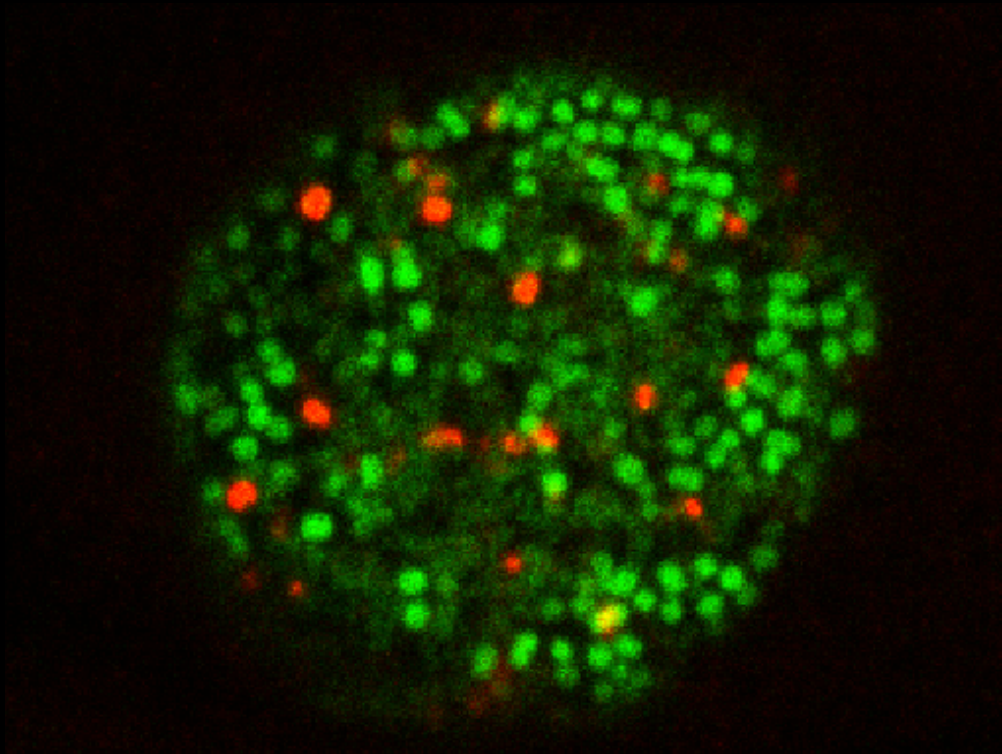
Identification and characterization of *Chlamydia trachomatis* type III secretion substrates

Maria Sarmento de Matos Paiva Raposo da Cunha



Dissertation presented to obtain the Ph.D degree in Biology

Instituto de Tecnologia Química e Biológica António Xavier | Universidade Nova de Lisboa

Oeiras,
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Maria da Cunha and Luís Jaime Mota. The *Chlamydia trachomatis* type III secretion substrates CT142, CT143, and CT144 could be part of a protein complex in the lumen of the inclusion (*manuscript in preparation*).

ABSTRACT

Chlamydiae are a large group of Gram-negative obligate intracellular bacteria that only grow within a membrane-bound vacuole in eukaryotic host cells. All *Chlamydiae* share a unique biphasic developmental cycle, in which the non-replicative elementary bodies (EBs) invade host cells and remain restricted within the bacterial vacuole, known as inclusion. Soon after invasion, EBs develop into replicative RBs that grow and divide by binary fission. Later in the cycle, RBs undergo a secondary differentiation into EBs, which are released from the host cell to initiate new rounds of invasion.

The most famous representative of *Chlamydiae* is *Chlamydia trachomatis*, an important human pathogen. Different *C. trachomatis* serovars are capable of significant differences in terms of infection, being the most frequent cause of infectious blindness (trachoma) in the developing world and of sexually transmitted bacterial diseases worldwide. Like all *Chlamydiae*, *C. trachomatis* encodes for a type III secretion system (T3SS), which is used to manipulate host cells through the delivery of effector proteins into their cytosol or membranes. Although a limited number of *C. trachomatis* effectors have been characterized, several *C. trachomatis* genes have been hypothesized to encode for effector proteins. However, this work started under the assumption that many *C. trachomatis* type III secretion (T3S) effectors remained to be identified.

To identify previously uncharacterized *C. trachomatis* T3S substrates, we performed a screen using *Yersinia enterocolitica* as heterologous host. We identified 23 *C. trachomatis* proteins whose first 20 amino acids were sufficient to drive T3S of the mature form of β-lactamase

TEM-1 by *Y. enterocolitica*. In addition, we found that 10 of these 23 proteins (CT053, CT105, CT142, CT143, CT144, CT161, CT338, CT429, CT656, and CT849) were also type III secreted in their full-length versions by *Y. enterocolitica*, providing additional support that they are T3S substrates. Real-time quantitative PCR analysis of genes encoding the 10 likely T3S substrates of *C. trachomatis* showed that all (except *ct161*) were expressed during infection of host cells. Furthermore, CT053, CT105, CT142, CT143, CT161, CT338, and CT429 were delivered by *Y. enterocolitica* into host cells, further suggesting they could be effector proteins.

Most *C. trachomatis* isolates maintain a highly conserved virulence plasmid, and recent work revealed that plasmid-encoded Pgp4 is a transcriptional regulator of plasmid genes and of multiple chromosomal genes, including *ct142*, *ct143* and *ct144*, which encode for proteins that were identified as possible effectors in our screen for T3S substrates. Altogether, this strongly suggests that these proteins could be virulence factors. We therefore aimed to characterize the expression and subcellular localization of CT142, CT143, and CT144 during infection of host cells by *C. trachomatis*. Transcription linkage showed that *ct142*, *ct143* and *ct144* are organized in an operon and that their expression in *C. trachomatis* is likely driven by σ^{66}, the homolog of the *Escherichia coli* main σ factor. Using anti-CT142 or anti-CT143 antibodies, expression of CT142 or CT143, respectively, was detected by immunoblotting from 20-30 h post-infection of HeLa cells with *C. trachomatis* serovar L2 strain 434/Bu. Immunofluorescence microscopy with anti-CT143 antibodies revealed that at 15 h post-infection CT143 co-localized with a cytosolic bacterial marker (Hsp60), while from 20 h post-infection CT143 appeared as intra-inclusion globular structures that did not overly co-

localize with the bacterial signal. This suggested that CT143 could be secreted into the lumen of the inclusion.

Using recently developed methods for transformation of *C. trachomatis*, we then constructed strains carrying plasmids expressing CT142, CT143, or CT144 with a C-terminal 2x hemagglutinin (2HA) epitope tag (CT142-2HA, CT143-2HA, CT144-2HA) under the control of the *ct142* promoter. Immunofluorescence microscopy of HeLa cells infected by these *C. trachomatis* strains revealed that CT142-2HA, CT143-2HA, or CT144-2HA localized within the bacteria at 15 h post-infection and within the lumen of the inclusion from 20 h post-infection, as had been observed for endogenous CT143. Moreover, immunofluorescence microscopy of HeLa cells infected by *C. trachomatis* carrying a plasmid simultaneously expressing CT142, CT143, and CT144-2HA under the control of the *ct142* promoter, revealed co-localization between CT144-2HA and CT143. Immunoprecipitation experiments using *Y. enterocolitica* as heterologous host indicated that CT142 could self-interact, bind to CT143, and possibly to CT144.

In conclusion, this work revealed several *C. trachomatis* proteins that could be effectors subverting host cell processes: CT053, CT105, CT142, CT143, CT144, CT161, CT338, CT429, CT656, and CT849. Three of these proteins were further characterized (CT142, CT143 and CT144) and we propose that they could form a complex in the inclusion lumen after their secretion by the bacteria. While the function of this putative complex remains to be determined, the timing of expression and localization of CT142, CT143, and CT144 suggests that these proteins could be involved in: (i) metabolic reactions within the inclusion lumen; (ii) host cell exit; or iii) host cell invasion.

RESUMO

O filo *Chlamydiae* inclui um grande número de bactérias, com reação de Gram-negativa, que vivem obrigatoriamente num compartimento vacuolar membranar (inclusão de *Chlamydia*) no interior de células eucarióticas. As bactérias pertencentes ao filo *Chlamydiae* têm um ciclo de desenvolvimento que inclui duas formas morfologicamente distintas: os corpos elementares (EBs*; elementary bodies*), capazes de entrar em células hospedeiras mas incapazes de se replicarem, e os corpos reticulares (RBs; *reticulate bodies*), incapazes de entrar em células hospedeiras mas com capacidade replicativa.

Chlamydia trachomatis é uma importante bactéria patogénica humana, que pertence às *Chlamydiae*. Esta espécie contém diferentes serovares que apresentam diferenças significativas em termos de infeção, sendo a maior causa bacteriana de doenças sexualmente transmitidas e de tracoma, uma doença ocular infeciosa que pode levar à cegueira, que é muito comum em países em desenvolvimento.

C. trachomatis, assim como todas as *Chlamydiae*, contém um sistema de secreção do tipo III que lhe permite injetar proteínas efetoras no citosol ou nas membranas das células hospedeiras, e deste modo manipular funções essenciais da célula hospedeira em seu benefício. No entanto, atualmente, apenas um pequeno número de proteínas efetoras foram identificadas em *C. trachomatis*.

No presente estudo, o principal objetivo foi identificar novas proteínas efetoras do sistema de secreção do tipo III de *C. trachomatis*. Para tal, efetuámos um rastreio utilizando a bactéria *Yersinia enterocolitica*

como hospedeiro heterólogo: i) identificámos 23 proteínas de *C. trachomatis* com sinais de secreção, localizados nos primeiros 20 aminoácidos de cada proteína, que são reconhecidos pelo sistema de secreção do tipo III de *Y. enterocolitica;* ii) destas 23 proteínas selecionadas, 10 delas são também transportadas para o meio extracelular, na sua sequência polipeptídica completa, pelo sistema de secreção do tipo III de *Y. enterocolitica*. Deste modo, neste rastreio, encontrámos 10 prováveis proteínas efetoras do sistema de secreção do tipo III de *C. trachomatis* (CT053, CT105, CT142, CT143, CT144, CT161, CT338, CT429, CT656, e CT849). Para além disso, CT053, CT105, CT142, CT143, CT161, CT338 e CT429 têm também a capacidade de serem transportadas para o citosol de células hospedeiras infectadas por *Y. enterocolitica.* Finalmente, com exceção do gene que codifica para CT161, todos os genes que codificam para estas proteínas são expressos em células infetadas por *C. trachomatis*.

A maior parte das estirpes de *C. trachomatis* contém um plasmídeo de virulência. Estudos recentes, mostraram que uma proteína (Pgp4) codificada no plasmídeo é um regulador transcricional, não apenas de genes do próprio plasmídeo, como também de genes cromossomais, incluindo *ct142*, *ct143* e *ct144*. O facto de CT142, CT143 e CT144 terem sido também detetados no nosso rastreio confere-lhes uma maior probabilidade de serem proteínas envolvidas na virulência de *C. trachomatis*. Foi assim realizada a caracterização de *ct142*, *ct143* e *ct144* em termos de organização genética. Verificou-se que estes genes se localizam num operão cujo local de início da transcrição foi determinado. Analisando a região promotora encontrámos regiões −10 e −35, em relação ao local de início da transcrição, com grande semelhança em relação a sequências de

consenso definidas para reconhecimento da RNA polimerase associada ao factor σ^{66}, homólogo do principal fator σ de *Escherichia coli*. Utilizando anticorpos anti-CT142 e anti-CT143 procedemos também a uma análise de expressão (CT142 e CT143) e localização subcelular (CT143) das proteínas em estudo. Verificámos, por *immunoblotting*, que ambas as proteínas são expressas em células HeLa infetadas pela estirpe 434/Bu do serovar L2 de *C. trachomatis*, a partir das 20 h após infeção. No caso de CT143, observámos, por microscopia de imunofluorescência, que às 15 h após infeção a proteína se localiza no interior da bactéria, enquanto que a partir das 20 h após infeção a proteína se localiza no lúmen da inclusão de *C. trachomatis*, em aglomerados que não co-localizam com marcadores bacterianos (Hsp60). Esta observação sugere que a proteína possa ser transportada para o lúmen da inclusão.

De seguida, empregando métodos recentemente descritos para transformação de *C. trachomatis,* construímos estirpes de *C. trachomatis*, contendo plasmídeos que expressam, a partir do promotor de *ct142,* as proteínas CT142, CT143 e CT144 contendo dois epitopo da proteína hemaglutinina (2HA) na sua extremidade carboxílica. Por microscopia de imunofluorescência, foram observadas células HeLa infetadas com cada uma das estirpes construídas. Verificou-se que CT142-2HA, CT143-2HA e CT144-2HA apresentam o mesmo tipo de localização no lúmen da inclusão que a proteína CT143 endógena: às 15 h após infeção as proteínas localizam-se no interior da bactéria enquanto que a partir das 20 h de infeção as proteínas localizam-se no lúmen da inclusão de *C. trachomatis*.

Para além disso, resultados apresentados neste trabalho apontam para que estas três proteínas possam formar um complexo proteico: i) estudos de co-immunoprecipitação usando *Y. enterocolitica* como hospedeiro heterólogo sugerem que CT142 poderá interagir com CT142, com CT143 e talvez com CT144 e ii) a partir de estudos de imunofluorescência em células infetadas com uma estirpe de *C. trachomatis* que expressa as três proteínas no mesmo plasmídeo (CT142-CT143-CT144-2HA), verificámos que CT144-2HA e CT143 co-localizam no lúmen da inclusão.

Em conclusão, este trabalho contribuiu para expandir os conhecimentos sobre as proteínas de *C. trachomatis* transportadas através de um mecanismo de secreção do tipo III, sendo que dez prováveis proteínas efetoras foram reveladas neste estudo: CT053, CT105, CT142, CT143, CT144, CT161, CT338, CT429, CT656, e CT849. De entre elas, CT142, CT143 e CT144 foram caracterizadas em mais detalhe e os resultados obtidos sugerem que estas proteínas sejam transportadas pelo sistema de secreção de *C. trachomatis* para o lúmen da inclusão e que aí formem um complexo proteico. Apesar da função deste presumível complexo não ter sido ainda determinada, o tipo de localização destas proteínas, assim como a altura do ciclo de desenvolvimento de *C. trachomatis* em que são expressas, sugerem que: i) as proteínas possam participar em reações metabólicas dentro do lúmen da inclusão; ii) estejam envolvidas no final do ciclo de desenvolvimento, no processo de libertação dos EBs ou iii) estejam envolvidas no início do ciclo de desenvolvimento, no processo de reconhecimento de novas células hospedeiras.

DISSERTATION OUTLINE

This dissertation is divided into five chapters. Chapter I consists of a general introduction on *Chlamydiae* and on *Chlamydia trachomatis* in particular, including a brief historic and taxonomic description as well as an epidemiologic characterization of the different *C. trachomatis* serovars. General characteristics on the biology of *C. trachomatis*, its developmental cycle and interactions with the host cell are addressed. This chapter also comprehends a section on the importance of secretion systems in pathogenic bacteria with a particular emphasis on the type III secretion system (T3SS) of *C. trachomatis*. I also briefly describe recently devised methods for genetic manipulation in *Chlamydia*.

The results presented on Chapter II consist of published material. They describe a screen where we used *Yersinia enterocolitica* as heterologous host to identify candidate chlamydial T3S effector proteins, first by identifying T3S signals in the selected proteins and secondly by analyzing the capacity of the proteins containing T3S signals to be secreted by *Y. enterocolitica* as full length proteins. We identified ten likely T3S substrates of *C. trachomatis* (CT053, CT105, CT142, CT143, CT144, CT161, CT338, CT429, CT656, and CT849) and could detect translocation into host cells by *Y. enterocolitica* of CT053, CT105, CT142, CT143, CT161, CT338, and CT429.

Chapter III and Chapter IV include results that are part of a manuscript in preparation. Chapter III comprises a characterization of the genes encoding three of the proteins that were highlighted in the screen described in Chapter II (CT142, CT143 and CT144) in terms of genetic organization and profile of expression. It also includes

results that confirm that *ct142*, *ct143* and *ct144* are transcriptionally regulated by the *C. trachomatis* virulence plasmid. Additionally, in this chapter we describe the production of antibodies against CT142 and CT143 that constituted valuable tools for further expression studies (CT142 and CT143) and subcellular localization studies (CT143) in HeLa cells infected by *C. trachomatis*. Both CT142 and CT143 were found to be expressed in infected cells. Furthermore CT143 was found to be secreted into the lumen of the inclusion where it appears as globular structures that do not co-localize with bacterial markers.

The results presented in Chapter IV, were based on the recently described transformation methods in *C. trachomatis* that allow ectopic expression of proteins from a plasmid. We observed that ectopically expressed CT142-2HA, CT143-2HA and CT144-2HA show a subcellular localization similar to the endogenous CT143, supplementing the results obtained in Chapter III. This chapter also includes immunoprecipitation experiments using *Y. enterocolitica* as heterologous host that suggest that CT142 can self-interact, bind to CT143, and perhaps also to CT144, implying that these proteins could form a complex in the lumen of the inclusion.

Finally, Chapter V consists of a general discussion regarding the importance of the results obtained and on the future directions of this work.

ABBREVIATIONS

Abbreviation	Full Form
2HA	Double hemagglutinin epitope tag
ADP	Adenosine diphosphate
AHNAK	Neuroblast differentiation-associated protein
ARP	Actin-related protein
ATCC	American type culture collection
ATPase	Adenosine triphosphatase
BHI	Brain heart infusion
cDNA	Complementary DNA
DMEM	Dulbecco's modified Eagle medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECACC	European Collection of Cell Cultures
ECDC	European Center for Disease Prevention and Control
EDTA	Ethylenediamine tetraacetic acid
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
FBS	Fetal bovine serum
GCIP	Grap2 cyclin D-interacting protein
gDNA	Genomic DNA
GFP	Green fluorescent protein
GST	Glutathione-S transferase
GTPases	Guanine triphosphate hydrolases
HA	Hemagglutinin epitope tag
HBSS	Hank's balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hsp60	Heat shock protein 60
LB	Lysogeny broth
LPS	Lipopolysaccharide
MACPF	Membrane attack complex/perforin domain
MAPK	Mitogen-activated protein kinase
MBP	Maltose-binding protein
MOI	Multiplicity of infection
mRNA	Messenger RNA

Abbreviation	Full Form
NK-κB	Nuclear factor κB
NSF	N-ethylmaleimide sensitive fusion protein
N-WASP	Wiskott-Aldrich syndrome protein
OD_{600}	Optical density at 600 nm
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
p.i.	Post-infection
PMSF	Phenylmethylsulphonyl fluoride
RACE	Rapid amplification of cDNA ends
rpm	Revolutions per minute
RPMI	Roswell park memorial institute medium
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RT-qPCR	Real-time quantitative PCR
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SET	*Drosophila* Su(var)3-9 and 'Enhancer of zeste'
SNARE	Soluble NSF Attachment Protein Receptor
SPG	Sucrose phosphate buffer
TLR	Toll-like receptor
VAMP	Vesicle-associated membrane protein
WAVE2	Wiskott-Aldrich syndrome protein
WHO	World Health Organization

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Chapter I

General Introduction

The author of this dissertation has written this chapter based on the referred bibliography.

1.1. *Chlamydiae*

The *Chlamydiae* are a separate phylum in the domain *Bacteria* comprising a large group of microorganisms that have in common an obligate intracellular lifestyle and a unique conserved biphasic developmental cycle. *Chlamydiae* depend on a eukaryotic host for replication, which takes place in a vacuolar compartment inside the host cell. In addition, they have evolved to colonize a wide variety of vertebrate and non-vertebrate hosts as well as free-living amoebae (Horn, 2008).

1.1.1. History and taxonomy

Chlamydial organisms were first described in 1907, after the identification of intracytoplasmic vacuoles containing large numbers of microorganisms in conjuctival cells from scrapings of trachoma cases (Halberstaedter & von Prowazec, 1907, 1909). At the time, these organisms were thought to be protozoa that were embedded or hidden in what appeared to be a mantle, thus naming them "chlamydozoa", after the Greek word "chlamys" which stands for mantle. After this report, similar type of vacuolar microorganisms were described in scrapings from adults with urethritis and cervicitis and newborns with nongonococcal conjunctivitis (Lindner, 1910), in samples obtained from patients with lymphogranuloma venereum (LGV) (Durand *et al.*, 1913) and in samples from humans and birds during a worldwide pandemic (1929-1930) of an atypical and acute pneumonia caused by contact with psittacine birds (Bedson *et al.*, 1930; Coles, 1930; Lillie, 1930). In 1935, Miyagawa considered these organisms to be viruses (Miyagawa *et al.*, 1935) as they could be passed through bacterial filters and were unable to grow on artificial media. This was only corrected in 1966 with the aid of electron

microscopy when *Chlamydiae* were finally classified as bacteria (Moulder, 1966). The main reason for this re-classification was the fact that DNA, RNA and ribosomes were observed in these microorganisms as well as a cell wall apparently similar to the one present in Gram-negative bacteria.

The *Chlamydiae* phylum includes a total of nine families (Taylor-Brown *et al.*, 2015). The *Chlamydiaceae* family, the most widely studied, consists of one single genus (*Chlamydia*) and was for a long time considered the only family in this order. The members of the other 8 families (*Parachlamydiaceae*, *Waddliaceae*, *Simkaniaceae, Rhabdochlamydiaceae*, *Criblamydiaceae*, *Piscichlamydiaceae, Chlavichlamydiaceae* and *Parilichamydiaceae*) were described more recently and have often been referred to as “*Chlamydia*-like” organisms, “*Chlamydia*-related” bacteria, or “Environmental *Chlamydia*”. Although many of these microorganisms were isolated from environmental sources, they have been found to infect a variety of hosts such as free-living amoeba, bivalves, spiders, reptiles and fishes. Additionally, some of these recently described species, have been described as emerging human pathogens: *Parachlamydia sp.* as mucosal pathogens in humans and other animals (Corsaro & Greub, 2006), *Simkania negevensis* associated with respiratory disease in humans (Husain *et al.*, 2007; Lieberman *et al.*, 2002) and *Waddlia chondrophila*, an abortive agent in cattle that has also been associated with miscarriages in humans (Horn, 2008; Lamoth *et al.*, 2015). However, none of the strains were ever isolated from human patients and the associations were mainly based on serological and molecular data (Horn, 2008; Lamoth *et al.*, 2015).

1.1.2. The *Chlamydiaceae* family

In 1999, Everett and co-workers made a controversial proposal to divide the *Chlamydiaceae* family in two genera: *Chlamydia* (*C. trachomatis*, *C. suis* and *C. muridarum*) and *Chlamydophila* (*C. pecorum*, *C. pneumoniae*, *C. caviae*, *C. felix*, *C. psittaci* and *C. abortus*) (Everett *et al.*, 1999). This division was based on differential clustering of the 16S rRNA. However, the majority of chlamydial researchers found this classification to be inadequate because it was based on limited sequence data and, most of all, it did not take into account the unique conserved biological properties of the species. As a consequence, in 2009 it was proposed that the *Chlamydiaceae* should be reunited into a single genus, *Chlamydia* (Greub, 2010; Sachse *et al.*, 2015; Stephens *et al.*, 2009).

There are currently 11 species of *Chlamydia* described that are characterized by major differences in host range, tissue tropism and disease pathology (reviewed in Nunes & Gomes, 2014; Sachse *et al.*, 2015) (Fig. 1.1). *C. trachomatis* is a human specific pathogen and is the most medically significant *Chlamydia* species, capable of causing ocular and genital infections (further detailed in section 1.1.3., below). However, to date, 10 additional *Chlamydia* species have been characterized and one new candidate species has been proposed:

- *C. pneumoniae* is the other major human pathogen and is a prevalent cause of respiratory infections (Blasi *et al.*, 2009; Kern *et al.*, 2009). It is distributed worldwide being responsible for 10-20% of community acquired pneumonia in adults, exacerbations of chronic bronchitis, pharyngitis and asthma (Blasi *et al.*, 2009; Kern *et al.*, 2009) and it is also associated with asthma in children (Asner *et al.*, 2014). It has also been linked to cardiovascular disease, atherosclerosis, central

nervous system disorders and Alzheimer's disease (Benagiano *et al.*, 2012; Roulis *et al.*, 2013). Furthermore, *C. pneumoniae* is able to infect other mammals (horses, marsupials) and reptiles leading to respiratory and vascular pathologies (Bodetti *et al.*, 2002).

- *C. psittaci* is primarily a pathogen of avian species causing respiratory diseases that have a considerable impact on poultry farming (Knittler *et al.*, 2014). It is able to infect approximately 450 different bird species, including psittacine birds, pigeons, ducks, geese and turkeys. Yet, transmission between humans and birds is possible through inhalation of droplets dispersed in the air containing the microorganism, causing severe respiratory diseases (Van Droogenbroeck *et al.*, 2009; Knittler *et al.*, 2014; Moroney *et al.*, 1998).
- *C. abortus* is of economic relevance since it is able to colonize the placenta of sheep and goats causing abortions (Longbottom *et al.*, 2013). Pregnant women who are exposed are also at risk of miscarriage (Longbottom & Coulter, 2003).
- *C. muridarum* infects mice and is able to colonize the lungs and genital tract (Ramsey *et al.*, 2009).
- *C. avium* was found in pigeons and members of the psittacine family of birds (parrots, parakeets and macaws) (Sachse & Laroucau, 2014; Sachse *et al.*, 2014). Infections appear to be asymptomatic but are widely disseminated.
- *C. gallinacea* was identified in domestic poultry (chicken, guinea fowl and turkey) (Sachse & Laroucau, 2014; Sachse *et al.*, 2014). As in *C. avium*, infections are widely disseminated but appear to be asymptomatic.

- *C. pecorum* is a pathogen of livestock (cattle, sheep, goats and pigs) (Bachmann *et al.*, 2014a; Fukushi & Hirai, 1992) causing polyarthritis, conjunctivitis, pneumonia and encephalomyelitis (Poudel *et al.*, 2012). It is also capable of infecting the marsupial Koala causing ocular and genital infections (Polkinghorne *et al.*, 2013).
- *C. felis* has been related to conjunctivitis in cats (Cai *et al.*, 2002) and a few cases of conjunctivitis in humans have also been reported (Hartley *et al.*, 2001).
- *C. caviae* infects guinea pigs and causes conjunctivitis and genital tract infections (Read *et al.*, 2003).
- *C. suis* is restricted to infections in swine and is associated with pneumonia, conjunctivitis and reproductive disorders (Donati *et al.*, 2014; Schautteet & Vanrompay, 2011).

Recently, a new candidate chlamydial species has been proposed, *C. ibidis*, which infects Feral African Sacred Ibises (Vorimore *et al.*, 2013). This strain appears to be relatively innocuous in avian hosts and has not been associated with zoonotic infections.

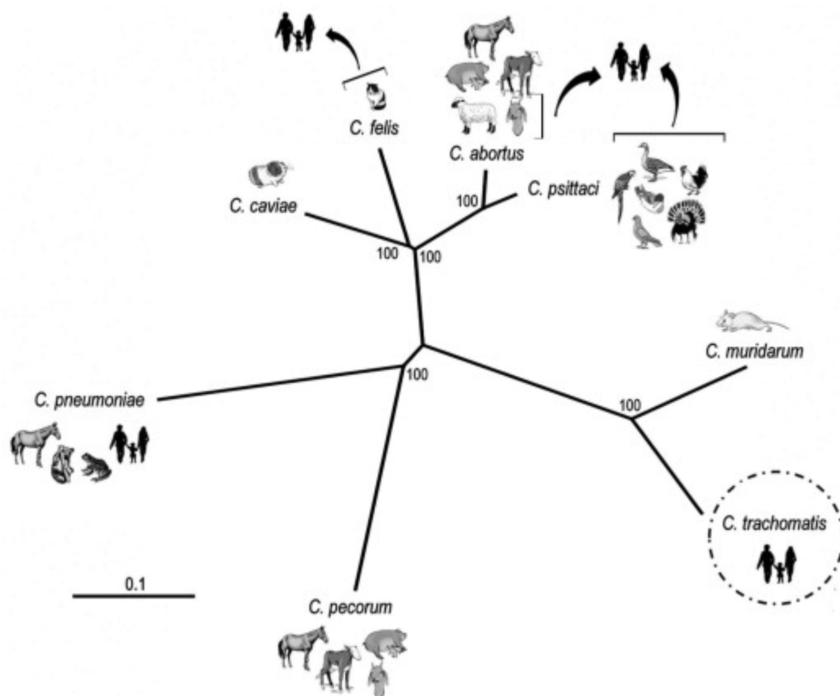

Figure 1.1. **Phylogenetic reconstruction of the *Chlamydiaceae* species whose genomes are fully sequenced**. Each chlamydial species is associated to its natural host and cases of zoonotic transmission are depicted with arrows. *C. suis*, *C. avium* and *C. gallinacea* are not represented due to lack of sequence data. The *Chlamydiaceae* tree topology is based on the accumulation of single-nucleotide polymorphisms (SNPs) on 600 orthologous genes shared among the species. (Reprinted from Nunes *et al*, 2014, with permission from Elsevier).

1.1.3. *Chlamydia trachomatis*

The earliest reliable descriptions of trachoma, the blinding disease, are found in Ebers papyrus that appear to have been written between 1553 and 1550 BC (Taylor, 2008). This suggests that *C. trachomatis* was present in early civilizations and consequently trachoma is most probably a disease that has been closely related to humans for millennia. The knowledge that *C. trachomatis* was also the cause of sexually transmitted diseases was much more recent than for trachoma. A significant advance in the knowledge of *C. trachomatis*

was the isolation of the microorganism in embryonated chickens eggs in China by T'ang and collaborators in 1957 (T'ang *et al.*, 1957). It then became possible to preserve and propagate these bacteria providing resources for future studies.

C. trachomatis isolates have been classified into 15 main serovars based on a micro-immunofluorescent test that detects differential serospecificity of its Major Outer Membrane Protein (MOMP) (Schachter, 1999; Yuan *et al.*, 1989). *C. trachomatis* serovars A-C and Ba infect ocular epithelial cells leading to conjunctivitis, with severe inflammation and scarring of the conjunctiva ultimately leading to trachoma, the world's leading cause of preventable blindness (Whitcher *et al.*, 2001; Wright *et al.*, 2008). *C. trachomatis* serovars A and B are found in underdeveloped countries, ranging from Latin America, to Sub-Saharan Africa or Asia. *C. trachomatis* serovar C is rarely isolated but it is present in communities of Australia Northern territory. *C. trachomatis* serovars D-K represent the genital track strains (non invasive ano-urogenital infections) and are the main cause of bacterial sexually transmitted disease worldwide (World Health Organization, 2011). Within this group *C. trachomatis* serovars E and F represent approximately 50% of the uro-genital tract infections in heterosexual populations while *C. trachomatis* serovar G appear to be more prevalent in rectal tissues of man who have sex with men (MSM) (Christerson *et al.*, 2012). In genital tract infections, *C. trachomatis* replicates mainly in epithelial cells of the urethra in men, or in epithelial cells of the endocervix of women causing inflammation, edema and mucosal discharge. Ascending uterine infections can lead to pelvic inflammatory disease, tubal scarring, ectopic pregnancies and infertility. *C. trachomatis* can also be transmitted from mother to baby causing neonatal conjunctivitis

(Bébéar, C., and de Barbeyrac, 2009; Shaw *et al.*, 2011). The more invasive *C. trachomatis* LGV strains belong to serovars L1, L2 and L3 and are responsible for a systemic disease (lymphogranuloma venereum) characterized by an infection of regional draining lymph nodes (Schachter, 1978). The *C. trachomatis* LGV strains are endemic in Africa, Southeast Asia, South America and the Caribbean.

1.1.3.1 Impact of C. trachomatis *infections on human health*

Taking into account the huge potential to cause human diseases as well as its ample tissue tropism, *C. trachomatis* has been considered a global health problem. Trachoma is endemic in 51 countries and is responsible for the visual impairment of 2.2 million people (1.2 million are irreversibly blind) (World Health Organization, 2014). The rate of cases of *C. trachomatis* sexually transmitted disease reported to the ECDC in Europe in 2012 were 184 per 100000 population (ECDC, 2014) and close to 100 million new cases of *C. trachomatis* sexually transmitted infections are believed to occur annually worldwide (World Health Organization, 2011). Despite being effectively treated with antibiotics [azithromycin and doxycycline are the most widely used (Kong & Hocking, 2015)], a major concern with chlamydial genital infections is that 70% to 85% of infected women and over 50% of infected men are asymptomatic (Shaw *et al.*, 2011).

Chlamydial infections represent a significant financial burden to health care systems in order to treat acute genital infections including pelvic inflammatory disease and tubal infertility. Moreover, chlamydial infections have also been associated to other diseases: are considered to be a risk factor for the transmission of HIV (Shaw *et al.*, 2011; Xiridou *et al.*, 2013) or human papillomavirus (HPV) (Luostarinen *et al.*, 2013; Silva *et al.*, 2014); could be linked with

cervical cancer in women (Chumduri *et al.*, 2013; González *et al.*, 2014; Shanmughapriya *et al.*, 2012; Weitzman & Weitzman, 2014) and may also be involved in chlamydia-related reactive arthritis (Taylor-Robinson & Keat, 2015).

1.1.3.2. Experimental models to study pathogenesis of C. trachomatis

C. trachomatis interaction with host cells has been studied mainly through the use of non-polarized cell cultures. The most widely used cell lines are HeLa 229, derived from human cervical carcinoma (Scherer *et al.*, 1953) and McCoy, a mouse fibroblast cell line (Gordon *et al.*, 1972). The use of cell cultures as a model to study *C. trachomatis* has contributed greatly not only to our knowledge of chlamydial biology but also for the characterization of a variety of interactions between the bacteria and its host cell (Scidmore, 2005). However, the studies of chlamydial pathogenesis as a whole cannot be done using tissue culture alone, thus animal models have been crucial for the study of *C. trachomatis* infections and vaccine development.

Diseases caused by *Chlamydia* are mainly based on chronic inflammation elicited by re-infection or persistent infection. The majority of information regarding host responses to *Chlamydia* infections has derived from murine, guinea pig, pig or non-human primate models (De Clercq *et al.*, 2013; Neuendorf *et al.*, 2015; O'Meara *et al.*, 2014).

Regarding pathogenesis, animal models of chlamydial ocular and genital infections lead to pathologies that are highly similar to those observed in humans (scarring sequelae in ocular infections or lower genital tract infections that can lead to upper genital tract

inflammatory pathologies). However, none of the available animal models have been found to perfectly reproduce the immune responses that occur during human *C. trachomatis* infections, which has greatly limited the development of an effective human vaccine against *C. trachomatis* (De Clercq *et al.*, 2013). Attempts to develop a human vaccine against *C. trachomatis* go back to the 1960's when clinical trials with inactivated EBs took place. An initial early protection in vaccinated individuals was achieved but upon second challenge it resulted in more severe symptoms when compared to the placebo group. This constituted a major obstacle for further clinical development of vaccines against *C. trachomatis*. However, advances in the development of a candidate vaccine have taken place recently (Kari *et al.*, 2011a; Mabey *et al.*, 2014; Stary *et al.*, 2015).

1.1.3.3. C. trachomatis *genomic studies*

The *C. trachomatis* serovar D was the first chlamydial genome to be sequenced (Stephens *et al.*, 1998) and revealed a single 1,042,519 bp (~1 Mbp) circular chromosome and a 7,493 bp plasmid. It has 894 gene-coding sequences and the average size of the genes is 1050 bp. This is a relatively small genome when compared to free-living bacteria or even to the related *Chlamydia*-like bacteria (~2 Mbp), suggesting that during adaptation to its host *Chlamydia* lost a large number of genes (Mendonça *et al.*, 2011).

Genome analysis revealed that *Chlamydia* possess the minimal machinery required for DNA replication, transcription and translation, for type III secretion machinery, for basic lipid metabolism and for essential functions in aerobic respiration (Stephens *et al.*, 1998; Yao *et al.*, 2015). However, *Chlamydia* have several incomplete metabolic pathways such as the tricarboxilic acid cycle or the biosynthesis of

aminoacids (Stephens *et al.*, 1998), therefore relying on the host to obtain basic nutrients.

Genome sequences from all the different *C. trachomatis* serovars are now available and they all share a high degree in similarity regarding size and synteny. Most genes are shared between the different chlamydial species and there is a high level of genomic similarity (> 98%) between different serovars. This observation is striking when considering the divergence in tissue tropism and disease outcome. In fact, a small region in the genome of about 10 to 50 kb (45-49 genes for *C. trachomatis*), the Plasticity Zone (PZ), contains the highest degree of genetic divergence between the different serovars and is likely responsible for most of the phenotypic and host specificity differences observed (Nunes & Gomes, 2014). Genes located in this PZ include: i) the *trpRBA* operon, (incomplete *trp* operon) present only in the genital strains, allowing these bacteria to synthesize tryptophan from indole, a product that can be found within the genital tract microflora. This mechanism is thought to be of great importance for the survival of genital strains since the presence IFN-γ elicited by the host immune response limits the availability of tryptophan (Caldwell *et al.*, 2003); ii) the gene encoding the chlamydial cytotoxin, *ct166*, which is involved in the disassembly of cytoskeleton actin filaments during bacterial internalization (“cytopathic effect”) (Bothe *et al.*, 2015; Thalmann *et al.*, 2010). This gene may be active, truncated or deleted among *C. trachomatis* strains. Ocular and genital strains retain remnants of a larger ancestral cytotoxin gene that is still intact in other *Chlamydia* spp. (Bachmann *et al.*, 2014b; Lo *et al.*, 2012; Nunes & Gomes, 2014) (see section 1.4.9.4, below). In the case of the LGV strains *ct166* is completely deleted (Carlson *et al.*, 2004); iii) gene encoding for the putative membrane attack complex/perforin

(MACPF) (Nunes & Gomes, 2014) or iv) gene encoding phospholipase D enzymes (PLD), that could be involved in the acquisition of host lipids (Nelson *et al.*, 2006).

In addition, there are other genes outside the PZ that contribute to the variability among different *C. trachomatis* serovars. These include genes encoding for: the highly variable Pmps (polymorphic outer membrane proteins) (Becker & Hegemann, 2014; Crane *et al.*, 2006; Gomes *et al.*, 2006), the Inc family of proteins (see section 1.4.8.7, below), the TTS effectors (see section 1.4.8, below) and biotin and pyrimidine pathways (Nunes & Gomes, 2014).

1.1.3.4. The C. trachomatis *plasmid*

Chlamydial plasmids are highly conserved and have so far found to be present in seven species of *Chlamydia*: *C. trachomatis*, *C. psitacci*, *C. pneumoniae*, *C. suis*, *C. felis*, *C. muridarum* and *C. caviae* (Lovett *et al.*, 1980; Pickett, 2005). Their length is about 7.5 kb, they are present in the quantity of 4 -10 copies per cell and are non integrative and non-conjugative (Pickett, 2005; Rockey, 2011).

The *C. trachomatis* plasmid has been the most studied and is the most conserved among *Chlamydia* species (Ferreira *et al.*, 2013). It contains non-coding RNAs (Abdelrahman *et al.*, 2011) as well as 8 ORFs that have recently been described (Gong *et al.*, 2013; Song *et al.*, 2013):

- ORF 1 (*pgp7*) and ORF2 (*pgp8*) encode for proteins that are homologues of an integrase and recombinase, respectively, and are involved in regulation of plasmid replication;
- ORF 3 (*pgp1*) encodes for a homologue of a DNA helicase;

- ORF4 (*pgp2*) encodes for a protein of unknown function;
- ORF5 (*pgp3*) encodes for a protein that has been demonstrated to localize in the inclusion lumen and cytosol of infected cells (Li *et al.*, 2008), indicating it might be a secreted protein (see section 1.4.10.5., below); Pgp3 has been reported to represent a major virulence factor in *C. muridarum* pathogenesis in mice (Liu *et al.*, 2014a) and more recently has been found to neutralize the anti-chlamydial activity of human cathelicidin LL-37, a host anti-microbial peptide secreted by both genital tract epithelial cells and infiltrating neutrophils (Hou *et al.*, 2015).
- ORF 6 (*pgp4*) encodes for a protein that has been found to act as a transcriptional regulator of plasmid encoded *pgp3* and also of multiple chromosomal genes, including *glgA,* that is involved in the accumulation of glycogen in the inclusion (Song *et al.*, 2013);
- ORF7 (*pgp5*) from *C. muridarum* encodes for a protein that has been proposed to be involved in the suppression of plasmid dependent gene expression, suggesting that Pgp5 can negatively regulate the same plasmid dependent genes that are upregulated by Pgp4 (Liu *et al.*, 2014b);
- ORF8 (*pgp6*) encodes for a protein that may be involved in plasmid replication.

It has been proposed that *pgp1*, *pgp2*, *pgp6-8* are required for plasmid maintenance while *pgp3-5* are not required for the maintenance of the plasmid but likely represent the primary virulence genes.

Naturally occurring plasmidless clinical isolates are rare. *C. trachomatis* serovar L2 strain 25667R is a naturally occurring plasmidless strain that has been well characterized (Carlson *et al.*, 2008). When compared to the serovar L2 strain 434/Bu the majority of the *in vitro* virulence characteristics were similar, including growth kinetics, plaquing efficiency and plaque size. The only *in vitro* phenotypic differences were the lack of accumulation of glycogen granules in the inclusion lumen and the absence of the typical Brownian-like movement characteristic of *C. trachomatis* strains. However, most *in-vivo* infections with plasmid deficient strains in murine or non-human primate models are either asymptomatic or show significantly reduced pathology (Kari *et al.*, 2011a; O'Connell *et al.*, 2007; Olivares-Zavaleta *et al.*, 2010). These observations, together with the strong selection observed to maintain the plasmid implies a fundamental role for the plasmid in pathogenesis. The molecular basis of plasmid-mediated virulence is not clearly understood but is thought to involve plasmid related gene products (including chromosomal genes that are transcriptionally regulated by Pgp4) that could function as TLR2 (O'Connell *et al.*, 2011) or TNF-α (Dong *et al.*, 2014; Murthy *et al.*, 2011) receptor antagonists.

1.2. The *Chlamydia* developmental cycle

During its developmental cycle (Fig. 1.2), *Chlamydia* exhibits two main forms: an infectious, non-replicating elementary body (EB) of approximately 0.3 μm of diameter, and a non-infectious but actively replicating reticulate body (RB) of approximately 1 μm of diameter (Fig. 1.3) (AbdelRahman & Belland, 2005). An intermediate form (IF) has also been observed by electron microscopy and described as a differentiation step between the RB and EB in the later stages of the

cycle (Fig. 1.3) (Berger *et al.*, 1999; Wilkat *et al.*, 2014). Another morphologic form might also appear during the developmental cycle, the aberrant body (AB). It is non infectious and non replicative and is associated with persistent infections (discussed ahead in section 1.2.7.).

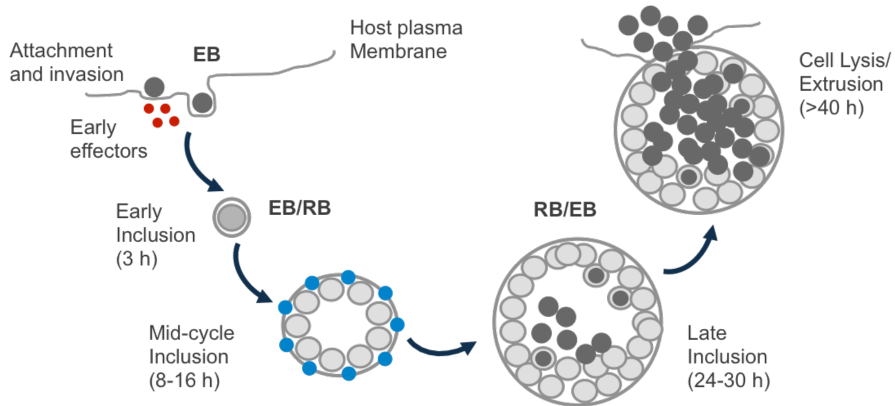

Figure 1.2. Schematic representation of the *Chlamydia* developmental cycle. The cycle begins with the attachment of EBs to the plasma membrane of host epithelial cells, in a process where early bacterial effectors (depicted in red) have been shown to play a major role. The EB is involved in a host-derived membrane forming an early inclusion, where the EB is converted into an RB. The inclusion membrane is rapidly modified by insertion of bacterial proteins (depicted in blue). RBs replicate by binary fission, which is accompanied by inclusion expansion through acquisition of host-derived nutrients. Late in the cycle, replication becomes asynchronous and RBs start re-differentiating back into EBs. The cycle is completed with the release of the infectious EBs by cell lysis or extrusion, allowing for other rounds of infection. (Adapted from Valdivia, 2008).

The chlamydial cell wall includes general features of a Gram-negative cell envelope: inner membrane, periplasmic space with peptidoglycan (PG) and an outer membrane containing lipopolysaccharides (LPS). However, the ‘Chlamydial Anomaly’ debated for a long time the presence of PG in *Chlamydia*. It was puzzling that *Chlamydia* had in its genome genes that encode for peptidoglycan biosynthesis and exhibited susceptibility to anti-peptidoglycan antibiotics, but

conversely, attempts to detect or purify PG in chlamydial species were proven unsuccessful. Recently, however, the presence of PG in *Chlamydia* was finally established (Jacquier *et al.*, 2015; Liechti *et al.*, 2014; Packiam *et al.*, 2015).

1.2.1. The elementary body

The EBs (infectious form) are small, round and have adapted to extracellular survival (Fig. 1.3). They possess an electron dense nucleoid, a structure that is unique to *Chlamydiae*. Two histone-like proteins Hc1 and Hc2 (Wagar & Stephens, 1988) are thought to contribute to the nucleoid structure by inducing conformational changes in the DNA (Barry *et al.*, 1992, 1993; Brickman *et al.*, 1993). The location of the nucleoid in the cell suggests an association with the bacterial inner-membrane. EBs have a relatively hydrophobic surface and a net negative surface charge at neutral pH. The outer membrane of EBs is formed of a rigid network of inter and intra-molecular disulphide bonds between the cysteine-rich proteins that are part of the chlamydial outer-membrane complex (OmcA, OmcB and MOMP) (Aistleitner *et al.*, 2015; Wang *et al.*, 2014):

- OmcA and OmcB are present exclusively in EBs. OmcA is a lipoprotein with a structure similar to murein lipoproteins found in other Gram-negative bacteria (Everett *et al.*, 1994). OmcB, is the second most abundant outer membrane protein thought to contribute to cell-wall rigidity and osmotic stability of the EBs. It is thought to be localized at the inner surface of the outer membrane but also to be surface exposed (Fadel & Eley, 2007)
- MOMP (Major Outer Membrane Protein) is the chlamydial protein that is present in largest quantity in EBs and RBs and

share the characteristics of other Gram-negative bacterial porins (Feher *et al.*, 2013).

Other components of the outer membrane include the group of Polymorphic Membrane Proteins (Pmps), which can be differentially expressed and distributed in the surface of the outer membrane. These have also been described as antigenic determinants (Vasilevsky *et al.*, 2016).

1.2.2. The reticulate body

RBs are the replicating form of the bacteria and divide by binary fission during the mid-phase of the development cycle. They are larger than EBs with diffuse fibrillar nucleic acids loosely packed in the cytoplasm, which contrasts with the highly condensed nucleoid structure of the EBs (Fig. 1.3) (Barry *et al.*, 1992; Hackstadt *et al.*, 1985; Moulder, 1991). Additionally they have a high metabolic activity, as they are responsible for uptake and transport of nutrients and abundant protein synthesis (Omsland *et al.*, 2012).

In comparison to EBs, the disulphide-bond network is reduced in RBs: OmcA and OmcB are absent and MOMP is present in a reduced form (Newhall & Jones, 1983), leading to a more flexible and osmotically fragile life stage. The permeability of the outer membrane is enhanced by the reduction state of disulfide bonds between cysteine residues of MOMP (Aistleitner *et al.*, 2015).

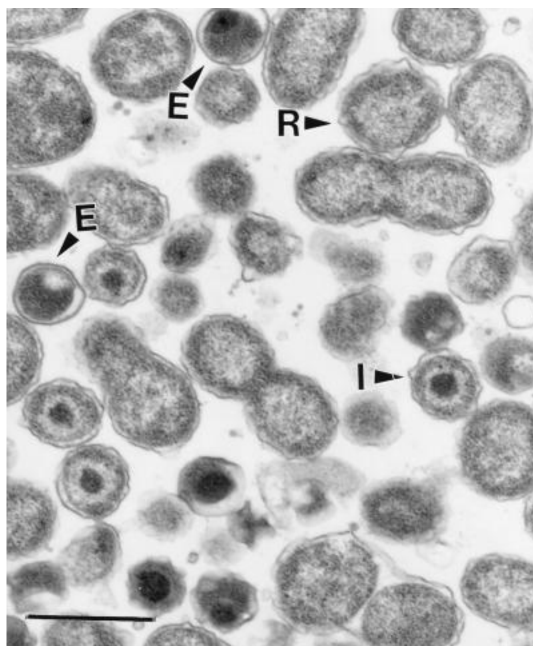

Figure 1.3. Transmission electron micrograph representing chlamydial particles at various stages of development. **R**, large reticulate bodies that undergo binary fission; **I**, intermediate bodies that constitute a differentiation step between RBs and EBs and **E**, condensed elementary bodies. Scale bar, 700 nm. (Reprinted from Berger *et al*, 1999, with permission from the American Society for Microbiology).

1.2.3. Attachment and entry

The infectious form of *C. trachomatis*, the EB, attaches to and is internalized by the host cell. Multiple redundant mechanisms are likely to occur to ensure chlamydial entry and they depend not only on the chlamydial species but also on the host cell type being invaded. The interaction of EBs with host cells is thought to occur in a two step process: a initial step that consists of a reversible, electrostatic interaction of heparan sulphate-like glycosaminoglycans (Davis & Wyrick, 1997; Su *et al.*, 1996; Zhang & Stephens, 1992), followed by a high affinity irreversible binding of the EB to one or several cellular receptors. Several surface proteins of EBs have been proposed to play a role at this stage of infection as adhesins or ligands for

receptor interactions. These include MOMP (Su *et al.*, 1996), OmcB (Fadel & Eley, 2007) or PmpD (Wehrl *et al.*, 2004).

Chlamydiae are able to infect a wide variety of cultured cell types, suggesting that receptor(s) mediating invasion are ubiquitous or that multiple receptors can be used (Bastidas *et al.*, 2013). To date several host receptors have been proposed for *Chlamydia* entry: heparan sulphate (Chen & Stephens, 1994, 1997; Chen *et al.*, 1996; Wuppermann *et al.*, 2001), Toll-like receptor 2 (TLR2) (O'Connell *et al.*, 2006), TLR4 (Heine *et al.*, 2003), mannose-6-phosphate receptor, the estrogen receptor (reviewed in Cocchiaro & Valdivia, 2009), growth factors and their respective receptors (Elwell *et al.*, 2008; Kim *et al.*, 2011), as well as the protein disulfide isomerase (PDI) (when present at the cell surface) (Abromaitis & Stephens, 2009). Clathrin (Hybiske & Stephens, 2007a) and cholesterol-rich microdomains (Jutras *et al.*, 2003; Norkin *et al.*, 2001; Stuart *et al.*, 2003) are additional host factors that may play a role in chlamydial uptake into non-phagocytic cells.

To date, a small number of chlamydial proteins delivered into host cells have been identified to play a major role in the entry step (discussed ahead in section 1.4.8.): TarP (Translocated Actin-Recruiting Phosphoprotein) is delivered into the host cell and contributes to bacterial internalization by directly nucleating actin polymerization (Jewett *et al.*, 2006) or by indirectly inducing the reorganization of the actin cytoskeleton at attachment sites (Bastidas *et al.*, 2013; Carabeo *et al.*, 2004, 2007); CT694, was found to interact with human AHNAK, an actin-binding protein, and interfere with actin mobilization (Hower *et al.*, 2009); and TepP (Translocated

Early Phosphoprotein), a scaffolding protein that is thought to mediate signaling events (Chen *et al.*, 2014).

1.2.4. Intracellular development

Upon uptake, the EBs are sequestered in a membrane bound compartment that initially resembles the host plasma membrane. The disulphide bonds present in EBs are reduced during internalization (Hackstadt *et al.*, 1985) followed by nucleoid decondensation and initiation of bacterial transcription. New bacterial proteins start being produced within 15 minutes and RNA expression can be detected as early as one hour post-infection (Belland *et al.*, 2003; Plaunt & Hatch, 1988). RBs replicate by binary fission inside the inclusion. Very rapidly bacterial Inclusion membrane (Inc) proteins (discussed ahead in section 1.4.8.7) are produced and inserted in this newly formed membrane compartment. These proteins share a common bi-lobular hydrophobicity motif that likely spans the inclusion membrane. They are thought to recruit to the inclusion key regulators of membrane trafficking, such as Rab GTPases (Rzomp *et al.*, 2003, 2006) or SNAREs, (Delevoye *et al.*, 2008; Paumet *et al.*, 2009; Südhof & Rothman, 2009), which may function to regulate trafficking and or fusogenic properties of the inclusion (Moore & Ouellette, 2014). The modification of the inclusion membrane circumvents normal trafficking through the host endocytic pathway and by this manner chlamydial inclusions are dissociated from late endosomes and lysosomes (Fields & Hackstadt, 2002; Scidmore *et al.*, 2003), thus escaping from one of the major host defense mechanism.

As early as 2 h after entry into host cells, early inclusions are transported along microtubules in a dynein-dependent manner to the perinuclear region of the host cell, near the centrosome (Grieshaber

et al., 2003). They remain in close proximity to the Golgi apparatus and are able to intersect a subset of Golgi-derived vesicles containing sphingomyelin (Hackstadt *et al.*, 1996) and cholesterol (Carabeo *et al.*, 2003).

Chlamydial infection of human epithelial cells induces Golgi fragmentation and generates ministacks surrounding the bacterial inclusion (Heuer *et al.*, 2009) that most likely provide the main source of lipids for the inclusion. Alternative lipid transport pathway include interception of (i) multivesicular bodies (MVBs), endocytic organelles that sort endocytosed proteins and lipids to different destinations (Woodman & Futter, 2008), (ii) lipid droplets (LDs), eukaryotic neutral lipid storage organelles (Kumar *et al.*, 2006) and peroxisomes that contribute to the synthesis of plasmogens (Boncompain *et al.*, 2014). In fact, intact LDs and peroxisomes have been found to be transported into the inclusion lumen (Boncompain *et al.*, 2014; Cocchiaro *et al.*, 2008).

Additionally, the inclusion is thought to interact with other host organelles including the endoplasmic reticulum (ER) (Agaisse & Derré, 2014), lysosomes (Ouellette *et al.*, 2011), and mitochondria (Derré *et al.*, 2007; Matsumoto *et al.*, 1991) (Fig. 1.4). Lysosomes might be a source of essential amino acids, while interaction with mitochondria were only seen in *C. psittaci* and *C. caviae*.

Taken together all these interactions allow for the acquisition of the necessary nutrients for bacterial replication as well as for inclusion stability and expansion.

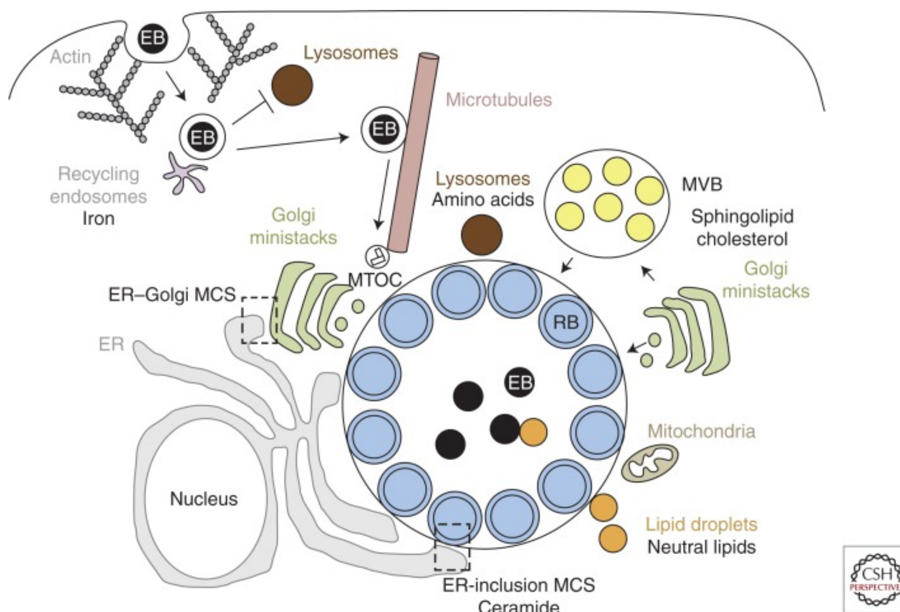

Figure 1.4. Model for chlamydial and host-cell interactions. The initial cell invasion step involves actin recruitment and remodeling. Upon uptake, EBs are surrounded by a membrane bound vacuolar compartment. The early inclusion is rapidly modified by the insertion of the bacterial Inc proteins that inhibit the association with the endocytic pathway or lysosomes. Early inclusions are transported along microtubules to the perinuclear region, where EBs are converted in RBs that divide and promote inclusion growth. Chlamydial infections generate Golgi ministacks surrounding the bacterial inclusion that most likely provide the main source of lipids for the chlamydial inclusion. Alternative lipid transport pathways include interception of: multivesicular bodies (MVBs) and Lipid Droplets (LDs) Additionally, the inclusion is thought to interact with the endoplasmic reticulum (ER), lysosomes and mitochondria. (Reprinted from Bastidas *et al*, 2013, with permission from Elsevier).

1.2.5. Inclusion expansion

The inclusion must expand to accommodate the higher number of bacteria as RBs divide. After establishing near the perinuclear region, the inclusion is surrounded by host actin and intermediate filaments that form a dynamic scaffold. This provides structural integrity to the chlamydial vacuole and minimizes immune detection (Kumar & Valdivia, 2008). Following a period of rapid cell division, RBs begin to

re-differentiate back into EBs. Expression of late-cycle genes occurs at this point, including genes that encode for components of the outer membrane complex (e.g. OmcA and OmcB) and proteins involved in the condensation of the chromosome (e.g. HctA and HctB).

1.2.6. Exiting the host cell

Once the developmental cycle is completed, EBs must be released to initiate new rounds of infection. The release of EBs might occur by two mutually exclusive mechanisms: cell lysis or extrusion (Hybiske & Stephens, 2007b; Todd & Caldwell, 1985) (Fig. 1.5). Lysis consists of an ordered sequence of inclusion, nucleus and plasma membrane ruptures. This calcium dependent-mechanism is mediated by proteases and is accompanied by death of the host cell. Extrusion, on the other hand, results in the pinching of the inclusion into separable compartments, protrusion out of the cell within a membrane compartment and ultimately detachment from the host cell, leaving the original cell and inclusion intact. The amount of bacteria packaged within extrusions is variable and the rate of extrusion is slow when compared with cell lysis. Extrusion requires actin polymerization, N-WASP, Myosin II and Rho GTPases. Differences in cell exit mechanisms strongly correlate with *in vivo* host cell invasiveness and consequently this is possibly an important determinant of pathogenesis: more aggressive strains such as the LGV strains tend to be more lytic, whereas non-LGV genital or ocular strains tend to be less lytic and exit cells predominantly by extrusion.

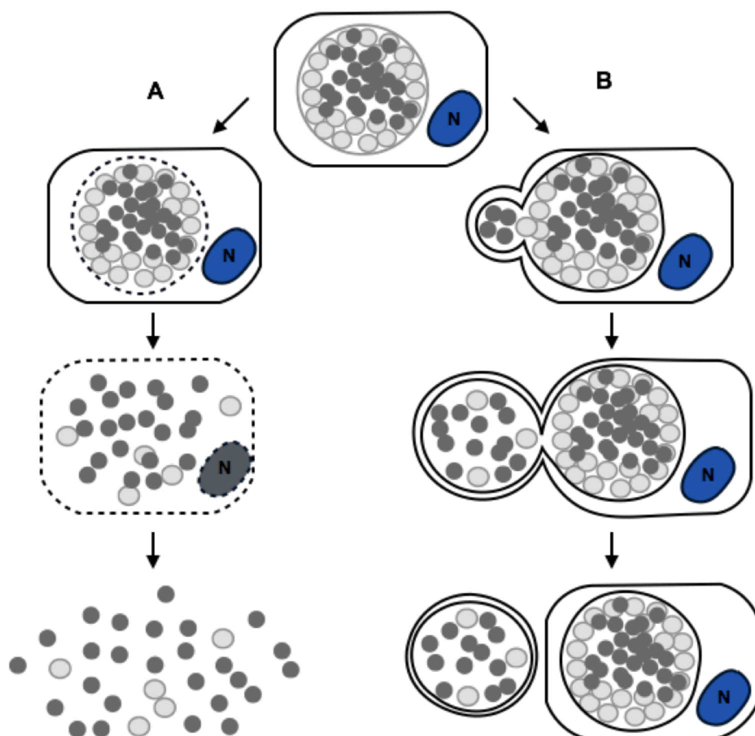

Figure 1.5. Alternative mechanisms for host cell exit. Once the cycle is completed EBs must be released to initiate new rounds of infection. This can occur by two mutually exclusive ways. **(A)** Cell lysis, which consists of an ordered event of membrane permeabilizations and **(B)** Extrusion that results in the pinching of the inclusion into two separate compartments. (Adapted from Stephen and Hybiske, 2007).

1.2.7. Persistence

Persistence in *Chlamydia* has been described as a viable but non-cultivable growth stage resulting in a long-term infection within the host cell (Hogan *et al.*, 2004). It is important to note that persistent forms of *Chlamydiae* have been primarily studied in culture. During the persistent stage, chlamydial metabolism is slowed and cell division as well as RB-EB differentiation is halted. This state is characterized by morphologically enlarged and aberrant RBs, often called aberrant bodies (ABs) that are viable but non-infectious

(Fig. 1.6). ABs have lower levels of outer membrane components MOMP and OmcB (Beatty *et al.*, 1993; Hogan *et al.*, 2003) and enhanced expression of heat shock proteins (Beatty *et al.*, 1994).

Several factors have been described to induce a persistence state (reviewed in Schoborg, 2011): cytokines (IFN-γ); nutrient starvation; growth in non-permissive cells; iron deprivation; antibiotics; heat-shock; co-infection with Herpes Simplex Virus (HSV); or chlamydiophage infection. However, persistence is a reversible state since bacteria can be maintained for a long time in culture, but once the inducer is removed their normal replicative cycle is restored.

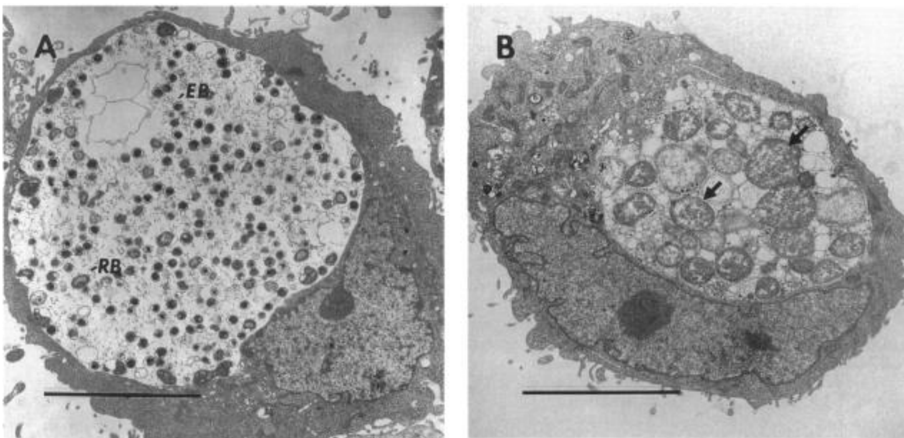

Figure 1.6. Electron micrographs of *C. trachomatis* infected cells showing normal or aberrant chlamydial inclusions, **48 h post-infection. (A)** A typical inclusion, with EBs and RBs. (B) Treatment of cells with IFN-γ results in smaller inclusions containing enlarged and aberrant RBs, the Aberrant Bodies (ABs) (highlighted with arrows) that are viable but non-infectious. Scale bars, 5 µm. (Reprinted from Beatty *et al*, 1994, with permission from American Society for Microbiology).

Even though the concept of persistence has been recognized for decades, definitive *in vivo* evidence is not conclusive. Aberrant inclusions have been observed *in vivo* but whether or not *Chlamydia*

enter this developmental state to establish chronic infections has not yet been determined (Hogan *et al.*, 2004; Wyrick, 2010).

An old paradigm in the field is that in virtually all animal hosts, including birds and mammals, *Chlamydia* spp. reside in the gastrointestinal (GI) tract for long periods of time in the absence of a clinical disease (Rank & Yeruva, 2014). Based on this, a different view on chlamydial persistence related to the survival of *Chlamydia* as commensal bacteria in the GI tract has been proposed (Rank & Yeruva, 2014).

1.2.8. Regulation of gene expression during the developmental cycle

Chlamydia genes are expressed in three major temporal classes that correspond to the main stages of the developmental cycle (Belland *et al.*, 2003; Nicholson *et al.*, 2003; Shaw *et al.*, 2000):

- Early genes are transcribed as early as 1 to 3 h after bacterial entry when the EB is beginning to convert into an RB and play a role in establishing the initial intracellular niche.
- Mid cycle genes include the majority of the chlamydial genes and are thought to play a role in RB growth and replication.
- Late genes include a small number of genes that are involved in the final stages of infection, namely in the RB to EB conversion and EB function.

Transcription in *Chlamydia* is thought to be regulated by three main mechanisms: alternative forms of RNA polymerase (Barta *et al.*, 2015; Belland *et al.*, 2003; Douglas & Hatch, 2000; Shaw *et al.*, 2000; Yu & Tan, 2003), DNA supercoiling (Cheng & Tan, 2012; Hatfield & Benham, 2002; Niehus *et al.*, 2008; Orillard & Tan, 2015) and

transcription factors (Bao *et al.*, 2012; Rosario & Tan, 2012; Rosario *et al.*, 2014). There are also reports of interactions of type III secretion chaperones (see section 1.4.7. below) with RNA polymerase (Hanson *et al.*, 2015), regulation by small RNAs (Tattersall *et al.*, 2012) and transcription from tandem promoters (Rosario & Tan, 2016). Together, the mechanisms of regulation of gene expression result in a hierarchically organized and timely regulated transcription of genes.

1.3. Modification of the host response during infection

In the initial stages of infection, *Chlamydia* is recognized by host pattern recognition receptors (PRRs) through specific pathogen-associated molecular patterns (PAMPs). Candidate chlamydial PAMPs described to date include bacterial LPS (Ingalls *et al.*, 1995) which are recognized by TLR4 (Heine *et al.*, 2003) or Hsp60 which is recognized by TLR4 or TLR2 (Bulut *et al.*, 2002, 2009; Costa *et al.*, 2002; Kol *et al.*, 1999; Vabulas *et al.*, 2001). Additionally, several studies indicate that cytosolic PRR may also be activated by PG or by the highly crossed-linked and immunogenic outer membrane proteins (MOMP, OmcB or OmcA) (reviewed in Bastidas *et al.*, 2013). Recognition of PAMPs by their receptors leads to the activation of diverse inflammatory signaling pathways, including the NF-κB pathway or MAPK pathway, with subsequent production of cytokines and chemokines.

Upon infection there is an acute localized inflammatory response mediated mainly by polymorphonuclear lymphocytes (PMNs) recruited by cytokines and chemokines that are released by epithelial cells (Kelly & Rank, 1997; Rank *et al.*, 2000, 2008). Early responses also target the site of infection for immature dendritic cells (DC) that

constitute an important step for the adaptive responses (Rank *et al.*, 2010). The recruitment of immune cells that mediate innate and adaptive immune response upon infection with *C. trachomatis* are in the majority of cases sufficient to clear primary infections. However, inflammatory responses from recurring infections seem to be the main cause for the detrimental scarring and pathologies observed (Darville & Hiltke, 2010; Stephens, 2003).

During the intracellular stages of *C. trachomatis* infection the accessibility of PAMPs is minimized by the recruitment of actin and intermediate filaments to the periphery of the inclusion (Kumar & Valdivia, 2008). Moreover, *Chlamydia* has the ability of subverting host signaling pathways in order to avoid activation of innate immune responses and promote bacterial and host survival. Two well-studied examples of this mechanism are the modulation of NF-kB responses and the inhibition of apoptosis.

NF-kB acts as a central regulator of immune responses. The NF-kB subunits p65 (relA) and p50 form a heterodimeric complex that is translocated into the nucleus where it acts as a transcription factor of cytokines and other antimicrobial factors (Hayden *et al.*, 2006). Different chlamydial proteins have been described to interfere with the NF-kB signaling pathway during infection:

i) ChlaDub1 (deubiquitinase) inhibit ubiquitin-mediated protein degradation of the NF-kB retention factor (IκB) and consequently block nuclear translocation of NF-kB (Le Negrate *et al.*, 2008);
ii) CT441 (a protease) blocks NF-kB activation directly through the proteolysis of the RelA subunit (Lad *et al.*, 2007a, b).

iii) iii) CPAF (Chlamydial Protease-Like Activity Factor) has been implicated in the degradation of p65 during infection (Christian *et al.*, 2010).

It is thought that *Chlamydia* regulates apoptosis in a temporal manner: preventing host cell death early in the developmental cycle, while promoting its occurrence in the final stages of the cycle. Several mechanisms have been proposed to interfere with the apoptotic pathway (reviewed in Bastidas *et al.*, 2013): CPAF mediated degradation of the pro-apoptotic BH3 proteins and subsequent inhibition of cytochrome *c* release; recruitment of certain BH3-only proteins away from their functional site at the mitochondria; upregulation and stabilization of Mcl-1 (major anti-apoptotic protein) or activation of caspase 3. The molecular basis for most of the anti-apoptotic pathways still remains to be elucidated but clearly *Chlamydia* seems to have adopted redundant mechanisms.

1.4. The *Chlamydia* type III secretion system

1.4.1. The general importance of secretion systems in pathogenic bacteria

Type III, type IV and type VI secretion systems are specialized nanomachines used by many Gram-negative bacteria to deliver effector proteins into eukaryotic or prokaryotic target cells (Costa *et al.*, 2015). While these secretion systems are often associated with bacterial virulence, the most widespread function of type IV secretion systems (T4SSs) is the transfer of DNA between bacteria during conjugation (Ilangovan *et al.*, 2015), the most common function of type VI secretion systems appears to be bacterial antagonism (Basler, 2015; Cianfanelli *et al.*, 2015), and type III secretion systems

(T3SSs) are also used in the establishment of symbiotic relationships between bacteria and eukaryotic hosts (Galán *et al.*, 2014). Nevertheless, it is unquestionable that T3SSs, T4SSs, and T6SSs have an essential role in bacterial virulence.

In terms of bacterial virulence, T3SSs and T4SSs have been well characterized and they have been reported in a wide range of obligate intracellular, facultative intracellular and extracellular pathogens, such as *Brucella* spp. (T4SS; Boschiroli *et al.*, 2002), *Chlamydia trachomatis* (T3SS; Stephens *et al.*, 1998), *Coxiella burnetti* (T4SS; Seshadri *et al.*, 2003), enterohemorrhagic and enteropathogenic *Escherichia coli* (EHEC and EPEC, respectively; T3SS; Jarvis *et al.*, 1995; McDaniel *et al.*, 1995), *Helicobacter pylori* (T4SS; Censini *et al.*, 1996), *Legionella pneumophila* (T4SS; Segal *et al.*, 1998; Vogel *et al.*, 1998), *Pseudomonas aeruginosa* (T3SS; Yahr *et al.*, 1996) *Salmonella* spp. (T3SS; Galán, 1996; Ochman *et al.*, 1996; Shea *et al.*, 1996), *Shigella* spp. (T3SS; Maurelli *et al.*, 1985; Parsot *et al.*, 1995; Sasakawa *et al.*, 1988), or *Yersinia* spp, (T3SS; Perry *et al.*, 1998).

As mentioned above, T6SSs have been mainly described as a way to target other bacterial cells with lethal toxins. However, T6SS effectors have also been implicated in the subversion of eukaryotic host cells (Hachani *et al.*, 2015). Many bacterial species possess this type of secretion system, including the human pathogens *S. enterica* and *P. aeruginosa* or the plant pathogen *Rhizobium leguminosarum* (Filloux *et al.*, 2008).

In addition, Gram-positive bacteria such as *Mycobacterium tuberculosis* and *Staphylococcus aureus* have also been described to

possess specialized secretion systems that transport proteins across their rigid cell wall. This secretion system has been referred to as type VII secretion system (Abdallah *et al.*, 2007). In *M. tuberculosis* at least one protein delivered by this system has been associated with modulating host cellular responses, such as suppression of pro-inflammatory responses and induction of apoptosis or cell necrosis (Simeone *et al.*, 2009, 2012).

Evolution of protein delivery systems such as T3SS, T4SS or T6SS were most likely driven by the need to deliver multiple proteins in a coordinated way in order to modulate host cellular processes. Protein effectors are known to target most cellular compartments, organelle membranes, the nucleus and cytoskeleton components (Tosi *et al.*, 2013). General knowledge on effector functions is crucial for understanding cellular physiopathology associated with bacterial pathogens as well as fundamental aspects of host-pathogen interactions.

1.4.2. Type III secretion systems

T3SS are present in many Gram-negative bacteria that are pathogens of animals and plants [reviewed by (Cornelis, 2006; Galán *et al.*, 2014)]. They consist of: i) the components of a secretion machinery known as injectisome (basal structure and a 2-3 nm hollow needle or a long pilus protruding from the bacterial surface); ii) a translocon; iii) effector proteins, which have the capacity to modulate a variety of cellular functions (Galán, 2009) but display little sequence similarity between different bacterial species; iv) specific T3S chaperones; v) regulatory proteins. While a subset of proteins that compose the injectisome are highly conserved across different bacterial species, effector proteins are normally species-specific.

T3SSs have been extensively studied in *Yersinia* spp., *Salmonella* spp. or *Shigella* spp., which led to the comprehensive understanding of the assembly and function of the injectisome as well as to the identification of numerous effector proteins. Early phylogenetic analysis revealed that T3SSs are evolutionary related to the flagellar apparatus (Pallen *et al.*, 2005). More recently, it has been described that T3SSs suffered loss of essential flagellar motility genes and acquired components that enabled protein secretion across the bacterial envelope and translocation through eukaryotic membranes (Abby & Rocha, 2012).

The injectisome is a multi-protein complex that allows translocation across the two bacterial membranes. The translocon is a pore complex formed within a host cell membrane by two type III secreted proteins (the translocators) and is connected to the needle through a tip complex (formed by another type III secreted translocator protein), thereby creating a continuous channel between the cytosol of the bacteria and the host cell that enables the one-step delivery of effector proteins. Secretion of some T3S substrates requires specific strictly bacterial cytosolic chaperones, characterized by an acidic pI (4-5), a low molecular mass (15-20 kDa), formation of dimers, and lack of ATPase activity. The activity of T3SSs is regulated at transcriptional and post-translational levels to ensure its functioning at the right time and place and in the appropriate environmental conditions. It is normally thought that most T3SSs are activated upon contact of the bacteria with a host cell membrane, although, for example, the T3SS of intravacuolar *Salmonella* appears to be activated by pH sensing (Yu *et al.*, 2010).

1.4.3. Discovery and unique features of the *Chlamydia* T3SS

Genome sequence revealed that all *Chlamydia* species, including the environmental *Chlamydia*, contain a set of genes capable of encoding a complete T3SS (Horn *et al.*, 2004; Read *et al.*, 2000, 2003; Stephens *et al.*, 1998). Unlike other Gram-negative bacteria where the T3S genes are located on a virulence plasmid or arranged in pathogenicity islands, in *Chlamydia* the genes coding for components of the T3SS are grouped in three major loci. Furthermore, in other Gram-negative bacteria T3S genes have often a G + C content lower than their respective genomes, while in *Chlamydia* the G + C content does not differ from the remaining genome. Additionally there is no evidence of transposons, insertion sequence elements or horizontal gene transfer that would suggest recent acquisition of the T3SS (reviewed in Mueller *et al.*, 2014).

Recent cryo-electron tomography studies in *C. trachomatis* revealed polarity in EBs (Nans *et al.*, 2014, 2015a): one pole is characterized by an inner membrane tubular invagination while the opposite pole exhibits an asymmetric widening of the periplasmic space that contains the T3SSs. This pole faces the target cells, promoting contact of T3SS with host plasma membrane. Interestingly, once internalized in early vacuoles both EB polarity and T3SS are lost.

Additionally, cryo-electron tomography data has revealed the first intact structures of *C. trachomatis* T3SS (Nans *et al.*, 2015b) and revealed key structural distinctions between the chlamydial T3SS when compared with the same system in other bacteria, in particular the basal body is more elongated with a pronounced convex structure (with a length of 34 nm and a diameter that ranges from 14 to 20 nm) while the needle is shorter and is associated with an additional ring-

like structure on the outer membrane surface. Furthermore, this work compared *C. trachomatis* T3SS structures in the presence or absence of host cell membranes, revealed a compaction of the basal body in the presence of host cell membrane that suggested a pump-action conformational change related to effector injection.

The nominations of the different components of the T3SS in *Chlamydia* is as follows: contact dependent secretion (Cds) refers to the components of the basal apparatus; secreted or mobile components are sometimes denoted as *Chlamydia* outer proteins (Cops) and cytoplasmic T3S chaperones are referred to as specific *Chlamydia* chaperone (Scc), SycE-like chaperones (Slc) or multicargo chlamydial specific chaperone (Mcsc). The major components of the *Chlamydia* T3SS have been reviewed in (Dai & Li, 2014; Ferrell & Fields, 2015; Mueller *et al.*, 2014) and are schematically represented in Fig. 1.7.

1.4.4. The *C. trachomatis* injectisome

Chlamydia genomes encode proteins homologous to essential components of the injectisomes of other bacteria (*Yersinia*, *Salmonella*) where the T3SSs have been studied in further detail. Largely based on this, it has been possible to establish a function/localization for each of the Cds proteins of *C. trachomatis* (Fig. 1.7):

- CdsQ (C-Ring protein) is predicted to be at the base of the injectisome and it has been shown by yeast two-hybrid to interact with multiple chlamydial proteins (Spaeth *et al.*, 2009). The base of the secretion apparatus is proposed to serve as docking site for chaperone-substrate complexes (Spaeth *et*

al., 2009) and may play a central role in regulating the recognition of effector proteins.

- The basal body is formed by three structural proteins: CdsC, CdsD and CdsJ (Betts-Hampikian & Fields, 2010; Meriläinen *et al.*, 2016; Nans *et al.*, 2015b). CdsJ is a lipoprotein, predicted to span the periplasmic space and associate with integral membrane proteins CdsR-V. It is also thought to interact with the inner membrane protein CdsD.
- CdsV is predicted to be inserted in the inner membrane but has also been found to face the cytoplasmic domain of the inner membrane (Nans *et al.*, 2015b) where it may interact with other components of the apparatus, chaperones or even effector proteins.
- The outer membrane ring of the injectisome, necessary for the needle to cross the outer membrane, is composed of the secretin CdsC that is thought to form a rosette-like hexameric structure (Nans *et al.*, 2015b). However, CdsC is phylogenetically separated from other secretins and contains a large *Chlamydia*-specific N-terminal domain (Abby & Rocha, 2012).
- CdsF is able to polymerize into multi-subunit complexes forming the needle-like projection (Betts *et al.*, 2008). In other T3SSs a tip protein located at the terminus of the needle serves a role in activating the system and/or allowing for translocon assembly (Mueller *et al.*, 2008). However, to date there is no evidence of a corresponding structure in *Chlamydia*.
- CdsU is suggested to contribute to changes in substrate specificity (Ferrell & Fields, 2015). CdsN is most probably an

ATPase (Nans *et al.*, 2015b; Stone *et al.*, 2008) while CdsL functions as its regulator (Stone *et al.*, 2011).

- CT398, recently named CdsZ, (Barta *et al.*, 2015) was found to interact with CdsL as well as with the alternative sigma-factor RpoN (σ^{54}), implying that CdsZ may interact with RNA polymerase.

1.4.5. The *C. trachomatis* translocon

CopB and CopD are T3S substrates (Fields *et al.*, 2005; Ho & Starnbach, 2005) and are components of the invasion-related translocon that forms a pore in the host cell membrane (Bulir *et al.*, 2014, 2015) (Fig. 1.7), allowing the translocation of subsequent secreted effectors across the host membrane. Interestingly, *Chlamydia* encodes for potential duplicators of the translocator proteins: CopB2 and CopD2. The expression levels of CopB and CopB2 vary during the developmental cycle (late cycle and mid-cycle, respectively), raising the possibility for the existence of two different translocons: CopB acting during early and late stages of infection while CopB2 acting during mid-developmental cycle (Chellas-Géry *et al.*, 2011).

1.4.6. The *C. trachomatis* gatekeeper

CopN is an homolog of the *Yersinia* T3S substrate YopN (Fields & Hackstadt, 2000) that has been shown to associate with other proteins and block secretion prior to activating signals (Silva-Herzog *et al.*, 2011), functioning as a gatekeeper (Archuleta & Spiller, 2014; Barison *et al.*, 2013). Once activated, the T3S apparatus is thought to secrete CopN, followed by the translocator proteins. Secretion of the translocators results in a complete T3SS at which point effectors can be injected into the host cell (Fig. 1.7). Moreover, CopN has been

shown to localize at the inclusion membrane and might also function as an effector protein by modulating host microtubules within infected eukaryotic cells (Archuleta *et al.*, 2011; Fields & Hackstadt, 2000; Huang *et al.*, 2008).

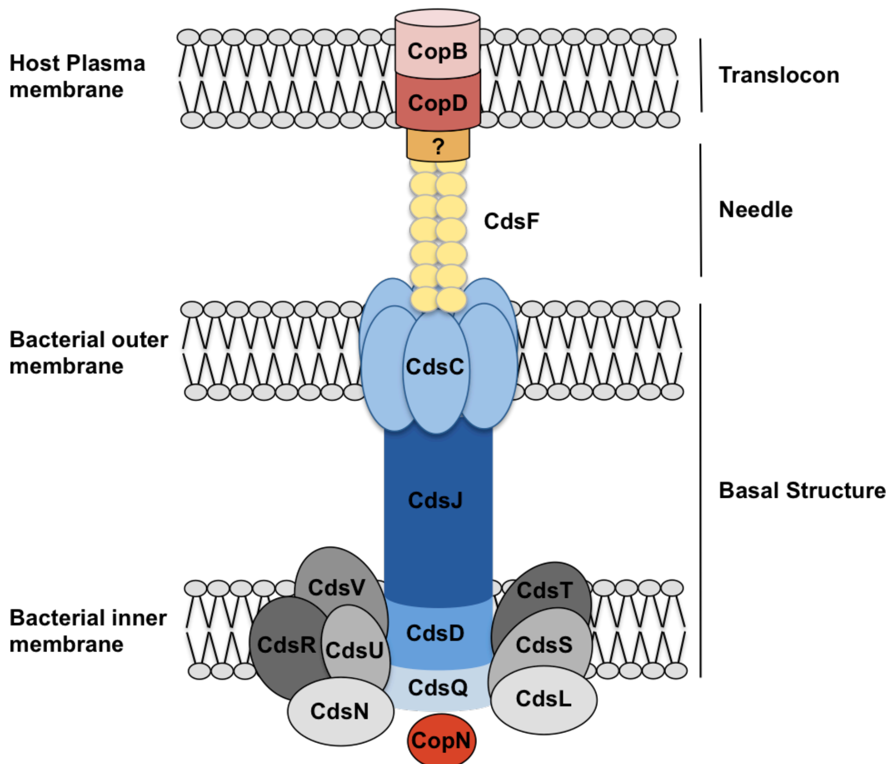

Figure 1.7. Schematic representation of the predicted composition of T3S apparatus in *Chlamydia*. The needle-like structure protrudes from the bacterial surface and enables translocation of effector proteins from the bacterial cytoplasm into the host cell cytosol or membranes. (Adapted from Betts-Hampikian & Fields, 2010; Peters *et al.*, 2007; Spaeth *et al.*, 2009).

1.4.7. The *C. trachomatis* T3S chaperones

Several roles have been associated with T3S chaperones, including: i) protecting their substrates from degradation, ii) keeping their substrates in a partially unfolded secretion-competent state, iii)

preventing premature/unproductive interactions with the respective substrate and iv) directly promoting the secretion of their substrates via direct interactions with components of the injectisome. In addition T3S chaperones have also been shown to modulate RNA polymerase through interactions with σ^{66} subunit (Hanson *et al.*, 2015; Rao *et al.*, 2009). It has also been speculated that T3S chaperones might help to establish a hierarchy in secretion of protein substrates.

T3S chaperones have been divided into 3 classes: class I chaperones bind effectors, class II chaperones bind pore-forming translocators, while class III chaperones bind subunits of the injectisome or flagellar substructures. Class I chaperones are the best studied. They share low sequence similarity between each other but share a conserved three-dimensional (3D) structure. This group can be further divided depending on the number of effectors with which they can associate. Currently, there are only two chaperones identified that are dedicated to effectors (Slc1/CT043 and Mcsc/CT260) and both are multicargo chaperones. Slc1/CT043 is a known chaperone for TarP, CT694 and TepP (Brinkworth *et al.*, 2011; Chen *et al.*, 2014; Pais *et al.*, 2013) and Mcsc/CT260 has been shown to bind and stabilize at least Cap1 and two other Inc proteins, CT225 and CT618 (Spaeth *et al.*, 2009). It is surprising, however, that given the number of chlamydial candidate effectors indicated to date there is such a limited number of chaperones described.

CT584 has also been indicated as a potential chaperone (Pais *et al.*, 2013) that interacts with CT082, an identified T3S substrate (Hovis *et al.*, 2013; Pais *et al.*, 2013). Other studies based on biophysical characterizations have also suggested Cpn0803 (the *C. pneumoniae* orthologue of CT584) to act as the tip complex protein (Markham *et*

al., 2009). However, subsequent determination of the three-dimensional structure of the protein did not support the hypothesis (Stone *et al.*, 2012).

Class II chaperones described to date include Scc2/CT576, chaperone for CopB (Fields *et al.*, 2005) and Scc3/CT862 chaperone for CopB2 (Fields *et al.*, 2005). In addition CP0432 (Scc1) and CP0033 (Scc4) (the *C. pneumoniae* orthologues of CT088 and CT663, respectively) promote CopN secretion as a heterodimeric chaperon (Silva-Herzog *et al.*, 2011), while Scc3 (CT862) inhibits CopN secretion (Silva-Herzog *et al.*, 2011).
Finally, CdsE and CdsG belong to Class III chaperones and have been identified as chaperones for CdsF, the needle component (Betts *et al.*, 2008).

1.4.8. The *C. trachomatis* T3S effector proteins

In *C. trachomatis* T3S effectors have been shown to be translocated into the host cell cytosol or into the inclusion membrane (Betts *et al.*, 2009; Valdivia, 2008). Even though *C. trachomatis* is thought to have 5-10% of its genome encoding for proteins with putative T3S signals (Arnold *et al.*, 2009; Samudrala *et al.*, 2009), only very few T3S effectors have been characterized to date:

1.4.8.1 TarP

TarP (Translocated Actin Recruiting Phosphoprotein) is conserved among all *Chlamydia* spp., was identified using the heterologous T3SS of *Y. enterocolitica* and is the most thoroughly studied *Chlamydia* effector: it is synthesized at late stages of the developmental cycle when it is packaged into EBs; it is secreted within minutes after contact with the host cell (Clifton *et al.*, 2004) and

is mainly involved in actin recruitment and remodeling events that occur in the invasion step of the chlamydial developmental cycle. *C. trachomatis* TarP exhibits different functional domains (N-terminal tyrosine repeat units and C-terminal actin binding domains) that vary in number across different serovars. This characteristic may account for differences in tissue tropism and invasiveness (Jewett *et al.*, 2010; Lutter *et al.*, 2010).

Tyrosine phosphorylation at the N-terminus of TarP is *C. trachomatis*-specific and correlates with interactions with several signaling pathways, such as EGF receptor, MAPK cascades, Rac/WAVE2/ARP 2/3 signaling and with humoral and cellular immune signaling (Clifton *et al.*, 2004; Jewett *et al.*, 2008; Jiwani *et al.*, 2012; Mehlitz *et al.*, 2010). On the other hand, TarP-mediated actin nucleation and actin filament biding is restricted to the C-terminal half of the protein. (Carabeo *et al.*, 2004; Jewett *et al.*, 2006; Jiwani *et al.*, 2013; Lane *et al.*, 2008).

1.4.8.2. CT694

CT694 is present only in *C. trachomatis* and *C. muridarum* and was identified by transcriptome analyses to be expressed late in the developmental cycle (Belland *et al.*, 2003). This suggested that CT694 could play a role in the early events of host cell entry, such as TarP. CT694 has been shown to cause endocytic and other toxic effects in yeast (Sisko *et al.*, 2006). In addition, using the heterologous T3SS of *Y. enterocolitica* and further characterization, CT694 was identified as an invasion-related effector found to be secreted minutes after contact with the host cell (Hower *et al.*, 2009). It was described to interact with actin indirectly, by interfering with AHNAK-mediated actin mobilization. Interaction with AHNAK is

through a C-terminal domain and affects the formation of host cell actin stress fibers. It also contains a membrane localization domain (not necessary for AHNAK binding) that resembles membrane localization domains found in effector proteins from *Yersinia*, *Pseudomonas* and *Salmonella* spp. CT694 may influence actin dynamics by manipulating the activity of Rho GTPases (Bullock *et al.*, 2012). It has been hypothesized that CT694 may play a similar role as the *Salmonella* SopE effector (containing an analogous membrane localization domain) (Zhou & Galán, 2001) which implies that CT694 could negatively regulate the actin cytoskeleton alterations caused by the activity of TarP.

1.4.8.3. TepP

TepP/CT875 (Translocated early phosphoprotein) was identified in a search to identify new interacting partners of the chaperone Slc1. This protein is highly conserved in all *C. trachomatis* serovars; it shares 49% identity with its orthologue in *C. muridarum* and shares less than 25% identity with potential orthologues of other *Chlamydia* spp.. TepP was found to be translocated early during bacterial entry and it is tyrosine-phosphorylated upon translocation into host cells by host kinases (Chen *et al.*, 2014). It has been shown to act downstream of TarP by recruiting scaffolding proteins like Crk to the nascent inclusion which in turn might recruit other proteins mediating a series of signaling events that are important in establishing an early replicative niche and adequate immune environment within infected cells.

1.4.8.4. CT619, CT620, CT621, CT711 and CT712

All pathogenic *Chlamydia* species contain four or five proteins with a domain of unknown function (DUF582) unique to the genus *Chlamydia* (CT619, CT620, CT621, CT711 and CT712 in *C. trachomatis*) and were all identified as putative T3S substrates based on amino-terminal signal recognition by the heterologous T3SS of *S. flexneri* (Muschiol *et al.*, 2011; Subtil *et al.*, 2005). In addition, *C. trachomatis* CT620, CT621, and CT711 were shown to be expressed in the mid and late stages of the chlamydial developmental cycle. Immunolocalization studies detected CT620 and CT621 both in the cytoplasm and nucleus of the host cell, showing these proteins are undoubtedly effectors that could target nuclear cell functions. Additionally, CT620 and CT621 were also found to be secreted into the lumen of the inclusion, where they do not associate with bacterial markers (Muschiol *et al.*, 2011). Based on these observations it is very likely that CT619, CT711, and CT712 are also *C. trachomatis* effector proteins.

1.4.8.5. NUE/CT737

In silico analysis of CT737 identified a conserved SET domain, present in proteins that are involved in chromatin remodeling. SET domain proteins are present in all eukaryotes (Dillon *et al.*, 2005), but are also present in pathogenic bacteria or viruses that interact with eukaryotic cells (Alvarez-Venegas *et al.*, 2007; Hamon *et al.*, 2007). CT737 was identified as a histone methyltransferase conserved among all *Chlamydia* spp., and as a putative T3S substrate based on amino-terminal signal recognition by the heterologous T3SS of *S. flexneri*. In addition, CT737 was found to be secreted by *C. trachomatis* and translocated into the host nucleus where it associates with chromatin (Pennini *et al.*, 2010).

1.4.8.6. CT847

CT847 is conserved among all *Chlamydia* spp. and was identified using the T3SS of *Y. enterocolitica* as heterologous host. Yeast-two hybrid studies using CT847 as bait to screen a HeLa cDNA library identified an interaction with mammalian Grap2 cyclin D-interacting protein (GCIP), a multi-functional ubiquitously expressed protein that is degraded during chlamydial infection (Chellas-Géry *et al.*, 2007).

1.4.8.7. Inc proteins

The Inc family of proteins contain two major characteristics: an N-terminal T3S signal that is necessary for their translocation out of the bacteria (Almeida *et al.*, 2012; Dehoux *et al.*, 2011; Subtil *et al.*, 2001) and a large bi-lobed hydrophobic domain of 40-60 residues (Bannantine *et al.*, 1998) that is predicted to enable the insertion of these proteins into the inclusion membrane. It is thought that at least one segment of these proteins is facing the cytoplasm of the host cell where interactions with host proteins are likely to occur. A bioinformatics analysis of chlamydial genomes identified 59 putative Incs in *C. trachomatis* and 107 in *C. pneumoniae*, thus the *inc* genes are thought to represent 7-10% of the genomes of *Chlamydia* (Dehoux *et al.*, 2011). These proteins are thought to have a central role in the biogenesis and maintenance of the inclusion. Depending on the host protein they associate with, these proteins should play a major role in avoiding endocytic membrane fusion that would prevent destruction of the bacteria or, on the contrary, promoting exocytic vesicle fusion with nutrient rich membrane compartments (see section 1.2.4., below). It has also been proposed that some Inc proteins, lacking a long stretch of amino acids predictably facing the host cell cytosol, might interact with other Inc proteins in the inclusion membrane and form supramolecular complexes and thus provide a

structural role in maintaining the integrity of the inclusion rather than an effector function (Gauliard *et al.*, 2015; Moore & Ouellette, 2014).

It is important to note that this group of proteins is not conserved among all chlamydial species. However, very few variations among Inc proteins are likely to contribute to *C. trachomatis* serovar-specific differences in inclusion maintenance that might contribute to tropism and invasiveness specific characteristics (Almeida *et al.*, 2012; Lutter *et al.*, 2012).

Thus far, the role of a number of *C. trachomatis* Inc proteins has been elucidated:

- IncA/CT119 is involved in the homotypic fusion of *C. trachomatis* inclusions by interaction with SNARE proteins (Delevoye *et al.*, 2008; Suchland *et al.*, 2000).
- IncB/CT232, CT101, CT222 and CT850 were found to localize in inclusion membrane microdomains that are enriched in activated Srk kinases and in cholesterol. These microdomains are also in close association with the microtubule network and with centrosomes (Mital *et al.*, 2010).
- CT850 has been reported to interact with dynein (Mital *et al.*, 2015).
- CT229 and CT813 are thought to play a role in intercepting host vesicular trafficking by their respective interaction with host protein Rab4 (Rzomp *et al.*, 2006) and VAMP7-8 (Delevoye *et al.*, 2008).
- CT813 was recently renamed InaC (inclusion membrane protein for actin assembly) (Kokes *et al.*, 2015). It was described to bind host ARF and 14-3-3 proteins and to

modulate F-actin assembly and Golgi redistribution around the chlamydial vacuole.

- IncD/CT115 has been shown to mediate the recruitment of lipid protein CERT and the ER-protein VAPB to contact sites between the inclusion membrane and the ER (Agaisse & Derré, 2014).
- IncE has been recently found to bind to the SNX5/6 components of the retromer, which re-localizes SNX5/6 to the inclusion membrane and promotes inclusion membrane tubulation (Mirrashidi *et al.*, 2015).
- IncG/CT118 was also found to interact with the mammalian signal transducer protein 14-3-3-β at the inclusion membrane (Scidmore & Hackstadt, 2001).
- IPAM/CT223 (inclusion protein acting on microtubules), recruits the centrosomal protein CEP170 to hijack the microtubule organizing functions of the host cell (Dumoux *et al.*, 2015).
- CT228 recruits elements of the myosin phosphate pathway and is associated to regulation of host cell exit (Lutter *et al.*, 2013).

1.4.9. Probable *C. trachomatis* T3S effector proteins

There is an additional group of proteins that due to multiple lines of evidence are generally pointed out as likely T3SS effectors, but in fact they have not clearly been demonstrated to be so. This group of proteins also includes CT619, CT711, and CT712 (discussed above, section 1.4.8.4.).

1.4.9.1. CADD/CT610

CADD/CT610 (*Chlamydia* protein associating with death domains) has orthologues in *C. trachomatis*, *C. pneumoniae* and *C. muridarum*. It induces apoptosis in a variety of mammalian cell lines when ectopically expressed. Immunolocalization studies revealed that this protein localizes at the periphery of the inclusion, suggesting that it could be secreted from the bacteria (Stenner-Liewen *et al.*, 2002). In fact, it was identified as putative T3SS substrate based on amino-terminal signal recognition by the heterologous T3SS of *S. flexneri* (Subtil *et al.*, 2005).

1.4.9.2. Cap1

Cap1/CT529 (class I accessible protein-1) localizes to the inclusion membrane but does not possess the bilobal hydrophobic domain characteristic of the Inc family of proteins (Fling *et al.*, 2001).

1.4.9.3. ChlaDub1 and ChlaDub2

ChlaDub1/CT868 and ChlaDub2/CT867, are predicted cysteine proteases, likely to have deubiquitinating and deneddylating activities on host cell proteins (Misaghi *et al.*, 2006). They are present in all *Chlamydia* species except for *C. pneumoniae* and their catalytic domains share similarities to other eukaryotic ubiquitin-like proteases. Additionally ChlaDub1/CT868 has also been found to have the capacity to suppress the NF-κB pathway (Le Negrate *et al.*, 2008) (see also section 1.3., above).

1.4.9.4. Cytotoxin CT166

CT166 is present in the plasticity zone (PZ) of *C. trachomatis* genital serovars D-K, in *C. muridarum* and in *C. caviae*. Protein database alignment revealed homologies with the N-terminal

glucosyltransferase domain of *Clostridium difficile* large cytotoxins A and B. Ectopic expression of CT166 results in an immediate cytotoxic phenotype in host cells (Belland *et al.*, 2001). CT166 has been suggested to induce actin reorganization, that may involve the glycosylation of Rac1 (Thalmann *et al.*, 2010).

1.4.9.5. Lda1/CT156, Lda2/CT163 and Lda3/CT473

The Lda proteins (Lipid droplet associated) were found to be translocated into the eukaryotic host and have affinity for host cell lipid droplets (LD). They are proposed to contribute to the recruitment of LD into the inclusion lumen (Kumar *et al.*, 2006). Lda1/CT156 and Lda2/CT163 are *C. trachomatis* specific while Lda3/CT473 and Lda4 are present in all *Chlamydia* species.

1.4.9.6. Pls1 and Pls2

Pls1/CT049 and Pls2/CT050 (Pmp-like secreted proteins), are conserved among *Chlamydia* spp., do not contain classical signal peptides but are secreted into the inclusion lumen, where they localize in globular structures that closely associate with the inclusion membrane, not co-localizing with bacterial markers. They appear to be important for the development of the inclusion but their functional mechanisms remain unknown (Jorgensen & Valdivia, 2008).

1.4.9.7. Chlamydial glycogen enzymes

Recently, the bacterial glycogen metabolism enzymes (GlgA, GlgB, GlgX, GlgP and MalQ) were reported to be secreted into the inclusion lumen through T3SS (Gehre *et al.*, 2016), suggesting that *de novo* synthesis of glycogen may occur in the lumen of the inclusion.

1.4.10. *C. trachomatis* T3S-independent effector proteins

Chlamydial effector proteins might also reach the cytosol of infected cells via T3S-independent mechanisms. The proteases CPAF/CT858 (Chlamydial protease-like activity factor) and Tsp/CT441 are identified chlamydial effectors that before reaching the cytosol of host cells, are translocated into the lumen of the inclusion by a type II secretion system (Betts *et al.*, 2009; Zhong, 2011).

1.4.10.1. CPAF

CPAF has been identified as a factor involved in the maintenance and growth of the chlamydial inclusion by cleaving host proteins (the cytoskeleton intermediate filament vimentin, the nuclear envelope lamin-associated protein [LAP1]) and also by disrupting cell division (Brown *et al.*, 2012, 2014; Kumar & Valdivia, 2008; Snavely *et al.*, 2015). It has also been shown to target bacterial factors (Hou *et al.*, 2013; Jorgensen *et al.*, 2011). However, the molecular mechanisms by which CPAF acts are still not clearly understood.

1.4.10.2. CT441

Tsp/CT441 (tail specific protease) is a PDZ domain protein containing tail specific protease. Tsp was not detected outside the inclusion using standard immunofluorescence assays but it was found to cleave the host NF-κB in *C. trachomatis* infected cells (Lad *et al.*, 2007a, b), suggesting that an undetectable amount of Tsp might be secreted into the host cell cytosol.

1.4.10.3. CT823

cHtrA/CT823 (chlamydial high temperature requirement protein A protease) also contains an N-terminal signal sequence and by immunofluorescence assays was found to localize both inside the

inclusion and in the cytoplasm of infected cells (Zhong, 2011). However, a host cell target for cHtrA has not been identified.

1.4.10.4. CT311 and CT795

Additionally, two other proteins of unknown function, CT311 and CT795, are also predicted to reach the lumen of the inclusion via-sec dependent pathway and, using standard immunofluorescence assays, were found to localize both inside the inclusion and in the cytoplasm of infected cells, showing a pattern of distribution similar to CPAF (Lei *et al.*, 2011; Qi *et al.*, 2011).

1.4.10.5. Pgp3

Pgp3 (plasmid ORF5) is the only plasmid protein found to be secreted into the inclusion lumen and cytosol of infected cells, showing a similar cytosolic distribution as CPAF (Li *et al.*, 2008). It is not clear how Pgp3 is secreted out of the bacteria since no signal peptide has been detected and it has never been characterized as being a T3SS substrate (see also section 1.1.3.4., above).

1.4.11. The chlamydial T3SS during the developmental cycle

Recently a working model for the T3SS during the chlamydial developmental cycle has been proposed (Ferrell & Fields, 2015). In this model the authors hypothesize the existence of four different phases of the T3SS during the chlamydial developmental cycle (Fig. 1.8):

- T3SS in recently converted elementary bodies (EBs): EBs possess a complete assembled T3SS. Before reaching a new cell, early secretion of effectors is prevented by the intermolecular disulphide bonding among needle protein (CdsF) subunits in EBs (Betts *et al.*, 2008; Betts-Hampikian & Fields, 2011) as well as by positioning of the gatekeeper protein (CopN) on the bacterial cytoplasmic side of the apparatus.
- T3SS in invading EBs: during the invasion step secretion activity is activated upon contact with the host plasma membrane. This results in secretion of CopN, deployment of the translocon proteins CopB and CopD and consequent secretion of early effectors.
- T3SS in RBs: secretion in intracellular RBs is maintained by association of RBs with the inclusion membrane and is accompanied by *de novo* expression of T3SS genes.
- T3SS in RB-EB conversion: RB to EB conversion is associated with detachment of the RBs from the inclusion membrane and consequent loss of secretion capacity by association of CopN to the cytoplasmic side of the T3SS, preventing further secretion from the recently converted EB.

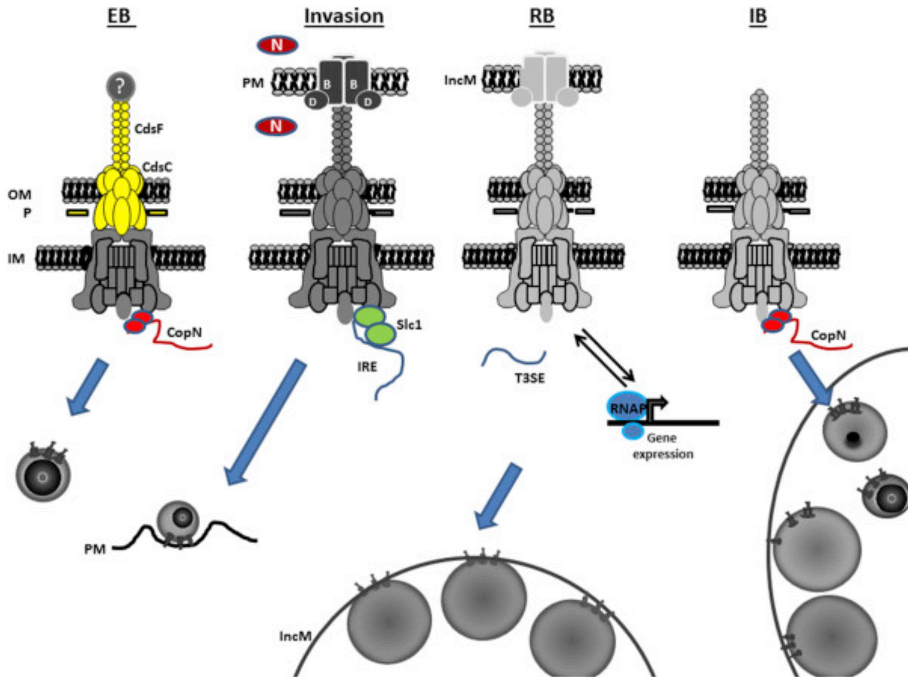

Figure 1.8. Proposed model for T3S during chlamydial developmental cycle. Infectious EBs contain a complete T3SS that is activated upon contact with the host cell plasma membrane (PM). This results in secretion of CopN followed by CopB and CopD (translocator proteins). The chaperone Slc1 is thought to mediate hierarchical secretion of T3SS invasion related effectors. During intracellular development the association of RBs with the inclusion membrane mediates secretion and new T3SS apparatuses are synthesized (light gray). T3S is halted when CopN re-associates with the cytoplasmic components of the apparatus, which coincides with RB dissociation from the inclusion membrane and respective RB-EB conversion. (Reprinted from Ferrel & Fields, 2015, with permission from Elsevier).

1.5. Genetic manipulation of *C. trachomatis*

Like other intracellular obligate microorganisms it has been very challenging to develop genetic tools to manipulate *C. trachomatis.* Host cell restrictive growth constitutes a major barrier to genetic transformation, making simple procedures such as cloning extremely difficult. In the past 10 years, however, significant advances in the genetic manipulation of intracellular pathogens have emerged (Beare *et al.*, 2011). *Coxiella burnetti*, the causative agent of Q-fever (Maurin & Raoult, 1999), is the example that has the most complete set of genetic tools available. The reason for having such a complete array of genetic tools is due mainly to the development of a medium that supports axenic growth of *Coxiella* (Omsland *et al.*, 2009). Attempts for developing axenic growth media for *Chlamydia* have taken place (Omsland *et al.*, 2012), but so far they were only successful in supporting metabolic activity of EBs and RBs, not yet supporting replication or differentiation between the different chlamydial forms.

Advances in genetic manipulation of *C. trachomatis* also started taking place in the last decade. After the event of *C. trachomatis* genome sequencing (Stephens *et al.*, 1998) it was noticed that DNA repair and recombination systems were well represented in the genome, indicating that *C. trachomatis* is capable of recombination. The first attempt to obtain *C. trachomatis* transformants was in 1994, when Priscilla Wyrick and collaborators tried electroporation of EBs with a shuttle vector based on the chlamydial plasmid and an *E. coli* cloning vector. No stable transformants were isolated although inclusions were present for up to 4 passages under chloramphenicol selection (Tam *et al.*, 1994). In 2009, Binet and Maurelli attempted an approach using electroporation of EBs. The idea was to obtain

recombinants of *C. psittaci* generated by homologous recombination. However, this technique was limited to the 16S rRNA region and only allowed integration of a short 1kb marker at very low frequencies (Binet & Maurelli, 2009).

In 2011, Ian Clarke and his collaborators developed a reproducible, plasmid-based genetic transformation system for *C. trachomatis* based on chemical transformation ($CaCl_2$ instead of electroporation) and using penicillin for selection (Wang *et al.*, 2011). Selection with penicillin is useful in *Chlamydia* because non-transformed strains form aberrant inclusions (easily distinguished under a light inverted microscope) (see Fig 1.6) in the presence of the antibiotic. In contrast, transformed bacteria having the β-lactamase gene in a plasmid can recover from the aberrant state and consequently form normal inclusions.

In 2013, Agaisse and Derré took a step further and developed a series of versatile shuttle vectors (p2TK-SW2) that could be used to express *C. trachomatis* proteins fused to three different fluorescent tags (GFP, m-Cherry or CFP) and under control of the *incDEFG* operon promoter and terminator (Agaisse & Derré, 2013). Other groups also created different shuttle vectors, based on a Tet-inducible promoter (Bauler & Hackstadt, 2014; Wickstrum *et al.*, 2013).

Further advances in genetic manipulation of *C. trachomatis* have taken place: chemical mutagenesis has been described and a library of mutants has been generated (Kari *et al.*, 2011b; Kokes *et al.*, 2015; Nguyen & Valdivia, 2012); specific chlamydial genes have been knocked down using dendrimer-delivered antisense RNA (Mishra *et al.*, 2012) development of a group II intron based approach (Johnson

& Fisher, 2013) or more recently a novel suicide vector was described that is dependent on inducible expression and allows rapid fluorescent reported allelic exchange mutagenesis (FRAEM). This method has the great advantage of allowing the monitoring of mutagenesis by fluorescent microscopy (Mueller *et al.*, 2016).

A broader approach to chlamydia genetics, using all the recently developed methods will definitively help to elucidate the role of many proteins whose function remains unknown. In consequence, many aspects regarding the chlamydial developmental cycle that are still unclear are most likely to be solved.

1.6. Aims of this project

It is believed that *C. trachomatis* may possess more than 100 T3S substrates (Valdivia, 2008) and that these proteins play a key role during the *C. trachomatis* developmental cycle. These potential effectors are presumably translocated into host epithelial cells at various stages of infection to mediate cell invasion, establishment of a protective replicative vacuole, evasion of innate immune responses or host cell exit. The majority of T3S effector proteins recognized to date are involved in the entry step or belong to the Inc family of effectors. Many other effector proteins are predicted to exist and to play a role in other stages of the chlamydial developmental cycle.

The overall purpose of this project was to provide insights on the identity and function of novel chlamydial T3S effectors. In particular, the specific objectives were:

- Screen for candidate chlamydial T3S effectors by using *Y. enterocolitica* T3SS as a heterologous system.
- Identify novel chlamydial T3S effectors in infected cells by analyzing the subcellular localization of candidate effectors.
- Use recently developed transformation methods in *C. trachomatis* in an attempt to further characterize the newly identified proteins.

REFERENCES

Abby, S. S. & Rocha, E. P. C. (2012). The non-flagellar type III secretion system evolved from the bacterial flagellum and diversified into host-cell adapted systems. *PLoS Genet* **8**(9), e1002983.

Abdallah, A. M., Gey van Pittius, N. C., Champion, P. A. D., Cox, J., Luirink, J., Vandenbroucke-Grauls, C. M. J. E., Appelmelk, B. J. & Bitter, W. (2007). Type VII secretion-*Mycobacteria* show the way. *Nat Rev Microbiol* **5**(11), 883–891.

Abdelrahman, Y. M., Rose, L. A. & Belland, R. J. (2011). Developmental expression of non-coding RNAs in *Chlamydia trachomatis* during normal and persistent growth. *Nucleic Acids Res* **39**(5), 1843–1854.

AbdelRahman, Y. M. & Belland, R. J. (2005). The chlamydial developmental cycle. *FEMS Microbiol Rev* **29**(5), 949–959.

Abromaitis, S. & Stephens, R. S. (2009). Attachment and Entry of *Chlamydia* Have Distinct Requirements for Host Protein Disulfide Isomerase. *PLoS Pathog* **5**(4), e1000357.

Agaisse, H. & Derré, I. (2013). A *C. trachomatis* Cloning Vector and the Generation of *C. trachomatis* Strains Expressing Fluorescent Proteins under the Control of a *C. trachomatis* Promoter. *PLoS One* **8**(2), e57090.

Agaisse, H. & Derré, I. (2014). Expression of the effector protein IncD in *Chlamydia trachomatis* mediates recruitment of the lipid transfer protein CERT and the endoplasmic reticulum-resident protein VAPB to the inclusion membrane. *Infect Immun* **82**(5), 2037–2047.

Aistleitner, K., Anrather, D., Schott, T., Klose, J., Bright, M., Ammerer, G. & Horn, M. (2015). Conserved features and major differences in the outer membrane protein composition of chlamydiae. *Environ Microbiol* **17**(4), 1397–1413.

Almeida, F., Borges, V., Ferreira, R., Borrego, M. J., Gomes, J. P. & Mota, L. J. (2012). Polymorphisms in Inc proteins and differential expression of *inc* genes among *Chlamydia trachomatis* strains correlate with invasiveness and tropism of Lymphogranuloma venereum isolates. *J Bacteriol* **194**(23), 6574–6585.

Alvarez-Venegas, R., Sadder, M., Tikhonov, A. & Avramova, Z. (2007). Origin of the bacterial SET domain genes: Vertical or horizontal? *Mol Biol Evol* **24**(2), 482–497.

Archuleta, T. L. & Spiller, B. W. (2014). A gatekeeper chaperone complex directs translocator secretion during type three secretion. *PLoS Pathog* **10**(11), e1004498.

Archuleta, T. L., Du, Y., English, C. A., Lory, S., Lesser, C., Ohi, M. D., Ohi, R. & Spiller, B. W. (2011). The *Chlamydia* effector chlamydial outer protein N (CopN) sequesters tubulin and prevents microtubule assembly. *J Biol Chem* **286**(39), 33992–33998.

Arnold, R., Brandmaier, S., Kleine, F., Tischler, P., Heinz, E., Behrens, S., Niinikoski, A., Mewes, H. W., Horn, M. & Rattei, T. (2009). Sequence-Based Prediction of Type III Secreted Proteins. *PLoS Pathog* **5**(4), e1000376.

Asner, S. A., Jaton, K., Kyprianidou, S., Nowak, A.-M. L. & Greub, G. (2014). *Chlamydia pneumoniae*: possible association with asthma in children. *Clin Infect Dis* **58**(8), 1198–2009.

Bachmann, N. L., Fraser, T. A., Bertelli, C., Jelocnik, M., Gillett, A., Funnell, O., Flanagan, C., Myers, G. S. A., Timms, P. & Polkinghorne, A. (2014a). Comparative genomics of koala, cattle and sheep strains of *Chlamydia pecorum*. *BMC Genomics* **15**(667), 1–14.

Bachmann, N. L., Polkinghorne, A. & Timms, P. (2014b). *Chlamydia* genomics: Providing novel insights into chlamydial biology. *Trends Microbiol* **22**(8), 464–472.

Bannantine, J. P., Rockey, D. D. & Hackstadt, T. (1998). Tandem genes of *Chlamydia psittaci* that encode proteins localized to the inclusion membrane. *Mol Microbiol* **28**(5), 1017–1026.

Bao, X., Nickels, B. E. & Fan, H. (2012). *Chlamydia trachomatis* protein GrgA activates transcription by contacting the nonconserved region of 66. *Proc Natl Acad Sci* **109**(42), 16870–16875.

Barison, N., Gupta, R. & Kolbe, M. (2013). A sophisticated multi-step secretion mechanism: How the type 3 secretion system is regulated. *Cell Microbiol* **15**(11), 1809–1817.

Barry, C. E., Hayes, S. F. & Hackstadt, T. (1992). Nucleoid condensation in *Escherichia coli* that express a chlamydial histone homolog. *Science* **256**, 377–379.

Barry, C. E., Brickman, T. J. & Hackstadt, T. (1993). Hc1-mediated effects on DNA structure: a potential regulator of chlamydial development. *Mol Microbiol* **9**(2), 273–283.

Barta, M. L., Battaile, K. P., Lovell, S. & Hefty, P. S. (2015). Hypothetical protein CT398 (CdsZ) interacts with σ(54) (RpoN)-holoenzyme and the type III secretion export apparatus in *Chlamydia trachomatis*. *Protein Sci* **24**, 1617–1632.

Basler, M. (2015). Type VI secretion system: secretion by a contractile nanomachine. *Philos Trans R Soc Lond B Biol Sci* **370**, 20150021.

Bastidas, R. J., Elwell, C. A., Engel, J. N. & Valdivia, R. H. (2013). Chlamydial Intracellular Survival Strategies. *Cold Spring Harb Perspect Med* **3**, a010256.

Bauler, L. D. & Hackstadt, T. (2014). Expression and Targeting of secreted proteins from *Chlamydia trachomatis*. *J Bacteriol* **196**(7), 1325–1334.

Beare, P. A., Sandoz, K. M., Omsland, A., Rockey, D. D. & Heinzen, R. A. (2011). Advances in Genetic Manipulation of Obligate Intracellular Bacterial Pathogens. *Front Microbiol* **2**, 1–13.

Beatty, W. L., Byrne, G. I. & Morrison, R. P. (1993). Morphologic and antigenic characterization of interferon gamma-mediated persistent *Chlamydia trachomatis* infection *in vitro*. *Proc Natl Acad Sci U S A* **90**, 3998–4002.

Beatty, W. L., Morrison, R. P. & Byrne, G. I. (1994). Persistent chlamydiae: from cell culture to a paradigm for chlamydial pathogenesis. *Microbiol Rev* **58**(4), 686–699.

Bébéar, C., and de Barbeyrac, B. (2009). Genital *Chlamydia trachomatis* infections. *Clin Microbiol Infect* **15**, 4–10.

Becker, E. & Hegemann, J. H. (2014). All subtypes of the Pmp adhesin family are implicated in chlamydial virulence and show species-specific function. *MicrobiologyOpen* **3**(4), 544–556.

Bedson, S. P., Western, G. T. & Simpson, S. L. (1930). Observations on the aetiology of psitacosis. *Lancet i* 235–236.

Belland, R. J., Scidmore, M. A., Crane, D. D., Hogan, D. M., Whitmire, W., McClarty, G. & Caldwell, H. D. (2001). *Chlamydia trachomatis* cytotoxicity associated with complete and partial cytotoxin genes. *Proc Natl Acad Sci* **98**(24), 13984–139899.

Belland, R. J., Zhong, G., Crane, D. D., Hogan, D., Sturdevant, D., Sharma, J., Beatty, W. L. & Caldwell, H. D. (2003). Genomic transcriptional profiling of the developmental cycle of *Chlamydia trachomatis*. *Proc Natl Acad Sci* **100**(14), 8478–8483.

Benagiano, M., Munari, F., Ciervo, A., Amedei, A., Paccani, S. R., Mancini, F., Ferrari, M., Della Bella, C., Ulivi, C. & other authors. (2012). *Chlamydophila pneumoniae* phospholipase D (CpPLD) drives Th17 inflammation in human atherosclerosis. *Proc Natl Acad Sci U S A* **109**(4), 1222–1227.

Berger, L., Volp, K., Mathews, S., Speare, R. & Timms, P. (1999). *Chlamydia pneumoniae* in a free-ranging giant barred frog (Mixophyes iteratus) from Australia. *J Clin Microbiol* **37**(7), 2378–2380.

Betts, H. J., Twiggs, L. E., Sal, M. S., Wyrick, P. B. & Fields, K. A. (2008). Bioinformatic and biochemical evidence for the identification of the type III secretion system needle protein of *Chlamydia trachomatis*. *J Bacteriol* **190**(5), 1680–1690.

Betts, H. J., Wolf, K. & Fields, K. A. (2009). Effector protein modulation of host cells: examples in the *Chlamydia* spp. arsenal. *Curr Opin Microbiol* **12**, 81–87.

Betts-Hampikian, H. J. & Fields, K. A. (2011). Disulfide bonding within components of the *Chlamydia* type III secretion apparatus correlates with development. *J Bacteriol* **193**(24), 6950–6959.

Betts-Hampikian, H. J. & Fields, K. A. (2010). The chlamydial type III secretion mechanism: Revealing cracks in a tough nut. *Front Microbiol* **1**, 1–13.

Binet, R. & Maurelli, A. T. (2009). Transformation and isolation of allelic exchange mutants of *Chlamydia psittaci* using recombinant DNA introduced by electroporation. *Proc Natl Acad Sci U S A* **106**, 292–297.

Blasi, F., Tarsia, P. & Aliberti, S. (2009). *Chlamydophila pneumoniae*. *Clin Microbiol Infect* **15**, 29–35.

Bodetti, T. J., Jacobson, E., Wan, C., Hafner, L., Pospischil, A., Rose, K. & Timms, P. (2002). Molecular evidence to support the expansion of the hostrange of *Chlamydophila pneumoniae* to include reptiles as well as humans, horses, koalas and amphibians. *Syst Appl Microbiol* **25**, 146–152.

Boncompain, G., Muller, C., Meas-Yedid, V., Schmitt-Kopplin, P., Lazarow, P. B. & Subtil, A. (2014). The intracellular bacteria *Chlamydia* hijack peroxisomes and utilize their enzymatic capacity to produce bacteria-specific phospholipids. *PLoS One* **9**(1), e86196.

Boschiroli, M. L., Ouahrani-Bettache, S., Foulongne, V., Michaux-Charachon, S., Bourg, G., Allardet-Servent, A., Cazevieille, C., Liautard, J. P., Ramuz, M. & other authors. (2001). The *Brucella suis* virB operon is induced intracellularly in macrophages. *Proc Natl Acad Sci U S A* **99**(3), 1544–1549.

Boschiroli, M. L., Ouahrani-Bettache, S., Foulongne, V., Michaux-Charachon, S., Bourg, G., Allardet-Servent, A., Cazevieille, C., Lavigne, J. P., Liautard, J. P. & other authors. (2002). Type IV secretion and *Brucella* virulence. *Vet Microbiol* **90**, 341–348.

Bothe, M., Dutow, P., Pich, A., Genth, H. & Klos, A. (2015). DXD motif-dependent and-independent effects of the *Chlamydia trachomatis* cytotoxin CT166. *Toxins* **7**, 621–637.

Brickman, T. J., Barry, C. E. & Hackstadt, T. (1993). Molecular cloning and expression of hctB encoding a strain-variant chlamydial histone-like protein with DNA-binding activity. *J Bacteriol* **175**(14), 4274–4281.

Brinkworth, A. J., Malcolm, D. S., Pedrosa, A. T., Roguska, K., Shahbazian, S., Graham, J. E., Hayward, R. D. & Carabeo, R. A. (2011). *Chlamydia trachomatis* Slc1 is a type III secretion chaperone that enhances the translocation of its invasion effector substrate TARP. *Mol Microbiol* **82**(1), 131–144.

Brown, H. M., Knowlton, A. E. & Grieshaber, S. S. (2012). Chlamydial infection induces host cytokinesis failure at abscission. *Cell Microbiol* **14**(10), 1554–1567.

Brown, H. M., Knowlton, A. E., Snavely, E., Nguyen, B. D., Richards, T. S. & Grieshaber, S. S. (2014). Multinucleation during *C. trachomatis* infections is caused by the contribution of two effector pathways. *PLoS One* **9**(6), e100763.

Bulir, D. C., Waltho, D. A., Stone, C. B., Mwawasi, K. A., Nelson, J. C. & Mahony, J. B. (2014). *Chlamydia pneumoniae* CopD translocator protein plays a critical role in type III secretion (T3S) and infection. *PLoS One* **9**(6), e99315.

Bulir, D. C., Waltho, D. A., Stone, C. B., Liang, S., Chiang, C. K. W., Mwawasi, K. A., Nelson, J. C., Zhang, S. W., Mihalco, S. P. & other authors. (2015). Chlamydia Outer Protein (Cop) B from *Chlamydia pneumoniae* possesses characteristic features of a type III secretion (T3S) translocator protein. *BMC Microbiol* **15**(163), 1–9.

Bullock, H. D., Hower, S. & Fields, K. A. (2012). Domain analyses reveal that *Chlamydia trachomatis* CT694 protein belongs to the membrane-localized family of type III effector proteins. *J Biol Chem* **287**(33), 28078–28086.

Bulut, Y., Faure, E., Thomas, L., Karahashi, H., Michelsen, K. S., Equils, O., Morrison, S. G., Morrison, R. P. & Arditi, M. (2002). Chlamydial heat shock protein 60 activates macrophages and endothelial cells through Toll-like receptor 4 and MD2 in a MyD88-dependent pathway. *J Immunol* **168**(3), 1435–1440.

Bulut, Y., Shimada, K., Wong, M. H., Chen, S., Gray, P., Alsabeh, R., Doherty, T. M., Crother, T. R. & Arditi, M. (2009). Chlamydial heat shock protein 60 induces acute pulmonary inflammation in mice via the Toll-like receptor 4- and MyD88-dependent pathway. *Infect Immun* **77**(7), 2683–2690.

Cai, Y., Fukushi, H., Koyasu, S., Kuroda, E., Yamaguchi, T. & Hirai, K. (2002). An etiological investigation of domestic cats with conjunctivitis and upper respiratory tract disease in Japan. *J Vet Med Sci* **64**(3), 215–219.

Caldwell, H. D., Wood, H., Crane, D., Bailey, R., Jones, R. B., Mabey, D., Maclean, I., Mohammed, Z., Peeling, R. & other authors. (2003). Polymorphisms in *Chlamydia trachomatis* tryptophan synthase genes differentiate between genital and ocular isolates. *J Clin Invest* **111**(11), 1757–1769.

Carabeo, R. A., Grieshaber, S. S., Hasenkrug, A., Dooley, C. A. & Hackstadt, T. (2004). Requirement for the Rac GTPage in *Chlamydia trachomiatis* invasion of non-phagoycytic cells **5**, 418–425.

Carabeo, R. A., Dooley, C. A., Grieshaber, S. S. & Hackstadt, T. (2007). Rac interacts with Abi-1 and WAVE2 to promote an Arp2/3-dependent actin recruitment during chlamydial invasion. *Cell Microbiol* **9**(9), 2278–2288.

Carabeo, R. A., Mead, D. J. & Hackstadt, T. (2003). Golgi-dependent transport of cholesterol to the *Chlamydia trachomatis* inclusion. *Proc Natl Acad Sci U S A* **100**(11), 6771–6776.

Carlson, J. H., Hughes, S., Hogan, D., Cieplak, G., Sturdevant, D. E., McClarty, G., Caldwell, H. D. & Belland, R. J. (2004). Polymorphisms in the *Chlamydia trachomatis* cytotoxin locus associated with ocular and genital isolates. *Infect Immun* **72**(12), 7063–7072.

Carlson, J. H., Whitmire, W. M., Crane, D. D., Wicke, L., Virtaneva, K., Sturdevant, D. E., Kupko, J. J., Porcella, S. F., Martinez-Orengo, N. & other authors. (2008). The *Chlamydia trachomatis* plasmid is a transcriptional regulator of chromosomal genes and a virulence factor. *Infect Immun* **76**(6), 2273–2283.

Censini, S., Lange, C., Xiang, Z., Crabtree, J. E., Ghiara, P., Borodovsky, M., Rappuoli, R. & Covacci, A. (1996). *cag*, a pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated virulence factors. *Proc Natl Acad Sci U S A* **93**, 14648–14653.

Chellas-Géry, B., Wolf, K., Tisoncik, J., Hackstadt, T. & Fields, K. A. (2011). Biochemical and localization analyses of putative type III secretion translocator proteins CopB and CopB2 of *Chlamydia trachomatis* reveal significant distinctions. *Infect Immun* **79**(8), 3036–3045.

Chellas-Géry, B., Linton, C. N. & Fields, K. A. (2007). Human GCIP interacts with CT847, a novel *Chlamydia trachomatis* type III secretion substrate, and is degraded in a tissue-culture infection model. *Cell Microbiol* **9**(10), 2417–2430.

Chen, J. C. & Stephens, R. S. (1994). Trachoma and LGV biovars of *Chlamydia trachomatis* share the same glycosaminoglycan-dependent mechanism for infection of eukaryotic cells. *Mol Microbiol* **11**(3), 501–507.

Chen, J. C. & Stephens, R. S. (1997). *Chlamydia trachomatis* glycosaminoglycan-dependent and independent attachment to eukaryotic cells. *Microb Pathog* **22**, 23–30.

Chen, J. C., Zhang, J. P. & Stephens, R. S. (1996). Structural requirements of heparin binding to *Chlamydia trachomatis*. *J Biol Chem* **271**(19), 11134–11140.

Chen, Y.-S., Bastidas, R. J., Saka, H. A., Carpenter, V. K., Richards, K. L., Plano, G. V & Valdivia, R. H. (2014). The *Chlamydia trachomatis* type III secretion chaperone Slc1 engages multiple early effectors, including TepP, a tyrosine-phosphorylated protein required for the recruitment of CrkI-II to nascent inclusions and innate immune signaling. *PLoS Pathog* **10**(2), e1003954.

Cheng, E. & Tan, M. (2012). Differential effects of DNA supercoiling on *Chlamydia* early promoters correlate with expression patterns in midcycle. *J Bacteriol* **194**(12), 3109–3115.

Christerson, L., Bom, R. J. M., Bruisten, S. M., Yass, R., Hardick, J., Bratt, G., Gaydos, C. A., Morré, S. A. & Herrmann, B. (2012). *Chlamydia trachomatis* strains show specific clustering for men who have sex with men compared to heterosexual populations in Sweden, the Netherlands, and the United States. *J Clin Microbiol* **50**(11), 3548–3555.

Christian, J., Vier, J., Paschen, S. A. & Häcker, G. (2010). Cleavage of the NF-κB family protein p65/RelA by the chlamydial protease-like activity factor (CPAF) impairs proinflammatory signaling in cells infected with *Chlamydiae*. *J Biol Chem* **285**(53), 41320–41327.

Chumduri, C., Gurumurthy, R. K., Zadora, P. K., Mi, Y. & Meyer, T. F. (2013). *Chlamydia* Infection Promotes Host DNA Damage and Proliferation but Impairs the DNA Damage Response. *Cell Host Microbe* **13**, 746–758.

Cianfanelli, F. R., Monlezun, L. & Coulthurst, S. J. (2015). Aim, Load, Fire: The Type VI Secretion System, a Bacterial Nanoweapon. *Trends Microbiol* **24**(1), 51–62.

De Clercq, E., Kalmar, I. & Vanrompay, D. (2013). Animal models for studying female genital tract infection with *Chlamydia trachomatis*. *Infect Immun* **81**(9), 3060–3067.

Clifton, D. R., Fields, K. A., Grieshaber, S. S., Dooley, C. A., Fischer, E. R., Mead, D. J., Carabeo, R. A. & Hackstadt, T. (2004). A chlamydial type III translocated protein is tyrosine-phosphorylated at the site of entry and associated with recruitment of actin. *Proc Natl Acad Sci U S A* **101**(27), 10166–10171.

Cocchiaro, J. L., Kumar, Y., Fischer, E. R., Hackstadt, T. & Valdivia, R. H. (2008). Cytoplasmic lipid droplets are translocated into the lumen of the *Chlamydia trachomatis* parasitophorous vacuole. *Proc Natl Acad Sci U S A* **105**(27), 9379–9384.

Cocchiaro, J. L. & Valdivia, R. H. (2009). New insights into *Chlamydia* intracellular survival mechanisms. *Cell Microbiol* **11**, 1571–1578.

Coles, A. C. (1930). Microorganisms in psittacosis. *Lancet i* 1011–1012.

Cornelis, G. R. (2006). The type III secretion injectisome. *Nat Rev Microbiol* **4**, 811–825.

Corsaro, D. & Greub, G. (2006). Pathogenic potential of novel *Chlamydiae* and diagnostic approaches to infections due to these obligate intracellular bacteria. *Clin Microbiol Rev* **19**(2), 283–297.

Costa, C. P., Kirschning, C. J., Busch, D., Durr, S., Jennen, L., Heinzmann, U., Prebeck, S., Wagner, H. & Miethke, T. (2002). Role of chlamydial heat shock protein 60 in the stimulation of innate immune cells by *Chlamydia pneumoniae*. *Eur J Immunol* **32**, 2460–2470.

Costa, T. R. D., Felisberto-Rodrigues, C., Meir, A., Prevost, M. S., Redzej, A., Trokter, M. & Waksman, G. (2015). Secretion systems in Gram-negative bacteria: structural and mechanistic insights. *Nat Rev Microbiol* **13**, 343–359.

Crane, D. D., Carlson, J. H., Fischer, E. R., Bavoil, P., Hsia, R., Tan, C., Kuo, C. & Caldwell, H. D. (2006). *Chlamydia trachomatis* polymorphic membrane protein D is a species-common pan-neutralizing antigen. *Proc Natl Acad Sci U S A* **103**(6), 1894–1899.

Dai, W. & Li, Z. (2014). Conserved type III secretion system exerts important roles in *Chlamydia trachomatis*. *Int J Clin Exp Pathol* **7**(9), 5404–5414.

Darville, T. & Hiltke, T. J. (2010). Pathogenesis of genital tract disease due to *Chlamydia trachomatis*. *J Infect Dis* **201**(S2), S114–S125.

Davis, C. H. & Wyrick, P. B. (1997). Differences in the association of *Chlamydia trachomatis* serovar E and serovar L2 with epithelial cells *in vitro* may reflect biological differences *in vivo*. *Infect Immun* **65**(7), 2914–2924.

Dehoux, P., Flores, R., Dauga, C., Zhong, G. & Subtil, A. (2011). Multi-genome identification and characterization of chlamydiae-specific type III secretion substrates: the Inc proteins. *BMC Genomics* **12**, 109.

Delevoye, C., Nilges, M., Dehoux, P., Paumet, F., Perrinet, S., Dautry-Varsat, A. & Subtil, A. (2008). SNARE protein mimicry by an intracellular bacterium. *PLoS Pathog* **4**(3), e1000022.

Derré, I., Pypaert, M., Dautry-Varsat, A. & Agaisse, H. (2007). RNAi screen in *Drosophila* cells reveals the involvement of the Tom complex in *Chlamydia* infection. *PLoS Pathog* **3**(10), e155.

Dillon, S. C., Zhang, X., Trievel, R. C. & Cheng, X. (2005). The SET-domain protein superfamily: protein lysine methyltransferases. *Genome Biol* **6**, 227.

Donati, M., Huot-Creasy, H., Humphrys, M., Di Paolo, M., Di Francesco, A. & Myers, G. S. A. (2014). Genome Sequence of *Chlamydia suis* MD56, Isolated from the Conjunctiva of a Weaned Piglet. *Genome Announc* **2**(3), e00425–14.

Dong, X., Liu, Y., Chang, X., Lei, L. & Zhong, G. (2014). Signaling via tumor necrosis factor receptor 1 but not Toll-like receptor 2 contributes significantly to hydrosalpinx development following *Chlamydia muridarum* infection. *Infect Immun* **82**(5), 1833–1839.

Douglas, A. L. & Hatch, T. P. (2000). Expression of the transcripts of the sigma factors and putative sigma factor regulators of *Chlamydia trachomatis* L2. *Gene* **247**, 209–214.

Van Droogenbroeck, C., Beeckman, D. S. A., Verminnen, K., Marien, M., Nauwynck, H., Boesinghe, L. de T. de & Vanrompay, D. (2009). Simultaneous zoonotic transmission of *Chlamydophila psittaci* genotypes D, F and E/B to a veterinary scientist. *Vet Microbiol* **135**, 78–81.

Dumoux, M., Menny, A., Delacour, D. & Hayward, R. (2015). A *Chlamydia* effector recruits CEP170 to reprogram host microtubule organization. *J Cell Sci* **128**, 3420–3434.

Durand, N., Nicolas, J. & Favre, M. (1913). Lymphogranulomatose inguinale subaigue d´origine génitale probable, peut être vénérienne. *Bull la société des Med des Hôpitaux Paris* **35**, 274–288.

ECDC. **(2014).** Sexually transmitted infections in Europe 2012. *www.ecdc.europa.eu*.

Elwell, C. A., Ceesay, A., Kim, J. H., Kalman, D. & Engel, J. N. (2008). RNA interference screen identifies Abl kinase and PDGFR signaling in *Chlamydia trachomatis* entry. *PLoS Pathog* **4**(3), e1000021.

Everett, K. D., Bush, R. M. & Andersen, A. A. (1999). Emended description of the order *Chlamydiales*, proposal of *Parachlamydiaceae* fam. nov. and *Simkaniaceae* fam. nov., each containing one monotypic genus, revised taxonomy of the family *Chlamydiaceae*, including a new genus and five new species, and standards. *Int J Syst Bacteriol* **49** (Pt 2), 415–440.

Everett, K. D. E., Desiderio, D. M. & Hatch, T. P. (1994). Characterization of lipoprotein EnvA in *Chlamydia psittaci* 6BC. *J Bacteriol* **176**(19), 6082–6087.

Fadel, S. & Eley, A. (2007). *Chlamydia trachomatis* OmcB protein is a surface-exposed glycosaminoglycan-dependent adhesin. *J Med Microbiol* **56**, 15–22.

Feher, V. A., Randall, A., Baldi, P., Bush, R. M., de la Maza, L. M. & Amaro, R. E. (2013). A 3-Dimensional Trimeric β-Barrel Model for *Chlamydia* MOMP Contains Conserved and Novel Elements of Gram-Negative Bacterial Porins. *PLoS One* **8**(7), e68934.

Ferreira, R., Borges, V., Nunes, A., Borrego, M. J. & Gomes, J. P. (2013). Assessment of the load and transcriptional dynamics of *Chlamydia trachomatis* plasmid according to strains' tissue tropism. *Microbiol Res* **168**, 333–339.

Ferrell, J. C. & Fields, K. A. (2015). A working model for the type III secretion mechanism in *Chlamydia*. *Microbes Infect* **18**, 84–92.

Fields, K. A. & Hackstadt, T. (2000). Evidence for the secretion of *Chlamydia trachomatis* CopN by a type III secretion mechanism. *Mol Microbiol* **38**(5), 1048–1060.

Fields, K. A. & Hackstadt, T. (2002). T he Chlamydial Inclusion : Escape from the Endocytic Pathway 1. *Rev Lit Arts Am* **18**, 221–245.

Fields, K. A., Fischer, E. R., Mead, D. J. & Hackstadt, T. (2005). Analysis of putative *Chlamydia trachomatis* chaperones Scc2 and Scc3 and their use in the identification of type III secretion substrates. *J Bacteriol* **187**(18), 6466–6478.

Filloux, A., Hachani, A. & Bleves, S. (2008). The bacterial type VI secretion machine: Yet another player for protein transport across membranes. *Microbiology* **154**, 1570–1583.

Fling, S. P., Sutherland, R. A., Steele, L. N., Hess, B., D'Orazio, S. E., Maisonneuve, J., Lampe, M. F., Probst, P. & Starnbach, M. N. (2001). CD8+ T cells recognize an inclusion membrane-associated protein from the vacuolar pathogen *Chlamydia trachomatis*. *Proc Natl Acad Sci U S A* **98**(3), 1160–1165.

Fukushi, H. & Hirai, K. (1992). Proposal of *Chlamydia pecorum* sp. nov. for *Chlamydia* strains derived from ruminants. *Int J Syst Bacteriol* **42**(2), 306–308.

Galán, J. E. (1996). Molecular genetic basis of *Salmonella* entry into host cells. *Mol Microbiol* **20**(2), 263–271.

Galán, J. E., Lara-Tejero, M., Marlovits, T. C. & Wagner, S. (2014). Bacterial type III secretion systems: specialized nanomachines for protein delivery into target cells. *Annu Rev Microbiol* **68**, 415–438.

Galán, J. E. (2009). Common Themes in the Design and Function of Bacterial Effectors. *Cell Host Microbe* **5**, 571–579.

Gauliard, E., Ouellette, S. P., Rueden, K. J. & Ladant, D. (2015). Characterization of interactions between inclusion membrane proteins from *Chlamydia trachomatis*. *Front Cell Infect Microbiol* **5**, 1–11.

Gehre, L., Gorgette, O., Perrinet, S., Prevost, M.-C., Ducatez, M., Giebel, A. M., Nelson, D. E., Ball, S. G. & Subtil, A. (2016). Sequestration of host metabolism by an intracellular pathogen. *elife* **5**, e12552.

Gomes, J. P., Nunes, A., Bruno, W. J., Borrego, M. J., Florindo, C. & Dean, D. (2006). Polymorphisms in the nine polymorphic membrane proteins of *Chlamydia trachomatis* across all serovars: evidence for serovar Da recombination and correlation with tissue tropism. *J Bacteriol* **188**(1), 275–286.

Gong, S., Yang, Z., Lei, L., Shen, L. & Zhong, G. (2013). Characterization of *Chlamydia trachomatis* Plasmid-Encoded Open Reading Frames. *J Bacteriol* **195**(17), 3819–3826.

González, E., Rother, M., Kerr, M. C., Al-Zeer, M. A., Abu-Lubad, M., Kessler, M., Brinkmann, V., Loewer, A. & Meyer, T. F. (2014). *Chlamydia* infection depends on a functional MDM2-p53 axis. *Nat Commun* **5**(5201), 1–10.

Gordon, F. B., Woolridge, R. L., Quan, A. L., Gillmore, J. D., Arm, H. G., Yang, Y. F. & Magruder, G. B. (1972). Field studies on McCoy cell cultures for detection of *Chlamydia trachomatis*. *Southeast Asian J Trop Med Public Health* **3**, 69–78.

Greub, G. (2010). International Committee on Systematics of Prokaryotes. Subcommittee on the taxonomy of the *Chlamydiae*: minutes of the inaugural closed meeting, 21 March 2009, Little Rock, AR, USA. *Int J Syst Evol Microbiol* **60**, 2694.

Grieshaber, S. S., Grieshaber, N. A. & Hackstadt, T. (2003). *Chlamydia trachomatis* uses host cell dynein to traffic to the microtubule-organizing center in a p50 dynamitin-independent process. *J Cell Sci* **116**, 3793–3802.

Hachani, A., Wood, T. E. & Filloux, A. (2015). Type VI secretion and anti-host effectors. *Curr Opin Microbiol* **29**, 81–93.

Hackstadt, T., Todd, W. J. & Caldwell, H. D. (1985). Disulfide-mediated interactions of the chlamydial major outer membrane protein: role in the differentiation of chlamydiae? *J Bacteriol* **161**(1), 25–31.

Hackstadt, T., Rockey, D. D., Heinzen, R. A. & Scidmore, M. A. (1996). *Chlamydia trachomatis* interrupts an exocytic pathway to acquire endogenously synthesized sphingomyelin in transit from the Golgi apparatus to the plasma membrane. *EMBO J* **15**(5), 964–977.

Halberstaedter, L. & von Prowazec, S. (1907). Zur atiologie des trachoms. *Deutsh Medizinische Wochenschrift* **33**, 1285–1287.

Halberstaedter, L. & von Prowazec, S. (1909). Uber Clamydozoenbefunde bei blenorrhea neonatorum nongonorrhoica. *BerlKlinWchenschr* **46**, 1839–1844.

Hamon, M. A., Batsché, E., Régnault, B., Tham, T. N., Seveau, S., Muchardt, C. & Cossart, P. (2007). Histone modifications induced by a family of bacterial toxins. *Proc Natl Acad Sci U S A* **104**(33), 13467–13472.

Hanson, B. R., Slepenkin, A., Peterson, E. M. & Tan, M. (2015). *Chlamydia trachomatis* type III secretion proteins regulate transcription. *J Bacteriol* **197**(20), 3238–3244.

Hartley, J. C., Stevenson, S., Robinson, A. J., Littlewood, J. D., Carder, C., Cartledge, J., Clark, C. & Ridgway, G. L. (2001). Conjunctivitis due to *Chlamydophila felis* (*Chlamydia psittaci* feline pneumonitis agent) acquired from a cat: case report with molecular characterization of isolates from the patient and cat. *J Infect* **43**, 7–11.

Hatfield, G. W. & Benham, C. J. (2002). DNA topology-mediated control of global gene expression in *Escherichia coli*. *Annu Rev Genet* **36**, 175–203.

Hayden, M. S., West, A. P. & Ghosh, S. (2006). NF-κB and the immune response. *Oncogene* **25**, 6758–6780.

Heine, H., Muller-Loennies, S., Brade, L., Lindner, B. & Brade, H. (2003). Endotoxic activity and chemical structure of lipopolysaccharides from *Chlamydia trachomatis* serotypes E and L2 and *Chlamydophila psittaci* 6BC. *Eur J Biochem* **270**, 440–450.

Heuer, D., Rejman Lipinski, A., Machuy, N., Karlas, A., Wehrens, A., Siedler, F., Brinkmann, V. & Meyer, T. F. (2009). *Chlamydia* causes fragmentation of the Golgi compartment to ensure reproduction. *Nature* **457**(5), 731–735.

Ho, T. D. & Starnbach, M. N. (2005). The *Salmonella enterica* Serovar Typhimurium-Encoded Type III Secretion Systems Can Translocate *Chlamydia trachomatis* Proteins into the Cytosol of Host Cells. *Infect Immun* **73**(2), 905–911.

Hogan, R. J., Mathews, S. A., Kutlin, A., Hammerschlag, M. R. & Timms, P. (2003). Differential expression of genes encoding membrane proteins between acute and continuous *Chlamydia pneumoniae* infections. *Microb Pathog* **34**, 11–16.

Hogan, R. J., Mathews, S. A., Mukhopadhyay, S., Summersgill, J. T. & Timms, P. (2004). Chlamydial Persistence: Beyond the Biphasic Paradigm. *Infect Immun* **72**(4), 1843–1855.

Horn, M. (2008). *Chlamydiae* as symbionts in eukaryotes. *Annu Rev Microbiol* **62**, 113–131.

Horn, M., Collingro, A., Schmitz-Esser, S., Beier, C. L., Purkhold, U., Fartmann, B., Brandt, P., Nyakatura, G. J., Droege, M. & other authors. (2004). Illuminating the evolutionary history of *Chlamydiae*. *Science* **304**, 728–730.

Hou, S., Lei, L., Yang, Z., Qi, M., Liu, Q. & Zhong, G. (2013). *Chlamydia trachomatis* outer membrane complex protein B (OmcB) is processed by the protease CPAF. *J Bacteriol* **195**(5), 951–957.

Hou, S., Dong, X., Yang, Z., Li, Z., Liu, Q. & Zhong, G. (2015). Chlamydial plasmid-encoded virulence factor Pgp3 neutralizes the antichlamydial activity of human cathelicidin LL-37. *Infect Immun* **83**(12), 4701–4709.

Hovis, K. M., Mojica, S., Mcdermott, J. E., Pedersen, L., Simhi, C., Rank, R. G., Myers, G. S. A., Ravel, J., Hsia, R. C. & Bavoil, P. M. (2013). Genus-optimized strategy for the identification of chlamydial type III secretion substrates. *Pathog Dis* **69**, 213–222.

Hower, S., Wolf, K. & Fields, K. A. (2009). Evidence that CT694 is a novel *Chlamydia trachomatis* T3S substrate capable of functioning during invasion or early cycle development. *Mol Microbiol* **72**(6), 1423–1437.

Huang, J., Lesser, C. F. & Lory, S. (2008). The essential role of the CopN protein in *Chlamydia pneumoniae* intracellular growth. *Nature* **456**(7218), 112–115.

Husain, S., Kahane, S., Friedman, M. G., Paterson, D. L., Studer, S., McCurry, K. R., Wolf, D. G., Zeevi, A., Pilewski, J. & Greenberg, D. (2007). *Simkania negevensis* in bronchoalveolar lavage of lung transplant recipients: a possible association with acute rejection. *Transplantation* **83**(2), 138–143.

Hybiske, K. & Stephens, R. S. (2007a). Mechanisms of *Chlamydia trachomatis* entry into nonphagocytic cells. *Infect Immun* **75**(8), 3925–3934.

Hybiske, K. & Stephens, R. S. (2007b). Mechanisms of host cell exit by the intracellular bacterium *Chlamydia*. *Proc Natl Acad Sci U S A* **104**(27), 11430–11435.

Ilangovan, A., Connery, S. & Waksman, G. (2015). Structural biology of the Gram-negative bacterial conjugation systems. *Trends Microbiol* **23**(5), 301–310.

Ingalls, R. R., Rice, P. A., Qureshi, N., Takayama, K., Lin, J. S. & Golenbock, D. T. (1995). The inflammatory cytokine response to *Chlamydia trachomatis* infection is endotoxin mediated. *Infect Immun* **63**(8), 3125–3130.

Jacquier, N., Viollier, P. H. & Greub, G. (2015). The role of peptidoglycan in chlamydial cell division: towards resolving the chlamydial anomaly. *FEMS Microbiol Rev* **39**(2), 262–275.

Jarvis, K. G., Girón, J. A., Jerse, A. E., McDaniel, T. K., Donnenberg, M. S. & Kaper, J. B. (1995). Enteropathogenic *Escherichia coli* contains a putative type III secretion system necessary for the export of proteins involved in attaching and effacing lesion formation. *Proc Natl Acad Sci U S A* **92**, 7996–8000.

Jewett, T. J., Fischer, E. R., Mead, D. J. & Hackstadt, T. (2006). Chlamydial TarP is a bacterial nucleator of actin. *Proc Natl Acad Sci U S A* **103**(42), 15599–15604.

Jewett, T. J., Dooley, C. A., Mead, D. J. & Hackstadt, T. (2008). *Chlamydia trachomatis* TarP is phosphorylated by src family tyrosine kinases. *Biochem Biophys Res Commun* **371**(2), 339–344.

Jewett, T. J., Miller, N. J., Dooley, C. A. & Hackstadt, T. (2010). The conserved TarP actin binding domain is important for chlamydial invasion. *PLoS Pathog* **6**(7), e1000997.

Jiwani, S., Ohr, R. J., Fischer, E. R., Hackstadt, T., Alvarado, S., Romero, A. & Jewett, T. J. (2012). *Chlamydia trachomatis* TarP cooperates with the Arp2/3 complex to increase the rate of actin polymerization. *Biochem Biophys Res Commun* **420**(4), 816–821.

Jiwani, S., Alvarado, S., Ohr, R. J., Romero, A., Nguyen, B. & Jewett, T. J. (2013). *Chlamydia trachomatis* TarP harbors distinct G and F actin binding domains that bundle actin filaments. *J Bacteriol* **195**(4), 708–716.

Johnson, C. M. & Fisher, D. J. (2013). Site-specific, insertional inactivation of *incA* in *Chlamydia trachomatis* using a group II intron. *PLoS One* **8**(12), e83989.

Jorgensen, I. & Valdivia, R. H. (2008). Pmp-like proteins Pls1 and Pls2 are secreted into the lumen of the *Chlamydia trachomatis* inclusion. *Infect Immun* **76**(9), 3940–3950.

Jorgensen, I., Bednar, M. M., Amin, V., Davis, B. K., Ting, J. P. Y., McCafferty, D. G. & Valdivia, R. H. (2011). The *Chlamydia* protease CPAF regulates host and bacterial proteins to maintain pathogen vacuole integrity and promote virulence. *Cell Host Microbe* **10**, 21–32.

Jutras, I., Abrami, L. & Dautry-Varsat, A. (2003). Entry of the lymphogranuloma venereum strain of *Chlamydia trachomatis* into host cells involves cholesterol-rich membrane domains. *Infect Immun* **71**(1), 260–266.

Kari, L., Whitmire, W. M., Olivares-Zavaleta, N., Goheen, M. M., Taylor, L. D., Carlson, J. H., Sturdevant, G. L., Lu, C., Bakios, L. E. & other authors. (2011a). A live-attenuated chlamydial vaccine protects against trachoma in nonhuman primates. *J Exp Med* **208**(11), 2217–2223.

Kari, L., Goheen, M. M., Randall, L. B., Taylor, L. D., Carlson, J. H., Whitmire, W. M., Virok, D., Rajaram, K., Endresz, V. & other authors. (2011b). Generation of targeted *Chlamydia trachomatis* null mutants. *Proc Natl Acad Sci* **108**(17), 7189–7193.

Kelly, K. A. & Rank, R. G. (1997). Identification of homing receptors that mediate the recruitment of CD4 T cells to the genital tract following intravaginal infection with *Chlamydia trachomatis*. *Infect Immun* **65**(12), 5198–208.

Kern, J. M., Maass, V. & Maass, M. (2009). Molecular pathogenesis of chronic *Chlamydia pneumoniae* infection: a brief overview. *Clin Microbiol Infect* **15**, 36–41.

Kim, J. H., Jiang, S., Elwell, C. A. & Engel, J. N. (2011). *Chlamydia trachomatis* co-opts the FGF2 signaling pathway to enhance infection. *PLoS Pathog* **7**(10), e1002285.

Knittler, M. R., Berndt, A., Böcker, S., Dutow, P., Hänel, F., Heuer, D., Kägebein, D., Klos, A., Koch, S. & other authors. (2014). *Chlamydia psittaci*: new insights into genomic diversity, clinical pathology, host-pathogen interaction and anti-bacterial immunity. *Int J Med Microbiol* **304**, 877–93.

Kokes, M., Dunn, J. D., Granek, J. A., Nguyen, B. D., Barker, J. R., Valdivia, R. H. & Bastidas, R. J. (2015). Integrating Chemical Mutagenesis and Whole-Genome Sequencing as a Platform for Forward and Reverse Genetic Analysis of *Chlamydia*. *Cell Host Microbe* **17**, 1–10.

Kol, A., Bourcier, T., Lichtman, A. H. & Libby, P. (1999). Chlamydial and human heat shock protein 60s activate human vascular endothelium, smooth muscle cells, and macrophages. *J Clin Invest* **103**, 571–7.

Kong, F. Y. S. & Hocking, J. S. (2015). Treatment challenges for urogenital and anorectal *Chlamydia trachomatis*. *BMC Infect Dis* **15**, 293.

Kumar, Y. & Valdivia, R. H. (2008). Actin and intermediate filaments stabilize the *Chlamydia trachomatis* vacuole by forming dynamic structural scaffolds. *Cell Host Microbe* **4**(2), 159–169.

Kumar, Y., Cocchiaro, J. & Valdivia, R. H. (2006). The Obligate Intracellular Pathogen *Chlamydia trachomatis* Targets Host Lipid Droplets. *Curr Biol* **16**, 1646–1651.

Lad, S. P., Li, J., da Silva Correia, J., Pan, Q., Gadwal, S., Ulevitch, R. J. & Li, E. (2007a). Cleavage of p65/RelA of the NF-kappaB pathway by *Chlamydia*. *Proc Natl Acad Sci U S A* **104**(8), 2933–2938.

Lad, S. P., Yang, G., Scott, D. A., Wang, G., Nair, P., Mathison, J., Reddy, V. S. & Li, E. (2007b). Chlamydial CT441 Is a PDZ domain-containing tail-specific protease that interferes with the NF-kappaB pathway of immune response. *J Bacteriol* **189**(18), 6619–6625.

Lamoth, F., Pillonel, T. & Greub, G. (2015). *Waddlia*: An emerging pathogen and a model organism to study the biology of *Chlamydiae*. *Microbes Infect* **17**, 732–737.

Lane, B. J., Mutchler, C., Al Khodor, S., Grieshaber, S. S. & Carabeo, R. A. (2008). Chlamydial entry involves TarP binding of guanine nucleotide exchange factors. *PLoS Pathog* **4**(3), e1000014.

Lei, L., Qi, M., Budrys, N., Schenken, R. & Zhong, G. (2011). Localization of *Chlamydia trachomatis* hypothetical protein CT311 in host cell cytoplasm. *Microb Pathog* **51**(3), 101–109.

Li, Z., Chen, D., Zhong, Y., Wang, S. & Zhong, G. (2008). The chlamydial plasmid-encoded protein Pgp3 is secreted into the cytosol of *Chlamydia*-infected cells. *Infect Immun* **76**(8), 3415–3428.

Lieberman, D., Dvoskin, B., Lieberman, D. V, Kahane, S. & Friedman, M. G. (2002). Serological evidence of acute infection with the Chlamydia-like microorganism *Simkania negevensis* (Z) in acute exacerbation of chronic obstructive pulmonary disease. *Eur J Clin Microbiol Infect Dis* **21**(4), 307–309.

Liechti, G. W., Kuru, E., Hall, E., Kalinda, A., Brun, Y. V, VanNieuwenhze, M. & Maurelli, A. T. (2014). A new metabolic cell-wall labelling method reveals peptidoglycan in *Chlamydia trachomatis*. *Nature* **506**(7498), 507–510.

Lillie, R. D. (1930). Psittacosis: ricketsia-like inclusions in man and in experimental animals. *Public Heal Rep* **45**, 773–778.

Lindner, K. (1910). Zur atiologie der gonokokken-freien urethritis. *Wien Klin Wochenschr* **8**, 283–284.

Liu, Y., Huang, Y., Yang, Z., Sun, Y., Gong, S., Hou, S., Chen, C., Li, Z., Liu, Q. & other authors. **(2014a).** Plasmid-encoded Pgp3 is a major virulence factor for *Chlamydia muridarum* to induce hydrosalpinx in mice. *Infect Immun* **82**(12), 5327–5335.

Liu, Y., Chen, C., Gong, S., Hou, S., Qi, M., Liu, Q., Baseman, J. & Zhong, G. (2014b). Transformation of *Chlamydia muridarum* reveals a role for Pgp5 in suppression of plasmid-dependent gene expression. *J Bacteriol* **196**(5), 989–998.

Lo, C.-C., Xie, G., Bonner, C. A. & Jensen, R. A. (2012). The alternative translational profile that underlies the immune-evasive state of persistence in *Chlamydiaceae* exploits differential tryptophan contents of the protein repertoire. *Microbiol Mol Biol Rev* **76**(2), 405–443.

Longbottom, D. & Coulter, L. J. (2003). Animal chlamydioses and zoonotic implications. *J Comp Pathol* **128**, 217–244.

Longbottom, D., Entrican, G., Wheelhouse, N., Brough, H. & Milne, C. (2013). Evaluation of the impact and control of enzootic abortion of ewes. *Vet J* **195**, 257–259.

Lovett, M., Kuo, C.-C., Holmes, K. & Falkow, S. (1980). Plasmids of the genus *Chlamydia. Curr Chemoth Infect Dis* **2**, 1250–1252.

Luostarinen, T., Namujju, P. B., Merikukka, M., Dillner, J., Hakulinen, T., Koskela, P., Paavonen, J., Surcel, H.-M. & Lehtinen, M. (2013). Order of HPV/*Chlamydia* infections and cervical high-grade precancer risk: a case-cohort study. *Int J cancer* **133**, 1756–1760.

Lutter, E. I., Bonner, C., Holland, M. J., Suchland, R. J., Stamm, W. E., Jewett, T. J., McClarty, G. & Hackstadt, T. (2010). Phylogenetic analysis of *Chlamydia trachomatis* TarP and correlation with clinical phenotype. *Infect Immun* **78**(9), 3678–3688.

Lutter, E. I., Martens, C. & Hackstadt, T. (2012). Evolution and conservation of predicted inclusion membrane proteins in *Chlamydiae*. *Comp Funct Genomics* **2012**, Article ID362104.

Lutter, E., Barger, A., Nair, V. & Hackstadt, T. (2013). *Chlamydia trachomatis* Inclusion Membrane Protein CT228 Recruits Elements of the Myosin Phosphatase Pathway to Regulate Release Mechanisms. *Cell Rep* **3**, 1921–1931.

Mabey, D. C. W., Hu, V., Bailey, R. L., Burton, M. J. & Holland, M. J. (2014). Towards a safe and effective chlamydial vaccine: lessons from the eye. *Vaccine* **32**, 1572–1578.

Markham, A. P., Jaafar, Z. A., Kemege, K. E., Middaugh, C. R. & Hefty, P. S. (2009). Biophysical characterization of *Chlamydia trachomatis* CT584 supports its potential role as a type III secretion needle tip protein. *Biochemistry* **48**(43), 10353–10361.

Matsumoto, A., Bessho, H., Uehira, K. & Suda, T. (1991). Morphological studies of the association of mitochondria with chlamydial inclusions and the fusion of chlamydial inclusions. *J Electron Microsc (Tokyo)* **40**(5), 356–363.

Maurelli, A. T., Baudry, B., D'Hauteville, H., Hale, T. L. & Sansonetti, P. J. (1985). Cloning of plasmid DNA sequences involved in invasion of HeLa cells by *Shigella flexneri*. *Infect Immun* **49**(1), 164–171.

Maurin, M. & Raoult, D. (1999). Q fever. *Clin Microbiol Rev* **12**(4), 518–553.

McDaniel, T. K., Jarvis, K. G., Donnenberg, M. S. & Kaper, J. B. (1995). A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens. *Proc Natl Acad Sci U S A* **92**, 1664–1668.

Mehlitz, A., Banhart, S., Mäurer, A. P., Kaushansky, A., Gordus, A. G., Zielecki, J., MacBeath, G. & Meyer, T. F. (2010). Tarp regulates early *Chlamydia*-induced host cell survival through interactions with the human adaptor protein SHC1. *J Cell Biol* **190**(1), 143–157.

Mendonça, A. G., Alves, R. J. & Pereira-Leal, J. B. (2011). Loss of genetic redundancy in reductive genome evolution. *PLoS Comput Biol* **7**(2), e1001082.

Meriläinen, G., Koski, M. K. & Wierenga, R. K. (2016). The extended structure of the periplasmic region of CdsD, a structural protein of the Type III Secretion System of *Chlamydia trachomatis*. *Protein Sci* **25**, 987–998.

Mirrashidi, K. M., Elwell, C. A., Verschueren, E., Johnson, J. R., Frando, A., Von Dollen, J., Rosenberg, O., Gulbahce, N., Jang, G. & other authors. (2015). Global Mapping of the Inc-Human Interactome Reveals that Retromer Restricts *Chlamydia* Infection. *Cell Host Microbe* **18**(1), 109–121.

Misaghi, S., Balsara, Z. R., Catic, A., Spooner, E., Ploegh, H. L. & Starnbach, M. N. (2006). *Chlamydia trachomatis*-derived deubiquitinating enzymes in mammalian cells during infection. *Mol Microbiol* **61**(1), 142–150.

Mishra, M. K., Gérard, H. C., Whittum-Hudson, J. A., Hudson, A. P. & Kannan, R. M. (2012). Dendrimer-enabled modulation of gene expression in *Chlamydia trachomatis*. *Mol Pharm* **9**, 413–421.

Mital, J., Miller, N. J., Fischer, E. R. & Hackstadt, T. (2010). Specific chlamydial inclusion membrane proteins associate with active Src family kinases in microdomains that interact with the host microtubule network. *Cell Microbiol* **12**(9), 1235–1249.

Mital, J., Lutter, E. I., Barger, A. C., Dooley, C. a & Hackstadt, T. (2015). *Chlamydia trachomatis* inclusion membrane protein CT850 interacts with the dynein light chain DYNLT1 (Tctex1). *Biochem Biophys Res Commun* **462**, 165–170.

Miyagawa, Y., Mitamura, T., Yaoi, H., Ishii, N. & Okanishi, J. (1935). Studies on the virus of lymphogranuloma inguinale Nicolas, Favre and Durand. Cultivation of the virus on chorioallantoic membrane of the chicken embyo. (Fourth Report). *Jpn J Exp Med* **13**, 733–738.

Moore, E. R. & Ouellette, S. P. (2014). Reconceptualizing the chlamydial inclusion as a pathogen-specified parasitic organelle: an expanded role for Inc proteins. *Front Cell Infect Microbiol* **4**, 157.

Moroney, J. F., Guevara, R., Iverson, C., Chen, F. M., Skelton, S. K., Messmer, T. O., Plikaytis, B., Williams, P. O., Blake, P. & Butler, J. C. (1998). Detection of Chlamydiosis in a Shipment of Pet Birds, Leading to Recognition of an Outbreak of Clinically Mild Psittacosis in Humans. *Clin Infect Dis* **26**(6), 1425–1429.

Moulder, J. W. (1991). Interaction of Chlamydiae and host cells *in vitro*. *Microbiol Rev* **55**(1), 143–190.

Moulder, J. W. (1966). The relation of the psittacosis group (*Chlamydiae*) to bacteria and viruses. *Ann Rev Microbiol* **20**, 107–130.

Mueller, C. A., Broz, P. & Cornelis, G. R. (2008). The type III secretion system tip complex and translocon. *Mol Microbiol* **68**(5), 1085–1095.

Mueller, K. E., Plano, G. V. & Fields, K. A. (2014). New frontiers in type III secretion biology: The Chlamydia perspective. *Infect Immun* **82**(1), 2–9.

Mueller, K. E., Wolf, K. & Fields, K. A. (2016). Gene Deletion by Fluorescence-Reported Allelic Exchange Mutagenesis in *Chlamydia trachomatis*. *MBio* **7**(1), e01817–15.

Murthy, A. K., Li, W., Chaganty, B. K. R., Kamalakaran, S., Guentzel, M. N., Seshu, J., Forsthuber, T. G., Zhong, G. & Arulanandam, B. P. (2011). Tumor necrosis factor alpha production from CD8+ T cells mediates oviduct pathological sequelae following primary genital *Chlamydia muridarum* infection. *Infect Immun* **79**(7), 2928–2935.

Muschiol, S., Boncompain, G., Vromman, F., Dehoux, P., Normark, S., Henriques-Normark, B. & Subtil, A. (2011). Identification of a family of effectors secreted by the type III secretion system that are conserved in pathogenic chlamydiae. *Infect Immun* **79**(2), 571–580.

Nans, A., Saibil, H. R. & Hayward, R. D. (2014). Pathogen-host reorganization during *Chlamydia* invasion revealed by cryo-electron tomography. *Cell Microbiol* **16**(10), 1457–1472.

Nans, A., Ford, C. & Hayward, R. D. (2015a). Host-pathogen reorganisation during host cell entry by *Chlamydia trachomatis*. *Microbes Infect* **17**, 727–731.

Nans, A., Kudryashev, M., Saibil, H. R. & Hayward, R. D. (2015b). Structure of a bacterial type III secretion system in contact with a host membrane *in situ*. *Nat Commun* **6**, 10114.

Le Negrate, G., Krieg, A., Faustin, B., Loeffler, M., Godzik, A., Krajewski, S. & Reed, J. C. (2008). ChlaDub1 of *Chlamydia trachomatis* suppresses NF-kappaB activation and inhibits IκBα ubiquitination and degradation. *Cell Microbiol* **10**(9), 1879–1892.

Nelson, D. E., Crane, D. D., Taylor, L. D., Dorward, D. W., Goheen, M. M. & Caldwell, H. D. (2006). Inhibition of chlamydiae by primary alcohols correlates with the strain-specific complement of plasticity zone phospholipase D genes. *Infect Immun* **74**(1), 73–80.

Neuendorf, E., Gajer, P., Bowlin, A. K., Marques, P. X., Ma, B., Yang, H., Fu, L., Humphrys, M. S., Forney, L. J. & other authors. **(2015).** *Chlamydia caviae* infection alters abundance but not composition of the guinea pig vaginal microbiota. *Pathog Dis* **73**(4), 1–12.

Newhall, W. J. & Jones, R. B. (1983). Disulfide-linked oligomers of the major outer membrane protein of *Chlamydiae*. *J Bacteriol* **154**(2), 998–1001.

Nguyen, B. D. & Valdivia, R. H. (2012). Virulence determinants in the obligate intracellular pathogen *Chlamydia trachomatis* revealed by forward genetic approaches. *Proc Natl Acad Sci* **109**(4), 1263–1268.

Nicholson, T. L., Olinger, L., Chong, K., Schoolnik, G. & Stephens, R. S. (2003). Global stage-specific gene regulation during the developmental cycle of *Chlamydia trachomatis*. *J Bacteriol* **185**(10), 3179–3189.

Niehus, E., Cheng, E. & Tan, M. (2008). DNA supercoiling-dependent gene regulation in *Chlamydia*. *J Bacteriol* **190**(19), 6419–6427.

Norkin, L. C., Wolfrom, S. A. & Stuart, E. S. (2001). Association of caveolin with *Chlamydia trachomatis* inclusions at early and late stages of infection. *Exp Cell Res* **266**, 229–238.

Nunes, A. & Gomes, J. P. (2014). Evolution, phylogeny, and molecular epidemiology of *Chlamydia*. *Infect Genet Evol* **23**, 49–64.

O'Connell, C. M., Ingalls, R. R., Andrews Jr., C. W., Scurlock, A. M. & Darville, T. (2007). Plasmid-deficient *Chlamydia muridarum* fail to induce immune pathology and protect against oviduct disease. *J Immunol* **179**, 4027–4034.

O'Connell, C. M., AbdelRahman, Y. M., Green, E., Darville, H. K., Saira, K., Smith, B., Darville, T., Scurlock, A. M., Meyer, C. R. & Belland, R. J. (2011). Toll-like receptor 2 activation by *Chlamydia trachomatis* is plasmid dependent, and plasmid-responsive chromosomal loci are coordinately regulated in response to glucose limitation by *C. trachomatis* but not by *C. muridarum*. *Infect Immun* **79**(3), 1044–1056.

O'Connell, C. M., Ionova, I. A., Quayle, A. J., Visintin, A. & Ingalls, R. R. (2006). Localization of TLR2 and MyD88 to *Chlamydia trachomatis* inclusions: Evidence for signaling by intracellular TLR2 during infection with an obligate intracellular pathogen. *J Biol Chem* **281**(3), 1652–1659.

O'Meara, C. P., Andrew, D. W. & Beagley, K. W. (2014). The mouse model of *Chlamydia* genital tract infection: a review of infection, disease, immunity and vaccine development. *Curr Mol Med* **14**(3), 396–421.

Ochman, H., Soncini, F. C., Solomon, F. & Groisman, E. A. (1996). Identification of a pathogenicity island required for *Salmonella* survival in host cells. *Proc Natl Acad Sci U S A* **93**, 7800–7804.

Olivares-Zavaleta, N., Whitmire, W., Gardner, D. & Caldwell, H. D. (2010). Immunization with the attenuated plasmidless *Chlamydia trachomatis* L2(25667R) strain provides partial protection in a murine model of female genitourinary tract infection. *Vaccine* **28**, 1454–1462.

Omsland, A., Cockrell, D. C., Howe, D., Fischer, E. R., Virtaneva, K., Sturdevant, D. E., Porcella, S. F. & Heinzen, R. A. (2009). Host cell-free growth of the Q fever bacterium *Coxiella burnetii. Proc Natl Acad Sci U S A* **106**(11), 4430–4434.

Omsland, A., Sager, J., Nair, V., Sturdevant, D. E. & Hackstadt, T. (2012). Developmental stage-specific metabolic and transcriptional activity of *Chlamydia trachomatis* in an axenic medium. *Proc Natl Acad Sci U S A* **109**(48), 19781–19785.

Orillard, E. & Tan, M. (2015). Functional analysis of three topoisomerases that regulate DNA supercoiling levels in *Chlamydia*. *Mol Microbiol* **99**(3), 484–496.

Ouellette, S. P., Dorsey, F. C., Moshiach, S., Cleveland, J. L. & Carabeo, R. A. (2011). *Chlamydia* species-dependent differences in the growth requirement for lysosomes. *PLoS One* **6**(3), e16783.

Packiam, M., Weinrick, B., Jacobs, W. R. & Maurelli, A. T. (2015). Structural characterization of muropeptides from *Chlamydia trachomatis* peptidoglycan by mass spectrometry resolves ‘chlamydial anomaly’. *Proc Natl Acad Sci U S A* **112**(37), 11660–11665.

Pais, S. V., Milho, C., Almeida, F. & Mota, L. J. (2013). Identification of Novel Type III Secretion Chaperone-Substrate Complexes of *Chlamydia trachomatis*. *PLoS One* **8**(2), e56292.

Pallen, M. J., Beatson, S. A. & Bailey, C. M. (2005). Bioinformatics, genomics and evolution of non-flagellar type-III secretion systems: A Darwinian perpective. *FEMS Microbiol Rev* **29**, 201–229.

Parsot, C., Ménard, R., Gounon, P. & Sansonetti, P. J. (1995). Enhanced secretion through the *Shigella flexneri* Mxi-Spa translocon leads to assembly of extracellular proteins into macromolecular structures. *Mol Microbiol* **16**(2), 291–300.

Paumet, F., Wesolowski, J., Garcia-Diaz, A., Delevoye, C., Aulner, N., Shuman, H. A., Subtil, A. & Rothman, J. E. (2009). Intracellular bacteria encode inhibitory SNARE-like proteins. *PLoS One* **4**(10), e7375.

Pennini, M. E., Perrinet, S., Dautry-Varsat, A. & Subtil, A. (2010). Histone methylation by NUE, a novel nuclear effector of the intracellular pathogen *Chlamydia trachomatis*. *PLoS Pathog* **6**(7), e1000995.

Perry, R. D., Straley, S. C., Fetherston, J. D., Rose, D. J., Gregor, J. & Blattner, F. R. (1998). DNA sequencing and analysis of the low-Ca2+-response plasmid pCD1 of *Yersinia pestis* KIM5. *Infect Immun* **66**(10), 4611–4623.

Peters, J., Wilson, D. P., Myers, G., Timms, P. & Bavoil, P. M. (2007). Type III secretion à la *Chlamydia*. *Trends Microbiol* **15**(6), 241–251.

Pickett, M. A. (2005). The plasmids of *Chlamydia trachomatis* and *Chlamydophila pneumoniae* (N16): accurate determination of copy number and the paradoxical effect of plasmid-curing agents. *Microbiology* **151**, 893–903.

Plaunt, M. R. & Hatch, T. P. (1988). Protein synthesis early in the developmental cycle of *Chlamydia psittaci*. *Infect Immun* **56**(12), 3021–3025.

Polkinghorne, A., Hanger, J. & Timms, P. (2013). Recent advances in understanding the biology, epidemiology and control of chlamydial infections in koalas. *Vet Microbiol* **165**, 214–223.

Poudel, A., Elsasser, T. H., Rahman, K. S., Chowdhury, E. U. & Kaltenboeck, B. (2012). Asymptomatic endemic *Chlamydia pecorum* infections reduce growth rates in calves by up to 48 percent. *PLoS One* **7**(9), e44961.

Qi, M., Lei, L., Gong, S., Liu, Q., DeLisa, M. P. & Zhong, G. (2011). *Chlamydia trachomatis* secretion of an immunodominant hypothetical protein (CT795) into host cell cytoplasm. *J Bacteriol* **193**(10), 2498–2509.

Ramsey, K. H., Sigar, I. M., Schripsema, J. H., Denman, C. J., Bowlin, A. K., Myers, G. A. S. & Rank, R. G. (2009). Strain and virulence diversity in the mouse pathogen *Chlamydia muridarum*. *Infect Immun* **77**(8), 3284–3293.

Rank, R. G., Bowlin, A. K. & Kelly, K. A. (2000). Characterization of lymphocyte response in the female genital tract during ascending chlamydial genital infection in the guinea pig model. *Infect Immun* **68**(9), 5293–5298.

Rank, R. G. & Yeruva, L. (2014). Hidden in plain sight: chlamydial gastrointestinal infection and its relevance to persistence in human genital infection. *Infect Immun* **82**(4), 1362–1371.

Rank, R. G., Whittimore, J., Bowlin, A. K., Dessus-Babus, S. & Wyrick, P. B. (2008). *Chlamydiae* and polymorphonuclear leukocytes: unlikely allies in the spread of chlamydial infection. *FEMS Immunol Med Microbiol* **54**(1), 104–113.

Rank, R. G., Lacy, H. M., Goodwin, A., Sikes, J., Whittimore, J., Wyrick, P. B. & Nagarajan, U. M. (2010). Host chemokine and cytokine response in the endocervix within the first developmental cycle of *Chlamydia muridarum*. *Infect Immun* **78**(1), 536–544.

Rao, X., Deighan, P., Hua, Z., Hu, X., Wang, J., Luo, M., Wang, J., Liang, Y., Zhong, G. & other authors. **(2009).** A regulator from *Chlamydia trachomatis* modulates the activity of RNA polymerase through direct interaction with the beta subunit and the primary sigma subunit. *Genes Dev* **23**, 1818–1829.

Read, T. D., Brunham, R. C., Shen, C., Gill, S. R., Heidelberg, J. F., White, O., Hickey, E. K., Peterson, J., Utterback, T. & other authors. **(2000).** Genome sequences of *Chlamydia trachomatis* MoPn and *Chlamydia pneumoniae* AR39. *Nucleic Acids Res* **28**(6), 1397–1406.

Read, T. D., Myers, G. S. A., Brunham, R. C., Nelson, W. C., Paulsen, I. T., Heidelberg, J., Holtzapple, E., Khouri, H., Federova, N. B. & other authors. **(2003).** Genome sequence of *Chlamydophila caviae* (*Chlamydia psittaci* GPIC): examining the role of niche-specific genes in the evolution of the *Chlamydiaceae*. *Nucleic Acids Res* **31**(8), 2134–2147.

Rockey, D. D. (2011). Unraveling the basic biology and clinical significance of the chlamydial plasmid. *J Exp Med* **208**(11), 2159–2162.

Rosario, C. J., Hanson, B. R. & Tan, M. (2014). The transcriptional repressor EUO regulates both subsets of *Chlamydia* late genes. *Mol Microbiol* **94**(4), 887–897.

Rosario, C. J. & Tan, M. (2012). The early gene product EUO is a transcriptional repressor that selectively regulates promoters of *Chlamydia* late genes. *Mol Microbiol* **84**(6), 1097–1107.

Rosario, C. J. & Tan, M. (2016). Regulation of *Chlamydia* gene expression by tandem promoters with different temporal patterns. *J Bacteriol* **198**(2), 363–369.

Roulis, E., Polkinghorne, A. & Timms, P. (2013). *Chlamydia pneumoniae*: modern insights into an ancient pathogen. *Trends Microbiol* **21**(3), 120–128.

Rzomp, K. A., Moorhead, A. R. & Scidmore, M. A. (2006). The GTPase Rab4 interacts with *Chlamydia trachomatis* inclusion membrane protein CT229. *Infect Immun* **74**(9), 5362–5373.

Rzomp, K. A., Scholtes, L. D., Briggs, B. J., Whittaker, G. R. & Scidmore, M. A. (2003). Rab GTPases are recruited to chlamydial inclusions in both a species-dependent and species-independent manner. *Infect Immun* **71**(10), 5855–5870.

Sachse, K. & Laroucau, K. (2014). Avian chlamydiosis: two more bacterial players discovered. *Vet J* **200**, 347–348.

Sachse, K., Laroucau, K., Riege, K., Wehner, S., Dilcher, M., Creasy, H. H., Weidmann, M., Myers, G., Vorimore, F. & other authors. **(2014).** Evidence for the existence of two new members of the family *Chlamydiaceae* and proposal of *Chlamydia avium* sp. nov. and *Chlamydia gallinacea* sp. nov. *Syst Appl Microbiol* **37**, 79–88.

Sachse, K., Bavoil, P. M., Kaltenboeck, B., Stephens, R. S., Kuo, C.-C., Rosselló-Móra, R. & Horn, M. (2015). Emendation of the family *Chlamydiaceae*: proposal of a single genus, *Chlamydia*, to include all currently recognized species. *Syst Appl Microbiol* **38**, 99–103.

Samudrala, R., Heffron, F. & McDermott, J. E. (2009). Accurate prediction of secreted substrates and identification of a conserved putative secretion signal for type iii secretion systems. *PLoS Pathog* **5**(4), e1000375.

Sasakawa, C., Kamata, K., Sakai, T., Makino, S., Yamada, M., Okada, N. & Yoshikawa, M. (1988). Virulence-associated genetic regions comprising 31 kilobases of the 230-kilobase plasmid in *Shigella flexneri* 2a. *J Bacteriol* **170**(6), 2480–2484.

Schachter, J. (1978). Chlamydial infections (first of three parts). *N Engl J Med* **298**, 428–435.

Schachter, J. (1999). Infection and disease epidemiology. In *Chlamydia Intracell Biol Pathog Immun*. Edited by R. S. Stephens. ASM Press, Washington, DC. pp. 139–169.

Schautteet, K. & Vanrompay, D. (2011). *Chlamydiaceae* infections in pig. *Vet Res* **42**(29), 1–10.

Scherer, W. F., Syverton, J. T. & Gey, G. O. (1953). Studies on the propagation *in vitro* of poliomyelitis viruses. IV. Viral multiplication in a stable strain of human malignant epithelial cells (strain HeLa) derived from an epidermoid carcinoma of the cervix. *J Exp Med* **97**, 695–710.

Schoborg, R. V. **(2011).** *Chlamydia* persistence-a tool to dissect chlamydia-host interactions. *Microbes Infect* **13**(7), 649–662.

Scidmore, M. A. & Hackstadt, T. (2001). Mammalian 14-3-3-β associates with the *Chlamydia trachomatis* inclusion membrane via its interaction with IncG. *Mol Microbiol* **39**(6), 1638–1650.

Scidmore, M. (2005). Cultivation and laboratory maintenance of *Chlamydia trachomatis*. *Curr Protoc Microbiol* 1–25.

Scidmore, M. A., Fischer, E. R. & Hackstadt, T. (2003). Restricted fusion of *Chlamydia trachomatis* vesicles with endocytic compartments during the initial stages of infection. *Infect Immun* **71**(2), 973–984.

Segal, G., Purcell, M. & Shuman, H. A. (1998). Host cell killing and bacterial conjugation require overlapping sets of genes within a 22-kb region of the *Legionella pneumophila* genome. *Proc Natl Acad Sci U S A* **95**, 1669–1674.

Seshadri, R., Paulsen, I. T., Eisen, J. A., Read, T. D., Nelson, K. E., Nelson, W. C., Ward, N. L., Tettelin, H., Davidsen, T. M. & other authors. **(2003).** Complete genome sequence of the Q-fever pathogen *Coxiella burnetii*. *Proc Natl Acad Sci U S A* **100**(9), 5455–5460.

Shanmughapriya, S., Senthilkumar, G., Vinodhini, K., Das, B. C., Vasanthi, N. & Natarajaseenivasan, K. (2012). Viral and bacterial aetiologies of epithelial ovarian cancer. *Eur J Clin Microbiol Infect Dis* **31**, 2311–2317.

Shaw, E. I., Dooley, C. A., Fischer, E. R., Scidmore, M. A., Fields, K. A. & Hackstadt, T. (2000). Three temporal classes of gene expression during the *Chlamydia trachomatis* developmental cycle. *Mol Microbiol* **37**(4), 913–925.

Shaw, K., Coleman, D., O'Sullivan, M. & Stephens, N. (2011). Public health policies and management strategies for genital *Chlamydia trachomatis* infection. *Risk Manag Healthc Policy* **4**, 57–65.

Shea, J. E., Hensel, M., Gleeson, C. & Holden, D. W. (1996). Identification of a virulence locus encoding a second type III secretion system in *Salmonella typhimurium*. *Proc Natl Acad Sci U S A* **93**, 2593–2597.

Silva, J., Cerqueira, F. & Medeiros, R. (2014). *Chlamydia trachomatis* infection: Implications for HPV status and cervical cancer. *Arch Gynecol Obstet* **289**, 715–723.

Silva-Herzog, E., Joseph, S. S., Avery, A. K., Coba, J. A., Wolf, K., Fields, K. A. & Plano, G. V. (2011). Scc1 (CP0432) and Scc4 (CP0033) function as a type III secretion chaperone for CopN of *Chlamydia pneumoniae*. *J Bacteriol* **193**(14), 3490–3496.

Simeone, R., Bottai, D. & Brosch, R. (2009). ESX/type VII secretion systems and their role in host-pathogen interaction. *Curr Opin Microbiol* **12**, 4–10.

Simeone, R., Bobard, A., Lippmann, J., Bitter, W., Majlessi, L., Brosch, R. & Enninga, J. (2012). Phagosomal rupture by *Mycobacterium tuberculosis* results in toxicity and host cell death. *PLoS Pathog* **8**(2), e1002507.

Sisko, J. L., Spaeth, K., Kumar, Y. & Valdivia, R. H. (2006). Multifunctional analysis of *Chlamydia*-specific genes in a yeast expression system. *Mol Microbiol* **60**(1), 51–66.

Snavely, E. A., Kokes, M., Dunn, J. D., Saka, H. A., Nguyen, B. D., Bastidas, R. J., Mccafferty, D. G. & Valdivia, R. H. (2015). Reassessing The Role Of The Secreted Protease **71**(3), 336–351.

Song, L., Carlson, J. H., Whitmire, W. M., Kari, L., Virtaneva, K., Sturdevant, D. E., Watkins, H., Zhou, B., Sturdevant, G. L. & other authors. (2013). *Chlamydia trachomatis* plasmid-encoded Pgp4 is a transcriptional regulator of virulence-associated genes. *Infect Immun* **81**(3), 636–644.

Spaeth, K. E., Chen, Y. S. & Valdivia, R. H. (2009). The *Chlamydia* type III secretion system C-ring engages a chaperone-effector protein complex. *PLoS Pathog* **5**(9), e1000579.

Stary, G., Olive, A., Radovic-Moreno, A. F., Gondek, D., Alvarez, D., Basto, P. A., Perro, M., Vrbanac, V. D., Tager, A. M. & other authors. (2015). A mucosal vaccine against *Chlamydia trachomatis* generates two waves of protective memory T cells. *Science* **348**(6241), aaa8205.

Stenner-Liewen, F., Liewen, H., Zapata, J. M., Pawlowski, K., Godzik, A. & Reed, J. C. (2002). CADD, a *Chlamydia* protein that interacts with death receptors. *J Biol Chem* **277**(12), 9633–9636.

Stephens, R. S., Kalman, S., Lammel, C., Fan, J., Marathe, R., Aravind, L., Mitchell, W., Olinger, L., Tatusov, R. L. & other authors. (1998). Genome sequence of an obligate intracellular pathogen of humans: *Chlamydia trachomatis*. *Science* **282**, 754–759.

Stephens, R. S. (2003). The cellular paradigm of chlamydial pathogenesis. *Trends Microbiol* **11**(1), 44–51.

Stephens, R. S., Myers, G., Eppinger, M. & Bavoil, P. M. (2009). Divergence without difference: phylogenetics and taxonomy of *Chlamydia* resolved. *FEMS Immunol Med Microbiol* **55**, 115–119.

Stone, C. B., Johnson, D. L., Bulir, D. C., Gilchrist, J. D. & Mahony, J. B. (2008). Characterization of the putative type III secretion ATPase CdsN (Cpn0707) of *Chlamydophila pneumoniae*. *J Bacteriol* **190**(20), 6580–6588.

Stone, C. B., Bulir, D. C., Emdin, C. A., Pirie, R. M., Porfilio, E. A., Slootstra, J. W. & Mahony, J. B. (2011). *Chlamydia pneumoniae* CdsL Regulates CdsN ATPase Activity, and Disruption with a Peptide Mimetic Prevents Bacterial Invasion. *Front Microbiol* **2**, 21.

Stone, C. B., Sugiman-Marangos, S., Bulir, D. C., Clayden, R. C., Leighton, T. L., Slootstra, J. W., Junop, M. S. & Mahony, J. B. (2012). Structural characterization of a novel *Chlamydia pneumoniae* type III secretion-associated protein, Cpn0803. *PLoS One* **7**(1), e30220.

Stuart, E. S., Webley, W. C. & Norkin, L. C. (2003). Lipid rafts, caveolae, caveolin-1, and entry by *Chlamydiae* into host cells. *Exp Cell Res* **287**, 67–78.

Su, H., Raymond, L., Rockey, D. D., Fischer, E., Hackstadt, T. & Caldwell, H. D. (1996). A recombinant *Chlamydia trachomatis* major outer membrane protein binds to heparan sulfate receptors on epithelial cells. *Proc Natl Acad Sci U S A* **93**, 11143–11148.

Subtil, A., Parsot, C. & Dautry-Varsat, A. (2001). Secretion of predicted Inc proteins of *Chlamydia pneumoniae* by a heterologous type III machinery. *Mol Microbiol* **39**(3), 792–800.

Subtil, A., Delevoye, C., Balañá, M. E., Tastevin, L., Perrinet, S. & Dautry-Varsat, A. (2005). A directed screen for chlamydial proteins secreted by a type III mechanism identifies a translocated protein and numerous other new candidates. *Mol Microbiol* **56**(6), 1636–1647.

Suchland, R. J., Rockey, D. D., Bannantine, J. P. & Stamm, W. E. (2000). Isolates of *Chlamydia trachomatis* that occupy nonfusogenic inclusions lack IncA, a protein localized to the inclusion membrane. *Infect Immun* **68**(1), 360–367.

Südhof, T. C. & Rothman, J. E. (2009). Membrane fusion: grappling with SNARE and SM proteins. *Science* **323**(5913), 474–477.

T'ang, F. ., Chang, Y. T., Huang, Y. T. & Wang, K. C. (1957). Studies on the aetiology of trachoma with special reference to isolation of the virus in chick embryo. *ChinMedJ* **75**, 429–447.

Tam, J. E., Davis, C. H. & Wyrick, P. B. (1994). Expression of recombinant DNA introduced into *Chlamydia trachomatis* by electroporation. *Can J Microbiol* **40**(7), 583–591.

Tattersall, J., Rao, G. V., Runac, J., Hackstadt, T., Grieshaber, S. S. & Grieshaber, N. A. (2012). Translation Inhibition of the Developmental Cycle Protein HctA by the Small RNA IhtA Is Conserved across *Chlamydia*. *PLoS One* **7**(10), e47439.

Taylor, H. R. (2008). *Trachoma. A Blinding Scourge from the Bronze age to the Twenty first Century.* Haddington Press. Australia.

Taylor-Brown, A., Vaughan, L., Greub, G., Timms, P. & Polkinghorne, A. (2015). Twenty years of research into Chlamydia-like organisms: A revolution in our understanding of the biology and pathogenicity of members of the phylum *Chlamydiae*. *Pathog Dis* **73**, 1–15.

Taylor-Robinson, D. & Keat, A. (2015). Observations on *Chlamydia trachomatis* and other microbes in reactive arthritis. *Int J STD AIDS* **26**(3), 139–144.

Thalmann, J., Janik, K., May, M., Sommer, K., Ebeling, J., Hofmann, F., Genth, H. & Klos, A. (2010). Actin re-organization induced by *Chlamydia trachomatis* serovar D-evidence for a critical role of the effector protein CT166 targeting Rac. *PLoS One* **5**(3), e9887.

Todd, W. J. & Caldwell, H. D. (1985). The Interaction of *Chlamydia trachomatis* with Host Cells: Ultrastructural Studies of the Mechanism of Release of a Biovar II Strain from HeLa 229 Cells. *J Infect Dis* **151**(6), 1037–1044.

Tosi, T., Pflug, A., Discola, K. F., Neves, D. & Dessen, A. (2013). Structural basis of eukaryotic cell targeting by type III secretion system (T3SS) effectors. *Res Microbiol* **164**, 605–619.

Vabulas, R. M., Ahmad-Nejad, P., Da Costa, C., Miethke, T., Kirschning, C. J., Häcker, H. & Wagner, H. (2001). Endocytosed HSP60s Use Toll-like Receptor 2 (TLR2) and TLR4 to Activate the Toll/Interleukin-1 Receptor Signaling Pathway in Innate Immune Cells. *J Biol Chem* **276**(33), 31332–31339.

Valdivia, R. H. (2008). *Chlamydia* effector proteins and new insights into chlamydial cellular microbiology. *Curr Opin Microbiol* **11**, 53–59.

Vasilevsky, S., Stojanov, M., Greub, G., Baud, D., Vasilevsky, S., Stojanov, M., Greub, G. & Baud, D. (2016). Chlamydial polymorphic membrane proteins : regulation , function and potential vaccine candidates. *Virulence* **7**(1), 11–22.

Vogel, J. P., Andrews, H. L., Wong, S. K. & Isberg, R. R. (1998). Conjugative transfer by the virulence system of *Legionella pneumophila*. *Science* **279**, 873–876.

Vorimore, F., Hsia, R.-C., Huot-Creasy, H., Bastian, S., Deruyter, L., Passet, A., Sachse, K., Bavoil, P., Myers, G. & Laroucau, K. (2013). Isolation of a New *Chlamydia* species from the Feral Sacred Ibis (Threskiornis aethiopicus): *Chlamydia ibidis*. *PLoS One* **8**(9), e74823.

Wagar, E. A. & Stephens, R. S. (1988). Developmental-form-specific DNA-binding proteins in *Chlamydia* spp. *Infect Immun* **56**(7), 1678–1684.

Wang, X., Schwarzer, C., Hybiske, K., Machen, T. E. & Stephens, R. S. (2014). Developmental stage oxidoreductive states of *Chlamydia* and infected host cells. *MBio* **5**(6), e01924–14.

Wang, Y., Kahane, S., Cutcliffe, L. T., Skilton, R. J., Lambden, P. R. & Clarke, I. N. (2011). Development of a transformation system for *Chlamydia trachomatis*: Restoration of glycogen biosynthesis by acquisition of a plasmid shuttle vector. *PLoS Pathog* **7**(9), e1002258.

Wehrl, W., Brinkmann, V., Jungblut, P. R., Meyer, T. F. & Szczepek, A. J. (2004). From the inside out - processing of the chlamydial autotransporter PmpD and its role in bacterial adhesion and activation of human host cells. *Mol Microbiol* **51**(2), 319–334.

Weitzman, M. D. & Weitzman, J. B. (2014). What's the damage? The impact of pathogens on pathways that maintain host genome integrity. *Cell Host Microbe* **15**(3), 283–294.

Whitcher, J. P., Srinivasan, M. & Upadhyay, M. P. (2001). Corneal blindness: a global perspective. *Bull World Health Organ* **79**, 214–221.

Wickstrum, J., Sammons, L. R., Restivo, K. N. & Hefty, P. S. (2013). Conditional gene expression in *Chlamydia trachomatis* using the tet system. *PLoS One* **8**(10), e76743.

Wilkat, M., Herdoiza, E., Forsbach-Birk, V., Walther, P. & Essig, A. (2014). Electron tomography and cryo-SEM characterization reveals novel ultrastructural features of host-parasite interaction during *Chlamydia abortus* infection. *Histochem Cell Biol* **142**, 171–184.

Woodman, P. G. & Futter, C. E. (2008). Multivesicular bodies: co-ordinated progression to maturity. *Curr Opin Cell Biol* **20**, 408–414.

World Health Organization. **(2011).** Prevalence and incidence of selected sexually transmitted infections. *WHO, Geneva, Switz*.

World Health Organization. **(2014).** Alliance for the Global Elimination of Blinding Trachoma by the year 2020. *WHO, Geneva, Switz*.

Wright, H. R., Turner, A. & Taylor, H. R. (2008). Trachoma. *Lancet* **371**, 1945–1954.

Wuppermann, F. N., Hegemann, J. H. & Jantos, C. A. (2001). Heparan sulfate-like glycosaminoglycan is a cellular receptor for *Chlamydia pneumoniae*. *J Infect Dis* **184**(2), 181–187.

Wyrick, P. B. (2010). *Chlamydia trachomatis* Persistence *in vitro*: an overview. *J Infect Dis* **201**(S2), S88–S95.

Xiridou, M., Vriend, H. J., Lugner, A. K., Wallinga, J., Fennema, J. S., Prins, J. M., Geerlings, S. E., Rijnders, B. J. A., Prins, M. & other authors. **(2013).** Modelling the impact of *Chlamydia* screening on the transmission of HIV among men who have sex with men. *BMC Infect Dis* **13**(436), 1–10.

Yahr, T. L., Goranson, J. & Frank, D. W. (1996). Exoenzyme S of *Pseudomonas aeruginosa* is secreted by a type III pathway. *Mol Microbiol* **22**(5), 991–1003.
Yao, J., Cherian, P. T., Frank, M. W. & Rock, C. O. (2015). *Chlamydia trachomatis* Relies on Autonomous Phospholipid Synthesis for Membrane Biogenesis. *J Biol Chem* **290**(31), 18874–18888.
Yu, H. H. Y. & Tan, M. (2003). Sigma-28 RNA polymerase regulates hctB, a late developmental gene in *Chlamydia*. *Mol Microbiol* **50**(2), 577–584.
Yu, X.-J., McGourty, K., Liu, M., Unsworth, K. E. & Holden, D. W. (2010). pH sensing by intracellular *Salmonella* induces effector translocation. *Science* **328**, 1040–1043.
Yuan, Y., Zhang, Y. X., Watkins, N. G. & Caldwell, H. D. (1989). Nucleotide and deduced amino acid sequences for the four variable domains of the major outer membrane proteins of the 15 *Chlamydia trachomatis* serovars. *Infect Immun* **57**(4), 1040–1049.
Zhang, J. P. & Stephens, R. S. (1992). Mechanism of *C. trachomatis* attachment to eukaryotic host cells. *Cell* **69**(5), 861–869.
Zhong, G. (2011). *Chlamydia trachomatis* secretion of proteases for manipulating host signaling pathways. *Front Microbiol* **2**, 14.
Zhou, D. & Galán, J. (2001). *Salmonella* entry into host cells: the work in concert of type III secreted effector proteins. *Microbes Infect* **3**, 1293–1298.

Chapter II

Identification of type III secretion substrates of *Chlamydia trachomatis* using *Yersinia enterocolitica* as a heterologous system

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The author of this dissertation participated in all the experiments described in this Chapter, except for the RT-qPCR assays illustrated in Fig. 4 that were done by Vítor Borges at INSA Ricardo Jorge.

2.1. ABSTRACT

Chlamydia trachomatis uses a type III secretion (T3S) system to manipulate host cells, through the delivery of effector proteins into their cytosol and membranes. In this Chapter, we aimed to find previously unidentified *C. trachomatis* T3S substrates. We first analyzed the genome of *C. trachomatis* strain L2 434/Bu for genes encoding mostly uncharacterized proteins that did not appear to possess a signal of the general secretory pathway and which had not been previously experimentally shown to be T3S substrates. We selected several genes with these characteristics and analyzed T3S of the encoding proteins using *Yersinia enterocolitica* as a heterologous system. We identified 23 *C. trachomatis* proteins whose first 20 amino acids were sufficient to drive T3S of the mature form of β-lactamase TEM-1 by *Y. enterocolitica*. We found that 10 of these 23 proteins were also type III secreted in their full-length versions by *Y. enterocolitica*, providing additional support that they are T3S substrates. Seven of these 10 likely T3S substrates of *C. trachomatis* were delivered by *Y. enterocolitica* into host cells, further suggesting that they could be effectors. Finally, real-time quantitative PCR analysis of expression of genes encoding the 10 likely T3S substrates of *C. trachomatis* showed that 9 of them were clearly expressed during infection of host cells. In conclusion, using *Y. enterocolitica* as a heterologous system, we identified 10 likely T3S substrates of *C. trachomatis* (CT053, CT105, CT142, CT143, CT144, CT161, CT338, CT429, CT656, and CT849) and could detect translocation into host cells of CT053, CT105, CT142, CT143, CT161, CT338, and CT429. Therefore, we revealed several *C. trachomatis* proteins that could be effectors subverting host cell processes.

2.2. INTRODUCTION

Chlamydia trachomatis is an obligate intracellular human pathogen that undergoes a developmental cycle involving the inter-conversion between two morphologically distinct forms: a non-replicative infectious form, the elementary body (EB), and a replicative non-infectious form, the reticulate body (RB) (AbdelRahman & Belland, 2005). Throughout its developmental cycle, *C. trachomatis* uses a type III secretion system (T3SS) to translocate several effector proteins across the host cell plasma membrane and the inclusion membrane (Betts *et al.*, 2009; Valdivia, 2008). These T3S effectors are thought to play a central role in bacterial invasion (Jewett *et al.*, 2010; Lane *et al.*, 2008) and exit of host cells (Lutter *et al.*, 2013), and in the subversion of various host cell processes (Chellas-Géry *et al.*, 2007; Delevoye *et al.*, 2008; Derré *et al.*, 2011; Hower *et al.*, 2009; Mital *et al.*, 2010; Pennini *et al.*, 2010; Rzomp *et al.*, 2006; Scidmore & Hackstadt, 2001). There are, however, chlamydial effectors, such as CPAF/CT858 or CT441, which are not T3S substrates (Betts *et al.*, 2009).

Given their likely central role during infection, considerable efforts have been placed in identifying chlamydial effectors. This is not a trivial task because the amino acid sequence of most effectors does not display significant similarity to proteins of known function. Additionally, T3S substrates, which should comprise the bulk of *Chlamydia* effectors, contain no easily recognizable secretion signal. Moreover, in spite of the recent development of systems for transformation of *Chlamydia* (Gérard *et al.*, 2013; Wang *et al.*, 2011), for a long time no methods have been available for genetic manipulation of these bacteria. To overcome these obstacles,

chlamydial effectors have been searched: i) by systematic phenotypic analyses of yeast *Saccharomyces cerevisiae* expressing individual chlamydial proteins (Sisko *et al.*, 2006); ii) by using *Salmonella* (Ho & Starnbach, 2005) *Shigella* (Furtado *et al.*, 2013; Muschiol *et al.*, 2011; Pennini *et al.*, 2010; Subtil *et al.*, 2005), or *Yersinia* (Chellas-Géry *et al.*, 2007; Clifton *et al.*, 2004; Fields & Hackstadt, 2000; Hovis *et al.*, 2013; Hower *et al.*, 2009; Pais *et al.*, 2013) as genetically tractable heterologous host bacteria carrying well characterized T3SSs; or iii) by complex computational predictions of T3S signals (Arnold *et al.*, 2009; Löwer & Schneider, 2009; Samudrala *et al.*, 2009). The subsequent use of specific antibodies enabled to detect translocation into host cells of some of the *C. trachomatis* proteins singled out in these searches, such as in the case of Tarp/CT456 (Clifton *et al.*, 2004), CT694 (Hower *et al.*, 2009), CopN/CT089 (Fields & Hackstadt, 2000), Cap1/CT529 (Fling *et al.*, 2001), CT620 (Muschiol *et al.*, 2011), CT621 (Hobolt-Pedersen *et al.*, 2009; Muschiol *et al.*, 2011), CT711 (Muschiol *et al.*, 2011), lipid-droplet associated (Lda) proteins Lda1/CT156, Lda2/CT163, and Lda3/CT473 (Kumar *et al.*, 2006), Nue/CT737 (Pennini *et al.*, 2010), or of a group of proteins containing a hydrophobic motif thought to mediate their insertion into the inclusion membrane (Inc proteins) (Li *et al.*, 2008; Mital *et al.*, 2010). Moreover, the direct use of antibodies raised against particular *C. trachomatis* proteins (CT311, CT622, CT795, GlgA/CT798, HtrA/CT823, or Pgp3) revealed their presence in the host cell cytosol or nucleus of infected cells (Gong *et al.*, 2011; Lei *et al.*, 2011, 2013; Li *et al.*, 2008; Lu *et al.*, 2013; Qi *et al.*, 2011). Finally, the *in vitro* deubiquitinase activity of *Chla*DUB1/CT868 and of *Chla*DUB2/CT867 (Misaghi *et al.*, 2006), and the capacity of *Chla*DUB1/CT868 to suppress the NF-κB pathway in transfected cells (Le Negrate *et al.*, 2008), indicate that these two proteins should be effectors.

In this Chapter, we have surveyed the genome of *C. trachomatis* mostly for genes encoding uncharacterized proteins that were not described before as T3S substrates. We then used *Yersinia enterocolitica* as a heterologous system to identify 10 novel likely T3S substrates of *C. trachomatis* and real-time quantitative PCR (RT-qPCR) to show that 9 of the genes encoding these proteins are clearly expressed during the bacterial developmental cycle. Furthermore, we showed that 7 of the 10 likely T3S substrates of *C. trachomatis* could be translocated into host cells by *Y. enterocolitica*. Therefore, we identified several novel putative effectors of *C. trachomatis*.

2.3. MATERIALS AND METHODS

Cell culture, bacterial strains and growth conditions

HeLa 229 (ATCC) cells were maintained in DMEM (Life Technologies) supplemented with 10% (v/v) FBS (Life Technologies) at 37°C in a humidified atmosphere of 5% (v/v) CO_2. *C. trachomatis* serovar L2 strain 434/Bu (L2/434; from ATCC) was propagated in HeLa 229 cells using standard techniques (Scidmore, 2005). *Escherichia coli* TOP10 (Life Technologies) was used for construction and purification of the plasmids. *Y. enterocolitica* ΔHOPEMT (MRS40 pIML421 [$yopH_{\Delta 1\text{-}352}$, $yopO_{\Delta 65\text{-}558}$, $yopP_{23}$, $yopE_{21}$, $yopM_{23}$, $yopT_{135}$]), deficient for the *Yersinia* Yop T3S effectors H, O, P, E, M, and T, but T3S-proficient (Iriarte & Cornelis, 1998) and T3S-deficient *Y. enterocolitica* ΔHOPEMT ΔYscU (MRS40 pFA1001 [$yopH_{\Delta 1\text{-}352}$, $yopO_{\Delta 65\text{-}558}$, $yopP_{23}$, $yopE_{21}$, $yopM_{23}$, $yopT_{135}$, $yscU_{\Delta 1\text{-}354}$) (Almeida *et al.*, 2012) were used for T3S assays. The *yscU* gene encodes an essential component of the *Y. enterocolitica* T3S system, and the $yscU_{\Delta 1\text{-}354}$ mutation is non-polar (Sorg *et al.*, 2007). *E. coli* or *Y. enterocolitica* were routinely grown in liquid or solid LB medium with the appropriate antibiotics and supplements. Plasmids were introduced into *E. coli* or *Y. enterocolitica* by electroporation.

DNA manipulations, plasmids, and primers

The plasmids used in this work and their main characteristics are detailed Table A1 (Annexes). The DNA primers used in their construction are shown in Table A2 (Annexes). Plasmids were constructed and purified with proof-reading Phusion DNA polymerase (Thermo Fisher Scientific), restriction enzymes (Thermo Fisher Scientific), T4 DNA Ligase (Life Technologies), DreamTaq DNA polymerase (Thermo Fisher Scientific), DNA clean & concentrator^{TM}-5

Kit and Zymoclean^{TM} Gel DNA Recovery kit (Zymo Research), and purified with GeneElute Plasmid Miniprep kit (Sigma-Aldrich), according to the instructions of the manufacturers. In brief, to analyze T3S signals we constructed plasmids harboring hybrid genes encoding the first 20 amino acids of each *C. trachomatis* protein, and the mature form of TEM-1 β-lactamase (TEM-1) (Charpentier & Oswald, 2004). These hybrids were made using as vector plasmid pLJM3, a low-copy plasmid which enables expression of the cloned genes driven by the promoter of the *Y. enterocolitica yopE* gene (Marenne *et al.*, 2003), either by overlapping PCR or by using a cloning strategy previously described for the construction of plasmids encoding Inc-TEM-1 hybrid proteins (Almeida *et al.*, 2012). To analyze secretion of full-length *C. trachomatis* proteins, we constructed plasmids expressing the proteins C-terminally tagged with a HA epitope. For this, the genes were amplified by PCR from chromosomal DNA of *C. trachomatis* L2/434 using a reverse primer with a sequence complementary to the transcribed strand of the DNA encoding the HA-epitope. PCR products digested with the appropriate enzymes were ligated into pLJM3 (Marenne *et al.*, 2003). The accuracy of the nucleotide sequence of all the inserts in the constructed plasmids was checked by DNA sequencing.

Y. enterocolitica T3S assays

T3S assays were done as previously described (Sorg *et al.*, 2007). We used *Y. enterocolitica* ΔHOPEMT or ΔHOPEMT ΔYscU strains carrying the plasmids described in Table A1 (Annexes). The proteins in bacterial pellets and culture supernatants were analyzed by immunoblotting, and the amounts of protein in bacterial pellets and/or culture supernatants were estimated from images of immunoblots with Image Lab (Bio-Rad). Where appropriate, we calculated the

percentage of secretion as the ratio between the amounts of secreted protein (in the culture supernatant fraction) relative to the total amount of protein (in the culture supernatant and in the bacterial pellet fractions). The results from the quantifications are the average ± SEM from at least three independent experiments. Detailed results for each protein analyzed are in Table A4 (Annexes) and Table A5 (Annexes).

Y. enterocolitica translocation assays

Analyses of protein translocation into host cells by *Y. enterocolitica* were done essentially as previously described (Denecker *et al.*, 2002; Grosdent *et al.*, 2002). In brief, *Y. enterocolitica* strains were grown in BHI (Scharlau) medium overnight at 26°C with continuous shaking (130 rpm). Bacteria were then diluted to OD_{600} of 0.2 in fresh BHI and cultured in the same conditions for 2 h. Subsequently, the *yop* regulon was induced by incubation for 30 min in a shaking water bath (130 rpm) at 37°C. Bacteria were then washed with DMEM supplemented with 10% (v/v) FBS and added to HeLa 229 cells, grown overnight in 24-well plates ($1x10^5$ cells/well), by using a multiplicity of infection of 50. The infected cells were incubated at 37°C in a humidified atmosphere of 5% (v/v) CO_2. After 3 h of incubation, extracellular bacteria were killed by adding gentamicin (50 µg/ml), and the cells were incubated in the same conditions for additional 2 h. The infected cells were then harvested on ice, washed with PBS, ressuspended in PBS containing 0.1% (v/v) Triton X-100 and a protease inhibitor cocktail (Sigma-Aldrich), and incubated for 10 min on ice. The samples were then centrifuged (15,000 *g* for 15 min at 4°C) and Triton-soluble and Triton-insoluble HeLa cell lysates were loaded on 12% (v/v) SDS-PAGE. After electrophoresis, the gels were processed for immunoblotting using 0.2 µm pore-size nitrocellulose membranes (BioRad).

Immunoblotting

The following antibodies were used for immunoblotting: rat monoclonal anti-HA (3F10; Roche; 1:1000), mouse monoclonal anti-TEM-1 (QED Bioscience; 1:500), rabbit polyclonal anti-SycO (1:1000) (Letzelter *et al.*, 2006), and mouse monoclonal anti-tubulin (clone B-5-1-2; Sigma-Aldrich; 1:1000). Immunoblot detection was done with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare and Jackson ImmunoResearch), Western Lightning *Plus*-ECL (Perkin Elmer), and a ChemiDoc XRS+ system (BioRad) or exposure to Amersham Hyperfilm ECL (GE Healthcare). All quantitative analyses were done with immunoblot images obtained using ChemiDoc XRS+ (BioRad).

Real-time quantitative PCR

The expression of the newly identified candidate T3S substrates during the developmental cycle of *C. trachomatis* L2/434 was estimated by determining mRNA levels at different times post-infection by RT-qPCR. These experiments were done as previously described (Almeida *et al.*, 2012). Primers (Table A2; annexes) were designed (Borges *et al.*, 2010) using Primer Express (Applied Biosystems). The RT-qPCR assays were done using the ABI 7000 SDS, SYBR green chemistry, and optical plates (Applied Biosystems), as previously described (Borges *et al.*, 2010). At each time point, raw RT-qPCR data for each gene were normalized against the data obtained for the 16S rRNA transcript, as it was previously demonstrated that this is an adequate endogenous control (Borges *et al.*, 2010). The final results were based on three independent experiments.

2.4. RESULTS

2.4.1 Selection of *C. trachomatis* proteins analyzed in this work

To search for previously unidentified T3S substrates of *C. trachomatis*, we first surveyed the genome of strain L2/434 for genes encoding mostly uncharacterized proteins, or with a putative biochemical activity compatible with the function of a T3S effector (e.g., proteases). Among these genes, we selected those encoding proteins not predicted to have a signal sequence characteristic of the general secretory pathway (according to Psortb v3.0) and that had not been previously analyzed experimentally for the presence of a T3S signal. This singled out 32 proteins (CT016, CT017, CT031, CT051, CT053, CT080, CT105, CT142, CT143, CT144, CT153, CT161, CT172, CT273, CT277, CT289, CT309, CT330, CT338, CT386, CT425, CT568, CT583, CT590, CT631, CT635, CT656, CT696, CT702, CT837, CT845, and CT849; we used the nomenclature of the annotated *C. trachomatis* serovar D strain UW3 (D/UW3) (Stephens *et al.*, 1998); the names of the corresponding genes as annotated for strain L2/434 (Thomson *et al.*, 2008) can be found in Table A4 (Annexes). Furthermore, for comparison purposes, we considered proteins that had been tested for the presence of a T3S signal using *Shigella flexneri* as a heterologous bacteria: eight proteins whose first ~40 amino acids of the corresponding *C. pneumoniae* homologs did not drive secretion of an adenylate cyclase (Cya) reporter protein by *S. flexneri* (CT066, CT429, GrgA/CT504, CT538, CT584, CT768, CT779, CT814), and three proteins whose N-terminal region of the *C. pneumoniae* homologs drove secretion of a Cya reporter protein by *S. flexneri* (CT203, CT577, CT863) (Subtil *et al.*, 2005). Please note that at the time this work was initiated GrgA/CT504 was an uncharacterized protein; however, it was

afterwards described as a transcriptional activator (Bao *et al.*, 2012). Finally, throughout this study we used as positive controls a *C. trachomatis* bona-fide T3S effector (CT694) (Hower *et al.*, 2009) and a *C. trachomatis* likely T3S substrate (CT082) that we had previously identified (Pais *et al.*, 2013), and which was recently independently confirmed (Hovis *et al.*, 2013), and as negative control a predicted ribosomal protein (RplJ/CT317).

In summary, in experiments that will be described below, we analyzed T3S signals in 46 *C. trachomatis* proteins (~5% of all proteins encoded by the L2/434 strain): 32 hypothetical proteins previously not analyzed experimentally for T3S signals, 11 proteins whose *C. pneumoniae* homologs were previously analyzed for T3S signals using *S. flexneri* as heterologous system, and 3 controls. In the selection of these proteins, we did not consider predictions made by any of the published *in silico* methods that suggest putative T3S substrates (Arnold *et al.*, 2009; Löwer & Schneider, 2009; Samudrala *et al.*, 2009; Wang *et al.*, 2013).

2.4.2 Identification of T3S signals in *C. trachomatis* proteins

To identify T3S signals in the selected 46 *C. trachomatis* proteins, we analyzed secretion of fusions to TEM-1 of the first 20 amino acids of each of these proteins by T3S-proficient *Y. enterocolitica* ΔHOPEMT. We previously showed that the first 20 amino acids of *C. trachomatis* T3S substrates were sufficient for an efficient secretion of TEM-1 fusion by *Y. enterocolitica* in a T3S-dependent fashion (data not shown). These experiments revealed 24 *C. trachomatis* proteins whose first 20 amino acids drove secretion of TEM-1 hybrid proteins by *Y. enterocolitica* (Fig. 2.1A). Owing to lack of expression, or very low expression levels, it was not possible to conclude if the TEM-1

hybrids comprising the N-terminal region of CT590, CT845 and CT863 were secreted (Fig. 2.1A). By individually introducing the plasmids encoding the TEM-1 hybrid proteins that were secreted into T3S-deficient *Y. enterocolitica* ΔHOPEMT ΔYscU and performing T3S assays, we confirmed that secretion of the proteins was dependent on a functional T3SS (Fig. 2.1B). The percentage of secretion of the different hybrid proteins that were secreted varied considerable, between 56% (SEM, 4) for $CT694_{20}$-TEM-1 to 5% (SEM, 2) for $CT143_{20}$-TEM-1 (Fig. 2.1A). Overall, this confirmed a T3S signal in CT203, which has been previously shown to be a T3S substrate (Subtil *et al.*, 2005), and revealed T3S signals in 23 previously unrecognized T3S substrates of *C. trachomatis*. The results obtained are summarized in Table A4, annexes.

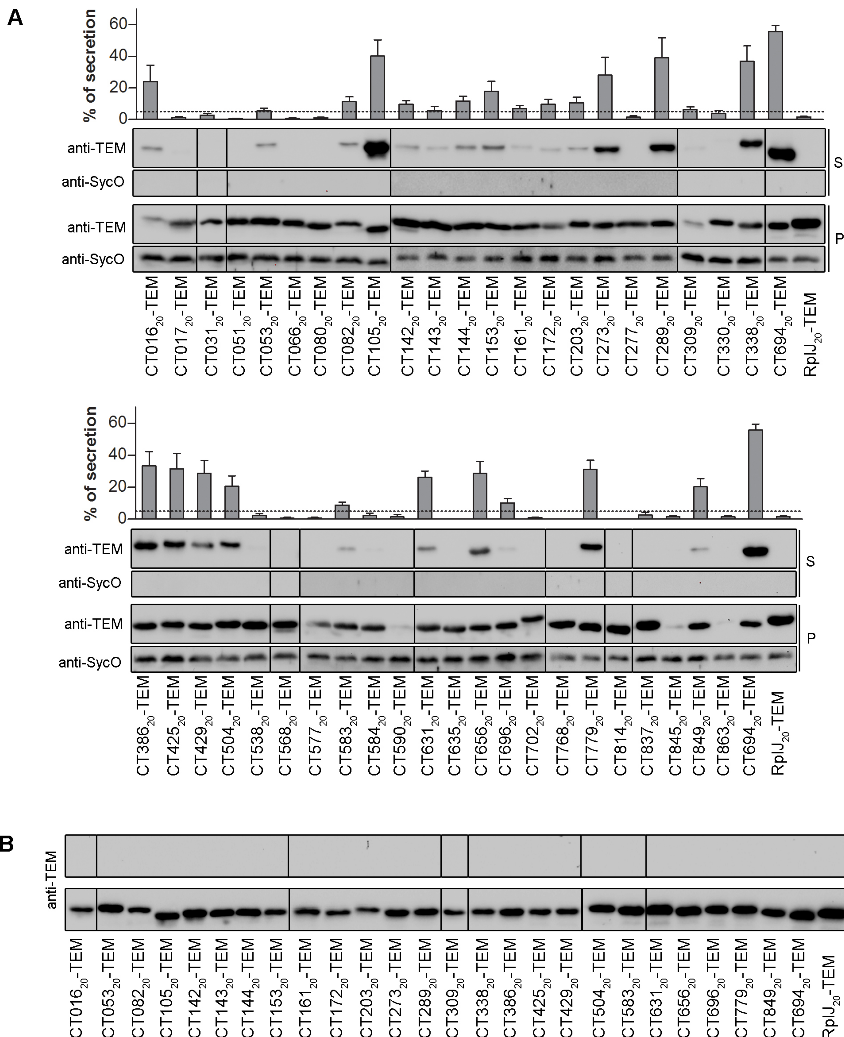
A
% of secretion
60
40
20
0
anti-TEM
anti-SycO
anti-TEM
anti-SycO
S
P
B
anti-TEM
S
P

Figure 2.1. Identification of T3S signals in *C. trachomatis* proteins using *Y. enterocolitica* as a heterologous system. *Y. enterocolitica* T3S-proficient (ΔHOPEMT) (A) and T3S-defective (ΔHOPEMT ΔYscU) (B) were used to analyze secretion of hybrid proteins comprising the first 20 amino acids of selected *C. trachomatis* proteins fused to the mature form of TEM-1 β-lactamase (TEM-1). Immunoblots show the result of T3S assays in which proteins in culture supernatants (S, secreted proteins) and in bacterial pellets (P, non-secreted proteins) from ~$2.5x10^8$ and ~$5x10^7$ bacteria, respectively, were loaded per lane. TEM-1 hybrids of the known *C. trachomatis* T3S substrates CT082 (Hovis *et al.*, 2013; Pais *et al.*, 2013) and CT694 (Hower *et al.*, 2009) were used as positive controls and a TEM-1 hybrid of the *C. trachomatis* ribosomal protein RplJ was used as a negative control. SycO is a strictly cytosolic *Yersinia* T3S chaperone (Iriarte & Cornelis, 1998; Letzelter *et al.*, 2006). Immunodetection of SycO ensured that the presence of TEM-1 hybrid proteins in the culture supernatants was not a result of bacterial lysis or contamination. The percentage (%) of secretion of each TEM-1 hybrid was calculated by densitometry, as the ratio between the amount of secreted and total protein. The threshold to decide whether a protein was secreted was set to 5% (dashed line), based on the % of secretion of a non-secreted *Yersinia* protein (da Cunha *et al.*, 2014). Data are the mean ± SEM from at least 3 independent experiments (see also Table A4, annexes).

2.4.3 Analysis of the secretion of the newly identified candidate T3S substrates of *C. trachomatis* as full-length proteins

We next analyzed if the 23 *C. trachomatis* proteins carrying newly identified T3S signals, and also CT203 and the controls (CT082, CT694 and RplJ), were secreted as full-length proteins by *Y. enterocolitica* ΔHOPEMT. The rationale for these experiments was that some proteins cannot be type III secreted even with a T3S signal grafted at their N-termini (Akeda & Galán, 2005; Feldman *et al.*, 2002; Lee & Schneewind, 2002; Sorg *et al.*, 2005), possibly because the secretion channel is too narrow (inner diameter of 2-3 nm (Cornelis, 2006)) to accommodate tightly folded proteins. Based on this, we predicted that if the full-length version of chlamydial proteins were type III secreted by *Yersinia* this would be an additional indication that they can be T3S substrates. However, lack of secretion of the full-length proteins would not preclude that they could be T3S substrates,

as they may require *Chlamydia*-specific chaperones, not present in *Yersinia* (Stebbins & Galán, 2001).
To analyze secretion of full-length *C. trachomatis* proteins by *Y. enterocolitica* we used plasmids expressing the chlamydial proteins with an HA tag at their C-termini. The plasmids were introduced into *Y. enterocolitica* ΔHOPEMT and T3S assays were performed. In these experiments, the percentage of secretion of the positive controls (CT694-HA and CT082-HA) was between 20-30% and the percentage of secretion of the negative control (RplJ-HA) was 0.13% (SEM, 0.05). Based on these results, in experiments involving full-length proteins of newly identified chlamydial T3S substrates we set a conservative threshold of 2% to decide whether a protein was secreted or not. This defined a group of 11 proteins that in their full-length version were secreted by *Y. enterocolitica* ΔHOPEMT: CT053-HA, CT105-HA, CT142-HA, CT143-HA, CT144-HA, CT161-HA, CT338-HA, CT429-HA, CT583-HA, CT656-HA, and CT849-HA (Fig. 2.2A and 2.2C). To test if secretion of these proteins was dependent on a functional T3SS, the plasmids carrying their encoding genes, as well as plasmids encoding positive controls CT694-HA or CT082-HA, were individually introduced into T3S-deficient *Y. enterocolitica* ΔHOPEMT ΔYscU. With the exception of CT583-HA, which for unknown reasons was very poorly expressed by *Y. enterocolitica* ΔHOPEMT ΔYscU, these assays indicated that the other 10 proteins analyzed were type III secreted (Fig. 2.2B).

Secretion of full-length CT153-HA, CT172-HA, CT203-HA, CT386-HA or CT425-HA by *Y. enterocolitica* could occasionally be seen by immunoblotting (Fig. 2.2A); however, the results were not always reproducible and their individual average percentage of secretion was in all cases below 2% (Fig. 2.2C). We did not detect significant

amounts of CT273-HA, CT289-HA, CT309-HA, or CT631-HA in culture supernatants (Fig. 2.2A), but as their levels of expression were either extremely low (CT273-HA, CT289-HA, and CT309-HA) or undetectable (CT631-HA) it was not possible to draw conclusions about secretion of these proteins. Furthermore, CT016-HA, and possibly CT696-HA (barely visible in Fig. 2.2A), were immunodetected in the culture supernatant fraction in a form that migrated on SDS-PAGE at a molecular weight much lower than the one predicted from their amino acid sequence (27 kDa and 46 kDa, respectively), while in the bacterial pellet fraction their migration on SDS-PAGE corresponded roughly to their predicted molecular weight (Fig. 2.2A). This suggests that the proteins could be cleaved during secretion, unstable in the culture supernatant, or their encoding genes possess internal Shine-Dalgarno sequences. Regardless of the exact reason, we could not confidently analyze whether CT016-HA and CT696-HA were secreted or not. The results obtained in this section are summarized in Table A5, annexes.

Overall, the full set of T3S assays revealed 10 proteins (CT053, CT105, CT142, CT143, CT144, CT161, CT338, CT429, CT656, and CT849) as newly identified likely T3S substrates of *C. trachomatis*, and therefore as possible effectors.

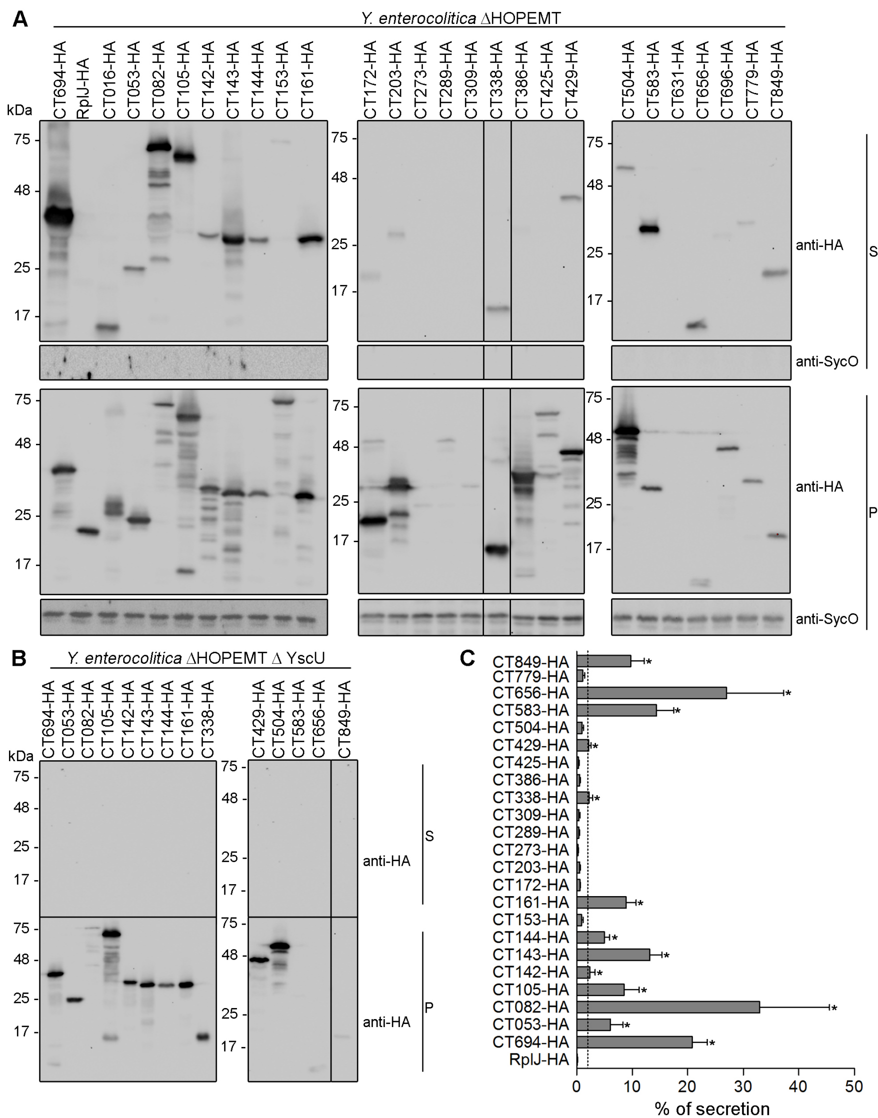
A
Y. enterocolitica ΔHOPEMT
CT694-HA
RplJ-HA
CT016-HA
CT053-HA
CT082-HA
CT105-HA
CT142-HA
CT143-HA
CT144-HA
CT153-HA
CT161-HA
CT172-HA
CT203-HA
CT273-HA
CT289-HA
CT309-HA
CT338-HA
CT386-HA
CT425-HA
CT429-HA
CT504-HA
CT583-HA
CT631-HA
CT656-HA
CT696-HA
CT779-HA
CT849-HA
kDa
75
48
25
17
anti-HA
anti-SycO
S
P
B
Y. enterocolitica ΔHOPEMT Δ YscU
CT694-HA
CT053-HA
CT082-HA
CT105-HA
CT142-HA
CT143-HA
CT144-HA
CT161-HA
CT338-HA
CT429-HA
CT504-HA
CT583-HA
CT656-HA
CT849-HA
C
CT849-HA
CT779-HA
CT656-HA
CT583-HA
CT504-HA
CT429-HA
CT425-HA
CT386-HA
CT338-HA
CT309-HA
CT289-HA
CT273-HA
CT203-HA
CT172-HA
CT161-HA
CT153-HA
CT144-HA
CT143-HA
CT142-HA
CT105-HA
CT082-HA
CT053-HA
CT694-HA
RplJ-HA
0
10
20
30
40
50
% of secretion

Figure 2.2. Analysis of the T3S of *C. trachomatis* full-length proteins by *Y. enterocolitica*. *Y. enterocolitica* T3S-proficient (ΔHOPEMT) (A) and T3S-defective (ΔHOPEMT ΔYscU) (B) were used to analyze secretion of full-length *C. trachomatis* proteins with a C-terminal HA epitope tag. Immunoblots show the result of T3S assays in which proteins in culture supernatants (S, secreted proteins) and in bacterial pellets (P, non-secreted proteins) from ~$5x10^8$ and ~$5x10^7$ bacteria, respectively, were loaded per lane. The known *C. trachomatis* T3S substrates CT082 (Hovis *et al.*, 2013; Pais *et al.*, 2013) and CT694 (Hower *et al.*, 2009) were used as positive controls, and the *C. trachomatis* ribosomal protein RplJ was used as a negative control. SycO is a strictly cytosolic *Yersinia* T3S chaperone (Iriarte & Cornelis, 1998; Letzelter *et al.*, 2006) and its immunodetection ensured that the presence of HA-tagged proteins in the culture supernatants was not a result of bacterial lysis or contamination. (C) The percentage (%) of secretion of each protein by *Y. enterocolitica* ΔHOPEMT was calculated by densitometry, as the ratio between the amount of secreted and total protein. The threshold to decide whether a protein was secreted was set to 2% (dashed line), based on the % of secretion of RplJ-HA. Data are the mean ± SEM from at least 3 independent experiments (see also Table A5, annexes).

2.4.4. CT053, CT105, CT142, CT143, CT161, CT338, and CT429 can be translocated into host cells by *Y. enterocolitica*

We next analyzed if the newly identified likely T3S substrates of *C. trachomatis* had the capacity of being translocated into host cells, by using *Y. enterocolitica* as a heterologous system. For this, *Y. enterocolitica* ΔHOPEMT harboring plasmids encoding C-terminal HA-tagged newly identified likely T3S substrates of *C. trachomatis* (CT053-HA, CT105-HA, CT142-HA, CT143-HA, CT144-HA, CT161-HA, CT338-HA, CT429-HA, CT656-HA, or CT849-HA), a positive control (CT694-HA) or a negative control (RplJ-HA), were used to infect human epithelial HeLa cells. We then used Triton X-100 fractionation of the infected cells followed by immunoblotting analysis of Triton-soluble and insoluble HeLa cell lysates to monitor protein translocation into host cells. As expected, we found CT694-HA in the Triton-soluble fraction, which showed that this protein was delivered into the cytoplasm of HeLa cells, and only detected RplJ-HA in the Triton-insoluble fraction (Fig. 2.3), which confirmed that this protein

remained within the bacteria (and that the fractionation procedure did not lyse the bacteria). Among the 10 likely T3S substrates of *C. trachomatis* under analysis, we could not detect CT656-HA or CT849-HA in both the Triton-soluble and Triton-insoluble fractions. It is possible that in the experimental conditions used in this study CT656-HA or CT849-HA are translocated in minute and undetectable amounts and/or that they are degraded either after translocation or within the bacteria. Regardless of the exact scenario, these results did not enable us to conclude about the capacity of CT656-HA and CT849-HA of being translocated into host cells. However, we could consistently detect CT053-HA, CT105-HA, CT142-HA, CT143-HA, CT161-HA, CT338-HA and CT429-HA in the Triton-soluble fraction (Fig. 2.3), indicating that these proteins were injected into the cytoplasm of HeLa cells by *Y. enterocolitica*. We could also occasionally detect small amounts of CT144-HA in the Triton-soluble fraction (barely visible in Fig. 2.3).

In summary, these experiments showed that CT053-HA, CT105-HA, CT142-HA, CT143-HA, CT161-HA, CT338-HA and CT429-HA have the capacity of being translocated into infected host cells further suggesting that the endogenous *C. trachomatis* proteins could be effectors. The results do not preclude that CT144, CT656 or CT849 could be effectors, but the evidence is not as strong as for the other 7 proteins.

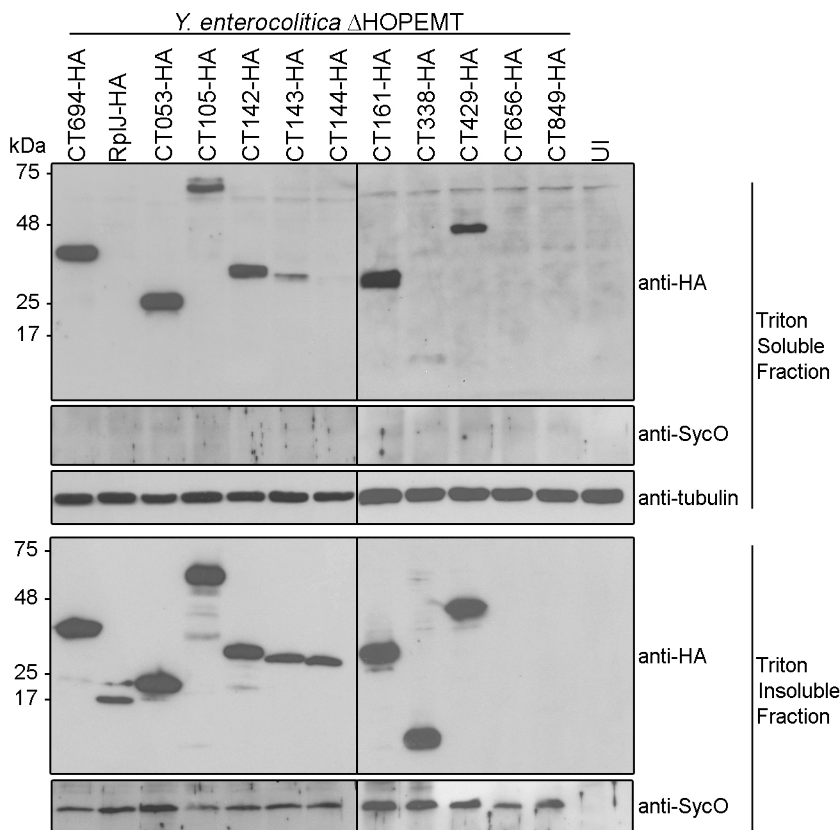

Figure 2.3. Translocation of *C. trachomatis* proteins into the cytoplasm of HeLa cells by *Y. enterocolitica*. HeLa cells were left uninfected (UI) or infected with *Y. enterocolitica* ΔHOPEMT strains expressing the indicated HA-tagged proteins. After 3 h of infection, extracellular bacteria were killed by the addition of gentamicin and the infected cells were incubated for additional 2 h. The infected cells were then fractionated into Triton-soluble and Triton-insoluble cell lysates that were subsequently analyzed by immunoblotting using anti-HA, anti-SycO and anti-tubulin antibodies, as indicated. Presence of HA-tagged proteins in the Triton-soluble cell lysates is indicative of translocation into the cytoplasm of HeLa cells. SycO is a strictly cytosolic *Yersinia* T3S chaperone (Iriarte & Cornelis, 1998; Letzelter *et al.*, 2006) and its immunodetection ensured that the presence of HA-tagged proteins in the Triton-soluble cell lysates was not a result of bacterial lysis during the fractionation. Additionally, the incapacity to detect HA-tagged RplJ (a *C. trachomatis* ribosomal protein) in the Triton-soluble cell lysates further indicated that this fraction did not contain bacteria or non-translocated bacterial proteins. Tubulin served as a loading control of the Triton-soluble cell lysates. The images shown are representative of three independent experiments.

2.4.5. Expression of genes encoding newly identified likely T3S substrates during development of *C. trachomatis*

To test if the newly identified likely T3S substrates, and possible effectors, of *C. trachomatis* (CT053, CT105, CT142, CT143, CT144, CT161, CT338, CT429, CT656, and CT849) were expressed during infection, and to gain insights of when they could be acting during the developmental cycle, we analyzed by RT-qPCR the mRNA levels of their encoding genes during the developmental cycle of strain L2/434, at 2, 6, 12, 20, 30 and 42 h post-infection. While *ct053*, *ct105*, *ct142*, *ct143*, *ct144*, *ct338*, *ct429*, *ct656*, and *ct849* displayed significant mRNA levels in more than one of the time-points analyzed, *ct161* showed only vestigial levels of expression throughout the cycle (Fig. 2.4). The mRNA levels of *ct105* and *ct338* were > 5-fold higher at 2-6 h post-infection than in any other of the time-points analyzed (Fig. 2.4), suggesting that the encoded proteins should function at early-cycle. The mRNA levels of *ct053* and *ct429* were higher between 6 and 20 h post-infection (Fig. 2.4), suggesting that the encoded proteins might act from early to mid cycle. The mRNA levels of *ct142*, *ct143*, *ct144* and *ct849* were higher at the later time points analyzed (30-42 h post-infection). However, while *ct142*, *ct143*, and *ct144* were expressed at similar levels at 30 and 42 h post-infection, *ct849* showed a distinct peak of expression at 30 h post-infection (Fig. 2.4). Therefore, CT142, CT143, CT144 could function either at late or early cycle, and CT849 might probably acts at late cycle. Finally, the mRNA levels of *ct656* were constant at all time-points analyzed (Fig. 2.4), suggesting that CT656 could function throughout the cycle. Regarding *ct161*, when comparing the higher mRNA levels detected for each of the genes analyzed, those of *ct161* were > 6-fold lower than those of any of the other genes tested (Fig. 2.4). Therefore, in the experimental conditions used, CT161 may not be expressed by

strain L2/434. In summary, the RT-qPCR experiments supported that CT053, CT105, CT142, CT143, CT338, and CT429, and also CT144, CT656, or CT849, could be *C. trachomatis* T3S effectors, possibly acting at different times of the developmental cycle.

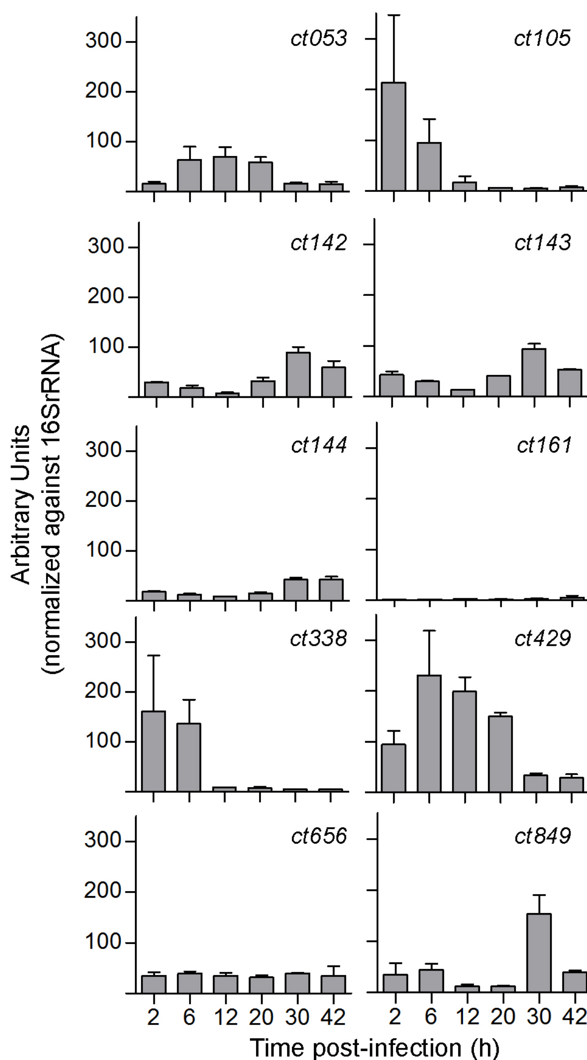

Figure 2.4. mRNA levels of newly identified putative effectors during the developmental cycle of *C. trachomatis*. The mRNA levels of *ct053*, *ct105*, *ct142*, *ct143*, *ct144*, *ct161*, *ct338*, *ct429*, *ct656*, and *ct849* were analyzed by RT-qPCR during the developmental cycle of *C. trachomatis* strain L2/434, at the indicated time-points. The expression values (mean ± SEM) resulted from raw RT-qPCR data (10^5) of each gene normalized to that of the 16s rRNA gene and are from three independent experiments.

2.5. DISCUSSION

Earlier studies using heterologous systems have led to the identification of several *bona-fide* or putative *C. trachomatis* T3S effectors (Chellas-Géry *et al.*, 2007; Fields & Hackstadt, 2000; Hovis *et al.*, 2013; Hower *et al.*, 2009; Muschiol *et al.*, 2011; Pais *et al.*, 2013; Pennini *et al.*, 2010; Song *et al.*, 2013; Subtil *et al.*, 2005). While these and other analyses covered a significant portion of all *C. trachomatis* proteins, we hypothesized that there could be previously unidentified T3S substrates. By combining basic bioinformatics searches, exhaustive T3S assays, translocation assays, and analyses of chlamydial gene expression in infected cells, we revealed 10 *C. trachomatis* proteins (CT053, CT105, CT142, CT143, CT144, CT161, CT338, CT429, CT656, and CT849) as likely T3S substrates and possible effectors. In particular, CT053, CT105, CT142, CT143, CT338, and CT429 were type III secreted by *Y. enterocolitica*, could be translocated into host cells, and their encoding genes were clearly expressed in *C. trachomatis* strain L2/434. Therefore, these 6 proteins have a high likelihood of being effectors. However, additional future studies are required to show that all of these 10 proteins are indeed translocated by *C. trachomatis* into host cells and to show that they are bona-fide effectors, i.e., that they interfere with host cell processes.

Among the likely T3S effectors of *C. trachomatis* that we identified, CT105 and CT142 have been previously singled out as possible modulators of host cell functions, based on the phenotypic consequences of their ectopic expression in yeast *S. cerevisiae* (Sisko *et al.*, 2006). In addition, the genes encoding CT142, CT143, and CT144 have been shown to be markedly transcriptionally

regulated by a protein (Pgp4) encoded by the *Chlamydia* virulence plasmid (Song *et al.*, 2013) . This plasmid is present in almost all *C. trachomatis* clinical isolates (Rockey, 2011), and studies in animal models of infection showed that it is a virulence factor *in vivo* (Kari *et al.*, 2011; Olivares-Zavaleta *et al.*, 2010). Additional studies are needed to understand if the putative effector function of CT142, CT143, and CT144 can partially explain the virulence role of the chlamydial plasmid. Furthermore, the predicted amino acid sequence of CT849 reveals a domain of unknown function (DUF720) that can only be found in *Chlamydia* proteins. In *C. trachomatis*, besides CT849, a DUF720 domain is found in CT847, a T3S effector that interacts with human Grap2 cyclin D-interacting protein (GCIP) (Chellas-Géry *et al.*, 2007), and in CT848, which has been indicated as a T3S substrate using *S. flexneri* as a heterologous system (Subtil *et al.*, 2005). Therefore, this further supports a possible role of CT849 as an effector. In contrast with CT105, CT142, CT143, CT144 or CT849, no significant information is available or could be retrieved about CT053, CT338, CT429, or CT656.

CT161 is a possible T3S substrate and effector, but we could not detect significant levels of *ct161* mRNA during the developmental cycle of strain L2/434. The *ct161* gene is localized within the “plasticity zone”, a chromosomal region of rare high genetic diversity among *C. trachomatis* strains. In fact, although *C. trachomatis* includes strains showing remarkably different tropisms (strains that can spread into lymph nodes and cause *lymphogranuloma venereum* [LGV], such as L2/434, and strains causing infections usually restricted to the mucosa of the conjunctiva and genitals), their genomes are all highly similar (Harris *et al.*, 2012). Preliminary data indicate that, contrarily to what is seen in LGV strains, the *ct161*

seems to be more expressed in some ocular and urogenital isolates (data not shown). We are currently investigating the possibility that *ct161* is a pseudogene in LGV strains, perhaps inactivated by a mutation in its promoter region. Interestingly, CT161 has been shown by yeast two-hybrid to bind CT274 (a possible chlamydial T3S chaperone) (Spaeth *et al.*, 2009). Another feature of this protein is that part of its amino acid sequence (residues 40-224, out of 246) shows 28% of identity to a region of Lda2/CT163 (residues 167-361, out of 548), a known *C. trachomatis* translocated protein (Kumar *et al.*, 2006).

Among the proteins for which we found a secretion signal but could not demonstrate their T3S as full-length proteins, we highlight CT153 and GrgA/CT504. Regarding CT153, this protein possesses a membrane attack complex/perforin (MACPF) domain (Ponting, 1999), and there is previous evidence that it may be translocated by *C. trachomatis* (Taylor *et al.*, 2010) , which is consistent with our data. The *ct504* gene has been recently shown to encode a transcriptional activator, GrgA (Bao *et al.*, 2012). Therefore, T3S of $CT504_{20}$-TEM-1 could be a false positive. However, if GrgA is a T3S substrate, as our data suggests, it could have a function within the host cell, and similarly to what has been described in the T3SSs of *Yersinia* (Pettersson *et al.*, 1996) or *Shigella* (Botteaux *et al.*, 2009; Parsot *et al.*, 2005), it could be discarded by secretion once its intra-bacterial regulatory activity needs to be shut down.

We found T3S signals in 56% proteins analyzed (26 out of 46, including controls). This high percentage of proteins showing a T3S signal suggests that some should be false positives. It is conceivable that within a single bacterium non-secreted proteins possess T3S

signals but are not targeted to the T3SS machinery because they also carry signals (e.g. DNA-, membrane-, or protein-binding) that preferentially direct them to other location within the bacterial cell. To help differentiating between true or false positives among chlamydial proteins carrying a T3S signal we analyzed their secretion as full-length proteins. This is because, as explained above in the Results section, not all proteins have folding characteristics compatible with T3S (Akeda & Galán, 2005; Feldman *et al.*, 2002; Lee & Schneewind, 2002; Sorg *et al.*, 2005). However, we cannot exclude that some of the *C. trachomatis* full-length proteins that were not type III secreted by *Yersinia* have a T3S chaperone that maintains them in a secretion-competent state (Stebbins & Galán, 2001) and enables their secretion during infection by *C. trachomatis*. Intriguingly, CT082 or CT694 have dedicated T3S chaperones, CT584 and Slc1, respectively (Pais *et al.*, 2013), and, in agreement with what we previously observed (Pais *et al.*, 2013), they were both secreted as full-length proteins in the absence of the chaperones. Considering that T3S chaperones have various functions (Feldman & Cornelis, 2003; Parsot *et al.*, 2003), the chaperone role of CT584 or Slc1 should be different from maintaining their substrates in a secretion-competent state.

Eleven of the *Chlamydia* proteins that we analyzed have been previously studied for T3S using *S. flexneri* has a heterologous system (Subtil *et al.*, 2005). In the majority of the cases the outcome of the experiments was identical; however, differently from what was shown in *Shigella*, we detected a T3S signal in the N-terminal of CT429 (which was also secreted as a full-length protein), GrgA/CT504, and CT779 and we did not detect a T3S signal in CT577. Evidence for a T3S signal in only one of the heterologous systems may suggest a false positive. There is a myriad of possible

explanations for these discrepancies, when considering that different heterologous systems (*Shigella* and *Yersinia*) and reporter proteins (Cya and TEM-1) were used, and that the N-terminal regions in the hybrid proteins consisted in different lengths of amino acids and were in some cases from different *Chlamydia* species.

We compared the data from our T3S assays (including the controls, CT082, CT694, and RplJ) with predictions of T3S substrates by *in silico* methods (Effective T3S (Arnold *et al.*, 2009), SIEVE (Samudrala *et al.*, 2009), Modlab (Löwer & Schneider, 2009), and T3_MM (Wang *et al.*, 2013)) using resources available in the Web (Effective T3S, Modlab and T3_MM, and Table 3 from Samudrala *et al.*, 2009) (SIEVE), as detailed in Table A6 (Annexes). When considering the analysis of T3S signals in TEM-1 hybrids, the vast majority of proteins (60%; 12 out of 20) in which we did not find a T3S signal were also predicted not to be secreted by each of the *in silico* methods. In contrast, the vast majority of proteins (58%; 15 out of 26) in which we detected a T3S signal were also predicted to be secreted by at least one of the *in silico* methods. The correlation between our experimental data and the *in silico* predictions was more striking when considering the T3S of full-length proteins. Among the 16 full-length proteins for which we could not find definitive evidence of T3S, 10 (i.e., 62.5%) were also predicted not to be secreted by each of the *in silico* methods, but among the 11 proteins that we showed or confirmed to be T3S substrates, 10 (i.e., 83%) were also predicted to be secreted by at least one of the *in silico* methods. Overall, this indicates some correlation between our experimental data and the *in silico* methods that predict T3S substrates. However, for many proteins, each of these *in silico* methods generates different predictions Table A6 (Annexes). It is possible that the quantitative

data on T3S such as the one we generated in this and in a previous study (Almeida *et al.*, 2012), can be used to normalize and improve the predictive value of such methods.

In conclusion, we found 10 *C. trachomatis* proteins (CT053, CT105, CT142, CT143, CT144, CT161, CT338, CT429, CT656, and CT849) with a high likelihood of being T3S substrates, and therefore possible effectors delivered by the bacteria into host cells. For 6 of these proteins (CT053, CT105, CT142, CT143, CT338, and CT429), the hypothesis that they could be effectors was supported by their capacity of being translocated into host cells and by the expression of their encoding genes by *C. trachomatis*. The identification of all *C. trachomatis* effectors is a crucial step towards a comprehensive understanding of the mechanisms by which this pathogen subverts host cells. The recently developed methods for genetic manipulation of *Chlamydia* indicate that it should be possible to ectopically express candidate effectors in *C. trachomatis* (Agaisse & Derré, 2013; Wang *et al.*, 2011), which would facilitate the analysis of their translocation into host cells. Our work highlights *C. trachomatis* proteins that should be prioritized in such studies, thus aiding the future identification of chlamydial effectors. Furthermore, the quantitative analysis of T3S of TEM-1 hybrid proteins that we carried out could help to further develop the *in silico* methods for identification of T3S substrates (Arnold *et al.*, 2009; Löwer & Schneider, 2009; Samudrala *et al.*, 2009; Wang *et al.*, 2013).

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REFERENCES

AbdelRahman, Y. M. & Belland, R. J. (2005). The chlamydial developmental cycle. *FEMS Microbiol Rev* **29**(5), 949–959.

Agaisse, H. & Derré, I. (2013). A *C. trachomatis* Cloning Vector and the Generation of *C. trachomatis* Strains Expressing Fluorescent Proteins under the Control of a *C. trachomatis* Promoter. *PLoS One* **8**(2), e57090.

Akeda, Y. & Galán, J. E. (2005). Chaperone release and unfolding of substrates in type III secretion. *Nature* **437**(6), 911–915.

Almeida, F., Borges, V., Ferreira, R., Borrego, M. J., Gomes, J. P. & Mota, L. J. (2012). Polymorphisms in Inc proteins and differential expression of *inc* genes among *Chlamydia trachomatis* strains correlate with invasiveness and tropism of Lymphogranuloma venereum isolates. *J Bacteriol* **194**(23), 6574–6585.

Arnold, R., Brandmaier, S., Kleine, F., Tischler, P., Heinz, E., Behrens, S., Niinikoski, A., Mewes, H. W., Horn, M. & Rattei, T. (2009). Sequence-Based Prediction of Type III Secreted Proteins. *PLoS Pathog* **5**(4), e1000376.

Bao, X., Nickels, B. E. & Fan, H. (2012). *Chlamydia trachomatis* protein GrgA activates transcription by contacting the nonconserved region of 66. *Proc Natl Acad Sci* **109**(42), 16870–16875.

Betts, H. J., Wolf, K. & Fields, K. A. (2009). Effector protein modulation of host cells: examples in the *Chlamydia* spp. arsenal. *Curr Opin Microbiol* **12**, 81–87.

Borges, V., Ferreira, R., Nunes, A., Nogueira, P., Borrego, M. J. & Gomes, J. P. (2010). Normalization strategies for real-time expression data in *Chlamydia trachomatis*. *J Microbiol Methods* **82**, 256–264.

Botteaux, A., Sory, M. P., Biskri, L., Parsot, C. & Allaoui, A. (2009). MxiC is secreted by and controls the substrate specificity of the *Shigella flexneri* type III secretion apparatus. *Mol Microbiol* **71**(2), 449–460.

Charpentier, X. & Oswald, E. (2004). Identification of the secretion and translocation domain of the enteropathogenic and enterohemorrhagic *Escherichia coli* effector Cif, using TEM-1 β-lactamase as a new fluorescence-based reporter. *J Bacteriol* **186**(16), 5486–5495.

Chellas-Géry, B., Linton, C. N. & Fields, K. A. (2007). Human GCIP interacts with CT847, a novel *Chlamydia trachomatis* type III secretion substrate, and is degraded in a tissue-culture infection model. *Cell Microbiol* **9**(10), 2417–2430.

Clifton, D. R., Fields, K. A., Grieshaber, S. S., Dooley, C. A., Fischer, E. R., Mead, D. J., Carabeo, R. A. & Hackstadt, T. (2004). A chlamydial type III translocated protein is tyrosine-phosphorylated at the site of entry and associated with recruitment of actin. *Proc Natl Acad Sci U S A* **101**(27), 10166–10171.

Cornelis, G. R. (2006). The type III secretion injectisome. *Nat Rev Microbiol* **4**, 811–825.

da Cunha, M., Milho, C., Almeida, F., Pais, S. V, Borges, V., Maurício, R., Borrego, M. J., Gomes, J. P. & Mota, L. J. (2014). Identification of type III secretion substrates of *Chlamydia trachomatis* using *Yersinia enterocolitica* as a heterologous system. *BMC Microbiol* **14**(40), 1-14.

Delevoye, C., Nilges, M., Dehoux, P., Paumet, F., Perrinet, S., Dautry-Varsat, A. & Subtil, A. (2008). SNARE protein mimicry by an intracellular bacterium. *PLoS Pathog* **4**(3), e1000022.

Denecker, G., Tötemeyer, S., Mota, L. J., Troisfontaines, P., Lambermont, I., Youta, C., Stainier, I., Ackermann, M., Cornelis, G. R. & Totemeyer, S. (2002). Effect of low- and high-virulence *Yersinia enterocolitica* strains on the inflammatory response of human umbilical vein endothelial cells. *Infect Immun* **70**(7), 3510–3520.

Derré, I., Swiss, R. & Agaisse, H. (2011). The lipid transfer protein CERT interacts with the *Chlamydia* inclusion protein IncD and participates to ER-*Chlamydia* inclusion membrane contact sites. *PLoS Pathog* **7**(6), e1002092.

Feldman, M. F. & Cornelis, G. R. (2003). The multitalented type III chaperones: All you can do with 15 kDa. *FEMS Microbiol Lett* **219**, 151–158.

Feldman, M. F., Müller, S., Wüest, E. & Cornelis, G. R. (2002). SycE allows secretion of YopE-DHFR hybrids by the *Yersinia enterocolitica* type III Ysc system. *Mol Microbiol* **46**(4), 1183–1197.

Fields, K. A. & Hackstadt, T. (2000). Evidence for the secretion of *Chlamydia trachomatis* CopN by a type III secretion mechanism. *Mol Microbiol* **38**(5), 1048–1060.

Fling, S. P., Sutherland, R. A., Steele, L. N., Hess, B., D'Orazio, S. E., Maisonneuve, J., Lampe, M. F., Probst, P. & Starnbach, M. N. (2001). CD8+ T cells recognize an inclusion membrane-associated protein from the vacuolar pathogen *Chlamydia trachomatis*. *Proc Natl Acad Sci U S A* **98**(3), 1160–1165.

Furtado, A. R., Essid, M., Perrinet, S., Balañá, M. E., Yoder, N., Dehoux, P. & Subtil, A. (2013). The chlamydial OTU domain-containing protein ChlaOTU is an early type III secretion effector targeting ubiquitin and NDP52. *Cell Microbiol* **15**(12), 2064–2079.

Gérard, H. C., Mishra, M. K., Mao, G., Wang, S., Hali, M., Whittum-Hudson, J. A., Kannan, R. M. & Hudson, A. P. (2013). Dendrimer-enabled DNA delivery and transformation of *Chlamydia pneumoniae*. *Nanomedicine Nanotechnology, Biol Med* **9**, 996–1008.

Gong, S., Lei, L., Chang, X., Belland, R. & Zhong, G. (2011). *Chlamydia trachomatis* secretion of hypothetical protein CT622 into host cell cytoplasm via a secretion pathway that can be inhibited by the type III secretion system inhibitor compound 1. *Microbiology* **157**, 1134–1144.

Grosdent, N., Maridonneau-Parini, I., Sory, M. P. & Cornelis, G. R. (2002). Role of Yops and adhesins in resistance of *Yersinia enterocolitica* to phagocytosis. *Infect Immun* **70**(8), 4165–4176.

Harris, S. R., Clarke, I., Seth-Smith, H. M. B., Solomon, A. W., Cutcliffe, L. T., Marsh, P., Skilton, R. J., Holland, M. J., Mabey, D. & other authors. **(2012).** Whole-genome analysis of diverse *Chlamydia trachomatis* strains identifies phylogenetic relationships masked by current clinical typing **44**(4), 1–16.

Ho, T. D. & Starnbach, M. N. (2005). The *Salmonella enterica* Serovar Typhimurium-Encoded Type III Secretion Systems can translocate *Chlamydia trachomatis* proteins into the cytosol of host cells. *Infect Immun* **73(2)**, 905–911.

Hobolt-Pedersen, A. S., Christiansen, G., Timmerman, E., Gevaert, K. & Birkelund, S. (2009). Identification of *Chlamydia trachomatis* CT621, a protein delivered through the type III secretion system to the host cell cytoplasm and nucleus. *FEMS Immunol Med Microbiol* **57**, 46–58.

Hovis, K. M., Mojica, S., Mcdermott, J. E., Pedersen, L., Simhi, C., Rank, R. G., Myers, G. S. A., Ravel, J., Hsia, R. C. & Bavoil, P. M. (2013). Genus-optimized strategy for the identification of chlamydial type III secretion substrates. *Pathog Dis* **69**, 213–222.

Hower, S., Wolf, K. & Fields, K. A. (2009). Evidence that CT694 is a novel *Chlamydia trachomatis* T3S substrate capable of functioning during invasion or early cycle development. *Mol Microbiol* **72**(6), 1423–1437.

Iriarte, M. & Cornelis, G. R. (1998). YopT, a new *Yersinia* Yop effector protein, affects the cytoskeleton of host cells. *Mol Microbiol* **29**(3), 915–929.

Jewett, T. J., Miller, N. J., Dooley, C. A. & Hackstadt, T. (2010). The conserved tarp actin binding domain is important for chlamydial invasion. *PLoS Pathog* **6**(7), e1000997.

Kari, L., Whitmire, W. M., Olivares-Zavaleta, N., Goheen, M. M., Taylor, L. D., Carlson, J. H., Sturdevant, G. L., Lu, C., Bakios, L. E. & other authors. (2011). A live-attenuated chlamydial vaccine protects against trachoma in nonhuman primates. *J Exp Med* **208**(11), 2217–2223.

Kumar, Y., Cocchiaro, J. & Valdivia, R. H. (2006). The Obligate Intracellular Pathogen *Chlamydia trachomatis* Targets Host Lipid Droplets. *Curr Biol* **16**, 1646–1651.

Lane, B. J., Mutchler, C., Al Khodor, S., Grieshaber, S. S. & Carabeo, R. A. (2008). Chlamydial entry involves TarP binding of guanine nucleotide exchange factors. *PLoS Pathog* **4**(3), e1000014.

Lee, V. T. & Schneewind, O. (2002). Yop Fusions to Tightly Folded Protein Domains and Their Effects on *Yersinia enterocolitica* Type III Secretion Yop Fusions to Tightly Folded Protein Domains and Their Effects on *Yersinia enterocolitica* Type III Secretion **184**(13), 3740–3745.

Lei, L., Qi, M., Budrys, N., Schenken, R. & Zhong, G. (2011). Localization of *Chlamydia trachomatis* hypothetical protein CT311 in host cell cytoplasm. *Microb Pathog* **51**(3), 101–109.

Lei, L., Dong, X., Li, Z. & Zhong, G. (2013). Identification of a Novel Nuclear Localization Signal Sequence in *Chlamydia trachomatis*-Secreted Hypothetical Protein CT311. *PLoS One* **8**(5), e64529.

Letzelter, M., Sorg, I., Mota, L. J., Meyer, S., Stalder, J., Feldman, M., Kuhn, M., Callebaut, I. & Cornelis, G. R. (2006). The discovery of SycO highlights a new function for type III secretion effector chaperones. *EMBO J* **25**(13), 3223–3233.

Li, Z., Chen, C., Chen, D., Wu, Y., Zhong, Y. & Zhong, G. (2008a). Characterization of fifty putative inclusion membrane proteins encoded in the *Chlamydia trachomatis* genome. *Infect Immun* **76**(6), 2746–2757.

Li, Z., Chen, D., Zhong, Y., Wang, S. & Zhong, G. (2008b). The chlamydial plasmid-encoded protein Pgp3 is secreted into the cytosol of *Chlamydia*-infected cells. *Infect Immun* **76**(8), 3415–3428.

Löwer, M. & Schneider, G. (2009). Prediction of type III secretion signals in genomes of gram-negative bacteria. *PLoS One* **4**(6), e5917.

Lu, C., Lei, L., Peng, B., Tang, L., Ding, H., Gong, S., Li, Z., Wu, Y. & Zhong, G. (2013). *Chlamydia trachomatis* GlgA Is Secreted into Host Cell Cytoplasm. *PLoS One* **8**(7), e68764.

Lutter, E., Barger, A., Nair, V. & Hackstadt, T. (2013). *Chlamydia trachomatis* Inclusion Membrane Protein CT228 Recruits Elements of the Myosin Phosphatase Pathway to Regulate Release Mechanisms. *Cell Rep* **3**, 1921–1931.

Marenne, M. N., Journet, L., Mota, L. J. & Cornelis, G. R. (2003). Genetic analysis of the formation of the Ysc-Yop translocation pore in macrophages by *Yersinia enterocolitica*: Role of LcrV, YscF and YopN. *Microb Pathog* **35**, 243–258.

Misaghi, S., Balsara, Z. R., Catic, A., Spooner, E., Ploegh, H. L. & Starnbach, M. N. (2006). *Chlamydia trachomatis*-derived deubiquitinating enzymes in mammalian cells during infection. *Mol Microbiol* **61**(1), 142–150.

Mital, J., Miller, N. J., Fischer, E. R. & Hackstadt, T. (2010). Specific chlamydial inclusion membrane proteins associate with active Src family kinases in microdomains that interact with the host microtubule network. *Cell Microbiol* **12**(9), 1235–1249.

Muschiol, S., Boncompain, G., Vromman, F., Dehoux, P., Normark, S., Henriques-Normark, B. & Subtil, A. (2011). Identification of a family of effectors secreted by the type III secretion system that are conserved in pathogenic *Chlamydiae*. *Infect Immun* **79**(2), 571–580.

Le Negrate, G., Krieg, A., Faustin, B., Loeffler, M., Godzik, A., Krajewski, S. & Reed, J. C. (2008). ChlaDub1 of *Chlamydia trachomatis* suppresses NF-κβ activation and inhibits IkBα ubiquitination and degradation. *Cell Microbiol* **10**(9), 1879–1892.

Olivares-Zavaleta, N., Whitmire, W., Gardner, D. & Caldwell, H. D. (2010). Immunization with the attenuated plasmidless *Chlamydia trachomatis* L2(25667R) strain provides partial protection in a murine model of female genitourinary tract infection. *Vaccine* **28**, 1454–1462.

Pais, S. V., Milho, C., Almeida, F. & Mota, L. J. (2013). Identification of Novel Type III Secretion Chaperone-Substrate Complexes of *Chlamydia trachomatis*. *PLoS One* **8**(2), e56292.

Parsot, C., Hamiaux, C. & Page, A. L. (2003). The various and varying roles of specific chaperones in type III secretion systems. *Curr Opin Microbiol* **6**, 7–14.

Parsot, C., Ageron, E., Penno, C., Mavris, M., Jamoussi, K., D'Hauteville, H., Sansonetti, P. & Demers, B. (2005). A secreted anti-activator, OspD1, and its chaperone, Spa15, are involved in the control of transcription by the type III secretion apparatus activity in *Shigella flexneri*. *Mol Microbiol* **56**(6), 1627–1635.

Pennini, M. E., Perrinet, S., Dautry-Varsat, A. & Subtil, A. (2010). Histone methylation by NUE, a novel nuclear effector of the intracellular pathogen *Chlamydia trachomatis*. *PLoS Pathog* **6**(7), e1000995.

Pettersson, J., Nordfelth, R., Dubinina, E., Bergman, T., Gustafsson, M., Magnusson, K. E. & Wolf-Watz, H. (1996). Modulation of virulence factor expression by pathogen target cell contact. *Science* **273**(5279), 1231–1233.

Ponting, C. P. (1999). Chlamydial homologues of the MACPF (MAC/perforin) domain. *Curr Biol* **9**(24), 911–913.

Qi, M., Lei, L., Gong, S., Liu, Q., DeLisa, M. P. & Zhong, G. (2011). *Chlamydia trachomatis* secretion of an immunodominant hypothetical protein (CT795) into host cell cytoplasm. *J Bacteriol* **193**(10), 2498–2509.

Rockey, D. D. (2011). Unraveling the basic biology and clinical significance of the chlamydial plasmid. *J Exp Med* **208**(11), 2159–2162.

Rzomp, K. A., Moorhead, A. R. & Scidmore, M. A. (2006). The GTPase Rab4 interacts with *Chlamydia trachomatis* inclusion membrane protein CT229. *Infect Immun* **74**(9), 5362–5373.

Samudrala, R., Heffron, F. & McDermott, J. E. (2009). Accurate prediction of secreted substrates and identification of a conserved putative secretion signal for type iii secretion systems. *PLoS Pathog* **5**(4), e1000375.

Scidmore, M. A. & Hackstadt, T. (2001). Mammalian 14-3-3-β associates with the *Chlamydia trachomatis* inclusion membrane via its interaction with IncG. *Mol Microbiol* **39**(6), 1638–1650.

Scidmore, M. (2005). Cultivation and laboratory maintenance of *Chlamydia trachomatis*. *Curr Protoc Microbiol* 1–25.

Sisko, J. L., Spaeth, K., Kumar, Y. & Valdivia, R. H. (2006). Multifunctional analysis of *Chlamydia*-specific genes in a yeast expression system. *Mol Microbiol* **60**(1), 51–66.

Song, L., Carlson, J. H., Whitmire, W. M., Kari, L., Virtaneva, K., Sturdevant, D. E., Watkins, H., Zhou, B., Sturdevant, G. L. & other authors. **(2013).** *Chlamydia trachomatis* plasmid-encoded Pgp4 is a transcriptional regulator of virulence-associated genes. *Infect Immun* **81**(3), 636–644.

Sorg, I., Wagner, S., Amstutz, M., Müller, S. A., Broz, P., Lussi, Y., Engel, A. & Cornelis, G. R. (2007). YscU recognizes translocators as export substrates of the Yersinia injectisome. *EMBO J* **26**(12), 3015–3024.

Sorg, J. A., Miller, N. C., Marketon, M. M. & Schneewind, O. (2005). Rejection of Impassable Substrates by *Yersinia* Type III secretion machines. *J Bacteriol* **187**(20), 7090–7102.

Spaeth, K. E., Chen, Y. S. & Valdivia, R. H. (2009). The *Chlamydia* type III secretion system C-ring engages a chaperone-effector protein complex. *PLoS Pathog* **5**(9), e1000579.

Stebbins, C. E. & Galán, J. E. (2001). Maintenance of an unfolded polypeptide by a cognate chaperone in bacterial type III secretion. *Nature* **414**, 77–81.

Stephens, R. S., Kalman, S., Lammel, C., Fan, J., Marathe, R., Aravind, L., Mitchell, W., Olinger, L., Tatusov, R. L. & other authors. **(1998).** Genome sequence of an obligate intracellular pathogen of humans: *Chlamydia trachomatis*. *Science* **282**, 754–759.

Subtil, A., Delevoye, C., Balañá, M. E., Tastevin, L., Perrinet, S. & Dautry-Varsat, A. (2005). A directed screen for chlamydial proteins secreted by a type III mechanism identifies a translocated protein and numerous other new candidates. *Mol Microbiol* **56**(6), 1636–1647.

Taylor, L. D., Nelson, D. E., Dorward, D. W., Whitmire, W. M. & Caldwell, H. D. (2010). Biological characterization of *Chlamydia trachomatis* plasticity zone MACPF domain family protein CT153. *Infect Immun* **78**(6), 2691–2699.

Thomson, N. R., Holden, M. T. G., Carder, C., Lennard, N., Lockey, S. J., Marsh, P., Skipp, P., O'Connor, C. D., Goodhead, I. & other authors. **(2008).** *Chlamydia trachomatis*: Genome sequence analysis of lymphogranuloma venereum isolates. *Genome Res* **18**, 161–171.

Valdivia, R. H. (2008). *Chlamydia* effector proteins and new insights into chlamydial cellular microbiology. *Curr Opin Microbiol* **11**, 53–59.

Wang, Y., Sun, M., Bao, H. & White, A. P. (2013). T3_MM: A Markov Model Effectively Classifies Bacterial Type III Secretion Signals. *PLoS One* **8**(3), 1–12.

Wang, Y., Kahane, S., Cutcliffe, L. T., Skilton, R. J., Lambden, P. R. & Clarke, I. N. (2011). Development of a transformation system for *Chlamydia trachomatis*: Restoration of glycogen biosynthesis by acquisition of a plasmid shuttle vector. *PLoS Pathog* **7**(9), e1002258.

Chapter III

Characterization of the *Chlamydia trachomatis ct142-ct143-ct144* operon and analysis of the expression and subcellular localization of CT142 and CT143

This Chapter contains data of a manuscript in preparation:

Maria da Cunha and Luís Jaime Mota. The *Chlamydia trachomatis* type III secretion substrates CT142, CT143, and CT144 could be part of a protein complex in the lumen of the inclusion.

The author of this dissertation participated in all experiments described in this Chapter.

3.1. ABSTRACT

Chlamydia trachomatis virulence is related to a type III secretion system (T3SS) and to a highly conserved virulence plasmid. In Chapter II, we described the identification of new candidate T3SS effector proteins of *C. trachomatis*, including CT142, CT143, and CT144. In this Chapter, transcription linkage analyses indicated that *ct*142, *ct143* and *ct144* are organized in an operon. Furthermore, the transcription start site of *ct142* was determined by 5´rapid amplification of cDNA ends (5´RACE). Real-time quantitative PCR revealed that *ct142*, *ct143*, and *ct144* showed 38-fold, 35-fold and 7-fold higher mRNA levels, respectively, at 30 h p.i. in wild-type *C. trachomatis* than in a strain lacking the virulence plasmid, as expected from the recently described role of this plasmid in promoting expression of these genes. Furthermore, by immunoblotting using antibodies against CT142 and CT143, we detected expression of the proteins at 30 h p.i. in extracts of HeLa cells infected by wild-type *C. trachomatis*, but not in cells infected by plasmidless *C. trachomatis*. Immunofluorescence microscopy using antibodies against CT143 revealed intra-inclusion globular structures that did not co-localize with the bacteria. Overall, these results support that expression of *ct142*, *ct143* and *ct144* is regulated by the *C. trachomatis* virulence plasmid and suggest that CT143 could be secreted into the lumen of the inclusion.

3.2. INTRODUCTION

The characteristic developmental cycle of the obligate intracellular bacterium *Chlamydia trachomatis* involves two morphological distinct forms, the infectious but non-replicative elementary bodies (EBs) and non-infectious but replicative reticulate bodies (RBs). During the cycle, *C. trachomatis* resides within host cells in a membrane-bound vacuole, known as inclusion. The inclusion constitutes a barrier between the host and the pathogen, thus restricting their interactions. One of the ways *Chlamydia* evolved to overcome this obstacle is to encode for a type III secretion system (T3SS), used by many bacterial pathogens to manipulate eukaryotic host cells by injecting them with effector proteins (Cornelis, 2006). It is believed that *C. trachomatis* may possess more that 100 T3SS substrates (Valdivia, 2008) and that these proteins play a key role during the *C. trachomatis* infectious cycle.

As until recently *C. trachomatis* had been genetically intractable, for a long time searching for its T3SS effectors relied on using heterologous bacteria (Chapter II; Fields & Hackstadt, 2000; Clifton *et al.*, 2004; Hower *et al.*, 2009; Subtil *et al.*, 2005). However, other methods are needed to confirm that a given protein is indeed a T3SS effector. Before transformation of *C. trachomatis* was described (Wang *et al.*, 2011), the main approach for these validation studies was to use specific antibodies against candidate effectors to analyze their expression and subcellular localization. For example, *C. trachomatis* CopN (CT089), Tarp (CT456), and CT694, or *Chlamydia caviae* CCA00037 (homologous to *C. trachomatis* CT671) were all first shown to be secreted by the *Yersinia enterocolitica* or *Shigella flexneri* T3SSs (Clifton *et al.*, 2004; Fields & Hackstadt, 2000; Hower

et al., 2009; Subtil *et al.*, 2005). Subsequently, by probing with specific antibodies, it was possible to show by immunofluorescence microscopy that these proteins are transported into host cells by detecting specific labeling at the inclusion membrane (Fields & Hackstadt, 2000), in close association with EBs during the initial invasion of host cells (Clifton *et al.*, 2004; Hower *et al.*, 2009), or in the host cell cytosol (Subtil *et al.*, 2005).

Most *C. trachomatis* isolates maintain a highly conserved plasmid (Lovett *et al.*, 1980; Pickett, 2005). Naturally occurring plasmidless clinical isolates are rare. Although *C. trachomatis* strains with and without the virulence plasmid display similar *in vitro* infectivity characteristics (Carlson *et al.*, 2008), recent studies have shown that plasmid deficient strains are attenuated for virulence in mouse and non-human primate models (Kari *et al.*, 2011; O'Connell *et al.*, 2007; Sigar *et al.*, 2014). Together with the strong selection to maintain the plasmid in *C. trachomatis* strains, these observations are indicative of an important role of the plasmid in *C. trachomatis* pathogenesis.

A recent work revealed that the plasmid encoded Pgp4 protein is a transcriptional regulator of plasmid genes and of multiple chromosomal genes, including *ct142*, *ct143* and *ct144* (Song *et al.*, 2013). In Chapter II, we described the identification of 10 *C. trachomatis* candidate T3SS effectors, including CT142, CT143 and CT144. Taken together, these observations strongly suggest that CT142, CT143 and CT144 could be virulence factors. In this Chapter, we describe a characterization of their encoding genes and of the expression and subcellular localization of CT142 and CT143 in infected cells.

3.3. MATERIALS AND METHODS

Cell culture, bacterial strains and growth conditions

HeLa 229 cells (ATCC) and HeLa HtTA-1 cells (ECACC) were maintained in DMEM (Life Technologies) supplemented with 10% (w/v) FBS (Life Technologies) at 37°C in a humidified atmosphere of 5% [v/v] CO_2. *C. trachomatis* serovar L2 strains 434/Bu (L2/434; from ATCC) and 25667R (L2/2566R; Peterson *et al.*, 1990; kindly provided by Agathe Subtil) were propagated in HeLa 229 cells using standard techniques (Scidmore, 2005). *Escherichia coli* TOP10 (Life Technologies) was used for construction and purification of plasmids. *E. coli* BL21 (DE3) (Novagen) was used for recombinant protein expression. *E. coli* was routinely grown in liquid or solid LB medium with the appropriate antibiotics and supplements.

DNA manipulations, plasmids, and primers

The plasmids used in this Chapter and their main characteristics are detailed in Table A1 (Annexes). The DNA primers used in their construction are described in Table A2 (Annexes). Plasmids were constructed and purified with proof-reading Phusion DNA polymerase (Thermo Fisher Scientific), restriction enzymes (Thermo Fisher Scientific), T4 DNA Ligase (Life Technologies), DreamTaq DNA polymerase (Thermo Fisher Scientific), DNA clean & concentrator™-5 Kit and Zymoclean™ Gel DNA Recovery kit (Zymo Research), and purified with GeneElute Plasmid Miniprep kit (Sigma Aldrich), according to the instructions of the manufacturers. The backbone plasmids used in this work were pGEX-4T-2 (GE Healthcare), pET-28b(+) (Novagen) and pMal-c (New England Biolabs), used for recombinant protein purification, and pEGFP-C1 used for transient transfection in mammalian cells. *C. trachomatis* genes were amplified

by PCR from genomic DNA of strain 434/Bu using custom oligonucleotide primers (see Table A2 in annexes). Purified PCR products and vector were digested with the appropriate restriction enzymes (see Table A2 in annexes) and ligated. The accuracy of the nucleotide sequence of all the inserts in the constructed plasmids was checked by DNA sequencing.

Infection of HeLa 229 cells with *C. trachomatis*

For immunofluorescence analysis, 5 x 10^4 HeLa 229 cells were seeded in 24 well plates (2 cm^2/well) previously filled with 13 mm glass coverslips (VWR). For immunoblotting 1 x 10^5 HeLa 229 cells were seeded in 24 well plates. Scaling-up was done accordingly (6 well plate (10cm^2/well), or tissue culture flasks with a surface area of 25cm^2 (T25), 75cm^2 (T75) or 175cm^2 (T175). The next day, cells were washed with HBSS and incubated 15 min at 37ºC in a humidified atmosphere of 5% [v/v] CO_2, while the *C. trachomatis* inocula (previously titrated infectious particles, as described in Scidmore, 2005) were prepared in SPG (0.2 mM sucrose, 17 mM Na_2HPO_4, 3 mM NaH_2PO_4, 5 mM L-glutamic acid) in the appropriate volume: 0.2 ml for 24 well plates, 0.5 ml for 6 well plates, 1 ml for T25, 3 ml for T75, or 6 ml for T175. HBSS was then removed and the *C. trachomatis* inocula were added and incubated for 1 h at 37ºC in a humidified atmosphere of 5% [v/v] CO_2. At this point, the inocula were removed and RPMI 1640 medium supplemented with 10% (w/v) FBS, 10 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamate, and 10 µg/ml of gentamicin was added. This step was considered time zero of the infection.

Transcription linkage analysis

HeLa cells were infected with *C. trachomatis* strain L2/434 for 40 h. EBs were purified as described in Scidmore (2005) and RNA was isolated from the EBs using an NZY total RNA kit (NZYTech). cDNA was then obtained by RT-PCR by using random hexamers and iSCRIPT (Bio-Rad). As controls for the PCR reactions we also used as template the product of a typical reverse transcription reaction but without iSCRIPT ($cDNA^-$) and *C. trachomatis* genomic DNA (gDNA) isolated from HeLa cells that were infected with *C. trachomatis* strain L2 434/Bu using a an NZY tissue gDNA kit (NZYTech). Specific primers-pair combinations (see Table A2 in Annexes) were designed in order to determine possible transcriptional linkages between *ct141*, *ct142*, *ct143*, *ct144* and *ct145*.

5´rapid amplification of cDNA ends (RACE)

The identification of the transcription start site (TSS) of *ct142* in *C. trachomatis* L2/434 was done by RACE, using a 5´/3´RACE kit (second generation; Roche). We used RNA isolated as described above from HeLa cells infected with *C. trachomatis* L2/434 for 40 h. The primers complementary to *ct142* that were used are listed in Table A2 (Annexes). Final PCR amplification of double stranded cDNA was done with Phusion DNA polymerase (Thermo Fisher Scientific). PCR products were purified after agarose gel electrophoresis, using a High Pure PCR purification kit (Roche), and then subject to DNA sequencing. All these manipulations were done according to instructions from the manufacturers.

Real-time quantitative PCR (RT-qPCR)

The expression of *ct142*, *ct143* and *ct144* was compared in *C. trachomatis* strains L2/434 and plasmidless L2/25667R by

determining mRNA levels at different times post-infection by RT-qPCR. These experiments were done as previously described (Borges *et al.*, 2010) and primers were designed using Primer Express (Applied Biosystems) (Borges *et al.*, 2010). The RT-qPCR assays were done using the Bio-RAD CFX Manager, SsoFast^{TM} Evagreen^{R} supermix (Bio-Rad), and optical plates. At each time point, raw RT-qPCR data for each gene were normalized against the data obtained for the 16S rRNA transcript, as it was previously demonstrated that this is an adequate endogenous control (Borges *et al.*, 2010). The final results were based on three independent experiments.

Recombinant protein expression and purification

E. coli BL21(DE3) carrying pGEX-4T-2 derived plasmids (encoding CT142, CT143, and CT144 with GST fused at their N-termini; GST-CT142, GST-CT143, and GST-CT144), pMal-c derived plasmids (encoding CT142, CT143, and CT144 with MBP fused at their N-termini; MBP-CT142, MBP-CT143, and MBP-CT144), and pET28b(+) derived plasmids (encoding CT142 and CT144 with a hexahistidine tag at their N-termini; 6xHis-CT142 and 6xHis-CT144) were used for recombinant protein expression by the auto-induction method (Studier, 2005). Bacterial cultures were grown for 4 h at 37ºC, with a shaking of 150 rpm, and then were shifted to 25ºC for an additional 24h, with a shaking of 150 rpm. The cells were harvested by centrifugation at 10500 *g* for 15 min at 4ºC.

Among the GST fusion proteins, in preliminary experiments neither GST-CT142 nor GST-CT144 were obtained in the soluble fraction, so only GST-CT143 was purified. The pellet of cells expressing GST-CT143 was resuspended in PBS containing 1% [v/v] Triton X-100, 10

mM DTT, lyzozyme (10 mg/ml) and benzonase® (Novagen). The cells were then lysed using BugBuster® (Novagen) according to the instructions of the manufacturer (1 µl of benzonase per 1 ml of BugBuster® reagent). The lysates were centrifuged at 10500 *g* for 30 min at 4ºC after which the supernatants were loaded onto equilibrated gluthathione sepharose beads (GE Healthcare) pre-packed in empty 1 ml columns (MoBiTec). The column was washed 3 times with 5 column volumes of binding buffer (PBS containing 0.05% [v/v], Triton X-100, and 1 mM DTT). After the washing step, the protein was eluted with 10mM gluthathione. All procedures were done with ice-cold buffers. This process yielded a significant portion of soluble GST-CT143 fusion protein of the expected molecular mass, as judged by SDS-PAGE.

For MBP fusion proteins, pellets of cells expressing MBP-CT142, MBP-CT143, or MBP-CT144 were resuspended in ice-cold column buffer (20 mM Tris-HCl [pH 7.4], 1 mM EDTA and 1 mM DTT). The cells were lysed by passing twice through a French Press in the presence of 1 mM PMSF. The lysates were then centrifuged at 10500 *g* for 30 min at 4ºC after which the supernatants were loaded onto equilibrated amylose resin (New England Biolabs), according to the instructions of the manufacturer. The column was washed 12 times with 1 ml of column binding buffer, after which the protein was eluted with column buffer containing 10 mM maltose.

For 6xHis-CT142 and 6xHis-CT144, both proteins remained insoluble after several conditions had been tested and therefore they were purified from inclusion bodies in denaturing conditions. For this, pellets of cells expressing 6xHis-CT142 or 6xHis-CT144 were

resuspended in a buffer containing 50mM Tris-HCl (pH 8.0), 100mM NaCl, lyzozyme (10 mg/ml) and Benzonase® (Novagen) and then lysed using BugBuster® reagent (Novagen) according to the instructions of the manufacturer (1µl of benzonase per 1 ml of BugBuster® reagent). The lysates were centrifuged at 10500 *g* for 30 min at 4ºC and the supernatant was discarded. Purification of inclusion bodies was done according to (Grenn & Sambrook, 2012). Briefly, pellets were washed in 9 volumes of a buffer containing 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.5% [v/v] Triton X-100, resuspended in 6 ml of a buffer containing 100 mM $Na_2H_2PO_4$, 10 mM Tris-HCl (pH 8.0) and 8 M urea, and incubated 30 min at 4ºC. The lysates were then centrifuged at 10500 *g* for 30 min at 4ºC and the supernatant was loaded onto equilibrated Ni-nitrilotriacetic acid (NTA) resin (Qiagen). Two washes were done with a buffer containing 100 mM $Na_2H_2PO_4$, 10 mM Tris-HCl (pH 6.3), and 8M urea, followed by consecutive elutions with a buffer containing 100 mM $Na_2H_2PO_4$, 10 mM Tris-HCl (pH 5.5), 8M urea and then with a buffer containing 100 mM $Na_2H_2PO_4$, 10mM Tris-HCl (pH 4.5), 8 M urea.

Purified proteins were dialysed using Snake Skin Dialysis Tubing (PierceNet) according to the manufacturer´s instruction.

Generation and purification of rabbit-anti CT143 antibodies

Purified GST-CT143 was used to immunize New Zealand White rabbits for the production of polyclonal antisera (Davids Biotechnologie, Regensburg, Germany). For purification, the anti-CT143 serum was firstly incubated with acetone powders (Spring & Wold, 1971) obtained from *E. coli* BL21 DE3 strain containing pGEX-4T-2, allowing the serum to be partly depleted of the anti-GST

antibodies or any contaminant *E. coli* protein in purified GST-CT143. Secondly, anti-CT143 serum was affinity purified on 1 mg of MBP-CT143 immobilized on a nitrocellulose membrane. The affinity-purified antibodies were concentrated using Amicon Ultra-4 (Millipore) and quantified using NanoDrop 1000 (Thermo Fisher Scientific).

Generation and purification of rabbit-anti CT142 antibodies

Polyclonal antibodies against CT142 were generated against three synthetic peptides conjugated to ovalbumin: peptide 1 (46-50 amino acid residues) NH_2-VKSISAKESFSVKRKC-COOH; peptide 2 (269-274 amino acid residues) NH_2-CKGGDYVDKSALSTLY-COOH; peptide 3 (124-140 amino acid residues) NH_2-QKLPLIGPSRLVYQSC-COOH (Metabion). The resulting antibodies were affinity purified on CNBr-sepharose matrix containing the respective peptides (Metabion).

Antibodies

The following primary antibodies were used: rabbit polyclonal anti-CT142 (this work; 1:200 dilution for immunoblotting); rabbit polyclonal anti-CT143 (this work; 1:200 dilution for immunoblotting and 1:50 dilution for immunofluorescence); mouse monoclonal anti chlamydial Hsp60 (A57-B9; Thermo Fisher Scientific; 1:1000 dilution for immunoblotting and 1:200 dilution for immunofluorescence); goat polyclonal anti- MOMP of *C. trachomatis* (ab34414; Abcam; 1:1000 dilution for immunoblotting and 1:500 dilution for immunofluorescence); mouse anti-CT442 antibody (Li et al., 2008; 1:200 dilution for immunofluorescence) mouse monoclonal anti-tubulin (clone B-5-1-2; Sigma Aldrich; 1:1000 for immunoblotting); goat polyclonal anti GFP (SICGEN; 1:1000 for immunoblotting).

For immunoblotting, the secondary antibodies used were all horseradish peroxidase (HRP)-conjugated (GE Healthcare and Jackson ImmunoResearch; used at 1:10000). For immunofluorescence, the following secondary antibodies were used: donkey anti-goat conjugated to cyanine 5 (Cy5) (Jackson ImmunoResearch Laboratories; 1:200), Alexa Fluor® -594 (AF594) conjugate AffiniPure donkey anti-goat (Jackson ImmunoResearch Laboratories; 1:200); Rhodamine Red^{TM}–X-Conjugated AffiniPure donkey anti-rabbit (Jackson ImmunoResearch Laboratories; 1:200); and goat anti-mouse Alexa Fluor® Fluor-488 (AF488) (Jackson ImmunoResearch Laboratories; 1:200).

Transient transfection of mammalian cells

HeLa HtTA-1 cells (ECACC) were transfected with 250 ng of plasmid DNA by using the jetPEI reagent (Polyplus-Transfection), as detailed by the manufacturer. Briefly, DNA and JetPei reagent were mixed and incubated for 30 min at room temperature. The mixture was then added drop-wise to cells seeded the previous day on 24 well plates. The plates were centrifuged for 180 *g* for 5 min and then incubated at 37°C in a humidified atmosphere of 5% [v/v] CO_2. After 4 h, fresh medium was added to each well. 24 h later, transfected cells were processed for immunoblotting.

Immunoblotting

To harvest infected or transfected HeLa cells, they were first washed once with PBS and then trypsinised with 50 µl (per well in a 24 well plate) of TrypLE™ Express (Life Technologies) by incubation during 5 min at 37ºC. The cells were then collected, pelleted by a brief centrifugation, washed 3 times with ice-cold PBS, and stored as cell pellets at -80ºC until use. Prior to immunoblotting the cell pellets were thawed, resuspended in an appropriate volume of SDS-PAGE loading buffer (Laemmli buffer 5 X: Tris-HCl 0.25M (pH 6.8), SDS 10 % [w/v], β-mercaptoethanol 0.5 M, Bromophenol Blue 0.5 % [w/v]) and the proteins in the extract were further denatured by an incubation of 5 minutes at 100ºC. Samples were resolved by 12% [v/v] SDS-PAGE and transferred to 0.2 µm nitrocellulose membranes (Bio-Rad). Membranes were incubated in blocking solution (PBS containing 4% [w/v] non-fat dried milk and 0.2% [v/v] Tween-20 for 1 h at room temperature, with gentle rocking. Primary antibodies were diluted in blocking solution in an appropriate volume to cover all the area of the membrane and incubated overnight at 4ºC, with gentle rocking. The following day membranes were washed twice (in PBS containing Tween-20 0.2% [v/v]) and then incubated with the respective HRP-conjugated secondary antibody. Western Lightning *Plus*-ECL (Perkin Elmer), and exposure to Amersham Hyperfilm ECL (GE Healthcare) were used for detection.

Immunofluorescence microscopy

Infected HeLa cells were fixed in either PBS containing 4% (w/v) paraformaldehyde (PFA) for 15 min or methanol (-20ºC) for 10 min. Cells were then washed with PBS and stored at 4ºC in PBS containing 10 mM NH_4Cl solution. Immunostaining was performed at room temperature. Antibodies were diluted in PBS containing 10%

[v/v] horse serum and (when fixation was done with PFA) 0.1% [v/v] Triton X-100 was added to allow permeabilization of cells. After immunolabeling, the cells were consecutively washed with PBS and H_2O. The coverslips were mounted using Aqua-poly/Mount (Polysciences) on microscopy glass slides, and the cells were examined by conventional fluorescence microscopy or by confocal microscopy. Quantitative analyses of immunofluorescence images were performed using Fiji software (Schindelin *et al.*, 2012).

3.4. RESULTS

3.4.1. *C. trachomatis ct142*, *ct143*, and *ct144* are organized in an operon

Previously, RT-qPCR revealed similar levels of *ct142*, *ct143* and *ct144* mRNA and an identical profile of expression of these genes (Chapter II; Fig. 2.4). These results and the localization of the genes in the *C. trachomatis* chromosome (Fig. 3.1A) suggested that *ct142*, *ct143* and *ct144* could be organized in an operon. To directly examine this, we performed transcription linkage analyses. RNA was isolated from EBs purified from HeLa 229 cells infected by *C. trachomatis* L2/434 for 40 h and cDNA was obtained by RT-PCR. Specific primer pairs (Table A2; Annexes) were used in conventional PCR reactions to determine possible transcriptional linkages between *ct142*, *ct143* and *ct144* (Fig. 3.1A) and genes upstream (*ct141*) and downstream (*ct145*). This showed that *ct142*, *ct143* and *ct144* are transcriptionally linked (Fig. 3.1A and 3.1B), indicating that they are organized in an operon. There was no detectable transcriptional linkage between *ct141* and *ct142* or between *ct144* and *ct145* (Fig. 3.1A and 3.1B). To precisely define the promoter of *ct142*, we determined its transcription start site (TSS) by 5´RACE using as template total RNA from HeLa 229 cells infected by *C. trachomatis* L2/434 for 40h, and primers complementary to *ct142* (Table A2; Annexes). By inspecting the nucleotide sequences immediately upstream from the determined TSS of *ct142*, for σ^{66}, σ^{54}, and σ^{28}-like promoters (Mathews & Timms, 2006), we only identified -10 and -35 regions possibly recognized by σ^{66} (Fig. 3.1A).

Figure 3.1. Characterization of *ct142*, *ct143*, and *ct144*. (A) Genetic organization of *ct142*, *ct143* and *ct144* depicting the fragments amplified in the transcription linkage analyses, the transcriptional start site (TSS) determined by 5´RACE as well as the deduced -10 and -35 σ^{66}-like promoter regions. (B) Transcription linkage analyses in *C. trachomatis* L2/434. cDNA+, PCR from cDNA generated with reverse transcriptase (RT) from total RNA isolated from EBs obtained from infected HeLa 229 cells at 20 h p.i.; cDNA-, as cDNA+ but without RT; gDNA, PCR from total DNA isolated from cells infected with the *C. trachomatis* strain L2/434.

3.4.2. Expression of *ct142*, *ct143* and *ct144* is downregulated in the plasmidless *C. trachomatis* L2/25667R strain

Recent work revealed that the plasmid encoded Pgp4 protein is a regulator of plasmid genes and of multiple chromosomal genes, including *ct142*, *ct143* and *ct144* (Song *et al.*, 2013). In order to confirm these observations, mRNA levels of *ct142*, *ct143* and *ct144* were analyzed by RT-qPCR during the developmental cycle of *C.*

trachomatis strains L2/434 and L2/25667R (lacking the virulence plasmid).

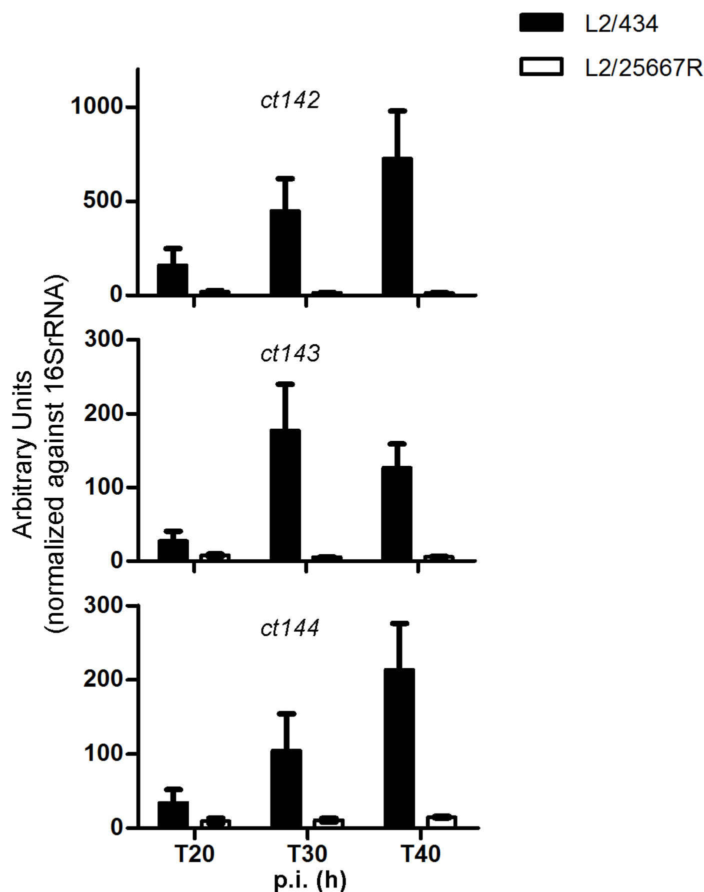


Figure 3.2. RT-qPCR analyzes of the expression of *ct142*, *ct143*, and *ct144* in *C. trachomatis*. The mRNA levels of *ct142*, *ct143* and *ct144* genes during the developmental cycle of *C. trachomatis* L2/434 and plasmidless L2/25667R were analyzed by RT-qPCR. The expression values resulted from raw RT-qPCR data of each gene normalized to that of the 16S rRNA gene.

As expected, this showed that the expression of *ct142*, *ct143* and *ct144* is significantly reduced in the strain without the plasmid (Fig. 3.2). In particular, at 30 h p.i., the mRNA levels of *ct142*, *ct143*, and *ct144* were about 38-fold, 35-fold, and 7-fold higher, respectively, in

strain L2/434 than in strain L2/25667R. Additionally, differently from what we observed in Chapter II and for unknown reasons, the mRNA levels of *ct142* in strain 434/Bu were 2.5-fold higher relative to mRNA levels of *ct143* and 4-fold higher relative to mRNA levels *ct144* at 30 h p.i..

Overall, this confirms a role of the virulence plasmid in the regulation of the expression of *ct142*, *ct143*, and *ct144*. Furthermore, the apparently higher mRNA levels of *ct142* relative to *ct143* and *ct144* indicate that expression of these genes might be subject to additional distinct regulatory mechanisms.

3.4.3. Generation of antibodies against CT142 and CT143

To analyze the expression of CT142, CT143 and CT144 in *C. trachomatis,* and their subcellular localization in infected cells*,* we aimed to obtain antibodies against these proteins. For this, we constructed plasmids encoding for GST-CT142, GST-CT143 or GST-CT144 fusion proteins. The plasmids were individually introduced in *E. coli* BL21 (DE3) and tested for expression and for solubility of the recombinant proteins in cell extracts. All proteins were expressed but only GST-CT143 was soluble (Fig 3.3A). Purified GST-CT143 (Fig. 3.3B) was used for generation of anti-CT143 antibodies, which were subsequently purified (see Materials and Methods).

Further attempts to obtain antibodies specifically recognizing CT142 and CT144 using fusion proteins (purified and soluble MBP-CT142 and MBP-CT144, or inclusion bodies containing 6x-His-CT142 and 6xHis-CT144) were unsuccessful (data not shown). Finally, we attempted to raise antibodies specifically recognizing CT142 by immunization of rabbits with 3 synthetic peptides. By this method we

were able to obtain antibodies against CT142 that performed well for immunoblotting, but not for immunofluorescence.

To analyze the specificity of the anti-CT142 and anti-CT143 antibodies, HeLa cells were transfected with plasmids encoding EGFP, EGFP-CT142 or EGFP-CT143. Extracts from the transfected cells were analyzed by immunoblotting comparing the anti-CT142 and anti-CT143 antibody relative to a commercial anti-GFP antibody (Fig. 3.3C). Using either anti-CT142 or anti-GFP we detected a band for a protein migrating on SDS-PAGE according to a molecular mass of approximately 70 kDa that should correspond to EGFP-CT142 (Fig. 3.3C). Equally, using either anti-CT143 or anti-GFP we detected a band for a protein migrating on SDS-PAGE according to a molecular mass of approximately 70 kDa that should correspond to EGFP-CT143 (Fig. 3.3C). However, only anti-GFP, but not anti-CT142 or anti-CT143, detected a protein in cells transfected with the plasmid encoding EGFP alone (Fig. 3.3C). Furthermore, anti-CT142 antibodies did not recognize GFP-CT143 and anti-CT143 antibodies did not recognize GFP-CT142 (Fig. 3.3C). These results indicated that the anti-CT142 and the anti-CT143 antibodies are specific and do not significantly recognise proteins endogenously expressed in HeLa cells.

We then analyzed the expression of CT142 and CT143 by immunoblotting using extracts of HeLa cells, either uninfected or infected by *C. trachomatis* L2/434 for 2, 8, 14, 20, 26, 32, 38, 40 h (Fig. 3.4 A and B). Bands that migrated on SDS-PAGE according to a molecular mass compatible with CT142 (31.5 kDa) and CT143 (32 kDa) could be detected from 20 h p.i., and more obviously from 26 h

p.i. However, at 20 h p.i. the signal detected with each antibody was very weak and in some cases not even detected (see Fig. 3.5).

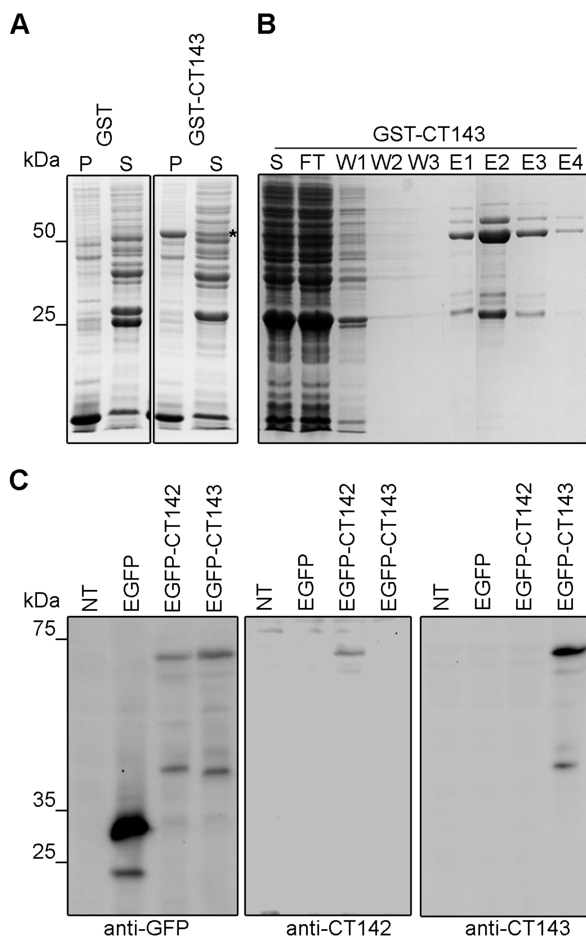


Figure 3.3. Generation and characterization of antibodies against CT142 and CT143. (A) *E. coli* BL21 (DE3) expressing either GST or GST-CT143 was grown for 24 h in auto-induction conditions at 25ºC. Protein extracts were prepared using BugBuster® (Novagen) and insoluble (P) and soluble fractions (S) were analyzed by SDS-PAGE. (B) GST-CT143 was purified from extracts of *E. coli* BL21 (DE3) (prepared as described in Materials and Methods) by gravity flow using a column packed with glutathione sepharose beads (GE Healthcare). Samples from the purification were collected and analyzed by SDS-PAGE: P: insoluble fraction; S: soluble fraction; FT: Flow through, W1-W3: wash fractions; E1-E4: Elution fractions. (C) Rabbits were immunized with purified GST-CT143 or with 3 synthetic peptides from CT142. The specificity of the resulting antibodies was determined by immunoblot analysis of extracts of HeLa cells either untransfected (NT) or transfected with plasmids encoding EGFP, EGFP-CT143 or EGFP-CT142. Anti-GFP, anti-CT142 or anti-CT143 antibodies were used as indicated.

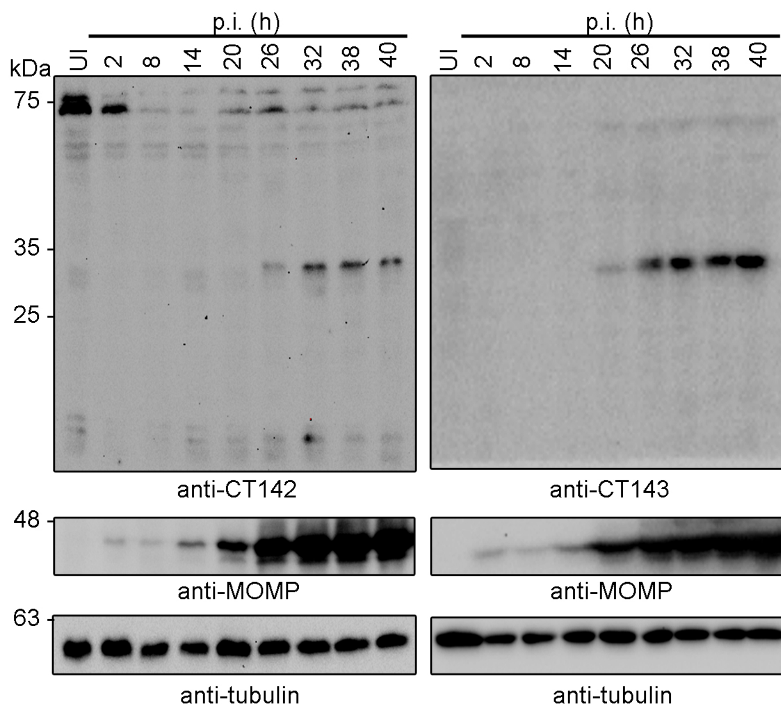


Figure 3.4. Expression of CT142 and CT143 in *C. trachomatis* L2/434. Expression of CT142 and CT143 during infection was assessed by immunoblotting with anti-CT142 and anti-CT143 antibodies, using lysates from HeLa cells either uninfected (UI) or infected by *C. trachomatis* L2/434 from 2 to 44 h p.i.. The level of *C. trachomatis* major outer membrane protein (MOMP) was monitored to assess the expression of chlamydial proteins, and the host cell protein tubulin was used as loading control.

3.4.4. Comparison of the expression of CT142 and CT143 in cells infected by *C. trachomatis* L2/434 or plasmidless L2/25667R

In order to ascertain the specificity of the signals obtained with anti-CT142 and anti-CT143 antibodies (Fig. 3.3 and 3.4), we analyzed extracts of HeLa cells infected by *C. trachomatis* L2/434 or L2/25667R (lacking the virulence plasmid) by immunoblotting. In extracts of HeLa cells infected by *C. trachomatis* L2/25667R, no bands migrating as proteins of about 32 kDa could be detected at any of the analyzed time points (Fig. 3.5). In contrast, in extracts from HeLa cells infected by *C. trachomatis* L2/434, we again detected

bands migrating as proteins of about 32 kDa, which indicated that the antibodies can specifically detect CT142 or CT143 (Fig. 3.5). In this experiment, the anti-CT142 and anti-CT143 signal was only visible from 30 h p.i.

Overall, this indicated that the anti-CT142 and anti-CT143 antibodies specifically detect CT142 and CT143, respectively, and further confirmed that expression of CT142 and CT143 is regulated by the *Chlamydia* virulence plasmid.

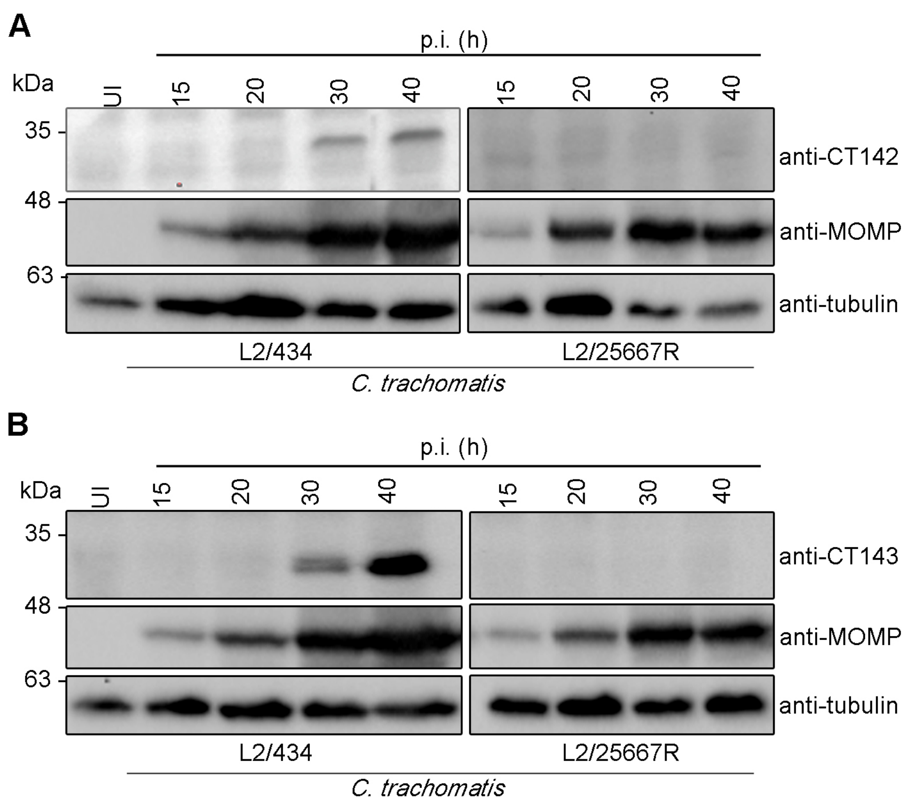


Figure 3.5. **Expression of CT142 and CT143 in *C. trachomatis* L2/434 and plasmidless L2/25667R.** Extacts of HeLa cells either left uninfected (UI) or infected by *C. trachomatis* L2/434 or *C. trachomatis* L2/25667 for 15, 20, 30 or 40 h were probed with anti-CT142 (A) or anti-CT143 (B) antibodies. The level of MOMP was monitored to assess the expression of chlamydial proteins, and the host cell protein tubulin was used as loading control.

3.4.5. Analysis of the subcellular localization of CT143 in HeLa 229 cells infected with *C. trachomatis*

To analyze the subcellular localization of CT143 in HeLa cells infected by *C. trachomatis* L2/434 we followed a time course of infection from 8 to 30 h p.i.. As mentioned above, in control experiments we could not detect an immunofluorescence-specific signal with the anti-CT142 antibody. At different time-points, the cells were fixed, immunolabeled for CT143 and Hsp60 (which recognizes a *Chlamydia* cytosolic molecular chaperone), and then analyzed by indirect immunofluorescence confocal microscopy (Fig. 3.6). The labeling for Hsp60 (shown in green) allowed us to monitor the inclusion, because it labels each bacterium inside the vacuole. At 8 h p.i. we already detected Hsp60 labeling, but CT143 was not detected (Fig. 3.6). At 15 h p.i. we detected small but defined inclusions. At this time point, the signal for CT143 was not very intense and overlapped with the Hsp60 signal. However, at 20 h p.i., the signal for CT143 did not overlap with the Hsp60 signal; instead we started to distinguish discrete structures (Fig 3.6) that at 30 h p.i. were much more abundant (Fig. 3.6). These structures did not seem to co-localize with the Hsp60 signal but appeared to be within the inclusion lumen. The distinct pattern of localization of CT143 at 15, 20, and 30 h p.i., as detected by the anti-CT143 antibody, is shown in more detail in Fig 3.7.

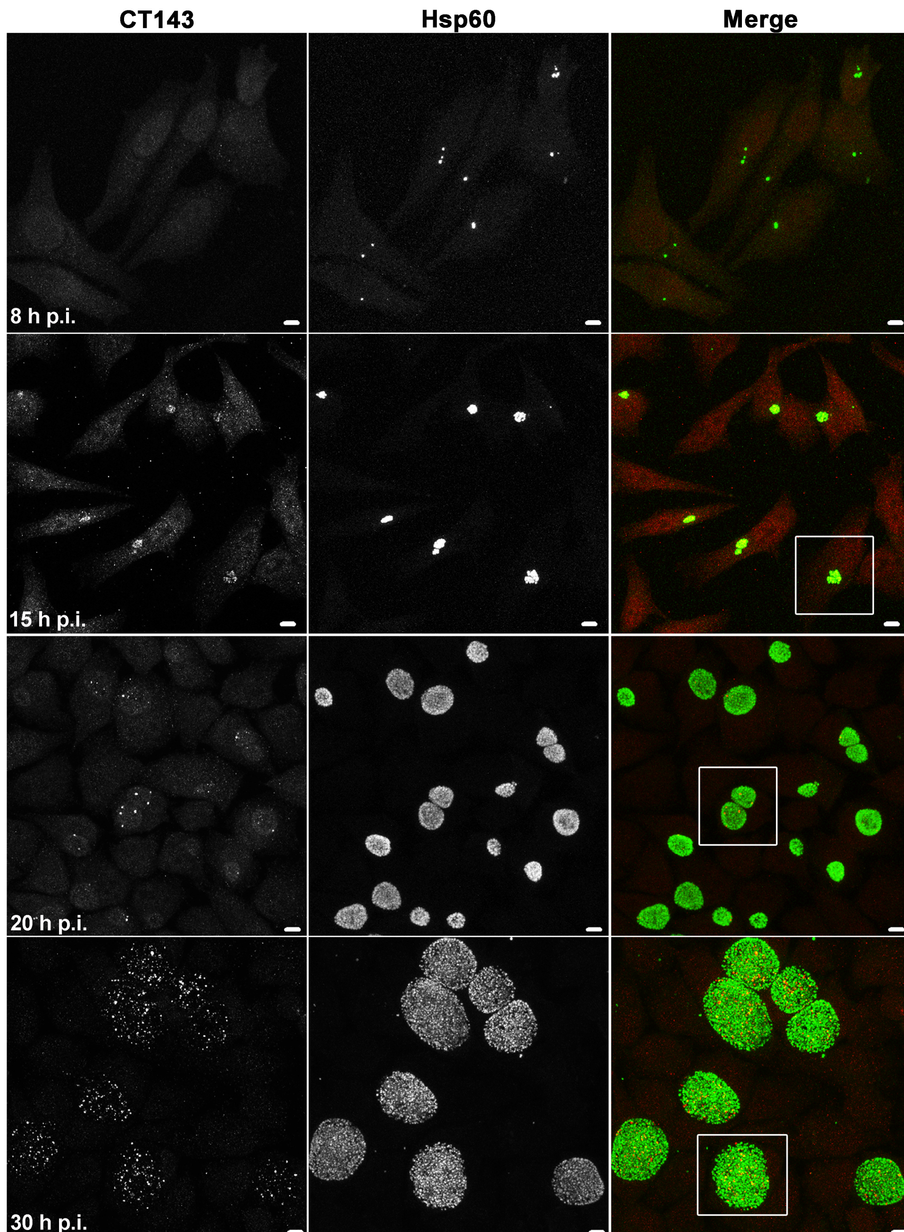


Figure 3.6. Subcellular localization of CT143 in HeLa cells infected with *C. trachomatis*. HeLa 229 cells were infected by *C. trachomatis* L2/434 for 8, 15, 20 and 30 h and then fixed with methanol. Fixed infected cells were immunolabeled with anti-CT143 and anti-Hsp60. Cells were analyzed by confocal immunofluorescence microscopy. Images are combined projections of multiple 0.2 μm z-sections. Scale bars represent 5 μm. The white box indicates the area selected to amplify the images (see Fig. 3.7).

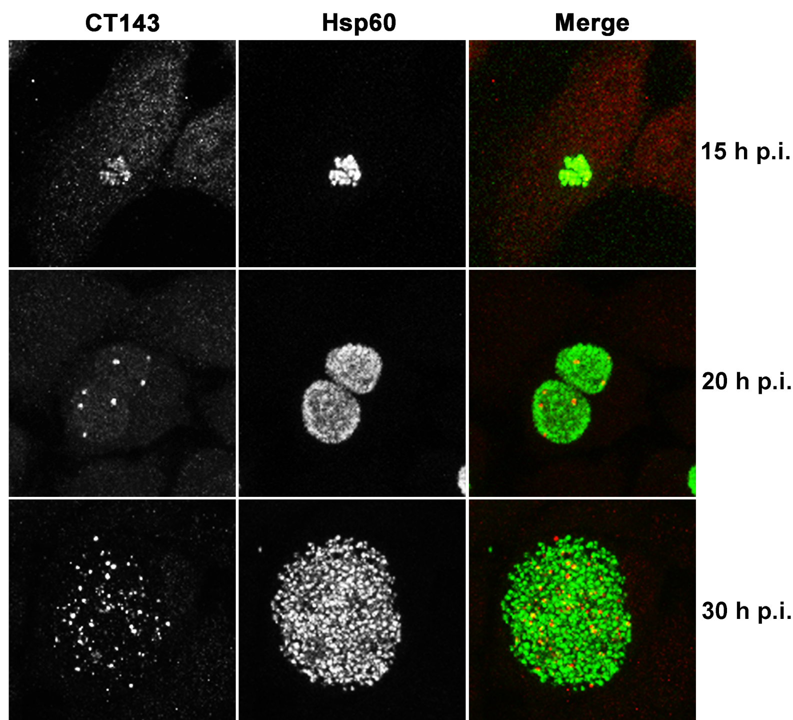


Figure 3.7. Comparison of localization of CT143 between 15, 20 and 30 h p.i.. Images from Fig. 3.6 representing HeLa 229 cells infected with *C. trachomatis* L2/434 for 15, 20, and 30 h were zoomed 3-fold in the area delimited by a white box (see Fig. 3.6.).

3.4.6. The immunofluorescence signal of CT143 is specific

To verify the specificity of the immunofluorescence signal obtained with the anti-CT143 antibody, we first performed an antibody competition assay. Prior to labeling, anti-CT143 was incubated without any protein or in the presence of an excess (8 µg) of MBP, MBP-CT142 or MBP-CT143. As observed, HeLa cells infected for 30 h by *C. trachomatis* L2/434 show a clear reduction of the immunofluorescence signal for CT143, only in the presence of MBP-CT143 (Fig. 3.8A). In addition, we compared the immunofluorescence signal of CT143 in HeLa 229 cells infected for 30 h by *C. trachomatis* strains L2/434 or plasmidless L2/25667R.

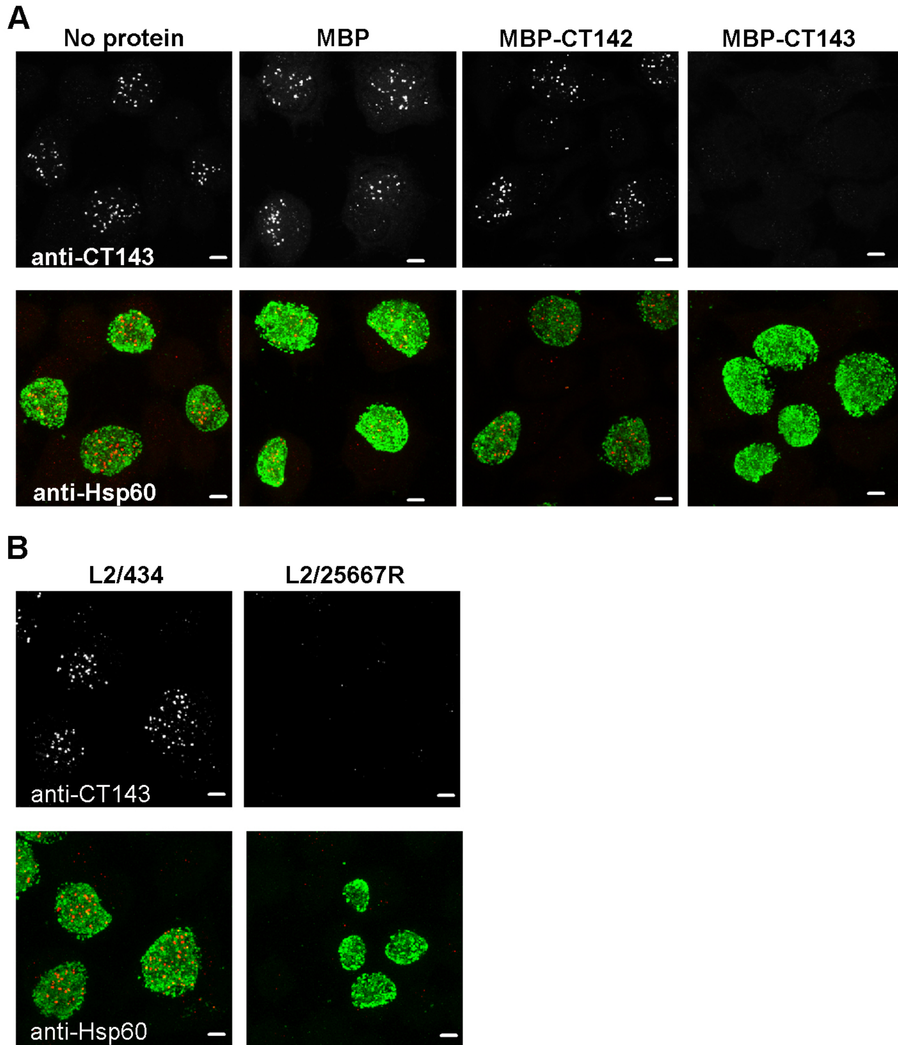
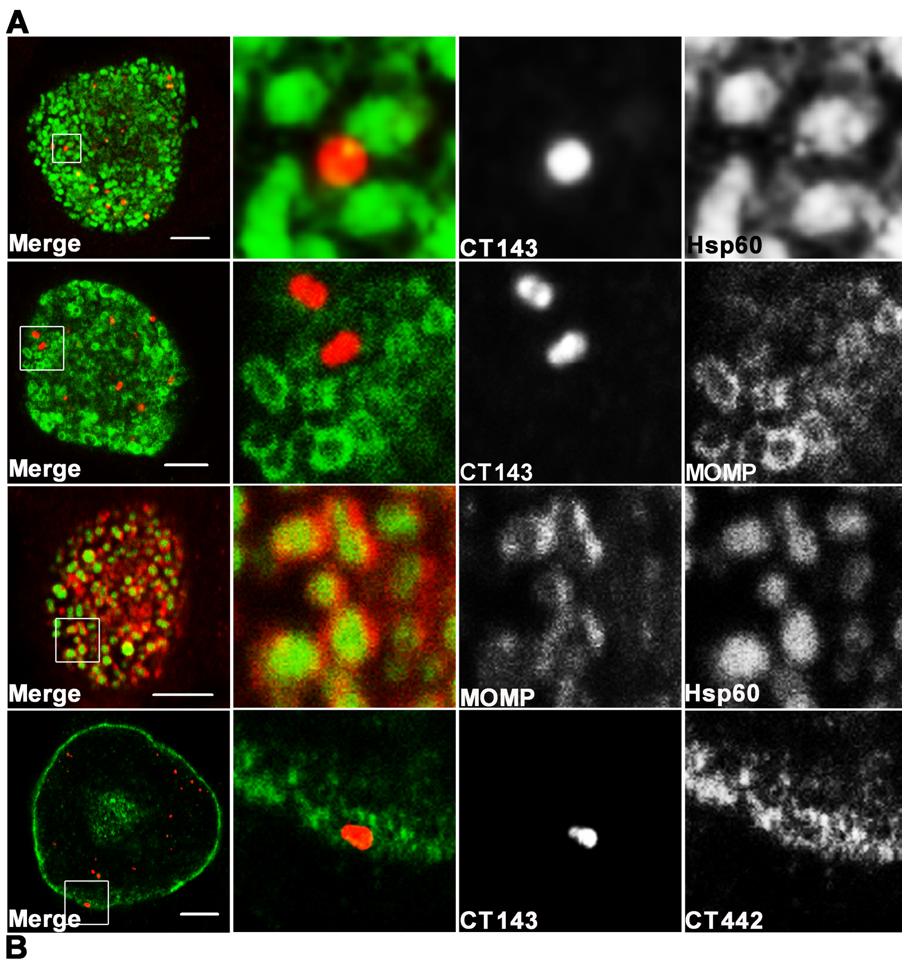


Figure 3.8. Specificity of the immufluorescence anti-CT143 signal. (A) HeLa 229 cells were infected with *C. trachomatis* L2/434 for 30 h and fixed with methanol. Before labeling with anti-CT143 and anti-Hsp60 antibodies, anti-CT143 was incubated in the presence of an excess (8 µg) of MBP, MBP-CT142 or MBP-CT143, as indicated. (B) HeLa 229 cells were infected with *C. trachomatis* L2/434 or plasmidless L2/25667R, fixed at 30 h p.i. with methanol and immunolabeled with anti-CT143 and anti-Hsp60. Cells were analyzed by confocal immunofluorescence microscopy. Images in panels A and B are combined projections of multiple 0.2 µm z-sections. Scale bars represent 5 µm.

This revealed that the CT143 immunofluorescence signal was drastically reduced in cells infected by the plasmidless *C. trachomatis* strain. Therefore, the immunofluorescence signal detected with the anti-CT143 antibody (Fig. 3.6 and 3.7) likely corresponds to CT143.

3.4.7. CT143 localizes at the inclusion lumen and appears as globular structures

In order to evaluate in more detail the localization of CT143 in HeLa cells infected by *C. trachomatis*, cells infected for 30 h were immunolabeled for CT143 as well as for other chlamydial proteins: anti-Hsp60, as before; MOMP, which recognizes *C. trachomatis* major outer membrane protein; and anti-CT442, which recognizes an inclusion membrane (Inc) protein (Bannantine *et al.*, 2000; Liu *et al.*, 2010) (Fig. 3.9). We further confirmed that CT143 appeared mostly as intra-inclusion globular structures that sometimes overlap with the chlamydial inclusion membrane (Fig. 3.9A). The immunofluorescence signal from the anti-CT143 antibody did not co-localize with the bacterial signal from the anti-MOMP or anti-Hsp60 antibodies (Fig. 3.9A). We then measured the area of each of the immunofluorescence signals analyzed (CT143, Hsp60 and MOMP), and confirmed that the area for CT143 is considerably smaller than the area for the cytosolic (Hsp60) and outer membrane (MOMP) bacterial markers (Fig. 3.9 B). This ruled out the possibility that the signal obtained with anti-CT143 could, for some unknown reason, correspond to labeling of only a few bacteria inside the inclusion. Overall, these results suggested that CT143 might be secreted into the lumen of the inclusion.



B

P<0.0001

P<0.0001

Area of signal (μm^2)

2.0
1.5
1.0
0.5
0.0

CT143 Hsp60 MOMP

Figure 3.9. Subcellular localization of CT143 relative to Hsp60, MOMP and Inc CT442 (A) HeLa 229 cells were infected with *C. trachomatis* L2/434 for 30 h, fixed with methanol, and immunolabeled for CT143 and anti-Hsp60 (upper panel), CT143 and MOMP (middle/upper panel), MOMP and Hsp60 (middle/lower panel), or CT143/Inc CT442 (lower panel). Cells were analyzed by confocal immunofluorescence microscopy. Images correspond to single *z* sections. Scale bars, 5 μm. The images were zoomed 5-fold in the area delimited by a white box. (B) Comparison of the area of the signal of CT143, MOMP and Hsp60. Measurement of the immunofluorescence area of signal for CT143, Hsp60 and MOMP was done using Fiji software (Schindelin *et al.*, 2012) for at least 120, 90, and 90 particles, respectively (chosen from 3 independent images). *P*-values were calculated by a two-tailed unpaired Student's *t*-test.

3.5. DISCUSSION

In this Chapter, we found that *C. trachomatis ct142*, *ct143* and *ct144* are organized in an operon and their expression in driven by a putative σ^{66} promoter upstream from the start codon of *ct142*. The expression profile of *ct142*, *ct143* and *ct144* (mid/late cycle) suggested two different timings for these proteins to act. One would be that these proteins could act mainly at mid and late cycle. Alternatively these proteins could accumulate adjacent to the EBs and have an important role in exit from the host cells or subsequent invasion. We also further confirmed previous studies indicating that expression of *ct142*, *ct143*, and *ct144* is dependent on the *C. trachomatis* virulence plasmid (Carlson *et al.*, 2008; Gong *et al.*, 2013), and more specifically on plasmid-encoded Pgp4 (Song *et al.*, 2013). The *ct142*, *ct143*, and *ct144* genes encode three proteins that were found in Chapter II to be candidate T3SS substrates. Using antibodies against CT142 and CT143 we confirmed that the proteins are expressed in *C. trachomatis* L2/434 during infection of HeLa cells. Furthermore, using anti-CT143 antibodies we showed that CT143 localizes in globular structures within the inclusion that do not co-localize with the signal for the bacteria, which suggest secretion of CT143 into the vacuolar lumen. Overall, these observations are consistent with a role of CT142, CT143 and CT144 in virulence (expression of the encoding genes is dependent on the virulence plasmid and at least CT142 and CT143 are produced in *C. trachomatis*), but they are at odds with the idea that they might be T3SS effectors, as in this scenario we would expect to find CT143 in the host cell cytosol.

To analyze the expression and subcellular localization of CT142, CT143 and CT144 it was essential to obtain antibodies against these proteins. Several attempts to raise antibodies against CT144 were unsuccessful, so this analysis could not be done for CT144. The reasons for our lack of success in obtaining antibodies for this protein are unclear. We can speculate that the protein has very low immunogenicity or that the antibodies generated against the fusion proteins used did not recognize any epitopes of the protein in its native conformation when expressed in the context of infection. Furthermore, the antibodies against CT142 could only be used in immunoblotting and not in immunofluorescence microscopy. To circumvent these difficulties, as described in the next Chapter, we took advantage of the newly described methods for introducing expression plasmids in *C. trachomatis* to construct strains expressing epitope-tagged CT142, CT143, and CT144 (see Chapter IV).

An important finding described in this Chapter was the localization of CT143 in the lumen of the inclusion. As mentioned above, this was surprising because, assuming it is a T3SS effector, we would expect to detect the protein within the cytoplasm of host cells. Therefore, different approaches were used to demonstrate the specificity of the immunofluorescence signal revealing this intra-lumenal localization. Furthermore, to rule out a possible artifact in the fixation process, we used both PFA or methanol as fixation agents and in both conditions we always detected the same globular structures when labeling with anti-CT143 antibodies (data not shown). We cannot exclude that CT143 is translocated into the host cell cytosol but in levels that are too low to be detected by immunofluorescence microscopy. Based on our findings we propose that at about 15 h p.i. CT143 begins to

accumulate inside the bacteria and at 20 h p.i. it starts being secreted into the lumen of the inclusion.

CT143 is not the first *C. trachomatis* protein found to localize inside the lumen of the inclusion. CT049 (Pls1) and CT050 (Pls2) (see section 1.4.9.6., below), paralogously related to the passenger domain of the polymorphic membrane protein PmpC, were observed by immunofluorescence microscopy as globular structures within the inclusion lumen and possibly at the inclusion membrane (Jorgensen & Valdivia, 2008). These proteins lack classical signal peptides and were not described as T3SS substrates. How these proteins are secreted into the lumen of the inclusion is not clearly understood. Two other proteins, CT620 and CT621 that are likely T3SS substrates belonging to a family of proteins containing a DUF582 domain of unknown function (Muschiol *et al.*, 2011) (see section 1.4.8.4., below), are expressed at middle and late phases of the infectious cycle. Immunolocalization examination in infected HeLa cells revealed that CT620 and CT621 localized in the host cell cytoplasm and nuclei as well as in the lumen of the inclusion, where they do not associate with bacterial markers (Muschiol *et al.*, 2011).

The possible function of CT143, CT049, CT050, CT620, and CT621 within the inclusion lumen is unclear. However, there is increasing evidence that the *Chlamydia* inclusion is not a closed compartment, but rather a complex and highly interactive space where contacts between bacterial and host molecules might occur. In fact, the inclusion lumen contains at least glycogen (Carlson *et al.*, 2008; Chiappino *et al.*, 1995), and lipid droplets (Cocchiaro *et al.*, 2008). The possible interaction of CT143, CT049, CT050, CT620, and CT621 with these molecules remains to be analyzed.

Obviously, a function of CT143 (and of CT142 and CT144) could be related to activities that have been attributed to the *Chlamydia* virulence plasmid, such as stimulation of pro-inflammatory cytokines (O'Connell *et al.*, 2011) infectivity (O'Connell *et al.*, 2007), accumulation of glycogen granules in the inclusion (Matsumoto *et al.*, 1998) or intra-inclusion Brownian-like movement (Carlson *et al.*, 2008). Furthermore, recent transcriptome analysis of HeLa cells infected with either a plasmid-bearing *C. trachomatis* strain or with its isogenic plasmid-deficient strain show that in the strain containing the plasmid there is a significant increase in the levels of expression of many human genes involved in inflammation, chemoattraction, cell growth and fibrosis (Porcella *et al.*, 2015). However, it is unclear if CT142, CT143 and CT144 are involved in such functions, and how this could be related to the localization of CT143 in the lumen of the inclusion. To help start addressing these and other questions, we thought it was important to determine the subcellular localization of CT142 and CT144, which is described in the following Chapter.

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REFERENCES

Bannantine, J. P., Griffiths, R. S., Viratyosin, W., Brown, W. J. & Rockey, D. D. (2000). A secondary structure motif predictive of protein localization to the chlamydial inclusion membrane. *Cell Microbiol* **2**(1), 35–47.

Borges, V., Ferreira, R., Nunes, A., Nogueira, P., Borrego, M. J. & Gomes, J. P. (2010). Normalization strategies for real-time expression data in *Chlamydia trachomatis*. *J Microbiol Methods* **82**, 256–264.

Carlson, J. H., Whitmire, W. M., Crane, D. D., Wicke, L., Virtaneva, K., Sturdevant, D. E., Kupko, J. J., Porcella, S. F., Martinez-Orengo, N. & other authors. **(2008).** The *Chlamydia trachomatis* plasmid is a transcriptional regulator of chromosomal genes and a virulence factor. *Infect Immun* **76**(6), 2273–2283.

Chiappino, M. L., Dawson, C., Schachter, J. & Nichols, B. a. **(1995).** Cytochemical localization of glycogen in *Chlamydia trachomatis* inclusions. *J Bacteriol* **177**(18), 5358–5363.

Clifton, D. R., Fields, K. A., Grieshaber, S. S., Dooley, C. A., Fischer, E. R., Mead, D. J., Carabeo, R. A. & Hackstadt, T. (2004). A chlamydial type III translocated protein is tyrosine-phosphorylated at the site of entry and associated with recruitment of actin. *Proc Natl Acad Sci U S A* **101**(27), 10166–10171.

Cocchiaro, J. L., Kumar, Y., Fischer, E. R., Hackstadt, T. & Valdivia, R. H. (2008). Cytoplasmic lipid droplets are translocated into the lumen of the *Chlamydia trachomatis* parasitophorous vacuole. *Proc Natl Acad Sci U S A* **105**(27), 9379–9384.

Cornelis, G. R. (2006). The type III secretion injectisome. *Nat Rev Microbiol* **4**, 811–825.

Fields, K. A. & Hackstadt, T. (2000). Evidence for the secretion of *Chlamydia trachomatis* CopN by a type III secretion mechanism. *Mol Microbiol* **38**(5), 1048–1060.

Gong, S., Yang, Z., Lei, L., Shen, L. & Zhong, G. (2013). Characterization of *Chlamydia trachomatis* Plasmid-Encoded Open Reading Frames. *J Bacteriol* **195**(17), 3819–3826.

Grenn, M. & Sambrook, J. (2012). *Molecular Cloning*, 4th Editio. Cold Spring Harbour Laboratory Press.

Hower, S., Wolf, K. & Fields, K. A. (2009). Evidence that CT694 is a novel *Chlamydia trachomatis* T3S substrate capable of functioning during invasion or early cycle development. *Mol Microbiol* **72**(6), 1423–1437.

Jorgensen, I. & Valdivia, R. H. (2008). Pmp-like proteins Pls1 and Pls2 are secreted into the lumen of the *Chlamydia trachomatis* inclusion. *Infect Immun* **76**(9), 3940–3950.

Kari, L., Whitmire, W. M., Olivares-Zavaleta, N., Goheen, M. M., Taylor, L. D., Carlson, J. H., Sturdevant, G. L., Lu, C., Bakios, L. E. & other authors. (2011). A live-attenuated chlamydial vaccine protects against trachoma in nonhuman primates. *J Exp Med* **208**(11), 2217–2223.

Liu, X., Afrane, M., Clemmer, D. E., Zhong, G. & Nelson, D. E. (2010). Identification of *Chlamydia trachomatis* outer membrane complex proteins by differential proteomics. *J Bacteriol* **192**, 2852–2860.

Lovett, M., Kuo, C.-C., Holmes, K. & Falkow, S. (1980). Plasmids of the genus *Chlamydia*. *Curr Chemoth Infect Dis* **2**, 1250–1252.

Mathews, S. & Timms, P. (2006). *In silico* Identification of Chlamydial Promoters and their Role in Regulation of Development. In *Chlamydia genomics Pathog*. Edited by W. P. Bavoil, PM. Norfolk, United Kingdom: Horizon Bioscience. pp. 133–156

Matsumoto, A., Izutsu, H., Miyashita, N. & Ohuchi, M. (1998). Plaque Formation by and Plaque Cloning of *Chlamydia trachomatis* Biovar Trachoma These include : Plaque Formation by and Plaque Cloning of *Chlamydia trachomatis* Biovar Trachoma **36**(10), 3013–3019.

Muschiol, S., Boncompain, G., Vromman, F., Dehoux, P., Normark, S., Henriques-Normark, B. & Subtil, A. (2011). Identification of a family of effectors secreted by the type III secretion system that are conserved in pathogenic *Chlamydiae*. *Infect Immun* **79**(2), 571–580.

O'Connell, C. M., Ingalls, R. R., Andrews Jr., C. W., Scurlock, A. M. & Darville, T. (2007). Plasmid-deficient *Chlamydia muridarum* fail to induce immune pathology and protect against oviduct disease. *J Immunol* **179**, 4027–4034.

O'Connell, C. M., AbdelRahman, Y. M., Green, E., Darville, H. K., Saira, K., Smith, B., Darville, T., Scurlock, A. M., Meyer, C. R. & Belland, R. J. (2011). Toll-like receptor 2 activation by *Chlamydia trachomatis* is plasmid dependent, and plasmid-responsive chromosomal loci are coordinately regulated in response to glucose limitation by *C. trachomatis* but not by *C. muridarum*. *Infect Immun* **79**(3), 1044–1056.

Peterson, E. M., Markoff, B. A., Schachter, J. & de la Maza, L. M. (1990). The 7.5-kb plasmid present in *Chlamydia trachomatis* is not essential for the growth of this microorganism. *Plasmid* **23**(2), 144–148.

Pickett, M. A. (2005). The plasmids of *Chlamydia trachomatis* and *Chlamydophila pneumoniae* (N16): accurate determination of copy number and the paradoxical effect of plasmid-curing agents. *Microbiology* **151**, 893–903.

Porcella, S. F., Carlson, J. H., Sturdevant, D. E., Sturdevant, G. L., Kanakabandi, K., Virtaneva, K., Wilder, H., Whitmire, W. M., Song, L. & Caldwell, H. D. (2015). Transcriptional Profiling of Human Epithelial Cells Infected with Plasmid-Bearing and Plasmid-Deficient *Chlamydia trachomatis*. *Infect Immun* **83**(2), 534–543.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S. & other authors. (2012). Fiji: an open-source platform for biological-image analysis. *Nat Methods* **9**(7), 676–682.

Scidmore, M. (2005). Cultivation and laboratory maintenance of *Chlamydia trachomatis*. *Curr Protoc Microbiol* 1–25.

Sigar, I. M., Schripsema, J. H., Wang, Y., Clarke, I. N., Cutcliffe, L. T., Seth-Smith, H. M. B., Thomson, N. R., Bjartling, C., Unemo, M. & other authors. **(2014).** Plasmid deficiency in urogenital isolates of *Chlamydia trachomatis* reduces infectivity and virulence in a mouse model. *Pathog Dis* **70**, 61–69.

Song, L., Carlson, J. H., Whitmire, W. M., Kari, L., Virtaneva, K., Sturdevant, D. E., Watkins, H., Zhou, B., Sturdevant, G. L. & other authors. **(2013).** *Chlamydia trachomatis* plasmid-encoded Pgp4 is a transcriptional regulator of virulence-associated genes. *Infect Immun* **81**(3), 636–644.

Spring, T. G. & Wold, F. (1971). The purification and characterization of *Escherichia coli* enolase. *J Biol Chem*.

Studier, F. W. (2005). Protein production by auto-induction in high density shaking cultures. *Protein Expr Purif* **41**, 207–34.

Subtil, A., Delevoye, C., Balañá, M. E., Tastevin, L., Perrinet, S. & Dautry-Varsat, A. (2005). A directed screen for chlamydial proteins secreted by a type III mechanism identifies a translocated protein and numerous other new candidates. *Mol Microbiol* **56**(6), 1636–1647.

Valdivia, R. H. (2008). *Chlamydia* effector proteins and new insights into chlamydial cellular microbiology. *Curr Opin Microbiol* **11**, 53–59.

Wang, Y., Kahane, S., Cutcliffe, L. T., Skilton, R. J., Lambden, P. R. & Clarke, I. N. (2011). Development of a transformation system for *Chlamydia trachomatis*: Restoration of glycogen biosynthesis by acquisition of a plasmid shuttle vector. *PLoS Pathog* **7**(9), e1002258.

Chapter IV

The *Chlamydia trachomatis* type III secretion substrates CT142, CT143 and CT144 could be part of a protein complex in the lumen of the bacterial vacuole

This Chapter contains data of a manuscript in preparation:
Maria da Cunha and Luís Jaime Mota. The *Chlamydia trachomatis* type III secretion substrates CT142, CT143, and CT144 could be part of a protein complex in the lumen of the inclusion.

The author of this dissertation participated in all experiments described in this Chapter.

4.1. ABSTRACT

Virulence of *C. trachomatis* is related to a type III secretion system (T3SS). In Chapter II, we described a screen for *C. trachomatis* T3SS substrates using *Yersinia enterocolitica* as heterologous host. Among others, we identified CT142, CT143, and CT144 as putative T3SS substrates. In Chapter III, we showed that *ct142*, *ct143* and *ct144* are organized in an operon. Furthermore, we described that CT142 and CT143 are expressed in *C. trachomatis* during infection of HeLa cells, and that endogenous CT143 appeared as intra-inclusion globular structures that did not overly co-localize with the bacterial signal. In this Chapter, using recently described methods for transformation of *C. trachomatis*, we constructed strains carrying plasmids expressing CT142, CT143, or CT144 with a C-terminal 2HA epitope tag (CT142-2HA, CT143-2HA, CT144-2HA) under the control of the *ct142* promoter. Immunofluorescence (IF) microscopy analyses of HeLa cells infected by the newly constructed strains revealed that CT142-2HA, CT143-2HA or CT144-2HA show a subcellular localization similar to endogenous CT143. Moreover, IF microscopy of HeLa cells infected by *C. trachomatis* carrying a plasmid simultaneously expressing CT142, CT143, and CT144-2HA under the control of the *ct142* promoter revealed co-localization between the IF signals of CT144-2HA and CT143. Immunoprecipitation experiments using *Y. enterocolitica* as heterologous host suggested that CT142 could self-interact, bind to CT143, and perhaps also to CT144. Based on these results, we propose that CT142, CT143, and CT144 could form a complex of unknown function in the inclusion lumen after their secretion by the bacteria.

4.2. INTRODUCTION

The obligate intracellular human pathogen *Chlamydia trachomatis* encodes for a type III secretion system (T3SS) (Stephens *et al.*, 1998), a virulence mechanism used by many bacterial pathogens to manipulate eukaryotic host cells by injecting them with T3SS effector proteins (Cornelis, 2006). In Chapter II, using *Yersinia enterocolitica* has heterologous host we identified 10 putative *C. trachomatis* proteins with a high likelihood of being T3S substrates, which included CT142, CT143 and CT144. In Chapter III, we analyzed the genetic organization of *ct142*, *ct143* and *ct144* as well as the expression of CT142 and CT143 in HeLa cells infected by *C. trachomatis*. Additionally, by immunofluorescence microscopy we observed that CT143 localizes in globular structures in the lumen of the chlamydial vacuole (known as inclusion), not overly co-localizing with bacterial cytosolic or outer membrane markers.

Like other intracellular obligate microorganisms, it has been very challenging to develop genetic tools to manipulate *C. trachomatis.* However, in recent years different methods were described allowing for introduction of plasmid DNA into *C. trachomatis*, for ectopic expression (Agaisse & Derré, 2013; Bauler & Hackstadt, 2014; Wang *et al.*, 2011) and for directed inactivation of *C. trachomatis* genes (Johnson & Fisher, 2013; Kari *et al.*, 2011; Kokes *et al.*, 2015; Mishra *et al.*, 2012; Nguyen & Valdivia, 2013).

Transformation of *C. trachomatis* allowed, very recently, for major advances in *Chlamydia* research. The identification of inclusion membrane proteins (Inc proteins) regarding their localization and interacting partners has benefitted greatly by the development of

transformation techniques (Agaisse & Derré, 2014; Almeida and Mota, unpublished; Bauler & Hackstadt, 2014; Dumoux *et al.*, 2015; Weber *et al.*, 2015). Furthermore, the newly identified TepP effector, was identified in a study that was the first example of genetic validation of the function of a T3SS effector in *C. trachomatis* (Chen *et al.*, 2014).

In this Chapter, we have used the recently developed methods for ectopic expression of proteins in *C. trachomatis*, to confirm and analyze the subcellular localization of CT142, CT143, and CT144 in infected cells.

4.3. MATERIALS AND METHODS

Cell culture, bacterial strains and growth conditions

HeLa 229 cells (ATCC) and Vero cells (ECACC) were maintained in DMEM (Life Technologies) supplemented with 10% [v/v] FBS (Life Technologies) (complete DMEM) at 37°C in a humidified atmosphere of 5% [v/v] CO_2. *C. trachomatis* serovar L2 strain 434/Bu (L2/434; ATCC) was propagated in HeLa 229 cells using standard techniques (Scidmore, 2005), *Escherichia coli* TOP10 (Life Technologies) was used for construction and purification of the plasmids. *Yersinia enterocolitica* ΔHOPEMT (MRS40 pIML421 [$yopH_{\Delta 1\text{-}352}$, $yopO_{\Delta 65\text{-}558}$, $yopP_{23}$, $yopE_{21}$, $yopM_{23}$, $yopT_{135}$]), deficient for the *Yersinia* Yop T3S effectors H, O, P, E, M, and T, but T3S-proficient (Iriarte & Cornelis, 1998) was used for co-immunoprecipitation assays. *E. coli* or *Y. enterocolitica* were routinely grown in liquid or solid Luria-Bertani (LB) medium with the appropriate antibiotics and supplements. Plasmids were introduced into *E. coli* or *Y. enterocolitica* by electroporation.

DNA manipulations, plasmids, and primers

The plasmids used in this Chapter and their main characteristics are detailed in Table A1 (Annexes). The DNA primers used in their construction are described in Table A2 (Annexes). DNA manipulations were done as described in Chapter III.

Infection of HeLa 229 cells with *C. trachomatis*

Infection of HeLa 229 cells with *C. trachomatis* was done as described in Chapter III.

Transformation of *C. trachomatis*

The following procedure was adapted from Derré *et al*, 2013. The plasmid DNA used for transformation of *C. trachomatis* was isolated from *E. coli* K12 ER2925 (*dam*$^-$ *dcm*$^-$) (New England Biolabs) using the NZYMidiprep Kit (NZTech). HeLa 229 cells were cultured as described above. Penicilin G and cyclohexamide were from Sigma-Aldrich. The optimal penicillin concentration to select transformants was 1 U/ml. Once established, the transformed *C. trachomatis* strains were cultured in the presence of 10 U/ml.

The procedure was initiated by the addition of 6 µg of plasmid DNA to 200 µl of $CaCl_2$ buffer (10 mM Tris, 50 mM $CaCl_2$ pH 7.4). Then, 20 µl of previously thawed and vortexed *C. trachomatis* EBs [prepared and titrated as described in Scidmore (2005)] were added to the DNA and $CaCl_2$ buffer mixture, and mixed by pipetting up and down. This mixture was then incubated for 30 min at room temperature. During this incubation period, $4x10^6$ HeLa cells were trypsinized, washed once in PBS, pelleted by centrifugation at 150 *g*, and resuspended in 200 µl of $CaCl_2$ buffer. After the 30 min incubation, the 200 µl of resuspended HeLa cells were added to the mixture of DNA and *C. trachomatis* EBs and incubated for an additional 20 min, pipetting up and down every 5 min. At the end of this incubation period, 200 µl of DNA and *C. trachomatis* EBs mixture was added very gently to an 8.5 cm^2 dish containing 3 ml of DMEM. After mixing well, the cells were incubated for about 2 days (44 h) at 37ºC in the presence of 5% [v/v] CO_2.

After this incubation of ~2 days, most of the cells were infected and wild-type inclusions were easily observed by phase-contrast microscopy. The cells were then quickly rinsed with 1 ml of sterile

H_2O and lysed by osmotic shock (10 min incubation in 2 ml of sterile H_2O). Cells were scrapped, centrifuged at 150 *g* for 5 min, and 750 µl of the supernatant was mixed in 3 ml of SPG (0.25 M sucrose, 10 mM sodium phosphate, 5 mM glutamic acid). This mixture (containing *C. trachomatis* EBs) was added to $4x10^6$ of HeLa cells previously washed with HBSS (Life Technologies), which had been seeded the day before in a tissue culture flask with a surface area of 75 cm^2 (T75). After 1 h of incubation, the supernatant was removed and 10 ml of complete DMEM containing 0.3 U/ml of penicillin and 1 µg/ml of cycloheximide was added. The cells were then incubated again for about 2 days (44 h) at 37ºC in the presence of 5% [v/v] CO_2.

After this second incubation of about 2 days, phase contrast microscopy typically revealed that the majority of the cells were infected, showing large but aberrant inclusions. At this point, the media was removed from the T75 flask and the infected cells were scrapped with 3 ml of ice-cold SPG. The cells were then lysed by sonication (using a UP 200S Ultrasonicator Processor, Hielscher; 2 pulses of 20 s with 100 W and a cycle of 0.5). Then, 3 ml of the supernatant was split in two parts of 1.5 ml that were used to infect two sets of $1.3x10^6$ HeLa cells seeded the day before in 2 tissue culture flasks with a surface area of 25 cm^2 (T25). After 1 h of incubation, the inoculum was removed and 10 ml of complete DMEM media containing 0.3 U/ml of penicillin and 1 µg/ml of cycloheximide was added. The cells were then incubated for about 3 days (72 h) at 37ºC in the presence of 5% [v/v] CO_2.

After this third incubation, it was usual not to detect many inclusions by phase contrast microscopy and those seen were usually aberrant. In any instance, the old media was removed from the two T25 flasks

and the cells were scrapped in 1.5 ml of ice-cold SPG (duplicate T25 flasks were lysed in the same 1.5 ml solution). Cells were lysed by sonication (as described above) and centrifuged (at 150 *g* for 5 min, 25°C) and the 1.5 ml lysate was used to infect 1.3×10^6 HeLa cells seeded the day before in 1 T25 flasks. After 1 h of incubation, the inoculum was removed and 10 ml of DMEM medium containing 1 U/ml of penicillin and 1 µg/ml of cycloheximide was added. The cells were incubated again for about 3 days (72 h) at 37ºC in the presence of 5% [v/v] CO_2.

After this fourth incubation, if the transformation was successful, wild-type inclusions were easily detected by phase-contrast microscopy. At this point, the old media was removed from the T25 flasks and the infected cells were scrapped in 1 ml of ice-cold SPG. Cells were again lysed by sonication (as described above), centrifuged (at 150 *g* for 5 min, 25°C), and 50 µl of the lysate was used to infect 1.3×10^6 HeLa cells seeded the day before in T25 flasks. At this point samples of the lysates were stored in aliquots in 10% [v/v] FBS at -80ºC. After 1 h of incubation, the inoculum was removed and 10 ml of DMEM medium containing 1 U/ml of penicillin and 1 µg/ml of cycloheximide was added. The cells were incubated again for about 3 days (72 h) at 37ºC in the presence of 5% [v/v] CO_2.

C. trachomatis transformants were selected after 2 additional passages in 1 U/ml of penicillin and 1 extra passage in 10 U/ml of penicillin. The amplification process was repeated until enough infectious particles were recovered to generate a frozen stock and proceed to clonal isolation (see below). The list of *C. trachomatis* strains obtained by transformation in this work is summarized in Table A3 (Annexes).

Clonal isolation of *C. trachomatis* strains by plaque assay purification

The following procedure was adapted from Nguyen & Valdivia (2013). First, $4x10^5$ Vero cells were seeded per well in a 6-well plate. Cells were incubated at 37ºC in the presence of 5% [v/v] CO_2 for about 24 h and allowed to form a confluent and homogeneous monolayer. Aliquots of infectious particles of the transformed *C. trachomatis* strains were thawed on ice and six 10-fold serial dilutions in a volume of 1 ml were done. Prior to infection, Vero cells were washed once with HBSS. Each well was infected with 500 µl of the respective dilution and swirled to ensure an even mixture. The infected plates were centrifuged at 2700 *g* for 30 min at 15ºC. The media was then replaced with complete DMEM containing 1 U/ml of penicillin and 1 µg/ml of cycloheximide and the cells were incubated at 37ºC in the presence of 5% [v/v] CO_2 for 24 h.

An overlay solution of 0.54 % (w/v) Seakam® agarose (Lonza) in DMEM containing 10% (v/v) FBS, 1 U/ml of penicillin and 1 µg/ml of cycloheximide was prepared and kept in a warm water bath at 55ºC before adding to the infected cells. The media was carefully aspirated from each well of the 6-well plates with the infected Vero cells and 2 ml of the overlay solution was quickly added to each well. Agarose was allowed to completely solidify at room temperature for 15 min. Additionally, after the overlay solution had solidified, 2 ml of complete DMEM was added on top. Cells were then incubated at 37ºC in the presence of 5% [v/v] CO_2 for 6 days. At this point, plaques were usually visible to the eye.

Isolated plaques were picked using a 200 µl sterile barrier pipette tip. Tips were then placed in a 1.5 ml tube previously loaded with 100 µl

of complete DMEM containing 1 U/ml of penicillin and 1 µg/ml of cycloheximide. Media was carefully removed from a 96-well plate seeded the day before with $1x10^4$ Vero cells per well, and the 100 µl of complete DMEM containing each plaque were added. After inoculating the wells, the 96-well plate was centrifuged at 2200 *g* for 30 min at 15ºC. Cells were incubated at 37ºC in the presence of 5% [v/v] CO_2 for 2 days.

Each well of the 96-well plate containing Vero cells infected with plaque purified *C. trachomatis* were lysed by osmotic shock through incubation in 100 µl of H_2O for 10 min. After this, 100 µl of SPG 2X (0.4 mM sucrose, 34 mM Na_2HPO_4, 6 mM NaH_2PO_4, 10 mM L-glutamic acid) was added to each well. This 200 µl solution containing the infectious particles was then added to each well of a 24 well plate seeded the previous day with $2x10^5$ HeLa cells per well. Infection proceeded as described above. This way each plaque-purified clone was propagated into HeLa cells providing a higher amount of viable bacteria to be stored. Stock aliquots were stored in 10% [v/v] FBS at -80ºC.

Quantification of infectious progeny

HeLa cells infected by *C. trachomatis* strains for different times were lysed by osmotic shock (10 min incubation in sterile H_2O). Dilutions of these lysates in SPG were used to infect freshly seeded HeLa cells. The newly infected cells were fixed after 24 h p.i., *C. trachomatis* bacteria were immunolabeled using anti-MOMP antibodies (see below), and the number of inclusion forming units (IFUs/ml) was calculated after determination of the number of infected cells/field of view by immunofluorescence microscopy (see Chapter III) (Scidmore, 2005).

Antibodies

The following primary antibodies were used: rabbit polyclonal anti-CT143 (generation and purification described in chapter III; 1:50 dilution for immunofluorescence); rat monoclonal anti-HA (3F10; Roche; 1:1000 dilution for immunoblotting and 1:200 dilution for immunofluorescence); mouse monoclonal anti chlamydial Hsp60 (A57-B9; Thermo Fisher Scientific; 1:1000 dilution for immunoblotting and 1:200 dilution for immunofluorescence); goat polyclonal anti-major outer membrane protein (MOMP) of *C. trachomatis* (ab34414; Abcam; 1:1000 dilution for immunoblotting); mouse monoclonal anti-tubulin (clone B-5-1-2; Sigma Aldrich; 1:1000 for immunoblotting); rabbit polyclonal anti-Myc (ab9106; Abcam; 1:1000 for immunoblotting); mouse monoclonal anti HA (HA-7; Sigma Aldrich; used for co-immunoprecipitation) and mouse monoclonal anti Myc (9E10; Sigma Aldrich; used for co-immunoprecipitation).

For immunoblotting the secondary antibodies used were all horseradish peroxidase (HRP)-conjugated (GE Healthcare and Jackson ImmunoResearch; used at 1:10000).

For immunofluorescence microscopy, we used the following secondary antibodies: Rhodamine Red^{TM}–X-Conjugated AffiniPure Donkey anti-rabbit (Jackson ImmunoResearch Laboratories; 1:200); goat anti-mouse Alexa Fluor® 488 (AF488) (Jackson ImmunoResearch Laboratories; 1:200); anti-rat conjugated to rhodamine RedX (Jackson ImmunoResearch Laboratories; 1:200) and AF488 AffiniPure Donkey anti-rat (Jackson ImmunoResearch Laboratories; 1:200).

Immunoblotting and immunofluorescence microscopy

Immunoblotting and immunofluorescence microscopy were done as described in Chapter III.

Co-immunoprecipitation

Co-immunoprecipitation experiments were done essentially as described in (Pais *et al.*, 2013). Strains used included derivatives of *Y. enterocolitica* ΔHOPEMT carrying pBAD/Myc-His A (Life Technologies) encoding C-terminally Myc-tagged *C. trachomatis* CT142 under the control of the *E. coli* arabinose promoter (P_{BAD}) and pBBR1MCS-2 (Kovach *et al.*, 1995) derivatives encoding C-terminally hemaglutittinin (HA)-tagged CT142, CT143 and CT144 under the control of the promoter of the *Yersinia* T3SS effector gene *yopE* (P_{yopE}). Overnight cultures were diluted to an OD_{600} of 0.1 in BHI supplemented with 5 mM of $CaCl_2$ and the appropriate antibiotics (non-secreting conditions). The bacterial cultures were incubated for 2 h at 26ºC, at 150 rpm, and then shifted to 37ºC (to induce expression from the *yopE* promoter) and incubated for an additional 4 h at 150 rpm. At the time of the temperature shift, 0.2% (w/v) L-arabinose was added to the cultures to induce expression under control of pBAD. After the 4 h incubation at 37ºC, bacterial cultures were transferred to ice and the equivalent of 5 OD_{600} units were centrifuged at 4500 *g* for 10 min at 4ºC. The bacterial cells were washed once with ice-cold PBS and lysed in co-immunoprecipitation (co-IP) buffer (20 mM Tris-HCl, pH 8.0; 150 mM NaCl; 1% [v/v] Triton X-100) using the BugBuster® reagent (Novagen). After centrifugation at 17000 *g* for 10 min at 4ºC, the lysate supernatant was pre-cleared with 20 µl of Pierce Protein G agarose (Thermo Fisher Scientific), which had been previously washed with ice-cold co-IP buffer, by incubation for 1 h with end-over-end rotation. A sample of the pre-

cleared lysate was collected for further analysis (input), and the remaining was incubated with 10 µl of Pierce Protein G agarose that had been previously incubated with 5 µg of mouse monoclonal anti HA (HA-7; Sigma Aldrich) or with 5 µg of mouse monoclonal anti Myc (9E10; Sigma Aldrich) for 1 h 30 min at 4ºC end-over-end rotation. The IP reactions were carried out overnight at 4ºC with end-over-end rotation. The samples were then centrifuged at 500 *g* for 2 min at 4ºC and washed 3 times with ice-cold co-IP buffer. Finally, 25 µl of Laemmli SDS-PAGE loading buffer (Laemmli buffer 5 X: Tris-HCl 0.25M (pH 6.8), SDS 10 % [w/v], β-mercaptoethanol 0.5 M, Bromophenol Blue 0.5 % [w/v]) was added to the agarose beads and the samples were incubated for 5 min at 100ºC (output). Input and output samples were analyzed by immunoblotting.

4.4. RESULTS

4.4.1. Ectopic expression of CT143-2HA in *C. trachomatis* from the *incD* promoter

To understand if the observed localization of endogenous CT143 in the inclusion lumen could be recapitulated by epitope-tagged and ectopically expressed CT143, we constructed a derivative of *C. trachomatis* L2/434 carrying a plasmid encoding CT143 with a C-terminal 2HA epitope-tag (CT143-2HA). In this plasmid, *ct143* is expressed under the control of the promoter of the gene encoding *C. trachomatis* inclusion membrane D (IncD; P_{incD}) and its transcription is also halted by the *incD* terminator. These elements (P*incD* and *incD* terminator) have been employed successfully to express different fluorescent proteins (GFP, mCherry and CFP) in *C. trachomatis* by the same cloning vector we used (p2TK2-SW2) (Agaisse & Derré, 2013).

We first analyzed the expression of CT143-2HA in *C. trachomatis*. For this, HeLa 229 cells were inoculated with the *C. trachomatis* recombinant strain expressing CT143-2HA, or with the parental L2/434 strain. Whole cell extracts were prepared at 5, 20, 30 and 40 h p.i., and expression of CT143-2HA was analyzed by immunoblotting with anti-HA antibodies (Fig.4.1A). While no HA signal was detected in extracts of cells infected by the parental L2/434 strain, expression of CT143-2HA could be detected from 20 h p.i. in extracts of cells infected by the recombinant strain (Fig.4.1A). Although CT143-2HA could also be detected at 30 and 40 h p.i., slightly higher levels of CT143-2HA were detected at 20 h p.i. (Fig.4.1A).

Next, we examined the subcellular localization of CT143-2HA in HeLa cells infected as described above by the recombinant *C. trachomatis* strain, but fixed at 20 and 30 h p.i. The cells were then immunolabeled with anti-Hsp60 (Hsp60 is a cytosolic *C. trachomatis* housekeeping chaperone, and therefore this antibody labels the bacteria) and anti-HA antibodies, followed by appropriate fluorophore-conjugated antibodies. The cells were then analyzed by immunofluorescence microscopy, which both at 20 h p.i. and at 30 h p.i. revealed a perfect co-localization between CT143-2HA and Hsp60 (Fig. 4.1B). Similar results were obtained with *C. trachomatis* strains expressing CT142-2HA or CT144-2HA under control of P_{incD} (data not shown). At earlier time-points of infection (1 h p.i. and 3 h p.i) it appeared that the CT143-2HA immunofluorescence signal was adjacent, but not co-localizing with the bacteria (i.e., with Hsp60 immunofluorescence signal), but this was not investigated in further detail (data not shown).

4.4.2. Ectopic expression of CT142-2HA, CT143-2HA and CT144-2HA in *C. trachomatis* from the *ct142* promoter

Because the signal observed for CT143-2HA in the strain expressing the protein in a plasmid under the control of the P_{incD} differed drastically from what we had seen for the endogenous protein (compare Fig. 4.1B with Figs. 3.7 and 3.9 in Chapter III), we wondered if this was due to the higher levels of expression of the ectopically expressed protein (the plasmid has a copy number of 4-6) or because of the different promoters driving expression of CT143 (P_{incD} and promoter of the *ct142-ct143-ct144* operon, P_{ct142}).

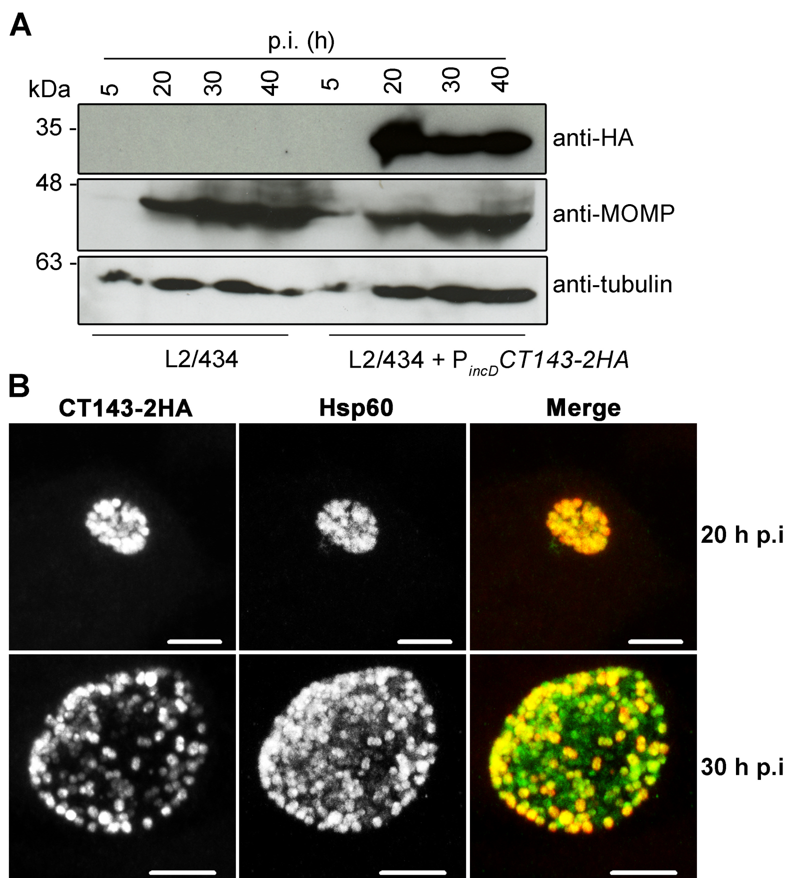


Figure 4.1. Expression and subcellular localization of CT143-2HA, when expressed under control of the *incD* promoter. HeLa 229 cells were infected by either parental *C. trachomatis* L2/434 strain or recombinant *C. trachomatis* expressing CT143-2HA under the control of the *incD* promoter. (A) At 5, 20, 30, and 40 h p.i. the cells were processed for immunoblotting using anti-HA antibodies. Expression of *C. trachomatis* MOMP was monitored to control the number of bacteria on each lane, and tubulin was used as loading control of host cell proteins. (B) At 20 and 30 h p.i. cells infected by the recombinant strain were fixed with methanol, immunolabeled with anti-HA and anti-Hsp60 antibodies, and with appropriate fluorophore-conjugated antibodies. Stained cells were analyzed by confocal immunofluorescence microscopy. Images are combined projections of multiple 0.2 µm *z*-sections. Scale bars, 5 µm.

We therefore constructed four *C. trachomatis* recombinant strains derived from L2/434 strain, each encoding a plasmid derived from p2TK2-SW2 (Agaisse & Derré, 2013) (Fig 4.2A):

i) a strain harbouring a plasmid encoding CT142-2HA expressed from P_{ct142};
ii) a strain harbouring a plasmid encoding CT143-2HA expressed from the P_{ct142};
iii) a strain harbouring a plasmid encoding CT144-2HA expressed from P_{ct142};
iv) a strain harbouring a plasmid encoding untagged CT142 and CT143, and CT144-2HA.

In all four plasmids, transcription is driven from the P_{ct142}, as in the chromosome of *C. trachomatis*, and transcription is halted by the *incD* terminator (Agaisse & Derré, 2013).

We performed control experiments to confirm that the constructed strains were not affected in their growth characteristics and expressed the expected proteins. First, a one-step growth curve analysis, in which we quantified the number of infectious progeny at different times of infection of HeLa cells, revealed that the four newly constructed strains grew at rates comparable to the parental L2/434 strain (Fig. 4.2B). This indicated that the overall physiology of the strains was not affected by the plasmids or by overexpression of CT142, CT143, and/or CT144. Second, by immunoblotting using extracts of HeLa cells infected for 30 h by the L2/434 parental strain or by each recombinant strain (using anti-HA antibodies), we confirmed that the HA-tagged proteins were expressed and that they migrated on SDS-PAGE according to their predicted molecular mass (CT142-2HA, 33.5 kDa; CT143-2HA, 34 kDa; CT144-2HA, 34 kDa) (Fig. 4.2C). However, in the strain expressing CT142-2HA we detected 2 different bands (Fig. 4.2C), an observation that needs to be further verified.

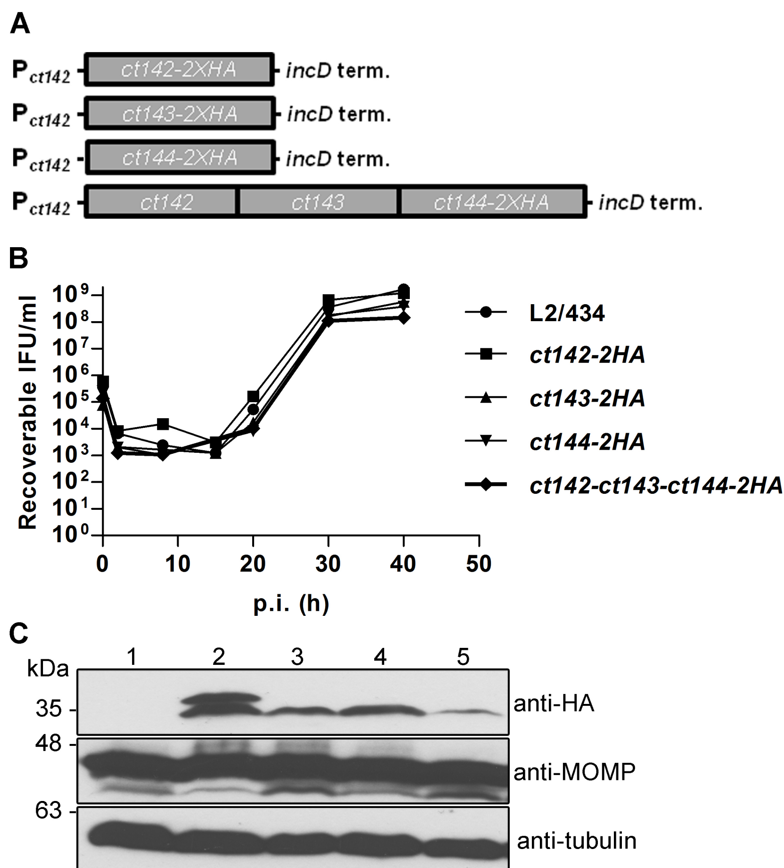


Figure 4.2. ***C. trachomatis*** **strains ectopically expressing CT142-2HA, CT143-2HA and/or CT144-2HA from the** ***ct142*** **promoter.** (A) Schematic representation of the genes present in the plasmid of each recombinant *C. trachomatis* strain. (B) Parental *C. trachomatis* L2/434 strain and the derived transformed strains exhibit similar growth kinetics. One step growth curves were conducted in HeLa 229 cells infected with the respective strains at a MOI of 50 and recoverable IFUs were determined at various times p.i., as detailed in Materials and Methods. (C) Expression of CT142-2HA, CT143-2HA and CT144-2HA during infection was assessed by immunoblotting with anti-HA antibodies, using extracts from HeLa cells infected for 30 h with: (1) parental strain L2/434; (2) strain bearing plasmid encoding CT142-2HA; (3) strain bearing plasmid encoding CT143-2HA; (4) strain bearing plasmid encoding CT144-2HA; (5) strain bearing plasmid encoding CT142-CT143-CT144-2HA. Expression of *C. trachomatis* MOMP was monitored to control the number of bacteria on each lane, and tubulin was used as loading control of host cell proteins.

Next, we followed the expression of CT142-2HA, CT143-2HA, and CT144-2HA by immunoblotting of extracts of HeLa cells either uninfected or infected for different times (2, 8, 15, 20, 30 and 40 h) with each of the *C. trachomatis* strains expressing these proteins. Expression of CT142-2HA, CT143-2HA and CT144-2HA could be detected from 20 h p.i., but the protein levels were higher at 30 or 40 h p.i. (Fig. 4.3). For CT142 and CT143 the profile of expression observed by imunoblotting was similar to that observed for the endogenous proteins (see Fig. 3.4; Chapter III).

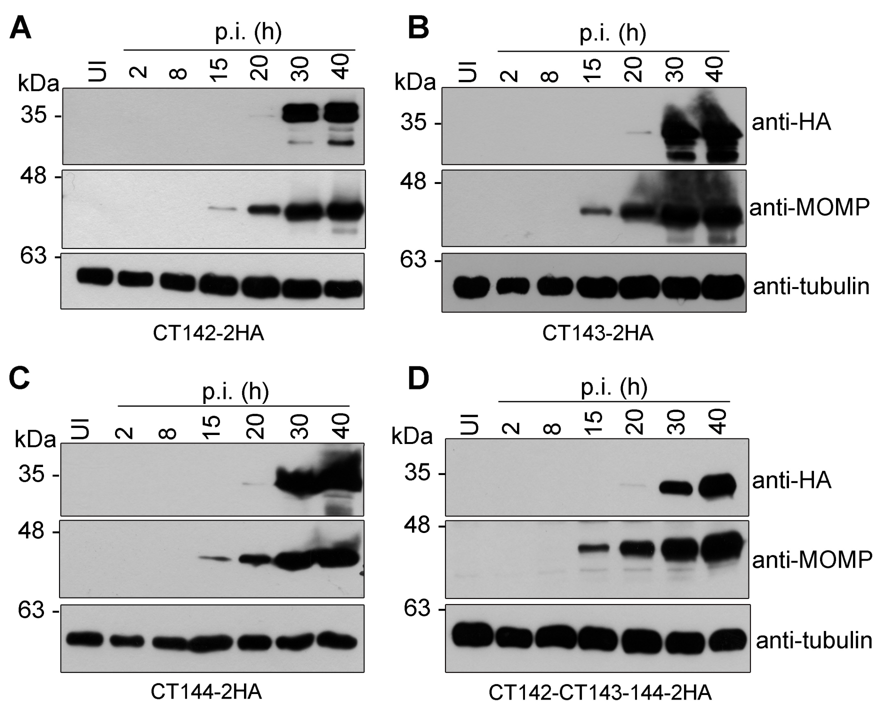
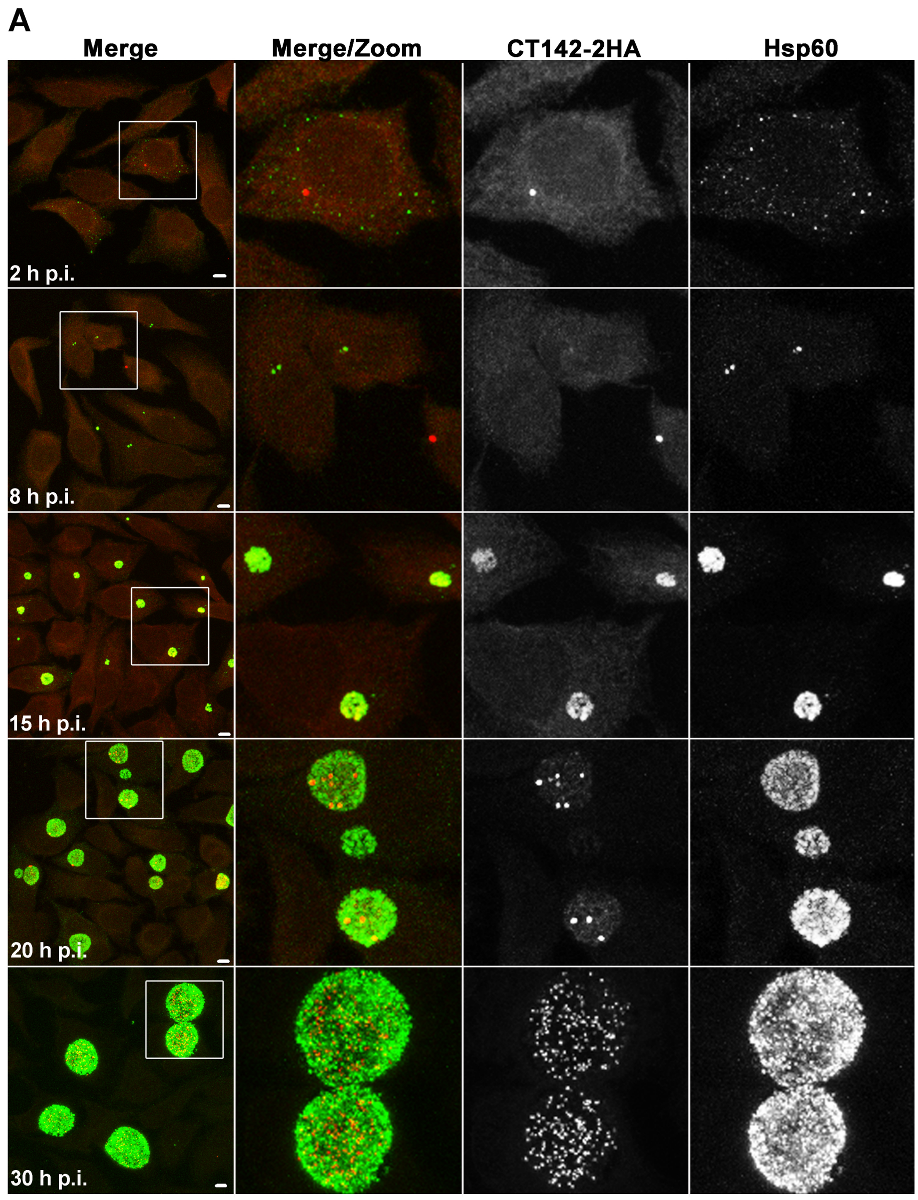


Figure 4.3 Kinetics of expression of CT142-2HA, CT143-2HA, and CT144-2HA in recombinant *C. trachomatis* strains. Expression of CT142-2HA (A), CT143-2HA (B) CT144-2HA (C and D) at the depicted times of infection by bacteria expressing the indicated proteins in a plasmid was assessed by immunoblotting with anti-HA antibodies to probe extracts of HeLa cells either left uninfected (UI) or that had been inoculated with strains expressing the indicated proteins. Expression of *C. trachomatis* MOMP was monitored to control the number of bacteria on each lane, and tubulin was used as loading control of host cell proteins.

4.4.3. Analysis of the subcellular localization of CT142-2HA, CT143-2HA, and CT144-2HA in infected HeLa 229 cells

To analyze the subcellular localization of CT142-2HA, CT143-2HA and CT144-2HA expressed in the plasmid from the P_{ct142}, we inoculated HeLa cells with each of the *C. trachomatis* strains represented in Fig. 4.2A. The cells were fixed at 2, 8, 15, 20, and 30 h p.i. and were then immunolabeled for HA (to visualize CT142, CT143, or CT144) and for Hsp60, followed by the appropriate fluorophore-conjugated secondary antibodies. The cells were then analyzed by immunofluorescence microscopy (Fig. 4.4 A-D). In all cases, at 2 and 8 h p.i. the Hsp60 labeling could already be seen (small green dots; Fig. 4.4). Although at these time-points we also sometimes detected a signal for HA (small red dots; Fig. 4.4), we cannot rule out that this signal is due to background. At 15 h p.i. we could observe in all cases small but defined chlamydial inclusions (Fig. 4.4). Furthermore, at this time point, in all cases, we could detect a signal for HA that was clearly distinct from background and that overlapped with the signal for Hsp60 (Fig. 4.4). However, at 20 h p.i., the signal for CT142-2HA (Fig. 4.4A), CT143-2HA (Fig. 4.4B) or CT144-2HA (Fig. 4.4C and 4.4D), while still clearly distinct from background, did not overlap with the signal of Hsp60. At this time point (20 h p.i.), and in all cases, we started to distinguish globular structures revealed by the HA signal (Fig. 4.4) that at 30 h p.i. were much more abundant (Fig. 4.4). These globular structures did not seem to co-localize with the Hsp60 signal, but appear to be within the inclusion lumen.


A
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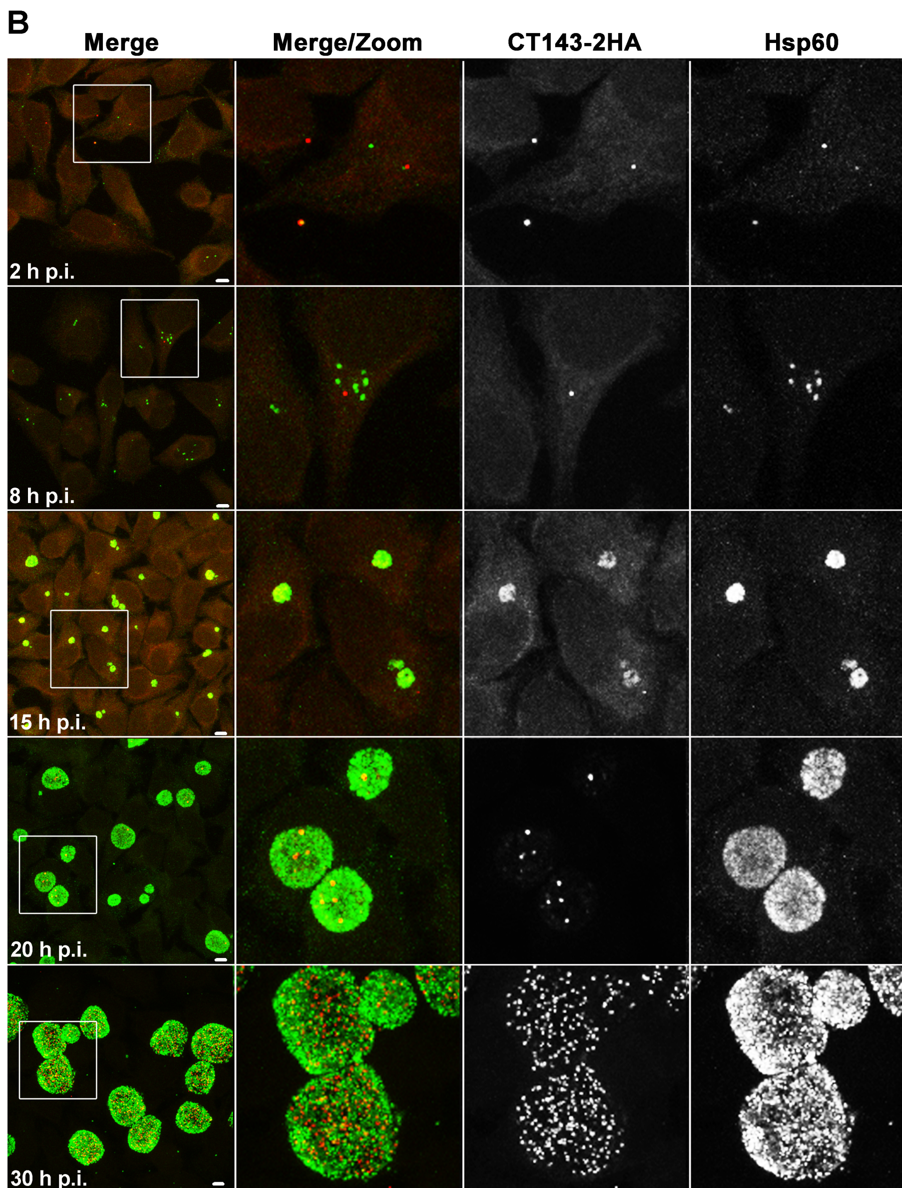
Merge/Zoom
CT142-2HA
Hsp60
2 h p.i.
8 h p.i.
15 h p.i.
20 h p.i.
30 h p.i.


B
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CT143-2HA
Hsp60
2 h p.i.
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20 h p.i.
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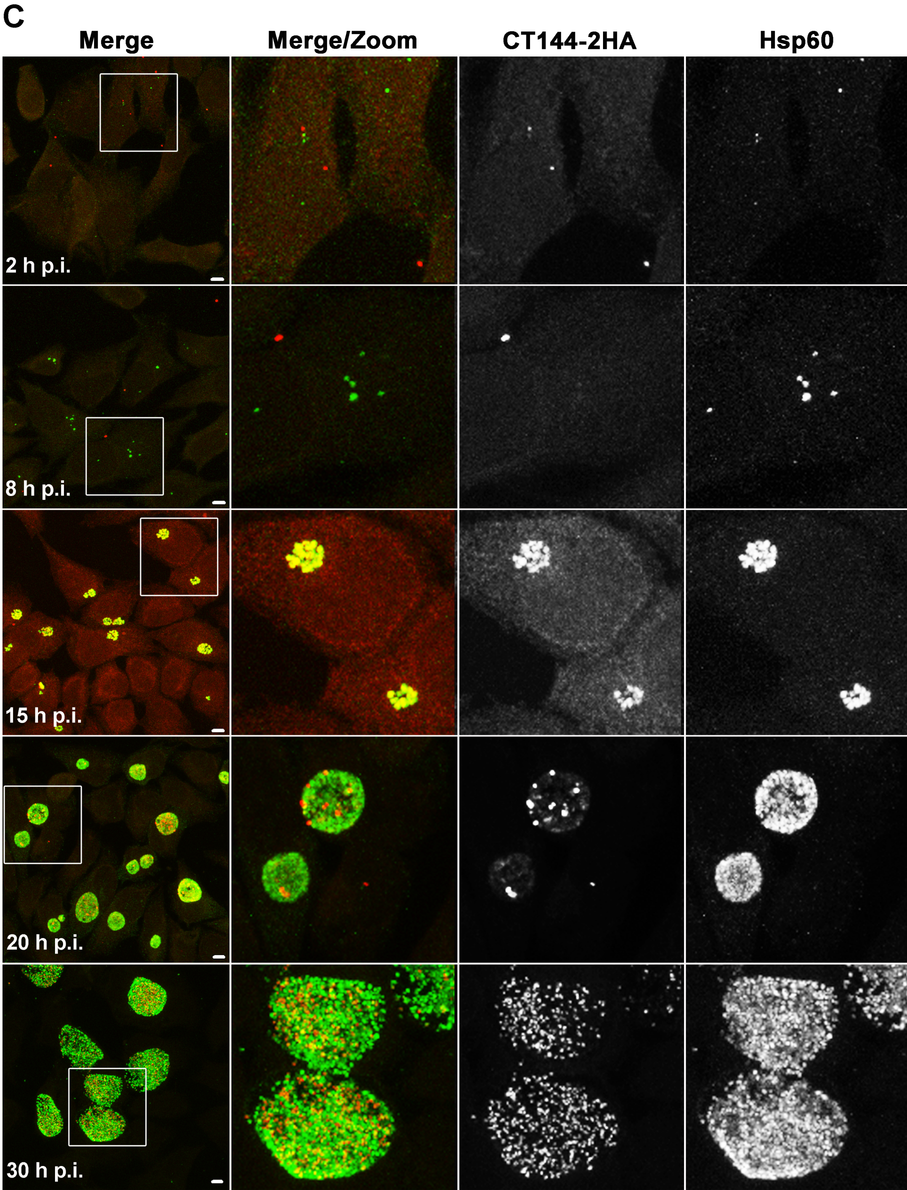
C
Merge
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CT144-2HA
Hsp60
2 h p.i.
8 h p.i.
15 h p.i.
20 h p.i.
30 h p.i.

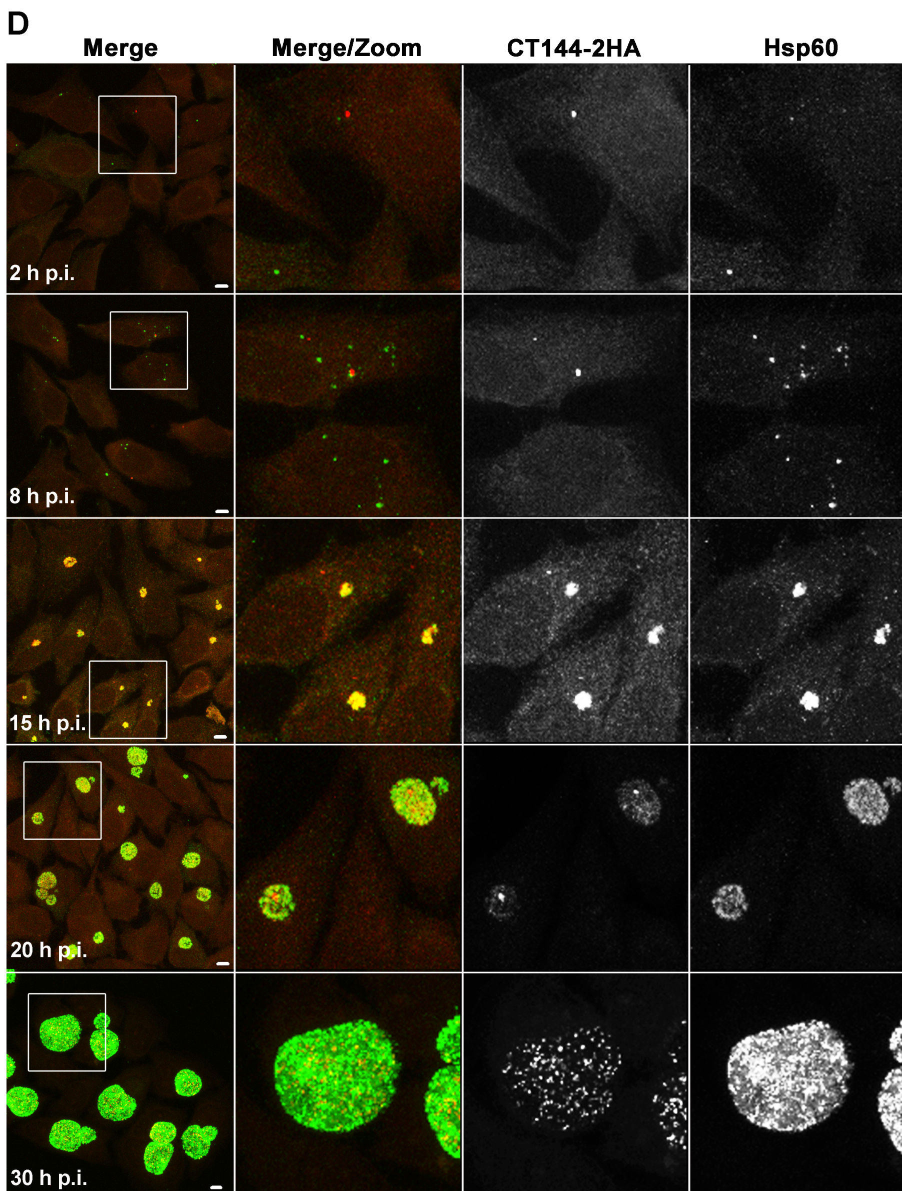

Figure 4.4. Subcellular localization of CT142-2HA, CT143-2HA and CT144-2HA in infected HeLa cells. HeLa 229 cells were infected for 2, 8, 15, 20 or 30 h with derivatives of *C. trachomatis* L2 strain 434/Bu ectopically expressing CT142-2HA (A), CT143-2HA (B), CT144-2HA (C), or CT142-CT143-CT144-2HA (D) from a plasmid. At these time-points the cells were fixed with methanol, immunolabeled with anti-HA and anti-Hsp60 antibodies, and appropriate fluorophore-conjugated secondary antibodies, and analyzed by confocal immunofluorescence microscopy. Images are combined projections of multiple 0.2 µm *z*-sections. Scale bars, 5 µm. In the area delimited by a white square (left panel), images were magnified 3X and the respective amplification is shown with both channels (merge/zoom) or with each channel individually (Hsp60 and HA).

This indicated a shift in the subcellular localization of each of the proteins (CT142-2HA, CT143-2HA, CT144-2HA) from the bacteria (i.e., co-localization with Hsp60) at 15 h p.i. to the lumen of the inclusion (i.e. no co-localization with Hsp60 but within the boundaries of its labeling) at 20 h p.i. and thereafter.

We then analyzed in more detail the HA immunofluorescence signal observed at 30 h p.i. in HeLa cells infected with the *C. trachomatis* strains ectopically expressing CT142-2HA, CT143-2HA, or CT144-2HA (Fig. 4.5). This confirmed that the globular structures revealed by the HA immunofluorescence signal of each of the ectopically expressed proteins did not overlap with the Hsp60 signal (Fig 4.5). Additionally, the area defined by the HA immunofluorescence signal for each ectopically expressed protein was smaller than the area defined by the Hsp60 immunofluorescence signal, suggesting that the observed globular structures are not bacteria (Fig. 4.4D). The observations illustrated in Fig. 4.4 and Fig. 4.5 essentially re-capitulated those previously done for endogenous CT143 using an anti-CT143 antibody (see Fig. 3.6, Fig. 3.7 and Fig. 3.9; Chapter III).

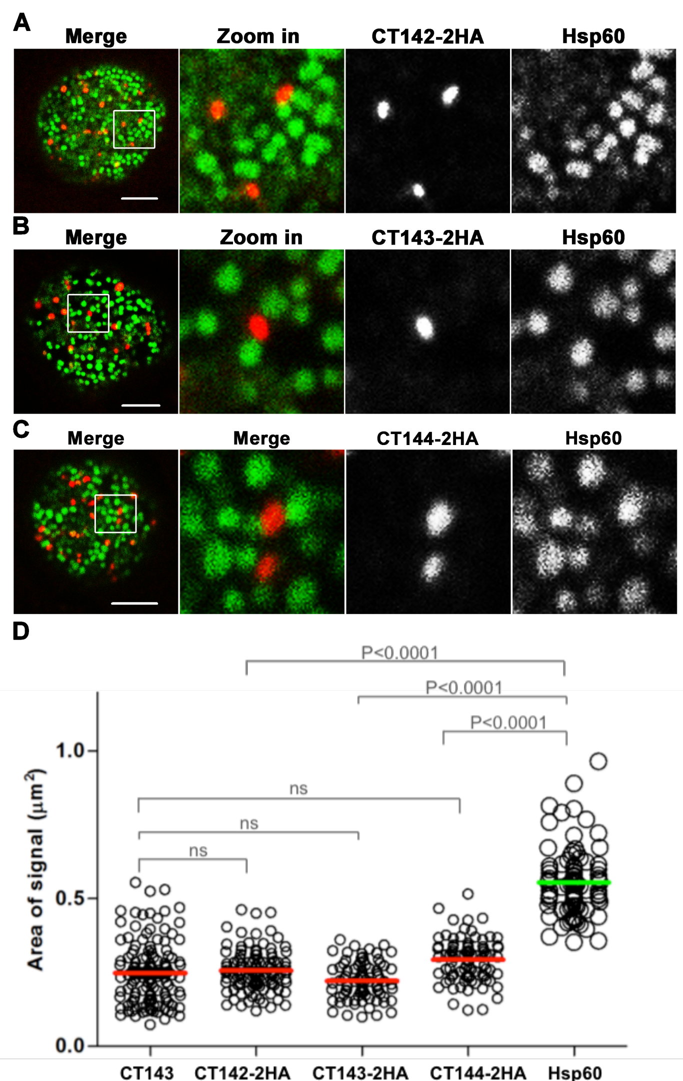

Figure 4.5. Subcellular localization of CT142-2HA, CT143-2HA, and CT144-2HA in HeLa cells infected for 30 h. HeLa cells infected for 30 h with *C. trachomatis* strains ectopically expressing CT142-2HA (A), CT143-2HA (B), or CT144-2HA (C), were fixed and immunolabeled with anti-HA and anti-Hsp60 antibodies as described in the legend of Figure 4.3. Cells were analyzed by confocal immunofluorescence microscopy. Images are single *z* sections. Scale bars, 5 μm. In the area delimited by a white square (left panel), images were magnified 3X and the respective amplification is shown with both channels (merge/zoom) or with each channel individually (Hsp60 and HA). (D) Comparison of the area of the immunofluorescence signal of CT143 (from Chapter III, used for comparative purposes), CT142-2HA, CT143-2HA, CT144-2HA, and Hsp60. Measurements were done using using Fiji software (Schindelin *et al.*, 2012) for at least 120 (CT143), 100 (CT142-2HA), 90 (CT143-2HA), 90 (CT144-2HA) and 90 (Hsp60) particles, chosen from 3 independent images. *P*-values were calculated by a two-tailed unpaired Student's *t*-test.

4.4.4. CT143 and CT144-2HA co-localize in the lumen of the inclusion

We next asked if the immunofluorescence signal of CT143 would co-localize with the immunofluorescence signal of CT144-2HA. For this, HeLa 229 cells were infected for 30 h by the *C. trachomatis* L2/434 derivative carrying a plasmid expressing CT142, CT143 and CT144-2HA. The cells were then fixed and immunolabeled using anti-CT143 and anti-HA antibodies, and appropriate fluorophore-conjugated antibodies. Analysis of these cells by immunofluorescence microscopy revealed a near perfect co-localization between CT143 and CT144-2HA (Fig. 4.6).

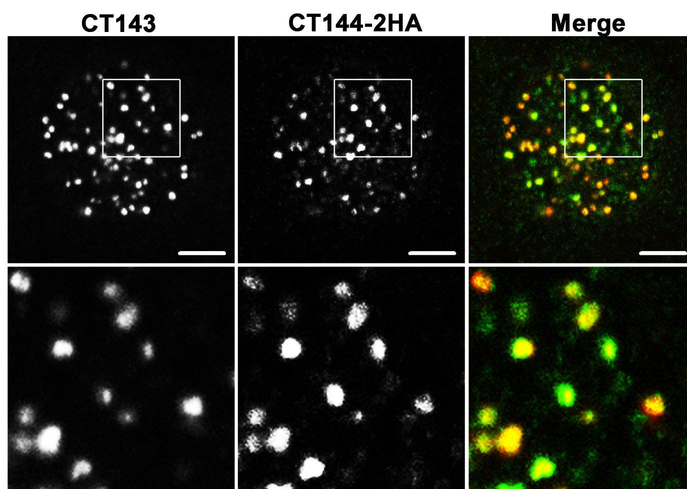

Figure 4.6. Subcellular localization of CT143 and CT144-2HA. HeLa 229 cells were infected for 30 by *C. trachomatis* L2/434 with a plasmid carrying p_{ct142}-*ct142-ct143-ct144-2HA* and then fixed with methanol. Fixed infected cells were immunolabeled with anti-CT143 and anti-HA antibodies, and with appropriate fluorophore-conjugated antibodies. Stained cells were analyzed by confocal immunofluorescence microscopy. Images are combined projections of multiple 0.2 µm *z*-sections. Scale bars, 5 µm. In the area delimited by a white square (upper panel) images were magnified 3X (lower panel).

4.4.5. Analysis of protein-protein interactions between CT142, CT143, and CT144

The appearance of endogenous CT143 and ectopically expressed CT142-2HA, CT143-2HA or CT144-2HA in infected cells as globular structures within the inclusion lumen, which at least in the case of CT144-2HA co-localize with CT143, led us to hypothesize that these structures could be a multi-protein complex. Therefore using *Y. enterocolitica* as heterologous expression host, we analyzed by co-immunoprecipitations (co-IP), if these proteins can interact.

Y. enterocolitica ΔHOPEMT strains carrying a plasmid encoding C-terminally Myc-tagged CT142 (CT142-Myc) under the control of the *E. coli* arabinose promoter (P_{BAD}) and a plasmid encoding CT142-HA, CT143-HA or CT144-HA under the control of the promoter of the *Yersinia* T3SS effector gene *yopE* (P_{yopE}) were grown in conditions where no protein secretion by the T3SS occurs (5 mM of free Ca^{2+} ions). The bacterial cells were lysed and proteins in the lysate supernatants (input) were immunoprecipitated with mouse monoclonal anti-HA (Fig. 4.7A) or anti-Myc (Fig. 4.7B) antibodies bound to Protein G agarose beads (output). The input and output fractions were analyzed by immunoblotting with rabbit polyclonal anti-Myc and rat monoclonal anti-HA antibodies.

When CT142-Myc and CT142-HA were co-expressed, CT142-Myc was pulled down by the IP with the HA antibody (Fig. 4.7A) and CT142-HA was pulled down by the IP with the Myc antibody (Fig. 4.7B), indicating that CT142 can interact with itself. In the same manner when CT142-Myc and CT-143-HA were co-expressed, CT142-Myc was pulled-down in the IP with HA antibody (Fig. 4.7A) and the CT143-HA was immunoprecipitated by the IP with the Myc

antibody (Fig. 4.7B), indicating that CT142 and CT143 can interact. CT144 was only obtained in small amounts in cell lysates, and the results obtained in the Co-IPs involving this protein were not conclusive. However, we occasionally could detect a pull-down of CT142-Myc by the IP of CT144-HA with the HA antibody (Fig. 4.7). These results indicated that CT142 can bind to itself and to CT143, and possibly also to CT144.

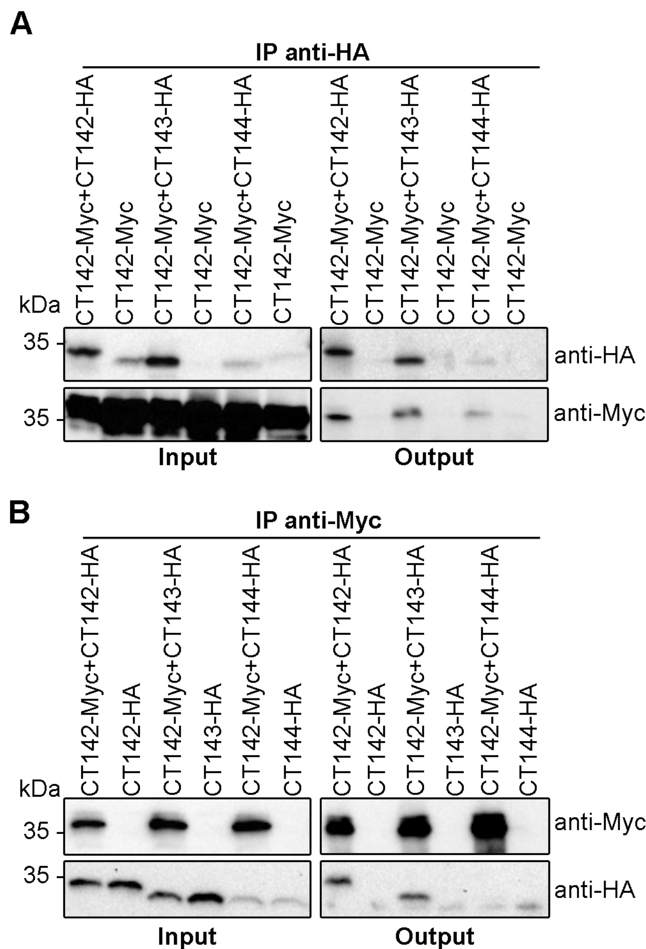

Figure 4.7 CT142 can interact with itself and with CT143. *Y. enterocolitica* ΔHOPEMT strains expressing the indicated proteins were grown in conditions blocking the activity of its T3SS (5 mM of Ca^{2+} ions). The bacterial cells were lysed and proteins in the lysate supernatants (input) were immunoprecipitated with mouse monoclonal anti-HA (A) or anti-Myc (B) antibodies bound to Protein G agarose beads (output). The input and output fractions from the immunoprecipitations (IPs) were analyzed by immunoblotting with rabbit polyclonal anti-Myc and rat monoclonal anti-HA antibodies.

4.5. DISCUSSION

In this Chapter, we used the recently developed methods for transformation of *C. trachomatis* to ectopically express CT142-2HA, CT143-2HA and CT144-2HA under the control of P_{ct142}. This allowed us to further confirm the localization of CT143 in globular structures within the inclusion lumen in infected HeLa cells from 20 h p.i., which was described for the endogenous protein in Chapter III. Furthermore, we could extend this observation to CT142 and CT144. In addition, the intralumenal globular structures labeled by anti-CT143 antibodies co-localized with those labeled by anti-HA antibodies in cells infected by a *C. trachomatis* strain co-expressing CT142, CT143 and CT144-2HA under the control of P_{ct142} in a plasmid. Finally, we showed that CT142 can interact with itself and with CT143, while an interaction between CT142 and CT144 was also detected (but was not reproducible). Overall, these results indicated that CT142, CT143, and CT144 could be secreted into the inclusion lumen where they might form large protein complexes of unknown function.

The subcellular localization of ectopically expressed CT142-2HA, CT143-2HA, and CT144-2HA in infected HeLa cells is quite distinct at 15 h p.i. and at 20 h p.i. While at 15 h p.i. CT142-2HA, CT143-2HA, and CT144-2HA apparently localize within the bacteria, at 20 h p.i. and thereafter, the proteins appear as globular structures within the inclusion lumen that do not co-localize with the bacteria. Similar observations had been made for endogenous CT143 (Chapter III). This result suggests that CT142, CT143 and CT144 are expressed at mid stages of the infection (15 h p.i.), after which they start being secreted from the bacteria into the lumen of the inclusion.

In Chapter II, using *Yersinia enterocolitica* as heterologous host we identified CT142, CT143 and CT144 as proteins with a high likelihood of being T3SS substrates. The T3SS injectisome is a complex nanomachine that allows bacteria to deliver protein effectors across eukaryotic cellular membranes (Cornelis, 2006). However, secretion of CT142, CT143 and CT144 differs from the classical T3SS mechanism described for effectors as they appear to be transported only across the two bacterial membranes into the lumen of the inclusion, not reaching the host cell cytosol. Several hypotheses could explain this observation: i) the classical T3S model might not apply to all type III secreted proteins in *Chlamydia*. In fact, other chlamydial proteins were found to be secreted into the inclusion lumen like CT620 and CT621 (Muschiol *et al.*, 2011), or CT049 and CT050 (Jorgensen & Valdivia, 2008); ii) CT142, CT143 and CT144 could be translocated across the inclusion membrane but its levels within the host cytosol cannot be detected by immunofluorescence microscopy. This could be tested by using the recently described BlaM-fusion reporter assays (Mueller & Fields, 2015) directly in HeLa cells infected with *C. trachomatis*; or iii) there are examples of T3S substrates that are not effectors as is the case of the components of the injectiosome and translocator proteins of the T3SS apparatus (Cornelis, 2006). In this scenario CT142, CT143 and CT144 could belong to this category of proteins.

Additionally, we observed that the subcellular localization of CT142-2HA, CT143-2HA, and CT144-2HA at 30 h p.i. was drastically different when they were expressed from the exogenous *C. trachomatis incD* promoter (not shown for CT142 and CT144) than when they were expressed from their own common endogenous *ct142* promoter. Upon expression from the *incD* promoter, these

proteins were apparently retained inside the bacteria while under the control of the *ct142* promoter they were found within the inclusion lumen as observed for endogenous CT143 (Chapter III). Such a dramatic change in localization of these proteins indicates that regulation of their presumed secretion into the lumen of the inclusion is tightly associated with the transcriptional control of their expression. In early time points (before 2 h p.i.), it is however possible that CT143-2HA expressed under the control of the P_{incD} promoter is secreted into the host cell cytosol, but these observations still need to be confirmed. These results suggest that different promoters might direct T3SS substrates during a specific time of the *C. trachomatis* cycle.

When expressing epitope-tagged CT142, CT143, and CT144 in *Y. enterocolitica,* we detected interactions between CT142-CT142, CT142-CT143 and possibly between CT142-CT144. Additionally, when ectopically expressed in *C. trachomatis*, CT143 and CT144-2HA also appear to localise in close proximity. These observations suggest that these proteins might form a complex in the inclusion lumen. In our model, we propose that CT142 could function as a nucleating protein (Fig. 4.8). The function of this complex, however, still remains to be elucidated.

In order to confirm the observed interactions, additional biochemical validation will be necessary. We have attempted to do pull down assays using extracts from HeLa 229 cells infected with *C. trachomatis* L2/434, the plasmidless *C. trachomatis* L2 strain 25667R or any of the strains ectopically expressing CT142-2HA, CT143-2HA or CT144-2HA and used HA antibodies cross-linked to a protein G resin. However, the results obtained were inconclusive (data not

shown). Alternatively, pull down assays could be done using purified GST-CT143, MBP-CT142, MBP-CT143 or MBP-CT144 immobilized to a resin and extracts of HeLa 229 cells infected with all the *C. trachomatis* strains mentioned above.

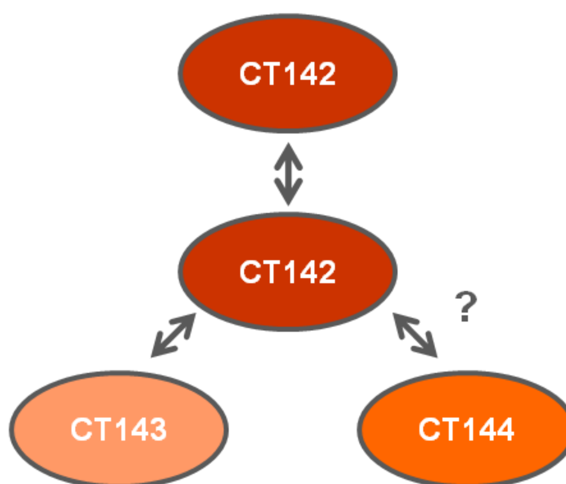

Figure 4.8 Model for possible interactions between CT142-CT142, CT143-CT143 and possibly CT142-CT144. Schematic representation of the predicted interactions between CT142, CT143 and CT144, based on the co-IP experiments.

Further experiments are also necessary to prove that a complex is formed and to understand the nature of its function. Ideally this could be done using knock-out mutants for CT142, CT143 and CT144 together with complementation plasmids encoding for the respective protein that was knocked-out. Another approach would be to construct a series of *C. trachomatis* recombinant strains derived from L2/434 strain, each encoding a plasmid derived from p2TK2-SW2 (Agaisse & Derré, 2013) in which *pgp4* was deleted and have all possible combinations of the three proteins: i) each of the three proteins expressed individually; ii) expression of pairwise combinations of the three proteins; or iii) the three proteins expressed

from the same plasmid. In all cases the expression would have to be driven from a conditional promoter (not regulated by *pgp4*, e.g P_{tet}; (Bauler & Hackstadt, 2014; Wickstrum *et al.*, 2013)) and proteins would be carrying an HA epitope. With this approach we could determine if CT142 is indeed necessary to nucleate the formation of the proposed complex. A broader approach to chlamydial genetics, using all the recently developed methods will definitively help us to elucidate the role for these novel likely virulence factors in the developmental cycle of *C. trachomatis*.

ACKNOWLEDGMENTS

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REFERENCES

Agaisse, H. & Derré, I. (2013). A *C. trachomatis* Cloning Vector and the Generation of *C. trachomatis* Strains Expressing Fluorescent Proteins under the Control of a *C. trachomatis* Promoter. *PLoS One* **8**(2), e57090.

Agaisse, H. & Derré, I. (2014). Expression of the effector protein IncD in *Chlamydia trachomatis* mediates recruitment of the lipid transfer protein CERT and the endoplasmic reticulum-resident protein VAPB to the inclusion membrane. *Infect Immun* **82**(5), 2037–2047.

Bauler, L. D. & Hackstadt, T. (2014). Expression and Targeting of secreted proteins from *Chlamydia trachomatis*. *J Bacteriol* **196**(7), 1325–1334.

Chen, Y.-S., Bastidas, R. J., Saka, H. A., Carpenter, V. K., Richards, K. L., Plano, G. V & Valdivia, R. H. (2014). The *Chlamydia trachomatis* type III secretion chaperone Slc1 engages multiple early effectors, including TepP, a tyrosine-phosphorylated protein required for the recruitment of CrkI-II to nascent inclusions and innate immune signaling. *PLoS Pathog* **10**(2), e1003954.

Cornelis, G. R. (2006). The type III secretion injectisome. *Nat Rev Microbiol* **4**, 811–825.

Dumoux, M., Menny, A., Delacour, D. & Hayward, R. (2015). A *Chlamydia* effector recruits CEP170 to reprogram host microtubule organization. *J Cell Sci* **128**, 3420–3434.

Iriarte, M. & Cornelis, G. R. (1998). YopT, a new *Yersinia* Yop effector protein, affects the cytoskeleton of host cells. *Mol Microbiol* **29**(3), 915–929.

Johnson, C. M. & Fisher, D. J. (2013). Site-specific, insertional inactivation of inca in *Chlamydia trachomatis* using a group II intron. *PLoS One* **8**(12), e83989.

Jorgensen, I. & Valdivia, R. H. (2008). Pmp-like proteins Pls1 and Pls2 are secreted into the lumen of the *Chlamydia trachomatis* inclusion. *Infect Immun* **76**(9), 3940–3950.

Kari, L., Goheen, M. M., Randall, L. B., Taylor, L. D., Carlson, J. H., Whitmire, W. M., Virok, D., Rajaram, K., Endresz, V. & other authors. (2011). Generation of targeted *Chlamydia trachomatis* null mutants. *Proc Natl Acad Sci* **108**(17), 7189–7193.

Kokes, M., Dunn, J. D., Granek, J. A., Nguyen, B. D., Barker, J. R., Valdivia, R. H. & Bastidas, R. J. (2015). Integrating Chemical Mutagenesis and Whole-Genome Sequencing as a Platform for Forward and Reverse Genetic Analysis of *Chlamydia*. *Cell Host Microbe* **17**, 1–10.

Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. a, Roop, R. M. & Peterson, K. M. (1995). Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* **166**, 175–176.

Mishra, M. K., Gérard, H. C., Whittum-Hudson, J. A., Hudson, A. P. & Kannan, R. M. (2012). Dendrimer-enabled modulation of gene expression in *Chlamydia trachomatis*. *Mol Pharm* **9**, 413–421.

Mueller, K. E. & Fields, K. A. (2015). Application of β-Lactamase Reporter Fusions as an Indicator of Effector Protein Secretion during Infections with the Obligate Intracellular Pathogen *Chlamydia trachomatis*. *PLoS One* **10**(8), e0135295.

Muschiol, S., Boncompain, G., Vromman, F., Dehoux, P., Normark, S., Henriques-Normark, B. & Subtil, A. (2011). Identification of a family of effectors secreted by the type III secretion system that are conserved in pathogenic *Chlamydiae*. *Infect Immun* **79**(2), 571–580.

Nguyen, B. D. & Valdivia, R. H. (2013). Forward genetic approaches in *Chlamydia trachomatis*. *J Vis Exp* **80**, e50636.

Pais, S. V., Milho, C., Almeida, F. & Mota, L. J. (2013). Identification of Novel Type III Secretion Chaperone-Substrate Complexes of *Chlamydia trachomatis*. *PLoS One* **8**(2), e56292.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S. & other authors. **(2012).** Fiji: an open-source platform for biological-image analysis. *Nat Methods* **9**(7), 676–682.

Scidmore, M. (2005). Cultivation and laboratory maintenance of *Chlamydia trachomatis*. *Curr Protoc Microbiol* 1–25.

Stephens, R. S., Kalman, S., Lammel, C., Fan, J., Marathe, R., Aravind, L., Mitchell, W., Olinger, L., Tatusov, R. L. & other authors. **(1998).** Genome sequence of an obligate intracellular pathogen of humans: *Chlamydia trachomatis*. *Science* **282**, 754–759.

Wang, Y., Kahane, S., Cutcliffe, L. T., Skilton, R. J., Lambden, P. R. & Clarke, I. N. (2011). Development of a transformation system for *Chlamydia trachomatis*: Restoration of glycogen biosynthesis by acquisition of a plasmid shuttle vector. *PLoS Pathog* **7**(9), e1002258.

Weber, M. M., Bauler, L. D., Lam, J. & Hackstadt, T. (2015). Expression and localization of predicted inclusion membrane proteins in *Chlamydia trachomatis. Infect Immun* **83**(12), 4710–4718.

Wickstrum, J., Sammons, L. R., Restivo, K. N. & Hefty, P. S. (2013). Conditional gene expression in *Chlamydia trachomatis* using the *tet* system. *PLoS One* **8**(10), e76743.

Chapter V

General Discussion

The author of this dissertation has written this chapter based on the referred bibliography and on results from chapters II to IV.

Gram-negative bacteria possess different macromolecular structures, known as type III, type IV, or type VI secretion systems, for delivery of effector proteins directly from the bacterial cytoplasm into eukaryotic or prokaryotic host cells (see Chapter I, section 1.4.1) (Costa *et al.*, 2015). The proteins that are delivered into host cells, the effector proteins, have the capacity to modulate a variety of cellular functions (Galán, 2009). Although many effectors have been elegantly described in many bacterial systems in the last decades, there are still a considerable number of candidate effectors in many bacteria that have not yet been described. This is indeed the case of *Chlamydia trachomatis* whose obligate intracellular lifestyle has further limited research in this area. In fact, relatively few chlamydial effectors have been characterized to date (see Chapter I, section 1.4.8).

The main goal of this project was to identify and characterize novel *C. trachomatis* type III secretion system (T3SS) effector proteins. The first part of the work involved a screen in the heterologous host *Yersinia enterocolitica* in order to identify novel chlamydial candidate effectors. It revealed 10 *C. trachomatis* proteins (CT053, CT105, CT142, CT143, CT144, CT161, CT338, CT429, CT656, and CT849) of yet unknown function that have a high likelihood of being effectors delivered by *C. trachomatis* into the host cell (Chapter 2). Among the proteins highlighted in the screen, CT142, CT143 and CT144 were chosen to be further characterized in this work. In addition to being putative T3SS effectors, it had been previously shown that expression of *ct142*, *ct143* and *ct144* is transcriptionally regulated by the *Chlamydia*-virulence plasmid (Carlson *et al.*, 2008; Song *et al.*, 2013), reinforcing the probability that these proteins are virulence factors. We showed that during infection of host cells by *C. trachomatis*,

CT142, CT143, and CT144 localize within the lumen of the bacterial vacuole but outside from the bacterial cells. Furthermore, we provide evidence that these proteins might interact with each other and form a protein complex within the lumen of the inclusion (Fig. 5.1). Altogether, this work contributed to increase our knowledge on the identity of *C. trachomatis* T3SS substrates, and supported that some of these substrates might be released into the inclusion lumen where they might have a functional role. This intriguing possibility needs to be further evaluated in the future.

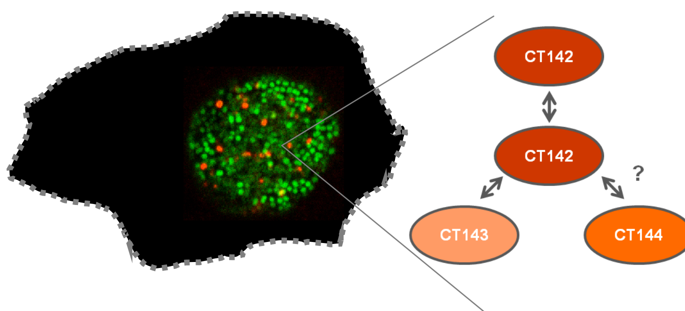

Figure 5.1. CT142, CT143 and CT144 could form a complex in the lumen of the inclusion. Schematic representation of the predicted interactions between CT142, CT143 and CT144, based on the co-immunoprecipitation experiments. We propose that the indicated proteins might form a complex in the lumen of the chlamydial inclusion.

5.1. Distribution of CT142, CT143, and CT144 among *Chlamydiae*

PSI-BLAST analysis (Altschul *et al.*, 1997) revealed that CT142, CT143 and CT144 are highly conserved in all *C. trachomatis* serovars (> 95% identity in CT142, > 96% identity in CT143 and > 90% identity in CT144) (data not shown) and are also conserved in *Chlamydiaceae* (Table 5.1). However, PSI-BLAST analysis failed to identify any significant sequence similarity with proteins from the other *Chlamydiae* families (Table 5.1).

Table 5.1. Identification of orthologues of *C. trachomatis* CT142 (CTL0397), CT143 (CTL0398), and CT144 (CTL0399) in other *Chlamydiae*[a]

	CTL0397 (CT142)		**CTL0398 (CT143)**		**CTL0399 (CT144)**	
	cover	Id	cover	Id	cover	Id
C. pneumoniae	96%	36%	96% 92%	35% 37%	99%	33%
C. muridarum	99%	71%	100%	74%	100%	68%
C. psittaci	83%	41%	93%	45%	98%	39%
C. abortus	99%	39%	93%	44%	98%	37%
C. caviae	96%	39%	93%	41%	96%	38%
C. pecorum	95%	34%	94%	37%	96%	40%
C. felis	95%	37%	97%	41%	98%	36%
C. avium	96%	42%	99%	39%	98%	36%
C. gallinacea	96%	41%	100%	42%	98%	37%
C. suis	100%	77%	100%	79%	99%	73%
Parachlamydiaceae	No Hits		No Hits		No Hits	
Waddliaceae	No Hits		No Hits		No Hits	
Simkaniaceae	No Hits		No Hits		No Hits	
Criblamydiaceae	No Hits		No Hits		No Hits	

[a] Orthologues of the *C. trachomatis* proteins CT142, CT143 and CT144 in other *Chlamydiae* were searched by PSI-BLAST (Altschul *et al.*, 1997). An individual PSI-BLAST search was performed between each protein (using the corresponding amino acid sequence from *C. trachomatis* serovar L2 strain 434/Bu: CTL0397, CTL0398 and CTL0399, respectively) and a representative strain from each *Chlamydia* spp. (*C. pneumoniae* strain CWL029, *C. muridarum* strain Nigg., *C. psittaci* strain Mat116, *C. abortus* strain S26/3, *C. caviae* strain GPIC, *C. pecorum* PV3056/3, *C. felis* Fe/C-56, *C. avium* strain 10DC88, *C. gallinacea* 08-1274/3, *C. suis* strain MD56). Another individual PSI-BLAST search was performed between each protein and members of the other *Chlamydiae* families. The families *Rhabdochlamydiaceae*, *Piscichlamydeacea, Chlavichlamydiaceae* and *Parilichlamydiaceae* were excluded from our search because they are not include in the genome database. Cover, indicates % of coverage, and Id indicates % of identity.

The syntenic organization of CT142, CT143, and CT144 is maintained in the majority of the *Chlamydia* spp. (Fig. 5.2). Interestingly, in *C. pneumoniae* there is a duplication of the orthologue of *ct143* and the order of the genes is altered (Table 5.1 and Fig. 5.2). The reason for this occurrence is not clearly understood. Nevertheless, the conserved nature of these genes is an indication of their importance in the biology of *Chlamydia*. In particular, the exclusive presence of CT142, CT143, and CT144 orthologues within *Chlamydia* spp. suggests a role of these proteins during infection of animal hosts.

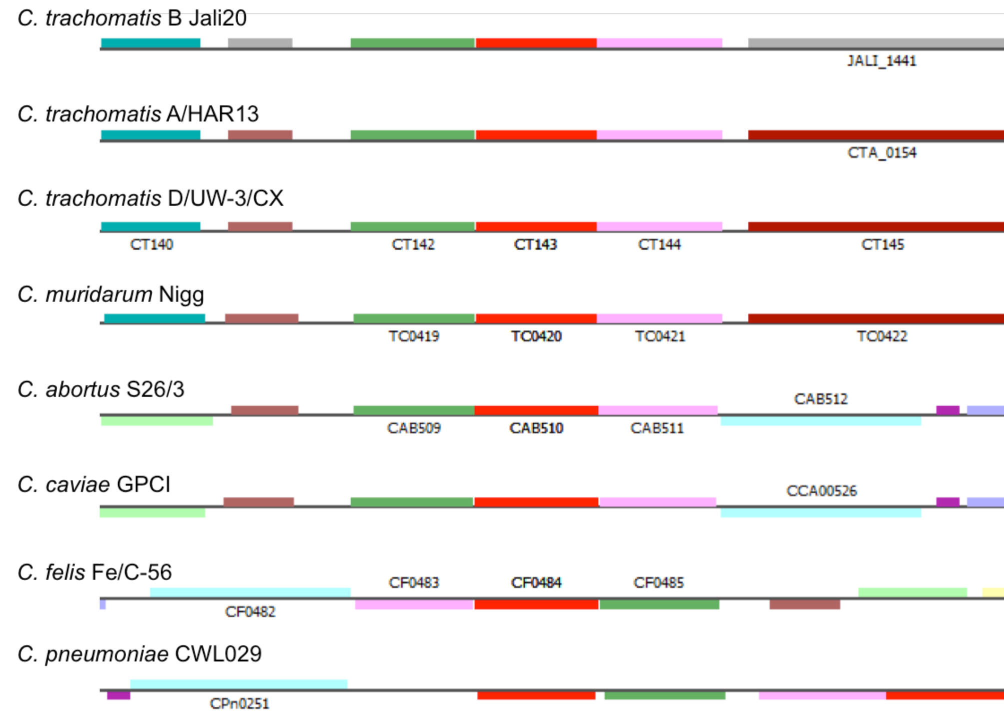

Figure 5.2. Genetic organization of *ct142* (depicted in green), *ct143* (depicted in red) and *ct144* (depicted in pink). Syntenic organization of the three genes is illustrated in *C. trachomatis* serovar A (strain A/HAR-13), *C. trachomatis* serovar B (strain B/Jali20/OT), *C. trachomatis* serovar D (strain D/UW-3/CX) as well as in *C. muridarum* Nigg, *C. abortus* S26/3, *C. caviae* GPIC, *C. felis* Fe/C-56 and *C. pneumoniae* CWLO29. Image from the ChlamydiaeDB website (no longer available).

5.2. Subcellular localization of CT142, CT143 and CT144

Our analyses of immunofluorescence microscopy of the subcellular localization of endogenous CT143 or ectopically expressed CT142-2HA, CT143-2HA and CT144-2HA in HeLa cells infected by *C. trachomatis* serovar L2 strain 434/Bu (L2/434, or each respective derivative recombinant strain) revealed these proteins within the bacteria (at 15 h p.i.) and outside of the bacteria within the inclusion lumen (from 20 h p.i.). To our surprise, we could never detect the proteins in the cytoplasm of the host cell, as would be expected for T3SS effectors. Other proteins have been found to localize at the inclusion lumen: Pls1/CT049 and Pls2/CT050 (Jorgensen & Valdivia, 2008), which have not been demonstrated to be T3SS substrates, CT620 and CT621, which have been shown to be T3SS substrates using *S. flexneri* as heterologous host (Muschiol *et al.*, 2011) and the chlamydial glycogen enzymes (GlgA, GlgB, GlgX, GlgP and MalQ) that have been recently described to possess T3S signals and to accumulate in the inclusion lumen (Gehre *et al.*, 2016). However, CT620, CT621 and GlgA have also been shown to localize within the host cell cytoplasm (Lu *et al.*, 2013; Muschiol *et al.*, 2011). Assuming that at least CT142, CT143, CT144, CT620, CT621 and the chlamydial glycogen enzymes are T3SS substrates, their localization within the inclusion lumen does not fit the classical model where T3SS effector proteins are translocated directly from the bacterial cytoplasm into the host cell cytosol. These observations raise the possibility that the *C. trachomatis* T3SS could have a different mode of action than in the organisms were it has been more thoroughly characterized.

A unique feature of the *C. trachomatis* T3SS is an apparent duplication of the hydrophobic translocator proteins that likely form a

pore within the host cell plasma membrane (during entry) and within the inclusion membrane (during the intracellular bacterial development and replication): CopB and CopB2, and CopD and CopD2. For CopB and CopB2, it has been found that CopB is detected early during infection, while CopB2 is detected throughout the developmental cycle (Chellas-Géry *et al.*, 2011). Likewise, earlier studies in *C. pneumoniae* revealed the presence of two distinct translocator chaperones (*Yersinia* spp., *lcrH* homologues), one acting on early stages of infection and the second during the developmental cycle (Ouellette *et al.*, 2005). Furthermore, in many T3SSs a hydrophilic translocator protein forms either a tip complex, such as LcrV in *Yersinia* spp. (Mueller, 2005), or a filament, such as EspA in *Escherichia coli* (Knutton *et al.*, 1998), which serve as link between the injectisome and the translocon (Mueller *et al.*, 2008). However, such hydrophilic translocator has not been found yet in *C. trachomatis.* Although CT584 has been initially proposed to form a tip complex in *Chlamydia* (Markham *et al.*, 2009), the recent determination of the three-dimensional structure of the protein indicates that this should not be the case (Stone *et al.*, 2012). While such link between the *C. trachomatis* injectisome and translocon might eventually be found, one can also speculate that in this bacterium some T3SS substrates are released into the inclusion lumen and either remain there (as could be the case of CT142, CT143, and CT144) or afterwards gain access to the host cell cytoplasm (as could be the case of CT620 and CT621).

It is clear that there are mechanisms for transport of proteins from the inclusion lumen into the host cell cytoplasm. For example, *C. trachomatis* effectors CPAF or Tsp/CT441 as well as the candidate effectors cHtrA, CT311 or CT795 are likely transported first by a Sec-

dependent mechanism (Chen *et al.*, 2010; Lei *et al.*, 2011; Qi *et al.*, 2011; Yang *et al.*, 2015b; Zhong, 2011) into the inclusion lumen and from there gain access to the host cell cytosol by an unknown mechanism. One hypothesis is that proteins located in the inclusion lumen may be exported into the host cell cytosol via outer membrane vesicles (OMVs) (Bonnington & Kuehn, 2014; Zhong, 2011).

According to the recently proposed working model for the T3SS during the chlamydial developmental cycle (Ferrell & Fields, 2015) (see Fig. 1.8, Chapter I), we could imagine a scenario where CT142, CT143, CT144, CT620, CT621, and possibly other mid/late cycle T3SS substrates, would be secreted into the inclusion lumen during the process of RB detachment from the inclusion membrane, before the event of association of the CopN gatekeeper to the bacterial cytoplasmic side of the apparatus. This would imply that these proteins would be secreted during the RB-EB conversion process. In contrast to the synchronized replication of RBs, it is known that the conversion of RBs to EBs occurs in a non-synchronized manner (Moulder, 1991). In fact, the timing of appearance of CT142, CT143 and CT144 in the lumen of the chlamydial inclusion could reflect the RB-EB conversion step: no protein detected in the inclusion lumen until approximately 20 h p.i., very low levels of protein observed at 20 h p.i. and an increment in amount of detected protein towards later times in the developmental cycle (30 and 40 h p.i.). According to this hypothesis, it would be possible to imagine T3S occurring during detachment of RBs from the inclusion membrane, which would consequently explain the presence of T3S substrates in the lumen of the inclusion. This model would clearly imply a complex regulatory mechanism that has not yet been characterized.

5.3. Possible functions for the proposed CT142-CT143-CT144 protein complex

It is possible that CT142, CT143 and CT144 are T3SS substrates but not T3SS effectors. In this scenario, these proteins could be components or regulators of the T3SS apparatus itself. Highly speculative possibilities would be that they from the needle tip complex or that they could be somehow involved in the activation of the system prior to the invasion step of the cycle. Alternatively, and bearing in mind the timing of expression and secretion of these T3SS substrates, the function of CT142, CT143, CT144, could be as effector proteins logically associated with late intracellular survival, host cell exit or the invasion step of the developmental cycle. Another obvious association for these proteins is the relation of activities that have been attributed to the *Chlamydia* virulence plasmid, such as stimulation of pro-inflammatory cytokines (O'Connell *et al.*, 2011) infectivity (O'Connell *et al.*, 2007), accumulation of glycogen granules in the inclusion (Matsumoto *et al.*, 1998) or intra-inclusion Brownian-like movement (Carlson *et al.*, 2008). Interestingly, recent work has shown that chlamydial exit from host cells is not only regulated by the plasmid transcriptional regulator Pgp4 but is also dependent on the chlamydial T3SS (Yang *et al.*, 2015a). It is therefore a possibility that CT142, CT143, and CT144 could play a role in the chlamydial exit from the host cell.

Final Remarks

The work developed in this thesis revealed ten novel candidate *C. trachomatis* effector proteins: CT053, CT105, CT142, CT143, CT144, CT161, CT338, CT429, CT656, and CT849. CT142, CT143 and CT144 were further described to be secreted into the lumen of the chlamydial inclusion but their function remains to be determined. These proteins, among others, might not fit the classical definition of ‘effector protein’, since direct interaction with the host cell has not been verified yet.

We believe that this study will lead way for future lines of research in the field of chlamydial effector proteins, namely in understanding how these proteins act and which cellular mechanisms are targeted by each protein. In fact, at least one candidate T3SS effector detected in our screen, CT105, has been recently demonstrated in our lab to be translocated into the host cell by *C. trachomatis* (Pais and Mota, unpublished). Others remain to be tested more thoroughly as possible effector proteins. Characterization of the whole set of chlamydial effector proteins, and understanding how they contribute to intracellular survival and to the subversion of the host cell, will definitively lead to a better understanding of *Chlamydia* pathogenesis.

REFERENCES

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**(17), 3389–3402.

Bonnington, K. E. & Kuehn, M. J. (2014). Protein selection and export via outer membrane vesicles. *Biochim Biophys Acta - Mol Cell Res* **1843**, 1612–1619.

Carlson, J. H., Whitmire, W. M., Crane, D. D., Wicke, L., Virtaneva, K., Sturdevant, D. E., Kupko, J. J., Porcella, S. F., Martinez-Orengo, N. & other authors. (2008). The *Chlamydia trachomatis* plasmid is a transcriptional regulator of chromosomal genes and a virulence factor. *Infect Immun* **76**(6), 2273–2283.

Chellas-Géry, B., Wolf, K., Tisoncik, J., Hackstadt, T. & Fields, K. A. (2011). Biochemical and localization analyses of putative type III secretion translocator proteins CopB and CopB2 of *Chlamydia trachomatis* reveal significant distinctions. *Infect Immun* **79**(8), 3036–3045.

Chen, D., Lei, L., Lu, C., Flores, R., DeLisa, M. P., Roberts, T. C., Romesberg, F. E. & Zhong, G. (2010). Secretion of the chlamydial virulence factor CPAF requires the Sec-dependent pathway. *Microbiology* **156**(10), 3031–3040.

Costa, T. R. D., Felisberto-Rodrigues, C., Meir, A., Prevost, M. S., Redzej, A., Trokter, M. & Waksman, G. (2015). Secretion systems in Gram-negative bacteria: structural and mechanistic insights. *Nat Rev Microbiol* **13**, 343–359.

Ferrell, J. C. & Fields, K. A. (2015). A working model for the type III secretion mechanism in *Chlamydia*. *Microbes Infect* **18**, 84–92.

Galán, J. E. (2009). Common Themes in the Design and Function of Bacterial Effectors. *Cell Host Microbe* **5**, 571–579.

Gehre, L., Gorgette, O., Perrinet, S., Prevost, M.-C., Ducatez, M., Giebel, A. M., Nelson, D. E., Ball, S. G. & Subtil, A. (2016). Sequestration of host metabolism by an intracellular pathogen. *elife* **5**, e12552.

Jorgensen, I. & Valdivia, R. H. (2008). Pmp-like proteins Pls1 and Pls2 are secreted into the lumen of the *Chlamydia trachomatis* inclusion. *Infect Immun* **76**(9), 3940–3950.

Knutton, S., Rosenshine, I., Pallen, M. J., Nisan, I., Neves, B. C., Bain, C., Wolff, C., Dougan, G. & Frankel, G. (1998). A novel EspA-associated surface organelle of enteropathogenic *Escherichia coli* involved in protein translocation into epithelial cells. *EMBO J* **17**(8), 2166–2176.

Lei, L., Qi, M., Budrys, N., Schenken, R. & Zhong, G. (2011). Localization of *Chlamydia trachomatis* hypothetical protein CT311 in host cell cytoplasm. *Microb Pathog* **51**(3), 101–109.

Lu, C., Lei, L., Peng, B., Tang, L., Ding, H., Gong, S., Li, Z., Wu, Y. & Zhong, G. (2013). *Chlamydia trachomatis* GlgA Is Secreted into Host Cell Cytoplasm. *PLoS One* **8**(7), e68764.

Markham, A. P., Jaafar, Z. A., Kemege, K. E., Middaugh, C. R. & Hefty, P. S. (2009). Biophysical characterization of *Chlamydia trachomatis* CT584 supports its potential role as a type III secretion needle tip protein. *Biochemistry* **48**(43), 10353–10361.

Matsumoto, A., Izutsu, H., Miyashita, N. & Ohuchi, M. (1998). Plaque Formation by and Plaque Cloning of *Chlamydia trachomatis* Biovar Trachoma These include : Plaque Formation by and Plaque Cloning of *Chlamydia trachomatis* Biovar Trachoma **36**(10), 3013–3019.

Moulder, J. W. (1991). Interaction of *Chlamydiae* and host cells *in vitro*. *Microbiol Rev* **55**(1), 143–190.

Mueller, C. A. (2005). The V-Antigen of *Yersinia* Forms a Distinct Structure at the Tip of Injectisome Needles. *Science* **310**, 674–676.

Mueller, C. A., Broz, P. & Cornelis, G. R. (2008). The type III secretion system tip complex and translocon. *Mol Microbiol* **68**(5), 1085–1095.

Muschiol, S., Boncompain, G., Vromman, F., Dehoux, P., Normark, S., Henriques-Normark, B. & Subtil, A. (2011). Identification of a family of effectors secreted by the type III secretion system that are conserved in pathogenic *Chlamydiae*. *Infect Immun* **79**(2), 571–580.

O'Connell, C. M., Ingalls, R. R., Andrews Jr., C. W., Scurlock, A. M. & Darville, T. (2007). Plasmid-deficient *Chlamydia muridarum* fail to induce immune pathology and protect against oviduct disease. *J Immunol* **179**, 4027–4034.

O'Connell, C. M., AbdelRahman, Y. M., Green, E., Darville, H. K., Saira, K., Smith, B., Darville, T., Scurlock, A. M., Meyer, C. R. & Belland, R. J. (2011). Toll-like receptor 2 activation by *Chlamydia trachomatis* is plasmid dependent, and plasmid-responsive chromosomal loci are coordinately regulated in response to glucose limitation by *C. trachomatis* but not by *C. muridarum*. *Infect Immun* **79**(3), 1044–1056.

Ouellette, S. P., Abdelrahman, Y. M., Belland, R. J. & Byrne, G. I. (2005). The *Chlamydia pneumoniae* type III secretion-related *lcrH* gene clusters are developmentally expressed operons. *J Bacteriol* **187**, 7853–6.

Qi, M., Lei, L., Gong, S., Liu, Q., DeLisa, M. P. & Zhong, G. (2011). *Chlamydia trachomatis* secretion of an immunodominant hypothetical protein (CT795) into host cell cytoplasm. *J Bacteriol* **193**(10), 2498–2509.

Song, L., Carlson, J. H., Whitmire, W. M., Kari, L., Virtaneva, K., Sturdevant, D. E., Watkins, H., Zhou, B., Sturdevant, G. L. & other authors. (2013). *Chlamydia trachomatis* plasmid-encoded Pgp4 is a transcriptional regulator of virulence-associated genes. *Infect Immun* **81**(3), 636–644.

Stone, C. B., Sugiman-Marangos, S., Bulir, D. C., Clayden, R. C., Leighton, T. L., Slootstra, J. W., Junop, M. S. & Mahony, J. B. (2012). Structural characterization of a novel *Chlamydia pneumoniae* type III secretion-associated protein, Cpn0803. *PLoS One* **7**(1), e30220.

Yang, C., Starr, T., Song, L., Carlson, J. H., Sturdevant, G. L., Beare, P. A., Whitmire, W. M. & Caldwell, H. D. (2015a). Chlamydial lytic exit from host cells is plasmid regulated. *MBio* **6**(6), e01648–15.

Yang, Z., Tang, L., Sun, X., Chai, J. & Zhong, G. (2015b). Characterization of CPAF critical residues and secretion during *Chlamydia trachomatis* infection. *Infect Immun* **83**(6), 2234–2241.

Zhong, G. (2011). *Chlamydia trachomatis* secretion of proteases for manipulating host signaling pathways. *Front Microbiol* **2**, 14.

Annexes

TABLE A1. Plasmids used in this work.

Name	Characteristics and use[a]	Source/Ref.	Chapter
pCX340	Derivative of pBR322. Expresses mature TEM-1 β-lactamase	(Charpentier & Oswald, 2004)	II
pLJM3	Low copy vector, derivative of pBBR1MCS-2 (Kovach *et al.*, 1995). Expresses YopE under the control of its own promoter (P_{yopE}).	(Marenne *et al.*, 2003)	II
pFA3	Derivative of pLJM3. Expresses TEM-1 under the control of P_{yopE}	(Almeida *et al.*, 2012)	II
pET28b(+)	*E. coli* expression vector allowing N or C-terminal His-tag fusions (Km^R)	Novagen	III
pGEX-4T-2	*E. coli* expression vector to generate fusions to the C-terminus of GST (Amp^R)	GE Healthcare	III
pMal-c	*E. coli* expression vector to generate fusions to the C-terminus of MBP (Amp^R)	New England Bioloabs	III
pEGFP-C1	Mammalian transfection vector (Km^R) to generate fusions to the C-terminus of EGFP.	Clontech	III
p2TK2-SW2	*C. trachomatis* expression vector. (Amp^R)	(Agaisse & Derré, 2013)	IV
pBAD/Myc-His A	pBR322-derived expression vector. *E. coli* arabinose inducible promoter P_{BAD} is used (Amp^R)	Invitrogen	IV
pBBR 1MCS-2	*E. coli* low-copy cloning and expression vector (Km^R)	(Kovach *et al.*, 1995)	IV
pCM13	Derivative of pLJM3. Expresses $CT016_{20}$-TEM-1 under the control of P_{yopE}	This study	II
pFA38	Derivative of pLJM3. Expresses $CT017_{20}$-TEM-1 under the control of P_{yopE}	This study	II
pFA39	Derivative of pLJM3. Expresses $CT031_{20}$-TEM-1 under the control of P_{yopE}	This study	II
pCM4	Derivative of pLJM3. Expresses $CT051_{20}$-TEM-1 under the control of P_{yopE}	This study	II
pCM5	Derivative of pLJM3. Expresses $CT053_{20}$-TEM-1 under the control of P_{yopE}	This study	II
pFA41	Derivative of pLJM3. Expresses $CT066_{20}$-TEM-1 under the control of P_{yopE}	This study	II
pCM14	Derivative of pLJM3. Expresses $CT080_{20}$-TEM-1 under the control of P_{yopE}	This study	II

TABLE A1. *Continued*.

Name	Characteristics and use[a]	Source/Ref.	Chapter
pRM10	Derivative of pLJM3. Expresses $CT082_{20}$-TEM-1 under the control of P_{yopE}	This study	II
pRM11	Derivative of pLJM3. Expresses $CT105_{20}$-TEM-1 under the control of P_{yopE}	This study	II
pRM12	Derivative of pLJM3. Expresses $CT142_{20}$-TEM-1 under the control of P_{yopE}	This study	II
pMC24	Derivative of pLJM3. Expresses $CT143_{20}$-TEM-1 under the control of P_{yopE}	This study	II
pMC25	Derivative of pLJM3. Expresses $CT144_{20}$-TEM-1 under the control of P_{yopE}	This study	II
pFA42	Derivative of pLJM3. Expresses $CT153_{20}$-TEM-1 under the control of P_{yopE}	This study	II
pCM6	Derivative of pLJM3. Expresses $CT161_{20}$-TEM-1 under the control of P_{yopE}	This study	II
pCM15	Derivative of pLJM3. Expresses $CT172_{20}$-TEM-1 under the control of P_{yopE}	This study	II
pCM16	Derivative of pLJM3. Expresses $CT203_{20}$-TEM-1 under the control of P_{yopE}	This study	II
pFA43	Derivative of pLJM3. Expresses $CT273_{20}$-TEM-1 under the control of P_{yopE}	This study	II
pCM17	Derivative of pLJM3. Expresses $CT277_{20}$-TEM-1 under the control of P_{yopE}	This study	II
pCM18	Derivative of pLJM3. Expresses $CT289_{20}$-TEM-1 under the control of P_{yopE}	This study	II
pRM13	Derivative of pLJM3. Expresses $CT309_{20}$-TEM-1 under the control of P_{yopE}	This study	II
pCM19	Derivative of pLJM3. Expresses $CT330_{20}$-TEM-1 under the control of P_{yopE}	This study	II
pCM20	Derivative of pLJM3. Expresses $CT338_{20}$-TEM-1 under the control of P_{yopE}	This study	II
pFA44	Derivative of pLJM3. Expresses $CT386_{20}$-TEM-1 under the control of P_{yopE}	This study	II

TABLE A1. *Continued.*

Name	Characteristics and use[a]	Source/Ref.	Chapter
pFA45	Derivative of pLJM3. Expresses $CT425_{20}$-TEM-1 under the control of P_{yopE}	This study	II
pFA46	Derivative of pLJM3. Expresses $CT429_{20}$-TEM-1 under the control of P_{yopE}	This study	II
pFA47	Derivative of pLJM3. Expresses $CT504_{20}$-TEM-1 under the control of P_{yopE}	This study	II
pFA48	Derivative of pLJM3. Expresses $CT538_{20}$-TEM-1 under the control of P_{yopE}	This study	II
pCM21	Derivative of pLJM3. Expresses $CT568_{20}$-TEM-1 under the control of P_{yopE}	This study	II
pFA49	Derivative of pLJM3. Expresses $CT577_{20}$-TEM-1 under the control of P_{yopE}	This study	II
pRM16	Derivative of pLJM3. Expresses $CT583_{20}$-TEM-1 under the control of P_{yopE}	This study	II
pFA50	Derivative of pLJM3. Expresses $CT584_{20}$-TEM-1 under the control of P_{yopE}	This study	II
pFA51	Derivative of pLJM3. Expresses $CT590_{20}$-TEM-1 under the control of P_{yopE}	This study	II
pFA52	Derivative of pLJM3. Expresses $CT631_{20}$-TEM-1 under the control of P*yopE*	This study	II
pFA53	Derivative of pLJM3. Expresses $CT635_{20}$-TEM-1 under the control of P_{yopE}	This study	II
pCM8	Derivative of pLJM3. Expresses $CT656_{20}$-TEM-1 under the control of P_{yopE}	This study	II
pRM20	Derivative of pLJM3. Expresses $CT696_{20}$-TEM-1 under the control of P_{yopE}	This study	II
pCM9	Derivative of pLJM3. Expresses $CT702_{20}$-TEM-1 under the control of P_{yopE}	This study	II
pFA57	Derivative of pLJM3. Expresses $CT768_{20}$-TEM-1 under the control of P_{yopE}	This study	II
pCM22	Derivative of pLJM3. Expresses $CT779_{20}$-TEM-1 under the control of P_{yopE}	This study	II

TABLE A1. *Continued*.

Name	Characteristics and use[a]	Source/Ref.	Chapter
pFA58	Derivative of pLJM3. Expresses $CT814_{20}$-TEM-1 under the control of P_{yopE}	This study	II
pFA59	Derivative of pLJM3. Expresses $CT837_{20}$-TEM-1 under the control of P_{yopE}	This study	II
pCM11	Derivative of pLJM3. Expresses $CT845_{20}$-TEM-1 under the control of P_{yopE}	This study	II
pFA60	Derivative of pLJM3. Expresses $CT849_{20}$-TEM-1 under the control of P_{yopE}	This study	II
pRM15	Derivative of pLJM3. Expresses $CT863_{20}$-TEM-1 under the control of P_{yopE}	This study	II
pFA37	Derivative of pLJM3. Expresses $RplJ_{20}$-TEM-1 under the control of P_{yopE}	This study	II
pFA55	Derivative of pLJM3. Expresses $CT694_{20}$-TEM-1 under the control of P_{yopE}	This study	II
pMC1	Derivative of pLJM3. Expresses CT016-HA under the control of P_{yopE}	This study	II
pMC7	Derivative of pLJM3. Expresses CT051-HA under the control of P_{yopE}	This study	II
pMC2	Derivative of pLJM3. Expresses CT053-HA under the control of P_{yopE}	This study	II
pMC3	Derivative of pLJM3. Expresses CT080-HA under the control of P_{yopE}	This study	II
pRM1	Derivative of pLJM3. Expresses CT082-HA under the control of P_{yopE}	This study	II
pRM7	Derivative of pLJM3. Expresses CT105-HA under the control of P_{yopE}	This study	II
pRM2	Derivative of pLJM3. Expresses CT142-HA under the control of P_{yopE}	This study	II, IV
pMC13	Derivative of pLJM3. Expresses CT143-HA under the control of P_{yopE}	This study	II, IV
pMC5	Derivative of pLJM3. Expresses CT144-HA under the control of P_{yopE}	This study	II, IV

TABLE A1. *Continued*.

Name	Characteristics and use[a]	Source/Ref.	Chapter
pFA62	Derivative of pLJM3. Expresses CT153-HA under the control of P_{yopE}	This study	II
pMC6	Derivative of pLJM3. Expresses CT161-HA under the control of P_{yopE}	This study	II
pMC14	Derivative of pLJM3. Expresses CT172-HA under the control of P_{yopE}	This study	II
pMC20	Derivative of pLJM3. Expresses CT203-HA under the control of P_{yopE}	This study	II
pFA63	Derivative of pLJM3. Expresses CT273-HA under the control of P_{yopE}	This study	II
pMC22	Derivative of pLJM3. Expresses CT277-HA under the control of P_{yopE}	This study	II
pMC18	Derivative of pLJM3. Expresses CT289-HA under the control of P_{yopE}	This study	II
pRM3	Derivative of pLJM3. Expresses CT309-HA under the control of P_{yopE}	This study	II
pMC15	Derivative of pLJM3. Expresses CT330-HA under the control of P_{yopE}	This study	II
pMC19	Derivative of pLJM3. Expresses CT338-HA under the control of P_{yopE}	This study	II
pFA64	Derivative of pLJM3. Expresses CT386-HA under the control of P_{yopE}	This study	II
pFA65	Derivative of pLJM3. Expresses CT425-HA under the control of P_{yopE}	This study	II
pFA66	Derivative of pLJM3. Expresses CT429-HA under the control of P_{yopE}	This study	II
pFA67	Derivative of pLJM3. Expresses CT504-HA under the control of P_{yopE}	This study	II
pMC7	Derivative of pLJM3. Expresses CT568-HA under the control of P_{yopE}	This study	II
pRM6	Derivative of pLJM3. Expresses CT583-HA under the control of P_{yopE}	This study	II

TABLE A1. *Continued*.

Name	Characteristics and use[a]	Source/Ref.	Chapter
pFA68	Derivative of pLJM3. Expresses CT631-HA under the control of P_{yopE}	This study	II
pMC8	Derivative of pLJM3. Expresses CT656-HA under the control of P_{yopE}	This study	II
pCM12	Derivative of pLJM3. Expresses CT694-HA under the control of P_{yopE}	This study	II
pRM18	Derivative of pLJM3. Expresses CT696-HA under the control of P_{yopE}	This study	II
pMC9	Derivative of pLJM3. Expresses CT702-HA under the control of P_{yopE}	This study	II
pMC10	Derivative of pLJM3. Expresses CT779-HA under the control of P_{yopE}	This study	II
pMC21	Derivative of pLJM3. Expresses CT845-HA under the control of P*yopE*	This study	II
pFA70	Derivative of pLJM3. Expresses CT849-HA under the control of P_{yopE}	This study	II
pRM5	Derivative of pLJM3. Expresses CT863-HA under the control of P_{yopE}	This study	II
pFA61	Derivative of pLJM3. Expresses RplJ-HA under the control of P_{yopE}	This study	II
pMC31	Derivative of pET28b(+). Encodes 6XHis-tagged CT142 (Km^R)	This study	III
pMC32	Derivative of pET28b(+). Encodes 6X His-tagged CT143. (Km^R)	This study	III
pMC33	Derivative of pET28b(+). 6X His-tagged CT144. (Km^R)	This study	III
pMC46	Derivative of pGEX-4T-2. Encodes GST-CT142. (Amp^R)	This study	III
pMC47	Derivative of pGEX-4T-2. Encodes GST-CT143. (Amp^R)	This study	III
pMC48	Derivative of pGEX-4T-2. Encodes GST-CT144. (Amp^R)	This study	III
pMC56	Derivative of pMal-c. Encodes MBP-CT143. (Amp^R)	This study	III
pCM33	Derivative of pMal-c. Encodes MBP-CT142 (Amp^R)	This study	III
pCM38	Derivative of pMal-c. Encodes MBP-CT144 (Amp^R)	This study	III
pSG1	Derivative of pEGFP-C1. Encodes EGFP-CT142 (Km^R)	This study	III

TABLE A1. *Continued*.

Name	Characteristics and use[a]	Source/Ref.	Chapter
pSG2	Derivative of pEGFP-C1. Encodes EGFP-CT143 (Km^R)	This study	III
pMC68	Derivative of p2TK2-SW2. Encodes CT142-2XHA under the control of P_{incD} (Amp^R)	This study	IV
pSVP253	Derivative of p2TK2-SW2. Encodes CT143-2XHA under the control of P_{incD} (Amp^R)	This study	IV
pMC69	Derivative of p2TK2-SW2. Encodes CT144-2XHA under the control of P_{incD} (Amp^R)	This study	IV
pMC70	Derivative of p2TK2-SW2. Encodes CT142-2XHA under the control of P_{ct142} (Amp^R)	This study	IV
pMC71	Derivative of p2TK2-SW2. Encodes CT143-2XHA under the control of P_{ct142} (Amp^R)	This study	IV
pMC72	Derivative of p2TK2-SW2. Encodes CT144-2XHA under the control of P_{ct142} (Amp^R)	This study	IV
pMC73	Derivative of p2TK2-SW2. Encodes CT142-CT143-CT144-2XHA under the control of P_{ct142} (Amp^R)	This study	IV
pSVP84	Derivative of pBAD/Myc-HisA. Encodes CT142-Myc-His	This study	IV

[a]Km^R, kanamycin resistance; Amp^R, ampicillin resistance

TABLE A2. DNA primers used in this work.

Code	Description	Sequence	Restriction enzyme[a]
28	Used to amplify the 5' region of genes cloned in pLJM3 to construct TEM-1 hybrids by overlapping PCR	GATTAAGTTGGGTAACGCC	-
60	Used to amplify *tem-1* to construct TEM-1 hybrids	CCCAAGCTTTTACCAATGCT TAATCAGTGAGG	HindIII
67	Used to amplify *tem-1* to construct TEM-1 hybrids	GATCCTCGAGTTACCAATG CTTAATCAGTGAGG	XhoI
103	Used to construct pFA38	GGAATTCCATATGCTCATTT TTGCCCTTTCTTGTGGGGC AGATGCCTGCTTATGTGCT GCGGATCTTTCCCACCCAG AAACGCTGGTG	NdeI
104	Used to construct pFA39	GGAATTCCATATGGCTAGA AAAGATCGTTTAACTAATGA AAGACTGAATAAGCTATTTG ATAGCCCCTTTCACCCAGA AACGCTGGTG	NdeI
106	Used to construct pFA41	GGAATTCCATATGGCTACG GCACAGATTACTATCCAAG AAGAAATAGAGCAGCTCAT AACTAAAGCGATTCACCCA GAAACGCTGGTG	NdeI
107	Used to construct pFA42	GGAATTCCATATGACTAAG CCTTCTTTCTTATACGTTAT TCAACCTTTTTCCGTATTTA ATCCACGATTACACCCAGA AACGCTGGTG	NdeI
108	Used to construct pFA43	GGAATTCCATATGGTCGAA ATTTTTAACTATAGTACCTC GGTATACGAGAAACACAGC TCTACGAACAAGCACCCAG AAACGCTGGTG	NdeI
109	Used to construct pFA44	GGAATTCCATATGCAAATTC CAAGAAGTGTTGGCACACA TGATGGTTCTTTTCACGCAG ATGAAGTGACGCACCCAGA AACGCTGGTG	NdeI
110	Used to construct pFA45	GGAATTCCATATGCGCAGA TCTGTTTGTTACGTTACTCC TTCAGTTGCTAGGGCTGGT CAAATTTCTACCCACCCAGA AACGCTGGTG	NdeI
111	Used to construct pFA46	GGAATTCCATATGACGACA TATCCTGTACCTCAAAATCC TCTTTTATTACGCGTTCTAC GCCTGATGGACCACCCAGA AACGCTGGTG	NdeI

TABLE A2. *Continued*.

Code	Description	Sequence	Restriction enzyme[a]
112	Used to construct pFA47	GGAATTCCATATGTATTTTA CAAGAGATCCAGTCATAGA GACTGTTATTACATCTAGAG AAGGATATAAGCACCCAGA AACGCTGGTG	NdeI
113	Used to construct pFA48	GGAATTCCATATGAATATTT CTGGAAGTATCAAACAAAAA CTTCTCCAGTTTTTGAAAAA GCAAAAATCCCACCCAGAA ACGCTGGTG	NdeI
114	Used to construct pFA49	GGAATTCCATATGAGTAAAA AACATAAGCACAAGCAAGC ACACACTTCTTCCAAACCCA AAGTAGAACCTCACCCAGA AACGCTGGTG	NdeI
115	Used to construct pFA50	GGAATTCCATATGACGACG AAACCCAAAACTCTAGAAAT CGATAACAACACGTTCCTG CTTTTGGAAGGCCACCCAG AAACGCTGGTG	NdeI
116	Used to construct pFA51	GGAATTCCATATGTCTCGTT TGGATGTTTCTGTATTTGAT TCCTTAGCTAATAAAGAAAA AGCTTCCTTACACCCAGAA ACGCTGGTG	NdeI
117	Used to construct pFA52	GGAATTCCATATGAAAACGT TAATTGATAACAACATCGTC AGATTCAAAAATATTTCTAA AACCAAACAGCACCCAGAA ACGCTGGTG	NdeI
118	Used to construct pFA53	GGAATTCCATATGAAAAATA ATTCCGCTCAAAAAATTATA GATTCTATAAAACAAATTCT CTCTATTTATCACCCAGAAA CGCTGGTG	NdeI
102	Used to construct pFA37	GGAATTCCATATGAAAGAA GAGAAAAAGTTGCTGCTTC GCGAGGTTGAAGAAAAGAT AACCGCTTCTCAACACCCA GAAACGCTGGTG	NdeI
120	Used to construct pFA55	GGAATTCCATATGAGTATTC GACCTACTAATGGGAGTGG AAATGGATACCCGTCTATTA ATCCTTCTAACCACCCAGAA ACGCTGGTG	NdeI
122	Used to construct pFA57	GGAATTCCATATGGATATTC CAGAACAGGGCTCAAATAC ACCAGAAGTAGAGCAAGCA GCTTGCTGCAATCACCCAG AAACGCTGGTG	NdeI
123	Used to construct pFA58	GGAATTCCATATGTTCAGAA GCCAAAAACCTAAAAAAAAT AAATGTTGCTTATGGTTGCG AGGCGTCCTACACCCAGAA ACGCTGGTG	NdeI

TABLE A2. *Continued*.

Code	Description	Sequence	Restriction enzyme[a]
124	Used to construct pFA59	GGAATTCCATATGGAAAAGACTCGTAAGTTTGAAAAAGCTTTAGAGAATTTAGAGCAGTTAAAAAAGATTCACCCAGAAACGCTGGTG	NdeI
125	Used to construct pFA60	GGAATTCCATATGTCAGCACCAACCTCACAGGTAGGAGACACACAATACGTCTCCTCGCTACCTCCTTTACACCCAGAAACGCTGGTG	NdeI
169	Used to construct pRM1	GGAATTCCATATGTCAATTTCTGGAAGTGG	NdeI
170	Used to construct pRM1	GATCCTCGAGTCAAGCATAATCAGGAACATCATACGGATATGAATCGCCGCCTGCATCCTC	XhoI
171	Used to construct pRM10 by overlapping PCR	CACCAGCGTTTCTGGGTGGATGGATGGGTCAAAATCAGG	-
172	Used to construct pRM10 by overlapping PCR	CCTGATTTTGACCCATCCATCCACCCAGAAACGCTGGTG	-
173	Used to construct pRM7	GGAATTCCATATGTCATTTGGTATTGGTAG	NdeI
174	Used to construct pRM7	GATCGGTACCCTAAGCATAATCAGGAACATCATACGGATAGATAGAGGAGCTTTGCACACC	KpnI
175	Used to construct pRM11 by overlapping PCR	GCCGTTTGTGTGGTTCATCACACCCAGAAACGCTGGTG	-
176	Used to construct pRM11 by overlapping PCR	CACCAGCGTTTCTGGGTGTGATGAACCACACAAACGGC	-
177	Used to construct pRM2	GGAATTCCATATGAGTGATTCTGACAAAATTATTAATG	NdeI
178	Used to construct pRM2	GATCGGTACCTTAAGCATAATCAGGAACATCATACGGATATCCTCCTATCTCTGGGTATACG	KpnI
179	Used to construct pRM12 by overlapping PCR	CGACTTTAATACAACTGTTCATCACCCAGAAACGCTGGTG	-
180	Used to construct pRM12 by overlapping PCR	CACCAGCGTTTCTGGGTGATGAACAGTTGTATTAAAGTCG	-
181	Used to construct pRM3	GGAATTCCATATGAATCAGTATTATTTTTATCC	NdeI
182	Used to construct pRM3	GATCAAGCTTCTAAGCATAATCAGGAACATCATACGGATACCATCTGATTCCTTTCTCC	HindIII
183	Used to construct pRM13 by overlapping PCR	CAACCCGAATCTTCTCCTCACCCAGAAACGCTGGTG	-

TABLE A2. ***Continued.***

Code	Description	Sequence	Restriction enzyme[a]
184	Used to construct pRM13 by overlapping PCR	CACCAGCGTTTCTGGGTGAGGAGAAGATTCGGGTTG	-
185	Used to construct pRM18	GGAATTCCATATGCTATTAGATTCTCGTTTCCC	NdeI
186	Used to construct pRM18	GATCCTCGAGCTAAGCATAATCAGGAACATCATACGGATAACGAGCTTCCTTACGGAAAGTTCC	XhoI
187	Used to construct pRM20 by overlapping PCR	CGTATCCTAGAATTAGTCATCCACCCAGAAACGCTGGTG	-
188	Used to construct pRM20 by overlapping PCR	CACCAGCGTTTCTGGGTGGATGACTAATTCTAGGATACG	-
193	Used to construct pRM5	GGAATTCCATATGGATACTCCCACACCCC	NdeI
194	Used to construct pRM5	GATCCTCGAGTTAAGCATAATCAGGAACATCATACGGATAGGGACGCATGTTGTAG	XhoI
195	Used to construct pRM15 by overlapping PCR	GCTTCTCTTAAGGGAGAACCACACCCAGAAACGCTGGTG	-
196	Used to construct pRM15 by overlapping PCR	CACCAGCGTTTCTGGGTGTGGTTCTCCCTTAAGAGAAGC	-
197	Use to construct pRM6	GGAATTCCATATGGGAAATATTAAAACCC	NdeI
198	Use to construct pRM6	GATCAAGCTTTTAAGCATAATCAGGAACATCATACGGATATCGATTTCTAGAGTTTTGGG	HindIII
199	Used to construct pRM16 by overlapping PCR	AAACCTACACCCGATAAAATGCACCCAGAAACGCTGGTG	-
200	Used to construct pRM16 by overlapping PCR	CACCAGCGTTTCTGGGTGCATTTTATCGGGTGTAGGTTT	-
206	Used to construct pFA61	GAATTCCATATGAAAGAAGAGAAAAAGTTGC	NdeI
207	Used to construct pFA61	GATCCTCGAGTTAAGCATAATCAGGAACATCATACGGATACTCTTGAGTTTTTTCTGC	XhoI
208	Used to construct pFA62	GAATTCCATATGACTAAGCCTTCTTTCTTATACG	NdeI
209	Used to construct pFA62	GATCCTCGAGTTAAGCATAATCAGGAACATCATACGGATAATAACCTGAAGATTTTTTAA	XhoI
212	Used to construct pFA63	GAATTCCATATGGTCGAAATTTTTAACTATAGTACC	NdeI
213	Used to construct pFA63	GATCCTCGAGTTAAGCATAATCAGGAACATCATACGGATAACCTTGTACGAATTGAAAAATACG	XhoI

TABLE A2. *Continued*.

Code	Description	Sequence	Restriction enzyme[a]
214	Used to construct pFA64	GAATTCCATATGCAAATTCC AAGAAGTGTTGGC	NdeI
215	Used to construct pFA64	CCCAAGCTTAAGCATAATCA GGAACATCATACGGATATA CTAATCTCTGCTGTTTTAAC	HindIII
216	Used to construct pFA65	GAATTCCATATGCGCAGAT CTGTTTGTTACG	NdeI
217	Used to construct pFA65	CCCAAGCTTAAGCATAATCA GGAACATCATACGGATAGT TAAGATCTATCCAAATAGG	HindIII
219	Used to construct pFA66	GAATTCCATATGACGACATA TCCTGTACC	NdeI
220	Used to construct pFA66	GATCCTCGAGTTAAGCATA ATCAGGAACATCATACGGA TATGAACGGCTCTTCTTACG	XhoI
221	Used to construct pFA67	GAATTCCATATGTATTTTAC AAGAGATCC	NdeI
222	Used to construct pFA67	GATCCTCGAGTTAAGCATA ATCAGGAACATCATACGGA TACTCTTCTGAAGAAATACT GTC	XhoI
223	Used to construct pFA68	GAATTCCATATGAAAACGTT AATTGATAAC	NdeI
224	Used to construct pFA68	CCCAAGCTTAAGCATAATCA GGAACATCATACGGATATA AACAAATAATTCCTTCAAAC TG	HindIII
227	Used to construct pFA70	GAATTCCATATGTCAGCAC CAACCTCACAGG	NdeI
228	Used to construct pFA70	CCCAAGCTTAAGCATAATCA GGAACATCATACGGATAAG ACAGGGGTTTATTTAATTGG	HindIII
229	Used to construct pMC1	GGAATTCCATATGAAAGTCA AAATTAATGATC	NdeI
230	Used to construct pMC1	GATCAAGCTTTTAAGCATAA TCAGGAACATCATACGGAT AAGTATAAAGAACAGCTTTC ACG	HindIII
233	Used to construct pMC7	GGAATTCCATATGAATAAAA AAGAACGAATTAATAAAAAA AACGC	NdeI
234	Used to construct pMC7	GATCGGTACCCTAAGCATA ATCAGGAACATCATACGGA TATACTATCTTATTTTTAATC	KpnI
235	Used to construct pMC2	GGAATTCCATATGAAAAGT GAGCGTTTAAAAAAATTAGA ATCAGAGC	NdeI
236	Used to construct pMC2	GATCAAGCTTTTAAGCATAA TCAGGAACATCATACGGAT ACCATTCATTCGCGTCAGG	HindIII
237	Used to construct pMC3	GGAATTCCATATGAAAAAAA GAAGCAGTCGC	NdeI

TABLE A2. *Continued*.

Code	Description	Sequence	Restriction enzyme[a]
238	Used to construct pMC3	GATCAAGCTTCTAAGCATAATCAGGAACATCATACGGATACAGTTTCACAGAATATCGCC	HindIII
241	Used to construct pMC13 and pMC32	GGAATTCCATATGAAGAAACCAGTATTTACAGG	NdeI
242	Used to construct pMC13	GATCCTCGAGTTAAGCATAATCAGGAACATCATACGGATAATCTGCCTCCTTATAAGAAG	XhoI
243	Used to construct pMC5 and pMC33	GGAATTCCATATGACAACACCAGATAATAATAC	NdeI
244	Used to construct pMC5	GATCAAGCTTTTAAGCATAATCAGGAACATCATACGGATAAGGAACAACAGGTAGCCG	HindIII
245	Used to construct pMC6	GGAATTCCATATGGCTAGAAAACCTTTAGTAG	NdeI
246	Used to construct pMC6	GATCAAGCTTTCAAGCATAATCAGGAACATCATACGGATAGTCATAAAAATTTTCCATTTCTG	HindIII
247	Used to construct pMC14	GGAATTCCATATGTTTTTCTTGGCAAAAAGAC	NdeI
248	Used to construct pMC14	GATCCTCGAGTCAAGCATAATCAGGAACATCATACGGATACTCTTGATAGTCTTGCATGG	XhoI
249	Used to construct pMC20	GGAATTCCATATGGAAATCTCCCATATTTTGG	NdeI
250	Used to construct pMC20	GATCCTCGAGCTAAGCATAATCAGGAACATCATACGGATATGACTCAAAAGGAAATC	XhoI
251	Used to construct pMC22	GGAATTCCATATGTCTGAGAGAAAGGTTGAG	NdeI
252	Used to construct pMC22	GATCCTCGAGCTAAGCATAATCAGGAACATCATACGGATAATGAGCAAGAGGAAATAGAC	XhoI
253	Used to construct pMC18	GGAATTCCATATGGTGATCCCTAAGGTGG	NdeI
254	Used to construct pMC18	GATCGGTACCTTAAGCATAATCAGGAACATCATACGGATATGGTTGCGTTGAGCCTCC	KpnI
255	Used to construct pMC15	GGAATTCCATATGCAGGAAATCTCGGTACC	NdeI
256	Used to construct pMC15	GATCCTCGAGTCAAGCATAATCAGGAACATCATACGGATATACAGATTCCCCAGGGATAAAAGG	XhoI
257	Used to construct pMC19	GGAATTCCATATGGTGTATAGTTATAAAGG	NdeI

TABLE A2. *Continued*.

Code	Description	Sequence	Restriction enzyme[a]
258	Used to construct pMC19	GATCGGTACCCTAAGCATAATCAGGAACATCATACGGATAAGGTTTTTGAGATAAAAGATACTG	KpnI
261	Used to construct pMC7	GGAATTCCATATGAACAGCGAGGGTAAGG	NdeI
262	Used to construct pMC7	GATCAAGCTTTTAAGCATAATCAGGAACATCATACGGATATCTTTCTAAACATAAACACC	HindIII
263	Used to construct pMC8	GGAATTCCATATGGACACGCAATTCATAGC	NdeI
264	Used to construct pMC8	GATCAAGCTTTCAAGCATAATCAGGAACATCATACGGATAATCTCTGTATACCGAACGC	HindIII
265	Used to construct pMC9	GGAATTCCATATGCACTACGAACCCTATGATG	NdeI
266	Used to construct pMC9	GATCAAGCTTTTAAGCATAATCAGGAACATCATACGGATATATGAAAGTAGACCATTTAGACC	HindIII
267	Used to construct pMC10	GGAATTCCATATGCATTCACTTACTGTTTTTCAAG	NdeI
268	Used to construct pMC10	GATCAAGCTTCTAAGCATAATCAGGAACATCATACGGATATGACTTCTGCATAGAGGC	HindIII
277	Used to construct pMC21	GGAATTCCATATGTCGCTAGGGACGACGATTG	NdeI
278	Used to construct pMC21	GATCAAGCTTCTAAGCATAATCAGGAACATCATACGGATAATCCTTACAGAGGCTCG	HindIII
325	Used to construct pMC24 by overlapping PCR	GGGATAAGTACAGAAGAAGGACACCCAGAAACGCTGGTG	-
326	Used to construct pMC24 by overlapping PCR	CACCAGCGTTTCTGGGTGTCCTTCTTCTGTACTTATCCC	-
327	Used to construct pMC25 by overlapping PCR	CCGACATTTGTACGTTTGAATCACCCAGAAACGCTGGTG	-
328	Used to construct pMC25 by overlapping PCR	CACCAGCGTTTCTGGGTGATTCAAACGTACAAATGTCGG	-
690	Used to construct pCM12	GAATTCCATATGAGTATTCGACCTACTAATGG	NdeI
691	Used to construct pCM12	CCCAAGCTTAAGCATAATCAGGAACATCATACGGATAGTCTAAGAAAACAGAAGAAG	HindIII
696	Used to construct pCM13 by overlapping PCR	CACCAGCGTTTCTGGGTGCCATCGAGCAGAAATGTATGG	-

TABLE A2. *Continued*.

Code	Description	Sequence	Restriction enzyme[a]
697	Used to construct pCM13 by overlapping PCR	CCATACATTTCTGCTCGATG GCACCCAGAAACGCTGGTG	-
700	Used to construct pCM4 by overlapping PCR	CACCAGCGTTTCTGGGTGT GTATTTCGTTGGATTTTCG	-
701	Used to construct pCM4 by overlapping PCR	CGAAAATCCAACGAAATAC ACACCCAGAAACGCTGGTG	-
702	Used to construct pCM5 by overlapping PCR	CACCAGCGTTTCTGGGTGC ATCCACTGGGTAAGATC	-
703	Used to construct pCM5 by overlapping PCR	GATCTTACCCAGTGGATGC ACCCAGAAACGCTGGTG	-
704	Used to construct pCM14 by overlapping PCR	CACCAGCGTTTCTGGGTGA TAGTTTCCCGTCTTACG	-
705	Used to construct pCM14 by overlapping PCR	CGTAAGACGGGAAACTATC ACCCAGAAACGCTGGTG	-
706	Used to construct pCM6 by overlapping PCR	CACCAGCGTTTCTGGGTGA TCACAAATAGTCCGATACTT GGG	-
707	Used to construct pCM6 by overlapping PCR	CCCAAGTATCGGACTATTT GTGATCACCCAGAAACGCT GGTG	-
708	Used to construct pCM15 by overlapping PCR	CACCAGCGTTTCTGGGTGC TTTTTGAGTAAGAAAGG	-
709	Used to construct pCM15 by overlapping PCR	CCTTTCTTACTCAAAAAGCA CCCAGAAACGCTGGTG	-
710	Used to construct pCM16 by overlapping PCR	CACCAGCGTTTCTGGGTGT TCTCTAGGAAGAACCCC	-
711	Used to construct pCM16 by overlapping PCR	GGGGTTCTTCCTAGAGAAC ACCCAGAA ACGCTGGTG	-
712	Used to construct pCM17 by overlapping PCR	CACCAGCGTTTCTGGGTGA AATTTTAATAATTTGTTACG	-
713	Used to construct pCM17 by overlapping PCR	CGTAACAAATTATTAAAATT TCACCCAGAAACGCTGGTG	-
714	Used to construct pCM18 by overlapping PCR	CACCAGCGTTTCTGGGTGA GTAAGCTTGTAACCCAG	-
715	Used to construct pCM18 by overlapping PCR	CTGGGTTACAAGCTTACTC ACCCAGAAACGCTGGTG	-
716	Used to construct pCM19 by overlapping PCR	CACCAGCGTTTCTGGGTGC AGAAGGCG CTGTATAAC	-

TABLE A2. *Continued*.

Code	Description	Sequence	Restriction enzyme[a]
717	Used to construct pCM19 by overlapping PCR	GTTATACAGCGCCTTCTGC ACCCAGAAACGCTGGTG	-
718	Used to construct pCM20 by overlapping PCR	CACCAGCGTTTCTGGGTGT CCTACAGTTTCAAAAGG	-
719	Used to construct pCM20 by overlapping PCR	CCTTTTGAAACTGTAGGACA CCCAGAAACGCTGGTG	-
722	Used to construct pCM21 by overlapping PCR	CACCAGCGTTTCTGGGTGT AGTAAAAAACAACGTTTTCG	-
723	Used to construct pCM21 by overlapping PCR	CGAAAACGTTGTTTTTTACT ACACCCAGAAACGCTGGTG	-
724	Used to construct pCM8 by overlapping PCR	CACCAGCGTTTCTGGGTGT GAAACAGAAGTAACTTC	-
725	Used to construct pCM8 by overlapping PCR	GAAGTTACTTCTGTTTCACA CCCAGAAACGCTGGTG	-
726	Used to construct pCM9 by overlapping PCR	CACCAGCGTTTCTGGGTGG CAGATGAGATGATCTAG	-
727	Used to construct pCM9 by overlapping PCR	CTAGATCATCTCATCTGCCA CCCAGAAACGCTGGTG	-
728	Used to construct pCM22 by overlapping PCR	CACCAGCGTTTCTGGGTGG GGGTAGGG CTTTTCTGTGTAGCG	-
729	Used to construct pCM22 by overlapping PCR	CGCTACACAGAAAAGCCCT ACCCCCACCCAGAAACGCT GGTG	-
732	Used to construct pCM11 by overlapping PCR	CACCAGCGTTTCTGGGTGC ATAGGCTGTAAAAGATC	-
733	Used to construct pCM11 by overlapping PCR	GATCTTTTACAGCCTATGCA CCCAGAAACGCTGGTG	-
177	Used to construct pMC31	GGAATTCCATATGAGTGATT CTGACAAAATTATTAATG	NdeI
416	Used to construct pMC31 and pMC46	GATCGAATTCTTATCCTCCT ATCTCTGGG	EcoRI
417	Used to construct pMC32 and pMC47	GATCCTCGAGTTAATCTGC CTCCTTATAAGAAG	XhoI
418	Used to construct pMC33	GATCAAGCTTTTAAGGAACA ACAGGTAGCCG	HindIII
490	Used to construct pMC46 and pCM33	CGCGGATCCAGTGATTCTG ACAAAATTATT	BamHI
491	Used to construct pMC47	GATCGAATTCAGAAACCAG TATTTACAGGGG	EcoRI

TABLE A2. *Continued.*

Code	Description	Sequence	Restriction enzyme[a]
492	Used to construct pMC48	CGCGGATCCACAACACCAG ATAATAATACTAT	BamHI
493	Used to construct pMC48	CCGCTCGAGTTAAGGAACA ACAGGTAGCCG	XhoI
920	Used to construct pCM33	GATCGTCGACTTATCCTCCT ATCTCTGGGTATACG	Sal I
1050	Used to construct pMC56	GATCGAATTCAAGAAACCA GTATTTACAGG	EcoRI
1051	Used to construct pMC56	GATCCTGCAG TTAATCTGCCTCCTTATAAG	PstI
893	Used to construct pCM38	GAT CGG ATC CAC AAC ACC AGA TAA TAA TAC	BamHI
919	Used to construct pCM38	GATCGTCGACTTAAGGAAC AACAGGTAGCCG	Sal I
622	Used to construct pSG1	GATCGAATTCTGATTCTGAC AAAATTATTA ATG	EcoRI
623	Used to construct pSG1	GATCGGATCCTTATCCTCCT ATCTCTGGG	BamHI
624	Used to construct pSG2	GATCCTCGAGCTAAGAAAC CAGTATTTACAGGG	XhoI
617	Used to construct pSG2	GATCGAATTCTTAATCTGCC TCCTTATAAGAAG	EcoRI
1546	Used to construct pMC68,pSVP253 and pMC69	GATC GG TAC C AAC GGA GCC TTC TAG CTA TTT TG	KpnI
1553	Used to construct pMC68 by overlapping PCR	ATC TGT CGA AGT GAG GTT T ATG AGT GAT TCT GAC AAA ATT ATT AAT G	-
1554	Used to construct pMC68 by overlapping PCR	C ATT AAT AAT TTT GTC AGA ATC ACT CAT A AAC CTC ACT TCG ACA GAT	-
1555	Used to construct pMC68 and pMC70	GATC GCG GCC GCG TCC TCC TAT CTC TGG GTA TAC G	NotI
1556	Used to construct pSVP253 by overlapping PCR	ATC TGT CGA AGT GAG GTT T ATG AAG AAA CCA GTA TTT ACA GG	-
1557	Used to construct pSVP253 by overlapping PCR	CC TGT AAA TAC TGG TTT CTT CAT A AAC CTC ACT TCG ACA GAT	-
1558	Used to construct pSVP253 and pMC71	GATC GCG GCC GCG ATC TGC CTC CTT ATA AGA AGA ACC	NotI
1559	Used to construct pMC68 by overlapping PCR	ATC TGT CGA AGT GAG GTT T ATG ACA ACA CCA GAT AAT AAT AC	-
1560	Used to construct pMC68 by overlapping PCR	GT ATT ATT ATC TGG TGT TGT CAT A AAC CTC ACT TCG ACA GAT	-
1561	Used to construct pMC68, pMC72 and pMC73	GATC GCG GCC GCG AGG AAC AAC AGG TAG CCG AAC C	NotI

TABLE A2. *Continued*.

Code	Description	Sequence	Restriction enzyme[a]
1652	Used to construct pMC70, pMC71, pMC72 and pMC73	GATCGGTACCGGACCTCAT AAAAATCCTATCAG	KpnI
1653	Used to construct pMC71 by overlapping PCR	CCCTGTAAATACTGGTTTCT TCATTTAATTGTTTCCAAGT TTTTATTTTG	-
1654	Used to construct pMC71 by overlapping PCR	CAAAATAAAAACTTGGAAAC AATTAAATGAAGAAACCAGT ATTTACAGGG	-
1655	Used to construct pMC72 by overlapping PCR	GTATTATTATCTGGTGTTGT CATTTAATTGTTTCCAAGTT TTTATTTTG	-
454	Used to construct pSVP84	GATC TC ATG AGT GAT TCT GAC AAA ATT ATT AAT GAT TGT CG	BspHI
455	Used to construct pSVP84	GATC GG TAC CAG TCC TCC TAT CTC TGG GTA TAC G	KpnI
1237	Used for trasncription linkage analysis (CT141-CT142)	GCAGGCGCTTCCACGGAAG G	-
1238	Used for trasncription linkage analysis (CT141-CT142)	GGGAGTCTTTCTCCGGGC	-
1239	Used for trasncription linkage analysis (CT142-CT143)	CTCAGGGACTACAGGCCTG C	-
1240	Used for trasncription linkage analysis (CT142-CT143)	CCCTTTAACTAGTGCACC	-
1241	Used for trasncription linkage analysis (CT143-CT144)	CCTATGTATCAGAATCGG	-
1242	Used for trasncription linkage analysis (CT143-CT144)	CCGGATAGCGCTGAACGC	-
1243	Used for trasncription linkage analysis (CT144-CT145)	GGCATCTCCTGCGGCTCCC G	-
1244	Used for trasncription linkage analysis (CT144-CT145)	GCGCAAGAGAAATAGTCCC	-
1255	Used for 5´RACE CT142 Sp1	CCACCCCCATCGCTTCCC	-
1256	Used for 5´RACE CT142 Sp2	CGAGACGATTGATACACC	-
1257	Used for 5´RACE CT142 Sp3	GGCTACTTGATAGATGCCT GG	-

TABLE A2. *Continued*.

Code	Description	Sequence	Restriction enzyme[a]
16SrRNA-9	Used for RT-qPCR	GCGAAGGCGCTTTTCTAATTTAT	-
1*6SrRNA*-10	Used for RT-qPCR	CCAGGGTATCTAATCCTGTTTGCT	-
CT053-A	Used for RT-qPCR	GGATGCAACTTGGCCTTGTT	-
CT053-B	Used for RT-qPCR	TCTTCCTGGTGTCTCTCGATTTCT	-
CT105-C	Used for RT-qPCR	ATGGAGCCGTTTGTGTGGTT	-
CT105-D	Used for RT-qPCR	CCTTCTTCGCTGTTACCCTCACT	-
CT142-A	Used for RT-qPCR	ATGCTTGCGAAAGGTGTGTACA	-
CT142-B	Used for RT-qPCR	CCATCGCTTCCCAACTCCTA	-
CT143-A	Used for RT-qPCR	TGACACATTAACCAGTCGGGATT	-
CT143-B	Used for RT-qPCR	GTATGTATTGTAGGCCCCGTAACC	-
CT144-E	Used for RT-qPCR	TTGTGGGAGAGCGAAACTTTC	-
CT144-F	Used for RT-qPCR	GAACCACACTCCAAGAGAGAGAAGA	-
CT338-C	Used for RT-qPCR	TCCCATGAGGAAATAAGCTTTGA	-
CT338-D	Used for RT-qPCR	GCGGACAGTCGTTGTTTTATCA	-
CT429-A	Used for RT-qPCR	GGTACATCTATTCGATGCCAAGGT	-
CT429-B	Used for RT-qPCR	TGACGCGCCTGTGTTAACTCT	-
CT656-A	Used for RT-qPCR	AGACCAGTTGCGGCAAGCT	-
CT656-B	Used for RT-qPCR	TGCCCTTTCTGTTCTCCCATT	-
CT849-A	Used for RT-qPCR	CAACAGCAATTAAACCAAGAAACG	-
CT849-B	Used for RT-qPCR	GCCAACGACAGCGTATTTGATT	-

[a]Restriction sites are underlined

TABLE A3. *C. trachomatis* strains used in this work

Strain (Code)	Plasmid used in transformation	Description	Source/ Reference	Chapter
L2 (434/Bu)	-	Wild-type	ATCC	II, III, IV
L2 (25667R)	-	Serovar L2 strain lacking the virulence plasmid	(Peterson *et al.*, 1990)	III
13	L2 (434/Bu) + pMC68	P_{incD}-*ct142-HA-incD term*	This Study	IV
4	L2 (434/Bu) + pSVP253	P_{incD}-*ct143-HA-incD term*	This Study	IV
9	L2 (434/Bu) + pMC69	P_{incD}-*ct144-HA-incD term*	This Study	IV
6	L2 (434/Bu) + pMC70	P_{ct142}-*ct142-HA-incD term*	This Study	IV
14	L2 (434/Bu) + pMC71	P_{ct142}-*ct143-HA-incD term*	This Study	IV
5	L2 (434/Bu) + pMC72	P_{ct142}-*ct144-HA-incD term*	This Study	IV
7	L2 (434/Bu) + pMC73	P_{ct142}-*ct142-ct143-ct144-HA-incD term*	This Study	IV

TABLE A4. Summary of results obtained from T3S signals

C. trachomatis protein name		T3S assays CTxxx$_{20}$-TEM-1 proteins				
		ΔHOPEMT				ΔHOPEMT ΔYscU
strain D/UW3	strain L2/434	% secretion average	SEM	*N*	Result	Result
CT016	CTL0271	24,0	10,3	4	S	NS
CT017	CTL0272	1,3	0,5	4	NS	NT
CT031	CTL0286	2,8	1,0	6	NS	NT
CT051	CTL0307	0,5	0,2	4	NS	NT
CT053	CTL0309	5,6	1,6	9	S	NS
CT066	CTL0322	0,6	0,6	4	NS	NT
CT080	CTL0336	1,0	0,5	4	NS	NT
CT082	CTL0338	11,3	3,1	5	S	NS
CT105	CTL0360	40,2	10,2	5	S	NS
CT142	CTL0397	9,6	2,4	6	S	NS
CT143	CTL0398	5,4	2,7	6	S	NS
CT144	CTL0399	11,8	3,0	5	S	NS
CT153	CTL0408	17,9	6,3	4	S	NS
CT161	CTL0417	6,9	1,8	4	S	NS
CT172	CTL0425	9,6	3,2	7	S	NS
CT203	CTL0455	10,6	3,6	5	S	NS
CT273	CTL0525	28,1	11,2	4	S	NS
CT277	CTL0529	1,4	0,9	4	NS	NT
CT289	CTL0541	39,0	12,6	4	S	NS
CT309	CTL0561	6,2	1,9	5	S	NT
CT330	CTL0584	3,9	1,9	6	NS	NT
CT338	CTL0592	36,8	9,8	5	S	NS
CT386	CTL0642	33,4	9,0	4	S	NS
CT425	CTL0684	31,5	9,6	4	S	NS
CT429	CTL0688	28,7	7,8	4	S	NS
CT504	CTL0766	20,7	6,5	4	S	NS
CT538	CTL0800	2,4	1,0	4	NS	NT
CT568	CTL0831	0,7	0,4	4	NS	NT
CT577	CTL0840	0,6	0,6	4	NS	NT
CT583	CTL0846	8,9	1,7	5	S	NS
CT584	CTL0847	2,4	1,3	5	NS	NT
CT590	CTL0853	1,5	1,4	4	NS (?)	NT
CT631	CTL0895	26,1	4,0	4	S	NS
CT635	CTL0003	0,0	0,0	4	NS	NT
CT656	CTL0025	28,6	7,5	4	S	NS
CT694	CTL0063	55,8	4,7	18	S	NS
CT696	CTL0065	10,2	2,8	6	S	NS
CT702	CTL0071	0,9	0,4	4	NS	NT
CT768	CTL0137	0,0	0,0	4	NS	NT
CT779	CTL0148	31,2	5,5	4	S	NS
CT814	CTL0185	0,0	0,0	4	NS	NT
CT837	CTL0209	2,5	1,6	4	NS	NT
CT845	CTL0217	1,5	0,8	4	NS (?)	NT
CT849	CTL0221	20,3	5,0	4	S	NS
CT863	CTL0238	1,4	0,9	4	NS (?)	NT
RplJ	RplJ	1,5	0,6	17	NS	NS

S, Secreted; NS, Not Secreted; NT, Not Tested; ND, Not Determined; (?), Unclear Result

TABLE A5. Summary of results obtained from T3S of *C. trachomatis* full-length proteins

C. trachomatis protein name		T3S assays $CTxxx_{FL}$-HA proteins				
		ΔHOPEMT				ΔHOPEMT ΔYscU
strain D/UW3	strain L2/434	% secretion average	SEM	*N*	Result	Result
CT016	CTL0271	ND	ND	ND	NS (?)	NT
CT017	CTL0272	ND	ND	ND	NT	NT
CT031	CTL0286	ND	ND	ND	NT	NT
CT051	CTL0307	ND	ND	ND	NT	NT
CT053	CTL0309	6,0	2,3	4	S	NS
CT066	CTL0322	ND	ND	ND	NT	NT
CT080	CTL0336	ND	ND	ND	NT	NT
CT082	CTL0338	32,9	12,6	5	S	NS
CT105	CTL0360	8,5	2,8	6	S	NS
CT142	CTL0397	2,3	1,0	4	S	NS
CT143	CTL0398	13,2	2,2	5	S	NS
CT144	CTL0399	5,0	0,9	4	S	NS
CT153	CTL0408	0,9	0,3	6	NS	NT
CT161	CTL0417	9,0	1,8	4	S	NS
CT172	CTL0425	0,6	0,1	5	NS	NT
CT203	CTL0455	0,5	0,1	5	NS	NT
CT273	CTL0525	0,2	0,1	2	NS (?)	NT
CT277	CTL0529	ND	ND	ND	NT	NT
CT289	CTL0541	0,4	0,1	3	NS (?)	NT
CT309	CTL0561	0,4	0,2	3	NS (?)	NT
CT330	CTL0584	ND	ND	ND	NT	NT
CT338	CTL0592	2,2	0,7	5	S	NS
CT386	CTL0642	0,5	0,1	7	NS	NT
CT425	CTL0684	0,3	0,1	7	NS	NT
CT429	CTL0688	2,2	0,4	6	S	NS
CT504	CTL0766	1,0	0,3	7	NS	NT
CT538	CTL0800	ND	ND	ND	NT	NT
CT568	CTL0831	ND	ND	ND	NT	NT
CT577	CTL0840	ND	ND	ND	NT	NT
CT583	CTL0846	14,4	3,1	8	S	NS (?)
CT584	CTL0847	ND	ND	ND	NT	NT
CT590	CTL0853	ND	ND	ND	NT	NT
CT631	CTL0895	ND	ND	ND	NS (?)	NT
CT635	CTL0003	ND	ND	ND	NT	NT
CT656	CTL0025	26,9	10,3	5	S	NT
CT694	CTL0063	20,8	2,7	10	S	NS
CT696	CTL0065	ND	ND	ND	NS (?)	NT
CT702	CTL0071	ND	ND	ND	NT	NT
CT768	CTL0137	ND	ND	ND	NT	NT
CT779	CTL0148	1,1	0,4	6	NS	NT
CT814	CTL0185	ND	ND	ND	NT	NT
CT837	CTL0209	ND	ND	ND	NT	NT
CT845	CTL0217	ND	ND	ND	NT	NT
CT849	CTL0221	9,7	2,5	5	S	NS
CT863	CTL0238	ND	ND	ND	NT	NT
RplJ	RplJ	0,1	0,0	13	NS	NS

S, Secreted; NS, Not Secreted; NT, Not Tested; ND, Not Determined; (?), Unclear Result

TABLE A6. Comparison of results obtained in analyses of T3S signals in proteins of *C. trachomatis* and to *in silico* prediction methods

Observed Results T3S assays			Prediction of T3S signals			
Protein	CTxxx20-TEM-1	CTxxxFL-HA	Effective T3S[a]	SIEVE[b]	Modlab[c]	T3_MM[d]
CT016	S	NS (?)	NS	NS	NS	NS
CT017	NS	NT	NS	NS	NS	NS
CT031	NS	NT	NS	NS	NS	NS
CT051	NS	NT	NS	NS	NS	S
CT053	S	S	NS	NS	NS	S
CT066	NS	NT	NS	NS	NS	S
CT080	NS	NT	NS	S	NS	NS
CT082	S	S	S	S	S	S
CT105	S	S	S	S	S	S
CT142	S	S	NS	S	NS	S
CT143	S	S	NS	NS	S	S
CT144	S	S	S	NS	NS	S
CT153	S	NS	NS	NS	NS	NS
CT161	S	S	NS	NS	NS	NS
CT172	S	NS	NS	NS	NS	NS
CT203	S	NS	NS	NS	NS	NS
CT273	S	NS (?)	S	NS	NS	S
CT277	NS	NT	NS	NS	NS	NS
CT289	S	NS (?)	NS	NS	NS	NS
CT309	S	NS (?)	S	S	NS	NS
CT330	NS	NT	NS	NS	NS	NS
CT338	S	S	NS	NS	NS	NS
CT386	S	NS	NS	NS	NS	NS
CT425	S	NS	NS	NS	NS	NS
CT429	S	S	NS	NS	NS	NS
CT504	S	NS	NS	NS	NS	NS
CT538	NS	NT	NS	NS	NS	S
CT568	NS	NT	NS	NS	NS	NS
CT577	NS	NT	S	NS	NS	S
CT583	S	S	NS	S	NS	S
CT584	NS	NT	NS	NS	NS	NS
CT590	NS (?)	NT	NS	NS	NS	NS
CT631	S	NS (?)	NS	NS	NS	S
CT635	NS	NT	NS	NS	NS	S
CT656	S	S	S	NS	S	S
CT694	S	S	S	S	S	S
CT696	S	NS (?)	NS	S	NS	NS
CT702	NS	NT	NS	NS	NS	NS
CT768	NS	NT	NS	NS	NS	S
CT779	S	NS	NS	NS	NS	S
CT814	NS	NT	NS	NS	NS	NS
CT837	NS	NT	NS	NS	NS	NS
CT845	NS (?)	NT	NS	NS	NS	NS
CT849	S	S	S	S	NS	S
CT863	NS (?)	NT	S	S	S	S
RplJ	NS	NS	NS	NS	NS	NS

S, Secreted; NS, Not Secreted; NT, Not Tested; ND, Not Determined; (?), Unclear Result. a) Using animal set and sensitive settings (cutoff 0.95) (Arnold *et al.*, 2009); b) Using information from Table 3 in (Samudrala *et al.*, 2009); c) Using Neural Network to predict T3S signal (threshold 0.4) (Löwer & Schneider, 2009); d) Using predefined settings (Wang *et al.*, 2013).

REFERENCES

Agaisse, H. & Derré, I. (2013). A *C. trachomatis* Cloning Vector and the Generation of *C. trachomatis* Strains Expressing Fluorescent Proteins under the Control of a *C. trachomatis* Promoter. *PLoS One* **8**(2), e57090.

Almeida, F., Borges, V., Ferreira, R., Borrego, M. J., Gomes, J. P. & Mota, L. J. (2012). Polymorphisms in Inc proteins and differential expression of *inc* genes among *Chlamydia trachomatis* strains correlate with invasiveness and tropism of Lymphogranuloma venereum isolates. *J Bacteriol* **194**(23), 6574–6585.

Arnold, R., Brandmaier, S., Kleine, F., Tischler, P., Heinz, E., Behrens, S., Niinikoski, A., Mewes, H. W., Horn, M. & Rattei, T. (2009). Sequence-Based Prediction of Type III Secreted Proteins. *PLoS Pathog* **5**(4), e1000376.

Charpentier, X. & Oswald, E. (2004). Identification of the secretion and translocation domain of the Enteropathogenic and Enterohemorrhagic *Escherichia coli* effector Cif, using TEM-1 β-lactamase as a new fluorescence-based reporter. *J Bacteriol* **186**(16), 5486–5495.

Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A, Roop, R. M. & Peterson, K. M. (1995). Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* **166**, 175–176.

Löwer, M. & Schneider, G. (2009). Prediction of type III secretion signals in genomes of gram-negative bacteria. *PLoS One* **4**(6), e5917.

Marenne, M. N., Journet, L., Mota, L. J. & Cornelis, G. R. (2003). Genetic analysis of the formation of the Ysc-Yop translocation pore in macrophages by *Yersinia enterocolitica*: Role of LcrV, YscF and YopN. *Microb Pathog* **35**, 243–258.

Peterson, E. M., Markoff, B. A., Schachter, J. & de la Maza, L. M. (1990). The 7.5-kb plasmid present in *Chlamydia trachomatis* is not essential for the growth of this microorganism. *Plasmid* **23**, 144–8.

Samudrala, R., Heffron, F. & McDermott, J. E. (2009). Accurate prediction of secreted substrates and identification of a conserved putative secretion signal for type III secretion systems. *PLoS Pathog* **5**(4), e1000375.

Wang, Y., Sun, M., Bao, H. & White, A. P. (2013). T3_MM: A Markov Model Effectively Classifies Bacterial Type III Secretion Signals. *PLoS One* **8**(3), e.