

NOVEL EFFECTOR PROTEIN AND TRANSCRIPTIONAL REGULATION OF
THE TYPE THREE SECRETION SYSTEM IN *CHLAMYDIA TRACHOMATIS*

By

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SUMMARY:

Chlamydia trachomatis is an obligate intracellular bacterial pathogen with a significant public health impact. A unifying characteristic of *Chlamydia* is the biphasic developmental cycle that is intimately linked to pathogenesis. Due to its intracellular lifestyle within a membrane bound vacuole, *Chlamydia* requires a mechanism to interact with and manipulate the host. To facilitate this interaction, *C. trachomatis* encodes a Type III Secretion System (T3SS) that is likely requisite for chlamydial growth and integral to the developmental cycle. Additionally, little is known about the mechanisms that regulate the developmental cycle, and the T3SS. This thesis is focused on a translocated T3SS effector protein that may serve in manipulation of the host, and the role of a transcriptional factor involved in regulating T3SS expression.

T3SSs are virulence determinants in many Gram-negative bacteria and responsible for numerous infectious processes. T3SSs are intricate bacterial machinery that carefully regulate secretion of bacterial proteins into the cytosol of eukaryotic host cells. Many of the T3SS genes in *C. trachomatis* are upregulated during the RB to EB conversion of the developmental cycle. A protein of unknown function, CT667, is transcriptionally-linked to known chlamydial T3SS homologues, suggesting that it too plays a role in type three secretion (T3S). Analysis of the protein sequence of CT667 revealed numerous tetratricopeptide repeat (TPR) domains. TPR domains are generally important for protein-protein interactions, and proteins containing these domains have diverse cellular roles such as cell-cycle

control, signal transduction, and protein stability. We hypothesize that CT667 is a T3SS secreted effector protein. Due to the current inability for genetic exchange in *C. trachomatis*, *Shigella flexneri* was initially used as a surrogate system to test our hypothesis. We demonstrate that not only is CT667 secreted in a type three dependent manner by *S. flexneri*, but is actually translocated in a type three dependent manner into eukaryotic host cells. After generating antibodies against CT667, the protein was also specifically detected in the cytosol of host cells during a *S. flexneri* infection. Expression profiling during a *C. trachomatis* infection revealed that CT667 transcripts are increased from 18 to 24 hpi and protein expression increases from 24 to 36 hpi. Using the antibodies generated against CT667, the protein could be detected in chlamydial inclusions at 36 hpi and could be visualized in the cytosol of host cells. This work suggests that CT667 may have effector function in the host cell and thus play a critical role in pathogenesis.

Chlamydia are propagated through a bi-phasic developmental cycle that is intimately linked to pathogenesis. Little is known about the mechanisms that control the developmental cycle; however, data suggest that regulation occurs at the transcriptional level. Very few known transcriptional regulators are encoded within *C. trachomatis*. An atypical response regulator, ChxR, has been demonstrated to activate transcription and is also upregulated during the RB to EB conversion of the *C. trachomatis* developmental cycle. We hypothesize that ChxR is an activator of multiple genes during this phase of the developmental cycle. ChxR levels were determined to increase from 24 to 36 hpi. Following formaldehyde crosslinking and

in vivo immunoprecipitation of ChxR, DNA specifically bound to ChxR was isolated. Specific gene targets of ChxR were hypothesized based on expression pattern during the developmental cycle as well as the presence of a conserved *cis*-acting motif recognized by ChxR. Promoter regions of many of these genes were successfully amplified from the DNA specifically bound to ChxR, suggesting regulation by the response regulator. The protein products of many of these genes are involved in T3SS and other integral processes. Regulation of these genes by ChxR further lends support that it functions as a transcriptional activator and plays a vital role in *C. trachomatis* pathogenesis. In the absence of a system for genetic exchange, this work demonstrates a novel *in vivo* approach to studying a transcriptional regulator in *Chlamydia*.

This work is dedicated
to my father for his
unconditional support
and love

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TABLE OF CONTENTS

I. <i>Chlamydia</i> Introduction	
History.....	12
Pathogenesis of <i>Chlamydia</i>	12
Developmental Cycle.....	14
<i>Chlamydia</i> Type III Secretion System.....	16
II. Type Three Dependent Translocation of a Putative Chlamydial Effector Protein, CT667.	
INTRODUCTION	22
MATERIALS AND METHODS	29
Construction of <i>S. flexneri</i> expression vectors and bacterial strains.....	29
SDS-PAGE and western blot analyses.....	30
Type Three Secretion Cloning Primers (Table 1).....	31
Preparation of electrocompetent <i>S. flexneri</i>	32
Transformation of CT667 on pHSI into <i>E. coli</i>	33
Transformation of CT667 on pHSI into <i>S. flexneri</i>	34
Chlamydial infection and EB seed Prep.....	34
RB harvest and CT667 protein expression profile.....	35
TCA precipitation.....	36
Congo Red induction.....	36
ReAsH labeling and technology.....	37
<i>S. flexneri</i> invasion assay.....	39

<i>S. flexneri</i> immunofluorescence assay.....	39
<i>C. trachomatis</i> immunofluorescence assay.....	40
RESULTS	40
CT667 has 3 TPR domains.....	41
CT667 is highly conserved among chlamydial species.....	44
Congo red induces CT667 translocation through <i>S. flexneri</i> T3SS.....	49
CT667 is translocated into Henle cells	52
Biological observations in intensity differences are significant	55
CT667 is detected in L929 cells through <i>S. flexneri</i> IFA.....	58
CT667 protein expression is upregulated at 24-36 hpi.....	61
CT667 is detected in inclusions at 36 hpi.....	64
DISCUSSION	69
III. <i>In vivo</i> analysis of the DNA binding activity of ChxR: a transcriptional regulator in <i>C. trachomatis</i> .	
INTRODUCTION	74
MATERIALS AND METHODS	79
Chlamydial Infection and EB seed Prep.....	79
RB harvest and ChxR protein expression profile.....	80
<i>In vivo</i> Immunoprecipitation of ChxR.....	80
<i>In vivo</i> crosslinking and Immunoprecipitation of ChxR.....	82
Primers for T3SS operon promoters (Table 2).....	84
Primers for constitutively expressed, midlate and late genes (Table 3).....	85

RESULTS	86
Protein Expression Profiling of ChxR.....	86
<i>In vivo</i> immunoprecipitation of ChxR.....	87
Immunoprecipitation and crosslinking can be used to isolate DNA.....	88
ChxR binding motif contains conserved recognition sequences.....	92
ChxR binds to the promoter regions of T3S operons.....	98
ChxR binds promoter regions of late and midlate genes.....	102
DISCUSSION	106
Bibliography.....	110

CHLAMYDIA INTRODUCTION

History

Chlamydiae are ancient obligate intracellular bacteria that have adapted unique mechanisms to invade eukaryotic host cells. *Chlamydiae* are highly evolved and comprise their own order, *Chlamydiales*. rRNA evidence suggests that chlamydiae diverged evolutionarily from other bacteria over two billion years ago (Horn, Collingro et al. 2004). The order Chlamydiales encompasses a large group of bacteria characterized by their obligate intracellular growth in eukaryotic cells as well as their unique biphasic developmental cycle (Stephens 2003). Over 700 million years ago, *Chlamydia* adopted its unique intracellular lifestyle as well as many of the virulence factors necessary for pathogenicity (Horn, Collingro et al. 2004).

Pathogenesis of *Chlamydia*

Within *Chlamydiaceae* there are 3 species currently demonstrated to have a significant impact on public health: *Chlamydia psittaci*, *Chlamydia pneumoniae*, and *Chlamydia trachomatis* (Belland, Ojcius et al. 2004). Infection with *C. pneumoniae* is one of the most common causes of atypical pneumonia. *C. pneumoniae* has also been implicated in coronary heart disease through its association of atherosclerotic plaque formation (Stassen, Vainas et al. 2008). *C. pneumoniae* has been isolated from over 50% of atherosclerotic plaques in animal models (Boland 2000). Heart disease is currently the leading cause of death in humankind in the United States,

further illustrating the importance of understanding *C. pneumoniae* pathogenesis (2007).

C. psittaci, a zoonotic pathogen, causes psittacosis, a severe pneumonia transmitted to humans through pet birds and poultry. Psittacosis causes general flu-like symptoms that can progress into acute pneumonia as well as other nonrespiratory conditions. Human exposure typically occurs through psittacine (parrot-like) birds when dried feces are aerosolized and inhaled (Smith, Bradley et al. 2005). Though *C. psittaci* is rarely fatal, it is considered by the Centers for Disease Control to be a category B biological threat agent.

C. trachomatis causes the most commonly reported infectious disease in the United States and the Centers for Disease Control estimates there are as many as 3 million new infections each year. *C. trachomatis* is known to cause both ocular and genital infections (Adderley-Kelly and Stephens 2005). *C. trachomatis* ocular infection is the leading cause of preventable blindness worldwide. Repeat ocular infection can lead to trachoma, characterized by inverting of the eyelashes, leading to corneal scratching and eventual scarring of the conjunctiva (Wright, Turner et al. 2008). The World Health Organization considers trachoma to be endemic in 56 countries, and estimates that 1 to 3 million people are blind as a result of *C. trachomatis* ocular infection (Resnikoff, Pascolini et al. 2004; Polack, Brooker et al. 2005). Trachoma is caused by serovars A, B, Ba, and C, and transmission is through flies and person to person from infected ocular secretions (Wright, Turner et al. 2008).

In the United States, *C. trachomatis* genital infection is the most common bacterial sexually transmitted disease (Adderley-Kelly and Stephens 2005). *C. trachomatis* genital infection can cause a variety of conditions including pelvic inflammatory disease (PID), ectopic pregnancy, and sterility (Stephens 2003). Infection with *C. trachomatis* frequently causes no overt symptoms; it is estimated that 75% of women and 50% of men are asymptomatic. *C. trachomatis* infection is one of the most common causes of PID, which remains one of the highest contributors to challenged reproductive capabilities in women. PID can occur through a chlamydial infection ascending to the upper reproductive tract resulting in severe scarring and possibly blockage of the fallopian tubes (Adderley-Kelly and Stephens 2005).

Developmental Cycle

Conserved in all *Chlamydiae* is the characteristic biphasic developmental cycle, requisite for intracellular survival and pathogenicity (Horn, Collingro et al. 2004). The developmental cycle in *Chlamydia* is carried out through two morphologically and phenotypically distinct forms: the elementary body (EB) and the reticulate body (RB) (Abdelrahman and Belland 2005). EBs are the infectious but metabolically inert form of *Chlamydia*, implicated in dissemination of the chlamydial infection. EBs are the small (0.1 to 0.3 μm), round, spore-like form of the organism that allow for extracellular survival (Matsumoto 1973) (Abdelrahman and Belland 2005). The EBs have an extremely condensed nucleoid region, and because very little

peptidoglycan is in their cell wall, structure is maintained through crosslinking of the outer membrane (Brickman, Barry et al. 1993) (Hatch 1999). Surface projections extending from the EB can be detected through freeze fracture procedures (Matsumoto 1973). These projections resemble those seen in other organisms containing T3SSs, and are speculated to represent the chlamydial T3SS needles that could facilitate the initial stages of entry (Abdelrahman and Belland 2005).

RBs are the metabolically active, non-infectious form of *Chlamydia* that arise following the internalization and establishment of EBs within the inclusion (Abdelrahman and Belland 2005). RBs are bound by an inner and outer membrane and are considerably larger than EBs at 1 μm in diameter and do not appear condensed but rather appear granular with a diffuse nucleoid region (Rockey 2000). RBs have surface projections, similar to EBs that are thought to represent the T3SS needles (Matsumoto 1973). However, there is a higher concentration of projections on the RB. In some species, such as *C. trachomatis*, during primary differentiation and binary fission, the RB is found to associate with the inclusion membrane (Matsumoto 1973; Abdelrahman and Belland 2005). This association could allow for interaction with the host across the inclusion membrane through the T3SS.

EBs begin the developmental cycle by attaching and invading eukaryotic host cells (**Fig. 1**). Upon entry, the EBs are internalized within a membrane bound parasitophorous vacuole termed an inclusion. The EBs then differentiate into RBs that begin to reproduce through binary fission. Through poorly defined mechanisms and unknown signals, secondary differentiation begins and RBs asynchronously convert

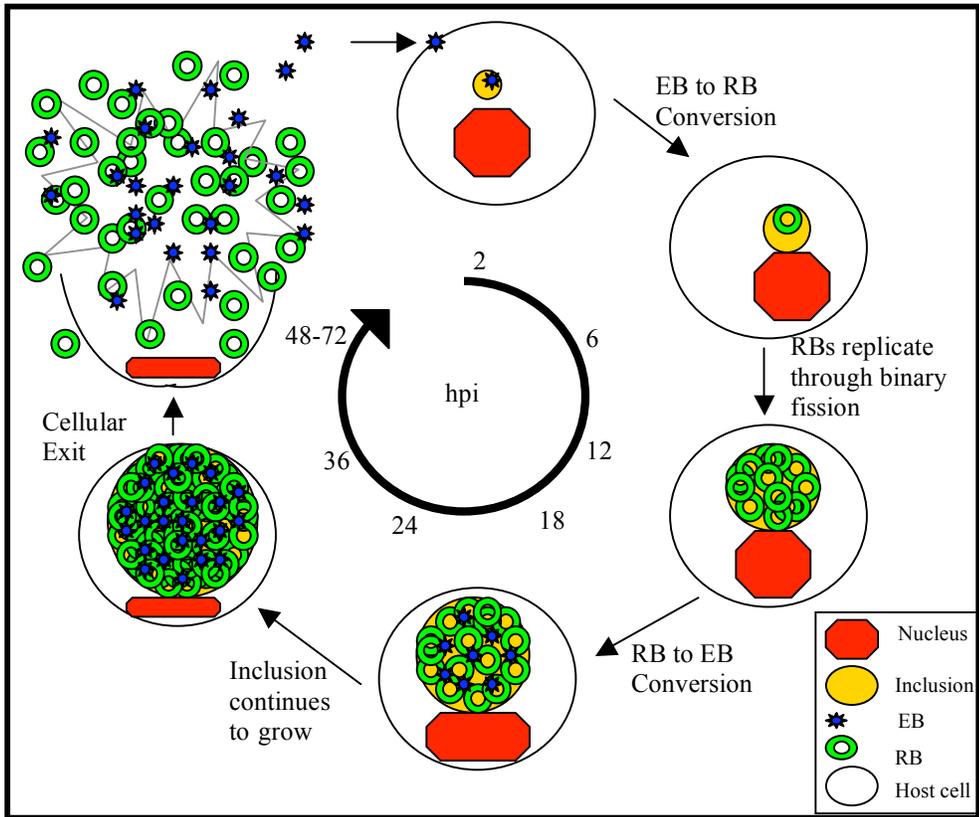
back into EBs (Abdelrahman and Belland 2005). This progression continues until highly controlled cellular exit occurs through either lysis, or a pinching of the inclusion termed extrusion (Hybiske and Stephens 2007). When the EBs are released, the developmental cycle initiates again and the EBs infect neighboring cells. Though this novel process is the unifying characteristic of all Chlamydiaceae, the developmental cycle does vary slightly depending on the species and strain. Nevertheless, functional characterization of components that mediate the cycle as well as elucidating the mechanisms that facilitate the infectious process, could lead to a greater understanding of the pathogenesis of *Chlamydia* as well as provide new targets for chemotherapeutic intervention.

Chlamydial Type III Secretion System

The mechanisms of pathogenesis are poorly understood; however, *C. trachomatis* encodes a type three secretion system (T3SS), that is likely requisite for intracellular growth and survival (Peters, Wilson et al. 2007). T3SSs are common virulence determinants of many Gram negative pathogens (Coburn, Sekirov et al. 2007). T3SSs facilitate the translocation of effector molecules into the cytosol of eukaryotic host cells by acting as a molecular syringe to direct the secretion through a needle into the target cell (Buttner and Bonas 2002).

Figure 1. *C. trachomatis* developmental cycle.

During infection with *C. trachomatis* L2/434/Bu, EBs enter host cells and form a parasitophorous vacuole termed an inclusion. The EB then converts into the metabolically active RB. RBs replicate through binary fission and fill the inclusion. The inclusion continues to grow as the RBs asynchronously convert back into the infectious EB form. The inclusion fills the host cell and the EBs and RBs are released from the cell from 48 - 72 hpi.

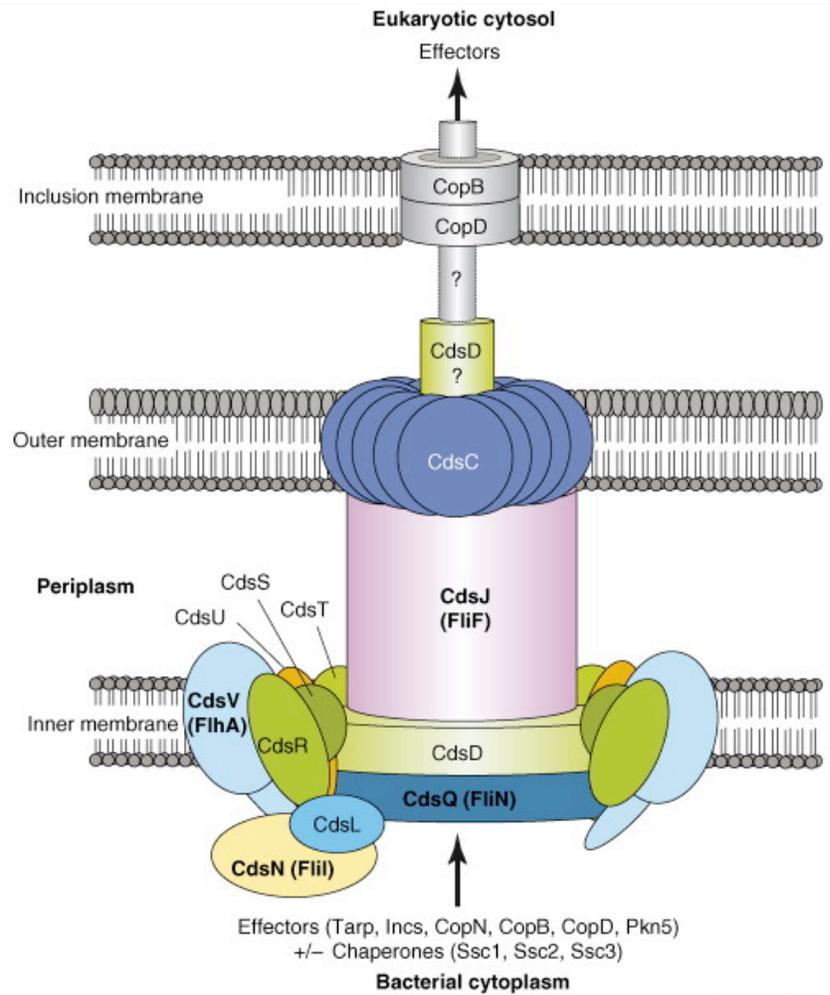


The chlamydial type III secretion apparatus (T3SA) is distinct from that of other Gram negative pathogens in that it is necessary to facilitate the organism's unique developmental cycle. The T3SS in *Chlamydia* allows for continuous interaction and association with the host during entry as well as later in the developmental cycle through the inclusion membrane (Peters, Wilson et al. 2007). The T3SS is made up of 20 – 25 proteins involved in formation of the needle apparatus or in chaperoning functions (Ghosh 2004) (**Fig. 2**). The T3SA within *Chlamydia* consists of several conserved proteins that make up the molecular syringe and are required for the assembly of a functional T3SS. The *Chlamydia* T3SS is proposed to be contact-dependent, allowing the RBs to replicate while in contact with the inclusion membrane. Detachment from the inclusion membrane is suggested to be involved in signaling of T3SS inactivation and late differentiation (Peters, Wilson et al. 2007).

The T3SS in *Chlamydia* is poorly characterized and relatively little is known about the chlamydial effector proteins. Chlamydial proteins that comprise the T3SS are termed contact dependent secretion (Cds) proteins and function in secretion of virulence factors into host cells (**Fig. 2**). Elucidating the role of other proteins involved in T3SS could provide new insight into the pathogenesis of *Chlamydia* as well new targets for therapeutic approaches to disease treatment.

Figure 2. *C. trachomatis* Type Three Secretion Apparatus.

The T3SA is a syringe-like structure that directs effector proteins through the needle and translocon pore into the host. Chlamydial proteins are indicated based on their predicted function.



(adapted from Peters, Wilson et al. 2007)

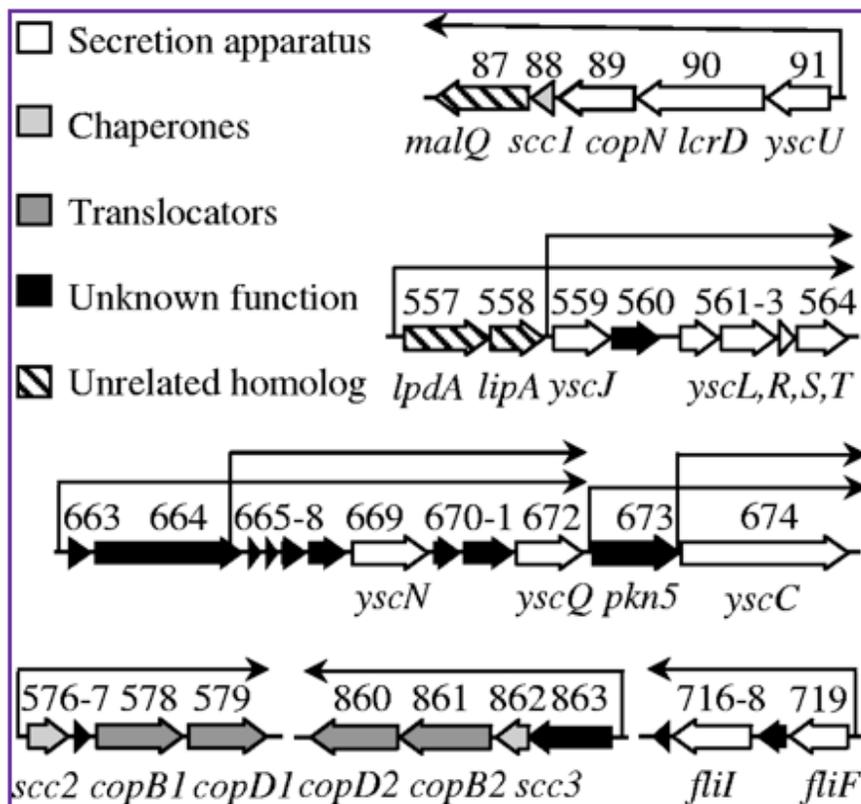
II. Type Three Dependent Translocation of a Putative Chlamydial Effector Protein, CT667.

INTRODUCTION

In *C. trachomatis* ten operons were identified within six loci known to encode homologues to the T3SS proteins. Thirteen proteins of unknown function are encoded by the genes within these operons (Hefty and Stephens 2007) (**Fig. 3**). Among these proteins of unknown function is CdsF, which was recently implicated as the needle protein of the T3SS through biochemical analysis (Betts, Twiggs et al. 2007). Immediately downstream of the needle protein is a protein of unknown function, CT667. Because it is transcriptionally-linked to the chlamydial T3SS homologues, it too likely plays role in T3S. As with many of the other proteins of unknown function, quantitative PCR (qPCR) data revealed that CT667 is upregulated during the RB to EB conversion of the developmental cycle (**Fig. 4**) (Hefty and Stephens 2007). During this conversion, a T3SS would provide a mechanism for interacting with the host through the inclusion membrane to facilitate the developmental cycle (Slepenkin, de la Maza et al. 2005). The presence of CT667 late in the developmental cycle suggests it could function in EBs and thus play a role in continuing the infectious process. Therefore, CT667 could contribute to chlamydial invasion during entry or intracellular survival of the organism.

Figure 3. *C. trachomatis* operon organization for six loci containing homologues to T3SS genes.

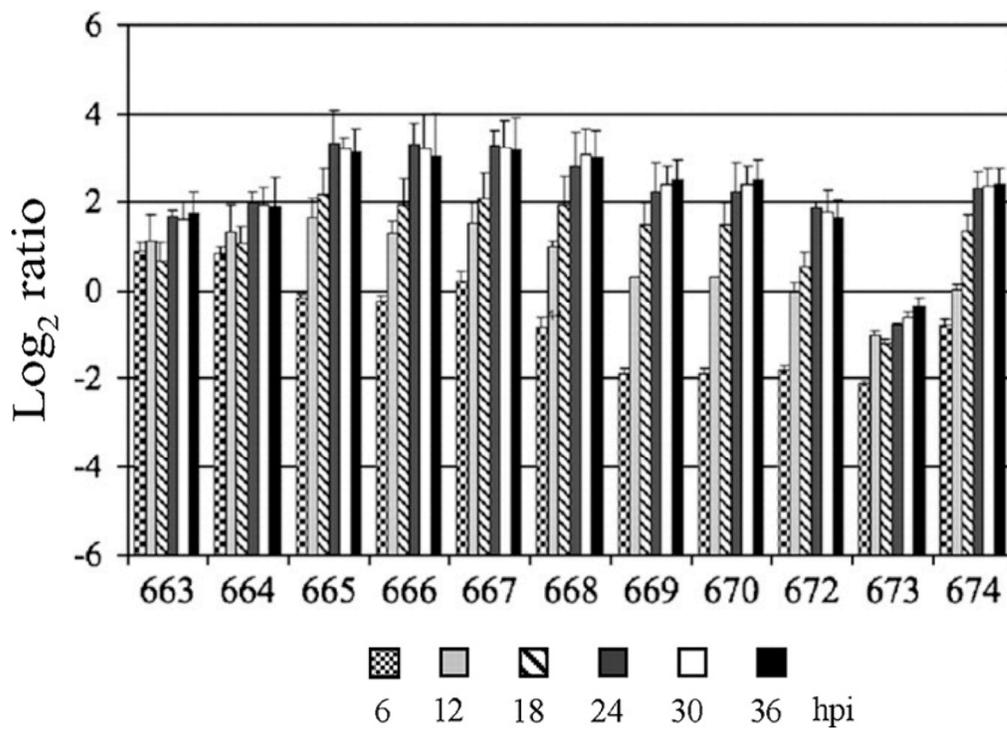
Block arrows indicate gene orientation and location. The open reading frames are denoted with numbers above the arrows. Above the genes, line arrows indicate orientation and location of the ten predicted operons.



(Adapted from Hefty and Stephens 2007)

Figure 4. Quantitative gene expression analysis of chlamydial T3SS loci.

Total RNA was isolated at 6, 12, 18, 24, 30, and 36 hours post infection (hpi). For each gene at each time point, the amount of cDNA was determined by qPCR. Transcript quantity was transformed to a \log_2 ratio of constitutively expressed *CT190* (*gyrB*). *CT667* transcript levels were upregulated from 18 - 24 hpi and remained at high levels through 36 hpi. Error bars indicate standard errors of the means.



(Adapted from Hefty and Stephens, 2007)

Preliminary Blast analysis of the CT667 protein sequence revealed three tetratricopeptide repeat (TPR) domains (**Fig. 5**). Proteins with TPR domains have been shown to have diverse cellular functions and many have been demonstrated to play a role in cell cycle control, signal transduction, protein transport, chaperone function and transcription (Blatch and Lassar 1999). CT667 is located immediately downstream of CdsF, the predicted needle protein of the chlamydial T3SS, and thus likely serves chaperoning function for the needle (**Fig. 3**). Chaperoning function for CT667 was further supported by a yeast 2-hybrid analysis which indicated an interaction with CdsF. Additionally, co-expression with CT665 and CT667 was required for CdsF protein stability and prevented degradation of the needle protein (Betts, Twiggs et al. 2007).

Frequently T3S proteins, including chaperones, have multiple functions in facilitating invasion and growth (Page and Parsot 2002). *Pseudomonas aeruginosa* T3SS protein, Pcr4, has three TPR domains and has multiple roles including chaperone function and regulation of T3S. It is also secreted in a type three dependent manner into host cells suggesting effector function (Yang, Shan et al. 2007). The *S. flexneri* effector protein, IpaD, is also a T3SS protein with dual function (Johnson, Roversi et al. 2007). IpaD secretion has been demonstrated to be requisite for *S. flexneri* translocon pore formation and IpaD also functions in conjunction with IpaB to control T3S by blocking the Mxi-Spa translocon (Menard, Sansonetti et al. 1994; Picking, Nishioka et al. 2005). Another protein, HilA in *Salmonella enterica*,

has 9 TPR domains and is a transcriptional regulator involved in activation of T3S (Thijs, De Keersmaecker et al. 2007).

Due to the presence of TPR domains and possible dual function, we hypothesize that CT667 is a chlamydial effector protein. In *C. trachomatis* there is currently no model for genetic exchange; therefore, a heterologous T3SS was employed to test the secretion capability of CT667 (Subtil, Parsot et al. 2001). Structural components of the chlamydial T3SA are conserved throughout other bacteria including *S. flexneri*, *Yersinia pestis*, and *Salmonella* spp. (Rosqvist, Hakansson et al. 1995) (Subtil, Parsot et al. 2001). *S. flexneri* has been previously employed as a heterologous system to elucidate chlamydial T3SS effector proteins (Subtil, Parsot et al. 2001). Therefore, we used *S. flexneri* as a surrogate system to study type three dependent secretion and translocation of CT667. *C. trachomatis* infected eukaryotic cells were also utilized to visualize CT667 within the inclusion membrane during late stages of the developmental cycle.

MATERIALS AND METHODS:

Construction of CT667 expression vectors and bacterial strains

The plasmid pHS1 (Amp^r), generated from puc 18 (Novagen, Madison, WI) for expression of CT667, was produced by Dr. Wendy Picking. *S. flexneri* IpgD⁻ and MxiH⁻ (SH116) strains were gifts from Dr. William Picking. *S. flexneri* IpgD⁻ and MxiH⁻ strains expressing six histidine tagged and CysCysProGlyCysCys (4 cys) tagged IpaD in pHS2 plasmid were also gifts from Dr. Picking.

To generate CT667 with a C-terminal histidine tag, the coding sequence of CT667 was copied from *C. trachomatis* L2/434/Bu using standard PCR amplification techniques. The 5' primer consisted of an EcoRI restriction endonuclease site and the first 18 bases of the appropriate coding sequence (**Table 1**). The 3' primer consisted of the HindIII restriction endonuclease site, six histidines, and bases 428 to 450 at the 3' end of the coding sequence. The resulting 484 bp fragment was ligated using the Quick Ligation Kit (Invitrogen, Carlsbad, CA) into pHS1 and transformed into *E. coli* strain TOP10. Transformants were screened for presence of the insert by PCR using vector specific primers. CT667 C-terminal histidine fragment in the pHS1 plasmid was verified for protein expression through western blot. Following plasmid purification using the Miniprep Kit (Qiagen, Valencia Ca), CT667 C-terminal histidine fragment in the pHS1 plasmid was transformed into *S. flexneri* strain IpgD⁻ and *S. flexneri* strain MxiH⁻. Transformants were screened for the presence of an insert by PCR using vector specific primers. An isogenic clone with the

EcoRI/HindIII CT667 C-terminal histidine fragment in the pHS1 plasmid was verified for protein expression through western blot.

For ReAsH technology experiments, CT667 with a C-terminal 4 cys was generated (Enninga, Mounier et al. 2005). The 5' primer consisted of an EcoRI restriction endonuclease site and the first 18 bases of the appropriate coding sequence. The 3' primer consisted of the HindIII restriction endonuclease site, CysCysProGlyCysCys and bases 428 to 450 at the 3' end of the coding sequence. The resulting 484 bp fragment was ligated using the Quick Ligation Kit (Invitrogen) into pHS1 and transformed into *E. coli* strain TOP10. Transformants were screened for presence of the insert by PCR using vector specific primers. CT667 C-terminal 4 cys fragment in the pHS1 plasmid was verified for protein expression through western blot. Following plasmid purification (Qiagen), CT667 C-terminal 4 cys in the pHS1 plasmid was transformed into *S. flexneri* strain IpgD⁻ and *S. flexneri* strain MxiH⁻. Transformants were screened for the presence of CT667 C-terminal 4 cys fragment in the pHS1 plasmid through incubation with the ReAsH reagent (Invitrogen) to verify labeling through fluorescence microscopy, as described below.

SDS-PAGE and Western Blot Analyses

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 12.5 % separating gel and 5 % stacking gel in a Mini-PROTEAN Tetra Cell System (Bio-Rad Laboratories, Hercules, CA). For western blot analysis, proteins were electrophoretically transferred from unstained gels to nitrocellulose

Table 1. Type Three Secretion Cloning Primers

Designation: CT667 EcoRI Sequence: GGAATTCC ATGGCAGATTTGGATGTA
Designation: CT667 HindIII C term his Sequence: CGCAAGCTTTT ATTGTGGTGGTGGTGGTGGTGGTGGTAAAGGCCACCGCTTTTAGC
Designation: CT667 HindIII C term flash Sequence: CCCAAGCTT CTAACAACAACCAGGACAACAACCACCATTTAAAGGCCACCGC TT
Designation: CT668 EcoR N term hisI Sequence: 5'- 3' CGCGAATTC CCACCACCACCACCACCACATGATAGATCCTCTTAAGCTTTTT
Designation: CT668 BamHI Sequence: 5'-3' GCGGGATC CTTATTAACCTAACTGGGTTGTCAT

membrane (Thermo Fisher Scientific, Pittsburg, PA) using the Mini-PROTEAN 3 Cell System (Bio-Rad Laboratories) at 125 v for 45 minutes. Protein binding sites on the nitrocellulose membrane were blocked by incubation with 5.0 % (w/v) non-fat dry milk in phosphate buffered saline (PBS) + 0.2 % Tween 20 solution for 30 minutes at room temperature. Anti-histidine monoclonal mouse antibodies (Novagen) were diluted 1:2000 in 5.0 % (w/v) non fat dry milk in PBS + 0.2 % Tween. Polyclonal rat antibodies generated against CT667 and polyclonal rabbit antibodies generated against chlamydial RNAP α subunit (RpoA) were diluted at 1:1000 in 5.0 % (w/v) non-fat dry milk in PBS + 0.2 % Tween. The nitrocellulose membrane was incubated at 4° C overnight with rocking. The primary antibodies were removed and the nitrocellulose membrane was washed twice for five minutes while rocking with PBS + 0.2 % Tween. The nitrocellulose membranes were then incubated with IR-tagged goat anti-rabbit IgG (Rockland, Gilbertsville, PA), goat anti-rat IgG (Rockland), or goat anti-mouse IgG (Rockland) diluted at 1:5000 in 5.0 % (w/v) non-fat dry milk in PBS + 0.2 % Tween. The nitrocellulose membrane was then washed twice for five minutes while rocking with PBS + 0.2 % Tween, once for five minutes with PBS, and once for five min with ultra pure water. The membrane was then visualized using the Odyssey Infared Scanner (LI-COR biosciences, Lincoln, Ne).

Preparation of Electrocompetent *S. flexneri*

Prior to procedure, 250 mL of 10 % glycerol in ultra pure water and 1.5 L of water was autoclaved. During the preparation of the cells, solutions, tubes, cells, and

equipment was kept at 4 °C or on ice. One liter of Tryptic Soy Broth (TSB) media with appropriate selection (ampicillin, 100 µg/mL or kanamycin, 50 µg/mL) was inoculated with 1 mL of *S. flexneri* (from overnight growth). The bacteria were grown at 37 °C with shaking (>200rpm) until OD₆₀₀ was between 0.4 - 0.7. The solution was separated into four, 250 mL sterile Oakridge tubes and incubated on ice for 15 minutes. After incubation, the bacterial cells were pelleted through centrifugation (1,600 x g, 10 min, 4 °C), the supernate was removed, and the cells were washed in 200 mL of cold water. The cells were collected through centrifugation (1,600 x g, 10 min, 4 °C) and the supernate was removed. The cells were then washed in 500 mL of cold water and combined into two, 250 mL centrifuge tubes. The cells were pelleted through centrifugation (1,600 x g, 10 min, 4 °C) and the supernate was removed. The cells were then washed in a total volume of 200 mL of cold 10 % glycerol and combined into one, 250 mL centrifuge tube. The cells were collected through centrifugation (1,600 x g, 10 min, 4 °C) and the supernate was removed. The cells were resuspended in 1.5 mL of cold 10 % glycerol, aliquotted on ice, and immediately stored at -80 °C.

Transformation of C-terminal his or C-terminal 4 cys CT667 on pHSI into *E. coli*

Chemically competent cells were thawed and stored on ice during the entire procedure. Two µL of purified pHS1 plasmid or five µL of ligation reaction was combined with 50 µL of chemically competent *E. coli* strain TOP10 cells (Invitrogen) and incubated on ice for 20 minutes. The mixture was heat shocked in a water bath at

42 °C for 45 seconds then immediately incubated on ice for one minute. Two hundred µL of Luria Burtani (LB) media with no antibiotics was added to each transformation reaction and incubated at 37 °C for 30 minutes. Two hundred µL of the solution was added to LB plates with 100 µg/mL ampicillin and incubated overnight at 37 °C.

Transformation of C-terminal his or C-terminal 4cys CT667 into *S. flexneri*

Electrocompetent cells were thawed and stored on ice during the entire procedure. Two µL of purified plasmid DNA or five µL of a ligation mixture was combined with 40 µL of electrocompetent *S. flexneri* and incubated on ice for three minutes. The mixture was added to a chilled electroporation cuvette (1 mm) and immediately placed in the Micropulser electroporator (Biorad). After electroporating according to manufacturers standards (1.8 kV), one mL of SOC media with no antibiotics was added to the cuvette. Cells were mixed in SOC media through pipetting, transferred to a 1.5 mL eppendorf tube, and incubated at 37 °C for 30 minutes. Two hundred µL of the solution was spread on Congo red plates with 100 µg/mL ampicillin and incubated overnight at 37 °C.

Chlamydial Infection and EB Seed Prep

L929 mouse fibroblast cells were grown to 8×10^5 cells/mL in a one liter spinner flask in RPMI (Mediatech Inc, Manassas, VA) supplemented with 50 µg/mL vancomycin and 5 % fetal bovine serum (FBS). Cells were infected with *C.*

trachomatis L2/434/Bu at a multiplicity of infection (MOI) of ~1. At 48 hpi, infected cells were transferred into four sterile 250 mL centrifuge tubes. Cells were collected through centrifugation (1,380 x g, 10 min, 15 °C) and the supernate was removed. Cells were washed in 200 mL HBSS and collected through centrifugation (1,380 x g, 10 min, 15 °C). The supernate was removed, the cells were resuspended in 50 mL HBSS, and separated into two sterile 40 mL Oak Ridge tubes. The cells were sonicated six times on setting six (Sonic Dismembrator 100) for 15 seconds while placing on ice in between sonications. The cellular debris was pelleted through centrifugation (150 x g, 10 min, 15 °C) and the supernate was transferred to sterile 40 mL Oak Ridge tubes. The supernate was centrifuged (28,000 x g, 30 min, 4 °C) and the resulting supernate was removed. The cells were resuspended in two mL SPG and sonicated two times on setting four (Sonic Dismembrator 100) for three seconds while placing on ice in between sonications. The chlamydial seed was aliquotted on ice and immediately transferred to -80 °C.

RB harvest and CT667 protein expression profile

L929 mouse fibroblast cells were grown to 8×10^5 cells/mL in a one liter spinner flask in RPMI (Mediatech Inc) supplemented with 50 µg/mL vancomycin and 5 % FBS. Cells were infected with *C. trachomatis* L2/434/Bu at an MOI of ~1. At 12, 24, and 36 hpi, 500 mL, 300 mL, and 200 mL respectively were transferred into sterile 250 mL centrifuge tubes. Cells were collected through centrifugation (1,400 x g, 10 min, 15 °C) and the supernate was removed. Cells were washed twice in 200

mL HBSS and collected through centrifugation (1,400 x g, 10 min, 15 °C). The supernate was removed and the cells were resuspended in a total volume of 30 mL HBSS at each time point and transferred to sterile 40 mL Oak Ridge tubes. The cells were sonicated two times on setting four (Sonic Dismembrator 100) for 15 seconds while placing on ice in between sonications. Disruption of the cells was verified through light microscopy. The sonicated debris was pelleted through centrifugation (150 x g, 10 min, 15 °C) and the supernate was transferred to sterile 40 mL Oak Ridge tubes. The supernate was centrifuged (12,000 x g, 15 min, 15 °C) and the RBs were resuspended in 450 µL PBS for TCA precipitation, as described below.

TCA precipitation

RBs from 12, 24, and 36 hpi in 450 µL PBS were combined with 50 µL of 100 % cold TCA. The samples were incubated on ice for 15 minutes then centrifuged (10,000 x g, 15 min, 4 °C). The supernate was removed and cells were washed two times in one mL cold 100 % ethanol and centrifuged (10,000 x g, 15 min, 4 °C). The supernate was removed and the tubes were inverted for 10 min to remove residual ethanol. Pellets were resuspended in 10 mM tris pH 8.0 and final sample buffer (FSB), then subjected to western blot analysis to determine protein expression profile.

Congo red induction

S. flexneri IgpD⁻ and MxiH⁻ strains containing pHS1 plasmid with the appropriate his-tagged protein were streaked onto TSB agar + 0.25 mg/mL Congo red

dye + 100 µg/mL ampicillin and incubated for 24 hrs. Isolated colonies were screened for the presence of the virulence plasmid through red/white screening and red colonies were selected and grown in TSB media supplemented with 100 µg/mL ampicillin and 50 µg/mL kanamycin overnight (37 °C, >200rpm). Bacteria were diluted 1:50 in 10 mL TSB media with 100 µg/mL ampicillin and 50 µg/mL kanamycin and grown (37 °C, >200rpm) to mid log-phase (OD₆₀₀ = 0.4 - 0.7). Bacteria were harvested by centrifugation (3,000 x g, 25 °C, 10 min) and the supernate was removed. Cells were washed with one milliliter cold PBS and harvested through centrifugation (11,000 x g, 4 °C, 1 min). Bacteria were then resuspended in 100 µL Congo red dye (3.5 mg/mL) in PBS (solution boiled for ~20 seconds and immediately stored at 37 °C until use) for 12 minutes in a 37 °C water bath. Following centrifugation (11,000 x g, 4 °C, 10 min), bacterial pellets and supernates were separated. The pellet was resuspended in 100 µL PBS, and five µL was added to 20 µL of FSB and boiled for five minutes, vortexed, and boiled again. Supernates were filtered through a 0.22 µm filter, and 20 µL of the supernatant and 10 µL of FSB were combined and boiled for five minutes, vortexed, and boiled again. Ten µL of the pellet sample and 25 µL of the supernatant sample were loaded onto an SDS-PAGE gel, followed by western blot analysis.

ReAsH Labeling and Technology

S. flexneri IgpD⁻ and MxiH⁻ strains containing pHS1 plasmid with 4 cys tagged CT667 and *S. flexneri* IgpD⁻ strain containing pHS1 plasmid were grown in

TSB media supplemented with 100 µg/mL ampicillin and 50 µg/mL kanamycin overnight (37 °C, >200rpm). Bacteria were diluted 1:50 in 10 mL TSB media with 100 µg/mL ampicillin and 50 µg/mL kanamycin and grown (37 °C, >200rpm) to mid log-phase ($OD_{600} = 0.4 - 0.7$). Bacteria were harvested by centrifugation (3,000 x g, 25 °C, 10 min), the supernate was removed, and the cells were resuspended in one mL PBS. The bacteria were labeled through the addition of one µl of ReAsH reagent (Invitrogen) to the mixture + one µM TCEP. The samples were covered in foil and incubated (37 °C, >200 rpm) for one hour. Henle cells were grown on coverslips in a four well plate to 1.2×10^5 cells/mL in DMEM (Mediatech Inc) supplemented with 100 µg/mL penicillin/streptomycin and 10 % FBS. Henle cells were washed three times with one mL DMEM (without serum and antibiotics). Each well was inoculated by adding three µl of mid log-phase ReAsH labeled bacteria directly to Henle cells in 300 µL of DMEM (without serum or antibiotics). Plates were centrifuged (2,000 x g, 5 min, 25 °C) to facilitate invasion and incubated in the CO₂ incubator for 30 minutes. Coverslips were washed three times with one mL DMEM then incubated in one mL DMEM in the CO₂ incubator at 37°C for two hours. Coverslips were washed three times with one mL PBS. Cells were fixed with 2 % formaldehyde (in PBS) for 15 minutes, and washed twice with one mL PBS. Coverslips were affixed to slides using Vectashield Mounting Medium for Fluorescence (Vector Laboratories, Burlingame, CA). Samples were visualized for ReAsH staining using epifluorescence microscopy.

S. flexneri invasion assay

S. flexneri strain IpgD⁻ with C-terminal his CT667 on pHS1 plasmid, MxiH⁻ with C-terminal his CT667 on pHS1 plasmid, and IpgD⁻ with pHS1 plasmid were diluted 1:100 from overnight growth in 10 mL of TSB media with 100 µg/mL ampicillin and 50 µg/mL kanamycin and grown to OD₆₀₀ = 0.4 - 0.7. L929 cells were grown on coverslips in a four well plate to 1.2 x 10⁵ cells/mL in RPMI (Mediatech Inc) supplemented with 50 µg/mL vancomycin and 5 % FBS. L929 cells were washed three times with one mL RPMI (without serum and antibiotics). Each well was inoculated by adding three µl of mid log-phase bacteria directly to L929 cells in 300 µl of RPMI (without serum or antibiotics). Plates were centrifuged (2,000 x g, 5 min, 25 °C) to facilitate invasion and incubated in the CO₂ incubator at 37 °C for 45 minutes. Coverslips were washed three times with one mL RPMI + 50 µg/mL gentamycin then incubated in one mL RPMI + 50 µg/mL gentamycin in the CO₂ incubator at 37°C for 2 hours. Coverslips were washed two times with one mL RPMI 50 µg/mL gentamycin. Cells were fixed in methanol for 10 minutes then washed and stored in one mL PBS prior to immunofluorescence assay as described below.

S. flexneri immunofluorescence assay

PBS was removed from L929 cells, and the coverslips were stained to visualize the intracellular presence of CT667. Coverslips were incubated in 20 µL of affinity purified rat anti-CT667 antibodies diluted 1:10 in PBS. After incubation in the dark for two hours at 24 °C, primary antibodies were removed by washing 15

times in PBS. Coverslips were then incubated in the dark for one hour with 10 μ L of goat anti rat Alexa Fluor 568 antibodies (Invitrogen) diluted 1:2000 in PBS and 10 μ L of rabbit anti-*Shigella* spp. Alexa Fluor 488 antibodies (US biologicals, Swampscott, Massachusetts) diluted 1:10 in PBS. Secondary antibodies were removed by washing 15 times in PBS. Samples were affixed to slides and stained with DAPI using Vectashield Mounting Medium for Fluorescence with DAPI (Vector Laboratories). Samples were visualized using epifluorescence microscopy.

C. trachomatis immunofluorescence assay

L929 mouse fibroblast cells were grown on coverslips in a four well plate to 1.2×10^5 cells/mL in RPMI (Mediatech Inc) supplemented with 50 μ g/mL vancomycin and 5 % FBS. Cells were infected with *C. trachomatis* L2/434/Bu and at 36 hpi cells were fixed with methanol, and the coverslips were stained to visualize the intracellular presence of CT667. Coverslips were incubated in 20 μ L of affinity purified rat anti-CT667 antibodies diluted 1:10 in PBS. After incubation in the dark for two hours at 24 °C, primary antibodies were removed by washing 15 times in PBS. Coverslips were then incubated in the dark for one hour with 20 μ L of goat anti-rat Alexa Fluor 568 conjugated antibodies (Invitrogen) diluted at 1:1000 in PBS. Secondary antibodies were removed by washing 15 times in PBS. Samples were affixed to slides and stained with DAPI using Vectashield Mounting Medium for Fluorescence with DAPI (Vector Laboratories). Samples were visualized using epifluorescence microscopy.

RESULTS

Sequence analysis reveals CT667 contains 3 TPR domains

Bacterial pathogens employ protein-protein interactions as a mechanism to facilitate manipulation of the host cell during infection (Bhavsar, Guttman et al. 2007). The tetratricopeptide repeat (TPR) motif is a structural motif known to mediate protein-protein interactions (Melville, Katze et al. 2000). Proteins containing these motifs have diverse biological roles including cell cycle control, signal transduction, protein stability, and effector function (Blatch and Lasse 1999). TPR motifs are made up of 34 semi-conserved amino acid residues in 3 - 16 tandem repeats. They are widespread among bacteria as well as eukaryotes. The structure of a TPR motif consists of two antiparallel alpha helices (Goebel and Yanagida 1991; D'Andrea and Regan 2003). The consensus positions in the motif were determined through sequence alignment. The motif is indicated by eight conserved amino acid residues four of those with an even higher level of conservation (Sikorski, Boguski et al. 1990). The eight amino acid residues have a high level of similarity in spacing, size and hydrophobicity (D'Andrea and Regan 2003).

Sequence analysis using BLAST revealed that CT667 contains three TPR domains (**Fig. 5b**). At each of the eight conserved positions, one of three residues can serve as the consensus residue (**Fig. 5a**). The first TPR motif in CT667 contains all four more highly conserved residues. The second TPR motif contains three of the

Figure 5. CT667 has three TPR motifs.

A. Canonical TPR motif. Eight conserved positions 4, 7, 8, 11, 20, 24, 27, and 32 are found within the domain. * denotes positions with more highly conserved residues. **B.** Conserved residues in CT667. Three TPR domains of CT667 were identified using BLAST. Conserved residues corresponding to the consensus TPR sequence at positions 4, 7, 11, and 32 are gray. More highly conserved residues corresponding to the consensus TPR sequence at positions 8, 20, 24, and 27 are underlined.

A.			*		*	*	*																													
<u>Position</u>		<u>4</u>	<u>78</u>	<u>11</u>		<u>20</u>	<u>24</u>	<u>27</u>	<u>32</u>																											
Conserved		W	LG	Y		A	F	A	P																											
residues		L	IA	L		S	Y	S	K																											
		Y	MS	F		E	L	L	E																											
B.																																				
TPR1	11	F	A	L	F	E	A	G	M	V	A	I	K	Q	G	D	E	A	S	A	K	A	L	F	Q	A	L	Q	V	L	D	P	E	H	44	
TPR2	45	T	A	H	E	L	G	S	G	L	L	H	L	H	K	M	E	L	T	K	A	E	V	L	F	R	A	I	V	E	K	D	P	E	N	79
TPR3	80	W	S	A	K	A	F	L	S	L	T	I	M	M	I	V	L	Q	Q	G	S	S	F	E	V	R	R	E	S	L	E	R	C	L	Q	113

four more highly conserved residues, and the third TPR motif contains two conserved residues and two highly conserved residues.

Computational prediction of CT667 tertiary structure using I-TASSER supported that the protein contained three individual TPR domains and the characteristic antiparallel alpha helices that comprise the motif (**Fig. 6**) (Zhang 2008). The presence of TPR domains within CT667 indicates a possible role in the host, as proteins with those domains have been implicated in multiple biological processes.

CT667 is highly conserved among chlamydial species.

Using the ClustalW sequence alignment program, the percent identity and similarity between CT667 from *C. trachomatis* L2/484/Bu, different *C. trachomatis* serovars and other chlamydial species was compared (**Fig. 7**). Among the three *C. trachomatis* serovars 100 % identity was found. Analysis of CT667 from *Chlamydia murdiriam*, *Chlamydia. caviae*, and *C. pneumoniae* also revealed high levels of similarity and identity to *C. trachomatis* L2. The conserved residues within the three TPR domains of CT667 from *C. trachomatis* L2 are also highly conserved among the different serovars and species (**Fig. 5 and 7**). CT667 from *C. murdiriam*, *C. caviae* and *C. pneumoniae* contained all the highly conserved residues and most of the conserved residues within the three TPR domains of CT667 from *C. trachomatis* L2. A high level of conservation further supports that CT667 plays an important role in the biology of *Chlamydia*.

Figure 6. Computational predicted CT667 tertiary structure.

The primary sequence of CT667 was used to model the tertiary structure using the ITASSER program. The model supported that the protein contained three individual TPR motifs as indicated in purple, red and yellow in the figure. Two anti-parallel alpha helices comprise a TPR motif.

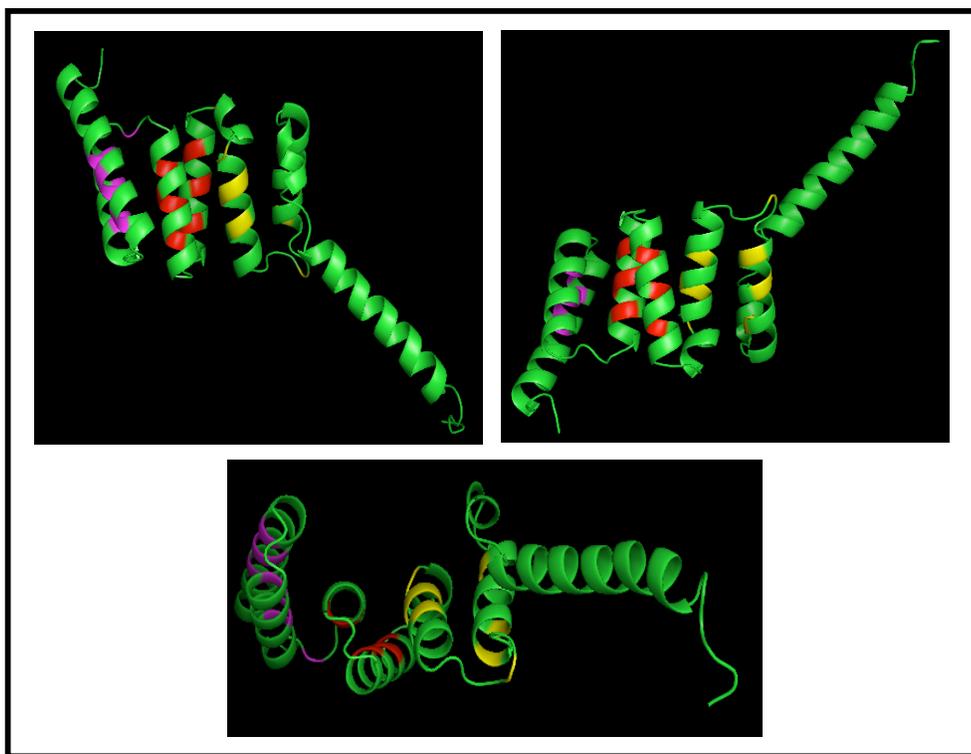


Figure 7. CT667 is highly conserved among chlamydial species.

Alignment was generated through ClustalW sequence alignment program. Analysis of CT667 from two different *C. trachomatis* serovars and chlamydial species revealed a high level of conservation. * denote conserved residues throughout all 5 species, and . denote similar residues. The first TPR motif is indicated in purple, the second TPR motif is indicated in green, and the third TPR motif is indicated in blue. Residues in the different serovars and species that correspond to the consensus positions found in CT667 from *C. trachomatis* L2 are indicated through the red rectangles. Percent identity and similarity to CT667 from *C. trachomatis* L2 was determined in comparison to the different *C. trachomatis* serovars and chlamydial species.

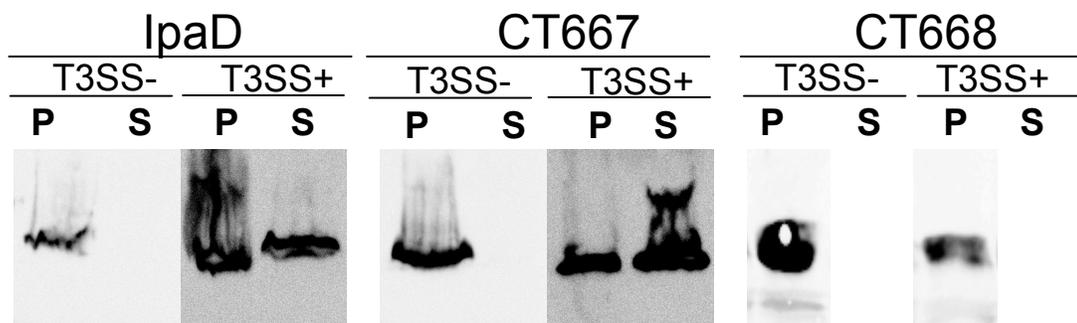
Congo red induces CT667 translocation through the *S. flexneri* T3SS.

In the absence of a system for genetic exchange in *Chlamydia*, a heterologous system was employed to test for T3S of effector proteins. The *S. flexneri* T3S machinery has been previously demonstrated to secrete chlamydial effector proteins (Subtil, Parsot et al. 2001). To analyze the secretion of CT667, *S. flexneri* competent in T3S (IpgD⁻ strain) and deficient in T3S (MxiH⁻ strain) were used. In the MxiH⁻ strain, MxiH, the needle monomer, cannot be made and a functional needle is not formed for subsequent secretion. T3S is normally induced by direct host cell contact, however; it has been well demonstrated that the addition of Congo red dye allows for artificial induction of T3S (Winstanley and Hart 2001).

The *S. flexneri* protein, IpaD, has been previously shown to be secreted through the T3SS upon the addition of Congo red dye and is used as a positive indicator of type three dependent secretion (Bahrani, Sansonetti et al. 1997). A chlamydial protein of unknown function, CT668, is transcriptionally-linked to known T3S homologues and also upregulated from 18 – 24 hpi (**Fig. 3 and 4**). CT668 was modeled through ITASSER and was predicted to be homologous to a transcriptional regulator in *Bacillus thuringiensis*. Therefore, CT668 is likely not a secreted effector protein and serves as negative secretion control. T3S competent (T3SS+) and T3S deficient (T3SS-) *S. flexneri* were grown to mid-log phase, incubated with Congo red dye, then separated into pellet and supernate fractions to test for the presence of CT667 (**Fig. 8**). Secreted proteins should be detected in the culture supernate, whereas proteins unable to be secreted will be absent. In the pellet fraction, IpaD,

Figure 8. CT667 is secreted in a type three dependent manner.

S. flexneri T3SS+ and T3SS- strains expressing IpaD, CT667, and CT668 were treated with Congo red dye to induce T3S. Immunoblots with monoclonal mouse anti-his antibodies were performed on pellets (P) and culture supernates (S). Immunoblot is representative of five experiments.



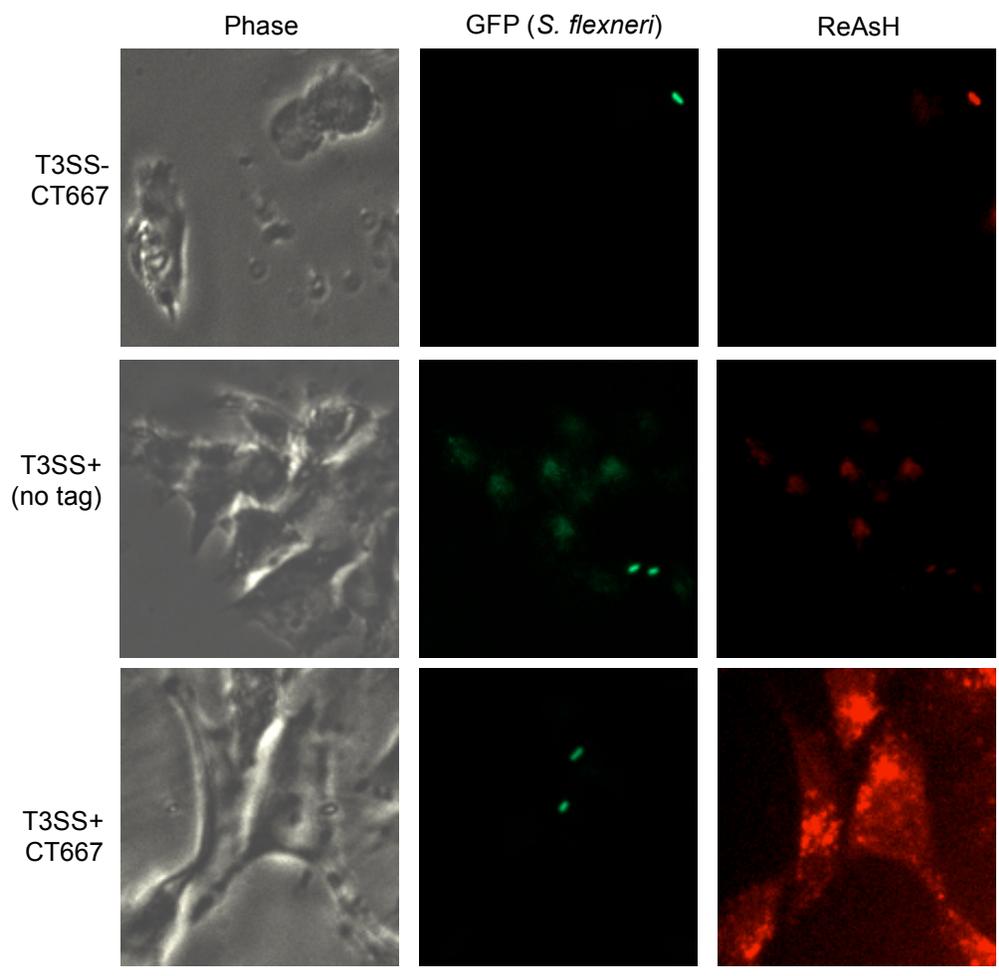
CT668, and CT667 were detected, indicating both the T3SS+ and T3SS- *S. flexneri* strains were expressing the proteins. In the culture supernate of the T3SS + *S. flexneri* strain, only CT667 and IpaD were detected in the supernate fraction, and no proteins were detected in the T3SS- strain. The presence CT667 in the culture supernate of the T3SS+ strain indicates type three dependent secretion of the protein through the heterologous *S. flexneri* T3SS.

CT667 is translocated into Henle cells

To test whether, in addition to being secreted, CT667 could also translocate into the cytosol of eukaryotic host cells, ReAsH technology was employed (**Fig. 9**). ReAsH is a fluorescent dye that has been previously used to stain and monitor the location of proteins (Adams, Campbell et al. 2002) (Enninga, Mounier et al. 2005). ReAsH by itself is a non-fluorescent molecule; however, when it forms a covalent bond with a small 4 cys tag, it becomes intensely fluorescent (Adams, Campbell et al. 2002). To test for the type three dependent translocation of the protein into Henle cells, CT667 with a C-terminal 4 cys tag was monitored following infection with T3SS+ (AgD⁻) and T3SS- (MxiH⁻) *S. flexneri*. Bacteria were labeled with GFP in order to visualize individual cells during infection. Human colonic epithelial cells (Henle) were infected with *S. flexneri* T3SS+ and T3SS- strains expressing CT667 and subjected to a *Shigella* invasion assay. A T3SS- *S. flexneri* strain expressing 4 cys tagged CT667 was used as a T3S control as the system is not functional in absence of the needle monomer. In the T3SS- strain, a GFP labeled bacterium is

Figure 9. ReAsH technology indicates CT667 is translocated in a type three dependent manner into Henle cells.

S. flexneri T3SS+ and T3SS- strains were labeled with ReAsH, and subjected to a *Shigella* invasion assay. *S. flexneri* are detected through green fluorescence and CT667 is detected through red fluorescence. **Top Panel:** Henle cells incubated with the T3SS- *S. flexneri* strain containing GFP and 4 cys tagged CT667. **Middle Panel:** Henle cells infected with the T3SS+ *S. flexneri* strain containing the vector without the 4 cys tagged CT667. **Bottom Panel:** Henle cells infected with the T3SS+ *S. flexneri* strain containing GFP and 4 cys tagged CT667. Images are representative of three experiments.



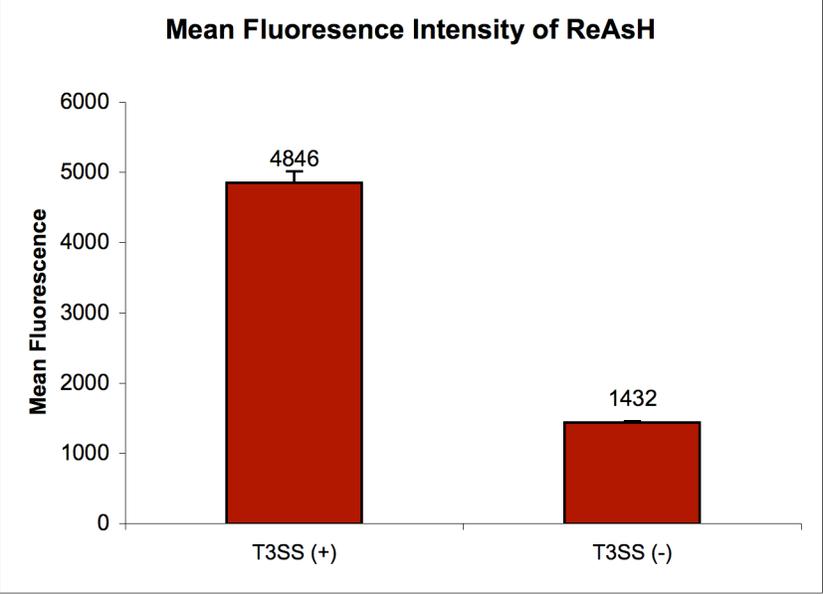
present and low-level background red fluorescence is detected in the cell (**Fig. 9 top panel**). T3SS+ *S. flexneri* containing the vector but lacking a 4 cys tagged CT667 was used as a negative control. A GFP labeled bacterium was detected in association with the Henle cell and a low-level fluorescence pattern was present, indicating background staining (**Fig. 9 middle panel**). In T3SS+ *S. flexneri* strain, GFP labeled bacteria containing 4 cys tagged CT667 are associated with the host cells. A red, punctate staining pattern is present in the cytosol of the host, indicating CT667 was translocated through the *S. flexneri* T3SS into the Henle cells (**Fig. 9 bottom panel**). The detection of CT667 only in the T3SS+ *S. flexneri* indicates CT667 is translocated in a type three dependent manner into eukaryotic host cells.

Biological observations in intensity differences are significant

The mean fluorescence intensity in individual Henle cells incubated with T3SS+ or T3SS- *S. flexneri* strains expressing CT667 and treated with ReAsH, was quantified. In 50 fields, fluorescence in individual cells was normalized with DAPI, and the amount of red fluorescence was quantified to determine the mean fluorescence intensity in multiple fields. Statistical analysis of mean red fluorescence intensity was compared and fluorescence in Henle cells incubated with the +T3SS *S. flexneri* was 3 times higher than the cells incubated with the T3SS- *S. flexneri* strain (**Fig. 10**). The difference in the red fluorescence intensity in the Henle cells treated with T3SS+ or T3SS- *S. flexneri* suggests that CT667 is being translocated into host cells. Bars represent standard error in intensity in individual cells. A two

Figure 10. Statistical analysis of mean red fluorescence intensity

The intensities of individual cells in 50 fields was normalized with DAPI then amount of red fluorescence was measured to quantify the mean fluorescence intensity in Henle cells incubated with the T3SS+ and T3SS- *S. flexneri* expressing 4 cys CT667. Bars represent standard error in intensity. Numbers above bars denote mean fluorescence intensity levels for each strain. A two-tailed Student T-test indicates difference in mean intensity to be statistically significant ($p < 0.001$). Statistical analysis was performed on a representative ReAsH experiment.



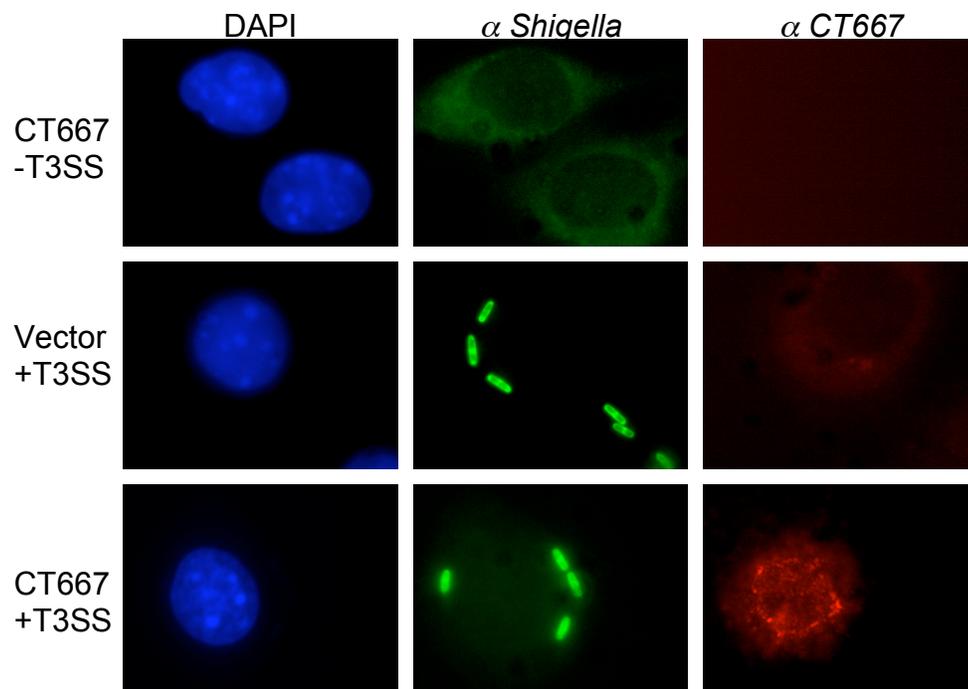
tailed Student T-test indicates difference in mean intensities to be statistically significant ($p < 0.001$).

CT667 is detected in L929 cells through *S. flexneri* immunofluorescence assay

The translocation of CT667 into eukaryotic host cells was further examined using immunofluorescence microscopy (**Fig. 11**). *S. flexneri* strains were grown to mid-log phase, incubated with L929 mouse fibroblast cells and subjected to a gentamycin protection invasion assay. After labeling the cells with rat anti-667 antibodies, Alexa Fluor 568 conjugated goat anti-rat IgG (Invitrogen), Alexa Fluor 488 conjugated rabbit anti-*Shigella* spp (US biologicals), and DAPI (Vector Laboratories), they were viewed through fluorescence microscopy. In the L929 cells incubated with the T3SS- *S. flexneri* expressing CT667, bacteria were not detected due to elimination of extracellular bacteria during the gentamycin protection assay (**Fig. 11, top panel**). In this condition, low-level background, red fluorescence in the host cytosol was observed. In L929 cells incubated with T3SS+ *S. flexneri* strain with vector only and no CT667 present, the bacteria were able to invade (**Fig. 11, middle panel**). There was low-level background red fluorescence indicating CT667 was not translocated into the cell. In L929 cells incubated with T3SS+ *S. flexneri* expressing CT667, bacteria were able to invade. A bright red punctate staining pattern was detected in the nuclear region of the L929 cell, indicating the protein was translocated (**Fig 11, bottom panel**). Lack of red fluorescence in the cells incubated with T3SS- *S. flexneri* expressing CT667, and presence of red fluorescence in the

Figure 11. CT667 was translocated into in L929 cells and visualized through immunofluorescence microscopy.

L929 mouse fibroblast cells were infected with *S. flexneri* strains and subjected to a gentamycin protection invasion assay. *S. flexneri* are detected through green fluorescence and CT667 is detected through red fluorescence. **Top Panel:** L929 cells incubated with the T3SS- *S. flexneri* strain expressing CT667. **Middle Panel:** L929 cells infected with the T3SS+ *S. flexneri* strain, containing the vector without CT667. **Bottom Panel:** L929 cells infected with the T3SS+ *S. flexneri* strain, expressing CT667. Images are representative of three experiments.



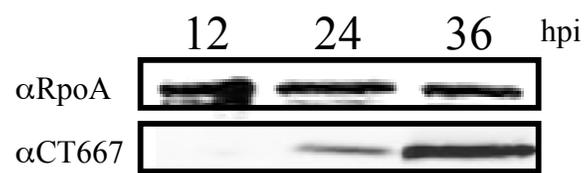
T3SS+ strain only when the protein was present suggests type three dependent translocation by the *S. flexneri* T3SS.

CT667 protein expression is upregulated at 24 – 36 hpi.

Quantitative gene expression analysis revealed that CT667 transcripts are upregulated during the RB to EB conversion of the *C. trachomatis* developmental cycle at 18 - 24 hpi, and remain highly expressed through 36 hpi (**Fig 4**) (Hefty and Stephens 2007). In order to detect and analyze CT667 in the cytosol of eukaryotic cells during a *C. trachomatis* infection, and account for post-transcriptional regulation, protein expression levels were assessed throughout the developmental cycle to determine the optimal time point to detect CT667. L929 cells were grown to 8×10^5 cells/mL, infected with *C. trachomatis* L2/434/Bu, enriched for RBs, and harvested at 12, 24, and 36 hpi. Protein levels were normalized with chlamydial RNAP (RpoA) (**Fig. 12**). CT667 was detected at 24 hpi and more highly expressed at 36 hpi. These data support that CT667 protein expression matches the transcriptional profile. Because CT667 expression was highest at 36 hpi, this time point was used for subsequent experiments. Additionally, the presence of CT667 later in the developmental cycle indicates it could be present in EBs and possibly contribute in facilitating the infectious process.

Figure 12. Protein expression profile of CT667 from purified RBs.

L929 mouse fibroblasts were infected *C. trachomatis* L2/434/Bu. Cells were enriched for RBs, collected at 12, 24 and 36 hpi, and analyzed through western blot analysis. Bacterial levels were normalized with RpoA. CT667 is expressed at 24 hpi and increases at 36 hpi.

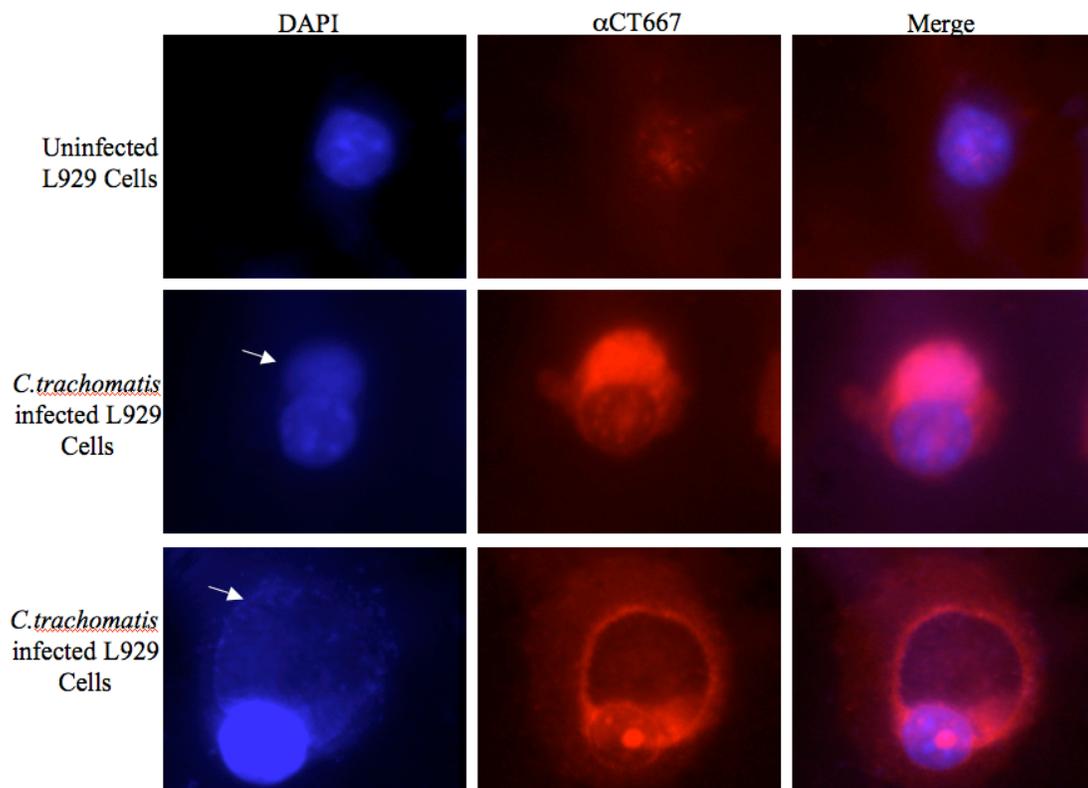


CT667 is detected in chlamydial inclusions at 36 hpi

To determine whether CT667 was present in eukaryotic host cells during a *C. trachomatis* infection, an immunofluorescence assay was performed on infected cells. L929 mouse fibroblasts were infected with *C. trachomatis* L2/434/Bu and at 36 hpi, in correlation with the protein expression profile, cells were stained using affinity purified rat anti-CT667 antibodies, goat anti-rat Alexa Fluor 568 conjugated antibodies (Invitrogen) and DAPI (Vector Laboratories) (**Fig. 13**). Host cell nuclei and chlamydial inclusions were stained with DAPI and visualized as blue fluorescence. CT667 was visualized through red fluorescence. CT667 was detected in the inclusion as a red fluorescence at 36 hpi. In cells that were incubated with *C. trachomatis* but did not become infected, no red fluorescence pattern was observed (**Fig. 13, top panel**). In cells infected with *C. trachomatis*, two different inclusion sizes were observed. In cells with smaller inclusions than those typically observed at 36 hpi, CT667 was detected throughout the inclusion. In this condition, minimal red fluorescence was observed in the cytosol of the L929 cells, possibly indicating translocation of the protein (**Fig. 13, middle panel**). In cells with larger inclusions, indicative of a 36 hour infection, CT667 appeared to be localized at the inclusion membrane (**Fig. 13, bottom panel**). There was also red staining observed in the nucleus and the cytosol of the L929 cell as well. The presence of CT667 in the inclusion membrane later in the developmental cycle could be in preparation for lysis. Due to the large inclusion size at this portion of the developmental cycle, CT667 could not be observed in the cytosol of the host in the majority of the cells; however,

Figure 13. CT667 is detected in *C. trachomatis* infected cells at 36 hpi.

L929 mouse fibroblasts were infected with *C. trachomatis* L2/434/Bu and stained for the intracellular presence of CT667 at 36 hpi. Host cell nuclei are stained with DAPI and visualized as blue fluorescence. Chlamydial inclusions are visualized with DAPI and denoted with white arrows. CT667 is visualized as red fluorescence.



in some cells where the cytosol could still be observed, red fluorescence was detected.

DISCUSSION

Elucidating the manner by which *Chlamydia* is able to invade and exist in an intracellular environment is crucial to understanding the pathogenesis of this organism. The chlamydial T3SS provides a mechanism for communication with the host through the secretion of effector proteins and is likely requisite for growth and pathogenesis. CT667 is transcriptionally-linked to T3SS homologues, suggesting a similar or biological function, and contains TPR domains, further supporting that it plays a significant cellular role. Frequently, proteins with TPR domains serve multiple functions including facilitating protein-protein interactions and chaperoning functions.

The gene encoding CT667 is located immediately downstream of predicted T3SS needle protein, CdsF, and data supports that it has chaperoning function (Betts, Twiggs et al. 2007). Our research has shown that CT667 can be secreted and translocated through a T3SS, supporting the hypothesis that it has effector function (**Fig. 8, 9, and 11**). Therefore, CT667 likely has dual function for the organism, similar to many other T3SS proteins.

Multiple functions for CT667 were supported by various localization patterns following translocation in the heterologous system (**Fig. 9 and 11**). In the ReAsH experiments, CT667 appeared to localize to the cytosol of Henle cells after being translocated by the *S. flexneri* T3SS (**Fig 9**). In contrast, CT667 appeared to have nuclear localization following translocation by the *S. flexneri* T3SS into L929 cells.

The reason for the differences in localization are not clear; however, the 4 cys tag present during the invasion assay into Henle cells could have had an effect on the secretion pattern of the protein. Additionally the differences between the Henle, human colonic epithelial cells, and L929, mouse fibroblast cells, could be responsible for the different localization patterns of CT667.

Though the ReAsH experiment results indicated an intracellular presence of CT667, the results were not completely reproducible and somewhat ambiguous (**Fig. 9, and 10**). Through ReAsH technology, CT667 was visualized as a red punctate staining pattern in Henle cells, though the staining pattern could not be directly correlated with *S. flexneri* infection (**Fig. 9**). GFP was used in attempts to visualize individual bacteria during invasion, but GFP was not stably maintained within *S. flexneri* and incomplete labeling was observed. As a result, cells with the red punctate staining pattern, indicative of CT667 intracellular presence, were seen in absence of associated *S. flexneri*. Though there was a statistically significant difference in the amount of red fluorescence in host cells exposed to the T3SS+ and T3SS- *S. flexneri* strains expressing CT667, the absence of associated bacteria could indicate continuous secretion of the protein verses translocation induced through host cell contact (**Fig. 10**).

In attempts to observe *S. flexneri* in association with target cells containing CT667, an immunofluorescence assay using *Shigella* specific antibodies and anti-CT667 antibodies, was performed (**Fig. 11**). Nuclear localization of the protein was observed through red fluorescence upon addition of T3SS+ strain expressing the

protein (**Fig. 11, bottom panel**). However, there were cells that appeared to have CT667 in the cytoplasm that were not infected with *S. flexneri*. Though the *Shigella*-specific antibodies provided a mechanism to visualize the bacteria during the infection, the gentamycin protection assay only allowed for the detection of intracellular bacteria. The presence of fluorescence in cells not infected with *S. flexneri* could have occurred because the bacteria associated with the cell and did not invade, but the protein was still translocated through the needle into the cytoplasm. The gentamycin protection assay eliminates all extracellular bacteria; therefore, a translocation event could have occurred, but *S. flexneri* would not be associated with the cell.

These results were further compromised by the occasional appearance of red fluorescence in the T3SS- *S. flexneri* strain, where it should not be possible for secretion to occur as a functional needle is not formed. This could be due to recombination events allowing the T3SS- *S. flexneri* strain to produce the MxiH needle monomer allowing for a functional T3SS to be formed, through which the protein could be secreted. This may indeed have happened as following transformation of CT667 into MxiH⁺ *S. flexneri*, a mixed population of both red and white colonies on Congo red plates were observed. Even after a white isogenic clone was selected through red/white screening on Congo red plates, subsequent plating of this *S. flexneri* strain would produce both red and white colonies. After performing an invasion assay, it was determined that some invasion into host cells was possible when a mixed population of colony types was observed.

Evidence suggests that the T3SS in *Chlamydia* functions during entry into the host cell as well as throughout the developmental cycle for maintenance of the intracellular lifestyle (Subtil, Blocker et al. 2000). When monitoring the location of CT667 during a chlamydial infection multiple localization patterns were observed similar to the heterologous system. In cells infected with *C. trachomatis* two different inclusion sizes were observed. Inclusion size is an indicator of the time point and progression of the developmental cycle. Though the immunofluorescence assay was performed at 36 hpi, the smaller inclusions could be representative of an earlier time point. During a chlamydial infection proteins can be secreted into the host during entry, into the inclusion lumen for maintenance of the vacuole, and into the inclusion membrane in preparation for lysis. (Jorgensen and Valdivia 2008) (Rockey, Scidmore et al. 2002) The different localization patterns of CT667 during a *C. trachomatis* infection could be due to the different functions of the T3SS throughout the developmental cycle.

The immunofluorescence assay performed on *C. trachomatis* infected cells was challenged by the large inclusion size in most cells at 36 hpi. Because CT667 protein expression is highest at 36 hpi, this time point was optimal for intracellular detection of the protein (**Fig. 13**). At 36 hpi during a *C. trachomatis* infection, the RBs are converting back into EBs and the inclusion is taking up the majority of the cell (**Fig. 1**). In cells with smaller inclusions, CT667 expression is detected throughout the inclusion, and a low-level red fluorescence could be observed in the cytosol, though the cytoplasmic space is still limited (**Fig. 13, middle panel**). This

fluorescence pattern may represent protein translocation, but it was not observed in all cells infected with *C. trachomatis*. In most cases, it was not possible to observe whether CT667 was in the cytosol of the cells with the large inclusions due to the limited cytoplasmic space (**Fig. 13, bottom panel**). Cells with large inclusions tend to adhere poorly to the coverslips, making it difficult to observe secretion of CT667. In a few cells that did remain flat on the coverslips, the cytosol did have a low-level red fluorescence pattern (**Fig. 13, bottom panel**). At 24 hpi the inclusion size would be small enough to still observe the cytoplasmic space; however, CT667 expression is not high enough at that time point to detect through immunofluorescence assay. An immunofluorescence assay was performed at 30 hpi, but there was very little cytoplasmic space left to observe within the cells, and CT667 expression was not high enough to detect (**data not shown**).

In the absence of a system for genetic exchange in *Chlamydia*, the direct role of CT667 in pathogenesis could not be established; however, the heterologous *S. flexneri* system provided a means to study the chlamydial T3SS protein. Through the Congo red induction data and the appearance of CT667 in the cytosol of eukaryotic host cells, this study does suggest that CT667 is secreted in a type three dependent manner and may have effector function (**Fig. 8, 9, and 10**). The chlamydial T3SS is likely requisite for growth and intracellular survival, and provides a mechanism for the organism to communicate with and manipulate the eukaryotic host through the inclusion membrane. As such, CT667 may be intimately linked to

the pathogenesis of the organism and serve multiple roles throughout the developmental cycle including facilitating infection.

III. *In vivo* analysis of the DNA binding activity of a transcriptional regulator in *C. trachomatis*, ChxR.

INTRODUCTION

Global gene expression profiling demonstrated that transcriptional regulation plays a vital role in the developmental cycle of *C. trachomatis*; however, little is known about the mechanisms that mediate this process (Koo and Stephens 2003). Microarray analyses indicate that approximately 70 % of the open reading frames in the genome were expressed at 6 hpi and levels remained constitutive throughout the developmental cycle. Approximately 20 % of the open reading frames were upregulated at 18 - 24 hpi, consistent with the phenotypic changes observed during the RB to EB conversion of the developmental cycle (Belland, Zhong et al. 2003; Nicholson, Olinger et al. 2003). Transcription of the constitutively expressed genes and many of the differentially expressed genes has been demonstrated to be dependent upon σ^{66} , a σ^{70} homologue (Fahr, Douglas et al. 1995).

Because σ^{66} regulates the genes that are on at 6 hpi as well as those turned on at 18 - 24 hpi, additional factors are hypothesized to be important in the upregulation of gene expression seen during the RB to EB conversion, though very few transcriptional regulators are encoded within *C. trachomatis* (Fahr, Douglas et al. 1995) (Hefty and Stephens 2007).. An atypical response regulator termed ChxR is

encoded within *C. trachomatis*. It has been demonstrated to activate transcription *in vitro*, and it is upregulated at 18 – 24 hpi (Koo and Stephens 2003).

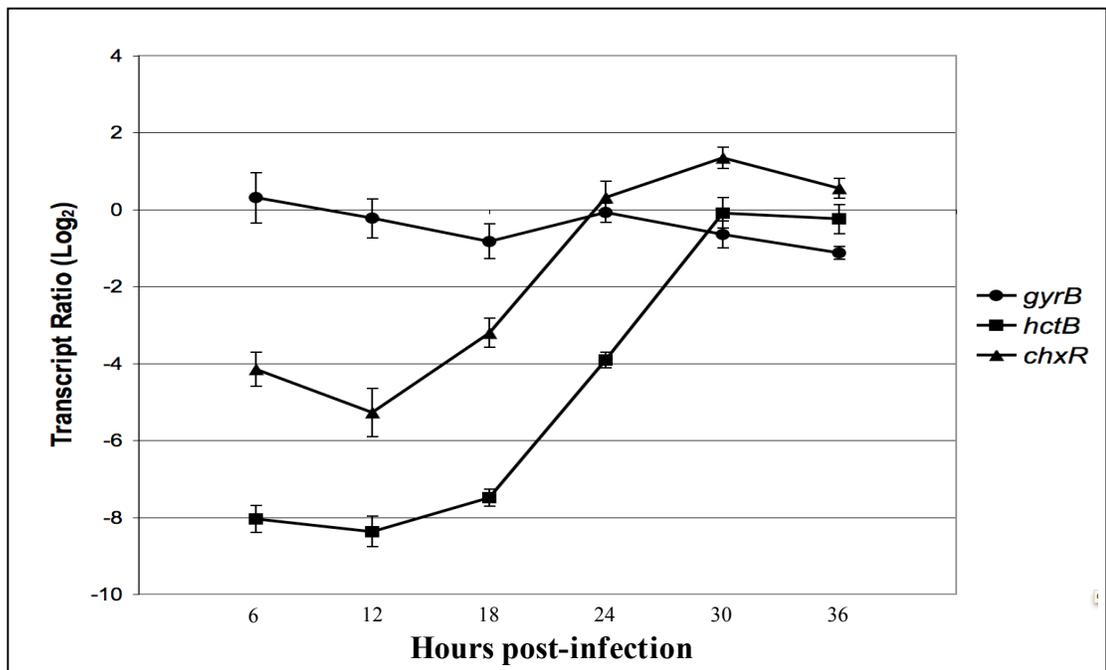
Two-component signal transduction systems are used to respond to environmental signals and mediate changes in transcription. They are integral to various bacterial processes, including virulence and development (Beier and Gross 2006). In a typical two-component signal transduction system, when an environmental signal is recognized, a sensor kinase is autophosphorylated and transfers the phosphoryl group to the response regulator. Upon addition of the phosphoryl group, the response regulator is activated and can in turn, activate transcription (Itou and Tanaka 2001). OmpR family response regulators contain a winged helix–turn–helix motif in the DNA binding domain and contain a conserved phosphorylated aspartate which allows for protein activation and transcriptional regulation of gene targets (Martinez-Hackert and Stock 1997).

Atypical response regulator homologues have been identified that do not retain the conserved aspartate at the phosphorylation site, and independent of phosphorylation, remain transcriptionally active (Rotter, Muhlbacher et al. 2006). Additionally, disruption of these response regulator homologues can cause severe impairment in growth or pathogenesis similar to the typical response regulators, suggesting they too play important biological roles (Beier and Gross 2006). ChxR, a unique member of the OmpR family of response regulators, is novel in that it contains a glutamate in place of the conserved aspartate. ChxR has no apparent cognate sensor kinase and is able to remain transcriptionally active in the absence of

phosphorylation. ChxR does retain the highly conserved winged helix-turn-helix motif that is able to bind DNA and has been demonstrated to activate transcription (Koo, Walther et al. 2006). *chxR* is also among those genes turned on during the RB to EB conversion of the developmental cycle (**Fig. 14**). Therefore, we hypothesize that ChxR is a transcriptional activator of multiple genes during the RB to EB conversion of the *C. trachomatis* developmental cycle.

Figure 14. qPCR data shows *chxR* is upregulated at 18 - 24 hpi.

Gyrase (*gyrB*) is a constitutively expressed gene involved in DNA replication and has similar transcript levels from 6 – 36 hpi. Histone like protein (HctB) is differentially regulated and *hctB* mRNA levels increase from 18 - 24 hpi. *chxR* transcript levels increase from 18 - 24 hpi and remain constant through 36 hpi.



(Courtesy of Megan Buechele)

MATERIALS AND METHODS

Chlamydial EB seed prep

L929 mouse fibroblast cells were grown to 8×10^5 cells/mL in a one liter spinner flask in RPMI (Mediatech Inc, Manassas, VA) supplemented with 50 μ g/mL Vancomycin and 5 % FBS. Cells were infected with *C. trachomatis* L2/434/Bu at an MOI of ~ 1 . At 48 hpi, infected L929 cells were transferred into four sterile 250 mL centrifuge tubes. Cells were collected through centrifugation (1,380 x g, 10 min, 15 °C) and the supernate was removed. Cells were washed in 200 mL HBSS and collected through centrifugation (1,380 x g, 10 min, 15 °C). The supernate was removed and the cells were resuspended in 50 mL HBSS, and separated into 2 sterile 40 mL Oak Ridge tubes. The cells were sonicated six times on setting six (Sonic Dismembrator 100) for 15 seconds each while placing on ice in between sonications. The cellular debris was pelleted through centrifugation (150 x g, 10 min, 15 °C) and the supernate was transferred to sterile 40 mL Oak Ridge tubes. The supernate was centrifuged (28,000 x g, 30 min, 4 °C) and the resulting supernate was removed. The chlamydial EBs were resuspended in two mL SPG and sonicated two times on setting four times for three seconds each while placing on ice in between sonications. The cells were and aliquotted on ice and immediately transferred to -80 °C.

Chlamydial RB harvest

L929 mouse fibroblast cells were grown to 8×10^5 cells/ mL in a one liter spinner flask in RPMI (Mediatech Inc) supplemented with 50 $\mu\text{g}/\text{mL}$ vancomycin and 5 % FBS. Cells were infected with *C. trachomatis* L2/434/Bu at an MOI of ~ 1 . At 12, 24, and 36 hpi, 500 mL, 300 mL, and 200 mL respectively were transferred into sterile 250 mL centrifuge tubes. L929 cells were collected through centrifugation (1,400 x g, 10 min, 15 °C) and the supernate was removed. Cells were washed twice in 200 mL HBSS and collected through centrifugation (1,400 x g, 10 min, 15 °C). The supernate was removed and the cells were resuspended in a total volume of 30 mL HBSS at each time point and transferred to sterile 40 mL Oak Ridge tubes. The cells were sonicated two times on setting four (Sonic Dismembrator 100) for 15 seconds each while placing on ice in between sonications. Disruption of the L929 cells was verified through light microscopy. The cellular debris was pelleted through centrifugation (150 x g, 10 min, 15 °C) and the supernate was transferred to sterile 40 mL Oak Ridge tubes. The supernate was centrifuged (12,000 x g, 15 min, 15 °C) and the pelleted cells from each time point were resuspended in 500 μL PBS.

In vivo Immunoprecipitation of ChxR

L929 mouse fibroblast cells were grown to 8×10^5 cells/ mL in a one liter spinner flask in RPMI (Mediatech Inc) supplemented with 50 $\mu\text{g}/\text{mL}$ vancomycin and 5 % FBS. Cells were infected with *C. trachomatis* L2/434/Bu at an MOI of ~ 1 . At 36 hpi, cells were collected and enriched for RBs using the RB harvest protocol.

Pelleted cells were resuspended in two mL of RIPA buffer (10 mM Tris-Cl pH 8, 1 mM EDTA, 1 % Triton, 0.1 % sodium deoxycholate, 0.1 % SDS, 140 mM NaCl, 5 mM DTT) with 15 μ L AEBSF (ThermoFisher Scientific) and incubated on ice for 45 minutes in the hood (rocked every 10 minutes by hand). Following lysis and incubation, cellular debris were centrifuged (14,000 x g, 15 min, 24 °C) and supernates were separated into 500 μ L aliquots and stored at -20 °C until use. For immunoprecipitation, 25 μ L of Protein G dynabeads (Invitrogen) were washed three times with one mL of RIPA Buffer then resuspended in 120 μ L of RIPA buffer. Ten μ g of affinity-purified rat anti-ChxR polyclonal antibodies were added to the beads and incubated at 4 °C for 24 hours while rotating. For antibody negative control, equal volume of RIPA buffer was substituted for anti-ChxR antibody. Beads were washed two times with one mL RIPA buffer, then 500 μ L of infected lysate was added to the beads. Samples were incubated at 4 °C for 24 hours while rotating. Beads were washed twice with one mL RIPA buffer, then resuspended in one mL RIPA buffer, and incubated at 25 °C for two hours while rotating. Beads were washed five times with one mL RIPA buffer and resuspended in 15 μ L FSB. Samples were boiled 15 min, vortexed, and boiled again for 15 min, then centrifuged (13,000 x g, 3 min, 25 °C). The supernate was collected and western blot analysis was performed using polyclonal rabbit anti ChxR antibodies diluted at 1:1000 in 5.0 % (w/v) non-fat dry milk in PBS + 0.2% Tween and IR tagged goat anti-rabbit IgG (Rockland) diluted at 1:5000 in 5.0 % (w/v) non fat dry milk in PBS + 0.2 % Tween.

In vivo immunoprecipitation of ChxR with formaldehyde crosslinking

L929 mouse fibroblast cells were grown to 8×10^5 cells/mL in a one liter spinner flask in RPMI (Mediatech Inc) supplemented with 50 $\mu\text{g/mL}$ vancomycin and 5 % FBS. Cells were infected with *C. trachomatis* L2/434/Bu at an MOI of ~ 1 . At 36 hpi, cells were collected and enriched for RBs using the RB harvest protocol. Pelleted cells were resuspended in 100 μL of 1 % formaldehyde, incubated on ice for 20 min (rocked every 5 min by hand), and quenched with 500 μM glycine (pH = 7) in equal volume. After crosslinking, 1.8 mL of RIPA buffer (10 mM Tris-Cl pH 8, 1 mM EDTA, 1 % Triton, 0.1 % sodium deoxycholate, 0.1 % SDS, 140 mM NaCl, 5 mM DTT) with 15 μL AEBSF (Thermofisher Scientific) was added and cells were incubated on ice for 45 minutes (rocked every 10 minutes by hand). The cells were sonicated six times on setting six (Sonic Dismembrator 100) for 15 seconds each while placing on ice in between sonications. Cells were centrifuged (14,000 $\times g$, 15 min, 24 $^{\circ}\text{C}$) and supernates were collected and separated into 500 μL aliquots and stored at -20 $^{\circ}\text{C}$ until use. For immunoprecipitation, 25 μL of Protein G dynabeads (Invitrogen) were washed three times with one mL of RIPA Buffer then resuspended in 120 μL of RIPA buffer. Ten μg of affinity purified rat anti-ChxR antibodies were added to the beads and incubated at 4 $^{\circ}\text{C}$ for 24 hours while rotating. For antibody negative control, equal volume of RIPA buffer was substituted for anti-ChxR antibodies. Beads were washed two times with one mL RIPA buffer, then 500 μL of infected lysate was added to the beads. Samples were incubated at 4 $^{\circ}\text{C}$ for 24 hours while rotating. Beads were washed twice with one mL RIPA buffer, then

resuspended in one mL RIPA buffer, and incubated at 25 °C for two hours while rotating. Beads were washed five times with one mL RIPA buffer and resuspended in 30 µL TE (10 mM Tris-Cl, 1 mM EDTA, pH 7.4) to elute the DNA. Samples were boiled five minutes to reverse the crosslinks and then centrifuged (13,000 x g, 3 min, 25 °C) to separate out the beads. The supernate was collected and DNA was amplified using PCR.

Table 2: Primers for T3SS operon promoters

Designation: ChxR Pro 5' Description: bases -291 > -270 Sequence 5'-3' CGATATCAACGGCTATAGAAGC
Designation: ChxR Pro 3' Description: bases +1 > -25 Sequence 5'-3'TAGATTACCTAATACAACAAAATAG
Designation: CT091 Pro 5' Description: bases-319 > -294 Sequence: 5'-3' GCGAATCCTGATTTCCCTACAT
Designation: CT091 Pro 3' Description: bases +50 > +21 Sequence: 5'-3'GCGTTTTCTAGCATCCCGAA
Designation: CT557 Pro 5' Description: bases Sequence: 5'-3'GCGCGGCCGTTACGTTCCCTATTTTTCTCTCC
Designation: CT557 Pro 3' Description: bases Sequence 5'-3'GCGCGGCCGATTTCCCTCTTCAATGAGTGTTTAGG
Designation: CT559 Pro 5' Description: bases Sequence: 5'-3'GCGCGGCCGCCATTCGTTTCGTTCTAGCTTTAAT
Designation: CT559 Pro 3' Description: bases Sequence 5'-3' GCGCGGCCGAACCCACGCACACCTTTCACTCACC
Designation: CT576 Pro 5' Description: bases -327 > -352 Sequence: 5' -3' GCGAATAAACGAATCCGAGAA
Designation: CT576 Pro 3' Description: bases > +50 Sequence: 5'-3' GCGTTTTATTAAAAGAGGCCG
Designation: CT663 Pro 5' Description: bases -353 > -332 Sequence: 5'-3' GCGTGGATACATACTTTTCG
Designation: CT663 Pro 3' Description: bases +71 > +50 Sequence: 5'-3' GCGATTCGGAAGCCACTCC
Designation: CT665 Pro 5' Description: bases Sequence: 5'-3'CAAGAAGTCAAGATATTGCAGGG
Designation: CT665 Pro 3' Description: bases Sequence 5'-3'CATAGATTTATTCCTACCTGGAAG
Designation CT863 Pro 5' Description: bases -321 > -287 Sequence: 5'-3'GCGGCAATAGGTTAATCGTCT
Designation: CT863 Pro 3' Description: bases +35 > +55 Sequence: 5'-3' GCGCCTTAAGAGAAGCGTTT

Table 3. Primers for constitutively expressed, midlate, and late genes

Designation: CT032/033 Pro 5'
Sequence 5'-3' ACATTCCTTAGATCTAGGTTCCC
Designation: CT032/033 Pro 3'
Sequence 5'-3' GCTATTGCTGTTTCGTAATAATAAGG
Designation: CT323/324 Pro 5'
Sequence: 5'-3' CATTCCCTAAAAAACCTAAAAAC
Designation: CT323/324 Pro 3'
Sequence: 5'-3' CATGGCCTATCTACTCTAC
Designation: CT444 Pro 5'
Sequence: 5'-3' AACTTCCAGACTCCTTTCTAG
Designation: CT444 Pro 3'
Sequence 5'-3' TCTGTTTGTTATCTATAGTTTAG
Designation: CT480 Pro 5'
Sequence: 5'-3' CATGCGTTGTCTCCTCTATTCG
Designation: CT480 Pro 3'
Sequence 5'-3' GAAGGGGAAGAAGGTGGAGACG
Designation: CT619/620 Pro 5'
Sequence: 5' -3' ATAAAGTCCTGAGTTTAACTCC
Designation: CT619/620 Pro 3'
Sequence: 5'-3' GCTCTATTTCTGTTTTTTGAAAC
Designation: CT733/734 Pro 5'
Sequence: 5'-3' CATATGATCCTCTGCTACTC
Designation: CT733/734 Pro 3'
Sequence: 5'-3' CATAAACCTGCCAAAATTA AAAATA
Designation: CT870/871 Pro 5'
Sequence: 5'-3' CGCAGGT TAAAAGGGGGATG
Designation CT870/871 Pro 5'
Sequence: 5'-3' AAAGGCAAAGCTGCAATAGAATTG

RESULTS

Protein Expression Profiling of ChxR indicates expression at 24 hpi and increases through 36 hpi.

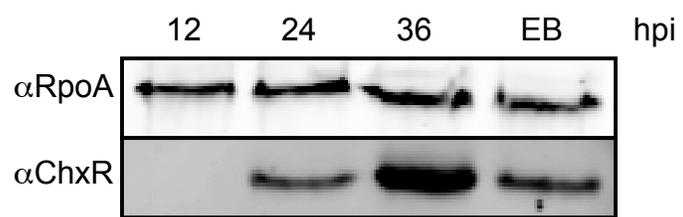
Through qPCR it was determined that *chxR*, is among those genes turned on during 18 - 24 hpi (**Fig. 14**) (Buechele and Hefty, unpublished). To determine if post-transcriptional mechanisms of ChxR regulation are occurring, ChxR protein levels throughout the developmental cycle were analyzed. L929 mouse fibroblasts were infected with *C. trachomatis* L2/434/Bu and collected and enriched for RBs at 12, 24, and 36 hpi. Chlamydial EBs were also analyzed for ChxR protein expression. Protein levels were normalized with chlamydial RNAP (RpoA) and samples were analyzed through western blotting (**Fig. 15**). Using polyclonal rat anti-ChxR antibodies, ChxR protein was detected at 24 hpi and expression reached peak levels at 36 hpi. Expression at 24 - 36 hpi suggests ChxR has functional activity during the middle and late portion of the *C. trachomatis* developmental cycle. ChxR was also detected in ultra-purified EBs suggesting a role in entry and infection. The presence of ChxR in both phenotypic stages further suggests that it may regulate genes throughout developmental cycle.

In vivo immunoprecipitation of ChxR from a *C. trachomatis* infected lysate

Prior *in vitro* studies demonstrated that ChxR is able to bind its own promoter and activate transcription (Koo, Walthers et al. 2006). In the absence of a system for

Figure 15. Protein expression profile of ChxR from purified RBs and EBs.

L929 mouse fibroblasts were infected *C. trachomatis* L2/484/Bu. Cells were enriched for RBs, collected at 12, 24 and 36 hpi, and subjected to western blot analysis. EBs were ultrapurified and collected at 48 hpi. **Top panel:** Bacterial levels were normalized with chlamydial RNAP (RpoA). **Bottom panel:** RBs and EBs were analyzed for ChxR levels using anti-ChxR antibodies



In vivo immunoprecipitation of ChxR from a *C. trachomatis* infected lysate

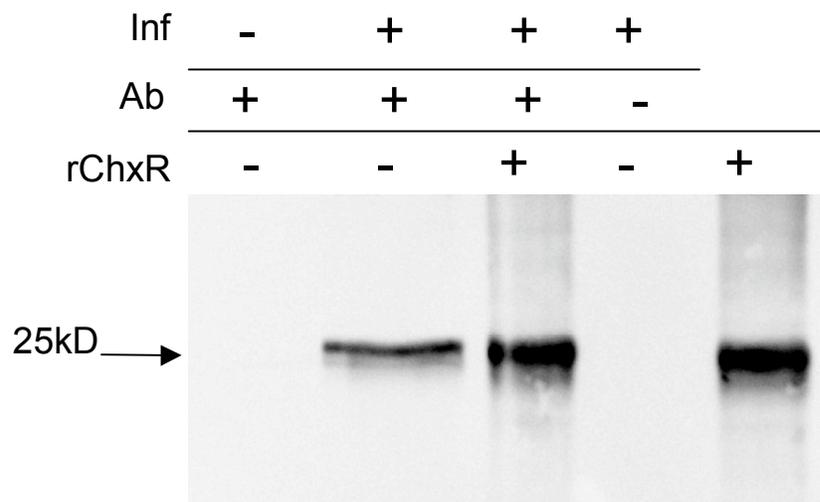
Prior *in vitro* studies demonstrated that ChxR is able to bind its own promoter and activate transcription (Koo, Walther et al. 2006). In the absence of a system for genetic exchange in *Chlamydia*, functional *in vivo* studies have proved difficult. To study whether or not immunoprecipitation could be used to study ChxR in an *in vivo* setting, ChxR first had to be immunoprecipitated during a *C. trachomatis* infection. To isolate ChxR from a *C. trachomatis* infected lysate, affinity purified rat anti-ChxR antibodies were used to pull-down ChxR at 36 hpi, at the time of optimal protein expression. Following immunoprecipitation, western blot analysis was performed, and ChxR was effectively detected from an infected lysate (**Fig. 16, lane 2**). Lack of ChxR detection in the absence of antibodies, supported the specificity of the antibodies (**Fig. 16, lane 4**). When uninfected L929 cells were used during the immunoprecipitation, ChxR was also not detected, further indicating this technique could be used to correlate a function for ChxR through an *in vivo* analysis of specific gene targets (**Fig. 16, lane 1**).

ChxR is associated with its own promoter *in vivo*

In an *in vitro* analysis, and a heterologous system, it was previously demonstrated that ChxR was an autoregulator and was able to bind to its promoter and activate transcription (Koo, Walther et al. 2006). To determine if ChxR associates with its promoter region *in vivo*, we used formaldehyde crosslinking and immunoprecipitation to isolate specific DNA sequences that were bound by ChxR

Figure 16. ChxR can be immunoprecipitated from an infected lysate.

L929 mouse fibroblast cells were infected with *C. trachomatis* L2/434/Bu. Cells were enriched for RBs and collected at 36 hpi. Immunoprecipitation using affinity purified rat-anti ChxR antibodies was used to isolate ChxR and samples were analyzed through western blot using rabbit anti-ChxR antibodies. **Lane 1:** In uninfected L929 mouse fibroblasts. **Lane 2:** *C. trachomatis* infected lysate immunoprecipitated with specific antibodies present. **Lane 3:** Recombinant ChxR immunoprecipitated through *in vitro* immunoprecipitation. **Lane 4:** *C. trachomatis* infected lysate with no antibodies present during immunoprecipitation. **Lane 5:** Recombinant ChxR.



that may be upstream of specific gene targets for ChxR (**Fig. 17**). At 36 hpi, ChxR was crosslinked to DNA with formaldehyde, immunoprecipitated, and the DNA bound by ChxR was eluted and amplified using PCR. To determine that DNA specifically bound by ChxR was isolated, amplification of the ChxR promoter region was used as a positive control because ChxR functions as an autoregulator (**Fig. 18, lane 3**). The specificity of the DNA isolated was supported through the absence of amplification in conditions where the antibody was not present during immunoprecipitation (**Fig. 18, lane 4**). For a negative control, CT863 promoter region was tested. *CT863* is hypothesized to be involved in *C. trachomatis* T3S; however, through qPCR is shown to be constitutively expressed and thus is not likely a gene target of ChxR (Hefty and Stephens 2007). Following immunoprecipitation with anti-ChxR antibody present, *CT863* promoter region DNA was not amplified (**Fig. 18, lane 7**). These data support that ChxR is associated with its own promoter *in vivo* and that immunoprecipitation can be used to isolate ChxR gene targets.

ChxR binding motif contains conserved sequences

OmpR family response regulator homologues have been demonstrated to bind DNA in tandem repeat sequences (Harlocker, Bergstrom et al. 1995). To identify the sequences that ChxR binds to activate transcription, the *chxR* promoter region was examined and a DNase protection assay revealed five putative binding sites (Koo, Walthers et al. 2006). Recently, the sequences of the protected sites were examined for a common DNA motif, and a repeat sequence was found in all five sites (Hickey,

Figure 17. Schematic of *in vivo* crosslinking with formaldehyde.

Protein G beads are used to bind affinity purified rat anti-ChxR antibodies. Through formaldehyde crosslinking ChxR is covalently bound to DNA. Heat is used to reverse the crosslinks and the DNA bound by ChxR is eluted in TE and beads are separated through centrifugation.

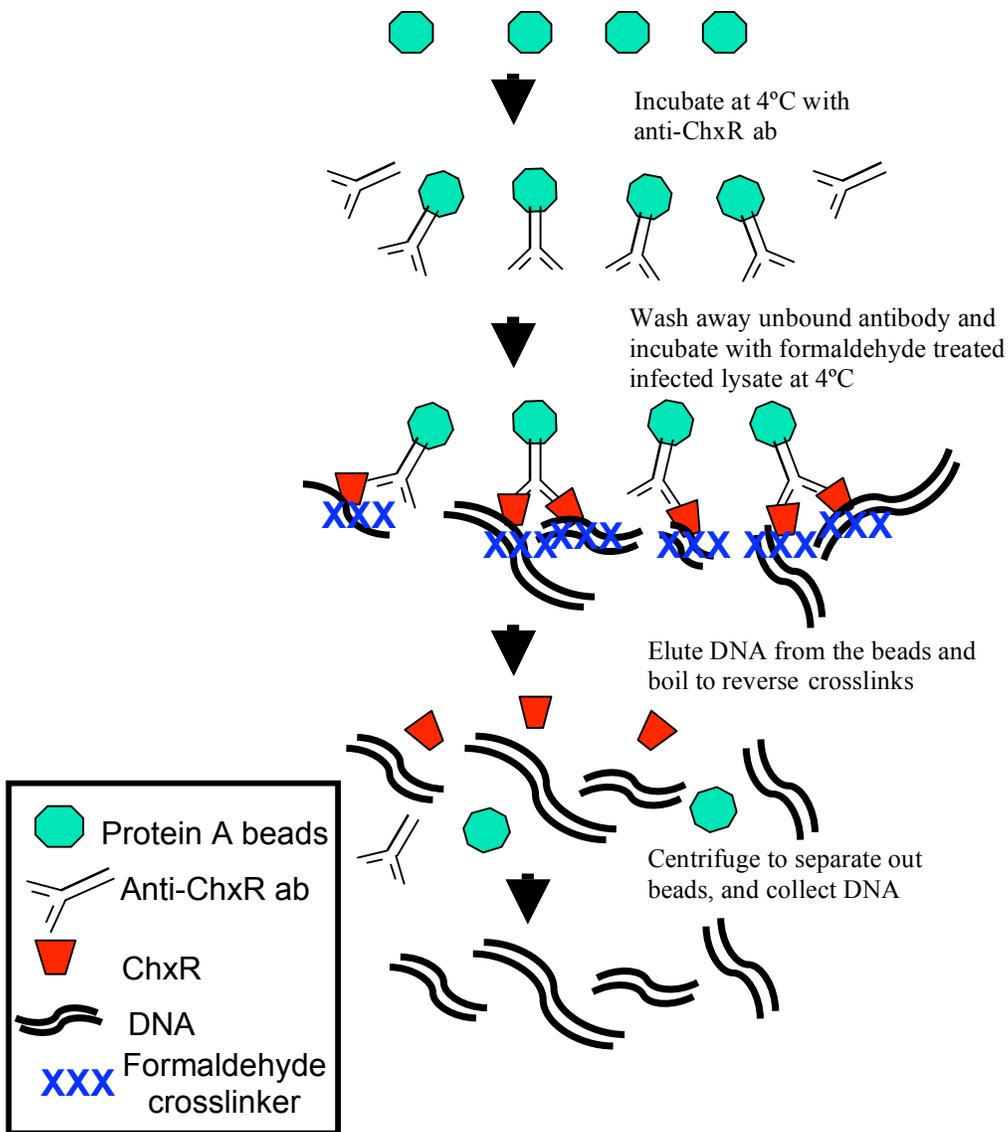
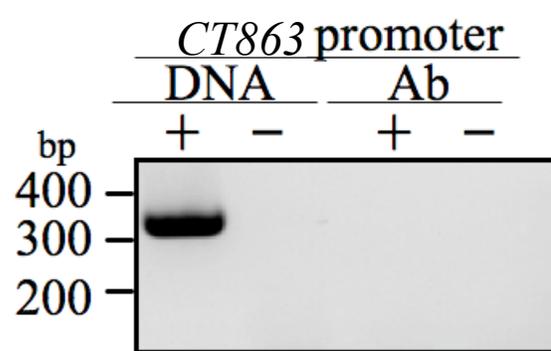
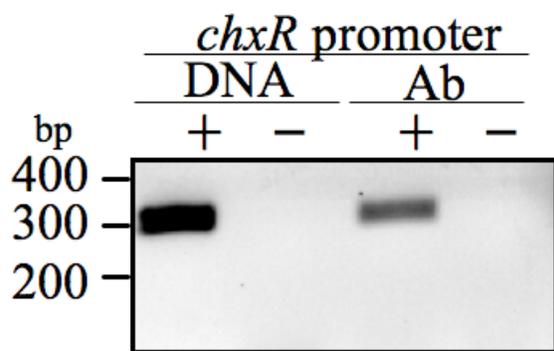


Figure 18. *In vivo* immunoprecipitation with formaldehyde crosslinking can be used to isolate ChxR gene targets.

Using formaldehyde crosslinking, specific gene targets of ChxR were isolated and amplified through PCR using primers for the *chxR* and *CT863* promoter region.

Lane 1: *C. trachomatis* L2/484/Bu genomic DNA amplified with *chxR* promoter region or *CT863* promoter region primers. **Lane 2:** No genomic DNA. **Lane 3:** PCR products obtained through using primers for *chxR* promoter region or *CT863* promoter region and DNA isolated from *in vivo* immunoprecipitation. **Lane 4:** No antibody present during immunoprecipitation.



Hefty et al 2009). Through defining the conserved *cis*- acting elements recognized by ChxR, additional gene targets could be predicted through analyzing promoter regions for the recognition sequence.

ChxR binds to the promoter region of many T3S operons

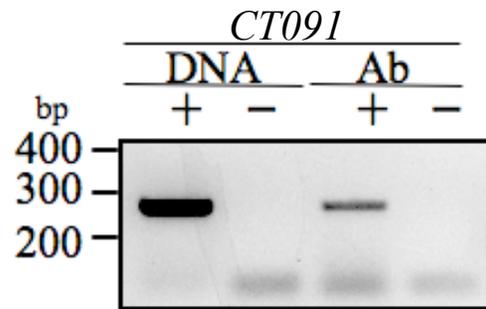
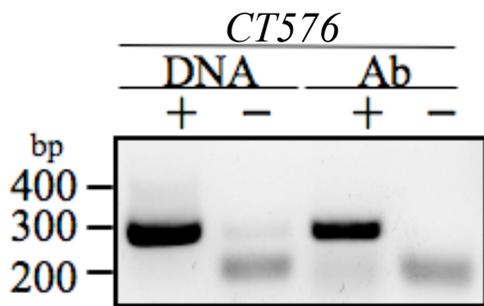
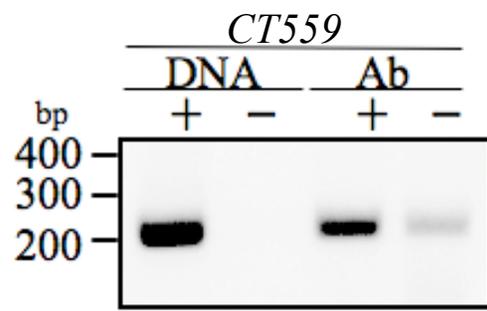
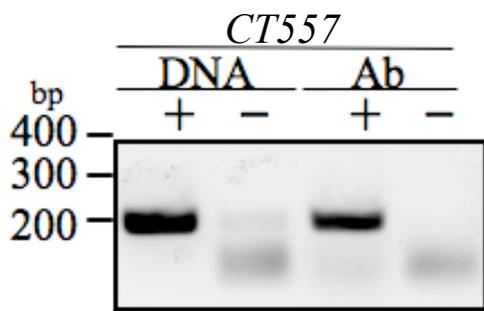
Many chlamydial T3S genes were determined through qPCR to be upregulated during the RB to EB conversion of the developmental cycle at 18 - 24 hpi (**Fig. 4**) (Hefty and Stephens 2007). Because ChxR transcriptional profiling indicates upregulation at this time (**Fig. 14**), we hypothesized ChxR may regulate expression of the chlamydial T3SS genes. The predicted binding sequence that ChxR recognizes was used to elucidate chlamydial T3SS genes that ChxR might also regulate. Sequence analysis of the T3S operon promoters, revealed regions with proposed ChxR binding sites (**Fig. 19**). Putative ChxR binding sequences were found in *CT091*, *CT557*, *CT559*, *CT576*, *CT665*, *CT669*, and *CT674*. DNA isolated from formaldehyde crosslinking and *in vivo* immunoprecipitation of ChxR was used to amplify regions upstream of the transcriptional start sites for the T3S promoters. Through PCR it was shown that DNA for *CT091*, *CT557*, *CT559* and *CT576* promoter elements could be amplified (**Fig. 20**). The ability of the promoter regions of these genes to be bound by ChxR suggests it serves as an activator of these T3SS genes. The protein products of T3S genes typically play a role in virulence for other Gram negative organisms. The activation of these genes by ChxR would further

Figure 19. Proposed ChxR recognition sequences within T3SS genes.

Line arrows underneath sequences denote the hypothetical orientation of ChxR at each proposed ChxR recognition sequence. Relative location of binding sequences to respective transcriptional start sites is indicated above each sequence.

Fig 20. Promoter region of T3S operons immunoprecipitated with ChxR.

Following *in vivo* immunoprecipitation and crosslinking, DNA was amplified using primers for T3S promoter regions. **Lane 1:** *C. trachomatis* L2/434/Bu genomic DNA. **Lane 2:** No genomic DNA. **Lane 3:** DNA isolated from *in vivo* immunoprecipitation using anti-ChxR antibodies. **Lane 4:** No antibody present during immunoprecipitation. Immunoprecipitations were performed in triplicate and similar results were obtained.



support the hypothesis that ChxR is a global regulator in *Chlamydia* and plays a key role in pathogenesis for the organism.

ChxR is associated with the promoter region of midlate and late genes.

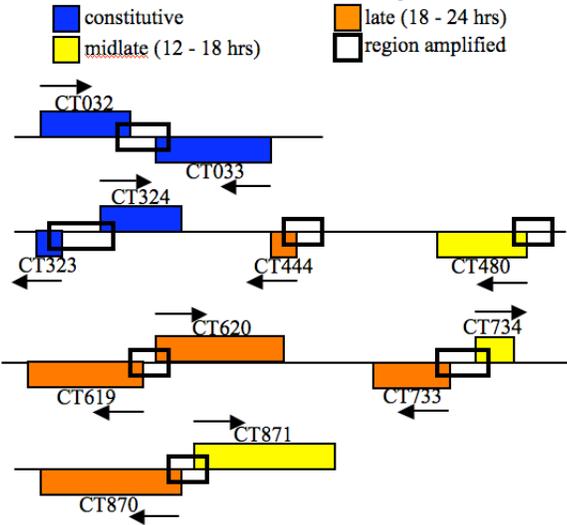
Constitutive genes are regulated by the σ^{70} homologue, σ^{66} , and are likely not targets of ChxR (Fahr, Douglas et al. 1995). ChxR is upregulated during the RB to EB conversion of the developmental cycle and thus could serve as a regulator for genes upregulated during the midlate (18 – 24 hpi) and late (>24 hpi) portion of the developmental cycle. Using the *C. trachomatis* microarray data, several of these genes were selected as possible targets of ChxR (**Fig. 21**). Specifically, the predicted promoter regions of divergent midlate or late genes were assessed for their ability to be bound by ChxR in attempts to elucidate two possible targets in a single region. Additionally, the individual promoter region for one midlate and late gene was also tested for the ability to immunoprecipitate with ChxR. DNA isolated from the formaldehyde crosslinking and *in vivo* immunoprecipitation of ChxR was used to amplify approximately 400 bps between the coding regions of the divergent genes or upstream of the transcriptional start sites of the individual genes. As predicted, the promoter regions of constitutively expressed *CT032/033* and *CT323/324* were not immunoprecipitated with ChxR. DNA from the predicted promoter regions for the midlate and late genes including *CT444*, *CT480*, *CT619/620*, and *CT733/734* was amplified (**Fig. 21**). The finding that the promoter regions of these genes immunoprecipitated with ChxR suggests the transcription of these genes may be

Figure 21. Promoter regions of midlate and late genes are immunoprecipitated with ChxR.

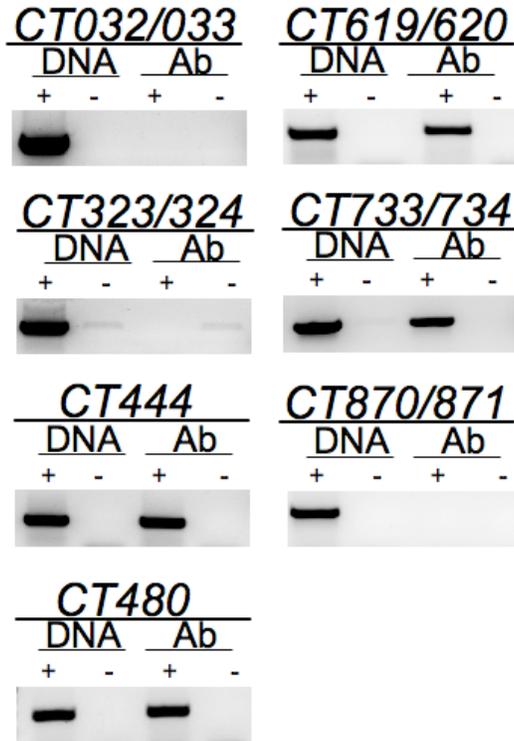
A. Model depicts constitutive, midlate, and late genes whose promoter regions were tested for their ability to be bound by ChxR through immunoprecipitation, formaldehyde crosslinking, and PCR. PCR was performed on the regions indicated (open boxes). Arrows denote direction of open reading frames. **B. Lane 1:** *C. trachomatis* L2/434/Bu genomic DNA. **Lane 2:** No genomic DNA. **Lane 3:** DNA isolated from *in vivo* immunoprecipitation using anti-ChxR antibodies. **Lane 4:** No antibody present during immunoprecipitation. Immunoprecipitations were performed in triplicate and similar results were obtained.

A.

C. trachomatis array data



B.



regulated by ChxR. However, *CT870/871* were not shown to associate with ChxR. The activation of specific genes through ChxR would further support the hypothesis that it is a global regulator in *Chlamydia* and plays a key role in pathogenesis for the organism.

DISCUSSION

Chlamydia are propagated through a bi-phasic developmental cycle and elucidating the mechanisms that control the cycle is of vital importance to understanding pathogenesis. In the absence of a system for genetic exchange in *Chlamydia*, the direct role of ChxR could not be studied. However, through determining the gene targets of ChxR, and exploring the role of the protein products of those genes, a function for ChxR can be assigned.

To isolate DNA specifically bound by ChxR, an *in vivo* immunoprecipitation technique with formaldehyde crosslinking was employed during optimal ChxR protein expression. Initial approaches at determining ChxR gene targets were aimed at cloning and sequencing the isolated DNA. The first approach included concentrating the isolated DNA with the Min-Elute Clean-up Kit (Invitrogen), polishing the ends of the DNA with T4 DNA polymerase (Promega), cloning into the pBlunt plasmid (Invitrogen), and transforming into *E. coli* Top10 cells (Invitrogen). Sequencing indicated that the resulting clones did not contain chlamydial DNA, but rather pieces of the pBlunt plasmid that were inadvertently ligated in through an unknown mechanism. Because the DNA was relatively low in concentration, another approach included amplifying the DNA prior to cloning through the REPLI-G kit (Invitrogen) or through PCR with random hexamers. Increasing the concentration did not aid in cloning the DNA, and despite the use of different vectors, cloning attempts

were ultimately not successful. Therefore individual promoters were tested via PCR to determine specific ChxR gene targets.

Because ChxR is upregulated during the RB to EB conversion of the developmental cycle, genes also upregulated at that time could serve as possible gene targets. Recent studies identified a DNA sequence proposed to be the sequence recognized by ChxR (Hickey, Hefty et al. 2009). This sequence was used to identify the promoter regions of T3SS operons that may be targets of ChxR (**Fig. 19**). Of the ten operons that contained homologs to T3SS genes (**Fig. 3**), seven contained genes that were differentially expressed and thus, possible targets for regulation by ChxR (Hefty and Stephens 2007)). Following formaldehyde crosslinking and *in vivo* immunoprecipitation of ChxR, DNA from the promoter regions of four of the T3SS operons was isolated, indicating it was able to associate with ChxR (**Fig. 20**).

The absence of the promoter region DNA from the other operons with differentially expressed genes does not definitely rule out regulation by ChxR. Perhaps there was a sensitivity issue that did not allow for amplification of these regions. However, the flagellar system is very similar to the T3SS and has a detailed expression pattern as the system is built (Blocker, Komoriya et al. 2003). Similarly, in other organisms containing T3SSs, the machinery is carefully regulated in a step-wise pattern (Eichelberg and Galan 1999). The chlamydial T3SS could also have a controlled sequential pattern of expression. The operons that we found to be regulated by ChxR include genes whose protein products are involved in making the basal body, spanning the periplasm, forming the translocon pore, and effector

function (**Fig. 2**). ChxR didn't associate with the operons containing genes whose protein products are outer membrane proteins, needle proteins, and many of the effector proteins. The chlamydial T3SS could have a differential expression pattern of expression of the components such as the apparatus, effectors, and regulators. The other operons could possibly be regulated by other factors to allow for this differential expression.

DNA isolated from the formaldehyde crosslinking and *in vivo* immunoprecipitation of ChxR was also used to amplify regions thought to contain the promoter regions for certain midlate and late genes (**Fig. 21**). *CT032/033* are constitutively expressed, convergent genes, and as such the region tested for amplification was not likely to be a binding site for activation through ChxR. This served as a negative control to ensure ChxR wasn't non-specifically binding DNA. *CT323/324* are constitutively expressed, divergent genes, and the region tested for amplification was likely to contain the promoter region for these genes. Though the predicted promoter region was tested, these genes were constitutively expressed and thus not hypothesized not be under the control of ChxR. As expected, this region was not amplified, further showing the specificity of the immunoprecipitation technique in isolating ChxR gene targets. Many of the midlate and late genes promoter regions tested for their ability to immunoprecipitate with ChxR were amplified. ChxR could serve as an activator of many other genes during the middle and late portion of the developmental cycle during the RB to EB conversion.

Antibiotic resistance has become increasingly more serious in recent years and new combinations of drugs are being used to treat infections that were once curable through routine antibiotic treatment. Current antibiotic agents are aimed at fundamental gene functions including DNA replication and others (Paterson 2008). New approaches to treat infection are under exploration in attempts to combat the problem. Targeting the regulation of virulence factors is one approach of recent interest in developing new therapies. A high through put screen of a small molecule library yielded a compound effective against virulence factor expression in *Vibrio cholera* (Hung, Shakhnovich et al. 2005). In *P. aeruginosa* a small molecule inhibitor of a two-component signal transduction system was identified and proposed as a potential therapy to treat infection (Roychoudhury, Zielinski et al. 1993). Very few transcription factors have been described in *Chlamydia* and elucidating the regulatory elements could provide new drug targets in the era of increasing antibiotic resistance.

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