
RNA	-ribonucleic acid
rRNA	-ribosomal ribonucleic acid
SDS	-sodium dodecyl sulfate
SOC	-super optimal broth with catabolic repressor
SPG	-sucrose-phosphate-glutamate
T1S	-type I secretion
T2S	-type II secretion
T3S	-type III secretion
T3SS	-type III secretion system
T4S	-type IV secretion
T5S	-type V secretion
T6S	-type VI secretion
TARP	-translocated actin recruitment protein
Tat	-twin-arginine pathway
TBST	-tris buffered saline with Tween-20
TCA	-trichloroacetic acid

CHAPTER ONE

1.0 INTRODUCTION

1.1 <u>The Chlamydiae Phylum</u>

1.1.1 *Chlamydiaceae*

Chlamydiaceae is a family of obligate intracellular, Gram-negative bacteria which infects a wide-host-range of multicellular organisms such as mammals (Betts-Hampikian & Fields, 2010), reptiles (Berger et al., 1999; Wilcke et al., 1983), and insects (Peters et al., 2007), as well as unicellular organisms such as free-living amoebae (Corsar & Venditti, 2006; Peters et al., 2007). These pathogens can infect a large array of eukaryotic cell types, causing various human and veterinary infections (Betts-Hampikian & Fields, 2010). The family can be divided into two genera, *Chlamydia* and *Chlamydophila*, and one candidate genus *Candidatus clavochlamydia* (Karlsen et al., 2008). Within the *Chlamydia* and *Chlamydophila* genera there are nine species; *Chlamydia trachomatis, Chlamydia muridarum, Chlamydia suis, Chlamydophila caviae, Chlamydophila abortus, Chlamydophila pecorum, Chlamydophila pneumoniae, Chlamydophila psittaci*, and *Chlamydophila felis* (Table 1.1)(Everett & Andersen, 1999).

Table 1.1: The family *Chlamydiaceae* as proposed by Everett *et al.* (1999)

Species	Typical Host (s)	Route of Entry
Chlamydia		
C. trachomatis	Human	Pharyngeal, ocular, genital, rectal
C. suis	Swine	Pharyngeal
C. muridarium	Mouse & hamster	Pharyngeal, genital
Chlamydophila		
C. psittaci	Birds	Pharyngeal, ocular, genital
C. pneumoniae	Human, koala, horse, frog	Pharyngeal, ocular
C. pecorum	Domestic animals, koalas	Oral
C. felis	House cat	Pharyngeal, ocular, genital
C. caviae	Guinea pig	Pharyngeal, ocular, genital, urethral
C. abortus	Mammals	Oral, genital

The *Chlamydophila* classification is not used in this thesis

1.1.2 Clarification of *Chlamydia* taxonomy

Classification of the *Chlamydiaceae* was emended in 1999 by Everett *et al.*, dividing the family into the distinct genera *Chlamydia* and *Chlamydophila,* based on the 16s rRNA sequence similarity (Everett et al., 1999). This genera separation however is not being used consistently in the scientific literature. More recently it has been suggested to abandon the name change and use a compromised taxonomy first proposed by Kalayoglu and Byrne (Kalayoglu & Byrne, 2006). This compromised taxonomy differs from the "official" taxonomy supported by the International Committee on Systematics of Prokaryotics, and combines both *Chlamydia* and *Chlamydophila* into the single genus of *Chlamydia* (Bavoil & Wyrick, 2006). This taxonomy had been adopted in this thesis, and all reference to *C. pneumoniae* in this document implies <u>*Chlamydia pneumoniae*</u>.

1.1.3 *Chlamydia pneumoniae*

Three specific species of the *Chlamydia* genus are associated with human disease; *Chlamydia trachomatis* (CT)*, Chlamydia psittaci* (Cpsi)*,* and *Chlamydia pneumoniae* (Cpn). *C. trachomatis* is the most common bacterial sexually transmitted infection and causes trachoma and genital infections, and is the number one cause of preventable blindness worldwide (Lutter et al., 2012). The pathogenic agent behind ornithosis or psitticosis in avian species is *C. psittaci*, but this pathogen can be zoonotically transmitted to humans if they come into close contact with infected animals (Voigt et al., 2012). *Chlamydia pneumoniae* is the final species of *Chlamydiaceae* family which is able to

cause human disease. This species causes a respiratory infection in humans and is a causative agent of pneumonia as well as other respiratory tract infections.

C. pneumoniae was the third species of *Chlamydia* isolated and was initially considered to be *C. psittaci.* However, upon comparing DNA homology of *Chlamydial* species and the unique elementary body structure determined by electron microscopy, it was classified as a distinct species (Chi et al., 1987). The TWAR organism was isolated from two distinct sources; an eye swab from a child in Taiwan in 1965, and then again from a throat swab of a University of Washington student in 1983 (Grayston et al., 1990). Using standard bacterial culturing techniques, *C. pneumoniae* tended to grow poorly in culture when compared to both *C. trachomatis* and *C. psittaci*, which likely led to its late discovery (Grayston et al., 1990). This bacterium can be transmitted via respiratory droplets from an infected individual to a non-infected individual, and has been implicated in epidemics within closed populations, such as isolated villages or military groups. Early serological studies suggested that approximately 1 in 9 *C. pneumoniae* infections resulted in pneumonia, indicating that many individuals likely experience an asymptomatic infection (Saikku et al., 1985).

1.1.4 *Chlamydia pneumoniae* genomics

Whole genome sequencing of the *C. pneumoniae* genome by Kalman *et al.* (1999) allowed for an in depth comparison to the well characterized organism, *C. trachomatis*. Although *C. pneumoniae* had an additional 187,711 nucleotides, with a whole genome

size of approximately 1.2 Mbp when compared to the genome of *C. trachomatis*, there

was approximately 80% functional conservation in the predicted coding sequences (Read

et al., 2000). This large proportion of gene similarity is accompanied by an average of

62% amino acid similarity between homologues of the two species (Kalman et al., 1999).

While both species are capable of producing ATP, *Chlamydia* is still thought to scavenge

energy from the host to fulfill its energy requirements (Stephens et al., 1998). Glycogen-

metabolizing genes have been found in Cpn, Cpsi and CT; however, only CT has been

shown to accumulate glycogen within the inclusion (Matsumoto et al., 1998). This

difference is correlated with the virulence plasmid found only in *C. trachomatis*, since

naturally occurring plasmid-lacking strains did not stain positive for glycogen when

stained with iodine (Carlson et al., 2008). Due to a remarkably stable genome with few

rearrangements, Chlamydial genes are relatively conserved between these two very

different species of *Chlamydia*, which emphasizes the idea that genome content was

likely established early in Chlamydial evolution. Minor differences in the genomes are

attributed to the differences in tissue tropism, life cycle and disease of each pathogen.

1.1.5 *Chlamydia pneumoniae* **pathogenesis**

Chlamydia pneumonia is a common human respiratory pathogen associated with

up to 10% of community-acquired pneumoniae cases each year in Canada, the U.S. and

Japan (Marrie et al., 2003; Miyashita et al., 2002). It has been estimated that presence of

C. pneumoniae antibodies in the general population is over 70% in adults, indicating that

most adults have been exposed to this pathogen at some point in their lives, however

many infections are likely asymptomatic (Leinonen, 1993; Saikku et al., 1985). *C. pneumoniae* may be associated with other respiratory conditions such as wheeze in children, asthma in adults and chronic obstructive pulmonary disease (COPD) (Gislason et al., 2010; Clementsen et al., 2000). Disease associated with this bacterium is generally acute, however growing evidence has linked chronic *C. pneumoniae* infections to many human diseases such as atherosclerosis (Grayston, 2005; Grayston, 2000), coronary artery disease (CAD) (Nieto et al., 1999), multiple sclerosis (Gerard et al., 2006), and Alzheimer's disease (Contini et al., 2008). Recently studies have focused on elucidation of the link between atherosclerosis and *C. pneumoniae*. Godzik *et al.* (1995) suggest that *C. pneumoniae* is a risk factor for atherosclerosis, demonstrated through the ability of the pathogen to grow within many different cell types including endothelial cells, macrophages, and aortic smooth muscle cells. It has been hypothesized that the contribution of the immune response by the host likely plays a role in the atherogenic response. However, Deniset *et al.* (2010) found that *C. pneumoniae* infection can stimulate thickening of arterial walls, independent of the host immune response. These results indicate that *C. pneumoniae* alone can induce a significant component of atherosclerosis, but further research into the specific mechanisms involved is required.

1.1.6 Challenges and limitations

Chlamydia is one of the few clinically relevant human pathogens which cannot be genetically modified. The lack of tools for genetic manipulation hampers efforts to discern the specific function of proteins. Growth of *Chlamydia* has also been an obstacle

in Chlamydial research. The obligate intracellular lifestyle of *Chlamydia*, means that the

bacteria needs to be grown and harvested from cultured eukaryotic cells, as opposed to

conventional bacteriological medium. This culturing technique can introduce

contamination, cause inactivation of the organism and is time consuming (Bavoli et al.,

2000). In the face of these obstacles, other scientific techniques are employed and the

function of proteins can be elucidated from sequence homology as well as unique protein

interactions that remain conserved in other bacterial species.

1.2 The Intracellular Life Cycle of *Chlamydia*

1.2.1 Developmental cycle

All *Chlamydial* species although unique in host range, tissue tropism and disease

pathology, require uptake by a eukaryotic cell to begin their biphasic developmental cycle

(Voigt et al., 2012). Initiation of the replication cycle begins with a small (~0.2 to 0.3

μm), infectious extracellular Elementary Body (EB) attaching to the host cell membrane

(Figure 1.1). Characteristically EBs have a highly condensed outer envelope composed

of intra- and intermolecular cysteine-rich disulfide bonds, and are considered

metabolically inactive (Hatch et al., 1986). This stable outer membrane known as the

Chlamydial outer membrane complex (COMC) is primarily composed of a single major

outer membrane protein (MOMP) in an abundance of ~60% (Liu et al., 2010). The rigid

shell can withstand mechanical agitation, likely an adaptation to facilitate EBs survival in

the extracellular environment while it passively waits for a suitable host. The

internalization of an EB is a critical step in Chlamydial pathogenesis and has been the

focus of much research, yet definitive mechanisms involved are still speculatory at best.

An involvement of clathrin in the uptake of EBs has been overwhelmingly accepted in

literature, yet it is not essential for internalization (Hybiske and Stephens, 2007a).

Another hypothesis is the involvement of a type III secretion effector known as

translocated actin-recruiting phosphoprotein (TARP). This protein is proposed to induce

the formation of actin pedestals at the host cell surface which aid in the internalization of

the EB into the cell (Clifton et al., 2004).   Along with their ability to initiate their own

uptake into the cell via pino- and phagocytosis (Reynolds & Pearce, 1991), EBs are able

to disrupt a vast array of host cell functions. These include the rearrangement of the actin

cytoskeleton, inhibition of early apoptosis (Peters et al., 2007), as well as an ability to

scavenge nutrients from the host by hijacking intracellular organelles (Saka & Valdiva,

2010). The internalized EBs are contained within a non-acidified membrane derived

vacuole known henceforth as an inclusion, which is arguably the most important driving

force in the formation of their peculiar ecological niche (Voigt et al., 2012).
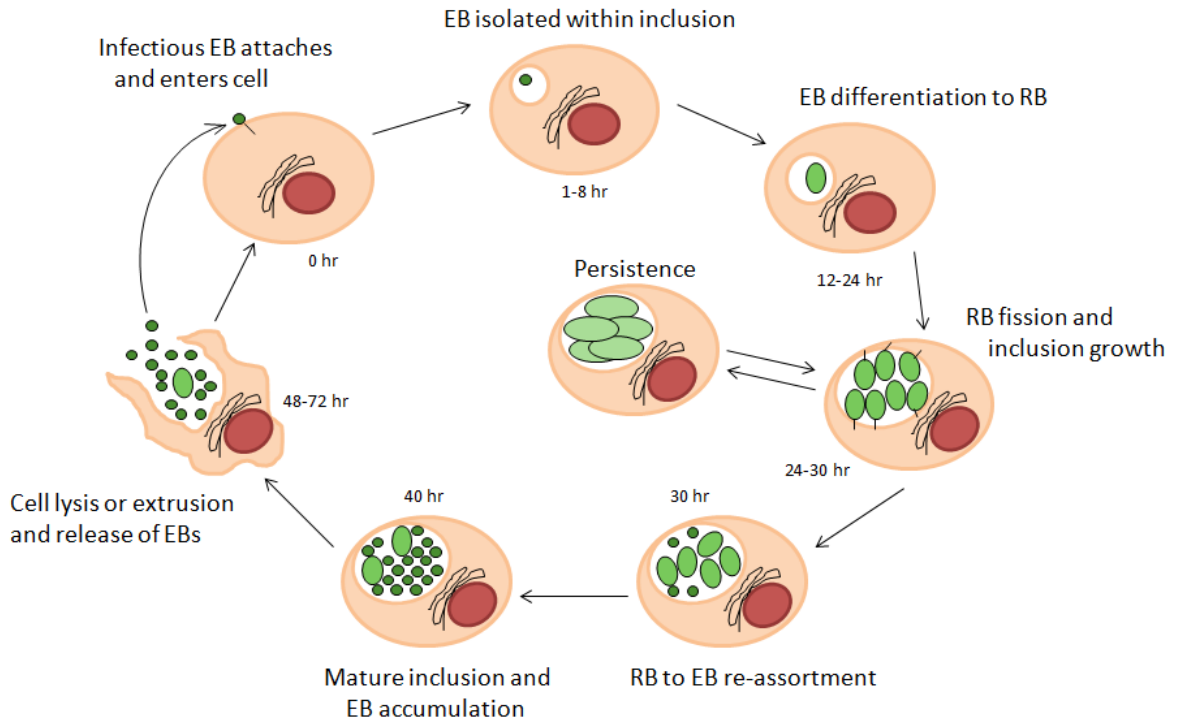
Figure 1.1: The life cycle of *Chlamydia pneumoniae* (adapted from: Hammerschlag, 2002). Diagram of an idealized Chlamydial developmental cycle. The small, infectious elementary bodies (EBs) are seen in dark green; the larger replicating reticulate bodies (RBs) are seen in a lighter green. T3SS are shown as the black lines protruding from *Chlamydia*. The nucleus (red sphere) and golgi apparatus inside the cell are shown. Large, oval, light green spheres are aberrant persistent bodies seen in the center of the diagram.

The EBs differentiate into a larger (~0.8 to 1 µm), non-infectious but metabolically active Reticulate Body (RB) (Erbenji, 1993; Beeckman et al., 2008). The increase in size is attributed to the reduction of the outer membrane protein disulfide bonds which allows for an increase in membrane permeability and the decondensation of the Chlamydial chromatin (Beatty et al., 1994; Hatch et al., 1986). Enhanced permeability allows for easier of uptake of nutrients and accounts for the fragile nature of the RB. Replication of RBs occurs through binary fission for several generations within the inclusion, and can fill the vacuole with up to 1000 progeny (Wyrick, 2000; Hogan et al., 2004). Upon replication of the RBs, the inclusion expands to occupy almost all intracellular space within the cell, sparse that of the nucleus and organelles (Hsia et al., 1997). The signal for the bacteria to switch from RBs back into EBs is unknown, however a "contact-dependent hypothesis" was proposed by Hoare *et al*. (2008). The RBs which are anchored to the inclusion membrane by their T3S injectisomes (explained in detail in section 1.4), are forced to reduce their contact with the membrane in order to compensate for the growing number of RBs. The RB crowding reduces the number of anchors associated with the inclusion. Once a specific number of injectisomes have detached from the membrane, the release of the bacteria is triggered by an unknown signal, and all remaining RBs disassociate from the membrane. This signal occurs approximately 24-48 hours post infection, and RBs differentiate into a transient form termed Intermediate Bodies (IB), then finally back into infectious EBs which exit the host cell via lysis or by extrusion around 72 hours (Hybiske & Stephens, 2007b). Infectious EBs disperse and infect surrounding host cells and the cycle of development will begin

again. The intracellular lifecycle of *Chlamydia* allows for persistent infection, which

antichlamydial antibiotics cannot eradicate (Herrmann, 2004).

1.2.2 Chlamydial persistence

Although *Chlamydia* can survive in the extracellular environment, this bacteria

requires uptake by a eukaryotic cell to initiate replication, and therefore has become

efficient at binding, invading and surviving in a wide array of eukaryotic cells.

Chlamydial persistence is defined as a long-term association between *Chlamydia* and the

host in which the organism remains viable, however is not actively replicating and thus is

culture-negative for infection (Beatty et al., 1994). This state of existence for the

bacterium is often compared to viral latency as *Chlamydia* takes up an altered replication

cycle and remains in a dormant state within the cell. Replication cycle plasticity is

thought to be a survival adaption which *Chlamydia* has evolved to help the pathogen

evade the host immune system by down regulating its metabolic activity. This strategy

has long been recognized as a key factor in Chlamydial pathogenesis (Meyer & Eddie,

1933). Persistence can be induced by factors such as nutrient deficiency, antimicrobial

agents, and immunologically induced persistence with central roles assigned to interferon

gamma (INF-γ) (Merigan & Hanna, 1966; Pantoja et al., 2001) and tumor necrosis factor

alpha (TNF-α) (Shemer et al., 1989). Upon the return of favorable conditions, *Chlamydia*

will reactivate and continue through the typical developmental cycle, reorganizing into its

infectious form (Beatty et al., 1994).

1.3 <u>Bacterial Secretory System Overview</u>

Both Gram-negative and Gram-positive bacteria have evolved various mechanisms to transport key molecules into the host cytoplasm, often termed secretory pathways. Secretion of proteins, DNA, and small molecules are particularly important for bacterial pathogenesis, adhesion, communication and nutrient acquisition as exemplified by the vast number of mechanisms bacteria have developed solely for this purpose. Since Gram-negative bacteria have two biological membranes as opposed to only one in Gram-positive species, they rely on various independent pathways to compensate for a more complex secretion topology. Six major secretion systems have been identified in these bacteria with three - namely type II, and type V - requiring the "general secretory pathway" (GSP or Sec pathway); while type I, type III and type IV systems are independent of the Sec pathway (Figure 1.2) (Beeckman & Vanrompay, 2010). The type VI secretion system is thought to also be Sec-independent, though this is a recently discovered system and more research is underway.
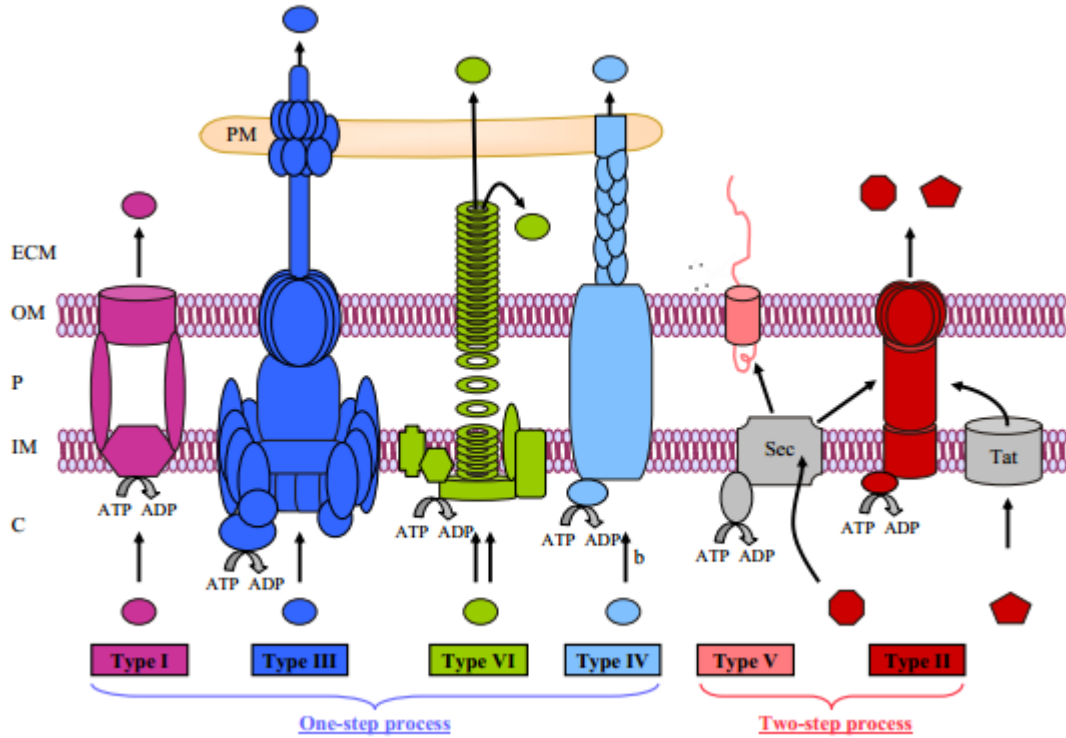
Figure 1.2: Major protein secretion systems in Gram-negative bacteria (adapted from Beeckman & Vanrompay, 2010). Legend: C, bacterial cytoplasm; IM, bacterial inner membrane; P, bacterial periplasm; OM, bacterial outer membrane; EMC, extracellular milieu; PM, host cell plasma membrane. Where appropriate, coupling of ATP hydrolysis to transport is highlighted. Arrows indicate the route followed by the transported proteins.

1.3.1 Type I secretion system

Type I secretion systems (T1SS), also known as the ATP binding cassette (ABC), is used by a wide variety of Gram-negative bacteria for the transport of ions, drugs and various proteins from inside the bacteria into the extracellular matrix (Jenewein et al., 2009). This secretion system is comprised of two integral membrane associated domains that form pore-like channels and two cytoplasmic domains which hydrolyze ATP, providing the power for the apparatus. Depending on where the substrate is required, ABC exporters can associate with membrane fusion proteins or outer membrane factors which allow the transportation across both bacterial membranes (Saier, 2006). T1SS transport a range of protein sizes as small as 10 kDa such as *Escherichia coli* peptide Colicin V, to others as large as the 900 kDa cell adhesion protein LapA of *Pseudomonas fluorescens* (Filloux, 2011).

1.3.2 Type II secretion system

Although general secretory systems are present in all living organisms, Type II secretion systems (T2SS) are limited to the Gram-negative proteobacteria phylum, and are most often involved in the transport of extracellular toxins and hydrolytic enzymes (Cianciotto, 2005). This pathway, often referred to as the main terminal branch of the general secretory pathway, is a two step, energy dependent process in which the Sec or Tat system is used for initial transport of the protein into the inner membrane (IM). The N-terminally associated secretion signal is cleaved, and the protein is transported across

the outer membrane by means of a specialized secretion apparatus not wholly conserved amongst bacterial species (Korotkov et al., 2012). *Vibrio cholerae* is a human pathogen which causes the disease cholera and requires the T2SS for virulence.

1.3.3 Type III secretion system

The term Type III secretion system (T3SS) is common amongst Gram-negative bacteria. This secretion system is thought to be one of the most complex and spans both the inner and outer bacterial membranes. Approximately 25 proteins make up the apparatus including the basal body apparatus, the filament of the needle and the tip complex which interacts with the host cell. The structure of the apparatus shows many similarities with bacterial flagella, used by bacteria for motility, and some of the T3SS genes even show sequence similarity to flagellar proteins. T3SS have been found to be evolutionarily related to the flagellar subunit export system, and the flagellar basal body may be the progenitor of the inner membrane components in the T3S apparatus (Nguyen et al., 2000). This secretion system is critical for virulence and transolcates proteins from the bacterial cytoplasm into the extracellular milieu or directly into the target host cell. Many species use the T3SS such as *Salmonella, Shigella, Pseudomonas*, *Yersinia*, *Escherichia coli*, as well as *Chlamydia* which will be discussed in further detail below.

1.3.4 Type IV secretion system

Type IV secretion systems (T4SS) are unique in the fact that they can transport proteins, but also nucleic acids and nucleic acid-protein complexes across the bacterial cell envelope. Many bacteria have homologous T4SS with similar function despite differences in their bacterial genes (Tseng et al., 2009). Although important in pathogenesis, not all bacteria contain this system (Baron, 2005). This secretion pathway is the only one of the six that can secrete into the periplasmic space or allow for direct translocation into the host cell (Thanassi et al., 2005). Another interesting feature of the system is the ability to secrete cargo into plant and animal cells, as well as yeast and other bacteria. Pertussis toxin from *Bordetella pertussis* is secreted through the outer membrane of the bacteria and into host cells via the T4SS (Rambow-Larsen & Weiss, 2004).

1.3.5 Type V secretion system

This system uses Sec signals to transport proteins from the bacterial cytosol into the periplasmic space, similar to T2SS; however the translocation of proteins through the outer membrane occurs in the absence of ATP. The type V secretion system (T5SS) is comprised of three comparatively simple systems - the autotransporter (V_a), two-partner secretion (V_b) and chaperone/usher pathways (V_c) - which allow for the secretion of proteins through the outer membrane without the input of energy (Thanassi et al., 1998). All T5SS proteins are composed of three domains; an N-terminal signal sequence for

transportation through the inner membrane, a passenger domain which will be exposed or secreted into the extracellular milieu, and a translocation unit required for the formation of a pore in the outer membrane (Desvaux et al., 2004). Many bacteria such as *Bordatella pertussis* as well as *Escherichia coli* use monomeric autotransporters, one of the first studied T5SS (Leo et al., 2012).

1.3.6 Type VI secretion system

Only recently discovered, the Type VI Secretion System (T6SS) was shown by John Mekalanos to be used by two bacterial pathogens, *Vibrio cholerae* and *Pseudomonas aeruginosa* (Pukatzki et al., 2006; Mougous et al., 2006). This system is thought to act like and inverted phage tail which ejects effector proteins from the membrane, and is encoded by at least a quarter of all known Proteobacteria species (Kanamaru, 2009). The T6SS is regulated largely through sensor kinase and Quorum Sensing pathways (Bernard et al., 2010). The role of the T6SS needs to be investigated further but appears to be a defense mechanism against other bacteria.

1.4 Chlamydial Type III Secretion

1.4.1 Evidence of Chlamydial type III secretion

As early as the 1970's, many research groups published electron microscopy images, depicting small needle-like projections protruding from both the inner and outer

membranes of Chlamydial species (Matsumoto, 1988). These non-uniformly distributed projections were found on the surface of infectious EBs but also on the metabolically active RBs. Based upon the observation that many Gram-negative bacteria employ a T3SS to inject virulence associated proteins into host cells, as well as the finding of type III secretion genes within the Chlamydial genome (Hsia et al., 1997), it was hypothesized by Bavoil & Hsia (1998) that the observed envelope-spanning components on *Chlamydia* were likely T3S machinery. Sequencing of the *Chlamydia trachomatis* genome, revealed a nearly complete set of genes for a T3S apparatus (Stephens et al., 1998). The organization of this system within the Chlamydial genome is unlike that of other bacteria such as *Salmonella, Shigella* and *Yersinia*. Most bacteria have their T3S genes clustered on "pathogenicity islands", while *Chlamydia*'s T3SS genes are distributed ten operons across six different loci (Hefty & Stephens, 2007).

As a minimalist bacterium, it is unlikely that *Chlamydia* carries within its genome genes which are not absolutely required for growth and survival. The presence of T3SS genes implies that the system is likely functional, however presence alone does not prove functionality. The first real evidence of a potentially functional T3SS within *Chlamydia* was provided by Fields & Hackstadt (2000), showing both gene expression and translocation of putative T3S proteins in orthologous bacterial secretion systems. Multiple components of the putative T3SS were expressed, and the successful translocation of CopN by the *Yersinia* T3S apparatus, provided support for a functional secretion system (Fields & Hackstadt, 2000). Since then other research has shown the translocation of multiple Chlamydial proteins in a number of other bacterial species, and

it has become widely accepted that all species of *Chlamydia* contain a functional T3SS (Subtil et al., 2001).

1.4.2 The Chlamydial type III secretion system

All *Chlamydia* genomes sequenced to date encode four different secretion systems; type II and type V, both Sec-dependent systems, as well as a type I and type III secretion systems which are independent of the Sec-system (Birkelund et al., 2006). The T3SS is structurally and functionally conserved amongst Chlamydial species, composed of approximately 25 proteins (Johnson et al., 2008). Known also as "contact dependent secretion" (Cds), the T3S pathway differs from other secretion systems in three major ways; there is no need for an amino terminal sequence indicative of Sec-dependent systems, no cleavage of the amino termini required to transolcate the proteins, and the type III pathway requires an extracellular signal for activation of the complete secretory apparatus (Collazo & Galan, 1997). This environmental signal can be temperature shifts or a pH change, which may occur when the bacteria is in close vicinity to the host cell (Ramamurthi & Schneewind, 2002). The T3S system functions as a "molecular syringe" otherwise known as an injectisome, enabling the injection of effector proteins from the bacteria into either the cytoplasm or the membrane of host cells (Gauthier et al., 2003; Cornelis, 2003). The assembly of the injectisome occurs in a step-wise highly regulated fashion.

1.4.3 Structure of the *Chlamydia pneumoniae* T3SS

In a recent review article by Betts-Hampikian & Fields (2010), the assembly of the T3SS was proposed to be initialized by three proteins: CdsC an outer membrane protein ring, CdsJ an inner membrane ring, and a bridge protein CdsD, which spans the inner membrane connecting the two rings (Figure 1.3). All three of these proteins contain Sec-dependent signals which could facilitate their export into the inner and outer bacterial membranes prior to the other structural proteins. The inner membrane components consist of: CdsR a proposed negative regulator of secretion, CdsS, CdsT, CdsU, and CdsV which form a central protein-conducting channel across the inner membrane (Beeckman & Vanrompay, 2010; Betts-Hampikian & Fields, 2010). CdsN is an inner membrane associated ATPase which is a critical energy dependent structure for the apparatus, and plays a role in releasing effectors from their chaperones, as well as unfolding of effectors (Akeda & Galan, 2005). CdsN hydrolyzes ATP in a time- and dose-dependent manner, and interacts with CdsL as well as CdsQ, a possible multi-cargo transport protein (Stone et al., 2008; Spaeth et al., 2009). The *Chlamydia* needle protein, CdsF, projects outward from the basal body and polymerizes to form the needle filament of the apparatus. CdsP is thought to control the length of the needle filament and might aid in the substrate switch from early proteins (needle) to late proteins (effectors) upon completion of the needle (Journet et al., 2003; Hayes et al., 2010). A functional T3SS requires a set of translocator proteins; CopB1/B2 and CopD1/D2 form a pore in the host cell cytoplasmic membrane, allowing for the needle to protrude into the eukaryotic cell (Betts-Hampikian & Fields, 2010). A tip protein caps the complex and might be

responsible for both host cell sensing as well as the correct insertion of translocators into the host cell membrane (Erhardt et al., 2010).

Once the T3S apparatus is assembled and upon contact with the host cell, secretory activity is initiated when an unknown signal transduces down the injectisome, causing unfolded effector molecules to travel through the hollow injectisome (Betts-Hampikian & Fields, 2010). These effector molecules are deposited within the host cell and exert a unique biological function, however many of these functions remain unknown or speculatory at best (Lugert et al., 2004; Johnson, 2009).
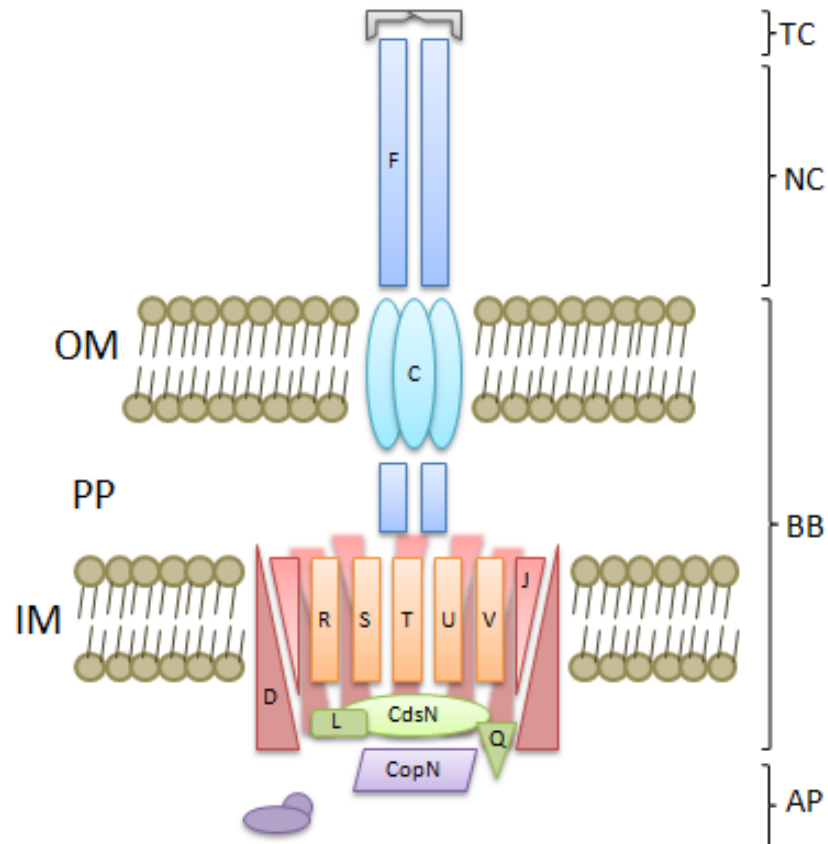
Figure 1.3: A cartoon representation of the type III secretion system in *Chlamydia pneumoniae* (adapted from Betts-Hampikian & Fields, 2010). Components include the tip (TC) and needle (NC) complexes, basal body (BB) which spans the inner membrane (IM), the periplasm (PP) and outer bacterial membrane (OM), as well as cytoplasmic ancillary proteins (AC). Correct stoichiometry of multimeric proteins is not indicated.

1.4.4 Temporal transcription of the Chlamydial T3SS genes

Temporal expression of the *Chlamydia* genes occurs throughout its biphasic developmental cycle, as studied by Shaw *et al*. (2000) using RT-PCR for the genes of *C. trachomatis*, and more recently *C. pneumoniae* (Slepenkin et al., 2003). The transcripts analyzed fell into three distinct temporal classes: "early cycle" genes detectable before 2 hours, "mid-cycle" genes present between 6 and 12 hours, and "late cycle" genes which are detectable around 20 hours (Figure 1.4) (Shaw et al., 2000). A fourth temporal class was added by Mäurer *et al*. (2007) known as "tardy" genes.   These "tardy" genes are defined as genes expressed in late cycle but have increasing expression levels towards late stage infection. Structural proteins were generally detected during early cycle including *cdsC*, *cdsS*, *cdsL*, and *cdsJ*, while genes involved in structural assembly as well as energy metabolism were expressed mid-cycle such as *cdsN*, *cdsR*, *cdsD* and *scc3*. Late cycle genes included *scc1*, *cdsT* and *copN*, while only one gene was placed in the tardy gene class known as *scc2* (Bailey, 2008; Mäurer et al., 2007; Slepenkin et al., 2003). A review of the literature indicates that differences in classification of these genes occurs frequently, and is likely due to both the method of detection as well as the multiplicity of infection used in each study.
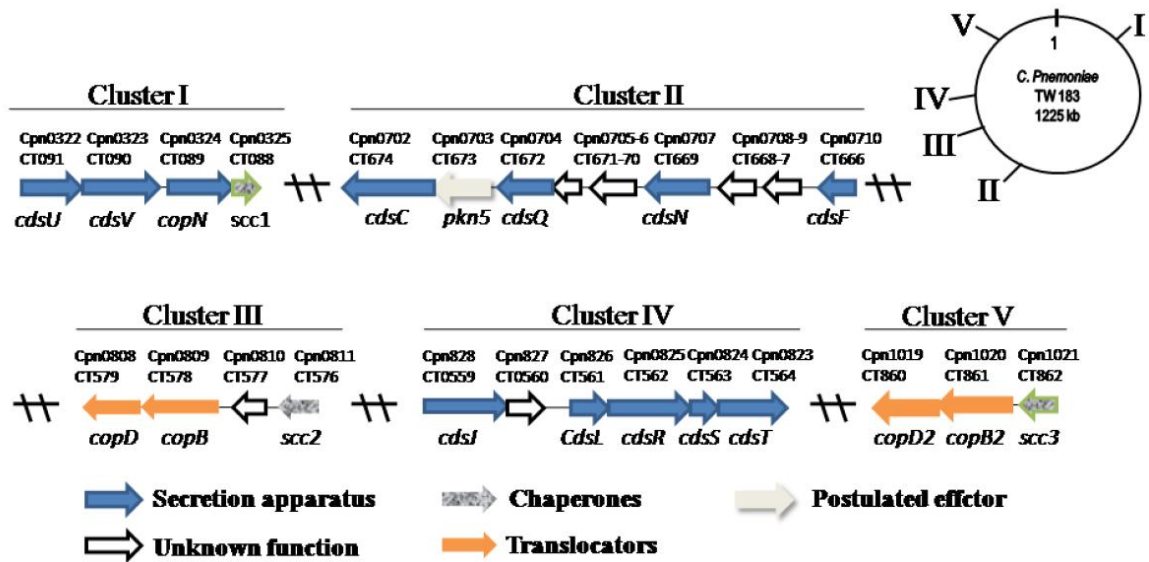
Figure 1.4: Organization of the T3SS gene clusters in *Chlamydia pneumoniae* TW183 (Bailey, 2008). The gene size is not displayed to scale and locations shown on the illustration may not be exact.

Expression of a large amount of T3S apparatus *cds* genes occurs during mid-cycle, approximately 12 hours post infection (Lugert et al., 2004; Fields et al., 2003). The question is then raised regarding how the invading bacterium is able to release effector proteins prior to the assembly of a functional T3SS, since the structural genes are transcribed mid-cycle. The answer to this has been the subject of much investigation by multiple groups including Vandahl *et al.* (2001), Fields *et al.* (2003), and Lugert *et al.* (2004). Our lab, along with that of Dr. Kenneth Fields have shown that EBs possess a pre-assembled T3S apparatus (Stone, unpublished data; Fields et al., 2003). The other Cds proteins would assemble mid-cycle when the RBs secretion apparatus is formed (Beeckman & Vanrompay, 2010).

1.4.5 Genetic redundancy within the *C. pneumoniae* genome

The *Chlamydia* genome not only contains genes encoding for a T3SS, but also a partial repertoire of flagellar proteins can be identified (Stone et al., 2010). Two paralogs exist in the Chlamydial genome for each of the gene families; *cdsJ/fliF*, *cdsN/fliI*, *cdsL/fliH* and *cdsV/flhA* (Kim, 2001). As a non-motile species which exhibits a minimalistic genome, any redundancy would mean selective disadvantage. In order to explain this genetic redundancy, two hypotheses have been proposed. First the flagellar proteins could be the archaic remains from an ancestral, motile *Chlamydia*-like bacterium; however this hypothesis is relatively unlikely due to the minimalistic Chlamydial genome. A more fitting proposal is that perhaps *Chlamydia* has modified the flagellar system only to retain the protein export function (Kim, 2001). Since both EBs

and RBs have T3S machinery protruding from their surfaces, it could also be postulated that a set of T3S proteins is used during one phase in the lifecycle, while the flagellar proteins could be used in the second. This hypothesis is supported by evidence that T3S proteins interact with flagellar components as shown by Stone *et al.* (2010) and Spaeth *et al.* (2009). A growing body of evidence supports the hypothesis that *Chlamydia* uses the flagellar proteins during invasion and replication.

Chlamydia encodes two sets of translocator proteins, CopB1 and CopD1 as well as CopB2 and CopD2. This redundancy has led to speculation that one set of translocators is used when the bacteria invades the host, while the other set might be used within the inclusion to allow for the bacteria to communicate with the host cell (Ouellette et al., 2005; Hefty & Stephens, 2007). It has been suggested that CopB1 and CopD1 are required for internalization, while CopB2 and CopD2 are required for translocation through the inclusion membrane. This hypothesis is supported by the duplication of the *Yersinia* LcrH homologue in *Chlamydia*, known as Scc2 and Scc3, which might function as chaperones for one or both sets of translocators. Some pathogens encode two unique T3SS such as *Salmonella (s.) enterica* pathogenicity islands SPI-1(*inv/spa*) and SPI-2 (*spi/ssa*) (Hensel et al., 1995). Since *Chlamydia* does not possess multiple T3SS, this redundancy built within the genome could effectively eliminate the need for a second T3SS within the same cell.

1.4.6 Chlamydial effector proteins

Type III secretion effector proteins are widely divergent in both sequence and function not only between T3S systems in other organisms, but also amongst Chlamydial species (Beeckman & Vanrompay, 2010). Since the Chlamydial effectors are distributed throughout the genome, identifying and characterizing them has been made especially difficult. Table 1.2 is a representative list of characterized Chlamydial effector proteins.

The first family of effector proteins identified within *Chlamydia* is a family of inclusion membrane proteins (Incs), characterized by a bi-lobed hydrophobic motif of 40-60 amino acids (Valdivia, 2008). The first identified Inc proteins, IncA, IncB, and IncC, are found in *C. trachomatis*, *C. psittaci*, and *C. pneumoniae*, though limited sequence homology exists (Subtil et al., 2001). This family is the best characterized in *Chlamydia* and are expressed approximately 2 hours post infection (Beeckman & Vanrompay, 2010). The Inc proteins comprise over 18% of the open reading frames (ORF) in *C. pneumoniae* and are located on the surface of the inclusion membrane; however little regarding their function is understood (Kostryukova et al., 2008).

Table 1.2 Characterized Chlamydial effector proteins

Effector	Location	Gene Number	Possible Function
IncA	Inclusion membrane	*Cpn0186* *Cpn0595*	Membrane protein
IncB	Inclusion membrane	*Cpn0291*	Membrane protein, host cell interactions
IncC	Inclusion membrane	*Cpn0292*	Membrane protein
Tarp	Surface of EBs	*Cpn0572*	Invasion, actin recruitment
Pkn5	Inclusion & host cell cyotsol	*Cpn0703*	Invasion, S/T kinase
Mip	EB, RB surface & inclusion membrane	*Cpn0661*	Induction of cytokine response
CADD	Inclusion	*Cpn0761*	Modulation of host cell apoptosis
CdsP	Bacteria & host cell cytosol	*Cpn0705*	Ruler protein
CopB1/2	Inclusion membrane & host cell membrane	*Cpn0809/1020*	Translocator protein
CopD1/2	Inclusion membrane & host cell membrane	*Cpn0808/1019*	Translocator protein
Cpn0803	Bacteria & inclusion/ host cell	*Cpn0803*	Might regulate PI levels in inclusion, or regulate GEFs in host
CopN	Outer membrane of the bacteria & host cell cytosol	*Cpn0324*	Plug protein, cell cycle arrest, inhibit MT assembly

Adapted from: Bailey. 2008; Beeckman & Vanrompay. 2010

Translocated actin-recruiting phosphoprotein (Tarp) recruits actin filaments to the site of host cell attachment, which creates an actin-rich pedestal-like structure facilitating the uptake of the bacteria into the host cell (Carabeo et al., 2002). As shown by Clifton and colleagues (2004), Tarp is secreted by the type III secretion system in *Yersinia* as well as the T3SS of Chlamydial species. The role of Tarp as a Chlamydial effector is critical during infection of the host however tyrosine phosphorylation at the site of entry may not be required for actin recruitment (Carabeo et al., 2002).

When the *C. trachomatis* genome was first sequenced by Stephens *et al.* (1998), Pkn5 was a predicted T3S substrate based on its location within one of the known T3S sub-clusters. Pkn5 is encoded upstream from CdsC (the outer membrane ring component) and is a protein with a large sequence homology to eukaryotic serine/threonine kinases (Subtil et al., 2000). Pkn5 has no real kinase activity in *C. trachomatis* and *C. pneumoniae*, however it might still promote or inhibit phosphorylation of host cell proteins despite the lack of intrinsic kinase activity (Herrmann, 2004). More recently the successful translocation of this protein by the SPI-2 T3S apparatus in *Salmonella* was shown, confirming its status as a type III secreted substrate in *Chlamydia* (Ho & Starnbach, 2005). Pkn5 has many hypothesized roles in the Chlamydial developmental cycle including modulation of the host cell cytoskeleton; however more research regarding this effector is underway.

The lipoprotein macrophage infectivity potentiator (Mip) in *Chlamydia* is homologous to Mip of *Legionella pneumophila* (Lundemose et al., 1993). This protein has been shown to be important in the invasion process of *Chlamydia* after inhibition of

Mip reduced the infectivity (Lundemose et al., 1993). In *C. pneumoniae,* Mip was surface localized on EBs and RBs, but has since been shown to be secreted into the host cell cytoplasm (Rockey et al., 1996; Herrmann et al., 2006). Although not a confirmed T3S effector protein, the translocation of Mip is thought to be a secreted by both the type II and type III secretion systems.

Chlamydial protein associating with death domains (CADD) is an effector protein, likely involved in modulation of host cell apoptosis (Beeckman & Vanrompay, 2010). It is associated with the inclusion membrane and might be secreted by the Chlamydial T3SS. CADD binds to death domains of tumor necrosis factor family of receptors, which allows the bacteria to modulate cellular apoptosis, a feature which helps the bacteria survive intracellularly (Stenner-Liewen et al., 2002).

CdsP (also known previously in literature as SctP) which has significant homology to YscP of *Yersinia*, is predicted to be the T3S ruler protein in *Chlamydia*. YscP is secreted by the *Yersinia* T3SS, while both CdsP orthologes in *C. trachomatis* and *C. caviae* were successfully translocated by *Shigella flexneri* (Subtil et al., 2005). This protein is predicted to be a molecular ruler which might control the length of the needle filament, and might also play a role in substrate switching between the *cds* genes and effectors. The mechanism of CdsP's action is currently unknown (Stone et al., 2008).

Included in the category of effector proteins are the Chlamydial translocator proteins; Chlamydial outer proteins D and B (CopD1/D2 and CopB1/B2). CopD and CopB are secreted initially, and inserted in the host cell outer membrane forming a pore

(Betts-Hampikian & Fields, 2010). Using translocator protein truncations, Fields *et al.* (2005) showed that the N-terminal regions of CopB and CopB2 were able to be translocated by the T3SS of *Yersinia*. CopD is translocated in the *Salmonella* SPI-2 T3SS, implying these effectors are also type III secreted in *Chlamydia* (Ho & Starnbach, 2005).

The crystal structure of another predicted effector protein, Cpn0803, was solved by Stone *et al.* (2012). This protein binds to T3S components including CdsN the ATPase, CdsQ a multicargo chaperone protein as well as CdsF the needle filament (Stone et al., 2012). Phosphatidylinositol and phosphatidic acid interact with Cpn0803 and could perhaps bind within the small hydrophobic pocket created when Cpn0803 dimerizes (Stone et al., 2012). The function of Cpn0803 is still currently unknown though it might be involved in regulating phosphatidylinositol levels within the inclusion membrane, or it could regulate guanine nucleotide exchange factors (GEFs) within the host cell.

Chlamydial outer protein N (CopN) is a surface localized protein, but more recently was implicated in various anti-host activities, indicative of an effector. Fields & Hackstadt (2000) showed that a CopN fusion protein was successfully translocated by the T3SS of *Yersinia* and is therefore likely secreted by the T3SS of *Chlamydia*. CopN will be discussed in further detail below in Section 1.5.

1.4.7 Chlamydial chaperone proteins

Production of effector proteins is essential for nutrient acquisition. If proteins misfold after production, their integrity and activity can be compromised. To combat this, small ancillary proteins physically bind effector proteins and prevent degradation while holding them in a secretion-competent state. In the T3SS field, the term chaperone is commonly used to describe small ancillary proteins which bind effectors. However these chaperones differ from traditional molecular chaperones as they lack ATPase or protein folding abilities (Thomas et al., 2012). Therefore the term "chaperone-like" protein was recently proposed, but the more accepted terminology has been adopted by this thesis and will refer to these ancillary proteins as chaperones.

T3S chaperones facilitate effector secretion by binding and transporting effectors to the base of the injectisome, keeping the effectors in a partially unfolded state prior to their secretion. Typically chaperones range in size from 15 to 20 kDa, have an acidic pI of approximately 4 to 5, and are often composed of alpha helices (Collazo & Galan, 1997). Chaperones can be grouped into different classes depending on their ability to bind effector proteins. Class I chaperones bind effector proteins and normally assemble as homodimers. Class II chaperones bind to the translocator proteins, while class III chaperones recognize needle-forming proteins and prevent early polymerization of the needle filament (Cornelis, 2006). There are two subtypes of class I chaperones, class IA that bind single effector proteins and are often located in the operon adjacent to their cognate effector, and class IB chaperones that bind multiple effector proteins and can be found in any operon which encodes for T3S machinery (Costa et al., 2012).

In *Yersinia* chaperones are known as specific Yersinia chaperones (Syc), while in *Chlamydia* they are denoted as specific Chlamydial chaperones (Scc). Most Chlamydial T3S chaperones were identified through sequence similarity between known chaperones in other bacterial systems. In *C. pneumoniae*, only nine chaperones were predicted based on genome sequence, with four being of particular interest. Scc1 was the first Chlamydial chaperone identified and is homologous to SycE (the chaperone of the effector YopE) in *Yersinia*. Scc2 and Scc3 have similarity to LcrH (also known as SycD) a chaperone for YopD (Beeckman & Vanrompay, 2010), while the last well characterized chaperone Scc4 is located in a similar gene position as *yscB* from *Yersinia* (Silva-Herzog et al., 2011).

With the discovery of new effector proteins, an emerging role for chaperones that bind multiple effector proteins has become apparent, as is the case for multiple cargo secretion chaperone (Mcsc) in *C. trachomatis* (Spaeth et al., 2009). Class IB chaperones might play a role in both creating and maintaining a secretion hierarchy, though currently data supporting this role are lacking (Parsot et al., 2003; Thomas et al., 2012). These chaperones might display a differential affinity for various effectors, potentially binding one preferentially over another. Selective binding has been overlooked in T3S literature and requires a more thorough investigation.

1.4.8 Regulation of the type III secretion system

The assembly of the type III secretion apparatus occurs in a highly regulated stepwise fashion beginning from the assembly of the basal apparatus and ending with the

polymerization of the needle filament. Currently it is unknown how the Chlamydial T3S

system's activity is regulated and what signals drive effector molecule switching.

Regulation within the pathogen occurs at the transcriptional level, the post-transcriptional

level, the translational level and by the T3SS components directly via conformational

changes (Deane et al., 2010). The first major molecular switch occurs between the

secretion of early and late substrates, needle and rod subunits respectively, and must

occur after the needle has reached its full length (Mota et al., 2005). This involves the

cessation of secretion of needle subunits CdsF, and the subsequent secretion of CdsP

subunits, which acts as the molecular ruler (Deane et al., 2010). The tip proteins must be

secreted after the needle is completed but before the secretion of late substrates

(Veenendaal et al., 2007). The second major switch can be described as the transition

from blocked effector secretion to active secretion. This transition is proposed to be

regulated by the molecular gatekeeper also known as CopN.

1.5 Plug Proteins of Type III Secretion Systems

Plug proteins are essential in all bacterial T3SS and can be found *Yersinia*,

Pseudomonas, *Shigella*, *Salmonella*, *Escherichia coli* and *Chlamydia*, amongst others

(Huang et al., 2008). These plug proteins are thought to be tethered to the base of the

injectisome by T3S chaperones and held in a partially unfolded state blocking the

syringe-like pore. An unknown signal initiates the switching of apparatus substrates to

that of effector proteins, and the plug protein detaches from their chaperones and is

secreted through the injectisome into the host cell. This secretion allows for the translocation of other effector proteins through the "open" conduit. Since the *Yersinia* T3SS has been studied more in depth than that of *Chlamydia*, the major hypotheses in literature for the regulation of secretion are generally proposed in this species.

1.5.1 The "gatekeeper" hypothesis

The YopN-TyeA complex blocks premature secretion of the effectors prior to the contact with the host cell. Cheng *et al*. (2001) believe that YopN, in conjunction with TyeA, regulate the T3S apparatus in *Y. enterocolitica,* with TyeA binding to residues 101-294 of YopN, tethering YopN and preventing it from moving up the injectisome so it forms a "lid" over the base of the injectisome (Figure 1.5a). In a second hypothesis known informally as the "plug" hypothesis, Ferracci *et al*. (2005) propose that YscB and SycN chaperone the YopN-TyeA complex to the base of the injectisome where once released from its chaperones, YopN undergoes a conformational change, partially unfolding to block T3S while still bound to TyeA (Figure 1.5b). Both scenarios require a low calcium signal which transduce down the injectisome and result in the dissociation of TyeA from YopN. Cheng *et al*. (2001) propose that TyeA binds YopN after YscB and SycN have brought YopN to the base, and that YopN is completely folded in its native form. In contrast, Ferracci *et al*. (2005) propose that the chaperones YscB and SycN deliver a preformed YopN-TyeA complex to the base, where the YopN N-terminus unfolds and is partially secreted into the injectisome.
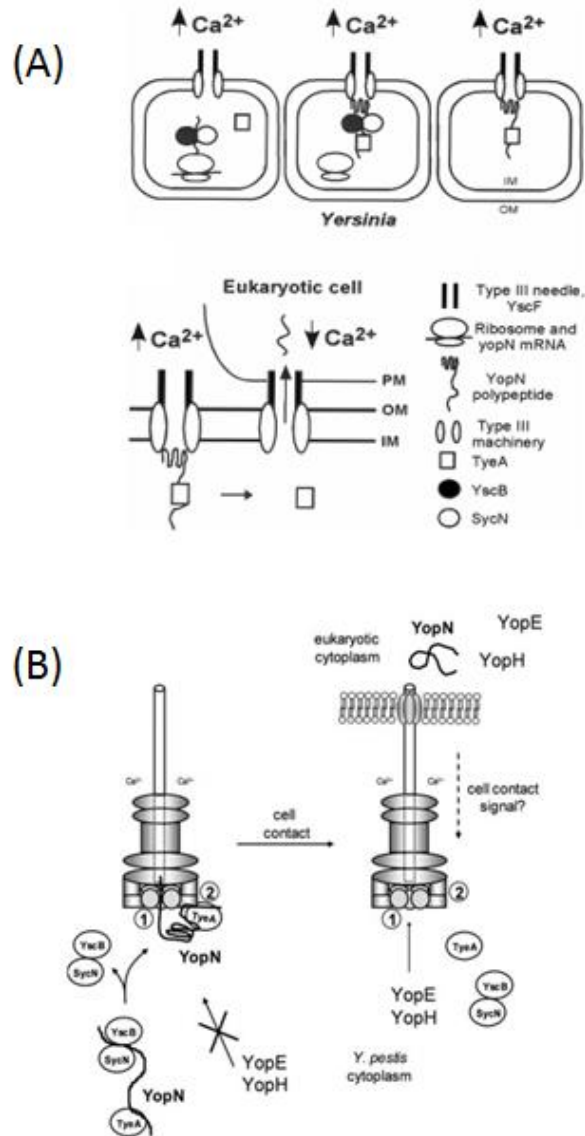
Figure 1.5: Proposed models for regulation of the type III secretion system in Yersinia. **(A)** The "lid" hypothesis proposed by Cheng *et al.* (2001) for the regulated transport of YopN by the type III machinery of *Yersinia enterocolitica*. **(B)** The "plug" hypothesis for the regulation of YopN secretion in *Yersinia pestis* proposed by Ferracci *et al.* (2005)

1.5.2 "Gatekeeper" proteins in type III secretion systems

Proteins similar to YopN and TyeA of *Yersinia* can be found in essentially all Gram-negative bacteria which utilize a T3S system, despite limited sequence homology. A non-exhaustive but representative comparison can be seen in Table 1.3.

YopN, the putative plug protein of *Yersina*, interacts with its heterodimeric chaperone pair SycN and YscB, along with another small molecule known as TyeA (Day & Plano, 1998). In *Pseudomonas* PopN interacts with Pcr1, similar to the interaction between YopN and TyeA, but also interacts with its own chaperones Pcr2 and PscB (Yang et al., 2007). These interactions however are not found in all Gram-negative bacteria, including *C. pneumoniae* since no TyeA-like molecule has been discovered. Plug proteins that lack a TyeA-like partner have been predicted to be hybrid or fusion proteins. These predicted fusion proteins, including CopN, MxiC, SepL and BopN, still function as down-regulators for the T3SS.

Table 1.3: Comparison of various bacterial putative plug proteins and their percent similarity based on YopN and TyeA of *Yersinia*

Protein(s)	Species	Chaperone(s)	Size (AA)	Size (kDa)	Similarity YopN/TyeA	Crystallization	Reference(s)
CopN	*Chlamydia* spp.	unknown	399	43.9	CopN: 50% to YopN / CopN: 32% to TyeA	No	Huang et al., 2008 / Hsia et al., 1997
YopN + TyeA	*Yersinia* spp.	YscB + SycN	294 / 92	32.3 / 10.1	N/A	$YopN_{76-293}$ + TyeA / $YopN_{32-277}$ + SycN + YscB	Schubot et al., 2005
MxiC	*Shigella* spp.	unknown	355	39.1	MxiC: 13% to YopN / MxiC: 13% to TyeA	$MxiC_{71-355}$	Deane et al., 2008
PopN + Pcr1	*Pseudomonas aeruginosa*	Pcr2 + PscB	288 / 92	31.7 / 10.1	PopN: 56% to YopN / Pcr1: 41% to TyeA	No	Yang et al., 2007
SepL	*Escherichia coli*	CesL	351	38.6	SepL: 11% to YopN / SepL: 21% to TyeA	No	Pallen et al., 2005 / Yonis et al., 2010
AopN + Acr1	*Aeromonas* spp.	Acr2	292 / 93	32.1 / 10.2	AopN: 60% to YopN / Acr1: 71% to TyeA	No	Burr et al., 2002
BopN	*Bordetella* spp.	BB1615	365	40.1	BopN: 41% to YopN / BopN: 20 % to TyeA	No	Panina et al., 2005

Percent similarity based on ClustalW2 alignments

1.5.3 Chlamydial outer protein N

Chlamydial outer protein N, also known as CopN (*Cpn0324*), is a putative member of a family of pathogenic proteins found in various Gram-negative bacteria. CopN is expressed in the late stages of infection, and is thought to be directed to the base of the injectisome by the chaperone Scc3 where it partially unfolds plugging the injectisome (Karyagina et al., 2009). Silva-Herzog *et al*. (2011) proposed that Scc1 (*CP0432*) and Scc4 (*CP0033*) forms a heterodimeric chaperone pair that binds to CopN within the N-terminal domain, whereas Scc3 was determined to bind to the C-terminus of CopN. In *Yersinia* the YopN/SycN/YscB/TyeA macromolecular complex plugs the pore of the injectisome and down-regulates secretion of the apparatus. Similarly in *Chlamydia* it is predicted that perhaps a complex of CopN/Scc1/Scc4 are able to block the premature secretion of the apparatus. This complex may or may not include Scc3 which has a slight inhibitory effect on CopN (Silva-Herzog et al., 2011).

CopN of *C. pneumoniae* is the first putative plug protein shown to have anti-host cell activity after secretion. Huang and colleagues (2008) discovered that CopN halts cell cycle progression at the G2/M phase, a process termed CopN-dependent mitotic arrest. The authors determined that this disruption in both yeast and mammalian cells was through interference of microtubule networks which form the spindle fibers. Small molecule inhibitors 0433YC1 and 0433YC2 alleviated the inhibition caused by CopN (Huang et al., 2008). Also CopN directly binds free αβ-tubulin and prevents microtubule polymerization (Archuleta et al., 2011). Since CopN from *C. pneumoniae* binds αβ-tubulin, CopN from *C. psittaci* and *C. trachomatis* were tested, and surprisingly, only *C.*

psiticci CopN was able to bind αβ-tubulin (Archuleta et al., 2011). The difference of

CopN between *C. pneumoniae*, *C. psittaci*, and *C. trachomatis* is attributed to the

difference in tissue tropism of each bacterium.

1.6 Thesis Objectives

My objective was to characterize the putative *Chlamydia pneumoniae* type III

secretion system plug protein, CopN (*Cpn0324*), which has an essential role in bacterial

pathogenesis. Its domain boundaries, protein-protein interactions, and a HeLa cell

secretion model were examined to determine if CopN could indeed be the plug protein of

Chlamydia pneumoniae.

The domains of CopN were assessed for individual protein interactions, using *in

vitro* techniques, such as GST pull-down assays, His$_6$ pull-down assays and a 96-well

indirect ELISA developed in lab. Secretion of CopN throughout one complete infection

cycle was tested using a HeLa cell model and a CopN specific antibody.

1.7 Hypothesis

I hypothesize that CopN (*Cpn0324*) of *Chlamydia pneumoniae* is the plug protein

of the type III secretion system. Furthermore I hypothesize that putative chaperones Scc1

and Scc4 (Silva-Herzog et al., 2011), interact with the N-terminal region of CopN, while

other type III secretion proteins interact within the remainder of the protein.

CHAPTER TWO

2.0 MATERIALS & METHODS

2.1 <u>Microbiological Methods</u>

2.1.1 Cultivation of *E. coli*

Escherichia coli XL-1 Blue, BL21(*DE3*) or Rosetta (pLysS) cells (Stratagene, La Jolla, CA, USA) were grown under aerobic conditions at 37°C in liquid Luria-Bertani broth (LB; 1% w/v bacto-tryptone, 0.5% w/v bacto-yeast extract, 1% w/v NaCl) shaking at 260 rpm or on solid LB agar plates (1.5% w/v) supplemented with antibiotics at 100 μg/mL for ampicillin or 30 mg/mL for kanamycin. Glycerol stocks of cultures for long-term storage were prepared by mixing stationary phase liquid culture with glycerol to a final concentration of 20% (v/v). Stocks were flash frozen in liquid nitrogen and kept at -80°C.

2.1.2 Transformation of *E. coli*

For transformations, 1 μL (50-150 ng) of prepared plasmid DNA was added to the desired chemically competent *E. coli* XL-1 Blue (Strategene, Mississauga, ON) cell line and incubated on ice for 30 minutes. Cells were then heat-shocked for 45 seconds in a 42°C water bath, and then returned onto ice for 2 minutes. After the addition of 250 μL of Super Optimal Broth with Catabolite Repression (SOC) medium (2% w/v bacto-tryptone, 0.5% w/v Yeast extract, 8.56 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 20 mM MgSO4, and 20 mM glucose), the cells were grown at 37°C for 1 hour with shaking at

260 rpm. The mixture was then plated on a pre-warmed LB agar plate containing the appropriate antibiotics and incubated overnight at 37°C to allow for colony growth.

2.1.3 HeLa cell culture

Cell culture was used throughout this work as a means of *Chlamydia pneumoniae* propagation, *in vitro Chlamydia* experiments as well as testing antibody cross reactivity. HeLa (ATCC; Cedarlane, Burlington, ON, CAN) cells were retrieved from stocks kept in liquid nitrogen, and thawed out in a 37°C water bath. Cells were transferred from the Nalgene 5000 Sterile Cryogenic Vials (Nalge Nunc International, Rodchester, NY, USA) to a 1.5 mL Eppendorf tube and centrifuged in a benchtop centrifuge for 5 minutes at 7,000 rpm in an Eppendorf 5415R centrifuge to pellet cells. Supernatant was removed and cells were resuspended in 1 mL of minimum essential medium (MEM; Invitrogen; Burlington, ON, CAN) containing Earle's salts and L-glutamine, and supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen; Burlington, ON, CAN). Resuspended HeLa cells were then added to a T75 canted neck flask containing 8 mL of MEM + 10% FBS, and allowed to grow until confluence in a 37°C incubator with 5% CO_2.

Splitting HeLa cells was carried out as follows unless otherwise noted. Spent medium was removed from flasks using a glass pipette and 2 mL of Trypsin (Invitrogen; Burlington, ON, CAN) was added, swirled around the flask then aspirated using a glass pipette. An additional 2 mL of Trypsin was added, swirled, and flasks were closed and put back in 37°C incubator for 5 minutes. Flasks were gently tapped to help the cell layer

release, and new MEM + 10% FBS was added to the flask. Cells and media were pipette up and down in the flask using the wall to gently separate the cells prior to aliquoting into new flasks until desired dilution was achieved. HeLa cells were kept in the 37^oC + 5% CO_2 incubator until confluent.

2.1.4 *Chlamydia pneumoniae* **growth and propagation**

Throughout this work, HeLa cells (ATCC; Cedarlane, Burlington, ON, CAN) were used as a host cell line for *Chlamydia pneumoniae* CWL029 (VR1310; ATCC) (GenBank accession #AE001363). HeLa cells were grown and maintained in MEM containing Earle's salts and L-glutamine, and supplemented with 10% heat-inactivated FBS at 37^oC and 5% CO_2. At 90-95% confluency, HeLa cell flasks were infected with 80 µL of previously harvested *C. pneumoniae* plus 10 mL of fresh MEM via centrifugation in a Beckman GPR Benchtop centrifuge at room temperature, for 1 hour at 2,500 rpm in a GH-3.7 rotor. Flasks were then incubated at 37^oC and 5% CO_2 for 1 additional hour, after which time the Chlamydial suspension was replaced by fresh MEM containing 1µL/mL cyclohexamide (Sigma; Burlington, ON,CAN) (1:1000 dilution). Infection flasks were then incubated at 37^oC and 5% CO_2 until desired time points achieved.

For the generation of Chlamydial stocks, *C. pneumoniae* infected cell cultures were scraped off the plastic flasks using rubber policemen, 72 hours post infection (hpi). Cell cultures along with media were transferred into 50 mL Falcon tubes containing sterile glass beads, and cell material was homogenized by vortexing for 2 minutes. Eukaryotic cell debris was pelleted by centrifugation at 1,500 rpm for 10 minutes at 4^oC

in a Beckman GPR Benchtop centrifuge. The *C. pneumoniae* supernatant was then

pelleted at 18,500 rpm for 45 minutes at 4°C by centrifugation in a Sorvall RC-5B

centrifuge in a Sorvall GSA rotor. *C. pneumoniae* elementary bodies were resuspended

in sucrose-phosphate-glutamate (SPG) plus MEM + 10% FBS (Invitrogen) media at a

final concentration of 50% and frozen as Chlamydial stocks at -20°C for 1 hour then

stored at -80°C for long term storage. Stocks were thawed out at 37°C in water bath prior

to use for propagation of *Chlamydia* in a new round of infection.

2.1.5 Fixing *C. pneumoniae* infected HeLa shell vials

HeLa 229 cells from T75 flasks that were ~90-95% confluent, were removed from

the flask as previously described (Materials & Methods 2.1.3) and 2x10^5 HeLa cells/ml

were added into sterile shell vials complete with round coverslips, along with 1 mL of

MEM + 10% FBS (Invitrogen). Shell vials were loosely capped and kept in the 37°C

incubator until confluent. Media was aspirated using a glass pipette, and 1 mL of new

media plus 80 μL of Cpn seed, and centrifuged for 1 hour at room temperature in a

Beckman GPR Centrifuge at 2,500 rpm with a GH-3.7 rotor. Cells were kept at 37°C for

one additional hour, then media was removed and 1 mL of new media plus 1μL/mL of

cyclohexamide (Sigma) (1:1000 dilution) was added to each vial. Vials were kept at

37°C in a CO_2 incubator until desired time points attained. To fix shell vials media was

aspirated from each and 1 mL of 1x phosphate buffered saline (PBS) was added then

aspirated off to wash. One mL of ice cold 100% methanol was added to each vial for 10

minutes then removed and washed additionally with 1 mL 1x PBS twice. Vials were either used immediately or stored at 4°C until further use.

2.1.6 Chlamydial DNA extraction

Chlamydia pneumoniae CWL029 genomic DNA was isolated using the NucliSENS MiniMag extraction kit (BioMerieux, Marcy l'Etoile, France) from Chlamydial stocks stored in 50% SPG and MEM + 10% FBS (Invitrogen) thawed out in a 37°C water bath. Chlamydial seed was added to lysis buffer in a 1:10 dilution and incubated at room temperature for 10 minutes, then briefly vortexed for 10 seconds. Silica was then added followed by vortexing of the sample and a second room temperature incubation for 10 minutes. The non-infectious specimen is then centrifuged in a GH-3.7 rotor for 2 minutes at 2,700 rpm on a Beckman GPR Benchtop centrifuge. The pellet is reuspended in 400 μl of wash buffer 1 and washed for 30 seconds on the Mini-mag (BioMerieux, Marcy l'Etoile, France). Each sample was washed in the same fashion a total of two times with wash buffer 1, as well as 500 μl of wash buffer 2, and 500 μl of wash buffer 3. After washing, 50 μl of elution buffer was added and centrifuged for 5 minutes at 1,400 rpm at 60°C on the Thermomixer 5436 Eppendorf (Captial Scientific Inc., Austin, TX, USA) centrifuge. The eluted DNA was stored at -20°C.

2.2 Molecular Biological Methods

2.2.1 Cloning procedures and plasmids

All constructs were amplified from *C. pneumoniae* CWL029 (VR1310; ATCC) (GenBank accession #AE001363) using thePlatinum® Pfx DNA Polymerase (Invitrogen). All gene specific primers were synthesized by Sigma-Aldrich Corporation and contained *attB*-recombination sites; Oligonucleotide sequences for primers used are listed in Appendix 6.2.

Purified PCR products were inserted into the *pDONR$_{201}$* vector (Gateway®; Invitrogen) using the *attB*-sites of the PCR product and the *attP*-sites on the donor vector. To generate entry (*pENT*) vectors containing kanamycin (kan) resistance along with the gene of interest flanked by *attL*-sites, PCR product (150 ng) was incubated with *pDONR$_{201}$* vector (150 ng) and BP clonase® II enzyme mix (Integrase, Integration Host Factors; Gateway®; Invitrogen) . The reaction proceeded at room temperature for one hour, and was then stopped with the addition of Proteinase K (0.2 µg/mL; Invitrogen) and a 10 minute incubation at 37°C. The entry vectors were propagated through transformation into *E. coli* XL-1 Blue (Stratagene) cells then plated on LB agar plates containing 30 mg/mL kanamycin to select for the *pDONR$_{201}$* vector. Small cultures containing LB, with 30 µg/mL kan and a single colony were grown overnight at 37°C while shaking at 260 rpm. Plasmids were extracted and purified using the GenElute Plasmid Miniprep kit (Sigma-Aldrich).

Purified entry vectors were used in a second recombination reaction with one of two *attR*-site containing destination vectors (*pDEST$_{15}$* or *pDEST$_{17}$*; Invitrogen) to create

expression vectors (*pEX$_{15}$* and *pEX$_{17}$* respectively). To generate exit (*pEX*) vectors

containing ampicillin resistance along with the gene of interest flanked by *attR*-sites,

pENT vector (150 ng) was incubated with 150 ng of *pDEST* (15 or 17) vector and LR

clonase® II enzyme mix (Integrase, Excisionase, Integration Host Factors; Gateway®;

Invitrogen). The reaction proceeded at room temperature for one hour, and was then

stopped with the addition of Proteinase K (0.2 mg/mL; Invitrogen) followed by a 10

minute incubation at 37°C. The recombination reaction created expression vectors

containing the gene of interest as well as an ampicillin resistance cassette with either a

GST or His$_6$-tag. The expression vectors were propagated in the same manner as

described above with the exception of the antibiotic used to select for the bacteria, which

was ampicillin at a concentration of 100 mg/mL. All constructs were verified by

sequencing by The Institute for Molecular Biology and Biotechnology – MOBIX Labs

(McMaster; Hamilton, ON, CAN).

2.2.2 Production of soluble recombinant protein

All constructs were expressed in *E. coli* BL21(*DE3*) or Rosetta (pLysS)

(Stratagene) cells and transformed with protein expression vectors. Transformed

expression plasmids were plated on LB plates containing 100 μg/mL ampicillin. LB broth

containing antibiotics at a final concentration of 100 μg/mL ampicillin, or 30 μg/mL

kanamyacin, were then inoculated with a 1:50 dilution of an overnight culture and grown

at 37°C shaking at 260 rpm until they reached an optical density optical density at 600nm

(OD$_{600}$) of approximately 0.7. Cultures were induced with 0.2 mM of isopropyl-β-D-

thiogalactopyranoside (IPTG; Sigma-Aldrich) and incubated for 2 hours at 37°C.

Bacteria were harvested by centrifugation at 7,000 rpm in a Sorvall GSA rotor for 5

minutes at 4°C in a Sorvall RC-5B centrifuge. Bacteria containing His$_6$-tagged protein

were resuspended in either 10 mL of ice-cold binding buffer (50 mM potassium

phosphate pH 7.2, 150 mM KCl, 1 mM MgCl2) for GST pull-down assays or 10 mL of

ice-cold Nickel A Buffer (20 mM Tris-HCl pH 7.2, 500 mM KCL, 10 mM imidizole,

10% (v/v) glycerol, 0.2% (v/v) β-mercaptoethanol (β-ME), 0.03% (v/v)

lauryldimethylamine-oxide (LDAO)) for purification on nickel-NTA agarose. Bacteria

containing GST-tagged protein were resuspended in 10 mL ice-cold 1x PBS (0.14M

NaCl, 2.68mM KCl, 8.1mM Na$_2$HPO$_4$, 1.47mM KH$_2$PO$_4$) and stored at 4°C until further

use. Both His and GST-tagged protein lysates contained 1 complete EDTA-free protease

inhibitor tablet (Roche; Mississauga, ON).

2.2.3 Purification of recombinant protein

Culture suspensions were sonicated on ice, on setting 5, for 4 x 10 second cycles

with 20 second breaks between each session (Fisher sonic dismembrator model 100,

Ottawa, ON, CAN). The lysates were centrifuged at 18,500 rpm in a Sorvall GSA rotor

for 45 minutes at 4°C in a Sorvall RC-5B centrifuge to remove intact cells and other

cellular debris. Pelleted material was discarded and supernatants were then filtered

through 0.45 μm Nalgene bottle top filter (Nalge Nunc International). The lysate is then

loaded into a Superloot column (GE Healthcare, Baie d'Urfe QUE, CAN) and purified

using affinity chromatography on the Akta FPLC (Amersham Biosciences, Uppsala,

SWE). Protein lysate was then bound to a primed His-Trap^{TM} FF column (GE

Healthcare) and subsequently washed with Nickel A buffer (20 mM Tris-HCl pH 7.2, 500

mM KCL, 10 mM imidizole, 10% (v/v) glycerol, 0.2% (v/v) β-mercaptoethanol (β-ME),

0.03% (v/v) lauryldimethylamine-oxide (LDAO)), then increasing amounts of Nickel B

buffer (20 mM Tris-HCl pH 7.2, 500 mM KCL, 300 mM imidizole, 10% (v/v) glycerol,

0.2% (v/v) β-mercaptoethanol (β-ME), 0.03% (v/v) lauryldimethylamine-oxide (LDAO)).

The bound protein was then eluted off the column in pure Nickel B buffer and caught in 1

mL fractions then quantified by comparison to BSA standards following SDS-PAGE and

Coomassie blue (225 ml methanol, 225 ml dH$_2$O, 50 ml Acetic acid, 1.25 g R-250

Brilliant Blue) staining. Purified protein is used immediately or stored at 4°C for no more

than 24 hours prior to use, or frozen down with glycerol at a final concentration of 20%

(v/v) at -80°C.

2.2.4 GST pull-down Assay

All soluble recombinant protein lysates containing GST-tagged proteins were

filtered through a Nalgene filter (0.45 μm; Nalge Nunc International) and incubated for 1

hour at 4°C on a rocking platform (Nutator®; TCS Scientific Corperation, New Hope,

PA, USA) with 500 μl of washed glutathione agarose beads (Sigma-Aldrich). The GST

protein bound to the beads was collected by centrifugation for 5 minutes at 4°C at 1000

rpm in a GH-3.7 rotor in a Beckman GPR Benchtop centrifuge. The supernatant was

removed and the beads were subsequently washed with ice-cold PBS then blocked

overnight rocking at 4°C with TBST + 4% BSA (Tris Buffered Saline with Tween-20 +

4% Bovine Serum Albumin). The beads were collected after blocking by centrifugation for 5 minutes at 4°C at 1,000 rpm in a GH-3.7 and 50 μL were incubated with 700 μL of *E. coli* lysate expressing the His$_6$-tagged proteins in 1.5 mL Eppendorf tubes for 1 hour at 4°C rocking. The beads were then harvested by centrifugation for 5 minutes at 4°C at 1,000 rpm in a GH-3.7 rotor and washed with increasing salt concentrations a total of 7 times to eliminate spurious protein interactions. Beads contained within 1.5 mL Eppendorf tubes were spun down for 8 seconds in an Eppendorf 5415R microcentrifuge reaching a speed no greater than 10,000 rpm. Supernatant was carefully removed as to not disturb the glutathione agarose beads and 600 μL of either low (0 mM NaCl + 50 mM Tris pH 7.2 + 1% Tween-20), medium (200 mM NaCl + 50 mM Tris pH 7.2 + 1% Tween-20) or high (500 mM NaCl + 50 mM Tris pH 7.2 + 1% Tween-20) salt conditions were added then gently inverted to mix. The tubes were centrifuged as described above for a total of 7 washes. After the final wash, the glutathione agarose beads were resuspended in 15 μl of 1x SDS-PAGE loading buffer (125mM Tris, pH 6.8, 2% β-mercaptoethanol, 20% glycerol, 4% SDS and 0.001% bromophenol blue) before being electrophoresed on a SDS-PAGE gel then transferred to a nitrocellulose membrane and probed for His$_6$-tagged protein via Western blot.

2.2.5 His$_6$ pull-down assay

Soluble lysates containing His$_6$-tagged proteins were filtered through a Nalgene filter (0.45 μm; Nalge Nunc International) and incubated for 1 hour at 4°C on a rocking platform (Nutator®; TCS Scientific Corperation) with 500 μl of washed and primed

Nickel-NTA Magnetic Agarose Beads (Ni-NTA; Qiagen, Valencia, CA, USA). The

His$_6$-tagged protein bound to the beads was collected by centrifugation for 5 minutes at

4°C at 1000 rpm in a GH-3.7 rotor in a Beckman GPR Benchtop centrifuge. The

supernatant was removed and the beads were washed with ice-cold PBS then blocked

overnight rocking at 4°C with TBST + 4% BSA (Tris Buffered Saline with Tween-20 +

4% Bovine Serum Albumin). The beads were collected after blocking by centrifugation

for 5 minutes at 4°C at 1,000 rpm in a GH-3.7 rotor and 50 μl were incubated with 700 μl

of *E. coli* lysate expressing the GST-tagged proteins in 1.5 mL eppendorf tubes for 1 hour

at 4°C rocking. The beads were then harvested by centrifugation for 5 minutes at 4°C at

1,000 rpm. Beads contained within 1.5 mL Eppendorf tubes were spun down for 8

seconds in an Eppendorf 5415R microcentrifuge reaching a speed no greater than 10,000

rpm. Supernatant was carefully removed as to not disturb the beads and 600 μl of either

low (0 mM NaCl + 50 mM Tris pH 7.2 + 1% Tween-20), medium (200 mM NaCl + 50

mM Tris pH 7.2 + 1% Tween-20) or high (500 mM NaCl + 50 mM Tris pH 7.2 + 1%

Tween-20) salt conditions were added then gently inverted to mix. The tubes were

centrifuged as described above for a total of 7 washes to eliminate spurious protein

interactions. After the final wash, the Ni-NTA beads were resuspended in 15 μl of 1x

SDS-PAGE loading buffer (125mM Tris, pH 6.8, 2% β-mercaptoethanol, 20% glycerol,

4% SDS and 0.001% bromophenol blue) before being electrophoresed on a SDS-PAGE

gel, transferred to a nitrous cellulose membrane, and probed for GST-tagged protein via

Western blot. All results were completed in triplicates for verification, and TCA

precipitations were carried out on all final wash solutions to ensure all unbound protein was properly washed from the Ni-NTA beads.

2.2.6 Trichloroacetic acid (TCA) precipitation

Supernatants from each of the final pull down washes were incubated with 10% (v/v) TCA (Fischer Scientific, Ottawa, ON, CAN) for 16 hours on a rocking platform (Nutator®; TCS Scientific Corperation) at 4°C. Precipitated proteins were then removed by centrifugation at 13,200 rpm for 40 minutes at 4°C in an Eppendorf 5415R microcentrifuge. Pelleted proteins were then washed with 100% acetone by resuspension followed by centrifugation for 20 minutes at 13,200 rpm in a microcentrifuge. Acetone was removed and 2 additional acetone washes were performed. Pellets were dried by evaporation for 20 minutes at room temperature. Dry pellets were resuspended in 1x SDS-PAGE loading buffer and the presence of His_6-tagged proteins (for GST pull-down assays) or GST-tagged proteins (for His_6 pull-down assays) was probed for by Western blot analysis.

2.2.7 Western blot analysis

All protein samples were resuspended in 1x SDS-PAGE (125mM Tris, pH 6.8, 2% β-mercaptoethanol, 20% glycerol, 4% SDS and 0.001% bromophenol blue) loading buffer, and were electrophoresed on an 11% acrylamide/bis-acrylamide SDS-PAGE gel, unless otherwise noted. The gel was electrophoresed for 20 minutes at 80 V to allow the

diffusion of proteins through the stacking gel, then for 80 minutes at 120 V until the protein have migrated fully through the resolving gel. Samples were then transferred to a nitrocellulose membrane using the iBlot® (Invitrogen) on the P3 transfer setting for 7 minutes. Membranes were blocked using a 5% low fat skim milk powder in PBS + 0.1% Tween-20 for one hour at room temperature on a shaking platform. Polyclonal mouse anti-His$_6$ antibody (Invitrogen) or polyclonal mouse anti-GST antibody (Invitrogen) were used to probe for His$_6$-tagged or GST-tagged proteins respectively, for 1 hour at room temperature on a shaking platform. Antibody dilutions were used at a concentration of 1:5,000 for mouse anti-His$_6$ and at a concentration of 1:4,000 for mouse anti-GST diluted in PBS + 0.1% Tween-20 with 5% low fat skim milk powder. Membranes were washed twice with 20 mL of PBS + Tween-20 for 5 minutes at room temperature, then bound antibodies were detected by incubating the membrane with polyclonal goat anti-mouse IgG antibody, conjugated to horseradish peroxidise (HRP) (Invitrogen), unless otherwise stated. This incubation was for 1 hour at room temperature, followed by a second PBS + 0.1% Tween-20 wash twice for 5 minutes each at room temperature. Goat anti-mouse IgG antibody was diluted 1:5,000 in PBS + 0.1% Tween-20 with 5% low fat skim milk powder. Blots were visualized using enhanced chemiluminescence (ECL) substrates (Sigma-Aldrich) and CL-X Posture^{TM} film (Thermo Scientific) developed in a Konica Minolta medical X-ray processor, model SRX-101A.

2.2.8 96-well indirect ELISA

Lysates of recombinant proteins fused to GST-tags, were bound to a glutathione-

agaorse 96-well plate by incubation at 4°C with 100 µL of protein. The wells were

washed 5 times with 1x PBS, and blocked for 1 hours with 100 µL TBST + 4% BSA.

Wells were washed 5 times with 1x PBS, then 200 µL of His$_6$-tagged protein was added

and the plate was incubated at 4°C for 2 hours. To eliminate spurious interactions, wells

were washed 5 times with high salt wash solution (500 mM NaCl + 50 mM Tris, pH 7.2 +

0.1% Tween-20) then a 1:5,000 dilution of mouse anti-His$_6$ (polyhistidine) antibody was

prepared and 100 µL was added to each well then incubated at room temperature for 1

hour. A PBS + 0.1% Tween-20 wash removed any remaining unbound antibody, and a

1:1,000 dilution of goat anti-mouse IgG antibody conjugated to HRP was prepared, and

100 µL added to each well followed by incubation at room temperature for 1 hour. A

second PBS + 0.1% Tween-20 wash was used to remove any unbound secondary

antibody, then 100 µL of o-Phenylenediamine was added to each well and the absorbance

of each sample was read at 450 nm in a ELx800 microplate reader (BioTek, Winooski,

VT, USA) after 40 minutes.

2.2.9 Limited proteolysis

The JBS Floppy Choppy *in situ* proteolysis kit (Jena Bioscience, Jena, GER) was

used to cleave CopN into stable domains or fragments which might yield better

diffracting crystals than the intact protein. The proteases used for *in situ* proteolysis were

as follows: α-Chymotrypsin from bovine pancreas, Trypsin from bovine pancreas,

Subtilisin from *Bacillis licheniformis*, and Papain from *papaya latex*. A 1:100 dilution

aliquot of each protease was created using the kit supplied buffer 10 mM HEPES pH 7.5

and 500 mM NaCl. A 100 µg quantity of protein was transferred to four new Eppendorf

tubes and 10 µl of each diluted protease was added to the appropriately labelled tube. The

tubes were incubated for 1 hour at room temperature, then reaction were quenched using

20 µl of 1x SDS-PAGE loading buffer (125mM Tris, pH 6.8, 2% β-mercaptoethanol,

20% glycerol, 4% SDS and 0.001% bromophenol blue). Products were analysed by SDS-

PAGE and prominent bands were excised from the gel and sent for Mass Spectrometry at

McMaster University. Truncated constructs of CopN from the resulting sequences were

cloned and henceforth known as $CopN_{\Delta83N}$ and $CopN_{84\text{-}388}$, products of the Trypsin and α-

Chymotrypsin digestion, respectively.

2.2.10 Chlamydial Immunofluorescence

To test for the presence of Chlamydial infection of HeLa cells, the Pathfinder

Chlamydia culture confirmation kit was used (Bio-Rad; Mississauga, ON, CAN), which

uses a genus specific fluorescein-conjugated murine monoclonal antibody to *Chlamydia*-

LPS. Shell vials with ~200,000 cells/mL were infected with *C. pneumoniae* and fixed as

previously described (Materials & Methods 2.1.5). The round coverslip from the shell

vial was placed on a regular rectangular slide in a slide chamber with the cells facing up.

Two drops of the Pathfinder Dx reagent were added to each slide, covering the entire

round coverslip. In complete darkness, the slide was incubated for 40 minutes in a $37^{o}C$

cell culture incubator. Slides were washed 5x in 1x PBS. Eight µL of mounting media

(1:1 of 1x PBS and 100% glycerol) was placed on a clean rectangular slide, and the round

coverslip was placed cell side down on the media. Infected HeLa cells were visualized using an Olympus BX51 fluorescent microscope with a FITC filterset and the UplanFl 60× NA 1.25 oil immersion objective (Olympus) and CCD camera (DP70; Olympus).

To assess the cross reactivity of the primary antibody, HeLa cell seeded shell vials were fixed as previously described (Materials & Methods 2.1.5). One mL of 0.1% Triton X-100 in 1x PBS was added to each shell vial for 5 minutes at room temperature, then aspirated off. One mL of 1x PBS was then added to each shell vial and aspirated off to rinse coverslips, for a total of 3 times. Coverslips were incubated in blocking buffer (1% BSA + 1x PBS) at room temperature for 1 hour, then blocking solution was aspirated off. Primary antibody was added to each vial diluted in blocking buffer for 1 hour at room temperature, at the desired dilution. Primary antibody was aspirated and coverslips were washed with 1 mL of 1x PBS as previously described for a total of 4 times. Secondary antibody with a FITC conjugate was diluted to desired concentration in blocking buffer for 1 hour at room temperature. Secondary antibody was discarded and washing of the coverslips was repeated. Using tweezers, coverslips were carefully removed from the shell vials and placed on a rectangular slide, cell side up. 2 drops of normal mouse gamma globulin DFA Direct Fluorescent-Antibody reagent (Diagnostic Hybrids; Athens, Ohio, USA) was added to the cells, then coverslips were rinsed with 1x PBS. Mount the coverslips, cell side down, with 8 μL of mounting media (1:1 of 1x PBS and 100% glycerol) on a cleaned rectangular slide. HeLa cells, either infected or mock infected, were visualized using an Olympus BX51 fluorescent microscope to assess any antibody cross reactivity.

2.2.11 CopN secretion time course in HeLa cells

Confluent HeLa 229 cell (ATCC) monolayers in T25 flasks were infected with 40 μl of *C. pneumoniae* CWL029 and centrifuged for 1 hour at 2,500 rpm in a GH-3.7 rotor in a Beckman GPR Benchtop centrifuge. Flasks were then incubated for 1 hour in a 37°C incubator with 5% CO_2 then all media was removed and replaced with fresh MEM + 10% FBS and cyclohexamide was added to each flask at a final concentration of 1 μg/mL. Flasks were allowed to remain in the cell culture incubator until desired time points were attained. Cells were scraped off the flask and all liquid was added to glass tube and cells were sheared with 40 strokes from a dounce homogenizer on ice. All liquid was centrifuged in a Beckman LE-80 Ultracentrifuge at 19,000 rpm in a Sw-28 rotor for 1 hour at 4°C. Supernatant was collected and TCA precipitated (Materials & Methods 2.2.6), then analyzed by SDS-PAGE and probed for by Western blot using rabbit-anti-CopN antibody (1:2,500), rabbit-anti-CdsD (1:500) or mouse-anti-β-actin antibody (1:5,000).

CHAPTER THREE

3.0 RESULTS

3.1 Predicted 3D-JigSaw Structure and ClustalW2 Alignment of CopN

Since no crystal structure has been determined for CopN of any Chlamydial species, x-ray crystallography was attempted for CopN. Full length CopN as well as various truncations including $CopN_{\Delta 40N}$, $CopN_{\Delta 83N}$, and $CopN_{84-388}$, were attempted using various x-ray crystallography screens; however no trials yielded protein crystals and further crystallography attempts were abandoned.

A ClustalW2 alignment showed that the sequence similarity of full length CopN and TyeA from *Yersinia*, was 32% but confined to the second half of the full length CopN protein (Figure 3.1a). To map where these residues were on the predicted crystal structure of CopN, the automatic comparative modelling server 3D-JigSaw was utilized. The predicted structure as well as PYMOL software was used to map the identical, conserved and semi-conserved residues (from the ClustalW2 server) on the CopN 98-399 structure (Figure 3.1b). Using the predicted crystal structure of CopN we were also able to compare it to the solved structure of YopN-TyeA of *Yersinia*, and MxiC of *Shigella* (Figure 3.2). Structural bias is encountered when a homologous protein structure is predicted from few known crystal structures, and therefore the structure of CopN should be viewed with caution in relation to that of MxiC and YopN-TyeA.

(A)

```
CopN   MAASGGTGGLGGTQGVNLAAVEAAAAKADAAEVVASQEGSEMNMIQQSQDLTNPAAATRT 60
TyeA   ------------------------------------------------------------

CopN   KKKEEKFQTLESRKKGEAGKAEKKSESTEEKPDTDLADKYASGNSEISGQELRGLRDAIG 120
TyeA   ------------------------------------------------------------

CopN   DDASPEDILALVQEKIKDPALQSTALDYLVQTTPPSQGKLKEALIQARNTHTEQFGRTAI 180
TyeA   ------------------------------------------------------------

CopN   GAKNILFASQEYADQLNVSPSGLRSLYLEVTGDTHTCDQLLSMLQDRYTYQDMAIVSSFL 240
TyeA   ----MAYDLSEFMG-----------------------DIVALVDKRWA----------- 21
            : :  .*: .                      :::::::.*::

CopN   MKGMATELKRQGPYVPSAQLQVLMTETRNLQAVLTSYDYFESRVPILLDSLKAEGIQTPS 300
TyeA   -----------G----------------IHDIEHLANAFS-----------------LPTP- 38
                  *              ::::: : .::.              : **

CopN   DLNFVKVAESYHKIINDKFPTASKVEREVRNLIGDDVDSVTGVLNLFFSALRQTSSRLFS 360
TyeA   EIK-VRFYQDLKRMFR-LFP-----------LG-----------------VFS 61
       ::: *:. :. :::::. **           :*                 :**

CopN   SADKRQQLGAMIANALDAVNINNEDYPKASDFPKPYPWS 399
TyeA   DEEQRQNLLQMCQNAID-MAIESEE-EELSELD------ 92
       . ::**:*  *  **:* : *:.*:  : *::
```

(B)

Figure 3.1: ClustalW2 alignment and 3D-JigSaw modelling of CopN from *Chlamydia pneumoniae* and TyeA from *Yersinia pestis*. (A) Sequence alignment of CopN from *Chlamydia pneumoniae* with TyeA from *Yersinia pestis* indicating residue homology of the C-terminal region. Asterisk (*) refers to identical amino acids, a double dot (:) refers to a conserved substitution and a single dot (.) refers to a semi-conserved substitution. (Chenna et al., 2003) **(B)** Cartoon ribbon diagram depicting CopN 98-399 aa using the 3D-JigSaw automatic comparative modelling server. Yellow sections indicate residues with sequence similarity to TyeA of *Yersinia pestis* (Bates et al., 2001).
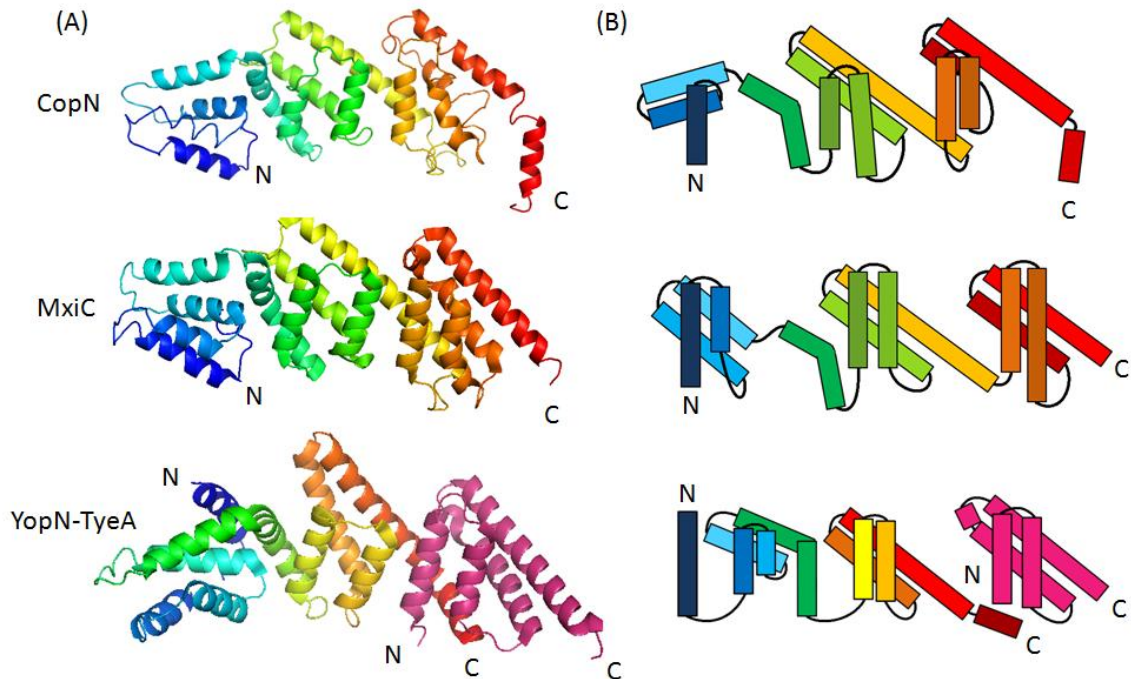
Figure 3.2: Comparison of the known plug protein crystal structures to the predicted secondary structure of CopN of *Chlamydia pneumoniae*. (A) Top: A cartoon ribbon diagram of the predicted secondary structure of CopN from *Chlamydia pneumoniae* using the 3D-JigSaw server. Only residues 98-399 are shown (Bates et al., 2001). **Middle:** A cartoon ribbon diagram of the crystal structure of MxiC from *Shigella flexneri* (Deane et al., 2008). Residues 74-355 of MxiC are shown. **Bottom:** A cartoon ribbon diagram of the crystal structure of YopN and TyeA from *Yersinia pestis* (Schubot et al., 2005). YopN residues 76-293 are shown along with TyeA residues 1-92. **(B)** A topological diagram of CopN 98-399 (top), MxiC 74-355 (middle) and the YopN-TyeA complex (bottom); YopN 76-293 and TyeA 1-92. All protein structures shown colored from blue at the N-terminus to red at the C-terminus with the exception of TyeA colored in magenta.

3.2 <u>Determination of CopN Domain Boundaries</u>

A method commonly employed to discern domain boundaries is limited proteolysis. Full length CopN was digested using Trypsin, α-Chymotrypsin, Subtilisin, and Papain all included in the Jena Biosciences Floppy Choppy *in situ* proteolysis kit. Digestion products were separated using SDS-PAGE and electrophoresed on an 11% gel, then stained with Coomassie blue dye (Figure 3.3). Bands of the largest molecular weight and most predominant, were excised from the gel and sent to McMaster University Center for Mass Spectrometry (MUCMS; Hamilton, ON, CAN) for analysis. The digestion product produced by the addition of Trypsin was a 35 kDa fragment of CopN comprised of amino acids 84-399, referred to as $CopN_{\Delta 83N}$ (Appendix 6.1.1). A similar sized product was obtained from the digestion using α-Chymotrypsin which spanned residues 84-388 of CopN and had a molecular weight of 31 kDa, referred to as $CopN_{84-388}$ (Appendix 6.1.2). The cleavage of CopN by both proteases at 83 amino acids defined the first boundary, while a second boundary was designated at residue 268 by Archuleta *et al.* (2011). Fragments corresponding to the mapped domains were cloned and used in further investigations of domain interaction studies; $CopN_{\Delta 83N}$ consisting of residues 84-399, $CopN_{85-268}$ consisting of residues 85-268, and finally $CopN_{270-399}$ consisting of residues 270-399.
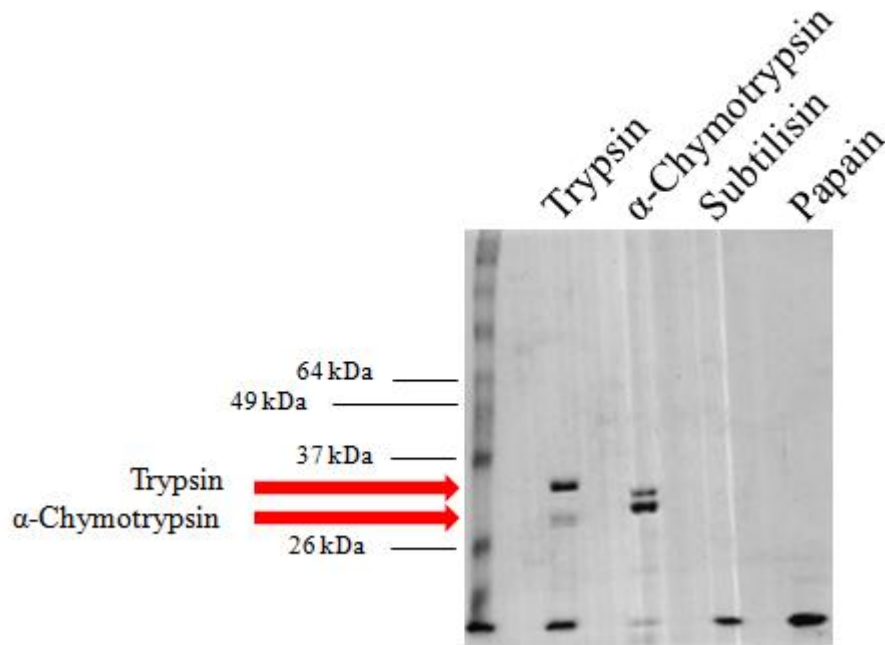
Figure 3.3: Limited proteolysis of full length CopN. Limited proteolysis of full length His$_6$-tagged CopN with Trypsin (lane 2), α-Chymotrypsin (lane 3), Subtilisin (lane 4) and Papain (lane 5) on an 11% SDS-PAGE gel. The Trypsin digestion produced fragment CopN$_{\Delta 83N}$ while the α-Chymotrypsin digestion produced fragment CopN$_{84-388}$ after identification by mass spectrometry. Benchmark prestained ladder seen in lane 1. The top red arrow indicate CopN$_{\Delta 83N}$ the cleavage product of the Trypsin digestion, while the bottom red arrow indicates CopN$_{84-388}$ the cleavage product of the α-Chymotrypsin digestion. Both bands were excised and sent to McMaster University Center for Mass Spectrometry (MUCMS).

3.3 Interaction of Flagellar and Type III Secretion Proteins with full length CopN

Previous experiments in our lab have suggested a large number of *in vitro* protein-protein interactions for CopN (Mahony, unpublished data). To investigate these interactions further a subset of proteins were tested for interaction using 96-well indirect ELISAs or by GST pull-down assays. Proteins were chosen based on their unique roles within the Chlamydial T3SS which included flagellar proteins, inclusion membrane proteins, translocator proteins as well as various T3S chaperones. ELISA data showed that GST-CopD1$_{158-206}$ interacted with His$_6$-CopN, while GST-CopD1$_{1-137}$ did not interact with His$_6$-CopN under high salt conditions, adsorbance values corresponding to 0.50 ± 0.098 and 0.16 ± 0.085 respectively (Figure 3.4). As a positive control, we demonstrated that GST-CopN interacted with His-Scc3 with an absorbance of 0.70 ± 0.086, and as a negative control GST alone did not interact with His-CopN, adsorbance of 0.14 ± 0.066. This screening method is still under revision to decrease background signal, and determine threshold of positive interactions.
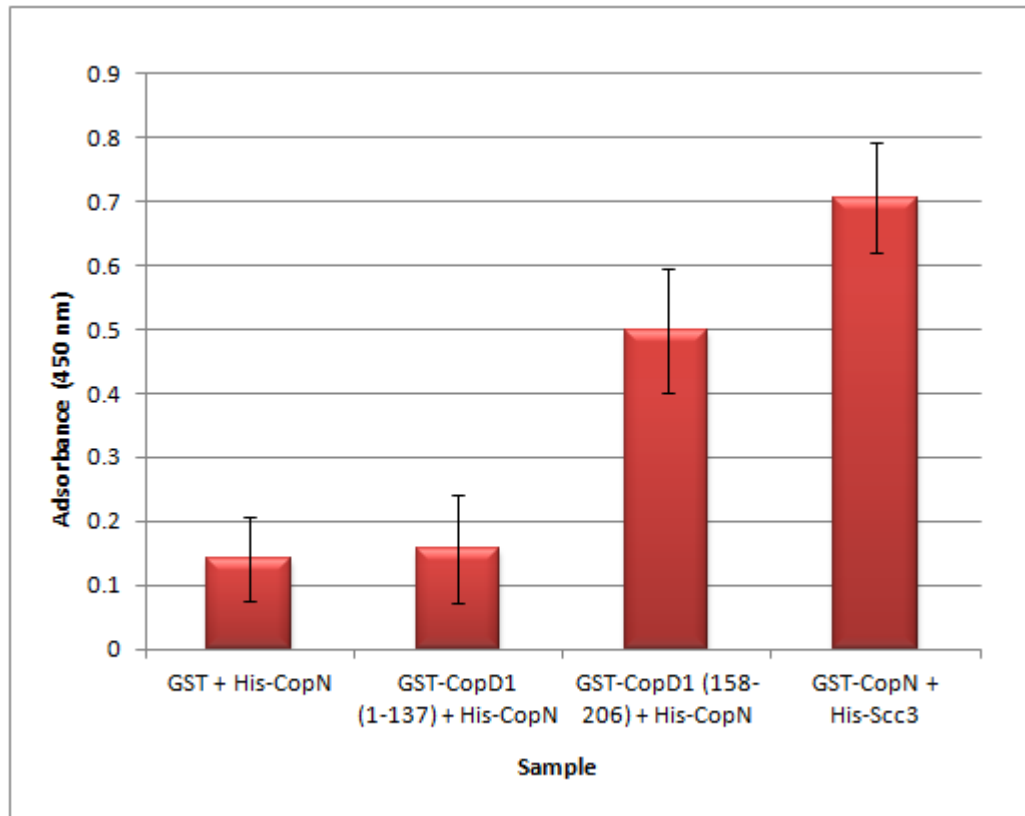
Figure 3.4: 96-well indirect ELISA interaction between CopN and CopD1 constructs. His_6-CopN interacts with GST-CopD1$_{158-206}$ seen in bar 3, one of the four putative translocators in *C. pneumoniae* however it does not interact with the N-terminal region of CopD1$_{1-137}$ seen in bar 2. GST alone did not interact with His_6-CopN (bar 1) and the positive interaction control is shown between GST-CopN and His_6-Scc3 (bar 4). Measurements were repeated in triplicates. Error bars represent 2 standard errors from the mean.

After screening the interaction of CopN and CopD1, this interaction as well as other ones were assessed using GST pull-down assays. Interactions between CopN and CopD1$_{158-206}$ as well as FliI and FliF were tested. All three proteins were bound to glutathione agarose beads and interacted with His$_6$-CopN in the presence of low (0 mM NaCl), medium (200 mM NaCl) and high (500 mM NaCl) salt wash solutions; only high salt wash solution results shown (Figure 3.5). No interactions between CopN and CdsF, CopB1$_{1-255}$, CopD1$_{1-137}$, or CopD1$_{227-444}$ was detected. Final washes of pull-downs were retained and precipitated using TCA, then probed for His$_6$-tagged proteins; all TCA precipitation results were negative for His$_6$-tagged protein. GST pull-downs showed an interaction between both His$_6$-tagged IncC and Scc3 with GST-tagged CopN. CopN interacted with His$_6$-IncC and His-Scc3 in the presence of low (0 mM NaCl), medium (200 mM NaCl) and high (500 mM NaCl) salt wash solutions (Figure 3.6). No interaction was detected between CopN and another inclusion membrane protein, IncB.
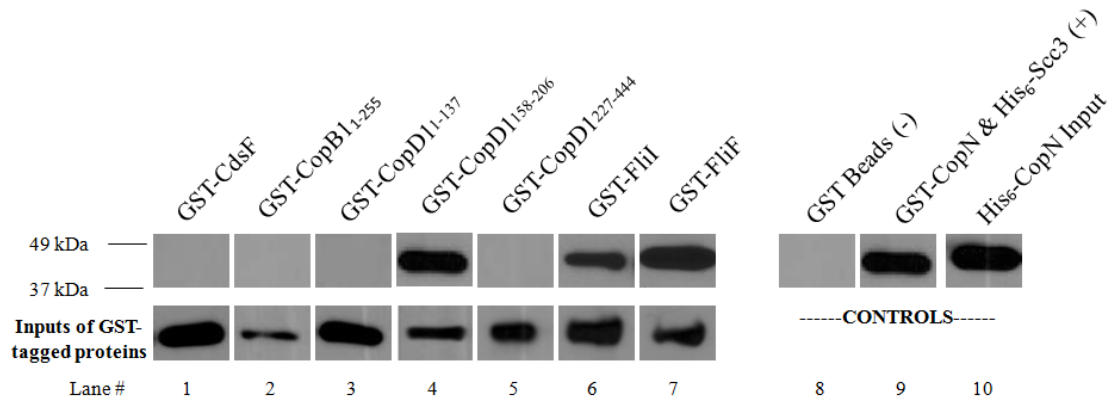
Figure 3.5: Interaction of full length His$_6$-CopN with T3S and flagellar proteins.
CopN interacts with CopD1$_{158\text{-}206}$ (lane 4) as well as FliI (lane 6) and FliF (lane 7), but not with CdsF (lane 1), CopB1$_{1\text{-}255}$ (lane 2) or the N-terminal or C-terminal regions of CopD1$_{1\text{-}137}$ or CopD1$_{227\text{-}444}$, (lane 3 and 5 respectively). CopD1$_{158\text{-}206}$, FliI and FliF bound to glutathione-agarose beads interacted with His$_6$-CopN in the presence of a high salt wash buffer (500mM NaCl). GST alone bound to glutathione agarose did not interact with His$_6$-CopN (lane 8), and GST-CopN interacted with His$_6$-Scc3 (lane 9) as the positive control. All results were completed in triplicate, and TCA precipitation results were negative. Inputs of all GST and His$_6$-tagged proteins included.
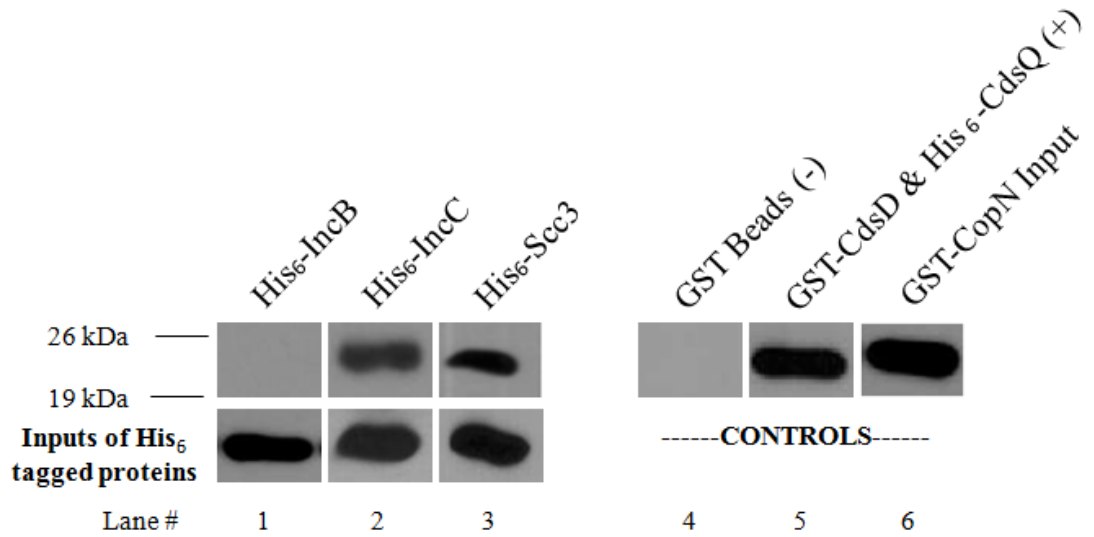
Figure 3.6: Interaction of full length GST-CopN with T3S components. CopN interacts with IncC (lane 2) and Scc3 (lane 3), but not with IncB (lane 1). CopN bound to glutathione-agarose beads interacted with His_6-IncC and His_6-Scc3 in the presence of a high salt wash buffer (500mM NaCl). GST alone bound to glutathione agarose did not interact with His_6-IncC (lane 4), and GST-CdsD interacted with His_6-CdsQ (lane 5) as the positive control. All results were completed in triplicate, and TCA precipitation results were negative. Inputs of all GST and His_6-tagged proteins included.

3.4 <u>Interaction of Flagellar and Type III Secretion Proteins using Various CopN Truncations</u>

The previously determined domain boundaries of CopN (Section 3.2) were used to create various CopN truncations including $CopN_{\Delta 83N}$, $CopN_{85-268}$ and $CopN_{270-399}$. These fragments were then assessed for specific interaction with FliI, FliF, $CopD1_{158-206}$, and Scc3.

3.4.1 $CopN_{\Delta 83N}$ fragment

To further explore the roles of the three domains within CopN, a combination of His_6 and GST pull-down assays were used to identify novel binding partners. As a way to rapidly localize individual binding sites of proteins on CopN, the construct $CopN_{\Delta 83N}$ was used. GST-tagged FliI, FliF and $CopD1_{158-206}$ all bound to glutathione agarose beads interacted with His_6-CopN in the presence of low (0 mM NaCl), medium (200 mM NaCl) and high (500 mM NaCl) salt wash solutions; only high salt wash solution results are shown (Figure 3.7). Similarly, GST-tagged CopN interacted with His_6-Scc3 under the same conditions. With the removal of the N-terminal 83 amino acids of CopN, interaction of FliI, FliF, $CopD1_{158-206}$ and Scc3 were retained with $CopN_{\Delta 83N}$. These results indicate that these flagellar and T3S proteins bind to the central region, the C-terminal region, or a combination of the two.
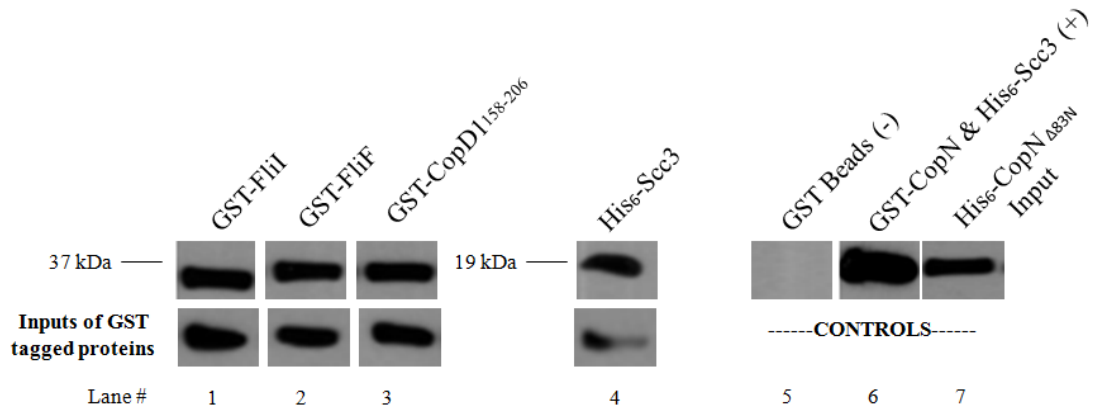
Figure 3.7: Interaction of CopN$_{\Delta83N}$ with flagellar & T3S proteins. GST-FliI (lane 1), GST-FliF (lane 2), & GST-CopD1$_{158-206}$ (lane 3) all bound to glutathione agarose beads interacted with His$_6$-CopN$_{\Delta83N}$ in the presence of a high salt wash buffer (500mM NaCl). GST-CopN$_{\Delta83N}$ bound to glutathione agarose beads also interacted with His$_6$-Scc3 (lane 4). GST alone bound to glutathione agarose beads did not interact with His$_6$-CopN$_{\Delta83N}$ (lane 5) while GST-CopN pulled His$_6$-Scc3 down as the positive control (lane 6). All results were completed in triplicate, and TCA precipitation results were negative. Inputs of all GST and His$_6$-tagged proteins included.

3.4.2 CopN$_{85-268}$ fragment

To determine the individual regions in which FliI, FliF, CopD1$_{158-206}$ and Scc3 interact with CopN, the previously described CopN truncations, CopN$_{85-268}$ and CopN$_{270-399}$ were used. GST-tagged FliI, FliF, and CopD1$_{158-206}$ did not interact with His$_6$-CopN$_{85-268}$ under high salt wash conditions, as determined by GST pull-down assay and Western blotting. Likewise GST-CopN$_{85-268}$ did not interact with His$_6$-tagged Scc3 in the presence of a high salt wash buffer (Figure 3.8).
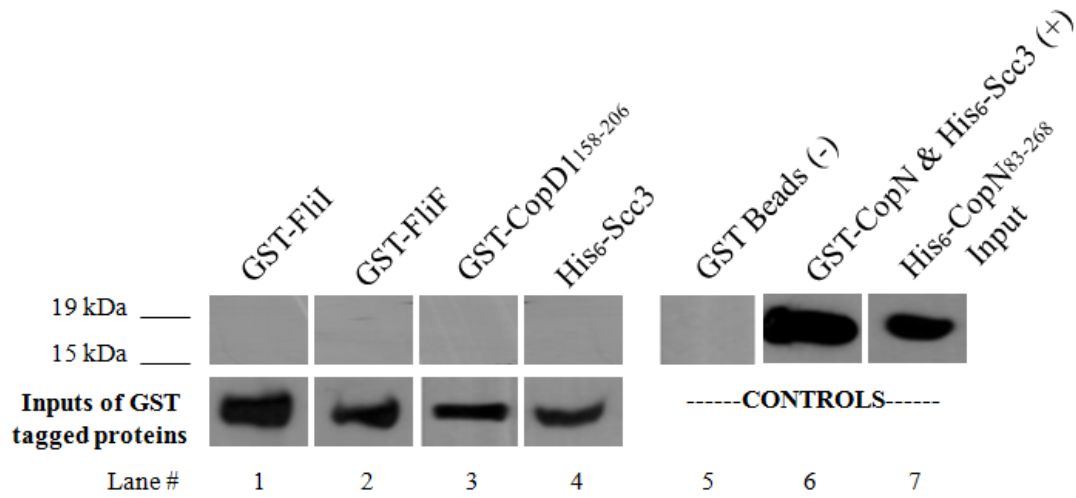
Figure 3.8: Interaction of CopN$_{85\text{-}268}$ with flagellar & T3S proteins. GST-FliI (lane 1), GST-FliF (lane 2), & GST-CopD1$_{158\text{-}206}$ (lane 3) all bound to glutathione agarose beads failed to interact with His$_6$-CopN$_{85\text{-}268}$ in the presence of a high salt wash buffer (500mM NaCl). GST-CopN$_{85\text{-}268}$ bound to glutathione agarose beads did also not interact with His$_6$-Scc3 (lane 4). GST alone bound to glutathione agarose beads did not interact with His6-CopN$_{85\text{-}268}$ (lane 5), while GST-CopN interacted with His$_6$-Scc3 as the positive control (lane 6). All results were completed in triplicates, and TCA precipitation results were negative. Inputs of all GST and His$_6$-tagged proteins included.

3.4.3 CopN$_{270-399}$ fragment

When the C-terminal domain of CopN, CopN$_{270-399}$ was incubated with FliI, FliF, CopD1$_{158-206}$ as well as Scc3, all proteins interacted. GST-tagged FliI, FliF, CopD1$_{158-206}$ interacted with His-CopN$_{270-399}$ in the presence of low (0 mM NaCl), medium (200 mM NaCl) and high (500 mM NaCl) salt wash solutions, only salt high wash solution results shown (Figure 3.9). This positive interaction was also determined for His$_6$-tagged Scc3 which interacted with GST-CopN$_{270-399}$ under the same conditions. These binding site localizations on the individual CopN domains might indicate a role for CopN interacting with various T3S substrates as well as flagellar substrates during the *C. pneumoniae* infectious cycle.
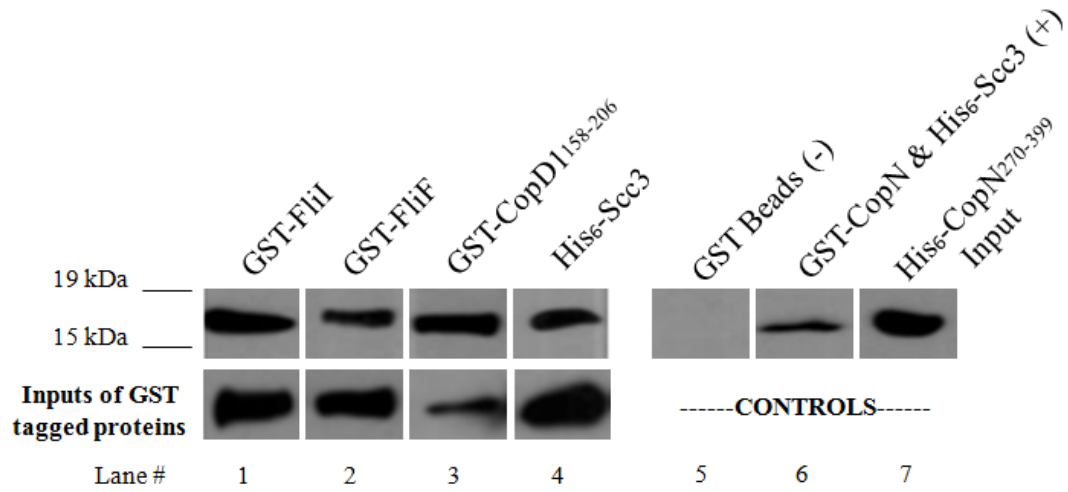
Figure 3.9: Interaction of CopN$_{270\text{-}399}$ with flagellar & T3S proteins. GST-FliI (lane 1), GST-FliF (lane 2), & GST-CopD1$_{158\text{-}206}$ (lane 3) all bound to glutathione agarose beads interacted with His$_6$-CopN$_{270\text{-}399}$ in the presence of a high salt wash buffer (500mM NaCl). GST-CopN$_{270\text{-}399}$ bound to glutathione agarose beads was also able to interact with His$_6$-Scc3 (lane 4). GST alone bound to glutathione agarose beads did not interact with His$_6$-CopN$_{270\text{-}399}$ (lane 5), while GST-CopN interacted with His$_6$-Scc3 as the positive control (lane 6). All results were completed in triplicate, and TCA precipitation results were negative. Inputs of all GST and His$_6$-tagged proteins included.

3.5 Interaction of the N-terminal Region of CopN with Scc1 and Scc4

During the course of this thesis, a heterodimeric chaperone pair was proposed for CopN of *C. pneumoniae* by Silva-Herzog *et al.* (2011). To validate these interactions His_6 pull-down assays were carried out using full length CopN, as well as the individual domain fragments. His_6-Scc1 bound to nickel-NTA beads interacted with GST-Scc4 and GST-CopN in the presence of low (0 mM NaCl), medium (200 mM NaCl) and high (500 mM NaCl) salt wash solutions; only high salt wash solution results shown (Figure 3.10a). His_6-Scc1 was unable to interact with GST-CopN$_{85-268}$ or GST-CopN$_{270-399}$ under the same conditions. As a positive control GST-CdsD bound His_6-CdsQ, while a negative control of His_6-Scc1 bound to nickel-NTA beads did not interact with GST alone. I previously determined that although Scc1 and Scc4 were able to bind to each other in the absence of CopN, neither protein was able to individually associate with CopN in the absence of the other chaperone (Figure 3.10b,c). This cooperative binding is seen amongst many heterodimeric chaperone pairs including SycN and YscB of *Yersinia*, as well as Pcr2 and PcsB of *Pseudomonas*.

A summary of the protein-protein interactions shown in this work, including that of the heterodimeric chaperone pair with CopN, can be seen in Figure 3.11.

(A)

CopN – full length
CopN85-268
CopN270-399
Nickle-NTA beads (-)
GST-CdsD & His6-CdsQ (+)
His6-Scc1 Input

GST-CopN → 82 kDa
64 kDa
GST-Scc4 → 49 kDa
37 kDa
26 kDa
19 kDa
15 kDa

Inputs of GST-CopN proteins

Inputs of GST-Scc4

------CONTROLS------

Lane # 1 2 3 4 5 6 7

(B)

GST- Scc4
GST Beads (-)
GST-CopN & His6-Scc3 (+)
His6-Scc1 Input

19 kDa
15 kDa

Inputs of GST tagged proteins

------CONTROLS------

Lane # 1 2 3 4

(C)

GST- Scc4
GST- Scc1
GST Beads (-)
GST-CopN & His6-Scc3 (+)
His6-CopN Input

49 kDa
37 kDa

Inputs of GST tagged proteins

------CONTROLS------

Lane # 1 2 3 4 5

Figure 3.10: Scc1 & Scc4 interact together with full length CopN but not with CopN$_{85\text{-}268}$ or CopN$_{270\text{-}399}$. **(A)** Scc1 & Scc4 interact together with full length CopN (lane 1) but not with CopN$_{85\text{-}268}$ (lane 2) or CopN$_{270\text{-}399}$ (lane 3). His$_6$-Scc1 bound to Nickel NTA beads interacted with both GST-CopN as well as GST-Scc4 in the presence of a high salt wash buffer (500mM NaCl). His$_6$-Scc1 bound to nickel-NTA beads did not interact with GST alone (lane 4), while GST-CdsD interacted with His-CdsQ as the positive control (lane 5). Red arrows indicate locations of GST-Scc4 (~39 kDa) seen in lanes 1, 2 & 3, and GST-CopN (~67 kDa) seen in lane 1 only. **(B)** Purified GST-Scc4 bound to glutathione agarose beads interacted with His$_6$-Scc1 (lane 1) in the presence of a high salt wash buffer (500 mM NaCl). GST-beads alone did not interact with His$_6$-Scc1 alone (lane 2) while GST-CopN was able to interact with His$_6$-Scc3 as the positive control (lane 3). **(C)** Purified GST-Scc4 and purified GST-Scc1 bound to glutathione agarose beads did not interact with His$_6$-CopN in the presence of a high salt wash buffer (500 mM NaCl), lane 1 and 2 respectively. GST-beads alone did not interact with His$_6$-CopN alone (lane 3) while GST-CopN interacted with His$_6$-Scc3 (lane 4) as the positive control. All results were completed in triplicate, and TCA precipitation results were negative. Inputs of all GST- and His$_6$-tagged proteins included.
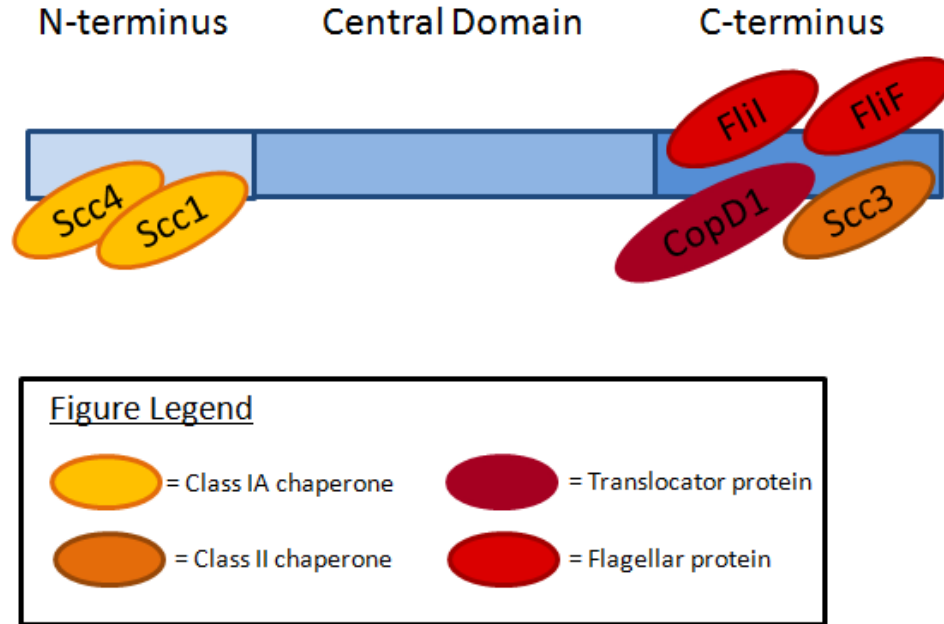
Figure 3.11: Cartoon representation of CopN protein interactions exhibited within the N-terminal, central and C-terminal domains. The heterodimeric chaperone pair of Scc1 and Scc4 interact in the CopN N-terminal domain (1-83 aa). The central domain of CopN (84-268 aa) did not interact with the small subset of proteins tested in this work. FliI and FliF both flagellar proteins, a translocator protein fragment of CopD1$_{158-206,}$ and a class II chaperone Scc3, all interact in the C-terminus of CopN (270-399 aa).

3.6 <u>Determination of CopN Secretion using a HeLa Cell Model</u>

Using an *in vitro* secretion assay, the presence of CopN inside the host cell cytoplasm was probed for, during the course of an entire infection cycle. HeLa cell monolayers were infected with *C. pneumoniae* and allowed to proceed to the time points indicated. Cell lysates were probed using a rabbit-anti-CopN antibody (provided by Dr. Christopher Stone), a mouse-anti-β-actin antibody as a positive control, or a rabbit-anti-CdsD antibody (provided by Dr. Dustin Johnson) as a negative secretion control (Figure 3.12 left panel). CopN was detected in the host cell cytoplasm immediately following infection (0 hours post infection; hpi), and at 66 and 72 hpi. Uninfected HeLa cells, a His$_6$-CopN input and a GST-CdsD input were all used to test for cross-reactivity with all three antibodies (Figure 3.12 right panel). The CopN antibody was also assessed for specificity and to determine the optimal concentration required for detection using immunofluorescent microscopy (Appendix 6.1.3).
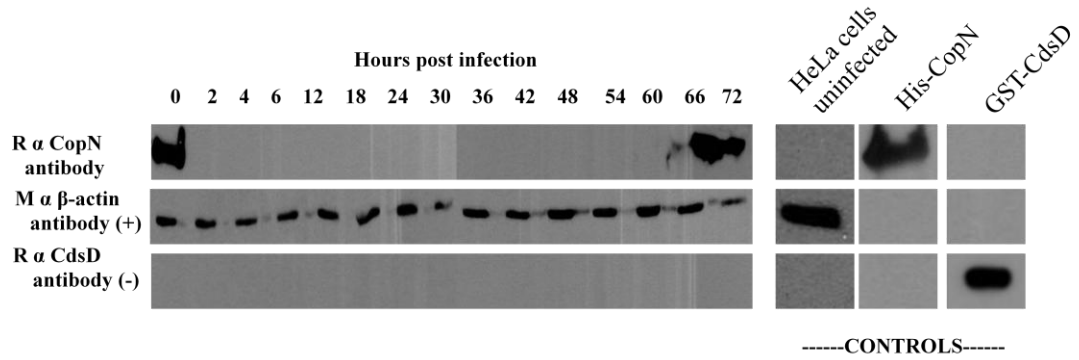
Figure 3.12: Detection of CopN throughout a *Chlamydia pneumoniae* infection cycle of a HeLa cell monolayer. Using a CopN specific antibody, CopN was detected within the host cell at 0 hours post infection, then was absent until 66 and 72 hour post infection. β-actin was detectable at every hour throughout infection, where as CdsD, a protein localized to the bacteria and not secreted into the host cell during infection, was undetectable at all time points. All ultra centrifuged supernatants were subject to trichloroacetic acid precipitation prior to being resuspended in loading dye and run on a 12% SDS polyacrylamide gel.

CHAPTER FOUR

4.0 GENERAL DISCUSSION

4.1 CopN May Be a Multi-domain Hybrid Protein

Proteins which are homologous to YopN and TyeA are found in virtually all Gram-negative bacteria which employ the T3SS for virulence. Many bacteria have a single protein with domains homologous to YopN and TyeA. Examples include SepL from *Escherichia coli*, BopN of *Bordetella*, and MxiC of *Shigella* (Silva-Herzog et al., 2011; Pallen et al., 2005). The length of CopN suggests that it is a hybrid protein, as it is approximately 100 residues longer than YopN. With the 92 residues of TyeA included, the size becomes similar. The sequence similarity between CopN to YopN is approximately 50%, while when compared to TyeA is 32% (Hsia et al., 1997). A more interesting finding is that the region of CopN which shows the greatest homology to TyeA is contained within the second half of the protein, and similarity increases towards C-terminal 399 amino acids (Figure 3.1).

Previously, a natural hybrid protein was discovered in *Yersinia* consisting of YopN and TyeA fused together (Ferracci et al., 2004). Since the 3' end of *yopN* overlaps the 5' end of *tyeA*, the resulting fusion protein after translation was speculated to be caused by a +1 translational frameshift, at a highly frameshift prone sequence motif (Ferracci et al., 2004). The functional protein consisted of the first 278 residues of YopN and residues 1-92 of TyeA. When the crystal structure of YopN and TyeA was solved, the C-terminal region of YopN was found to be in close proximity to the N-terminus of TyeA (Figure 3.2) (Schubot et al., 2005). Therefore the protein conformations would not have

to change drastically to accommodate the new hybrid protein. These observations along with the predicted secondary structure using 3D-JigSaw suggest that CopN may be a multi-domain hybrid protein.

4.2 Proteolytic Susceptibility of CopN Domains

As a first step in the biochemical characterization of CopN from *C. pneumoniae*, the domain structure of CopN was mapped using limited proteolysis. This technique is able to rapidly degrade unstructured regions within proteins and therefore elucidate possible regional boundaries (Archuleta et al., 2011). Digestions of Trypsin and α-Chymotrypsin yielded a similar fragment size results from cleavage at residue 84 of CopN. The N-terminal region of proteins is often observed to contain a binding site for the chaperone proteins (Sory et al., 1995; Galán & Wolf-Watz, 2006). When chaperones are not present, this N-terminal region tends to be unstructured. Since this region of CopN was highly susceptible to proteolytic degradation, it is likely that the first domain of CopN is contained within residues 1-83. Our search for the predicted second domain boundary was unsuccessful using this technique, however published data corroborating the first domain by Archuleta *et al.* (2011), also predicted a domain boundary at residue 268. Subsequently the predicted domain boundaries for CopN are an N-terminus consisting of residues 1-83, a protease resistant central domain located between residues 85-268 ($CopN_{85-268}$), and residues 270-399 ($CopN_{270-399}$) which comprise another protease resistant domain in the C-terminus (Archuleta et al., 2011). To elucidate the binding sites

of these domains, protein-protein interactions within each of the three regions of CopN were assessed.

4.3 The Specific Chlamydial Chaperones of CopN

Specific T3SS chaperones bind within the N-terminal region of many effectors (Sory et al., 1995). This chaperone binding domain (CBD) is downstream of the postulated N-terminal secretion signal, and is approximately 50-100 amino acids in length (Galán & Wolf-Watz, 2006). This region is not normally characterized by a conserved sequence motif within bacterial secretion systems, nor between bacterial species. Recently, the chemical nature of the N-terminal residues has been postulated to be potentially useful in predicting what substrates are secreted through the T3SS (Costa et al., 2012). An example is a high serine content in the first 50 residues of *Pseudomonas syringae* effectors (Collmer et al., 2002).

Like other effectors, injectisome plug proteins bind their chaperones in the N-terminal domain (Sory et al., 1995; Schubot et al., 2005; Ehrbar et al., 2006). Silva-Herzog *et al.* (2011) predicted Scc1 and Scc4 as the specific chaperones of CopN, based on genome location and GST pull-downs. The His_6 pull-downs I preformed using Scc1, Scc4, and CopN validate these results. Both putative chaperones yielded low amounts of soluble His_6-tagged protein, and were of a similar size. Scc1 expressed better than Scc4 and was chosen as the bait protein for the His_6 pull-down. Scc1 interacted with both Scc4 and full length CopN when incubated together, but not $CopN_{85-268}$ nor $CopN_{270-399}$ (Figure

3.10). It would have been ideal to assess the interaction directly within the first 83 residues of CopN, however the fragment of $CopN_{1-83}$ was insoluble and therefore this was not possible. Since the interaction of the chaperones with CopN occurred with full length protein, but not with truncated versions nor with $CopN_{\Delta 83N}$ (data not shown), the binding domain was localized to the N-terminal region. This interaction within the N-terminus supports the hypothesis that Scc1 and Scc4 are indeed the heterodimeric chaperone pair of CopN, and corroborates the results of Silva-Herzog *et al.* (2011).

4.4 <u>Interaction of Flagellar and Type III Secretion Proteins with CopN</u>

It is speculated that an elongated protein shape could provide a protein with an increased surface area to aid in the binding of one large protein, or in the binding of multiple smaller ones (Deane et al., 2008). The predicted structure of CopN was similar to that of other plug proteins, and like MxiC of *Shigella*, appears to have an elongated shape. Previous studies within our lab have shown interaction between CopN and CdsN, FliI, CdsU, Scc3, and CdsQ (Stone et al., 2008; Toor et al., 2012; Mahony, unpublished data). These observations led to the hypothesis that CopN is a highly promiscuous protein, possibly using the large amount of predicted surface area to bind many proteins within the bacteria prior to its secretion. Continuing to determine novel protein interactions may help to elucidate a model for the regulation of T3S systems in not only *C. pneumoniae* but also other Chlamydial species. Three novel protein interactions with CopN have been identified, namely $CopD1_{158-206}$, IncC and FliF, as well as two other previously identified interactions, FliI and Scc3 (Figure 3.11).

4.4.1 CopN and CopD: Interaction with the translocator protein

The plug protein of bacterial T3S systems has not been shown to interact with translocator proteins. In *Yersina* however TyeA, the small molecule that binds the C-terminal region of YopN, has been shown to interact with one of the translocators, YopD (Iriarte et al., 1998). TyeA bound to YopN is tethered to YopD at the base of the injectisome, and has been shown to be critical in the translocation of Yops. This 3-way interaction might also have a role in the release of chaperones from their effectors (Iriarte et al., 1998). Since TyeA has significant similarity to the C-terminal region of CopN, it was interesting to find this interaction between CopN and CopD1. Transmembrane proteins tend to be insoluble, therefore fragments of each translocator were made by David Bulir (PhD candidate, Mahony lab), which spanned regions of the protein predicted to be surface exposed. $CopB1_{1-255}$, $CopD1_{1-137}$, $CopD1_{158-206}$, and $CopD1_{227-444}$ yielded soluble protein and were used to examine potential interactions with CopN. Using a 96-well indirect ELISA to screen potential interactions, fragment $CopD1_{158-206}$ interacted with full length CopN, confirmed by GST pull-down. The interaction was subsequently localized by GST pull-down to the C-terminal region. If this interaction is similar to that of TyeA and YopD in *Yersinia* then it is hypothesized that CopD1 may tether CopN at the base of the injectisome prior to its secretion. It is speculated that a conformational change in CopN might allow for the release and secretion of CopD1 through the injectisome, prior to the secretion of the plug protein. The specific nature of the plug and translocator interaction needs to be further investigated.

4.4.2 CopN, FliI and FliF: Flagellar interactions

Flagellar proteins have previously been shown in *Chlamydia* to interact with other components of the T3SS (Stone et al., 2010). FliI which is paralogous to CdsN the ATPase of the T3SS, was shown to interact with CopN as well as CdsL, the ATPase regulator and tethering protein (Stone et al., 2010; Stone et al., 2011). Since FliI was previously determined to interact with full length CopN, I concentrated on localizing the interaction by using the fragments of CopN to determine the binding region. Both $CopN_{\Delta 83N}$ and $CopN_{270-399}$ interact with FliI, whereas $CopN_{85-268}$ did not. FliI is the flagellar ATPase and located within the bacterial cyotsol near the base of the injectisome. Its location at the base of the injectisome helps facilitate its role in chaperone release for T3S effectors, which are then unfolded and secreted through the needle (Stone et al., 2008). CopN is also shown to be associated with the base of the apparatus, and hypothesized to physically block the injectisome, which suggests that FliI, CdsN, or both may be important for chaperone/effector complex release for CopN.

The interaction of CopN with FliF, a CdsJ paralog, was an intriguing finding, as CdsJ homologs in other bacteria have not been shown to interact with the plug protein. Through GST pull-downs, it was determined that both CopN and $CopN_{\Delta 83N}$ interact with FliF. The localization of the binding site on CopN was determined to be within the C-terminal domain of CopN, residues 270-399. As an inner membrane component, FliF of the flagellar export system forms a scaffold for the other membrane components to assemble upon. CopN has been shown to be localized to the base of the injectisome and is likely within close proximity to FliF at the base of the apparatus. The interaction with

CopN suggests a different role for FliF compared to that of CdsJ which has not been shown to interact with CopN. At the base of the injectisome, CopN might associate with FliF during the RB phase of Chlamydial development during host cell reprogramming, associated with the inclusion membrane.

Since *Chlamydia* do not contain flagella, it is possible that flagellar and T3S proteins could assemble into a hybrid apparatus. The presence of FliI, FliI, FlhA and FliH in *Chlamydia* is not sufficient to form a functionally intact flagellar apparatus, thus it is possible that these proteins could form a fundamental base for a T3S apparatus (Kubori et al., 1992). A hybrid apparatus could explain the variation of interactions displayed by CopN, as both CdsN and FliI interact with the plug protein, but only FliF and not CdsJ have been shown to interact with CopN. The mismatched apparatus may have different energy requirements or perhaps slight structural differences compared to a regular T3S apparatus, which could account for these anomalies. Another possibility is that the remaining flagellar genes within *Chlamydia* are currently unannotated, and with the advancement of technology an entire flagellar repertoire could be uncovered (Stone et al., 2010).

4.4.3 CopN and IncC: An inclusion membrane interaction

As an inclusion membrane protein, IncC of *C. pneumoniae* is secreted early during infection and inserted into the membrane, where it modifies the inclusion surface (Fields et al., 2003). Both IncC and IncB in *C. trachomatis* have been shown to play a

role in upregulation of cytokine expression; however inclusion membrane proteins have a wide array of functions within the cell, many which are still cryptic (Kim et al., 2011). Interaction between inclusion membrane proteins and bacterial plug proteins has not been identified in literature. CopN was shown by GST pull-down assay to interact with IncC, but CopN did not interact with a second inclusion membrane protein, IncB. The interaction between CopN and at least one of the inclusion membrane proteins was interesting, though previously it was shown using indirect immunofluorescence that CopN was localized to both the bacteria and the inclusion membrane early in infection (Fields & Hackstadt, 2000). The interaction of CopN with IncC as opposed to IncB requires further investigation as little is known of why these proteins would interact within the host. The fragments of CopN previously described were to be probed for interaction with IncC, however the IncC construct's expression levels became too low for use in GST pull-down assays. This issue was overcome late in this work, but the IncC binding site on CopN was not identifiable.

4.4.4 CopN and Scc3: An old interaction revisited

Scc3 to date is one of the best characterized Chlamydial chaperones (Betts-Hampikian & Fields, 2010; Fields et al., 2005). Its homology to LcrH (also known as SycD) of *Yersinia* was determined soon after its discovery and the interaction of Scc3 with CopN was shown by pull-down assay (Slepenkin et al., 2005; Spaeth et al. 2009; Silva-Herzog et al., 2011). As a class II chaperone shown to be associated with the Chlamydial translocator proteins CopB1 and CopB2, it is interesting that Scc3 interacts

with CopN as it is not predicted to be a translocator protein. Another unusual feature

regarding the interaction is the domain in which the interaction occurs. In this work the

binding domain of Scc3 on CopN was localized to the C-terminal 129 residues of CopN,

using GST pull-down assays. As discussed in Section 4.3 chaperones typically bind the

N-terminal domain, as is the case for CopN's heterodimeric chaperone pair Scc1 and

Scc4. An interaction within the C-terminal region suggests a different role for Scc3. As

proposed by Silva-Herzog *et al*. (2011), Scc3 could be an inhibitor of CopN secretion. In

light of this data it is then suggested that the binding of Scc3 to CopN might promote the

down regulation function of the TyeA-like domain found in the C-terminus of CopN.

Further investigation into this regulation of CopN is warranted.

4.5 CopN Secretion in a HeLa Cell Model

CopN is contained within a Chlamydial open reading frame (ORF) first

recognized by Hsia *et al.* (1997). This gene cluster includes other T3S substrates flanking

the YopN orthologue such as Cds1, Cds2, CopN, and Scc1. Fields and Hackstadt (2000)

showed that CopN of *C. trachomatis* was secreted by *Chlamydia* and *Yersinia*

enterocolitica T3SS. Although CopN is transcribed during late-cycle, it is apparent that

CopN is present much earlier in the infection cycle based on specific RNA production

(Fields & Hackstadt, 2000), supporting the idea that a preassembled T3S apparatus is

formed prior to host cell contact. Due to the limitations of the Chlamydial genetic

system, little data is available on the secretion of type III specific substrates and even less

data is available on the secretion of CopN in *Chlamydia pneumoniae*. Making use of a

CopN specific antibody, I monitored the secretion of CopN every 6 hours throughout the

C. pneumoniae developmental cycle, using a secretion assay in a HeLa cell model.

I hypothesized that CopN of *C. pneumoniae* would be detected in the host cell

cytoplasm early on in the infection cycle secreted from EBs, then once again at mid-cycle

during the conversion of EBs to RBs. CopN was detected at the beginning of the cycle

immediately after infection (0 hpi), but was then absent until late cycle when it

reappeared in the host cell cytoplasm at 66 and 72 hpi (Figure 3.12). In early cycle as an

infectious EB attaches to a host cell, translocator proteins are secreted and inserted into

the host cell membrane allowing the apparatus to dock to the host. After insertion of the

T3S apparatus, more effector proteins will be secreted, including CopN, which will

manipulate intracellular processes to favor the internalization of the bacteria. The

detection of CopN early in the infection cycle is consistent with the hypothesis that CopN

is secreted from EBs. The appearance of CopN very late in the cycle is likely the product

of early Chlamydial lysis from a host cell. As infectious EBs are released from the host, a

new developmental cycle begins in HeLa cells which were previously uninfected.

Detection of CopN within the host cell was not seen during mid-cycle, indicating that

CopN may not be secreted into the host cell cytoplasm by RBs or is rapidly degraded or

bound to a host cell structure, such that it cannot be detected within the cytoplasm at these

later time points. Archuleta *et al.* (2011) showed that CopN inhibits the polymerization

of microtubules by binding directly to αβ-tubulin dimers which in turn inhibits cell cycle

progression. Once secreted into the eukaryotic cell, CopN may be rapidly degraded

through host cell mechanisms, and is not detected at mid-cycle time points. Little

research regarding the interaction of CopN on αβ-tubulin in eukaryotic cell models exists,

and further investigation is required.

4.6 <u>Summary</u>

4.6.1 CopN C-terminal interactions

The localization of CopD1$_{158-206}$, FliI, FliF and Scc3 to the 129 aa C-terminal

region of CopN is both novel and exciting. In order to support such a large array of

interactions, the protein binding domains must either overlap, or the interactions exhibited

are largely transient. If each protein is to be bound to CopN during one time period, each

protein may bind to a relatively small patch of residues on CopN without much overlap.

If the interactions displayed inside the bacteria are transient in nature and all proteins rely

on the same binding site, then perhaps CopN displays a hierarchy in which it binds certain

proteins preferentially over others. It seems unlikely that each protein has a designated

binding site within this small domain, and more likely that these interactions occur in a

transient manner. Taken together these results provide evidence for a functional domain

of the plug protein, CopN$_{270-399}$, which is able to associate with a large subset of proteins

including structural substrates, effectors and flagellar proteins.

4.6.2 CopN is secreted from EBs but not RBs

The data from the HeLa cell infection experiments show that CopN is secreted from EBs early on during the developmental cycle. If CopN was present at mid-cycle, it would provide evidence for CopN secretion by RBs; however this was not the case. This data is in contrast to that published by Fields & Hackstadt (2000) which showed that CopN was present in EBs, RBs, and whole-culture extracts at 20 hours post infection in *C. trachomatis*. The difference in these findings could be due to the species specific roles exhibited by CopN from *C. pneumoniae* and *C. trachomatis*. Another reason could be that RB associated CopN is not secreted into the host cell but rather interacting with the basal body of the apparatus and remains in the bacterial cytosol.

Huang *et al*. (2008) and Archuleta *et al*. (2011) showed that CopN was associated with cell cycle arrest. Huang *et al*. (2008) also showed that G2/M cell arrest occurred in mammalian cells 12 hours after transfection with CopN. At mid-cycle, the host cell would have already undergone cell cycle arrest, and it would be redundant for the bacteria to re-secrete CopN back into the host cell cytoplasm. Therefore CopN is only secreted into the host shortly after infection and not during mid-to-late cycle. This might not be the case for other Chlamydial CopN proteins, but suggests this role for CopN of *C. pneumoniae*.

4.6.3 Limitations in this study

Since the majority of this work was performed *in vitro* it is possible that the

protein interactions exhibited are artifacts of the artificial environment in which we

express and test these proteins (Mackay et al., 2006). False positive interactions can arise

if proteins are in conformations that are not physiologically relevant, or if these

experiments are carried out with proteins that are over expressed and incorrectly folded

(Mackay et al., 2006). False negative interactions are also a problem as the interaction of

two proteins might require other proteins to facilitate these interactions. The use of

strong salt solutions eliminates spurious interactions, and it is accepted in literature that

fusion proteins are in their native conformations when expressed on His_6 or GST-tags (Li,

2010). Corroborating *in vivo* data would be ideal for all the protein interactions presented

in this work. Co-immunoprecipitation experiments were attempted during the course of

this work, but mass spectrometry data returned no useful protein associations (data not

shown). As there is no genetic system for *Chlamydia*, the true role of each protein

examined in this work is based on observational data of protein-protein interactions, as

well as similarities within homologous T3SS.

4.6.4 CopN of *Chlamydia pneumoniae* and its function in the T3SS

The sequence homology to YopN of *Yersinia*, protein-protein interactions similar

to those of YopN and TyeA, as well as CopN's secretion by the T3SS from *C.*

pneumoniae into HeLa cells, lends support for the role of CopN from *Chlamydia*

pneumoniae as the plug protein within the T3SS. It was also shown that Scc1 and Scc4, the specific CopN chaperones, bind within the N-terminal region, while FliI, FliF, CopD1$_{158-206}$ and Scc3 bind to the C-terminal region of CopN.

The CopN N-terminal region consisting of residues 1-84, likely contains a secretion signal which allows for the T3SS of *Chlamydia pneumoniae* to both recognize and facilitate the secretion of CopN through the injectisome. This region also contains a chaperone binding domain in where Scc1 and Scc4 bind cooperatively to CopN. The heterodimeric chaperone pair provide stability for the N-terminus of CopN and may aid in keeping CopN in a partially unfolded state prior to secretion. The chaperones may also play a role in transporting CopN to the base of the injectisome, while it acts as a physical plug to prevent premature secretion of effector proteins.

Residues 270-399 of CopN interact with several proteins including two flagellar proteins, FliI and FliF, a translocator protein, CopD1, and a class II chaperone Scc3. Since these interactions were probed for in an artificial environment, it is hard discern the timing of each interaction as well as the duration of time each protein is associated with CopN. If these interactions do occur within the bacteria during the course of an infection, CopN might undergo a number of conformational changes when different proteins bind the C-terminal region. This might be important for CopN to delineate what effectors are to be secreted first; or perhaps CopN interacts with many proteins due to a secondary structure feature that is commonly recognized by T3S proteins. These suggestions are based on little data, and the C-terminus of CopN deserves further investigation to properly identify its function.

The role of the central domain of CopN comprised of residues 85-268 is currently a mystery. A possible role for this domain includes the physical blocking of the needle channel, inhibiting the premature secretion of effector proteins. However this is highly unlikely since no individual proteins (of the subset of known interactions tested within this work) appeared to bind specifically in this region. Once CopN is inside the host cell, it has been shown to bind to $\alpha\beta$-tubulin and cause cell cycle arrest (Archuleta et al., 2011; Huang et al., 2008). This domain might in fact be associated with the role of CopN as an effector protein within the eukaryotic cell. Since the association of CopN with the host cell has been largely unexplored, this avenue is worth pursuing.

4.7 <u>Future Directions</u>

When working with a particular system for a relatively short period of time, there are always avenues unveiled which are of interest, but for time's sake are not pursued. This section provides insight into a few potential directions for CopN research.

4.7.1 X-ray crystallography of CopN to determine the secondary structure

During the course of this work, x-ray crystallography of CopN was attempted but was unsuccessful. The failure to obtain protein crystals may be due to the disordered N-terminal region of the full length protein in the absence of the CopN chaperones. Although truncated versions of CopN were also tested, including $CopN_{\Delta 40N}$, $CopN_{\Delta 83N}$,

and CopN$_{83-388}$, any crystals obtained provided low resolution data. The macromolecular complex of YopN/SycN/YscB/TyeA was successfully crystallized (Schubot et al., 2005), as well as MxiC residues 71-355 (Deane et al. 2008). With the heterodimeric chaperone pair discovered by Silva-Herzog *et al.* (2011), co-crystallography between CopN and Scc1/Scc4 could be attempted. Along with being the only plug protein shown to have anti-host cell function, a solved structure of CopN/Scc1/Scc4 could lead to the elucidation of domain functionality which could be relevant in the context of other T3S plug proteins.

4.7.2 A genetic system in *C. trachomatis* to study the role of CopN

Recently Wang *et al*. (2011) provided the first glimpse of a potential genetic system for *Chlamydia*. The insertion of a plasmid into a plasmid-less strain of *C. trachomatis* could allow for various gene deletions, the effects of which could then be studied within a eukaryotic cell infection. This is the first report of a Chlamydial transformation system and could provide a means to study the function of T3S proteins in the EB and RB stages of infection. A Chlamydial transformation system could permit the elucidation of the role of individual CopN domains by the creation of domain knockouts.

4.8 Closing remarks

Working with *Chlamydia* is not an easy task. It forces you to think outside of the box as the majority of techniques used to study other bacterial T3SS cannot be used in

this pathogen. The use of traditional *in vitro* protein-protein interaction techniques, as well as a secretion assay were combined to characterize CopN the plug protein of the *Chlamydia pneumoniae* T3SS. CopN was characterized as whole protein as well as on an individual domain specific level. It was shown that CopN is secreted into the host cell rapidly after infection, implying a role for CopN in the host cell during the EB stage of infection, but not during the later RB stage when CopN was not detectable. As new techniques are created to study Chlamydial biology, the fundamental questions regarding its type III secretion system can approached and hopefully answered.

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5.0 REFERENCES

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APPENDICES

6.0 APPENDICES

6.1 Supplementary Figures

[1-316] mass = 35015.2

Small polar:	D(25)	E(19)	N(13)	Q(20)
Large polar:	K(18)	R(14)	H(3)	
Small non-polar:	S(30)	T(20)	A(27)	G(15)
Large non-polar:	L(35)	I(15)	V(17)	M(6) F(10) Y(12) W(1)
Special:	C(1)	P(15)		

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  1  K S E S T E E K P D T D L A D K Y A S G N S E I S G Q E L R   30
 31  G L R D A I G D D A S P E D I L A L V Q E K I K D P A L Q S   60
 61  T A L D Y L V Q T T P P S Q G K L K E A L I Q A R N T H T E   90
 91  Q F G R T A I G A K N I L F A S Q E Y A D Q L N V S P S G L  120
121  R S L Y L E V T G D T H T C D Q L L S M L Q D R Y T Y Q D M  150
151  A I V S S F L M K G M A T E L K R Q G P Y V P S A Q L Q V L  180
181  M T E T R N L Q A V L T S Y D Y F E S R V P I L L D S L K A  210
211  E G I Q T P S D L N F V K V A E S Y H K I I N D K F P T A S  240
241  K V E R E V R N L I G D D V D S V T G V L N L F F S A L R Q  270
271  T S S R L F S S A D K R Q Q L G A M I A N A L D A V N I N N  300
301  E D Y P K A S D F P K P Y P W S                             316
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Figure 6.1.1: Mass spectrometry sequencing results of CopN from the Trypsin digestion (CopN$_{\Delta 83N}$). Purified full length CopN from *Chlamydia pneumonia* was digested for 1 hour in Trypsin with 10 mM HEPES, pH 7.5 and 500 mM NaCl buffer, at a final concentration of 0.1 µg of protease, using the JBS Floppy Choppy in situ proteolysis kit. The reaction was quenched with 1X SDS-PAGE loading buffer and ran on an 11% SDS-PAGE gel, with the fragment excised and sent for mass spectrometry. Shown is the CopN sequence from 84 aa to 399 aa; known henceforth as CopN$_{\Delta 83N}$.

[1-282] mass = 31117.9

Small polar:	D(21)	E(18)	N(13)	Q(18)	
Large polar:	K(17)	R(13)	H(2)		
Small non-polar:	S(26)	T(17)	A(26)	G(14)	
Large non-polar:	L(30)	I(15)	V(16)	M(5)	F(9)	Y(10)	W(0)
Special:	C(0)	P(12)			

```
  1  K S E S T E E K P D T D L A D K Y A S G N S E I S G Q E L R  30
 31  G L R D A I G D D A S P E D I L A L V Q E K I K D P A L Q S  60
 61  T A L D Y L V Q T T P P S Q G K L K E A L I Q A R N T H T E  90
 91  Q F G R T A I G A K N I L F A S Q E Y A D Q L N V S P S G L  120
121  R Y T Y Q D M A I V S S F L M K G M A T E L K R Q G P Y V P  150
151  S A Q L Q V L M T E T R N L Q A V L T S Y D Y F E S R V P I  180
181  L L D S L K A E G I Q T P S D L N F V K V A E S Y H K I I N  210
211  D K F P T A S K V E R E V R N L I G D D V D S V T G V L N L  240
241  F F S A L R Q T S S R L F S S A D K R Q Q L G A M I A N A L  270
271  D A V N I N N E D Y P K                                      282
```
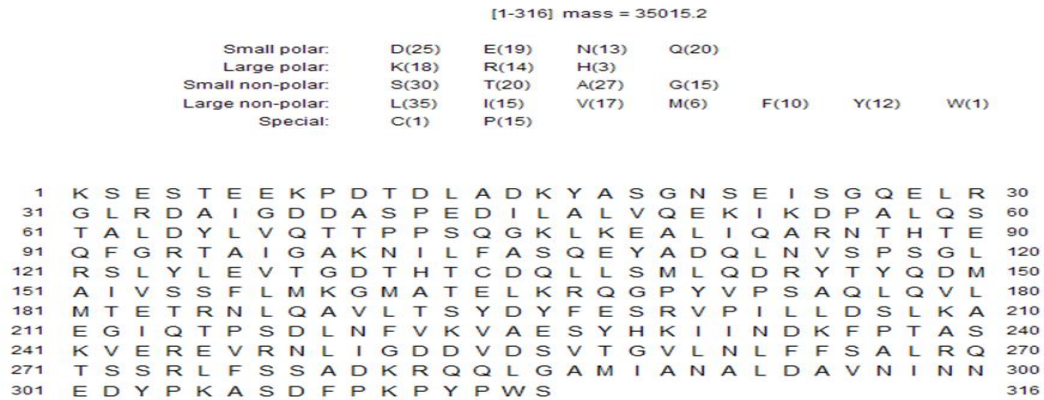
Figure 6.1.2: Mass spectrometry sequencing results of CopN from the α-Chymotrypsin digestion (CopN_{84-388}). Purified full length CopN from *Chlamydia pneumonia* was digested for 1 hour with α-Chymotrypsin with 10 mM HEPES, pH 7.5 and 500 mM NaCl buffer, at a final concentration of 0.1 µg of protease, using the JBS Floppy Choppy in situ proteolysis kit. The reaction was quenched with 1X SDS-PAGE loading buffer and ran on an 11% SDS-PAGE gel, with the fragment excised and sent for mass spectrometry. Shown is the CopN sequence from 83 aa to 388 aa; known henceforth as CopN_{84-388}.
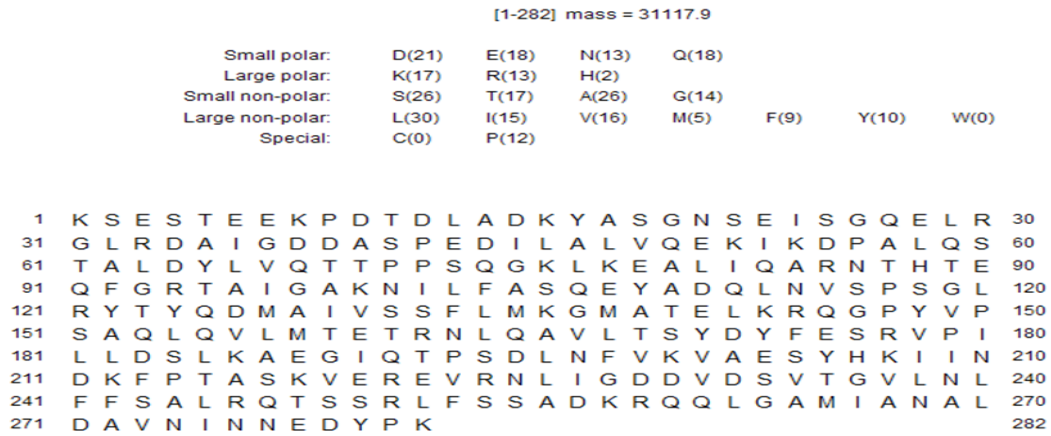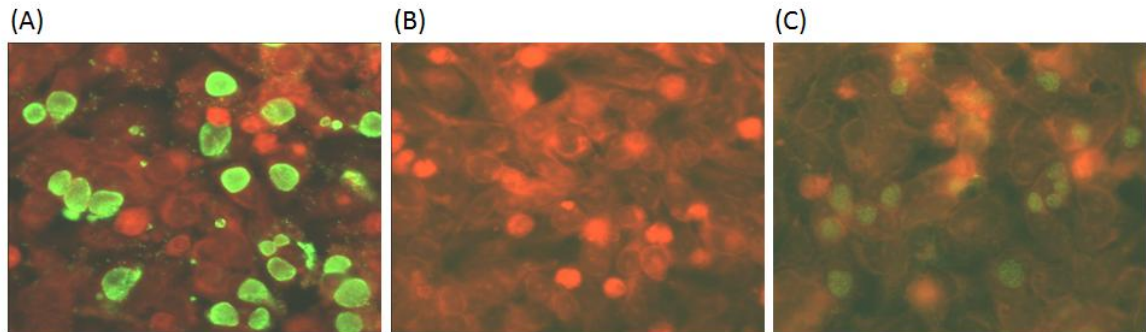
Figure 6.1.3: Fluorescent microscopy controls. Red indicates eukaryotic cells, while the green colors indicate *C. pneumoniae* **(A)** HeLa cells infected with Chlamydia pneumoniae at 72 hours post infection (hpi), detected using the Pathfinder Chlamydia culture confirmation kit, to test for presence of Chlamydial infection. **(B)** Rabbit-anti-CopN antibody at 1:25 dilution on an uninfected HeLa cell monolayer, to test antibody specificity. **(C)** Rabbit-anti-CopN antibody at 1:25 dilution, showing antibody localized to inclusions only.

6.2 <u>Oligonucleotide Primers</u>

Table 6.1: List of oligonucleotide primers used in this thesis

Primer Name	F / R	Oligonucleotide Primer Sequence (5'-3')
CopNFFL	F	ATG GCA GCA TCA GGA GGC ACA
CopNRFL	R	TTA TGA CCA AGG ATA GGG TTT AG
CopNF83N	F	ATG AAA TCT GAA TCT ACA GAA GAG A
CopNR83N	R	TTA TTT CTC AGC CTT TCC AGC
CopNR11C	R	TTA TTT GGG ATA ATC TTC ATT GTT TA
Scc4FFL	F	ATG TTG GAA AAA TTA ATA AA AAT T
Scc4RFL	R	TTA CTG TTT ACC TAG GCC AA
Scc1FFL	F	ATG CAA AAC CAA TAC GAG CA
Scc1RFL	R	TTA CGC GAC GTA GTA GAT TCC
CopN85FF	F	ATG GAA TCT ACA GAA GAG AAG CC
CopN268RR	R	TTA AGT TTC TGT CAT GAG AAC TTG
CopN270 FF	F	ATG GCA GTT CTT ACC TCG TAC G

All primers contained a Gateway sequence at the 5' end of both forward and reverse sequences, as follows unless otherwise stated:

Gateway Forward Sequence (+)

5' – GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT AGA TTA CGA TAT CCC AAC GAC CGA AAA CCT GTA TTT CA GGG C

Gateway Reverse Sequence (-)

5' GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC