DETERMINATION OF THE EFFECT OF JO146, A CTHTRA INHIBITOR, ON THE CHLAMYDIAL DEVELOPMENTAL CYCLE AND PERSISTENCE

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Abstract

Chlamydia trachomatis is the most common sexually transmitted bacterial pathogen worldwide. Among the many proteases expressed by bacteria, the High Temperature Requirement protein A (HtrA) family of proteins have been documented to have both chaperone and proteolytic activities. HtrA has been demonstrated to be important for both stress response and virulence of a number of bacterial pathogens. *C. trachomatis* HtrA (CtHtrA) has been documented to be elevated during heat stress and penicillin persistence models of this organism. However, the specific function of CtHtrA for the growth and pathogenicity of *C. trachomatis* has not been elucidated. Therefore, this PhD aimed to characterize the biological role of CtHtrA for developmental cycle during normal and stress conditions using the CtHtrA inhibitor compound, JO146, as well as genetic approaches. JO146 addition at 16 h PI (replicative phase) resulted in complete bacterial lethality as indicated by the loss of infectious progeny production at the end of the chlamydial developmental cycle. JO146 treatment resulted in marked decrease in the size of inclusions compared to the inclusions formed in the control, DMSO-treated cells when the cultures were examined for CtHtrA and MOMP through immunocytochemistry and confocal microscopy. Therefore, CtHtrA was important for the maintenance of cell morphology and infectious progeny production during the replicative phase of the chlamydial developmental cycle. The inhibitory effects of JO146 appeared to be partially reversible, signifying a bacteriostatic effect, as indicated by the recovery of infectious progeny when the compound was removed from the cultures. In addition, JO146 inhibited chlamydial growth without compromising the host cell viability. The inhibitory effect of JO146 was not mediated by host cell autophagy and lysosome pathways as indicated by the absence of sequestration between the chlamydial inclusions and the autophagy and lysosome markers LAMP1 and SQSTM1 during JO146 treatment.

It was identified that CtHtrA was essential during detrimental conditions such as heat stress and penicillin induced persistence where it is expected that extracytoplasmic proteins were compromised. JO146 addition during penicillin persistence resulted in slightly decreased infectious progeny yield. However, JO146

treatment during reversion and recovery to infectivity from penicillin persistence was completely lethal for *Chlamydia*. Therefore, CtHtrA was important either for the stress, or restoration of outer membrane protein assembly during recovery from penicillin persistence. Penicillin persistence may be a strategy for *Chlamydia* to reduce cellular and protein stress which may explain the reduced lethality of JO146 when the compound was added during persistence compared to when added during recovery from persistence.

Chemical mutagenesis coupled with whole genome sequencing of mutants that survived in continued sequential cultivation in the presence of JO146 was used to identify genetic loci that are involved in JO146 susceptibility that may link to CtHtrA function. Three clones that were less susceptible to JO146 and designated as 1A3, 1B3 and 2A3 were isolated and genotypically and phenotypically characterized. Genome sequence analysis revealed that the mutants acquired unique single nucleotide variants (SNVs) in the two genes, CT776 and CT206 which code for acyltransferases, signifying that these two loci were impacted by the function of JO146. CT776 and CT206 are both involved in fatty acid metabolism which was previously found to be highly essential for RB replication. Therefore, CT776 and CT206 may have compensated for CtHtrA inhibition by JO146 and may have indirect contributions to CtHtrA functions particularly for outer membrane protein biogenesis and maintenance of the bacterial envelope. Single base substitutions in these two loci may have conferred the mutants beneficial phenotypic characteristics. The mutants had higher yields of infectious progeny compared to wild-type *C. trachomatis* D (CtDpp) as shown in the growth analysis during normal conditions. Higher numbers of recoverable infectious EBs were also observed for the mutants with reduced susceptibility to JO146 during heat stress in combination with JO146 treatment and also during recovery from penicillin persistence.

Furthermore, results from this study demonstrated that JO146 was effective against currently circulating *C. trachomatis* clinical isolates representing different serovars. JO146 addition at mid-replicative phase resulted in loss of infectious progeny production and a reduction in inclusion vacuole size in *C. trachomatis* clinical isolates grown in McCoy B cells. Infectious progeny were recovered when JO146 was removed from the cultures, indicating that the compound had a bacteriostatic effect and should be left in the cultures until the end of the chlamydial

developmental cycle for it to be effective against *Chlamydia*. Western blot analysis showed a clear reduction on MOMP in the clinical isolates treated with JO146 compared to matched DMSO-treated controls.

Overall, this study demonstrated that inhibition of CtHtrA by the inhibitor JO146 during the replicative phase resulted in a disrupted chlamydial developmental cycle, reduction of inclusion vacuole size and prevented the formation of infectious progeny without being toxic to the host cells. CtHtrA was essential for chlamydial recovery from penicillin persistence and for heat stress conditions. The acyltransferases, CT776 and CT206 were the two identified loci in mutants with less susceptibility to JO146, indicating that these proteins which are involved in fatty acid synthesis might have an indirect impact on CtHtrA functions. JO146 was also effective against recent clinical isolates of *C. trachomatis*. These findings provide proof of concept that CtHtrA is a good, potential target for future development of anti-chlamydial therapeutics.

Research publications associated with this thesis

- **V.A. Ong**, A. Lawrence, P. Timms, L.A. Vodstrcil, S. Tabrizi, K.W. Beagley, J.A. Allan, J.S. Hocking, W.M. Huston. September 2015. *In vitro* susceptibility of recent *Chlamydia trachomatis* clinical isolates to the HtrA inhibitor JO146. This paper has been accepted for publication as a short communication in Microbes and Infection.
- **V.A. Ong**, J.W. Marsh, A. Lawrence, J.A. Allan, P. Timms, W.M. Huston. December 2013. The protease inhibitor JO146 demonstrates a critical role for CtHtrA for *Chlamydia trachomatis* reversion from penicillin persistence. Frontiers in Cellular and Infection Microbiology.3:100.
- S. Gloeckl*****, **V.A. Ong***, P. Patel*****, J.D.A. Tyndall, P. Timms, K.W. Beagley, J.A. Allan, C.W. Armitage, L. Turnbull, C.B. Whitchurch, M. Merdanovic, M. Ehrmann, J.C. Powers, J. Oleksyszyn, M. Verdoes, M. Bogyo, W.M. Huston. June 2013. Identification of a serine protease inhibitor which causes inclusion vacuole reduction and is lethal to *Chlamydia trachomatis*. Molecular Microbiology. 89(4): 676-689. (*These authors contributed equally to this paper.)

Research Presentations

- Institute of Health and Biomedical Innovation Annual Postgraduate Student Conference: IHBI Inspires 2014. Title of poster presentation: "Determination of *Chlamydia* CtHtrA function during penicillin persistence and heat stress using a chemical inhibitor". Mercure Gold Coast Resort, QLD Australia, November 19-20, 2014
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- Australian Society for Microbiology Annual Scientific Meeting 2013. Title of poster presentation: "Determination of *Chlamydia* HtrA function using a chemical inhibitor", Adelaide Convention Centre, Adelaide, South Australia, July 7- 12, 2013
- Institute of Health and Biomedical Innovation Annual Postgraduate Student Conference: IHBI Inspires 2012. Title of poster presentation: "Determination of *Chlamydia* HtrA function using a chemical inhibitor", Radisson Resort, Gold Coast, QLD Australia, November 22-23, 2012

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List of Abbreviations

16S rRNA	16S ribosomal ribonucleic acid protein
23S rRNA	23S ribosomal ribonucleic acid protein
AB	aberrant body
ABPs	activity-based probes
ATP	adenosine triphosphate
Bae	bacterial adaptive response
BZK	benzalkonium chloride
CAT	chlamydial IgG antibody test
CERT	ceramide transfer protein
CHG	chlorhexidine gluconate
CL	cardiolipin
CPAF	chlamydial protease/proteasome-like activity factor
Cpx	conjugative plasmid expression
CtD	*Chlamydia trachomatis* D (UW-3/Cx)
CtHtrA	*Chlamydia trachomatis* high temperature requirement protein A
DA-DA	D-alanine-D-alanine
DHFR	dihydrolate reductase
EB	elementary body
ECF	extracytoplasmic function
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's minimal essential medium

DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EGFR	epidermal growth factor receptor
ELISA	enzyme linked immunosorbent assay
EMS	ethyl methanesulfonate
FCS	fetal calf serum
FGF2	fibroblast growth factor 2
FGFR	fibroblast growth factor receptor
GET 2020	Global Alliance in the Elimination of Blinding Trachoma by year 2020
HeLa	Henrietta Lacks immortal cell line
HEp2	human epithelial type 2 cell line
HIV	human immunodeficiency virus
h PI	hours post-infection
HRP	horseradish peroxidase
HSP	heat shock protein
HSPGs	heparin sulfate proteoglycans
HtrA	high temperature requirement protein A
IκBα	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
IDO1	indoleamine 2,3-dioxygenase
IFU	inclusion forming unit
LAMP1	lysosomal-associated membrane protein
LGV	*Lymphogranuloma venereum*
MCR	MoPn with CF0001 resistance
MIF	microimmunoflourescence
MOI	multiplicity of infection

MOMP	major outer membrane protein
MSM	men having sex with men
NAATs	nucleic acid amplification tests
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
MCSs	membrane contact sites
MHC	major histocompatibility complex
MOI	multiplicity of infection
OMP	outer membrane protein
ORF	open reading frame
PBS	phosphate buffered saline
PBPs	penicillin binding proteins
PCR	polymerase chain reaction
PE	phosphoethanolamine
PFA	paraformaldehyde
PG	peptidoglycan
PI	protease inhibitor
PID	pelvic inflammatory disease
Psp	phage shock protein
PTG	phosphatidylglycerol
qPCR	quantitative polymerase chain reaction
RB	reticulate body
RCL	reactive centre loop
Rif^R	rifampicin resistance
RIP	regulated intramembrane proteolysis
RFX5	regulatory factor X 5
ROS	reactive oxygen species

rtPCR	real-time polymerase chain reaction
SAFE	surgery, antibiotics, facial cleanliness and environmental improvement
SDA	strand-displacement amplification
SDS	sodium dodecyl sulfate
SDS PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIRS	systemic inflammatory response syndrome
SNARE	soluble NSF (N-ethylmaleimide-sensitive factor) attachment protein receptors
SNPs	single nucleotide polymorphisms
SNVs	single nucleotide variants
SPG	sucrose phosphate glutamate
STIM1	stromal interaction molecule 1
SQSTM1	sequestosome 1
T3SS	type 3 secretion system
TILLING	targeting induced local lesions in genomes
TMA	transcription-mediated amplification
UGT	upper genital tract
USF-1	upstream stimulation factor
VAPA	VAMP (vesicle-associated membrane protein)-associated protein A
VAPB	VAMP (vesicle-associated membrane protein)-associated protein B
WGS	whole genome sequencing
WHO	World Health Organization
WIF	whole cell inclusion immunofluorescence

Statement of Original Authorship

The work contained in this thesis has not been previously submitted to meet requirements for an award at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made.

QUT Verified Signature

Signature:

Date: October 2015

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“If we did everything we are capable of doing, we would literally astound ourselves.”

- Ralph Waldo Emerson

Chapter 1: **Introduction**

1.1 BACKGROUND

Chlamydia (C.) trachomatis is an obligate bacterial intracellular pathogen that is the most prevalent bacterial sexually transmitted infections worldwide. *C. trachomatis* can result in serious, long-term sequelae such as tubal scarring, pelvic inflammatory disease, infertility and ectopic pregnancy in women and urethritis and epididymitis in men [1-6]. *C. trachomatis* is also the leading cause of preventable blindness, trachoma [7]. Pathogenic organisms employ a suite of metabolic mechanisms and virulence factors (including proteases) in order to successfully establish host-cell attachment and initiate subsequent infection. One biochemical activity that is often found in bacterial virulence factors is proteolysis. The high temperature requirement protein A (HtrA) is a highly conserved bacterial protease that has been generally described as a bacterial quality control protein that is critical for cellular survival during stress. HtrA has also been identified as essential for pathogenesis for several important infectious diseases [8-13]. However, much has yet to be uncovered about the role of CtHtrA in chlamydial infection. The present study aimed to identify the roles of CtHtrA for *C. trachomatis* pathogenicity and development especially during stress conditions by using the CtHtrA protease inhibitor, JO146.

1.2 CONTEXT

The works presented in this thesis clearly demonstrated that inhibition of CtHtrA using the chemical compound, JO146 led to loss of chlamydial inclusion morphology, decreased inclusion size, and complete loss of infectious progeny production when the compound was added at the mid-replicative phase of the developmental cycle. These effects occurred in the absence of host cell cytotoxicity and were partially reversible by removal of the compound. JO146 treatments during heat stress and during reversion from penicillin-induced persistence were also found to be lethal to *Chlamydia*. These data indicate that the function of CtHtrA appears to be essential during the replicative phase as well as during conditions when extracytoplasmic protein homeostasis is compromised. Mutants with reduced susceptibility to the inhibitor compound had higher infectious yield compared to wild type *C. trachomatis* D during heat stress and during recovery from penicillin-induced

persistence. One possible explanation could be that mutations in the acyltransferase genes observed in the mutants might have conferred them growth advantage over wild type *C. trachomatis* D, especially during harmful conditions. The current study also provided evidence that JO146 is effective against *C. trachomatis* clinical isolates, supporting the notion that CtHtrA might be a relevant target for future development of anti-chlamydial therapeutics.

1.3 PURPOSES

The overall objective of the study is to contribute to the knowledge about the *C. trachomatis* HtrA (CtHtrA) and its role in pathogenicity and normal developmental cycle of *C. trachomatis*.

The three specific aims of the study were investigated and are presented as three results chapters. The aims were:

1. To determine if CtHtrA is essential for chlamydial growth and persistence in human cell culture models using the inhibitor, JO146.
2. To isolate *C. trachomatis* genetic mutants resistant to the CtHtrA inhibitor (JO146) and characterize the phenotypic and genetic basis of this resistance.
3. To establish if JO146 is effective against currently circulating clinical isolates of *C. trachomatis*, thus validating if CtHtrA is a clinically relevant target for future development of anti-chlamydial drugs.

1.4 SCOPE

Previous investigations in the group identified JO146 in a library of serine protease inhibitors. The compound appeared to be specific for CtHtrA based on studies conducted within the group prior to the commencement of this PhD study. This project investigated the role of CtHtrA in chlamydial infection and pathogenesis using the inhibitor of CtHtrA activity, JO146, during a suite of *in vitro* experiments.

1.5 THESIS OUTLINE

Chapter Two is a review of the literature relevant to the project area.

Chapter Three provides a detailed account of the procedures that were followed in completing the experiments for this PhD project.

Chapter Four describes the application of the CtHtrA inhibitor, JO146 to cultures of *Chlamydia* to investigate CtHtrA function. Inclusion morphology and production of chlamydial infectious progeny upon treatment with the compound were determined. Evaluation of the role of host cell autophagy pathways on the inhibitory impact of JO146, as well as the host cell cytotoxicity of the compound was also conducted. The role of CtHtrA during stress conditions such as heat stress and penicillin induced persistence was also assessed by JO146 treatments during these conditions.

Chapter Five describes the isolation and characterization of *C. trachomatis* mutants resistant to JO146. Three mutants with reduced susceptibility to JO146 were isolated. The isolated mutants were phenotypically characterized. The mutants recovered more infectious progeny compared to the wild-type strain during normal and stress conditions. Comparative genome sequence analysis of the mutants with reference to wild type *C. trachomatis* D revealed mutations in acyltransferase genes, which are mainly involved in bacterial membrane biogenesis and catabolism, thus relating to CtHtrA functions.

Chapter Six describes the application of JO146 against currently circulating *C. trachomatis* clinical isolates representing different serovars. This work demonstrated that JO146 was effective in inhibiting chlamydial growth and resulted in decreased inclusion size for the clinical isolates examined, indicating that CtHtrA inhibition may form the basis for developing new class of anti-chlamydial drugs.

Chapters Four and Six have been accepted in peer reviewed international journals. Chapter Seven is a comprehensive discussion of the main findings and future directions of this study.

Chapter 2: **Literature Review**

2.1 INTRODUCTION

Chlamydia (*C.*) *trachomatis* is an obligate intracellular bacterial pathogen that causes sexually transmitted infections. *C. trachomatis* infection can result in serious complications such as pelvic inflammatory disease, infertility and ectopic pregnancy in women [14-17]. Clinical manifestations of *C. trachomatis* in men include epididymitis and urethritis [6]. Chlamydial infections can also cause chlamydial conjunctivitis and pneumonia in neonates passing through the birth canal of an infected woman [18]. Previous studies have demonstrated a number of virulence factors employed by *C. trachomatis* in order to successfully establish host-cell attachment, infection and propagation of the infection. One example family of virulence factors is the high temperature requirement protein A (HtrA) which plays a critical role in protein quality control. HtrA in organisms such as *Escherichia coli, Campylobacter jejuni* and *Shigella flexneri* efficiently cleaves E-cadherin on host cells to disrupt the epithelial barrier, a highly conserved and common mechanism in bacterial pathogenesis [10]. The potential role of HtrA in bacterial pathogenesis of *C. trachomatis* has been previously studied. Huston and co-workers [19] detected increased levels of HtrA in *C. trachomatis* (CtHtrA) during heat stress compared to acute (untreated) conditions. The reversibility of the heat-shock process was analysed by Kahane and Friedman [20], who reported that cultures that were incubated at 42^{o}C for 2, 5, and 9 hours recovered full infectivity when returned to 37^{o}C. These findings suggest a key role for CtHtrA in the heat stress response by *C. trachomatis* and its essential contribution in maintaining the viability of *C. trachomatis* especially during conditions detrimental to extracytoplasmic protein stability [19]. CtHtrA has also been found to be highly expressed during penicillin persistence but down regulated during IFN-γ persistence [19, 21, 22], indicating that CtHtrA may have potential functions during penicillin-induced persistence of *C. trachomatis*. Additionally, CtHtrA appears to be actively secreted into host cell cytosol [23]. Therefore, CtHtrA has the potential to be a significant factor for chlamydial development as well as for survival during stress conditions.

2.2 *C. TRACHOMATIS* EPIDEMIOLOGY AND DISEASE

2.2.1 *C. trachomatis* serovars and strains

C. trachomatis is the leading cause of sexually transmitted infections globally and the etiologic agent of preventable blindness in the developing world. *C. trachomatis* belongs to the family Chlamydiaceae, order Chlamydiales [24]. Based on phylogenetic analyses of 16S and 23S rRNA gene sequence, current taxonomic status of chlamydiae indicate that a single genus, *Chlamydia*, should be used for species of the genera *Chlamydia* and *Chlamydophila* [25]. These species are *Chlamydia trachomatis*, *C. suis*, *C. muridarum*, *C. pecorum*, *C. pneumoniae*, *C. psittaci*, *C. felis*, *C. abortus*, *C. caviae*, *C. avium*, and *C. gallinacea* [26-28]. Currently, the typing scheme for *C. trachomatis* is based on serospecificity of the major outer membrane protein (MOMP) [29, 30] that constitutes 60% of all the surface-exposed proteins [31] and functions as a porin [32]. *C. trachomatis* serovars can be divided into trachoma causing ocular infections, and urogenital strains causing ano-genital tract infections.

Genital Tract Infections

Nucleotide sequence differences between the serovars correlate with tissue tropism [33, 34]. Serovars A-C primarily infect ocular tissue, serovars D-K typically infect urogenital tissue, and serovars L1-L3 infect lymphatic tissue, which may involve perirectal and/or perianal lymphatic tissues [35, 36]. *C. trachomatis* particularly serovars D to K [37], are the most common causes of sexually transmitted diseases worldwide. In the United States, over 1.4 million cases of *C. trachomatis* infection were reported in 2012, the highest number of cases ever reported to CDC for any condition [38]. The actual infection rates are presumably underestimated because many infections are neither diagnosed nor treated due to the asymptomatic nature of the disease. As high as 50% and 80% asymptomatic cases of *Chlamydia* infections have been reported in men and women, respectively [4]. According to the National Notifiable Diseases Surveillance System of the Department of Health, Australia, chlamydial infections have exhibited a steady rise of prevalence, with 82,576 cases reported from the period April 2014 to April 2015 (http://www.health.gov.au/cdnareport). An age-structural heterosexual transmission model was developed to assess the impact of screening and treatment scenarios on age-dependent *Chlamydia* incidence and prevalence in Australian population [39].

The model predicts that *Chlamydia* prevalence in Australia would be reduced rapidly over 10 years in all age groups if 40% of men and women were screened yearly, with >50% of the reduction being achieved during the first 4 years, provided that 40% screening is achieved [39]. Figure 2.1 shows the predicted prevalence of *C. trachomatis* infections in different age groups in Australia after introduction of screening.

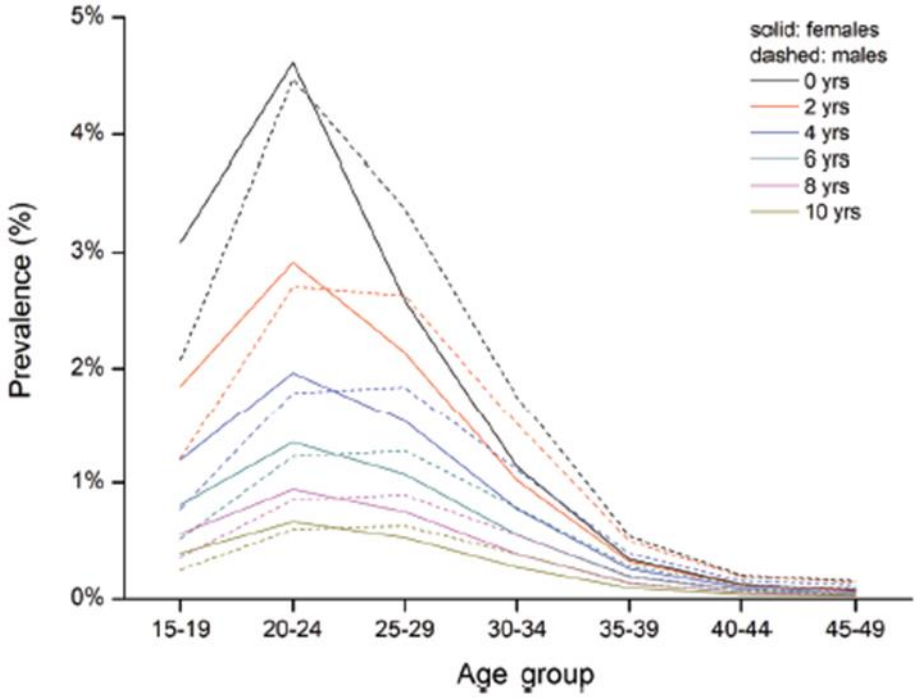

Figure 2.1. Predicted age-specific prevalence of *C. trachomatis* in Australia after introduction of the screening model

Age-specific prevalence in males (dashed lines) and females (solid lines) at 0, 2, 4, 6, 8, and 10 years after the introduction of screening, when 40% of both males and females are screened annually. Figure copied directly from Regan and co-workers, 2008 [39]

C. trachomatis is associated with non-gonococcal urethritis and epididymitis in men [6], and cervicitis, and pelvic inflammatory disease (PID) in women [15]. Some women develop disease sequelae such as tubal infection [16] or tubal factor pathology, one of the leading causes of ectopic pregnancy and infertility in women [17]. Women who had two and three or more chlamydial infections are also known to have elevated risks of hospitalizations for ectopic pregnancy and PID [40]. *C. trachomatis* infection has also been associated with increased risk of acquiring human immunodeficiency virus (HIV) [41].

Lymphogranuloma venereum (LGV) is an invasive infection caused by *C. trachomatis* serovars L1, L2, and L3 [42]. While infections with *C. trachomatis* serovars D-K are mostly associated with mild symptoms and are more often asymptomatic, infections with L genotypes are more invasive and cause ano-genital ulcer [43]. There have been recent outbreaks of LGV in Europe and South America particularly among men having sex with men (MSM) [43]. It was reported that 89.6% of patients with LGV were HIV-infected and that the epidemic of LGV in predominantly HIV-positive MSM is ongoing [43].

Ocular infections

C. trachomatis serovars A, B and C are the leading cause of infectious blindness worldwide, trachoma. Multiple episodes of reinfections cause inflammation and roughening of the conjunctival lining of the upper eyelid and can cause the eyelashes to scratch the cornea (trichiasis) [44]. Trachoma is endemic in 48 countries including large areas of the Middle East and Africa and smaller areas in south and central Asia, Latin America and Australasia [45]. More than 1 million people have become blind due to trachoma and 10 million people with trichiasis are in imminent danger of going blind [44]. There have been many programs established to control the rate of increase of trachoma infections worldwide. In 1998, the World Health Organization (WHO) and a consortium of nongovernmental organizations launched the Global Alliance in the Elimination of Blinding Trachoma by 2020 (GET 2020). The integrated strategy promoted by these organizations is known by the acronym SAFE (surgery, antibiotics, facial cleanliness and environmental improvement), an innovative public health approach designed to treat and prevent trachoma [44, 46]. At the 17th meeting of the WHO Alliance for GET 2020 in Geneva, Switzerland in 2013, a number of countries, Ghana, Islamic Republic of Iran, Morocco, Myanmar and Oman, have reported that they have reached the goal for eliminating blinding trachoma [47].

2.3 *CHLAMYDIA* DIAGNOSIS AND TREATMENT

2.3.1 Diagnostic tools for *C. trachomatis*

Effective diagnosis and treatment of patients with *C. trachomatis*, as well as their sexual partners, is important to prevent the spread and transmission of the

disease and reduce health burden. An array of diagnostic protocols has been developed for detection of *C. trachomatis*. Cell culture of cervical, vaginal swab, and urine was the main tool for diagnosis of *C. trachomatis* infection until the development of antigen and nucleic acid detection technologies [48, 49]. Cell culture is nearly 100% specific for *C. trachomatis* in urogenital specimens [reviewed by 50]. McCoy, HEp2 and HeLa cells are most commonly used for *C. trachomatis*, wherein clinical specimens are inoculated onto cycloheximide-treated monolayer of any of these appropriate cells followed by incubation for 48 to 72 hours and subsequent staining for cytoplasmic inclusions [reviewed by 49]. However, cell culture is not a recommended diagnostic method for routine use due to its complexity and technical skills required as well as the laborious nature of the technique. More sensitive techniques such as *C. trachomatis* nucleic acid amplification tests (NAATs) have been developed and implemented which are now commonly used.

A number of studies have found NAATs to be more sensitive than culture for detection of chlamydial infections [51-54]. Bachmann and co-workers [51] evaluated the performance of culture and NAATs employing transcription-mediated amplification (TMA), strand-displacement amplification (SDA) and PCR amplification for the detection of rectal gonococcal and chlamydial infections using rectal swab specimens. Their group found that each of these three NAATs is substantially more sensitive than is culture for the detection of *C. trachomatis*, with sensitivities ranging from 36.1% to 45.7% for culture and among NAATs from 91.4% to 95.8% for PCR to 100% for TMA [51]. Although DNA amplification technique is a sensitive and specific method for the diagnosis of *C. trachomatis* infection and is most relevant for detection of current infection, recent studies had also developed other rapid and sensitive screening techniques such as immunological diagnostic methods, particularly serological diagnostic tests [55-59] .

Serological detection of anti-chlamydial antibodies is an easier method particularly in developing countries. It is an easy and non-invasive method to diagnose disease sequelae, and other ELISA kits can also detect active infection [60, 61]. Accordingly, chlamydial serology is of great importance since a positive antibody result would be the sole non-invasive indicator of the presence of *C. trachomatis* in women who might be harbouring the pathogen only in the upper genital tract. In 1975, Wang and co-workers [55] developed a simplified

microimmunoflourescence (MIF) test with *C. trachomatis* antigens as a screening method for antibody in human sera. MIF detected antibodies to chlamydial elementary bodies and has long been considered the "gold standard" for the serodiagnosis for chlamydial infections [55, 56]. Another reference assay developed for *C. trachomatis* infections is the whole cell inclusion immunofluorescence (WIF) which was developed by Richmond and Caul in 1975 [57] wherein the whole chlamydial inclusion (LGV type 2 strain of *C. trachomatis*) acts as the antigen. WIF has been found to be more reliable in the diagnosis of UGT infection than MIF [62]. Since serological assays employ different *C. trachomatis* antigens, these tests have varying associations with degree of infection. For instance, the MIF-based serum chlamydial IgG antibody test (CAT) detect serum IgG antibodies which signify past infections [58, 63] while seropositivity with IgM as well as IgA showed significant correlation to proven current *C. trachomatis* infection [59].

C. trachomatis antibody diagnostic tests may have been greatly influenced by the type of chlamydial immunogens utilized by these methods. Varying immunogenic proteins utilized by different serological methods include the major outer membrane protein (MOMP) (Medac Diagnostics, Wedel, Germany), which is considered to be species and serovar specific [64], chlamydial heat shock protein (HSP) 60 and HSP10 [65, 66] and Pgp3, a 28 kDa polypeptide encoded by open reading frame (ORF) 5 of the chlamydial plasmid [67]. Pgp3 is a promising *C. trachomatis* immunogen since the plasmid is rarely found in *C. pneumoniae* isolates [68]. The antigen has recently been evaluated for determination of seroprevalence of chlamydia in young women in England using an indirect IgG ELISA [69]. Overall, efficient diagnostic tools for *C. trachomatis* infections include NAATs because they detect current, active infection, while past chlamydial infection can be detected by serological methods.

2.3.2 ***C. trachomatis*** **treatment**

Treatment of *C. trachomatis* infections varies according to different factors such as the site of infection, the age of the patient, and the severity of the infection [70]. The standard therapy for acute genital *C. trachomatis* infections is a single 1.0 g oral dose of the macrolide antibiotic azithromycin [71] or a 7-day course of doxycycline (100 mg twice a day). Previous studies have reported that both regimens were both safe and efficacious in treating *C. trachomatis* infections and have been

shown to result in satisfactory cure rates (93-97%) in clinical trials [72-74]. Horner [75] indicated that doxycycline may possibly be more efficacious than azithromycin in individuals with acute chlamydial infection. *In vitro* studies demonstrated that 1.0 μg ml^{-1} concentration of azithromycin resulted in an efficient suppression as indicated by the lack of detection of chlamydial HSP60 and MOMP [37]. However, a number of recently published studies challenge the efficacies of azithromycin therapy for chlamydial infections [75-78]. Batteiger and co-workers [78] conducted a cohort study among adolescent women and used a classification algorithm to characterize treatment outcomes among the study subjects after directly observed azithromycin treatment was given. Among women with paired, same-genotype episodes, as indicated by *ompA* genotyping, 31.6% were classified as possible/probable treatment failures while 68.64% were classified as possible/probable reinfections [78].

Another antimicrobial agent against *C. trachomatis* is rifalazil, one of the newest generations of ansamycins compounds [79]. Studies have shown that the ansamycin rifampicin and its derivatives have extraordinary potency against both *C. trachomatis* and *C. pneumoniae in vitro* with MICs reported at 0.0025 μg ml^{-1} for C. trachomatis and from 0.00125 to 0.0025 μg ml-1 for *C. pneumoniae* [80]. However, a single mutation in only one gene, *rpoB* which codes for the β subunit of RNA polymerase (RpoB) confer stable and high-level resistance in *C. trachomatis* to rifampicin [81]. By investigating the effect of serial passage on the development of genotypic and phenotypic resistance *in vitro*, Kutlin and co-workers [82] demonstrated that *C. trachomatis* is also able to acquire resistance to sub-inhibitory concentrations of rifampicin within six passages.

Incubation of chlamydial cultures with ciprofloxacin and ofloxacin, two antibiotics shown to have different efficacies in treating chlamydial infections in clinical trials, did not eradicate the pathogens from host cells but rather induced a state of chlamydial persistence [83]. This condition could, in turn, lead to absence of clinical manifestations, thus making it difficult for *Chlamydia* infections to be detected. As repeat infection is common in chlamydial infections, it is important to distinguish re-infection from antibiotic treatment failure to effectively evaluate or re-establish treatment recommendations and infection control strategies. It is therefore a concern that treatment failure with azithromycin, the most commonly used antibiotic

for chlamydial infections, could result in a proportion of cases not resolving the infection [78].

2.4 CHLAMYDIAL DEVELOPMENTAL CYCLE

The chlamydiae have a unique biphasic developmental cycle. The cycle begins when the extracellular, infectious, elementary bodies (EB) attach to and stimulate uptake by the host cell. The internalized EB remains within a host-derived parasitophorous vacuole termed the inclusion, and differentiate into larger, actively replicating reticulate bodies (RB). The RBs undergo several rounds of multiplication by binary fission and approximately 24 h PI (varies depending on the serovar from 18-24 h PI), RBs asynchronously commence the transition back to EBs [84, 85]. At approximately 44-48 h PI (varies depending on the species from 48 to 96 h PI), the EBs are released by either lysis or extrusion [86] of the inclusion vacuole and then infect neighbouring cells (Figure 2.2). Although EBs have long been referred to as metabolically inactive, Sixt and co-workers [87] recently reported metabolic capabilities of EBs of the amoeba symbiont, *Protochlamydia amoebophila*. Their study revealed uptake of D-glucose by host-free *P. amoebophila*, with the pentose-phosphate pathway identified as the major route of D-glucose catabolism, and that the availability of substrates prolonged the infectivity in both *P. amoebophila* and *C. trachomatis* EBs [87]. This is consistent with the results of Omsland and co-workers [88] who identified that *C. trachomatis* EBs possess metabolic activity in axenic culture, although the requirement for EBs and RBs differed. EBs preferentially used glucose-6-phosphate as an energy source while RBs utilized ATP [88]. These findings indicated that EBs are capable of metabolism and respiration outside of, and independent from, their natural host cells, and are important in understanding the extended survival of the infectious forms of *Chlamydia* [87].

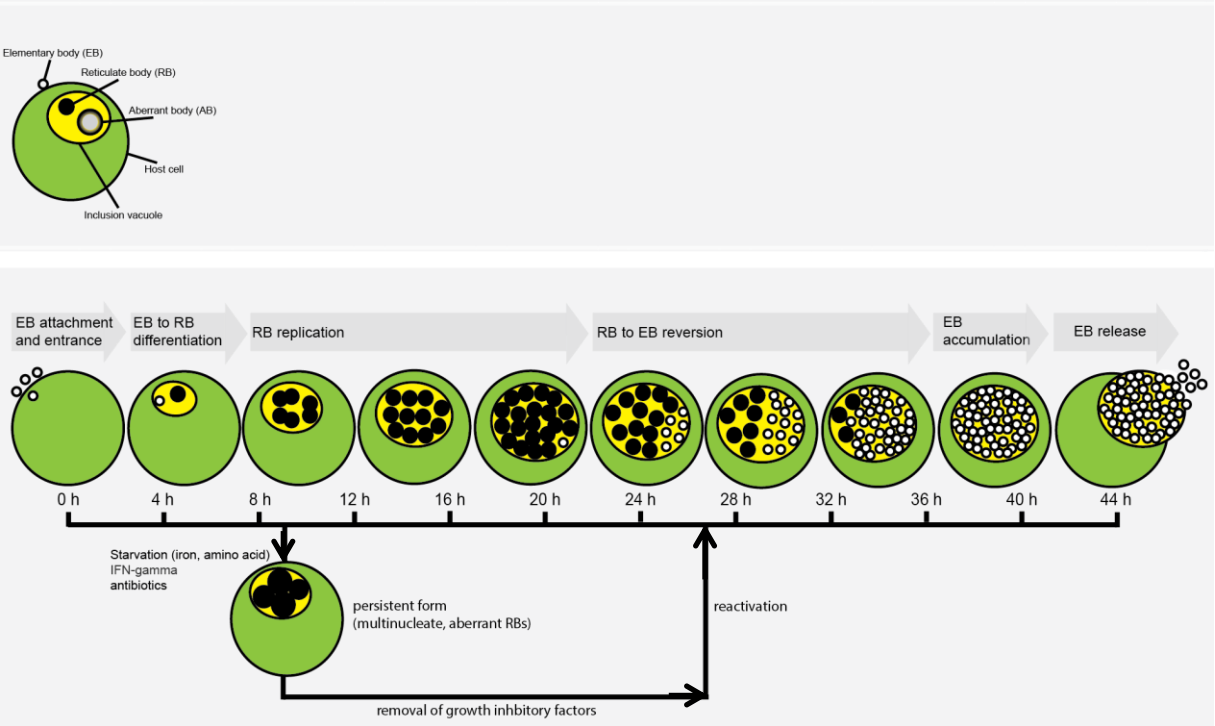

Figure 2.2. A schematic diagram of the developmental cycle of *Chlamydia*.

Chlamydia trachomatis has a biphasic developmental cycle. The cycle is initiated by the endocytic uptake into the host cell of an infectious elementary body (EB). Upon entry and internalization, the EB is surrounded by a host-derived parasitophorous vacuole termed an inclusion, which creates a permissive intracellular niche for the replication of *C. trachomatis*. Within the inclusion, the EB transforms into a larger metabolically active, replicative form called the reticulate body (RB). RBs divide by binary fission from 12-24 h PI. From 24 h PI, the RBs asynchronously transform back into infectious EBs, which are subsequently released by host cell lysis or by extrusion of the inclusion vacuole to infect neighbouring cells. During conditions deleterious to growth such as iron deprivation, nutrient depletion, and the presence of growth inhibitors such as IFN-γ and antibiotics such as penicillin, intracellular *C. trachomatis* enter into a non-replicating, viable but non-culturable state called persistence. Bacteria in the persistent form differentiate back into infectious forms after removal of the growth inhibitory factor.

Through the use of quantitative-competitive polymerase chain reaction (QC-PCR) and reverse transcription (RT) PCR, it was established that *C. trachomatis* doubles its DNA content every 2-3 hours, with DNA synthesis starting between 2 and 4 h after infection [84]. Miyairi and co-workers [89] identified the differences in length of developmental cycle between *C. trachomatis* serovars. The first 8 h after infection represent the lag phase for *C. trachomatis* D, which is associated with the period of attachment, endocytocis and primary differentiation of EB to RB [89]. Log phase, or replicative phase, is equivalent to the phase of RB replication before transition to EB. Genomic DNA copy number doubling rate determined that *C.*

trachomatis D generation time is similar to that of *C. trachomatis* L2 at 2.4 and 2.2 h, respectively [89]. Terminal differentiation from RBs to EBs started at 24 h PI and completion of developmental cycle occurred at 44-48 h PI for *C. trachomatis* D [89]. Chlamydiae become transcriptionally active as early as 1 h after infection with 3.2% of its genome being transcribed [90]. Temporal gene expression patterns that correspond with specific phases and activities in the chlamydial developmental cycle have been elucidated [84, 90]. The gene expression program which consisted of early genes (2 h after infection), mid-genes (2 or 6 h after infection but are present by 12 h) and late genes (20 h after infection) relate to the onset of transcription of developmentally expressed genes as the *Chlamydia* transitions between the different developmental phases namely, early, mid-cycle, and late [84]. Early genes include *dnaE*, the α subunit of DNA polymerase [91], *rpoB*, the β subunit of RNA polymerase [92], and *groEL*, a heat shock chaperone [93]. These genes are important in the establishment of the organism's intracellular niche in which chlamydiae then replicate [84]. The mid-cycle phase is primarily devoid of EBs and expressed genes products involve catabolic enzymes (including (*eno*)) or structural components (such as *ompA*) essential for RB replication by binary fission [84]. *ompA* encodes the major outer membrane protein (MOMP), which comprises 60% of the total outer membrane protein content of chlamydiae [31] and is primarily the antigenic protein to which typing of different *C. trachomatis* serovars has been based. In the final phase, late genes include *hctA* and *hctB* which encode the histone-like proteins Hc1 and Hc2, respectively, and are believed to mediate the condensation of DNA to form a nucleoid [94-96]. Other genes transcribed late in the developmental cycle are cysteine rich outer membrane proteins (OmcA and OmcB) which are transcribed at a point when RBs begin to differentiate back to infectious EBs [84].

2.4.1 *C. trachomatis* entry

The columnar cells of the endocervix of women and the urogenital epithelia of men are the primary site of chlamydial infections of the genital tract in humans [97]. A combination of host cell factors and bacterial proteins contributes to *Chlamydia* entry to the cell. The identity of specific receptor-ligand interactions have proven to be somewhat elusive largely due to the complexities caused by biovar- and serovar-specific mechanisms employed in chlamydial invasion [reviewed by 98]. Previous studies have demonstrated that alternative means of entry such as Fc-mediated

endocytosis of opsonised EBs [99] can lead to productive infection. This observation suggests that the vesicular interactions of the chlamydial inclusion are defined by parasite-directed modification of the endocytic vesicle rather than by the route of internalization.

It has also been reported that binding to the host heparan sulfate proteoglycans (HSPGs) is important in the initial reversible binding step for many *C. trachomatis* serovars [100]. Cell surface HSPGs facilitate the interactions of a large number of differentiation factors and growth factors with their receptors. It has been reported that the fibroblast growth factor 2 (FGF2) is crucial and necessary for successful binding of *C. trachomatis* to host cells in an HSPG-dependent manner [100]. FGF2 functions as a bridging molecule to facilitate interactions of EBs with the FGF receptor (FGFR) on the host cell surface and contributes to bacterial uptake to non-phagocytic cells [100]. The host cell epidermal growth factor receptor (EGFR) has also been reported to have important function to *C. trachomatis* attachment and development in the host cells [101].

In addition, phosphorylation of host cell proteins has been shown to be induced by chlamydial entry. Tyrosine phosphorylation events occur very early in infection and the phosphorylated proteins have been found to be relatively stable over the first several hours post infection [102, 103]. However, the identity and source of these phosphorylated proteins remains controversial. Swanson and co-workers [104] examined the tyrosine phosphorylation of a ~70-kDa protein complex and identified the polypeptide as the host protein ezrin, a member of the ezrin-radixin-moesin (ERM) protein family that serves as a physical link between host cell receptors and the actin cytoskeleton. They determined that ezrin is tyrosine phosphorylated upon *C. trachomatis* infection, an integral component in the pathogenesis of *C. trachomatis*. However, no tyrosine phosphorylation occurred after infection with *C. caviae*, a pathogen of guinea pigs, indicating that induction of tyrosine phosphorylation of ezrin occurred in a species-specific manner. A number of other tyrosine phosphorylated proteins including a triple band 68-66-64, 97 and 140 kDa [103], 75-85 and 100 kDa [102] were presumed to be of host cellular origin although definitive identification of these proteins was not achieved.

2.4.2 **Inclusion formation and nutrient acquisition**

Intracellular pathogens like *Chlamydia* establish direct membrane contact sites (MCSs) with organelles as well as utilize non-vesicular transport machinery of the host cell to mediate bacterial metabolism and signalling events crucial for chlamydial development. A number of previous studies have demonstrated the association of *C. trachomatis* inclusion with the endoplasmic reticulum (ER) [105-108]. The ER-Inclusion MCSs formed during infection are platforms composed of host and bacterial factors, namely, the mammalian ceramide transfer protein CERT, the *C. trachomatis* inclusion membrane protein IncD and the integral membrane proteins VAPA and VAPB [106, 107] which were proposed to mediate non-vesicular trafficking of lipids to the inclusion. A novel component of the ER-Inclusion MCSs, the ER calcium sensor stromal interaction molecule 1 (STIM1), was recently identified by Agaisse and co-workers [109]. STIM1 was found to co-localize with CERT and VAPB throughout the developmental cycle [109].

In all stages of the infection, *Chlamydia* secrete effector proteins that interact with host factors to establish and maintain an intracellular niche, and suppress the host's innate immune response [110]. Many of these secreted effector proteins are likely substrates of the type III secretion system [111]. Bacterial type III secretion system (T3SS) is a protein export system used by some Gram-negative bacteria to translocate proteins to the cytoplasm of the host cell by using a needle-like apparatus. The T3SS delivery system is consequently central to the translocation of effectors into target cells [112] thereby activating promotion of uptake into epithelial cells, cytokine production, and bacterial invasion of professional phagocytes [113]. Type III secretion apparatuses which have been found so far in the animal pathogens *Yersinia*, *Shigella*, *Salmonella*, enteropathogenic *Escherichia coli*, and *Pseudomonas* enable these bacteria to secrete pathogenicity proteins into the host cell cytosol [reviewed by 114]. Other bacterial pathogens infecting plant or human and animal hosts that depend on T3SS to cause disease include species of *Bordetella*, *Vibrio*, *Burkholderia*, *Aeromonas*, *Erwinia*, *Ralstonia*, *Pantoea*, and *Xanthomonas* [115, 116]. One of the well-studied *C. trachomatis* type III secreted effector proteins is called Tarp (translocated actin recruiting phosphoprotein).

Clifton and co-workers [117] reported that Tarp was of chlamydial origin. Tarp was found to be translocated and tyrosine phosphorylated at the site of entry and

associated with the recruitment of actin that coincides with endocytosis [118]. Thus, Tarp functions as a multivalent phosphorylation-dependent signalling hub that is important during the early phase of chlamydial infection [119]. It has been found that *C. trachomatis* Tarp is phosphorylated by src family tyrosine kinases [120]. Tyrosine phosphorylation occurs primarily within the repeat region of Tarp, while recruitment of actin is mediated by the C-terminal domain of the protein [118, 121].

The participation of the actin-containing cytoskeletal network in phagocytosis and endocytosis has been extensively studied [122]. In the initiation of *Chlamydia* infection, Tarp directly associates with actin, as suggested by the acceleration of actin polymerization in the absence of cellular factors such as Arp 2/3, a complex of seven proteins known to nucleate new actin filaments within eukaroyotic cells [121]. This direct association between mammalian actin and Tarp proteins suggests that chlamydiae directly manipulate the host cell's microfilament network. Thus, Tarp participates in the observed "parasite-specified phagocytosis" [123] by promoting the rapid polymerization of actin filaments required for EB uptake. This was corroborated by the findings of Clifton and co-workers [117] who identified that the translocation and phosphorylation of the *Chlamydia* effector protein CT456 (Tarp) by Type III secretion system is important in the initiation of chlamydial entry by recruiting actin at the site of chlamydial invasion. However, sequence analysis studies have found variations in the tyrosine-rich repeats and actin binding domains of Tarp between different *C. trachomatis* serovars. Through phylogenetic analysis of *tarP* from reference strains as well as ocular, urogenital and LGV clinical isolates, Lutter and co-workers [124] demonstrated that Tarps from LGV strains contained the highest number of tyrosine-rich repeat regions and the fewest predicted actin binding domains which is in contrast to the ocular strain that contained up to four actin binding domains and the fewest tyrosine-rich repeats. Their findings suggest that *C. trachomatis* Tarp varies in relation to disease and tissue tropism [124].

The role of actin-dependent mechanisms in chlamydial internalization has been studied using cytochalasin D as an inhibitor of host cell microfilament function. Studies using cytochalasin D showed consistency with Tarp variability since it was determined that the inhibitory effect of the toxin was dependent on the *C. trachomatis* serovar as well as on the time of treatment, which associated with stage of inclusion maturation [125, 126]. Other widely characterized host cell interactive

proteins translocated by T3SS are the membrane localizing proteins called Incs [127, 128]. Incs are a family of integral inclusion membrane proteins that share a large 40-60 amino acids bi-lobal hydrophobic motif [111]. Examples of Incs include IncA and IncG which mediate homotypic fusion of inclusions [129, 130] and localization of host protein 14-3-3β at the inclusion membrane [131, 132], respectively. IncA interacts directly with several host SNARE (soluble NSF (N-ethylmaleimide-sensitive factor) attachment protein receptors) proteins and plays a predominant role in SNARE recruitment [133, 134]. SNAREs are essential for membrane fusion, however, SNARE-like proteins encoded by intracellular bacteria such as *Chlamydia* inhibit SNARE-like mediated fusion and is one important mechanism employed by *Chlamydia* to manipulate membrane fusion of the host cells in order to escape lysosomal fusion and degradative pathway [134].

Considering the complex interactions manifested during invasion and early cycle development, it is probable that additional effector proteins are required at the time of chlamydial invasion. Hower and co-workers [135] identified CT694, a gene product transcribed late in the *C. trachomatis* developmental cycle which could represent additional EB-localized effector proteins capable of functioning during invasion or early cycle development. They demonstrated CT694, a T3SS substrate, as a novel chlamydial effector protein capable of contributing to pathogenic mechanisms as early as chlamydial entry, and that the protein is an early cycle-associated effector protein capable of functioning during early invasion or early cycle development [135]. In addition, it was observed that CT694 is unique to *C. trachomatis* and its interaction with host proteins such as AHNAK may be important for invasion [135]. Overall, these studies demonstrate that *C. trachomatis* invasion into host cells involve a suite of different host and bacterial factors as well as specific effector proteins that help establish infection and modulate cellular functions.

2.4.3 *C. trachomatis* Persistence

During conditions deleterious to growth, the chlamydiae can enter into persistence, which is a reversible state characterized by culture-negative but viable *Chlamydia* cells involving morphologically enlarged, aberrant, and non-dividing RBs [136, 137]. Arrested growth and the culture-negative state of chlamydiae during persistence is associated with a significant decrease in metabolic activity which

restricts growth and division and delays differentiation to cultivatable EBs, [reviewed by 138].

There is a paucity of information that definitively describes persistence *in vivo*. However, indirect evidence for this alternative growth *in vivo* has been reported. This includes recurrent same-serovar infections despite denials of re-exposure, detection of chlamydial nucleic acids and antigens in the absence of cultivability, as well as high serological titres for individuals who were tested culture-negative [137-142]. Through detailed morphologic and molecular analyses of cells and secretions from the endocervix, and identification of differing chlamydial growth patterns and contrasting responses to IFN-γ between two patients, Lewis and co-workers recently provided the first evidence of the existence of persistence in human genital tract [143]. This indicates that when the infection milieu resembles that of IFN γ-induced persistence *in vitro*, *C. trachomatis* persistent forms can be isolated from the human endocervix.

Although chlamydial persistence was recently demonstrated to occur *in vivo*, it was first described and reproduced using *in vitro* models. Several studies employing different experimental conditions have identified different inducers of chlamydial persistence including IFN-γ, nutrient/amino acid starvation, iron deprivation, antibiotics such as penicillin, the host cell differentiation state, and herpes simplex virus (HSV) infection [reviewed by 137]. The best characterized among these factors is IFN- γ-induced persistence.

Beatty and co-workers have determined using cell culture systems that low concentrations of IFN-γ completely inhibited chlamydial growth and differentiation and establish persistent chlamydial infection [144]. Upon infection of human epithelial cells, *Chlamydia* induces the production of antigen-specific IFN-γ-secreting CD4+ and CD8+ T cells [145]. In turn, IFN- γ activates the expression of indoleamine 2,3-deoxygenase (IDO) in the epithelial cells, an enzyme that catalyses the initial step of tryptophan degradation [146], thus affecting development of the tryptophan auxotroph *Chlamydia*. IFN-γ induced persistent chlamydiae are characterized by atypical inclusions containing aberrant bodies that are larger than typical RBs [144, 147]. Upon removal of IFN-γ or when tryptophan host pools are replenished, chlamydiae re-enter the active developmental cycle wherein the

persistent forms retransform back to normal RBs which then differentiate to infectious EBs [21, 144].

Very low tryptophan levels effectively suppress early chlamydial development by inhibiting both microtubule-organizing center (MTOC) trafficking and fusion of multiple inclusions in the cell [148]. However, Caldwell and co-workers [149] proposed that host-parasite relationship between chlamydial genital strains and the human epithelial cells involves the production of indole by components of the vaginal microflora thereby allowing the chlamydiae to escape IFN-γ-mediated tryptophan depletion and thus establish chlamydial persistent infection.

A number of previous studies have also determined and analysed antimicrobial agents as mediators of persistent chlamydial development. An example of which is the β-lactam antibiotic, penicillin. Penicillin does not inhibit conversion of infectious EBs to RBs but the transition from RB to EB becomes retarded [136]. RBs enlarge to become aberrant RBs within an inclusion in a viable but non-infectious state. The mechanism governing the formation of aberrant RBs is unknown although penicillin typically acts by blocking peptidoglycan (PG) biosynthesis through binding with penicillin binding proteins (PBPs) [150, 151]. *Chlamydia* has 3 PBPs and they are present in both EBs and RBs [152]. Each of the 3 PBPs binds to and is inhibited by beta-lactams [151, 152].

All attempts to detect and purify PG from *Chlamydia* had been unsuccessful [152-154]. However, a recent study by Leichti and co-workers [155] has put an end to the speculation concerning the chlamydial phenomenon that has long been debated by chlamydiologists. The group used a novel approach to metabolically label chlamydial PG using D-alanine-D-alanine (DA-DA) analogues as probes coupled with click chemistry assay to selectively capture functional groups of these peptides once incorporated into a macromolecule such as PG [155]. Fluorescent coupling to the dipeptide enabled detection and localization of peptidoglycan in *Chlamydia* for the first time [155].

In penicillin persistent *Chlamydia*, inclusions contain large aberrant RBs with multiple copies of the chromosomal DNA [156]. Whilst penicillin treatment blocks RB division [157], RBs continue to grow and expand and chlamydial chromosomal DNA and plasmid replication resumes at the same rate as the non-persistent culture [156, 158]. Upon removal of penicillin, the normal developmental cycle is recovered

although this is both dose and time dependent [136]. Skilton and co-workers [158] identified that the emergence of normal developmental cycle occurs 10-20 hours after removal of penicillin, although this does not happen in all inclusions. During resumption of normal developmental cycle, enlarged aberrant RBs do not revert back to smaller "normal' RBs but rather retained their large size and structure, and remained present and immobile within inclusions until cell death [158]. This finding is consistent with what was observed during normal developmental cycle resumption when penicillin was added at mid-log phase in the developmental cycle [159]. Reversion back to the active RB form occurs via a RB budding from the aberrant body, with this only productively occurring in some inclusions [136, 158]. Figure 2.3 shows electron micrographs of standard inclusions containing EBs and RBs as well as inclusions in penicillin-persistent *in vitro* cultures of *C. trachomatis* serovar E.

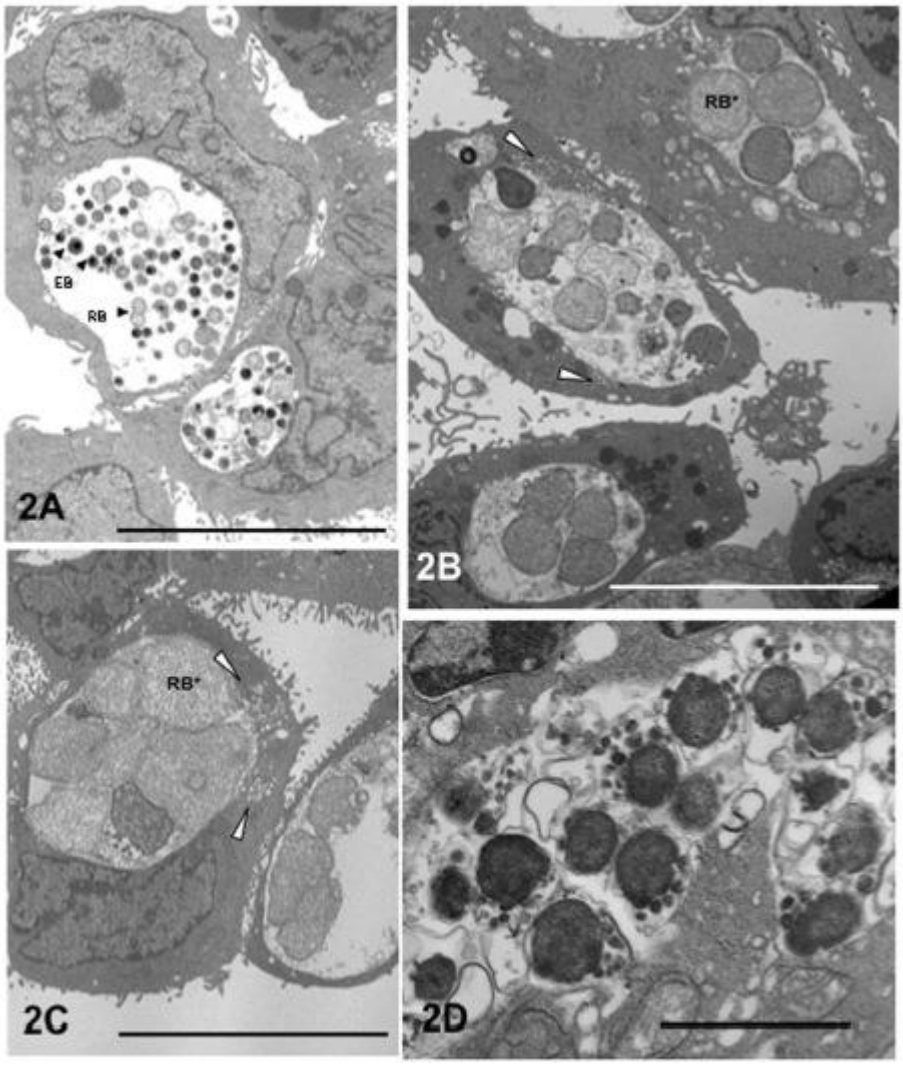

Figure 2.3. Electron micrographs of penicillin-persistent *Chlamydia*.

Electron photomicrographs showing penicillin-persistent *Chlamydia trachomatis* serovar E cultured in human endometrial epithelial cells *in vitro*. A, Untreated (control) cells containing RBs and elementary bodies (EBs) in a *C. trachomatis*–infected HEC-1B cell at 24 h PI. B and C, *C. trachomatis*–infected HEC-1B cells at 36 h PI, exposed to penicillin G (20 µg/mL) for a total of 35 h (penicillin was added at 1 h PI). The RBs are swollen, abnormal, and non-dividing (RB*). Arrowheads show blebbed vesicles resulting from excessive shedding of RB outer membrane into the inclusion lumen; these blebbed vesicles contain antigens such as the major outer membrane protein and lipopolysaccharide and somehow fuse with or cross the inclusion membrane and emerge into the cytoplasm of the infected host cell. D, *C. trachomatis*–infected HEC-1B cells pre-exposed to penicillin G (20 µg/mL) for 35 h, followed by removal of the antibiotic and continued cultivation of the infected cells for an additional 12 h. The miniature bodies indicate recovering RBs and signify their reprogrammed attempt to undergo cell division to produce progeny. Bars represent 10 µm at X2900 (A–C) or 2 µm at X10,000 (D) magnification. Image directly copied from Wyrick, 2010 [137].

2.4.4 **Methods to assess development and persistence in *Chlamydia***

A number of different methods can be used to assess persistence in *Chlamydia*. Byrne and co-workers [160] utilized reverse transcription (RT)-PCR from *C. pneumoniae* cultures treated with low and high doses of IFN-γ in order to analyse differential expression of *C. pneumoniae* genes that are involved in DNA replication and cytokinesis under non-persistent and persistent conditions. It was identified that transcripts from genes involved in bacterial chromosome replication, namely, *dnaA*, *polA*, *mutS*, and *minD* were expressed regardless of the presence of IFN-γ. In contrast, transcripts of the genes that encode products for bacterial replication, such as *ftsK* and *ftsW*, were reduced and lost in cells treated with low-dose and high doses of IFN-γ, respectively. This is corroborated by the findings of Skilton and co-workers [158] who demonstrated through time-lapse photomicroscopy, qPCR, electron microscopy and immunofluorescence assay that chlamydial chromosomal and plasmid DNA replication was unaffected by the addition of penicillin, and bacterial cytokinesis was arrested in penicillin-persistent cultures, resulting in enlarged RBs with multiple copies of the genome [158]. The same morphological effects were reported by Lambden and co-workers [156] for penicillin persistent culture. Beatty and co-workers demonstrated that treatment of *C. trachomatis* serovar A with low dose of IFN-γ resulted in slightly increased cHSP60 levels while MOMP levels decreased significantly. In *in vitro* cell culture systems, persistent *Chlamydia* are characterized by loss of infectivity while viability is retained as indicated by recovery of infectious EBs once the persistence-inducing agent is removed [142, 161]. Immunofluorescence assays also indicate the appearance of small, atypical inclusions containing fewer chlamydiae in persistent cultures [142].

2.5 GENETIC TOOLS FOR *C. TRACHOMATIS*

Studies using genetic manipulation of *C. trachomatis* have not been successful for a number of years due to the complex, obligate intracellular developmental cycle of the organism. This paucity of genetic tools has impeded the progress of chlamydial research. However, in the past five years, there have been exciting advances in *Chlamydia* genetics.

2.5.1 **Plasmid transformation system**

A highly conserved, cryptic plasmid of approximately 7.5 kb is present in almost all *C. trachomatis* strains [162, 163]. There are approximately 4 [164] to 10 [162, 165] plasmids per chlamydial particle. The presence of this plasmid has long been associated with glycogen synthesis. However, it is noteworthy that most *C. psittaci* and *C. pneumoniae* strains carry the plasmid, yet neither accumulates glycogen in the inclusions [68, 166, 167]. It has been determined that the plasmid is important for the pathophysiology of the disease [168], although it is not required for survival of the organism [169, 170]. In fact, naturally-occurring plasmid-free strains of *C. trachomatis* have been described.

O'Connell and Nicks [163] used novobiocin as a curing agent to inhibit chlamydial plasmid replication. They determined that plasmid-deficient derivatives of *C. muridarum* strain Nigg were unable to accumulate glycogen within intracytoplasmic inclusions and formed small plaques compared to wild-type strain [163]. They also identified a defect in the attachment and uptake that could be partially resolved by centrifugation [163] of the chlamydial inoculum onto the monolayer. Although the distinct role of the chlamydial plasmid remains unknown, it can be speculated that it functions in glycogen accumulation and efficiency of plaque formation [163].

A recent study by Song and co-workers [171] identified through transformation techniques and deletion mutagenesis, that the chlamydial ORF, pgp4, a transcriptional regulator of multiple chromosomal genes including the glycogen synthase, *glgA*, plays an important role in chlamydial virulence. A plasmid-based transformation system based on the conserved plasmid was developed by Wang and co-workers [172]. They developed the transformation protocol based on expression of β-lactamase that utilizes rescue from the penicillin-induced culture, which is characterized by failure of the host cells to lyse due to blockage of RB to EB differentiation. The vector, carrying both the endogenous chlamydial plasmid and an *E. coli* plasmid origin of replication, cured the endogenous chlamydial plasmid when introduced into *C. trachomatis* L2. Through penicillin resistance, Wang and co-workers were able to isolate transformants with restored ability to synthesize and accumulate glycogen in inclusions, a characteristic that is down-regulated in

plasmid-free strains [170]. The development of stable, reproducible transformation method permits future analysis of genome function by complementation.

2.5.2 **Homologous recombination**

Homologous recombination methods were also described for *C. trachomatis*. Notable recombination studies in *C. trachomatis* were performed by Binet and Maurelli [173]. The *E. coli* KsgA protein, which represents a family of rRNA adenine dimethylase, is functionally important for methylation, one of the post-transcriptional modifications of nucleotides. Binet and Maurelli [173] showed that the chlamydial KsgA protein is able to replace the orthologous enzyme in *E. coli* and was able to complement for the loss of *ksgA* in *E. coli*. Firstly, ATM809, an *E. coli* strain with deletion of the *ksgA* gene was constructed by λ red recombinase method described by Datsenko and Wanner [174]. ATM809 was then transformed with high copy number of plasmid pRAK316 (i.e. overexpressing *C. trachomatis* L2 KsgA, and was designated as ksgAL2). This resulted in, and was confirmed by, the restored sensitivity of ATM809 overexpressing KsgAL2 to kasugamycin (KSM), suggesting methylation of A1518 and A1519 in *E. coli* 16S rRNA [173]. It was also demonstrated that mutations in *C. trachomatis ksgA* negatively affected chlamydial growth in cell culture, indicating that this functional rRNA demethylase enzyme is important for normal development of *C. trachomatis* [173].

Another study by Binet and Maurelli constructed variants by homologous recombination by introducing the recombinant DNA to *C. psittaci* 6BC through electroporation [175]. Different concentrations of linearized or circular plasmids containing varying concentrations of rRNA regions homologous to the chromosomal copy were introduced into *C. psittaci* 6BC infectious particles [175]. Recombination/exchange of "heterologous" DNA sequences into the bacterial chromosome resulted in four nucleotide substitutions, three of which imparted bacterial resistance to kasugamycin (Ksm) and spectinomycin (Spc) [175]. Identification of double resistance and replacement of 16S rRNA gene as well as isolation of plaques resistant to the two antibiotics indicated successful, first step of genetic manipulation of *Chlamydia*. However, it is noteworthy that this stable transformation system involving homologous gene targeting has not been reported since.

2.5.3 **Chemical mutagenesis**

Recently, Kari and co-workers [176] generated *C. trachomatis* D *trpB* (CTD *trpB*-) null mutants by a process called targeting-induced local lesions in genomes (TILLING) [177] through ethyl methanesulfonate (EMS) mutagenesis coupled with digestion using the mismatch-specific endonuclease, CEL1 [178]. EMS is a mutagen that induces C-G to T-A transition mutations [179]. The optimal levels of mutagenesis was assessed by determining the percent killing (i.e., decrease in the number of infectious progeny) and the frequency of rifampicin resistant (RifR) strains [176, 180], which emerged as a result of point mutations in the β-subunit of RNA polymerase [181]. Kari and co-workers [176] determined that the infectivity of CTD *trpB*- was not rescued by exogenous indole, the substrate used by genital but not ocular *C. trachomatis* serovars to synthesize tryptophan and escape the antimicrobial effect of IFN-γ-induced tryptophan starvation [182]. Recently, reverse genetics approach was also employed by Bao and co-workers [183], who were able to isolate a *C. muridarum* (mouse pneumonitis pathogen, MoPn) mutant which was resistant to a T3SS inhibitor, CF0001, through continued cultivation of the strain in the presence of the inhibitor. By genomic sequencing of the mutant strain designated as MCR (MoPn with CF0001 resistance), the group were able to identify 4 single nucleotide polymorphisms (SNPs), however, the particularly affected gene that regulates the susceptibility to the inhibitor was not identified [183].

Nguyen and Valdivia [180] utilized forward genetic approaches by combining chemical mutagenesis, genome sequencing and subsequent DNA exchange (lateral gene transfer, [184]) among *Chlamydia* strains to identify genes responsible for certain phenotypes. Like Kari and co-workers, they also utilized EMS mutagenesis and used Rif^R to determine frequency of mutation. They were able to generate recombinant strains by co-infecting of host cells with mutants carrying antibiotic (spectinomycin, trimethoprim, or rifampicin) resistance markers and allowed natural DNA exchange among chlamydiae to occur. Recombinants that formed plaques in the presence of both antibiotics were then selected. Through whole genome sequencing (WGS), the group identified underlying genetic lesions in these mutants, which enable association between mutations in a common gene and a common plaque morphotype [180]. For instance, mutations in *glgB* led to the accumulation of

glycogen granule aggregates within inclusions and granular plaque morphology [180].

2.6 SMALL MOLECULE APPROACHES FOR *C. TRACHOMATIS*

Small molecule approaches have been used to investigate bacteria. These small molecule inhibitors typically are molecules that inhibit known virulence factors. One of the virulence systems widely characterized due to employment of such inhibitors is the *Yersinia* T3SS, an attractive target for therapeutic strategy for infectious diseases. Through chemical genetics approach, Kauppi and co-workers [185] screened a large library of synthetic compounds for the ability to inhibit *Yersinia* T3SS gene expression. They identified a group of inhibitors with general structure of acylated hydrazones of salicylaldehydes [185]. Such inhibitors include INP0010 and INP0400 which inhibits *C. pneumoniae* developmental cycle and prevents replication of *C. pneumoniae* and *C. trachomatis* in *in vitro* infection models respectively, thereby indicating an important role for T3SS in the chlamydial developmental cycle [186]. These findings were corroborated by Muschiol and co-workers [187] who showed that INP0400 inhibits *Chlamydia* growth in a dose-dependent manner. The group also demonstrated a link of T3SS to inclusion membrane proteins IncA and IncG since these two effector proteins failed to localize in the inclusion membrane in the presence of INP0400. Also through the use of *Yersinia* T3SS inhibitor, designated as compound 1 (C1) [185], Wolf and co-workers identified that T3SS activity and chlamydial development are interlinked processes [188]. Although *Chlamydia* remained viable and metabolically active, inhibition of T3SS resulted in stalled RB to EB differentiation [188]. Since inhibition of T3SS with these compounds was speculated to be coupled with iron deprivation, Bao and co-workers [183] recently developed benzylidene acylhydrazides (derivatives of acylhydrazides), which cannot chelate iron, and demonstrated a T3SS-independent anti-chlamydial effects. Small molecule approaches have also been employed to determine the role of lipooligosaccharides (LOS) in *C. trachomatis*. Through the use of small molecule inhibitors of LpxC, the enzyme that catalyses the first committed step in the biosynthesis of lipid A, the synthesis of lipooligosaccarides (LOS) in *C. trachomatis* is blocked [189]. In the absence of LOS, *C. trachomatis* remains viable but non-infectious and replicative RBs failed to express selected late-stage proteins and

transition to EBs [189]. Based on previous findings that 2-pyridone carboxylic acids block biofilm formation in *E. coli* and prevent infection of *L. monocytogenes*, Engström and co-workers [190] investigated the inhibitory effect of 2-pyridone amide inhibitors on *C. trachomatis*. Through isolating variants resistant to the inhibitor, they determined that 2-pyridone inhibitors, KSK120 and its analogues, prevent *C. trachomatis* infectivity by affecting the glucose-6-phosphate metabolism.

2.7 *C. TRACHOMATIS* PATHOGENIC FACTORS

Prevention of host cell apoptosis is one of the mechanisms used by *Chlamydia* to maintain infection and survival. Studies have previously reported anti-apoptotic effect of chlamydial infection that likely facilitates long-term survival in the infected host cell. Such anti-apoptotic activities include blockade of mitochondrial cytochrome c release and blocking the activation of caspases, inhibition of the activation of proapoptotic multidomain Bcl-2 proteins Bax and Bak, and degradation of pro-apoptotic BH3-only proteins [191-193]. BH3 is one of the four BH domains of a prototype of Bcl-2 proteins [194]. Philippe and co-workers [195] described the cellular function of BAD, a proapoptotic BH3-only protein in chlamydial host-cell survival. They demonstrated that the phosphorylation of BAD is accompanied by recruitment of this protein to the chlamydial inclusion, where BAD binds to a cellular adapter protein, 14-3-3-β which in turn, is attracted to the inclusion by a membrane protein produced by *Chlamydia*. These cellular mechanisms show that the chlamydial inclusion sequesters BAD away from mitochondria, where BAD could induce host-cell apoptosis. On the other hand, Fischer and co-workers [196] observed that blockade of apoptotic stimuli by destruction of BH3-only proteins (Bim/Bod, Puma and Bad) during infection occurred upstream of the mitochondrial activation of Bax/Bak. Bax and Bak are two proapoptotic members of Bcl-2 family that regulate mitochondrial cytochrome c release [197]. BH3-only molecules induce the activation of Bax and Bak, resulting in the permeabilization of the outer mitochondrial membrane and the efflux of cytochrome c [198]. Cytochrome c is an apoptogenic factor that participates in the formation of the apoptosome, leading to activation of down-stream effector caspases, including caspase-3, -6 and -7 which in turn, cleave a wide range of cellular molecules and also activate endonucleases [191]. Overall, the understanding of chlamydial interference in apoptosis is still to be fully elucidated.

Chlamydia also has mechanisms for the evasion of the host immune response. Previous studies have shown that among these immune evasion mechanisms is the inhibition of NF-κB activation, a crucial pathway for host inflammatory responses [199]. The *C. trachomatis* deubiquitinating and deneddylating [200] protease ChlaDub1 suppresses NF-κB activation by binding to the NF-κB inhibitory subunit IκBα, thereby impairing its ubiquitination and degradation [199], a prerequisite for NF-κB nuclear translocation/activation [201]. Additionally, Lad and co-workers [202] found that a bacterial tail-specific protease (Tsp) CT441 was responsible for chlamydial protease activity that cleaves the p65 protein, an important regulator of the NF-κB pathway of inflammatory response. Another virulence factor known to cleave host proteins is the chlamydial protease/proteasome-like activity factor (CPAF). CPAF was thought to have a wide range of host cell targets including those involved in apoptosis, cell cycle, cell structure, cellular adhesion, hypoxia signalling, and DNA repair [196, 203]. However, it was recently demonstrated that most of its host targets are artefact of inaccurate methods [204].

CPAF is secreted into the host cell cytosol by a Sec-dependent or type II secretion pathway [205]. Due to the protease's ability to degrade many different types of host proteins [206], it can alter multiple cellular functions. Chen and co-workers [207] recently examined 11 published CPAF substrates and found that there was no detectable proteolysis when CPAF activity was inhibited during cell harvest and lysate preparation. Although not ruling out the important role that CPAF plays during intracellular chlamydial infection, they concluded that the published cleavage and degradation of the host proteins in *Chlamydia*-infected cells are not likely to have occurred in intact cells but instead are due to *in vitro* proteolysis by CPAF during cell processing, thereby, highlighting the need to evaluate *Chlamydia* literature on CPAF [207].

Another virulence factor for *C. trachomatis* is its plasmid. The 7.5 kb *C. trachomatis* plasmid, originally described by Lovett and co-workers (1980) is found to be highly conserved within the species [68]. Although it is not required for survival of the organism [169, 170], the chlamydial plasmid is important for the pathophysiology of the disease [168]. This plasmid encodes 8 genes or coding sequences (CDS) and is considered a chlamydial virulence factor [208-211]. The presence or loss of the whole plasmid is not strictly a mutation. In fact, naturally-

occurring plasmid-free strains, human *C. trachomatis* lymphogranuloma venereum (LGV, L2, strain 25667R) [169] and trachoma isolate (serovar A, strain A2497P-) [212] have been described. Infections with these attenuated plasmid-deficient strains, particularly strain A2497P- elicited/caused no pathology in mice models but induced an anti-chlamydial immune response [212].

2.7.1 *Chlamydia* HtrA role in developmental cycle and stress

CtHtrA has been previously described to play critical roles during conditions deleterious to growth. Huston and co-workers [19] reported elevated levels of CtHtrA during heat stress, while major outer membrane protein (MOMP) levels decreased under the same conditions. Transmission electron microscopy revealed that heat-stressed cultures contained larger but fewer RBs with excess membranous-like material within the inclusion [19]. In addition, *C. trachomatis htrA* gene was able to heterologously complement the lethal heat stress phenotype of *E. coli htrA*- thus protecting *E. coli htrA*- from the detrimental effects of heat stress [19].

CtHtrA has been found to perform similar dual functions as chaperone and protease as those of its homologues in other bacteria. Also, CtHtrA was upregulated during penicillin-induced persistence but down-regulated during IFN-γ persistence [19] as indicated by transcriptional profiling conducted by Belland and co-workers [90]. This was corroborated by the findings of Huston and co-workers [19] who identified through immunocytochemistry and confocal microscopy analyses that CtHtrA and MOMP proteins were present during mid and later (20 h PI and 44 h PI, respectively) stages of penicillin persistence, with higher levels of CtHtrA detected at 44 h PI compared to the control, non-persistent cultures. This data indicated that CtHtrA may likely play an essential role during penicillin-induced persistence.

2.8 PROTEASES

Proteases are enzymes that oversee a range of regulatory mechanisms and cellular processes such as stability of the key metabolic enzymes, removal of terminally damaged polypeptides, and protein processing assembly [reviewed by 213]. Proteases are pivotal regulators in a multitude of biological and physiological processes such as digestion, blood clotting, pathogenic infections, conception, birth, growth, ageing and death, to name a few. Proteolytic enzymes are found ubiquitously

in both eukaryotic and prokaryotic organisms [214, 215]. Proteases can be divided into five classes according to the catalytic residue that affects enzymatic hydrolysis. These are the aspartic acid, cysteine, metallo, threonine, glutamic acid, and serine proteases [215, 216]. Serine proteases constitute almost one-third of all proteases and were named for the nucleophilic Ser residue at the active site [217]. Examples of serine proteases include chymotrypsin, trypsin, elastase, and thrombin. Therefore, based on the nature of P1 residue in their peptide substrates [218, 219], serine proteases can be divided into three classes namely, trypsin-like (positively charged residues Lys/Arg preferred at PI), elastase-like (small hydrophobic residues Ala, Val at PI) or chymotrypsin-like (large hydrophobic residues Phe/Tyr/Leu at PI) [217]. On the basis of three-dimensional structures, serine peptidases can be divided into clans that may share common ancestors [220]. Four of the most widely studied clans include chymotrypsin, subtilisin, carboxypeptidase C, and D-ala-D-ala peptidase A [219, 220]. HtrA belongs to the trypsin clan where the order of the catalytic triad is His-Asp-Ser [221]. An up to date list of families of proteases can be found in the MEROPS database [217, 220].

The most extensively studied and well-characterized bacterial proteases are the energy-dependent, intracellular proteases in *E. coli*, with the Clp protease and Lon protease being the two major groups. The multi-component Clp protease consists of a proteolytic component ClpP and the ATP-hydrolyzing, chaperonin-like ClpA [222]. Lon protease has been shown to play an essential role in the degradation of abnormally-folded proteins as well as unstable regulatory proteins [reviewed by 223]. The expression of serine proteases can be greatly associated with the pathogenicity of Gram-negative bacteria since they are believed to be linked to the resistance of microorganisms to lysosomal proteolytic activity by phagocytes and have been shown to play a role in skin and mucous membrane bacterial colonization [224].

2.8.1 **Mechanism of serine protease inhibitors**

The most common mechanism of protease inhibitors involves binding in and blocking access to the active site of their target protease [225]. Amide bond hydrolysis occurs in a groove or cleft where proteases bind their substrates [215]. Figure 2.4 shows a cartoon showing substrate/inhibitor residues (P) and protein binding sites (S) of proteases. S3, S2, S1, S1', S2' and S3' designate the enzyme sub-

sites occupied by amino acid side chain of substrates that bind to corresponding substrate/inhibitor residues P3, P2, P1, P1', P2' and P3' [215].

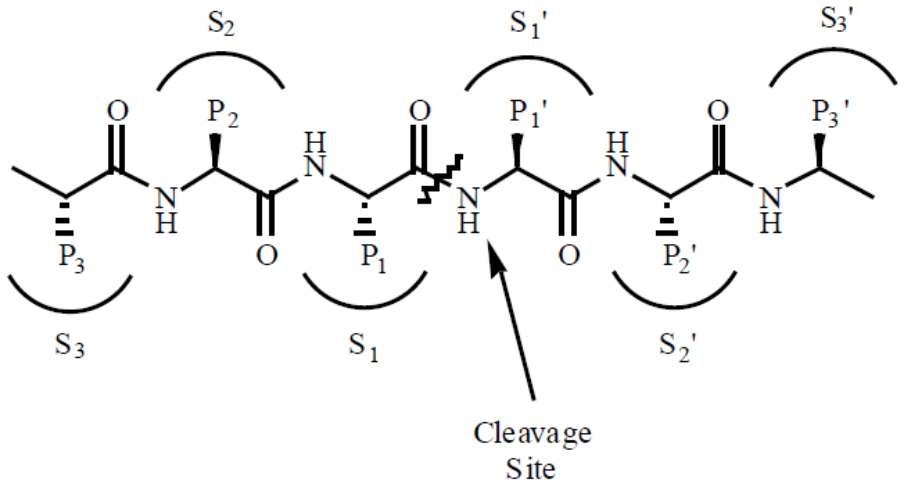

Figure 2.4. Cartoon showing substrate/inhibitor residues (P) and protease binding sites (S).

Prime and non-prime designations distinguish C-versus N-sides respectively of cleavage site. Figure copied directly from Abbenante and Fairlie, 2005 [215]

In serine protease inhibitors, the large reactive centre loop (RCL) is presented to the protease for proteolytic processing. Cleavage of the RCL leads to insertion of the N-terminal half of the protease, which is still attached to the protease as acyl enzyme intermediate, into the β-sheet of the body of the inhibitor [225]. This results in steric collisions and deformation of the active site, thus rendering the protease irreversibly and completely inactive [225]. In addition, the electrophilic property of phosphonate group in phosphonate peptide analogues, allow the phosphonate group to undergo a nucleophilic displacement reaction by the active site serine hydroxyl group [218, 226]. A trigonal bipyramidal molecular geometry is present in the substituent attached to the phosphorous atom, and the hydrolysis of peptide substrates lead to changes in bond angles and structural distortions, thereby inhibiting activity of the serine protease [226]. Phosphorylating agents and a series of mono-, di-, and tetrapeptide analogues with tetrahedral phosphonate moiety have also been reported as the prototypical inactivators of serine proteases [214].

2.8.2 **Proteases as target for inhibitors**

Due to their importance in health and disease processes, particularly in replication and transmission of infectious bacteria, protozoans and viruses, proteases are attractive targets for drug development. Protease inhibitors that have successfully progressed through clinical trials and are currently available as relatively safe and effective medicines for humans include ACE inhibitors for treating high blood pressure, human immunodeficiency virus (HIV) protease inhibitors (PIs) for treating people living with HIV/AIDS, thrombin inhibitors for treating stroke, and an elastase inhibitor for treating systemic inflammatory response syndrome (SIRS) [215]. HIV PIs are considered the first breakthrough in over a decade of HIV research [216] and are the most potent retroviral drugs in HIV clinical practice [227]. HIV PIs work by blocking HIV protease from making new HIV particles [216]. Most thrombin inhibitors such as melagatran and ximelagatran, both developed by AstraZeneca, are directed towards the inhibition of thrombin [215], a serine protease important in blood coagulation and fibrinolysis [228]. Argatroban and BIBR 1048 are thrombin inhibitors launched for treating arterial and venous thrombosis, respectively [215]. Argatroban is also used to treat peripheral vascular disease, thrombocytopenia, cerebral ischemia, and stroke [215]. A list of serine protease inhibitors and their clinical status as of 2005 is provided in Table 2.1.

Table 2-1. Serine protease inhibitors in clinical development.

Target	Indication	Drug Name	Company Name	Clinical Status
Thrombin	Venous Thrombosis	Ximelagatran	Astra Zeneca	Launched
	Thrombosis, general	Melagatran	Astra Zeneca	Pre-registration
	Arterial Thrombosis	Argatroban	Mitsubishi Pharma	Launched
	Venous Thrombosis	BIBR-1048	Boehringer Ingelheim	Phase III
	Thrombosis, general	MCC-977	Mitsubishi Pharma	Phase II
	Thrombosis, general	TGN-167, TGN-255	Trigen	Phase II
	Thrombosis, general	SSR-182289	Sanofi-Synthlabo	Phase I
	Thrombosis, general	AZD-0837	Astra Zeneca	Phase II
	Thrombosis, general	E-5555	Eisai	Phase II
	Venous Thrombosis	LB-30870	LG Life Sciences	Preclinical
Factor Xa	Thrombosis, Angina	DX-9065a	Daiichi	Phase II

Target	Indication	Drug Name	Company Name	Clinical Status
	Venous thrombosis	DPC-906	BMS	Phase II
	Thrombosis	CI-1031	Berlex Biosciences	Phase II
	Venous thrombosis	JTV-803	Japan Tobacco	Phase II
NS3-protease	Hepatitis C Virus Infection	BILN-2061, Ciluprevir	Boehringer-Ingleheim	Phase II
	Hepatitis C Virus Infection	VX-950	Vertex	Phase I
Elastase	SIRS, Inflammation,	Sivelestat, Elaspol	Ono	Launched (Japan)
	COPD	Midesteine	Medea Research	Pre-registration (Italy)
	COPD	AE-3763	Dainippon	Pre-clinical
	COPD	R-448	Roche	Phase I
Broad-Spectrum	Pancreatitis, Inflammation	Nafamostat, FUT-175	Japan Tobacco	Launched
	Pancreatitis	Camostat mesilate	Ono	Launched
Urokinase	Cancer, Gastrointestinal	WX-UK1	Wilex	Phase II
Chymase	Restenosis	NK-3201	Nippon Kayaku	Preclinical
DPP IV	Diabetes Type II	LAF-237	Novartis	Phase III
	Diabetes	MK-0431	Merck	PhaseII
	Diabetes	P32/98 (P3/01)	ProBiodrug	Phase I
	Diabetes	T-6666	Tanabe Seiyaku	Phase I
	Diabetes	NN-7201	Novo-Nordisk	Phase I
NS3/A4 serine-protease inhibitors	Hepatitis-C	Telaprevir (VX 9-50)	Vertex	Phase III
		Boceprevir (Sch503034)	Schering-Plough	Phase III
		ITMN-191	InterMune/Roche	Phase I
		B1201335	Boehringer	Phase I
		TMC435	Medivir/Tibotec	Phase II
		MK7009	Merck	Phase I

Table adapted from Arabshahi and co-workers [229] and Reiser and Timms [230].

2.8.3 **Activity-based approaches to investigate proteases**

Activity-based proteomics utilizing activity-based probes (ABPs) is a new subdivision of proteomics that defines protein activity as well as directly detects the enzymatic activities in complex proteomes [reviewed by 231]. Additionally, these chemical probes are designed to target and subsequently identify and/or purify functionally distinct families of enzymes as well as modify specific active-site

residues by using an activity-dependent chemical reaction, hence the name activity-based probes [reviewed by 232]. Distinct basic structures shared by all ABPs include those elements required for targeting, modification, and detection of labelled protein, which correspond to the reactive group, linker and tag, respectively. Development of selective activity probes that specifically target serine proteases has been previously described [233, 234].

2.8.4 **Functions of proteases**

One function of proteases is as quality control factors, recognizing regions that are commonly found on misfolded or unfolded proteins but not on native proteins [235]. These quality control factors not only recognize non-native conformations but also distinguish between substrates that can be refolded from severely damaged proteins that have to be degraded [221], thereby preventing the detrimental effects of protein accumulation and playing an essential role in bacterial survival under stressful conditions [236]. If these quality control options fail, damaged proteins accumulate as aggregates, a process known to be toxic to humans and has been implicated in causing amyloid [237] as well as neurodegenerative diseases [238]. Aggregation of misfolded proteins in bacteria results in the formation of disordered intracellular precipitates and is similarly detrimental and lethal.

Additionally, chaperones and proteases share common features because their substrates are similar even though they carry out antagonistic reactions [239].

2.8.5 **The HtrA Family**

Structure and functions of HtrA

Among the several putative proteases encoded by bacterial genomes, the high temperature requirement protein A (HtrA) family has been demonstrated to be important for virulence of a number of bacterial pathogens. The HtrA family of proteins is a group of heat shock-induced proteins exhibiting a temperature-dependent, ATP-independent dual chaperone-protease function in the periplasmic space of Gram-negative bacteria [194] with important roles in bacterial stress response and protein quality control. HtrA proteases are usually in a resting state that can be activated upon sensing of molecular stimulus (e.g. unfolded proteins) [240]. A well-characterized HtrA protease is DegP, a member of the widely conserved HtrA family of serine proteases (Figure 2.5). The DegP (HtrA) protein is essential for the

survival of *Escherichia coli* at elevated temperature and is involved in the degradation of misfolded proteins and correct processing of secreted proteins [241], and has been found to have homologues in almost all organisms [242]. Biochemical and structural studies of DegP from *E. coli* demonstrated that the resting hexameric DegP can be converted to proteolytically active 12-mer or 24-mer complexes by effector-binding to the PDZ domain of DegP [240]. HtrA proteins such as DegP are primarily composed of three distinct domains, an N-terminal protease comprising the catalytic triad which is composed of His_{105}, Asp_{135} and Ser_{210}, and two C-terminal PDZ domains, PDZ1 and PDZ2 [reviewed by 243]. These three domains and their trimeric assembly act to control and regulate entry of substrate into the central cavity and their interplay enables the protease to reversibly switch between active and inactive state as well as allowing the enzymatic activity to adjust according to the needs of the cell [reviewed by 243]. Other members of the HtrA family in *E. coli* include *degQ* and *degS*, which encode homologs of DegP [244]. Although neither gene is heat-inducible, DegQ has the properties of a serine endoprotease and can functionally substitute for DegP under some conditions, while DegS cannot substitute for DegP [244].

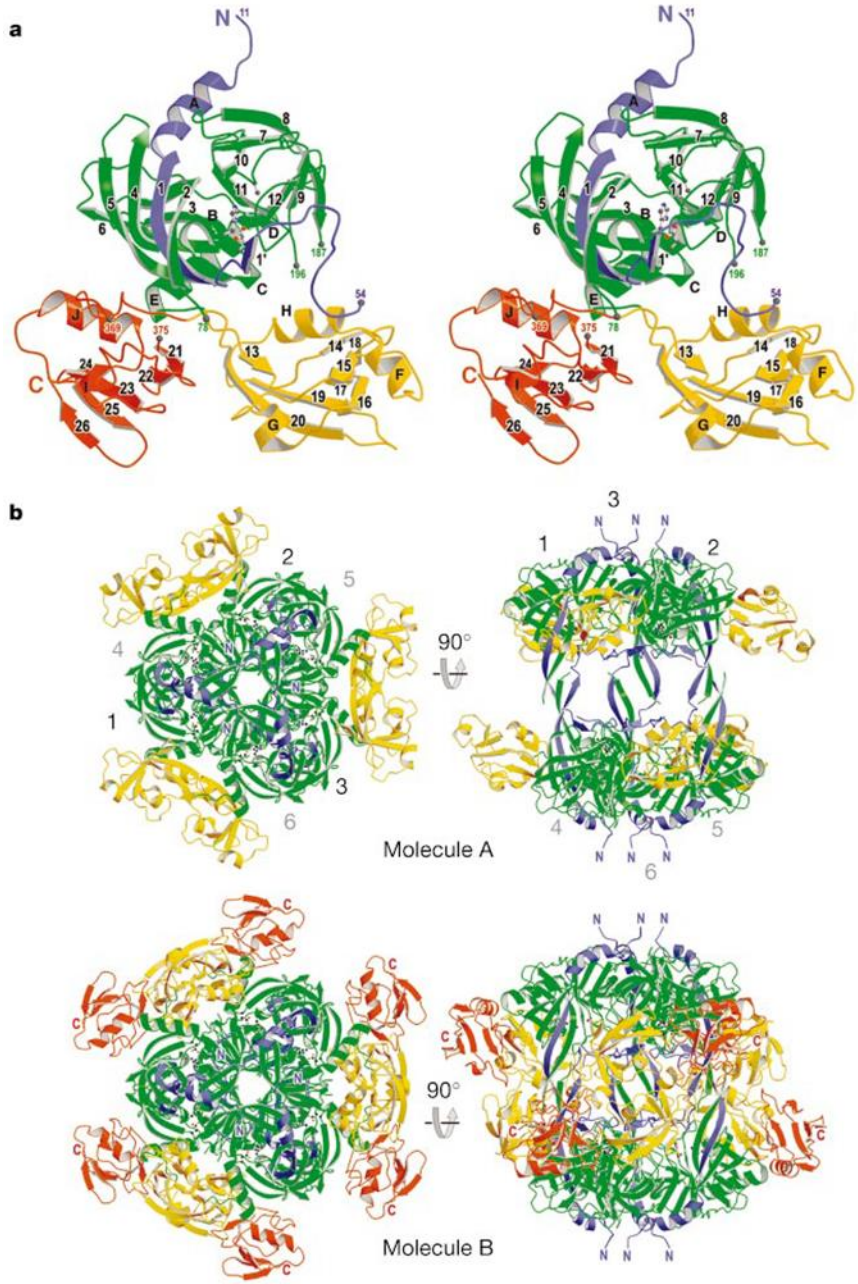

Figure 2.5. Structure of DegP.

a, Stereo ribbon presentation of DegP. The domains are represented by different colours, N terminal, purple; protease, green; PDZ1, yellow; PDZ2, red. The nomenclature of secondary structure elements are represented by letters for helices and numbers for strands. The termini of the protein as well as the regions that were not defined by electron density are indicated. b, Molecules A and B show the top and side views of DegP, respectively. Both hexamers are approximately equal in size, having a height of 105A° and a diameter of 120A°. Image directly copied from Krojer and co-workers, 2002 [245].

2.8.6 **HtrA as a pathogenic factor in bacteria**

The important contribution of HtrA to the pathogenicity and virulence of a number of bacterial pathogens have been previously described. Both the chaperone

and protease activities of *Salmonella enterica* serovar Typhimurium HtrA are essential for survival and successful infection of the organism in liver and spleen of mice [12] and are essential for the organism to divide and replicate within the host cell as well as to withstand oxidative killing in macrophages [246]. HtrA has been also found to play a crucial role in the virulence of *Streptococcus pneumoniae* as deletions of the *htrA* gene in murine models completely attenuated and dramatically reduced the virulence of strains D39 and TIGR4, respectively [11]. HtrA is also vital in host cell interaction and subsequent efficient bacterial adherence and invasion of gastrointestinal pathogens such as *Campylobacter jejuni* [8] and *Helicobacter pylori* [9]. Through the use of small molecule inhibitor of *H. pylori* HtrA (HpHtrA) activity, HHI, Hoy and co-workers [9] identified that HpHtrA was crucial for *H. pylori*-induced ectodomain E-cadherin cleavage in MKN-28 cells. However, it was identified that HtrA from *Neisseria gonorrhoeae*, the etiologic agent inducing gonorrhoea, did not cleave E-cadherin, suggesting structural differences in the active site pocket of HtrA in *N. gonorrhoeae* and the gastrointestinal pathogens [10]. Baek and co-workers [8] assessed the requirement for the dual (protease and chaperone) activities of *C. jejuni* HtrA in the interaction with host cells. Through comparison of an *htrA* mutant lacking protease activity, but retaining chaperone activity, with a Δ*htrA* mutant and a wild-type strain, it was revealed that the chaperone activity alone greatly stimulated adherence to the host cell and promote bacterial virulence of *C. jejuni* [8]. *Shigella flexneri* requires all three periplasmic chaperones DegP, a member of the HtrA family, Skp, and SurA for plaque formation and proper presentation of IcsA [247], an autotransporter involved in *S. flexneri* actin-based intracellular movement [248, 249]. HtrA in *Listeria monocytogenes*, a pathogen often sourced from contaminated foods, has been found to play essential role in degrading proteins that accumulate under stress conditions such as elevated-NaCl environments, heat shock and H_2O_2 stress [13].

In *C. trachomatis*, genomic transcriptional profiling performed by Belland and co-workers [90] determined that CtHtrA expression may occur throughout much of the developmental cycle from ~8 h PI to its highest levels from 24 to 40 h PI. In the preliminary work conducted by our team immediately prior to this PhD project, it was identified that inhibition of CtHtrA activity during mid-replicative phase, but not during early or late stages of the chlamydial developmental cycle was lethal for *C.*

trachomatis [250]. Direct immunofluorescence and/or functional implications indicated that CtHtrA was exported into the host cell cytosol and thus may play a role in chlamydial interactions with the host cell, and may be involved in chlamydial proteolysis strategy in manipulating the host cell signalling pathway [23, 251]. It was speculated that chlamydial proteases such as HtrA and CPAF were delivered to the periplasmic region by sec-dependent pathway and were further exported to the lumen of inclusion via outer membrane vesicles (OMVs) budding mechanism. This OMV budding process was supported by observations reported by Matsumoto and Manire [136] and the detection of extra-inclusion vesicles devoid of *Chlamydia* reported by Giles and co-workers [252], as well as the identification of globular structures containing Pmp-like secreted (Pls) proteins within the inclusion lumen and at the inclusion membrane of *C. trachomatis* as demonstrated by Jorgensen and Valdivia [253]. While the role of HtrA in the pathogenicity of a wide array of bacteria has been studied, the possible role of HtrA in *C. trachomatis* virulence is yet to be elucidated. CtHtrA deserves further study since the secretion of CtHtrA into the host cell cytosol suggests that CtHtrA may play an important role in chlamydial interactions with host cells [23]. *Chlamydia* HtrA may have similar virulence functions with both host and bacterial cell targets as the HtrA in other bacteria and therefore has a potential role in chlamydial infection and virulence.

2.8.7 ***Chlamydia trachomatis* HtrA (CtHtrA)**

It has been suggested that CtHtrA has similar physiological functions as those of homologs in other bacteria by both protease and chaperone activities such as extracytoplasmic protein quality control [19]. One of the unique characteristics of CtHtrA is its ability to differentially respond to the substrate which requires either chaperone or proteolysis activity, with preferences to cleave non-polar residues [254]. The cleaved peptides have been observed to always have at least one hydrophobic residue (termed I, L, A, V, or P) and through further analysis of CtHtrA substrate specificity, Huston and co-workers [254] demonstrated that I was the most preferred residue on the P1 site with V and L the next preferred residues for P1. This preference for V/I/A on the PI site of CtHtrA justifies and validates the substrate specificity of JO146, the CtHtrA tripeptide (Boc-Val-Pro-ValP(OPh)2) inhibitor used in the present study. Furthermore, molecular modelling studies on CtHtrA protein active site structure identified that mutation of the S1-S3 subsite residue V266

inhibits *in vivo* interactions of CtHtrA with a broad range of protein sequences with high affinity, suggesting the essential role of this residue during substrate binding [255]. A molecular model of the structure of CtHtrA protease domain is shown in Figure 2.6. Structural and amino acid sequence analyses demonstrated that CtHtrA has a conserved critical role during the replicative of the chlamydial developmental cycle in different species of *Chlamydia* infecting a range of different hosts: *C. trachomatis*, human, *C. suis*: pig, *C. pecorum*: cattle/sheep, *C. caviae*: guniea pig [256].

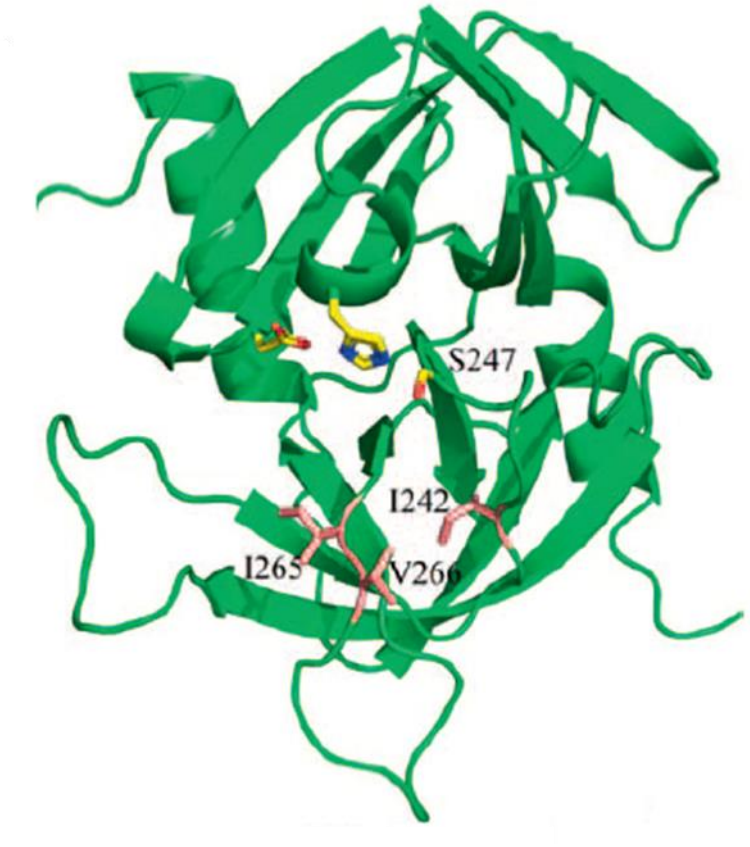

Figure 2.6. Structural model of the CtHtrA protease domain

In this figure, the catalytic triad S247, H143, D173 are shown in yellow. The flexible loop 1 (I242; S247) and loop 2 (I265; V266) encompassing the substrate-binding sites S1–S3 are also indicated. The residues predicted to be important for specificity (I242, V266 and I265) are shown in brown. Figure directly copied from Gloeckl and co-workers, 2012 [255].

In 2007, Huston and co-workers [257] demonstrated that CtHtrA is temperature activated at 34^{o}C and can function both as chaperone and protease at 37^{o}C in an ATP-independent manner, which is in contrast to the temperature-dependent "switch' mechanism of HtrA described in *E. coli*. [258]. CtHtrA is a

hexamer under native condition [257] and conversion to the 12/24-mers complexes is induced by the presence of substrates and activators [254]. Differences in the inhibitory molecules against CtHtrA compared to those against human HtrA and *E. coli* HtrA, indicate the potential consequent differences in access to the active site for CtHtrA [257]. It was confirmed by bioinformatics analyses of the CtHtrA sequence that the features previously reported in *E. coli* HtrA by Clausen and co-workers [221] such as excretion signal sequence to mediate periplasmic export, two C-terminal PDZ domains, and essential catalytic site serine residues, were also found in CtHtrA [257]. The interaction between the protein subunits such as the PDZ1 domain, loop L3, and loop LD, in the presence of substrates induces the formation of 12/24 mers [254]. The PDZ1-L3-LD interaction cascade is important for activating the proteolytic activity of CtHtrA [254, 259]. The same activation mechanism was reported for *E. coli* DegP [245]. Interestingly, CtHtrA and other chlamydial proteases RseP and Tsp have been found not to elicit a pathology-related serum IgG immune response in *C. trachomatis*-infected individuals [260].

It is pertinent to review all serine proteases in *Chlamydia* as it may have "off-target" impact other serine proteases. A complete list of bioinformatically annotated serine proteases in the genome is provided in Table 2.2. An amino acid sequence percent identity between *C. trachomatis* HtrA and its homologues in other *Chlamydia* species and some pathogens are presented in Table 2.3. Data from Table 2.3 were gathered through multiple alignment of the proteins and calculations of the different amino acid residues across the length of the longest protein. The highest percent identity was found to be between *C. trachomatis* HtrA and *C. pecorum* HtrA (Table 2.3). *C. trachomatis* HtrA and its homologue in *E. coli*, DegP have 45.1% amino acid sequence identity (Table 2.3).

Table 2-2. *C. trachomatis* serine proteases and their physiologic functions

Protease	Gene description	Base pairs	Gene number	Molecular weight (kDa)	Protein size (aa)	Function	References
lspA	lipoprotein signal peptidase	504	CT408	18.94	167	specifically catalyzes the removal of signal peptides from prolipoproteins	Denham *et. al.*, 2008
htrA	DO serine protease	1494	CT823	53.244	497	exhibits a dual chaperone-protease function; monitors and controls the state of cellular proteins; maintains viability of *C. trachomatis* during heat stress; secreted into host cell	Kim and Kim, 2005; Huston *et.al.*, 2008; Xiang *et. al.*, 2011
dacC	D-alanyl-D-alanine carboxypeptidase	1032	CT551	38.232	343		
lepB	signal peptidase I	1887	CT020	71.504	628		
lon	ATP-dependent protease La	2460	CT344	91.965	819	a stress response protease that mediates the selective degradation of mutant and abnormal protein plays a key role in maintaining cellular homeostasis and survival from DNA damaged induced by stress	Sisko *et. al.*, 2006
clpP	ATP-dependent Clp protease proteolytic subunit	612	CT706	22.049	203	plays a major role in the degradation of misfolded proteins; may play the role of a master protease which is attracted to different substrates by different specificity factors such as ClpA or ClpX	Thomsen *et. al.*, 2002
clpP_1	ATP-dependent Clp protease proteolytic subunit	579	CT431	21.162	192		
tsp	tail-specific protease	1935	CT441	73.306	644	cleaves the p65 protein, an important regulator of NF-kB transcription factor family	Lad *et. al.*, 2007
CPAF	chlamydial protease-like activity factor	1806	CT858	67.253 (processed to 30 and 40 kDa fragments)	601	degrades host cell transcription factors involved in MHC class I and II antigen presentation	Zong et. al., 2001, Shaw et al., 2002
sohB	protease	996	CT494	35.76	331	stands for suppressor of HtrA; sohB in *htrA*-mutant *E. coli* strains has been found to compensate for the missing HtrA	Baird *et. al.*, 1991

Table 2-3. A matrix of amino acid sequence percent identity between *C. trachomatis* HtrA (CtHtrA) and its homologues in other *Chlamydia* species and in some pathogens.

	E._coli DegP	*C. trachomatis* HtrA	*C. muridarum* HtrA	*C. pneumoniae* HtrA	*C. pecorum*_HtrA	*E. coli* DegQ	*C. suis* HtrA	*C. trachomatis* HtrA	*S._enterica*_DegP
C. trachomatis HtrA	45.1								
C_muridarum HtrA	44.3	93.4							
*C. pneumoniae*_HtrA	43.7	75.3	75.9						
C. pecorum HtrA	44.1	75.1	76.3	82.9					
Escherichia coli DegQ	63.6	43.3	42.9	42.5	42.9				
C. suis HtrA	44.1	93.0	93.6	76.1	75.3	43.5			
C. trachomatis HtrA	45.1	100.0	93.4	75.3	75.1	43.3	93.0		
Salmonella enterica DegP	93.0	45.1	44.5	44.3	44.1	63.6	44.5	45.1	
Shigella flexeneri DegP	99.4	45.3	44.1	43.5	44.3	63.8	44.3	45.3	92.4

2.8.8 **Identification of the CtHtrA inhibitor, JO146**

Prior to commencement of this PhD project, experiments were conducted in our team that led to the identification of the compound, JO146 as specific and selective to CtHtrA. Firstly, Gloeckl and co-workers [250] screened a library of 1090 serine protease inhibitors [261, 262] including isocoumarins and peptides with various electrophiles for the ability to block CtHtrA *in vitro* protease activity through CtHtrA protease assays with the substrate MCA-ENLHLPLPIIF-DNP previously described by Huston and co-workers [254]. A uniform concentration of 500 µM was initially used for all the compounds screened. The compounds which demonstrated complete lethality at 500 µM were then selected and further screened at different concentrations to identify compounds that were most effective at low concentrations *in vitro*. Using mass spectrometry, the top hits were validated for purity and structural integrity and this led to the selection of two compounds, JO146 and JCP83, as the most effective compounds that were structurally intact and pure. These two lead compounds were then tested against several other proteases to ensure they didn't have general serine protease inhibition. These proteases included chymotrypsin,

trypsin, elastase, *E. coli* DegP and DegS [263, 264] and recombinant forms of HTRA1 (human) and HTRA2 (human).

The two inhibitors (at a series of concentrations, namely, 0, 10, 50, 100, and 150 µM) were added to *C. trachomatis* HEp2 cultures ((multiplicity of infection, (MOI) of 0.3 and 3.0)) at different time points throughout the developmental cycle, namely, at 0, 8, 16, 20, 24, and 32 h PI. The resulting inclusion forming units at completion of the developmental cycle after each of these independent treatments was then assessed. It was determined that JO146 and JCP83 resulted in complete or significant loss of EB formation when added at mid-replicative phase [250]. When JO146 was added at 0, 8, 20, 24, and 32 h PI, infectious progeny yield was observed at the end of the chlamydial developmental cycle in cultures treated with 50 µM JO146. However, treatment with 50 µM JO146 at 16 h PI was completely lethal for *Chlamydia* with an MOI of 0.3. The activity was most effective at higher doses, with 100 and 150 µM JO146 treatments resulting in complete loss of infectious progeny yield for all the cultures except when added at 32 h PI, wherein infectious progeny were observed even at 150 µM JO146 treatments. At 16 h PI even 10 µM JO146 had a significant impact on the formation of EBs when added to *Chlamydia* cultures with MOI of 0.3. The host cell numbers were the same under each condition so these data indicate that the amount of *Chlamydia* present (MOI) associates with the effectiveness of the compounds [250].

It was identified that JO146 was more effective against CtHtrA, with an IC_{50} of 12.5 µM, compared to 47.19 µM of JCP83 [250]. JO146 was then chosen, over JCP83, as the CtHtrA inhibitor utilized and characterized in the said study. JO146 was most effective when added at 16 h PI, which is consistent with an effect on the mid-replicative phase of the chlamydial developmental cycle, but not when added during early or late phases of the developmental cycle [250]. 3D structure illumination microscopy (3D SIM) was used to further examine the chlamydial inclusion and morphology using immunofluorescence on cultures from 20 h PI (time of treatment), 22 and 28 h PI. JO146 treated inclusions failed to increase in size, exhibited a flatter appearance and eventually lost their morphology, with very few spherical cell shaped structures detected within the inclusions at 28 h PI. It was identified that the lethality of JO146 determined at 44 h PI was found to be maintained in the presence of cycloheximide, indicating that JO146 activity does not

require host cell protein synthesis [250]. Using the lactate dehydrogenase assay for cell lysis (LDH Assay) and through monitoring the metabolic turnover using the MTS incorporation assay (MTS Assay) after treatment with JO146 and DMSO control, it was determined that JO146 was not toxic to the host cells, HEp2 and McCoy cells [250]. The compound's stability during the mid-developmental cycle conditions where lethality was observed was tested by monitoring the *in vitro* stability of the JO146-activity-based probe (JO146-Cy5). Through SDS PAGE analysis after adding the probe, it was determined that JO146-Cy5 was stable throughout the experiment, suggesting that the critical nature of the timing of the compound addition for maximum effectiveness related specifically to a developmental cycle feature of *Chlamydia* rather than compound stability [250]. Wide-field microscopy analysis of lysotracker labelling of live host cells indicated that the JO146 inhibitory effect on *Chlamydia* was not mediated by recruitment of lysosomes to the chlamydial inclusions [250].

JO146-Cy5 activity-based probes identified three predominantly labelled proteins with one additional weakly labelled band. A dominant doublet of proteins around 48–50 kDa were labelled by JO146-Cy5 throughout the developmental cycle [250]. These labelled proteins are consistent with the banding pattern and molecular weight of CtHtrA as observed by Western blot done for this study and as consistent with previous reports using alternative antibodies [265]. Competitive binding assays confirmed that the JO146-Cy5 activity-based probe bound to the same targets as JO146.

A JO146-biotin activity-based probe was used to isolate the labelled proteins and confirm their identities by proteomics. Purified recombinant CtHtrA was incubated with the JO146-biotin activity-based probed and streptavidin magnetic bead binding was used to confirm that CtHtrA can be isolated using this methodology, and therefore that this activity-based probe binds to CtHtrA [250]. This approach was applied to chlamydial cell culture lysates to identify proteins labelled by JO146-biotin at 24 h PI. After proteins were isolated by the JO146-biotin activity-based probe streptavidin isolation, CtHtrA was identified by mass spectrometry (~48 kDa) as the only band which corresponds to this molecular mass with two peptides identified which correspond to CtHtrA, confirming that JO146-biotin binds to CtHtrA in a cell culture lysate [250].

Real-time widefield microscopy revealed that the inclusions of *C. trachomatis*-infected HEp2 cultures treated with JO146 at 16 h PI appeared to diminish in size and eventually could not be visualized [250]. In contrast, the inclusions in control cultures treated either with DMSO or media only increased in size over time [250].

The effectiveness of JO146 *in vivo* was evaluated using the mouse *C. muridarum* model of disease. It was initially determined that JO146 and JCP83 inhibited CmHtrA, with IC_{50} of 47 µM and 93.69 µM, respectively [250]. Treatment with JO146 and JCP83 of *C. muridarum*-infected McCoy cells resulted in 2–2.5 log reduction in infectious progeny production, with JO146 being slightly more effective. The *C. muridarum* life cycle is complete within 26-30 h PI and the most effective time (12 h PI) for JO146 treatment was consistent with the replicative phase. Examination of the chlamydial load in vaginal swabs from progesterone-synchronized mice treated vaginally with JO146 every second day of infection indicated a small but statistically significant decrease in the total viable *Chlamydia* shed compared with DMSO-treated mice [250].

Overall, these data indicate that JO146 is specific to CtHtrA and the compound is lethal to *Chlamydia* when added at mid-replicative phase of the developmental cycle [250]. In *C. trachomatis*, CtHtrA has been found to perform similar dual functions as chaperone and protease as those of its homologues in other bacteria. Also, CtHtrA is upregulated during penicillin-induced persistence and heat-shock conditions, suggesting its role in stress resistance [18]. However, while the role of HtrA in the pathogenicity of a wide array of bacteria has been studied, the role of CtHtrA in *C. trachomatis* developmental cycle is yet to be elucidated.

Chapter 3: **Materials and Methods**

3.1 GENERAL STOCKS AND SOLUTIONS

3.1.1 Supplemented Dulbecco's minimal essential medium (DMEM)

DMEM (Life Technologies, Eugene, OR, U.S.A.) was supplemented with 5% fetal calf serum (Lonza, Auckland, New Zealand), 10 µg ml^{-1} gentamicin (Invitrogen, Eugene, OR, U.S.A.), 100 µg ml^{-1} streptomycin sulphate (Sigma-Aldrich, St. Louis, MO, U.S.A.) and used in cell culture experiments with McCoy and HEp2 cell lines.

3.1.2 2X DMEM

2x DMEM was prepared by mixing 8.3 g powdered DMEM (Sigma-Aldrich, St. Louis, MO, U.S.A), 3.7 g $NaHCO_3$ (Merck, Darmstadt, Germany) and 4.5 g D-glucose (Merck, Darmstadt, Germany) in 300 ml distilled water. The solution was added with 10 ml 200 mM L-glutamine (Life Technologies, Eugene, OR, U.S.A), 10 ml 1 M HEPES (Calbiochem, CA, U.S.A.), 10 mL sodium pyruvate (Sigma-Aldrich, St. Louis, MO. U.S.A.), 1 ml 55 mM 2-mercaptoethanol (Life Technologies, Eugene, OR, U.S.A.) and 0.2 ml 50 mg ml^{-1} gentamicin (Invitrogen, Eugene, OR, U.S.A.). The solution was made up to a total volume of 500 ml with distilled water and was then filter-sterilized (0.2 µm pore-size, Corning, NY, U.S.A.) into 200 ml sterile Schott bottles. The solution was stored in aliquots at 4^oC.

3.1.3 Phosphate buffered saline (PBS)

PBS used for immunocytochemistry was made by 10x stock by combination of 81 g NaCl (Ajax Finechem, New South Wales, Australia), 4 g Na_2HPO_4 (Merck, Darmstadt, Germany) and 1.65 g NaH_2PO_4 (Merck, Darmstadt, Germany) in 1 L of Milli-Q (Biocel, Australia) water. The 10x stock was diluted to obtain 1x concentration with Milli-Q water. PBS used for cell culture was made from PBS tablets Dulbecco 'A' (Oxoid, Hampshire, England). One (1) tablet was suspended in every 100 ml Milli-Q water. The solution was autoclaved (Tomy SX-500 E, NY, U.S.A.) at 121^oC for 15 minutes.

3.1.4 **Sucrose phosphate glutamate (SPG) solution**

SPG was made by combining 13.68 ml of 0.5 M Na_2HPO_4 (Merck, Darmstadt, Germany), 6.32 ml of 0.5 M NaH_2PO_4 (Merck, Darmstadt, Germany), 85.575 g sucrose (Chem Supply, Adelaide, South Australia), 0.736 g L-glutamic acid (Life Technologies, Eugene, OR, U.S.A.) in 900 ml Milli-Q water. The pH was adjusted to 7 and the total volume of the solution was adjusted to 1 L with Milli-Q water. The solution was filter sterilized using 0.2 µm pore-size filters (Corning, NY, U.S.A.), aliquoted in 100 mL Schott bottles and stored at 4^oC.

3.1.5 **JO146**

For the purposes of this study, JO146 (chemical formula: $C_{31}H_{44}N_3O_7P$) was commercially sourced. The compound was synthesised, HPLC purified, and confirmed by MALDI-MS by GL Biochem (Shanghai, China). The compound was obtained in powder form (100 mg). Molecular weight of the compound is 607.67 g/mol. When dissolved in DMSO, the total concentration of stock JO146 is 0.166 M.

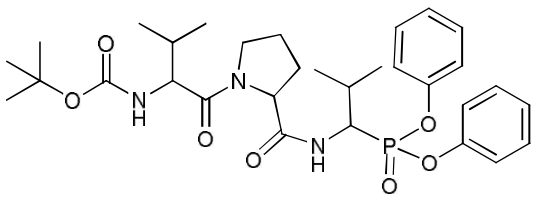

Figure 3.1. Chemical structure of JO146

JO146 is a tripeptide (Boc-Val-Pro-Val^P $(OPh)_2$) inhibitor with valine both in P1 and P3 sites for substrate specificity and proline in the P2 substrate specificity site. The phosphonate "warhead" acts irreversibly with the protease active site serine residue. Boc serves as a protecting group.

3.1.6 **Penicillin**

Benzylpenicillin sodium (BenPen, CSL Biosciences, Victoria, Australia) was obtained in powder form (600 mg). When dissolved in sterile water, the resulting concentration was 1.0 x 10^5 U ml^{-1}. Appropriate amount of the solution was dissolved in DMEM to obtain the concentration needed for cell cultures (typically 100 U ml^{-1}).

3.1.7 **1.1% agarose**

1.1% agarose was obtained for plaque purification of *Chlamydia*. 1.1g agarose LE (Roche Diagnostics GmbH, Mannheim, Germany) was dissolved in 100 ml sterile distilled water. The solution was aliquoted in sterile 50 ml Schott bottles and was autoclaved at 121^{o}C for 5 minutes.

3.2 CELL LINES

HEp2 (human epithelial type 2, ATCC® CCL-23™) and McCoy B (mouse fibroblasts, ATCC® CRL-1696™) cell lines were used in all cell culture experiments. The cell lines were regularly tested as mycoplasma free. HEp2 and McCoy cells were maintained in supplemented DMEM.

3.2.1 **Mycoplasma testing of cell lines**

Cells were grown in antibiotic-free media for 24 hours. The cells were harvested in PBS and DNA was extracted using a DNA extraction kit (Qiagen). Previously described primer set (UM 4, forward primer, and UM3, reverse primer) [266] were used to detect and amplify the 16S rRNA gene of mycoplasma in the cell lines. PCR was performed by 1 cycle of 95^{o}C for 5 min followed by 30 cycles each consisting of denaturation for 30 s at 94^{o}C, 30 s of annealing at 55^{o}C and 60 s extension at 72^{o}C. An amplicon size of approximately 270 bp (detected by agarose gel electrophoresis) would denote a positive contamination of mycoplasma in cell lines.

3.3 *CHLAMYDIA* STRAIN PROPAGATION

Chlamydia trachomatis serovar D (UW-3/Cx) was used in all experiments. In Chapter 5, plaque cloned *C. trachomatis D* was used as a control strain in relation to the *Chlamydia* clinical isolates. All *Chlamydia* strains were routinely cultured in DMEM and incubated at 37^{o}C, 5% CO_2. High yield stocks were prepared. Briefly, *Chlamydia* strains were used to infect T75 culture flasks with 90% confluent HEp2

or McCoy cells. The flasks were centrifuged at 500 × g for 30 minutes at 28^{o}C. Media was replaced with fresh media at 4 h PI. The cells were incubated up to end of chlamydial developmental cycle (i.e., 44-48 h PI) and were harvested by replacing the media with 3 ml SPG per flask. By using a cell scraper, the infected monolayer was gently removed from the flask. The cell lysate was transferred to a 50 ml Falcon tube containing sterile glass beads to an equivalent of 5 ml. The tubes were vortexed for approximately 2 minutes. The cell lysate was then transferred to a new Falcon tube and was centrifuged at 500 × g for 5 minutes at 4^{o}C to separate the host cell debris from *Chlamydia*. The supernatant was removed, transferred to a new falcon tube where it was mixed well, and then aliquoted in 1.5 ml cryovials and stored at -80^{o}C for further processing. To determine the titre of the stocks, *Chlamydia* was quantified from each stock or batch of SPG harvest by reinfecting fresh HEp2 or McCoy B cell monolayers in 96-well plates as described in section 3.4.

3.4 *CHLAMYDIA* QUANTIFICATION

HEp2 or McCoy cells were cultured in supplemented DMEM in 48 well cell culture plates. The host cells were infected with *C. trachomatis* D at 24 h post seeding using a stock of *Chlamydia* with known titre. The experiments were routinely performed using multiplicity of infection (MOI) of 0.3. Variations from this MOI are indicated in the appropriate sections. Treatments with JO146 or penicillin were performed accordingly at different doses and time points post infection as indicated for each experiment. *Chlamydia* was harvested at the completion of the chlamydial developmental cycle either by adding SPG equal to the amount of DMEM in the cultures or by replacing the media with SPG. The plates were stored at -80^{o}C. To determine the infectious viable yield for these cultures, the plates were thawed. The cells were then sonicated on ice 3 times per well (10 seconds each time) by using an ultrasonic processor (Microson, New York, U.S.A.). The harvested cultures were then serially diluted and cultured in fresh HEp2 or McCoy (the same cell line as the experiment) monolayers in 96 well plates. Briefly, *Chlamydia* from triplicate wells in 48 well plates were 3x serially diluted across the wells of 96 well plates. *Chlamydia* from one well of 48 well plate were used to reinfect 3 corresponding wells in 96 well culture plate, resulting to a total number of 9 independent wells per dilution per condition. Cultures were incubated for 4 hours at

37^{o}C, after which the media was changed with fresh DMEM containing 1 µg ml^{-1} cycloheximide (Sigma-Aldrich, St. Louis, MO. U.S.A.). At 30 h PI, cultures were fixed with absolute methanol or 4% paraformaldehyde (PFA, Electron Microscopy Sciences, Hatfield, Pennsylvania, U.S.A.) in PBS (PFA-PBS) for 10 minutes. The number of inclusions was determined by immunocytochemistry *C. trachomatis* HtrA or MOMP. Firstly, the cells were permeabilized with 0.5% Triton X-100 (Merck, Darmstadt, Germany) for 15 minutes, after which the permeabilization buffer was removed and replaced with blocking buffer. Non-reactive sites were blocked with 1% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, U.S.A.) in PBS for 2 hours. Then, the plates were incubated with anti-*C. trachomatis* HtrA (1:500 dilution in 1% BSA-PBS) antibody raised in rabbit [257], or mouse *C. trachomatis* MOMP (1:2,000 dilution in 1% BSA-PBS) (Biodesign International, Maine U.S.A.) and DAPI (1:40,000 dilution in blocking buffer) (4'6-diamidino-2-phenylindole, Life Technologies, Eugene, OR, U.S.A) for 1 hour. Unbound antibodies were removed by washing the wells with 0.2% Tween 20 (Merck, Darmstadt, Germany) in PBS (TPBS) for three times (5 minutes each time). Then, the wells were incubated with anti-rabbit or anti-mouse IgG, IgM AlexaFlour 488 conjugate as secondary antibody (1:500 dilution in blocking buffer) (Invitrogen, Eugene, OR, U.S.A.) for 45 minutes. The wells were again, washed with TPBS. Images were obtained using Nikon Eclipse Ti (Tokyo, Japan) fluorescence microscope. Inclusions were counted from triplicate fields of view per well from at least three independent culture wells, making it a total of 27 fields of view for each dilution. The size of the field of view on that particular microscope and camera was then extrapolated to the size of the wells to calculate the total number of inclusions per well. The volume of *Chlamydia* added to the well as well as the dilution wherein the inclusions were counted was accounted for to determine viable IFU ml^{-1}.

3.5 CONFOCAL MICROSCOPY

McCoy or HEp2 (25,000 cells well^{-1}) cells were cultured on coverslips (8 mm) in 48 well culture plates. The cultures were fixed with either 4% PFA-PBS (for *C. trachomatis* MOMP) or absolute methanol (for *C. trachomatis* HtrA) at designated time points post infection. The cells were permeabilized with 0.5% Triton X-100 (Merck, Darmstadt, Germany) for 15 minutes, after which the permeabilization

buffer was removed and replaced with blocking buffer, 1% BSA-PBS and the plates were incubated at 4^{o}C overnight. The cells were examined for the appropriate *C. trachomatis* and host cell proteins by incubating the coverslips with the primary antibody (*C. trachomatis* MOMP (1:2,000 in blocking buffer) (Biodesign International, Maine U.S.A) or HtrA (1:500 in blocking buffer) [257]; host cell LAMP1 (1:3,000 in blocking buffer) (Abcam, Victoria, Australia), SQSTM1 (1:5,000 in blocking buffer) (Abcam, Victoria, Australia); actin was examined with phalloidin 594 (2 µl per coverslip) (Invitrogen, Eugene, OR, U.S.A.) and nucleus was examined with DAPI (1:40,000 in blocking buffer) (Life Technologies, Eugene, OR, U.S.A) at room temperature for 2 hours. The cells were then washed with TPBS three times (5 min each time). Secondary antibody conjugated to Alexa Flour 488 (Invitrogen, Eugene, OR, U.S.A.) was then added to the wells at 1:500 (HtrA) or 1:2,000 (MOMP) dilutions in blocking buffer. After incubation at room temperature for 1 hour, the cells were washed thrice with TPBS (5 minutes each time). The final wash was removed and replaced with PBS. The coverslips were mounted onto labelled, clear white glass slide using a drop (approximately 20 µl) ProlongGold® antifade reagent (Invitrogen, Eugene, OR, U.S.A) for each coverslip. *C. trachomatis* cultures were examined using either Leica SP5 (Leica Microsystems, Wetzlar, Germany) or Olympus FV1200 (Olympus Corporation, Japan) confocal laser scanning microscopes. The settings were adjusted according to the emission/excitation wavelengths of the fluorophores. The excitation/emission (nm) wavelengths for the fluorophores were 358/461 for DAPI, 581/609 for phalloidin 594, and 495/519 for AlexaFlour fluorophores. Images were taken using 20x or 63x objectives using the suite of software on the microscope.

3.6 PLAQUE PURIFICATION OF *CHLAMYDIA*

Purification by plaque cloning was performed to isolate clones of *Chlamydia* suitable for the experiments. Briefly, 6-well plates were seeded with 1.2 x 10^{6} McCoy cells well^{-1} in 3 ml DMEM. Then, 24 hours after seeding, the monolayers were infected with the corresponding *Chlamydia* isolates at 10, 50, or 100 IFU well^{-1} by centrifugation at 500 × g for 30 minutes at 25^{o}C. The agarose overlay was prepared by mixing the following: 22.5 ml 2x DMEM, 22.5 mL 1.1% agarose, 5 ml

FCS (Lonza, Auckland, New Zealand) and 50 µl cyclohexemide (Sigma-Aldrich, St. Louis, MO, U.S.A.). The mixture was placed in a 45^{o}C water bath for 10 minutes. After centrifugation, the media in each well was replaced with 2 ml agarose overlay and was allowed to solidify. Meanwhile, the non-agarose overlay was prepared by obtaining a mixture containing the following components: 12.5 ml 2x DMEM, 10 ml dH_2O and 2.5 ml FCS (Lonza, Auckland, New Zealand), 25 µl cyclohexemide (Sigma-Aldrich, St. Louis, MO, U.S.A). After incubating the mixture in a 37^{o}C water bath for 5 minutes, 2 ml of the non-agarose overlay was then added to each well and the plates were stored at 37^{o}C, 5% CO_2. The plates were examined for presence of plaques every day for 14 days.

As soon as plaques appeared in the wells, the plaques were "picked" and used to reinfect fresh host cell monolayers to propagate the clonal *C. trachomatis* isolates. Briefly, to "pick' the strains from the plaques, the non-agarose overlay was carefully removed from the wells. Plaques that were not too close to each other were picked through the aid of sterile yellow P200 tips cut at the end. Each picked up plaque was then dispensed in 300 µl SPG in a 1.5 ml cryovial filled with sterile glass beads to an equivalent of 250 µl. The cryovials with *Chlamydia* were vortexed for 2 minutes after which, the cell lysates were used to reinfect fresh HEp2 or McCoy B cell monolayers in 96 well culture plates previously seeded with 40,000 host cells/well in 200 µL DMEM. The monolayers were infected by replacing the media with 100 µl of the cell lysate and spinning the plates at 500 × g for 1 hour at 25^{o}C. The plates were incubated at 37^{o}C, 5% CO_2 for 30 minutes. The media was changed with fresh media containing 1 µg ml^{-1} cyclohexemide (Sigma-Aldrich, St. Louis, MO, U.S.A.) and plates were incubated further. At 35 h PI, *Chlamydia* was harvested from the plates by replacing the media with 200 µl SPG well^{-1}. The infected monolayers were dislodged and elementary bodies (EBs) were released from the inclusions by vigorously pipetting the SPG up and down the wells using a multichannel pipette. The supernatant was transferred to a new 1.5 ml tube. These stocks were labelled passage 1. Then, 30 µl of the SPG harvest from passage 1 was used to reinfect fresh HEp2 or McCoy B cell monolayers in 96 well plates (seeded the previous day with 40,000 host cells well^{-1}). At 30 h PI, *Chlamydia* was harvested by replacing the media with SPG and vigorously pipetting up and down the wells as previously described. The SPG stock from this step was designated passage 2. *Chlamydia* from

stock 2 was then used to reinfect for passage 3. Briefly, 6-well plates seeded with 6.0 x 10^5 McCoy cells $well^{-1}$ were infected with *Chlamydia* from passage 2 (100 µl $well^{-1}$). DMEM from the wells seeded with the host cells were replaced with media containing *Chlamydia* and 1 µg ml^{-1} cyclohexemide (Sigma-Aldrich, St. Louis, MO, U.S.A). At 44 h PI, the cells were harvested by replacing the media with SPG (1 ml $well^{-1}$). By using cell scrapers, cells were gently scraped from the wells. The monolayers were completely dislodged by pipetting up and down the wells. The cell suspensions were pooled in a 50 ml tube containing glass beads to an equivalent of 3 ml. The 50 ml tubes containing the cell suspensions were vortexed for 2 minutes. The supernatant was gently transferred to a new 50 ml tube and were stored in aliquots (200 µl in 1.5 ml tube) at -80^{o}C. These stocks were designated as passage 3. For passage 4, T75 flasks (with 90-100% confluent host cells) were infected with stocks from passage 3 (200 µl $flask^{-1}$). The cells were harvested at 44 h PI by replacing the media with SPG and scraping the cells from the flasks using a cell scraper. The supernatant was transferred to a 50 ml tube containing glass beads and the tube was vortexed for 2 minutes. To separate the host cell debris, the 50 ml tubes were centrifuged at 500 × g for 5 minutes at 4^{o}C. The supernatant was transferred to a new 50 ml tube and mixed by pipetting up and down the tube. The cells were stored in aliquots at -80^{o}C. The stock of each clone was then quantified to determine IFU ml^{-1} using the method described in section 2.4. For all infection and reinfection steps involved in propagating plaque cloned isolates, the plates or flasks were spun at 500 × g for 30 minutes at 25^{o}C and were incubated at 37^{o}C, 5% CO_2 until harvest.

3.7 GENERATION OF *C. TRACHOMATIS* MUTANTS USING EMS MUTAGENESIS

Generation of *C. trachomatis* mutants as well as estimating the rate of mutations was performed following the procedure of Kari and co-workers [176] with minor modifications. Low-level ethyl methanesulfonate (EMS) was used as a mutagen since it has been shown to introduce C-G to T-A transition mutations. Briefly, a 6-well cell culture plate was seeded with 1.5 x 10^6 McCoy cells/well in 3 ml DMEM supplemented with 10 % FCS, 10 µg ml^{-1} gentamicin and 100 µg ml^{-1} streptomycin. 24 hours later, the cells were infected with *C. trachomatis* D at an MOI of 1.0 by centrifugation at 500 × g for 30 minutes at 25^{o}C. At 22 h PI, infected host

cells were exposed for one hour to EMS at 37^{o}C. Preliminary experiment using different concentrations of EMS namely, 2.0, 3.0 and 4.0 mg ml^{-1} was performed to evaluate the right amount of EMS to be used relative to "percent killing" and appropriate mutation frequency of 3-4 mutations per genome. After treatment with EMS, the media was removed and replaced with fresh media containing 1µg ml^{-1} cyclohexemide and the cells were incubated at 37^{o}C, 5% CO_2. Untreated (non-mutagenized) *C. trachomatis* D control was included. The cells were harvested in sucrose phosphate glutamate (SPG) buffer at 44 h PI. In brief, the media was removed from the wells and replaced with 1 ml well^{-1} SPG. The infected monolayers were harvested and stored in aliquots at -80^{o}C. Viable infectious yield and "percent killing" was determined by re-infecting fresh McCoy and HEp2 cells and number of IFUs was determined following the procedures previously described.

The rate of mutation was estimated by determining the frequency of rifampicin-resistant (Rif^{R}) mutants which emerged as a result of point mutations in β-subunit of RNA polymerase [181]. To determine the concentration of rifampicin to be used for identifying rifampicin resistant mutants, a preliminary experiment using different concentrations of rifampicin (0.05 µg ml^{-1} 0.1 µg ml^{-1}, 0.3 µg ml^{-1}) was conducted. This was performed by plaque assay on wild type, laboratory strain of *C. trachomatis* D (UW-3/Cx) as described in Materials and Methods Section 3.6 to confirm which concentration of rifampicin was inhibitory to the wild-type strain. 6-well plates seeded with 1.2 x 10^{6} McCoy cells well^{-1} were infected with a total of 2 x 10^{6} EBs by centrifugation at 500 × g for 30 minutes at 25^{o}C. Rifampicin was added to the non-agarose mixture to achieve the desired rifampicin concentration. The plates were examined for presence of plaques every day for 14 days. Once the concentration of rifampicin (0.1 µg ml^{-1} [176]) for determining mutation frequency was established, the plaque assay was again performed as previously described; however, this time, 2.0 x 10^{6} EMS-mutagenized *C. trachomatis* D strains were used to infect the monolayers. The number of plaques was noted and the rate of mutation was calculated.

The *C. trachomatis* genome contains 4.30 x 10^{5} G-C base pairs. Transition of five of these C-G pairs results in Rifampicin resistance [79, 81, 184, 267-269]. Therefore, we estimated the rate of mutation using this formula: [(number of plaques observed/2.0 x 10^{6} EBs) x 4.30 x 10^{5}]/5.

3.8 STATISTICAL ANALYSIS

All statistical analysis and graph construction was performed using PRISM Version 6.0 (GraphPad Software Inc.). Results are expressed as mean ± SEM. Two-way analysis of variance (ANOVA) with a post hoc Bonferroni multiple comparison test were used to assess the differences between the treatments at different time points relative to DMSO. Statistical significance was defined as $p < 0.05$.

Chapter 4: **Investigation of the physiological roles of CtHtrA during chlamydial development and stress using the inhibitor compound JO146**

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the two papers are included in this chapter.)

STATEMENT OF JOINT AUTHORSHIP

The authors listed below have certified that:

1. they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
2. they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. there are no other authors of the publication according to these criteria;
4. potential conflicts of interest have been disclosed to (a) granting bodies, and (b) the editor or publisher of **Molecular Microbiology**, and **Frontiers of Cellular Infection and Microbiology**, and;
5. they agree to the use of the publication in the student's thesis and its publication on the QUT ePrints database consistent with any limitations set by publisher requirements.

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Sarina Gloeckl	Conducted some of the major experiments, contributed to the intellectual input of the manuscript, contributed to manuscript drafting
Pooja Patel	Conducted some of the major experiments, contributed to the intellectual input of the manuscript, contributed to manuscript drafting
Joel D. Tyndall	Conducted some of the major experiments, provided feedback on experimental design, contributed to the intellectual input of the manuscript
Peter Timms	Provided feedback on experimental design, contributed to the intellectual input of the manuscript
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Melisa Merdanovic	Conducted the preliminary work for this paper, contributed to the intellectual input of the manuscript
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James C. Powers	Conducted the preliminary work for this paper, contributed to the intellectual input of the manuscript
Jozef Oleksyszyn	Conducted the preliminary work for this paper, contributed to the intellectual input of the manuscript

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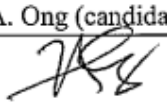
Martin Verdoes	Conducted the preliminary work for this paper, contributed to the intellectual input of the manuscript
Matthew Bogyo	Conducted some of the experiment for the paper, contributed to the intellectual input of the manuscript
Wilhelmina M. Huston	Contributed to research plan development and provided feedback on experimental design, data analysis and executions; contributed to the intellectual input of the manuscript, critically reviewed manuscript and approved final version of manuscript.

V.A. Ong, J.W. Marsh, A. Lawrence, J.A. Allan, P. Timms, W.M. Huston. 2013. The protease inhibitor JO146 demonstrates a critical role for CtHtrA for *Chlamydia trachomatis* reversion from penicillin persistence. Frontiers in Cellular and Infection Microbiology.3:100

Contributor	Statement of contribution
Vanissa A. Ong (candidate) Signature  Date 14/7/2015	Contributed to the design and implementation of overall project, conducted the majority of the laboratory work in the project, conducted the analysis and interpretation of the data and contributed to writing of the manuscript.
James W. Marsh	Conducted some of the experiments, contributed to the intellectual input of the manuscript, contributed to manuscript drafting
Amba Lawrence	Conducted some of the experiments, contributed to the intellectual input of the manuscript, contributed to manuscript drafting
John Allan	Provided feedback on experimental design, contributed to the intellectual input of the manuscript
Peter Timms	Provided feedback on experimental design, contributed to the intellectual input of the manuscript
Wilhelmina M. Huston	Contributed to research plan development and provided feedback on experimental design, data analysis and executions; contributed to the intellectual input of the manuscript, critically reviewed manuscript and approved final version of manuscript.

Principal Supervisor Confirmation

I have sighted email or other correspondence from all Co-authors confirming their certifying authorship.

Wilhelmina May Huston		14/7/2015
Name	Signature	Date

4.1 INTRODUCTION

Chlamydia (C.) trachomatis is an obligate intracellular bacterial pathogen. It is the most common sexually transmitted infection worldwide. Chlamydial infections are often asymptomatic [1-3] with as many as 50% and 80% of cases reported to be asymptomatic in men and women, respectively [4]. *Chlamydia* can cause long-term sequelae such as tubal scarring, ectopic pregnancy, infertility and pelvic inflammatory disease in women [5, 16, 18].

C. trachomatis exhibits a unique, bi-phasic developmental cycle. This cycle consists of an infectious spore-like extracellular form, termed the elementary body (EB), and an intracellular replicative form termed the reticulate body (RB), which replicates by binary fission prior to converting back to the infectious EB form (reviewed, [85]). The intracellular form is located within a unique vacuole inside the host cell that is called the inclusion vacuole. In addition to these two forms, the organism has a persistent phase of intracellular growth. Persistence is defined as viable but non-culturable *Chlamydia*. Persistent *Chlamydia* (also called aberrant bodies), are typified by a much larger chlamydial cell size, and the inclusions are morphologically distinct from the active replicating form with only a few cells visible per inclusion (and typically smaller inclusion vacuole sizes) [137, 153, 160, 270]. Persistence is induced by stressors such as immune pressure, amino acid deprivation, penicillin, iron limitation, or the presence of other intracellular pathogens [144, 160, 161, 271-274]. The ability to become persistent is thought to provide the organism with a survival mechanism to avoid any conditions where they would be unable to survive (reviewed, [270]). Whilst there are numerous means of inducing persistence and the chlamydial cellular morphology appears similar for each of these, there are distinct transcriptional and protein profiles associated with the different forms of persistence (reviewed, [137]).

Amino acid deprivation has been shown to induce persistence, which was able to be restored by cysteine and isoleucine [272, 275]. The best characterised mechanism of persistence is that induced by IFN-γ (interferon-gamma). IFN-γ (secreted by immune cells) induces a large range of responses in the epithelial cell [144, 271]. One of the proteins that is highly induced in human epithelial cells in response to IFN-γ is IDO1 (indoleamine 2,3-dioxygenase) [144, 271, 276]. This enzyme catabolises the host cell tryptophan resulting in reduced tryptophan supply

for the pathogen that is a tryptophan auxotrophic. *C. trachomatis* persistence induced by IFN- γ is able to be reversed by the removal of IFN- γ and addition of tryptophan during *in vitro* laboratory models [144]. IFN- γ aberrant bodies are typified by a loss of expression of genes required for cytokinesis with continuing chromosomal replication [21, 160]. Another commonly used laboratory model of persistence is that which occurs in response to cell wall targeting antibiotics. Models of this form of persistence typically involve penicillin. Penicillin persistence has been described to result in the *Chlamydia* cells rapidly ceasing cellular division, whilst chromosomal and plasmid replication continue at the same rate [160]. The removal of the penicillin then allows reversion back to the active RB form, which occurs via a RB budding from the aberrant body, with this only productively occurring in some inclusions and some aberrant bodies [158].

The unique, developmental cycle and the obligate intracellular nature of the *Chlamydia* have hitherto hampered efforts to develop traditional techniques to genetically manipulate the organism. However, recent reports demonstrate that genetic manipulation of *Chlamydia* is now possible. Wang and co-workers [172] demonstrated stable transformation of *C. trachomatis* L2/434/Bu through the use of shuttle vector based on the *E. coli* plasmid pBR325. The development of stable, reproducible transformation method permits analysis of genome function by complementation. Kari and co-workers [176] described targeting-induced local lesions in genome (TILLING)-based reverse genetic approach where a pool of ethyl methyl sulfonate (EMS)-generated mutants were screened for specific mutations. Nguyen and Valdivia [180] utilized forward genetic approaches by combining chemical mutagenesis and genome sequencing along with DNA exchange (lateral gene transfer) among *Chlamydia* strains to identify genes responsible for certain phenotypes. Such technologies clearly demonstrate that both reverse and genetic approaches can be performed to analyse *Chlamydia* which had been previously considered “unamenable” to genetic manipulations.

Chlamydial proteins have also been widely characterized through the advent of small molecule approaches. One of the most widely studied chlamydial virulence factors is the type III secretion system (T3SS). A peptide mimetic to the T3SS ATPase protein interaction domain could disrupt protein interactions of T3SS system and block chlamydial invasion [277]. Wolf and co-workers [188] have utilized a

T3SS-specific small molecule inhibitor to address contribution of the T3SS to the biology of *Chlamydia* [188]. Through the use of *Yersinia* T3SS inhibitor C1, it was identified that T3SS plays an important role in the progression of *C. trachomatis* developmental cycle, most notably in the RB to EB differentiation phase [188]. Overall, chemical and genetic approaches are proven powerful strategies to investigate the function of proteins within this unique organism.

A number of proteins are hypothesized to be important for chlamydial biology, virulence, and infection. An example of such an interesting protein is the high temperature requirement protein A (HtrA). HtrA has been described as having a number of virulence functions for pathogenic bacteria [9, 278]. It is a bacterial protease with essential roles for cell surface protein assembly and extracytoplasmic protein maintenance [279]. HtrA has been described in *C. trachomatis* using *in vitro* and microscopy methods, and in *Chlamydi*a it potentially functions both as a bacterial cell-associated protease and is secreted into host cell from 28 h post infection [19, 257, 265]. Our previous investigations into the biochemical mechanism of CtHtrA activation implicated outer membrane protein sequences with activation of the chaperone form, suggesting a potential role in surface protein assembly [254]. However, the function of CtHtrA for the pathogenicity of *C. trachomatis* is not well elucidated.

Therefore, this study aimed to characterize the biological impact and possible role of CtHtrA for chlamydial development using the CtHtrA inhibitor compound, JO146. CtHtrA has been documented to be important for stress tolerance and survival of *C. trachomatis* as it has been reported that CtHtrA is highly expressed during penicillin-induced persistence and down regulated during IFN-γ persistence [19, 21, 22]. Furthermore, CtHtrA has been previously found to be elevated during heat stress and has been determined by heterologous complementation experiments to protect *E. coli htrA*- against the detrimental effects of heat stress [19]. Therefore, the potential critical role that CtHtrA plays during detrimental conditions such as penicillin persistence and heat stress was also investigated.

4.2 MATERIALS AND METHODS

4.2.1 *Chlamydia* cultures and cells

Chlamydia culture was conducted essentially as described in Materials and Methods section 3.3. Briefly, *C. trachomatis* (serovar D UW-3/Cx) was routinely cultured in HEp2 cells in DMEM supplemented with 5% fetal calf serum, 10 µg ml^{-1} gentamicin, 100 µg ml^{-1} streptomycin sulphate and incubated at 37^{o}C, 5% CO_2. All cultures were conducted at a multiplicity of infection (MOI) of 0.3.

4.2.2 Growth curve and time frame required for lethality of JO146

The effect of the HtrA inhibitor on the growth and viability of *C. trachomatis* was determined by treating *C. trachomatis* -infected HEp2 cells either with DMSO or 0 µM, 10 µM, 50 µM, or 100 µM JO146 at 16 hours post-infection (h PI) and harvesting cultures in SPG buffer at 16, 20, 24, 28, 32, 36, 40, and 44 h PI. DMSO treatment was used as the negative control since JO146 was suspended in DMSO. The yield of infectious particles (i.e. infectious EBs able to form inclusions in the assay) at each time point was then assessed. The determination of inclusion forming units or infectious yield was conducted as described in Materials and Methods, Section 3.4.

4.2.3 Microscopy approaches to characterize chlamydial inclusion morphology

To determine the morphology of *C. trachomatis* at indicated time points and treatment, immunocytochemistry was performed as described in Materials and Methods Section 3.5. The cultures were examined using the Leica SP5 confocal microscope. Images were prepared using the supplied Leica software suite. Sizes of inclusions were measured using the tools available on the Leica application suite.

4.2.4 Assessment of the role of host cell pathways in JO146 lethality

The presence and localisation of specific host proteins such as LAMP1 and SQSTM1 relative to the chlamydial inclusions was examined using confocal microscopy and immunocytochemistry. Briefly, *C. trachomatis* D-infected HEp2 monolayers cultured on coverslips were treated with either 100 µM JO146 or DMSO

at 16 h PI. Following fixation with 4% paraformaldehyde (PFA) at 16, 20, and 24 h PI, the cells were stained with antibodies against LAMP1 (Abcam, Australia) or SQSTM1 (Abcam, Australia) and Alexa Flour 488 conjugates (Invitrogen, Eugene, OR, U.S.A.) were used as secondary antibodies. The cells were visualized using a Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany).

4.2.5 **Determination of cellular toxicity of JO146 on HEp2 cells**

The effect of JO146 on the viability of *C. trachomatis* - infected host cells was assessed by using LIVE/DEAD® Fixable Red Dead Cell Stain Kit (Life Technologies, Eugene, OR, U.S.A.). The assay is based on the reaction of a fluorescent dye with cellular amines. The reactive dye can permeate the compromised membranes of dead or necrotic cells and react with free amines both in the interior and on the cell surface, resulting in intense fluorescent staining. The control dead cell population (purposely killed by suspension in ethanol) was used as a positive control. In the present study, the intensity of fluorescence between the control samples, dead and live cells, differed by two logarithmic values. *C. trachomatis*-infected HEp2 cells treated either with 100 µM JO146 or DMSO at 16 h PI were fixed and assayed at 16, 18, 20, 22, 24, 32, 40 and 44 h PI. These samples were analysed using a FC500 Flow cytometer (Beckman Coulter, CA, U.S.A.) at 488 nm excitation and 585 nm emission wavelengths. Flow cytometry data was analysed using FlowJo version 7.6.4 (Tree Star Inc.).

4.2.6 **Determination of bacteriocidal or bacteriostatic effect of JO146**

JO146 was added to the cultures at 16 h PI. The compound was removed from the cultures at 20 and 24 h PI (i.e., after 4 and 8 hours exposure to the compound, respectively) by media washes (3 times with pre-warmed media). The cells were further incubated for harvesting at 44, 54, and 64 h PI. The infectious yield was then determined through titration in fresh HEp2 monolayers (as in Materials and Methods section 3.4).

4.2.7 **Determination of the effect of JO146 on heat stressed *C. trachomatis***

A variety of stress conditions were tested to test for a role for CtHtrA. Heat shock was conducted at 20 h PI for 4 h (20-24 h PI), as research in our team has previously shown an increase in CtHtrA protein at this time point during heat shock [19]. JO146 was added at 20 h PI, immediately at commencement of heat shock at 42°C, 5% CO_2 for 4 h. At the conclusion of the 4 h heat shock, JO146 was removed from the cultures by three washes in 37°C pre-warmed media, prior to returning the culture to 37°C for the remainder of the developmental cycle. In a separate experiment, the role of CtHtrA during recovery from heat shock was also analysed by the addition of JO146 immediately (at 24 h PI) after the 4 h heat shock treatment and was removed by media washes (three) at 28 h PI. The cultures were harvested at 44 h PI and viable infectious yield was determined as described in Materials and Methods Section 3.4. (see experiment outline figures at the beginning of Results sections).

4.2.8 **Effect of JO146 on penicillin-induced persistent *C. trachomatis* cultures**

Penicillin persistence was established by the addition of 100 U ml^{-1} of penicillin at 4 h PI and JO146 was added at 16 h PI to determine the impact of JO146 treatment during persistence. In order to measure the impact on infectious progeny yield, the cultures were allowed to revert from persistence by the removal of penicillin. Penicillin was removed by three sequential rounds of media washes and medium was replaced with penicillin-free media at 30 h PI. The infectious yield was subsequently measured at 68, 78, and 90 h PI. Cultures were also monitored for infectivity at 44 h PI without the removal of the penicillin to demonstrate lack of infectivity consistent with persistence (in conjunction with the ability to subsequently rescue these same culture conditions to detectable infectious particles by penicillin removal) [158]. Control cultures with no JO146 were included for each experiment. In order to assess the impact of JO146 on *Chlamydia* during reversion from penicillin persistence, a separate experiment was conducted where the persistence was induced in the cultures using penicillin (4 h PI, 100 U ml^{-1}); at 40 h PI the penicillin was removed (washes and media change). At 52 h PI when reversion is likely to be underway in most inclusions JO146 was added to the cultures. The cultures were

harvested and inclusion forming units were determined at 84, 90, and 100 h PI as described in Materials and Methods section 3.4. Control cultures without JO146 treatment were included for each experiment. Cultures for immunocytochemistry were conducted on glass coverslips, using the method previously described (Materials and methods section 3.5). A summary of the experimental methods used in this study is shown in Figure 4.1.

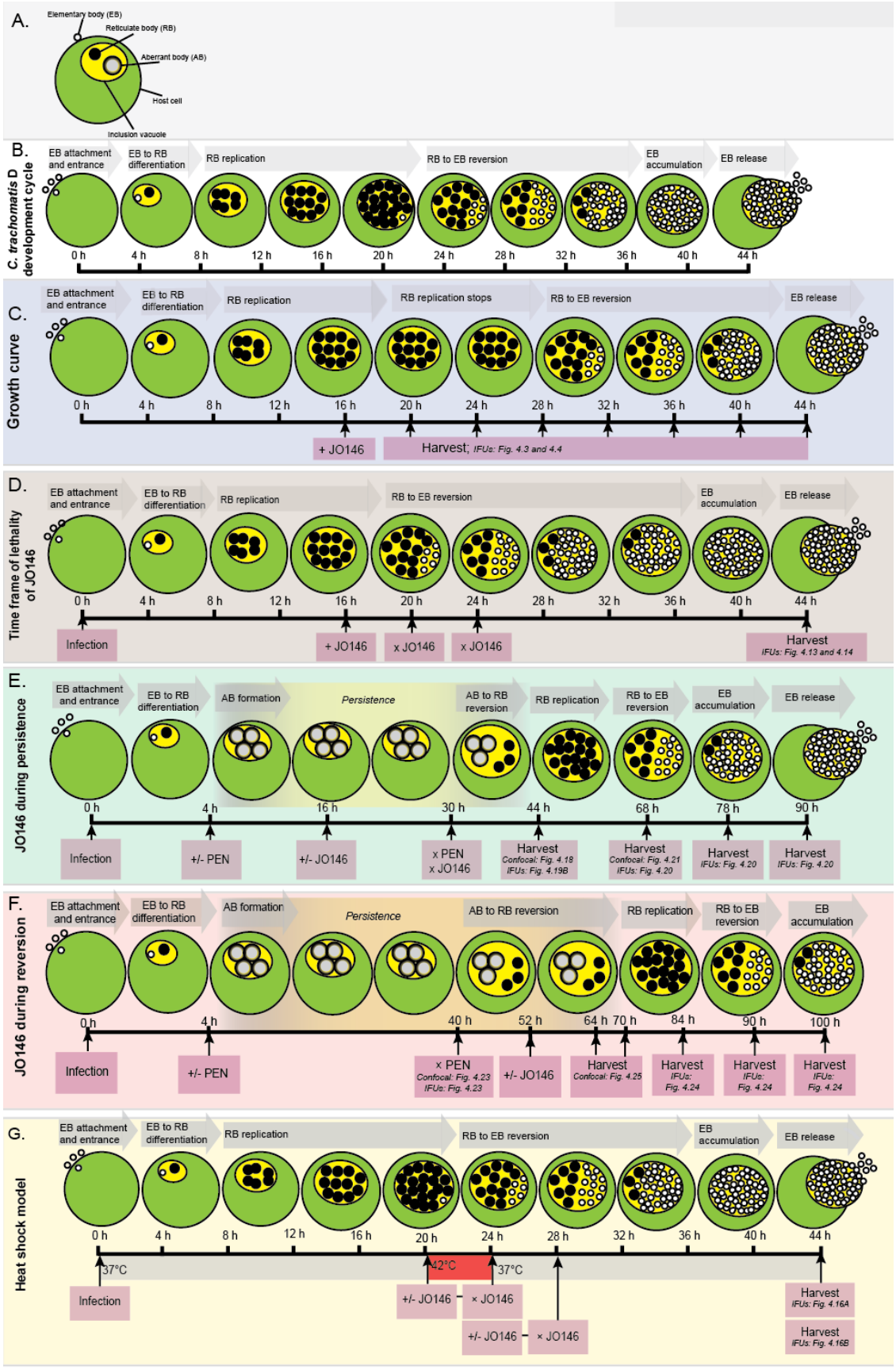


Figure 4.1 A summary of the experimental methods used in Chapter 4.

Flow chart of stress conditions tested and the corresponding expected growth phases of the *Chlamydia* that were tested during this project (A) Key to the components of a *Chlamydia*-infected host cell. Small, open circles: elementary bodies (EB); large black circles: reticulate bodies (RB); large grey circles: aberrant bodies (AB); green circle: host cell; yellow circle: chlamydial inclusion vacuole. (B) The *Chlamydia trachomatis* (serovar D) development cycle is represented by the cartoon. The relative time-points are shown below the cells and each stage of the cycle is shown in grey arrows above the cells. The five different sets of experiments (C-G) are presented in coloured backgrounds. (C) Experimental conditions used to assess the impact of JO146 after addition at 16 h PI. (D) Experimental plan to determine the time frame required for lethality of JO146, thus, assessing the bacteriostatic or bacteriocidal effect of the compound. (E) Experimental conditions used to assess the impact of JO146 addition during penicillin persistence. (F) Experimental plan to determine the impact of JO146 on *C. trachomatis* reversion from persistence. (G) Experimental plan to use the JO146 inhibitor to determine the role of CtHtrA for infectivity of *C. trachomatis* during heat stress conditions. The pink boxes represent the experimental actions taken, with arrows extending to the specific time-point for each action. The grey arrows indicate the stage of the development cycle represented by the cartoon. "PEN" represents penicillin. "+/−" indicates that separate experiments were conducted with or without the addition of penicillin ("+/− PEN") or the JO146 inhibitor ("+/− JO146"). "× PEN" indicates that penicillin was removed in the experiments where it was added. The yellow/orange highlighted section of the developmental cycle indicates the expected presence and relative duration of persistence when induced by the addition of penicillin at 4 h PI in the persistence experiments. The cartoon representations of the expected chlamydial developmental cycle phases and the associated time points under these experimental conditions are based on previously published data [89, 158, 160]. The appropriate experimental outline is presented at the beginning of each corresponding section in Results.

4.2.9 **Statistical analysis**

All statistical analysis was performed using PRISM Version 6.0 (GraphPad Software Inc.). Results are expressed as mean ± SEM. Two-way analysis of variance (ANOVA) with a post hoc Bonferroni multiple comparison test were used to assess the differences between the treatments at different time points relative to DMSO. Statistical significance was defined as $p < 0.05$.

4.3 RESULTS

4.3.1 Addition of JO146 at mid-replicative stage of the chlamydial developmental cycle is lethal for *C. trachomatis.*

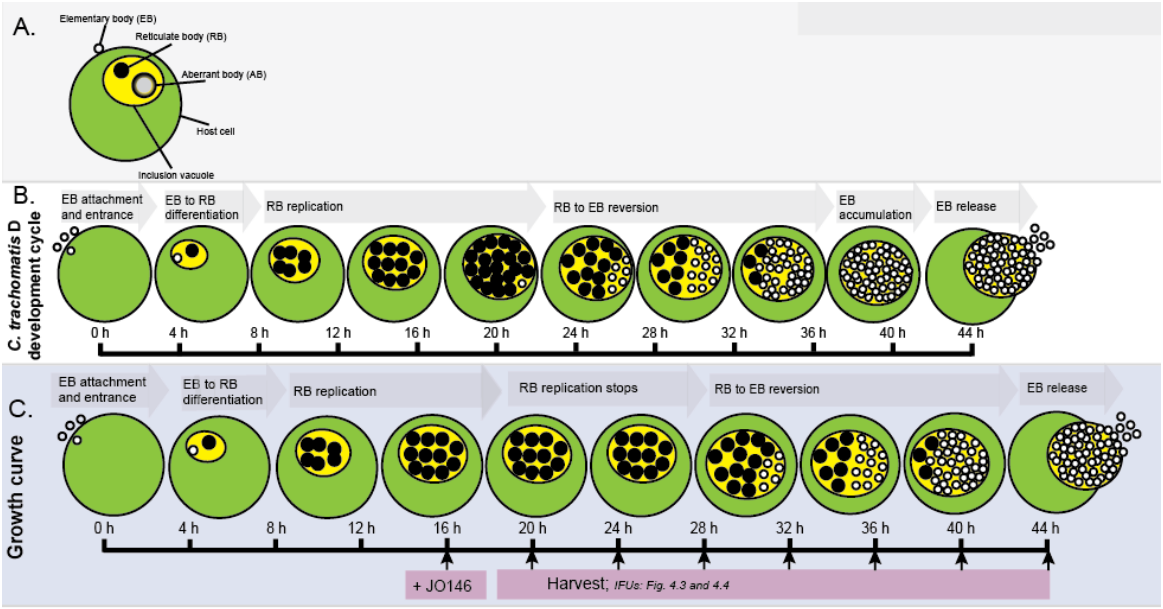


Figure 4.2. Schematic diagrams of the *C. trachomatis* inclusion and developmental cycle and the assessment of the inhibitory action of JO146 after addition at 16 h PI

(A) Key to the components of a *Chlamydia*-infected host cell. Small, open circles: elementary bodies (EB); large black circles: reticulate bodies (RB); large grey circles: aberrant bodies (AB); green circle: host cell; yellow circle: chlamydial inclusion vacuole. (B) The *Chlamydia trachomatis* (serovar D) development cycle is represented by the cartoon. The relative time-points are shown below the cells and each stage of the cycle is shown in grey arrows above the cells. The five different sets of experiments (C-G) are presented in coloured backgrounds. (C) Experimental conditions used to assess the impact of JO146 after addition at 16 h PI.

Concurrent experiments conducted in our team demonstrated that JO146 addition at mid-replicative phase (16 h PI) was most effective in preventing formation of infectious progeny when cultures were harvested at 44 h PI. The duration of JO146 treatment required to prevent infectious progeny formation was assessed. JO146 was added at 16 h PI and cells were harvested at different time points (16, 20, 24, 28, 32 and 36 h PI) until the end of the chlamydial developmental cycle. Inclusion forming units were then measured by re-culturing the harvests. No *Chlamydia* growth was observed during the first three time points (16, 20 and 24 h PI) in all treatments (Figure 4.3). At 28 h PI, inclusions were observed at 0 µM

JO146 and DMSO treated cells. Very few inclusions (1,137 IFU ml^{-1}) were observed at this time point in infected cells treated with 10 µM JO146.

An increasing number of inclusion forming units was observed over time in *C. trachomatis*-infected cells treated with 0 µM JO146, DMSO, and 10 µM JO146 as expected (Figure 4.3). Inhibition of CtHtrA function through the addition of the higher concentration of JO146 (100 µM) prevented the formation of infectious progeny at all time points post-infection. At 36 h PI, infectious progeny were identified in cells treated with 50 µM JO146 but not in cells treated with 100 µM JO146. Loss of inclusion forming units was also detected even at relatively lower concentration of JO146 treatment (10 µM) compared to DMSO-treated and untreated cells particularly at 28 hours PI wherein very few infectious particles were recovered (Figured 4.3). The number of IFU ml-^{1} observed in cells treated with 10, 50, and 100 µM JO146 were all significantly different at 28, 32, and 36 h PI compared to the DMSO control ($p < 0.001$). A second experiment was conducted that focused on extended time points. Chlamydial inclusions were observed from cultures harvested at 32 h PI in cells treated with 0 µM JO146 and DMSO (Figure 4.4). No *Chlamydia* inclusions were observed in *C. trachomatis*-infected host cells treated with 100 µM JO146 even at the end of the chlamydial developmental cycle (44 h PI) (Figure 4.4). The numbers of IFU ml^{-1} in DMSO-treated cells compared with 100 µM JO146 treated cells were significantly different at 32, 40 and 44 hours PI ($p < 0.001$) (Figure 4.4).

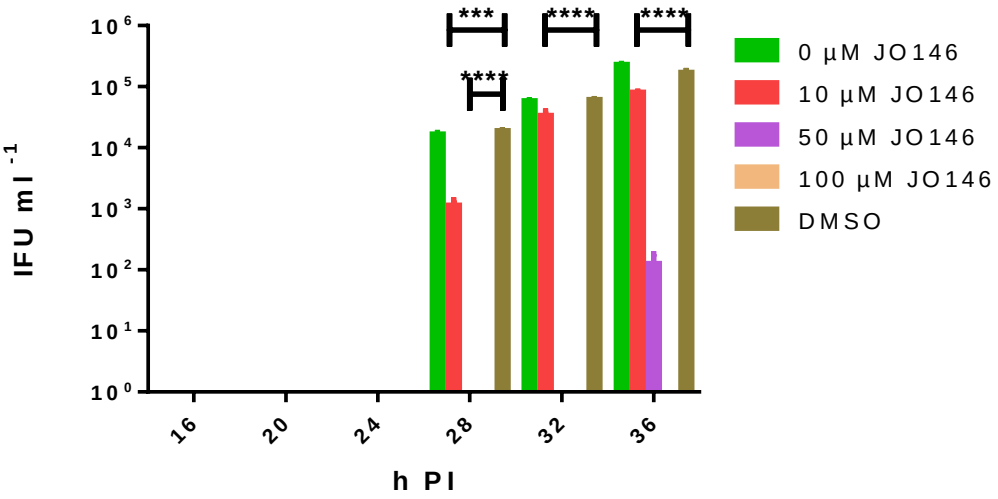


Figure 4.3. Yields of infectious progeny after JO146 treatment at 16 h PI of *C. trachomatis*

JO146 has a dose-dependent effect on the generation of formable EBs after treatment at 16 h PI. Each bar represents the number of inclusion forming units (y axis) at each of the hours PI (x axis). Data are presented as mean ± S.E.M, *** indicates $p < 0.001$, **** $p < 0.0001$, n=27.

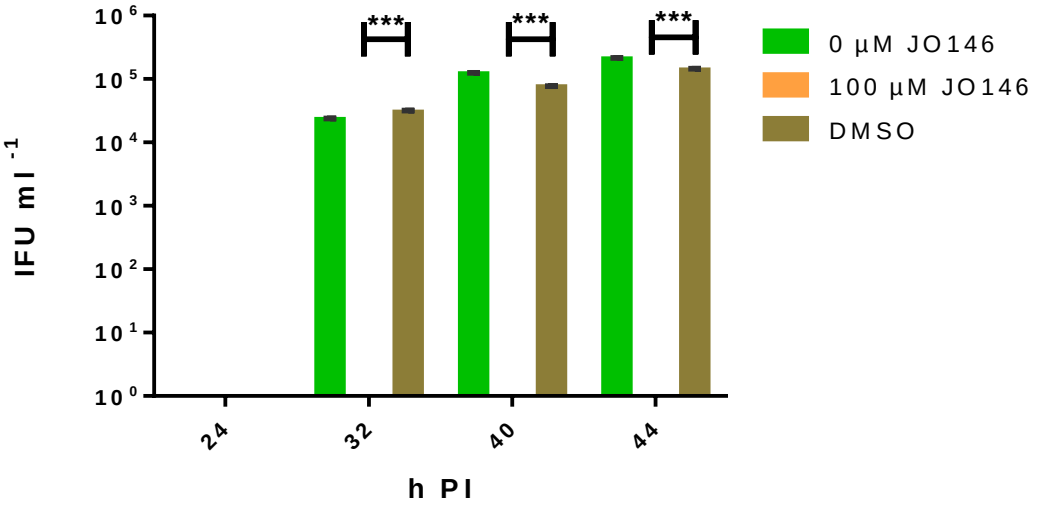


Figure 4.4. Growth analysis of *C. trachomatis* treated with JO146

Generation of infectious elementary bodies throughout the late stage of *C. trachomatis* developmental cycle indicated loss of EB formation after treatment with JO146 at 16 h PI. Data are presented as mean ± S.E.M, *** indicates $p < 0.001$, n=27.

4.3.2 Treatment with JO146 results in failure to increase the inclusion size and loss of chlamydial cellular morphology

Inclusion vacuole size and morphology after JO146 treatment was examined using confocal microscopy and immunocytochemistry. It was observed that although there was no difference in the size of inclusions at 20 h PI regardless of JO146 or DMSO treatment, *Chlamydia* inclusions from cultures in the presence of JO146 appeared smaller than those formed in DMSO treated cells both in cultures immunolabeled with CtHtrA and MOMP antibodies at 24 h PI (Figures 4.5 and 4.6).

There was a significant difference ($p < 0.001$) of the size of the inclusions formed particularly at 24 h PI between infected cells treated with JO146 (average inclusion size of 4.56 µm) and DMSO treated cells (average inclusion size of 9.31 µm) stained with antibodies for CtHtrA, (Figure 4.5). At 24 h PI, a significant difference ($p < 0.001$) in inclusion size was also observed between the inclusions formed in JO146-treated (average size of 4.35 µm) and DMSO-treated (average size of 8.66 µm) cells stained with anti-MOMP antibodies (Figure 4.6). Inclusions treated with JO146 also appeared hollow and flatter compared to DMSO-treated cells at 24

hours PI. Overall, immunocytochemistry and confocal microscopy using both *Chlamydia* HtrA and MOMP antibodies indicate that the chlamydial inclusions did not develop after addition of JO146, and were at a much smaller size compared to the cells treated with DMSO.

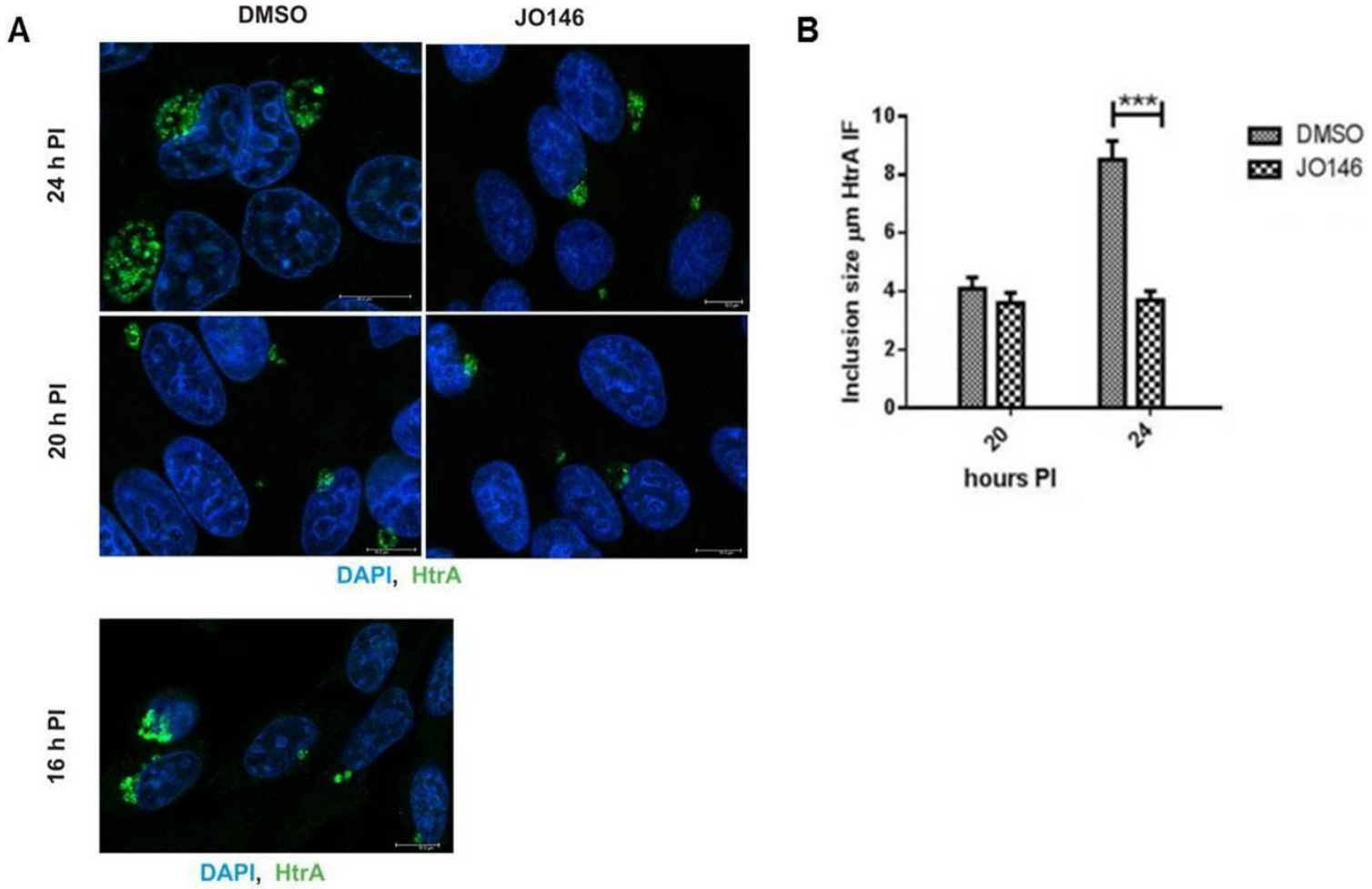


Figure 4.5. Immunofluorescence of *C. trachomatis* inclusions using anti- HtrA antibodies after treatment with JO146

(A) Immunofluorescence using anti-HtrA antibodies after JO146 (100 µM) treatment at 16 h PI. Scale bar (bottom right) indicates 10 µm. (B) Bar graph comparing the size of inclusions formed at 20 and 24 hours PI in DMSO- and JO146-treated *C. trachomatis*-infected HEp2 cells. Data are presented as mean ± S.E.M, *** indicates $p < 0.001$.

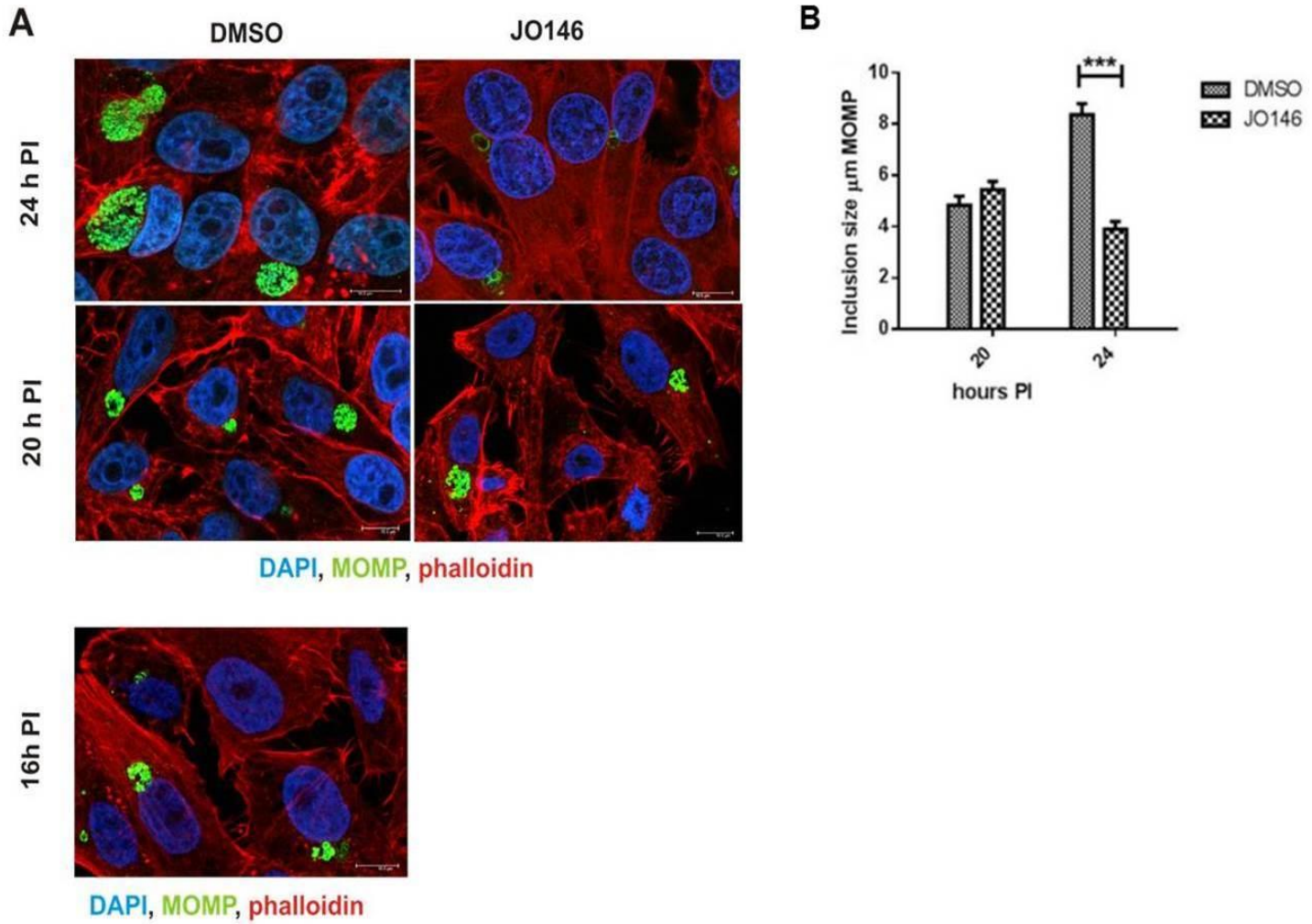


Figure 4.6. Immunofluorescence of *C. trachomatis* inclusions using anti-MOMP antibodies after treatment with JO146

(A) MOMP immunoflourescence after treatment of *C. trachomatis*-infected HEp2 cells with 100 µM JO146 at 16 h PI. Scale bar (bottom right) indicates 10 µm. (B) Bar graph comparing the size of inclusions formed at 20 and 24 hours PI in DMSO- and JO146-treated *C. trachomatis*-infected HEp2 cells. Data are presented as mean ± S.E.M, *** indicates $p < 0.001$.

4.3.3 **JO146 treatment does not lead to autophage/lysosome marker accumulation on the inclusion membrane**

To investigate if the marked decrease in inclusion size is due to host cell's main defence pathways, *C. trachomatis* infected HEp2 cells treated with JO146 were stained with anti-LAMP1 and anti-SQSTM1 antibodies. No co-localization between the autophage/lysosome markers and the *Chlamydia* inclusions (green) was observed in either treatment (100 µM JO146 or DMSO) at 20 and 24 h PI when JO146 was added at 16 h PI (Figure 4.7). If there was co-localization and therefore a potential role in the inclusion size decrease, it would have been expected to be seen at 20 h PI since at 24 PI, a big difference in inclusion sizes between the two treatments was observed.

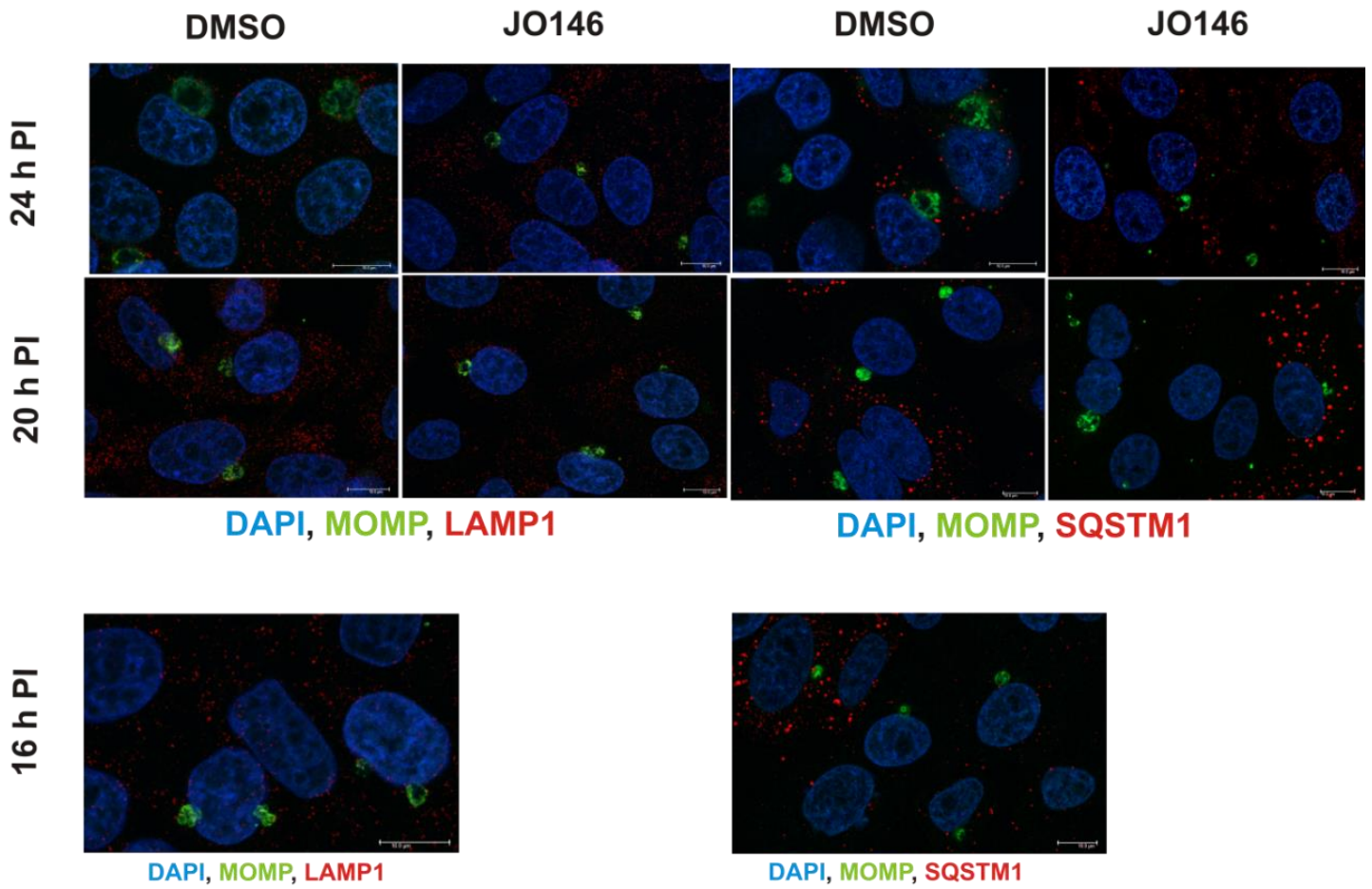


Figure 4.7. Confocal microscopy images of fixed cultures of *C. trachomatis* D-infected HEp2 cells using anti-LAMP1and anti-SQSTM1 antibodies

Immunofluorescence analysis using LAMP (left) and anti-SQSTM1 (right) (red) antibodies showed no sequestration of LAMP1 and SQSTM1 to *C. trachomatis* inclusions JO146 (100 µM) treatment. Scale bar (bottom right) indicates 10 µm.

4.3.4 **JO146 did not reduce viability of HEp2 host cells.**

Viability of the host cells was monitored using Live/Dead fixable flow cytometry assay to check whether the inhibitor compound (100 µM added at 16 h PI) was toxic to the host cells from the time of treatment until the end of the chlamydial developmental cycle. Figure 4.8 shows that the histogram marker encompasses 99.7% of the dead cell control population. Accordingly, this marker was used to "gate" the cell populations in each time point for each treatment. Only cells with fluorescence intensity falling within the histogram marker were considered dead and they were recorded in terms of percentage relative to the parent population.

Figure 4.9 shows the overlap of histograms of samples for every treatment and time point. The cells that were treated with JO146 had a higher percentage (1.74) of dead cells compared to the cells treated with DMSO (0.872) at the beginning of treatment (16 h PI). However, DMSO–treated cells had higher percentage of dead cells (2.11) than JO146-treated cells at 24 h PI (1.19). These results indicate that there was no correlation of the percentage of dead cells between cells treated with either JO146 or DMSO at all the time points indicated.

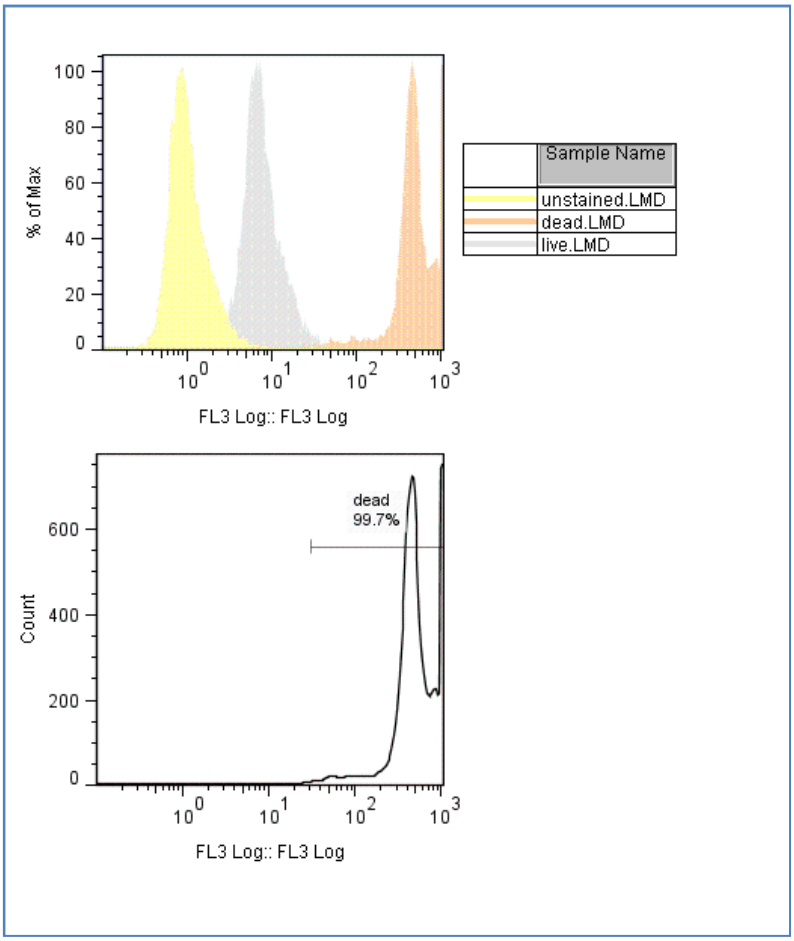


Figure 4.8. Histograms of the control samples

The histograms display relative fluorescence or light scatter intensity on the x-axis and the number of events on the y-axis (A). The histogram marker of interest was based on the dead cell control population and this gate was applied to the sample populations for each time point (B).

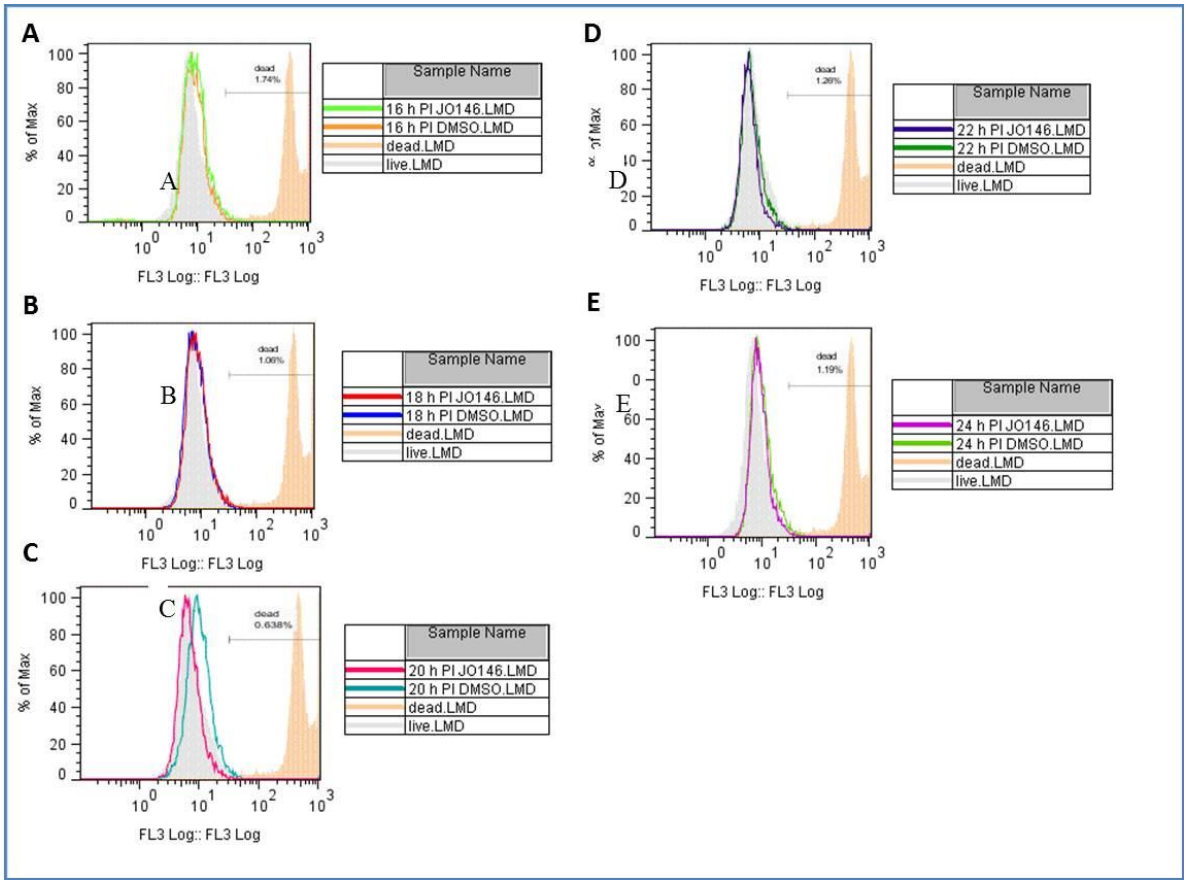


Figure 4.9. Overlay of histograms for every treatment for the different time points

Cells were assayed at 16 (A), 18 (B), 20 (C), 22 (D), and 24 (E) h PI. Dead and live control cell populations are represented by filled orange and grey histograms, respectively. Percentage values denote the number of necrotic cells in JO146-treated samples in each corresponding time point.

A second assay was performed to determine the effect of JO146 on host cell viability even up to the late stage of chlamydial developmental cycle. Live, dead, as well as mixed live and dead cell populations were included as controls (Figures 4.10). *C. trachomatis*-infected HEp2 cells were treated with DMSO or 100 µM JO146 at 16 h PI and were assayed at extended time points, namely, 24, 32, 40 and 44 h PI. Figure 4.11 shows the overlap of histograms of samples for every treatment and time point. At 24 h PI, a higher percentage of dead cells was observed in JO146-treated cells (0.958) compared to DMSO-treated cells (0.352). Eight hours later (32 h PI), less percentage of dead cells was observed on JO146-treated cells (0.423) than in DMSO-treated cells (0.547). A difference of 2 log in the Mean (FL3 Log) values between the dead and live cells, or an overlap of the histogram of samples on the

histogram of the dead cell control population was required to readily distinguish loss of viability of HEp2 cells. Therefore, based on the results obtained in this study, there was no correlation on the percentage of dead cells between each time point and for each sample regardless of JO146 treatment.

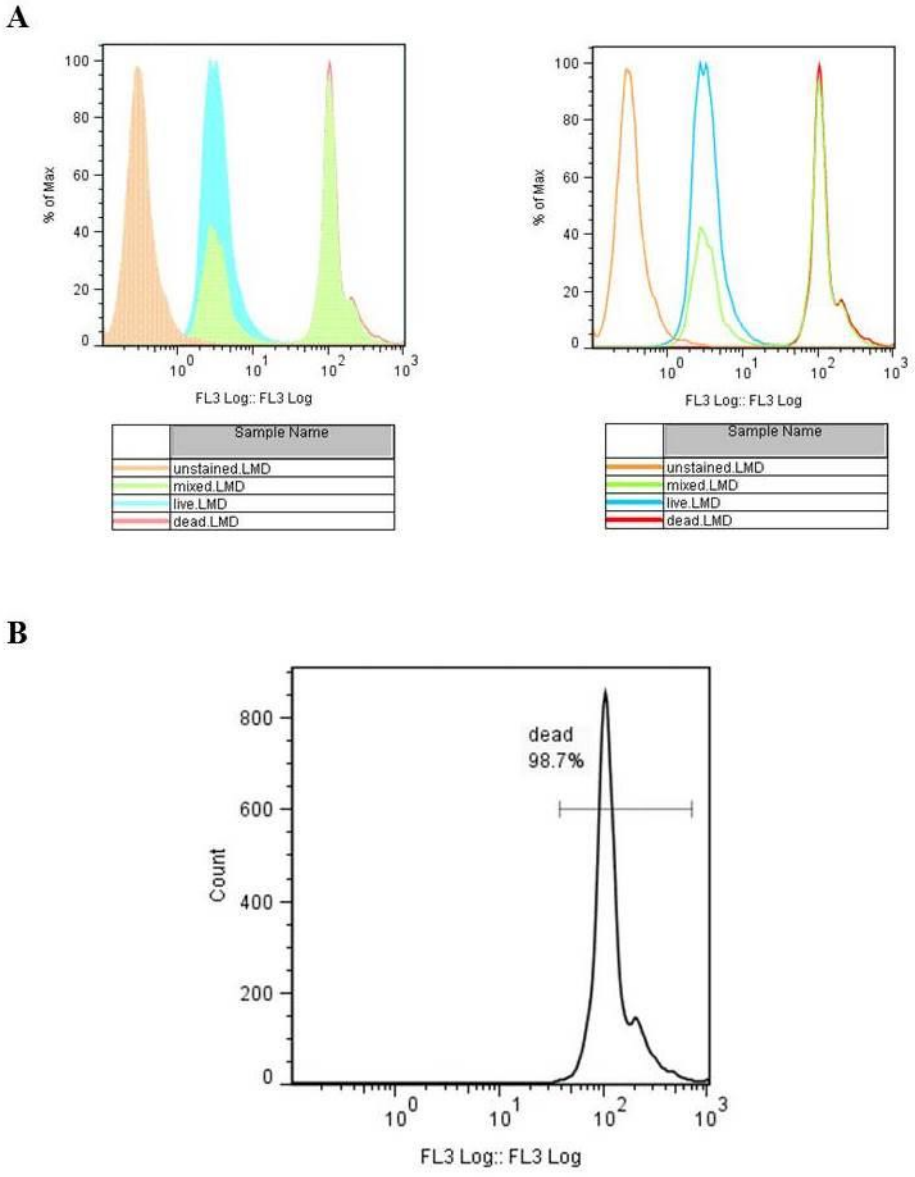


Figure 4.10. Histograms of the control samples for the second cytotoxicity assay to determine if JO146 is toxic to HEp-2 cells

The histograms display relative fluorescence or light scatter intensity on the x-axis and the number of events on the y-axis (A). Two peaks corresponding to live and dead cells can be observed in the mixed control sample (light green). Live control cell population is designated by the blue histogram. The histogram marker of interest was based on the dead cell control population (red) and this gate was applied to the sample populations for each time point (B).

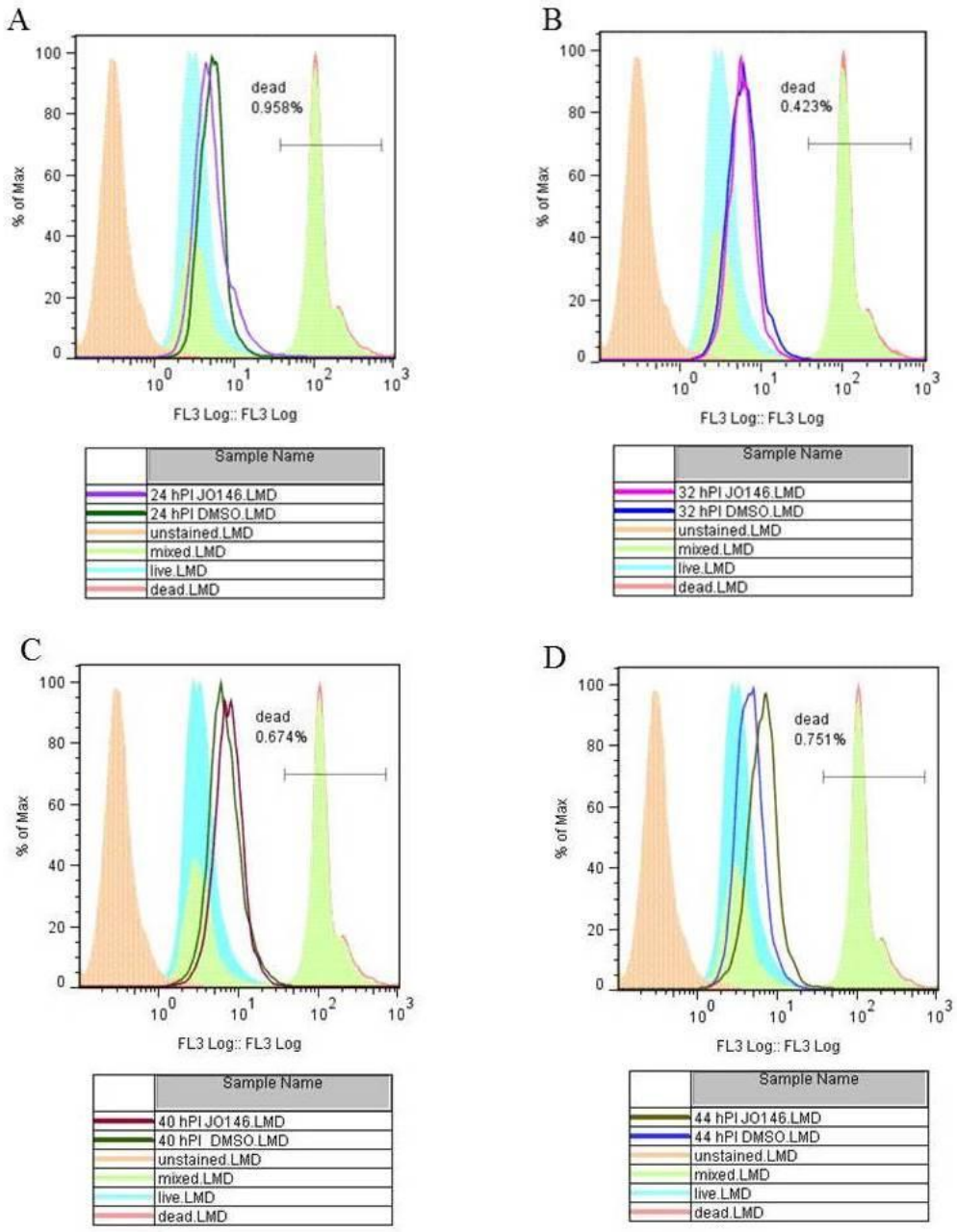


Figure 4.11. Overlay of histograms for every treatment for the different time points

Dead and live control cell populations are represented by filled red and blue histograms, respectively. Mixed live and dead cell population is represented by green histogram. Percentage values denote the number of necrotic cells in JO146-treated samples in each corresponding time point.

Tables 4.1 and 4.2 show the corresponding number of dead cells, expressed in percentage of the parent population for each time point and treatments, as well as the corresponding values for each parameter in the control cell populations. It was

observed that there was no significant difference in numbers of dead host cells between JO146 treated and DMSO controls (Figures 4.9 and 4.11).

Table 4-1. Number of necrotic cells expressed as percentage of the parent population in the time points indicated after JO146 treatment at 16 h PI

	Dead cells (% of parent)		**Mean FL3 Log***	
control dead cell population	99.7		565	
live dead cell population	1.71		11.6	
time point (h PI)	**DMSO**	**JO146**	**DMSO**	**JO146**
16	0.87	1.74	10.40	10.90
18	0.67	1.06	9.13	9.77
20	1.78	0.64	12.10	8.37
22	1.09	1.26	9.34	9.73
24	2.11	1.19	12.30	11.8

*Mean (FL3 Log) denotes the mean fluorescence intensity for each sample population.

Table 4-2. Number of necrotic cells expressed as percentage of the parent population in the time points indicated after JO146 treatment at 16 h PI

	Dead cells (% of parent)		**Mean FL3 Log***	
control dead cell population	98.7		134	
live dead cell population	0.35		4.13	
time point (h PI)	**DMSO**	**JO146**	**DMSO**	**JO146**
24	0.35	0.96	6.45	7.10
32	0.55	0.42	7.92	7.41
40	0.52	0.67	8.18	9.84
44	0.23	0.75	5.33	8.97

*Mean (FL3 Log) denotes the mean fluorescence intensity for each sample population.

4.3.5 **JO146 has a reversible inhibitory effect on *C. trachomatis*, suggesting a bacteriostatic effect.**

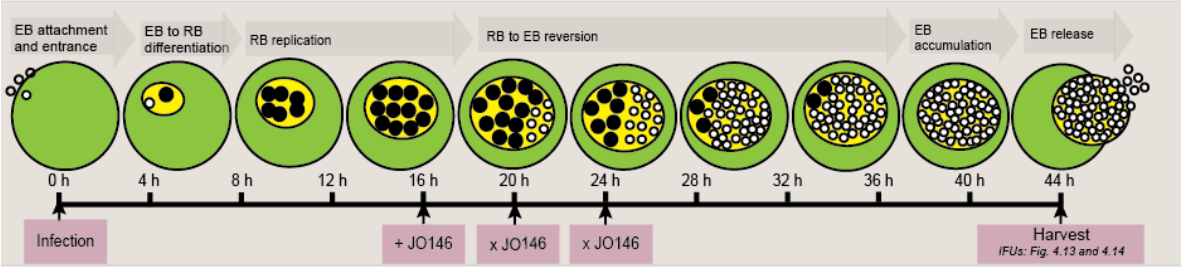


Figure 4.12. Experimental plan to assess the bacteriostatic or bacteriocidal effect of JO146

The lethality of JO146 when added at 16 h PI could be a consequence of a specific time frame or phase of the chlamydial developmental cycle. Given that JO146 at 100 µM could prevent infectious progeny formation at all time points tested, a shorter exposure time during the replicative phase of development was conducted to understand the maximum treatment duration for JO146 to be lethal. Therefore, JO146 was added and subsequently removed from the cultures after 4 and 8 hours exposure (20 and 24 h PI, respectively). Chlamydial inclusion forming units was then determined at 44, 54, and 64 h PI. JO146 treatment was still highly effective but not lethal when the compound was washed out after 4 and 8 h treatment (20 and 24 h PI respectively) with 1–2 log reduction in the formation of inclusion forming units (Fig. 4.13 and 4.14). The loss of infectious progeny when the compound was removed 4 and 8 h after addition was partially rescued (~ 0.5 log) by extended culture in the absence of the compound (to 54 and 64 h PI) (Fig. 4.13 and 4.14). The differences on the number of infectious progeny between cells treated with DMSO and 100 µM JO146 when the treatments were removed at both at 20 and 24 h PI were statistically significant.

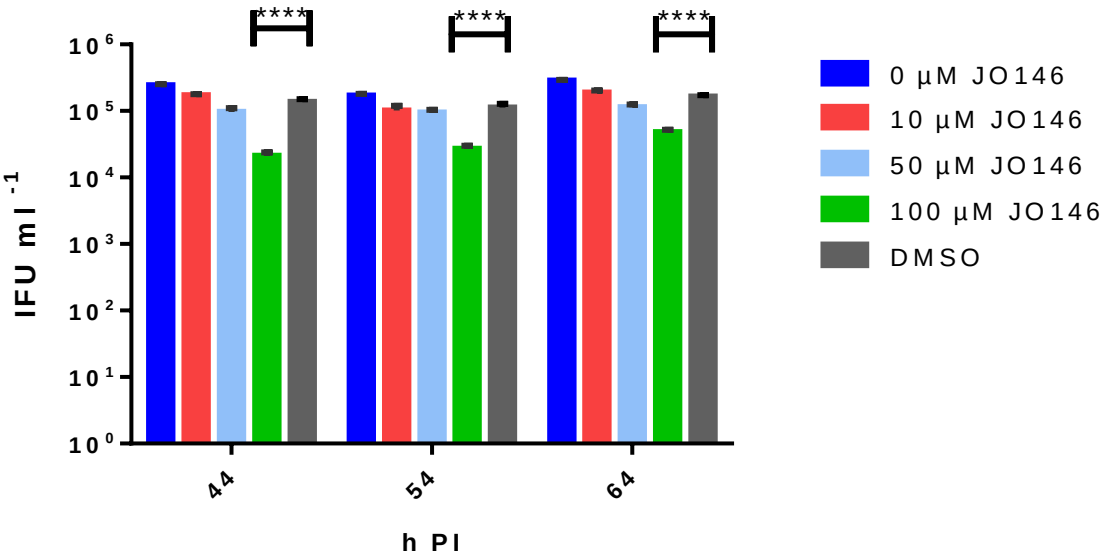


Figure 4.13. Infectious yield of *Chlamydia* after JO146 treatments 16 h PI when JO146 was washed out at 20 h PI

The cultures where harvested and the number of inclusion forming units was determined at 44, 54 and 64 h PI. The mean from three independent experiments are represented in the bar graphs and the error bars represent the standard error of the mean (n = 27). Statistics were conducted using Two-Way ANOVA relative to DMSO controls. **** indicates $p < 0.0001$. IFU ml^{-1} is indicated on the y-axis. Time point of harvest is indicated on the x-axis.

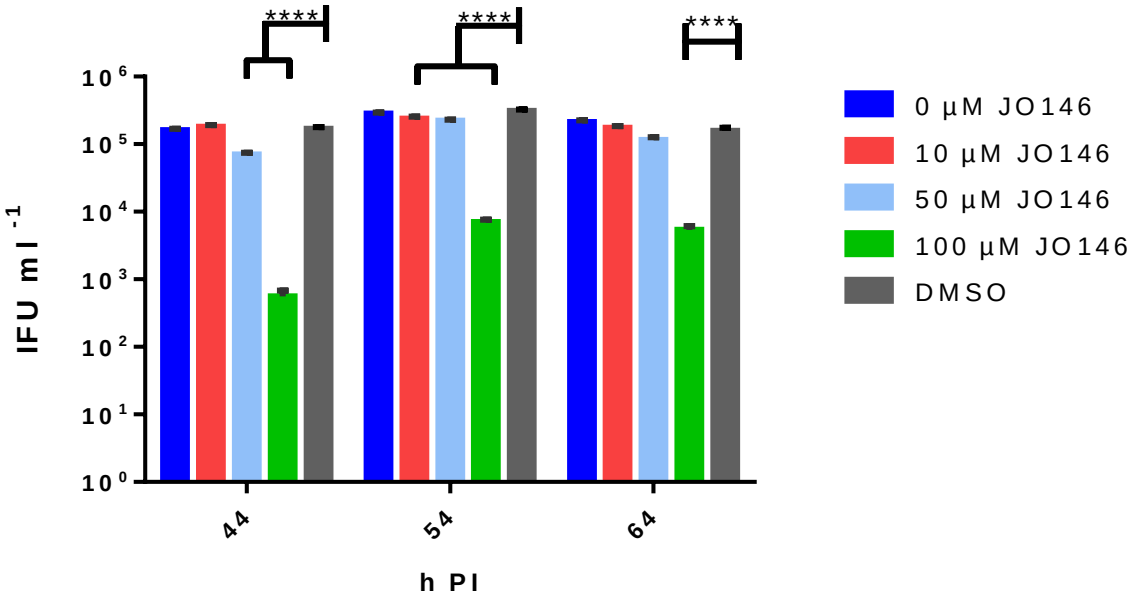


Figure 4.14. Infectious yield of *Chlamydia* after JO146 treatments 16 h PI when JO146 was washed out at 24 h PI

The cultures where harvested and number of inclusion forming units was determined at 44, 54 and 64 h PI. The mean from three independent experiments are represented in the bar graphs and the error bars represent the standard error of the mean (n = 27). Statistics were conducted using Two-Way ANOVA relative to DMSO controls. **** indicates $p < 0.0001$. IFU ml^{-1} is indicated on the y-axis. Time point of harvest is indicated on the x-axis.

4.3.6 JO146 treatment is lethal during heat stress and recovery from heat stress for *Chlamydia.*

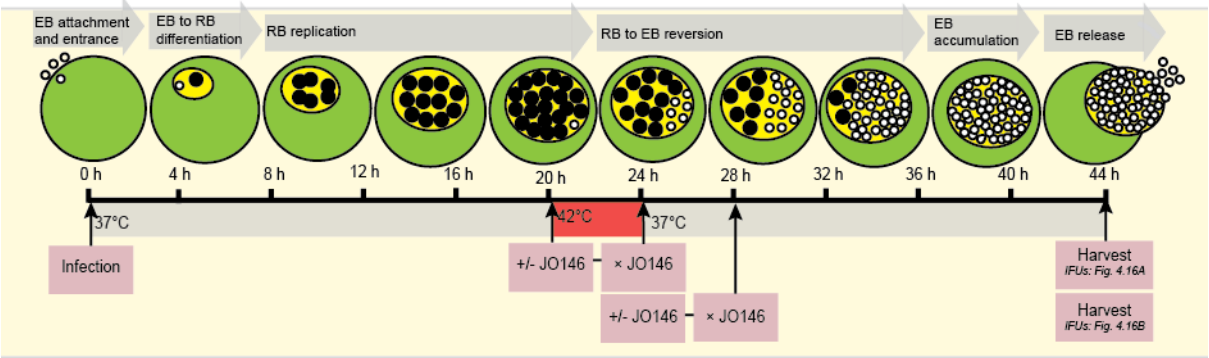


Figure 4.15. Experimental plan to use the JO146 inhibitor to determine the role of CtHtrA for infectivity of *C. trachomatis* during heat stress conditions

The impact of JO146 on heat stressed *Chlamydia* cultures was assessed to determine the role of HtrA for heat stress. JO146 was added either, during the heat shock and subsequently removed, or during the 4 h of post heat shock recovery and subsequently removed, and infectious yield was subsequently determined from the cultures after harvest at 44 h PI. It was previously demonstrated that the compound needed to be present for greater than 4 hours for complete lethality (Figure 4.3), therefore during this experiment, a higher concentration (150 μM) was used.

As shown in Figure 4.16A, the presence of JO146 during heat shock was completely lethal at 100 and 150 μM. 50 μM JO146 treatment during the 4 h heat shock also resulted in a marked loss of chlamydial infectious progeny (>2 log) (Figure 4.16A). Some JO146-induced reduction in infectious progeny was also observed in the controls which were not heat shocked (Figure 4.16A), and this is consistent with the previous observations that JO146 needs to be present throughout the replicative phase (not for 4 h only) to be completely lethal as shown in Figure 4.3 [250].

JO146 treatment during the first 4 h of recovery from heat shock was also completely lethal at higher concentrations (100 μM and 150 μM), with a minor impact on infectious yield observed with 50 μM JO146 (Figure 4.16B). There was a more noticeable reduction in viability for the heat shocked cultures compared to the controls during this experiment (Figure 4.16B). However, in spite of these differences it is clear that JO146 treatment both during heat stress and recovery from

heat stress was lethal for *Chlamydia* in spite of the relatively short treatment durations.

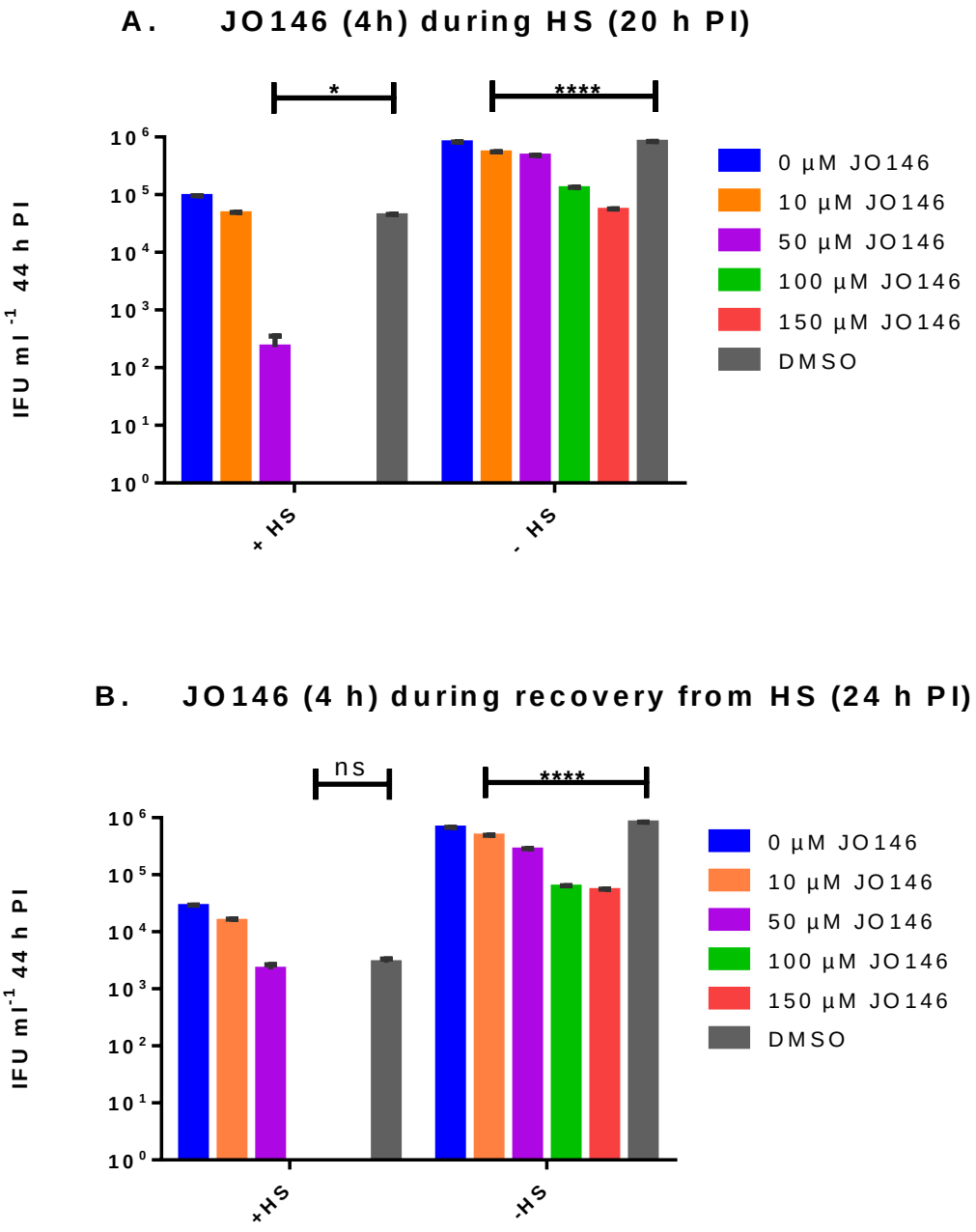


Figure 4.16. *C. trachomatis* infectious yield at 44 h PI after 4 h heat shock with JO146 treatment (20–24 h PI) or JO146 treatment during recovery from heat shock (24–28 h PI)

(A) The infectious yield after JO146 treatment for 4 h at 20 h PI with and without heat shock at 42°C. (B) Infectious yield after JO146 treatment for 4 h during recovery from heat shock (24–28 h PI) (n = 27). The concentration of JO146 is indicated by the coloured bars, legend to the right of each graph. IFU ml^{-1} is indicated on the y-axis. The presence or absence of heat stress is indicated on the x-axis. * indicates $p < 0.05$, ****$p < 0.0001$, ns indicates not significant.

4.3.7 **JO146 addition to *C. trachomatis* HEp2 cultures during penicillin persistence results in a reduced infectious yield after recovery.**

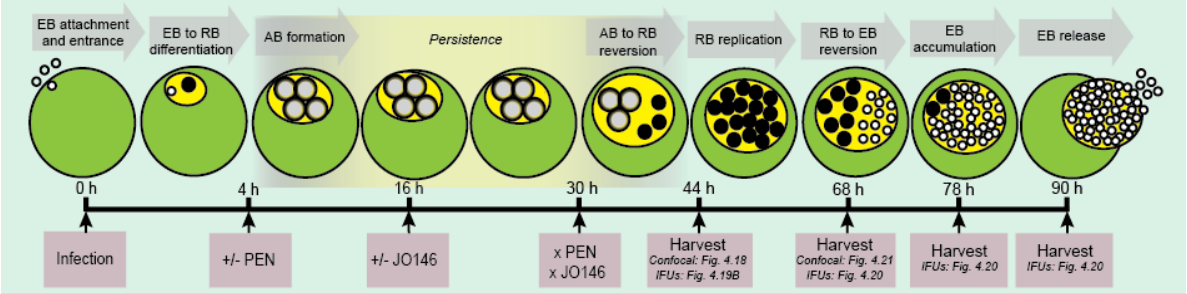


Figure 4.17. Experimental conditions used to assess the impact of JO146 addition during penicillin persistence

Previous studies have indicated that HtrA protein levels in *Chlamydia* are increased during penicillin persistence [19, 22], suggesting that the protease may be important during this phase. Therefore, the effect of JO146 during, and during reversion from, penicillin persistence was tested. Due to the bi-phasic nature of the chlamydial developmental cycle, it is not possible to measure the immediate impact on infectious progeny production during the replicative phase of growth. Therefore, for each of the penicillin persistence laboratory models conducted in this study, the infectious progeny yield was assessed once elementary bodies were expected to be formed, either at the conclusion of the developmental cycle, or once reversion from persistence and development of elementary bodies has occurred. The cultures were confirmed to be persistent by detection of loss of infectivity in the persistent cultures when control cultures were demonstrated to have infectious elementary bodies (as long as formation of infectious progeny was subsequently restored by removal of the growth inhibitory factor). The impact of JO146 addition during persistence (when aberrant bodies were present) was monitored by adding the compound at 16 h PI during persistence (penicillin was added at 4 h PI to induce persistence). Control cultures were included and harvested to measure infectious yield and also fixed and examined by confocal microscopy at 44 h PI. In the persistent cultures, media was changed with fresh media at 30 h PI to remove JO146 and penicillin. These cultures were harvested at 68, 78, and 90 h PI (or 38, 48, and 60 h after penicillin removal) to allow time for reversion from persistence and elementary body formation (as outlined in Figure 4.17). These cultures were tested for infectivity by measuring

inclusion forming units and morphology was examined using confocal laser scanning microscopy.

The cultures were firstly confirmed to be persistent at 44 h PI by monitoring infectivity and impact of JO146 treatment in the presence and absence of penicillin. As shown in Figure 4.18, the cultures treated with penicillin had much smaller inclusions compared to the controls at 44 h PI. The inclusions were also much less populated with cell shaped bodies consistent with a persistent phenotype (Figure 4.18 right column penicillin-treated compared to control left column). The increasing concentrations of JO146 resulted in a decreased inclusion vacuole size for both the penicillin treated and control cultures (Figure 4.19A). The penicillin treated cultures were confirmed to be persistent by a lack of infectious EBs at 44 h PI (Figure 4.19B) and supported by restoration of infectivity in subsequent experiments.

The infectious yield during the reversion from persistence from the cultures with and without JO146 treatment was determined (at 68, 78, and 90 h PI) when it was expected to see recovery to infectious elementary bodies [280]. As expected, and consistent with previous observation (Figure 4.3), JO146 was completely lethal on the control (non-persistent) culture at 50 and 100 µM, when formation of infectious progeny production was measured at 44 h PI (Figure 4.19B) with some recovery of infectious progeny observed at the later time points (extended culture conditions, Figure 4.20B) (as consistent with previous work from our team). In contrast, JO146 was not lethal when it was added at the same time point during penicillin induced persistence (Figure 4.20A). Cultures were treated with JO146 during persistence then rescued from the persistence by media change (30 h PI) to allow formation of EBs which could then be measured in the assay. JO146 treatment during the persistence phase resulted in a relatively minor loss of detectable inclusion forming units, with approximately a 1 log reduction of infectious yield observed at 100 µM JO146 when inclusion forming units were able to be detected at 68, 78, and 90 h PI (Figure 4.20A). The control cultures that were not persistent showed ~2-3 log reductions in infectivity with 100 µM JO146 treatment at the extended culture times of 68-90 h PI (Figure 4.20B). This observation of reduced effectiveness of JO146 over extended culture conditions is consistent with previous observations from our team [250]. Differences in the number of infectious progeny between cells treated with DMSO and cells treated with 100 µM JO146 was statistically significant for all the time

points indicated both in penicillin treated cultures and in control (no penicillin) cultures (Figures 4.20A and 4.20B).

The cultures were monitored by immunofluorescence during the reversion period and representative images from 68 h PI are shown in Figure 4.21 (left column control, right column penicillin). The penicillin treated cultures had smaller inclusions with atypical morphology (inclusions containing less cell-shaped bodies likely indicating there are still aberrant bodies present) at 68 h PI, with the inclusions generally appearing smaller in the presence of 100 µM JO146 (Figure 4.21, right column). The control cultures (no penicillin treatment) also showed a JO146 concentration dependent reduction of the inclusion sizes (Figure 4.21, left column). However, even though the inclusions appeared markedly smaller when recovering from penicillin persistence at 68 h PI, there was only ~one log (approximately 1.4 x 10^4 IFU ml^{-1} difference between cultures treated with 100 µM JO146 and DMSO) reduction in infectious progeny/inclusion forming yield compared to the controls (Figure 4.20A).

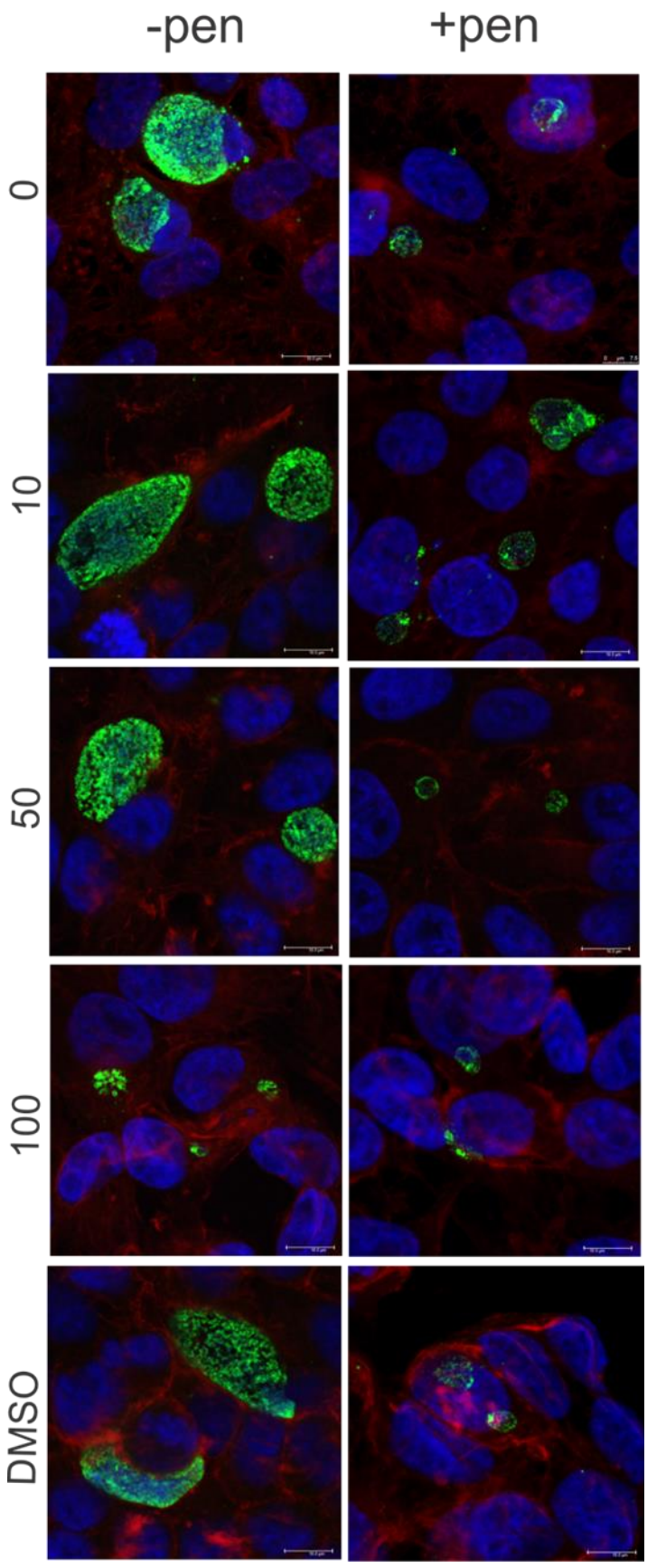


Figure 4.18. Confocal microscopy images of JO146 treated cultures during penicillin persistence at 44 h PI

The figure shows representative images from confocal laser scanning microscopy of cultures fixed at 44 h PI (+PEN: penicillin added, −PEN: no penicillin)., 100 Uml^{-1} penicillin was added at 4 h PI. Representative images from the control culture are shown in the left column (−PEN). Penicillin treated conditions are shown in the right column. JO146 treatment conditions are indicated to the right (0, 10, 50, 100 µM, DMSO). The image colours are as follows, green: MOMP (major outer membrane protein) is green, blue: cell nucleus (DAPI), and red: β-actin. Scale bar (bottom right) indicates 10 µm.

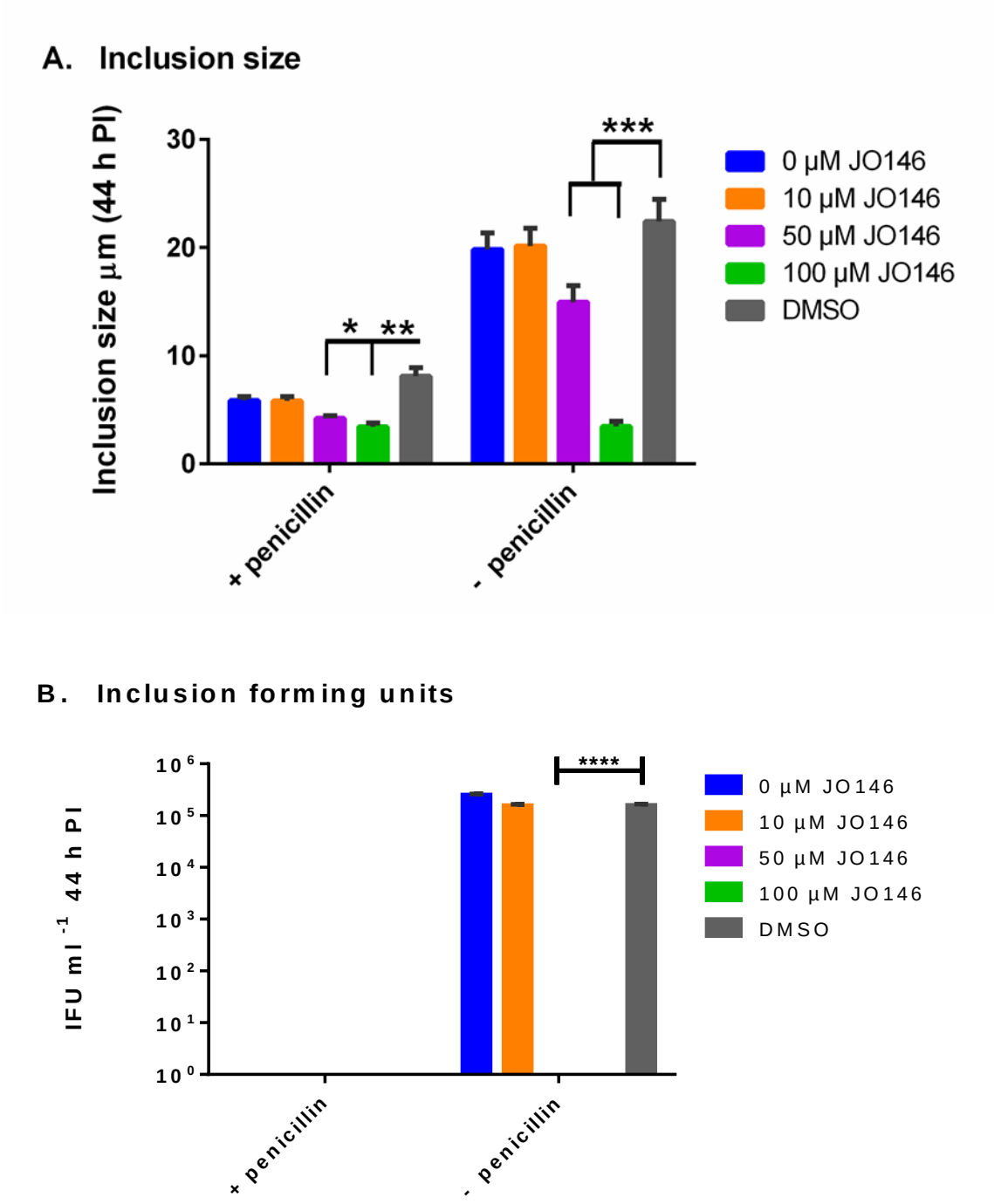


Figure 4.19. Inclusion sizes and infectious yield during penicillin persistence at 44 h PI with and without JO146 treatment

The figure shows morphological analysis of cultures during penicillin persistence and controls by measuring inclusion sizes. The infectious yield (44 h PI) in the presence or absence of 100 Uml^{-1} penicillin (4 h PI) is shown on the graph. (A) Inclusion sizes are shown from each condition. Inclusion sizes were measured from independent coverslips, n = 20. (B) Inclusion forming units with and without penicillin are shown graphically (n = 27). Statistics were conducted using Two-Way ANOVA relative to DMSO controls, $p < 0.05*$, $p < 0.01**$, $p < 0.001***$, $p < 0.0001****$.

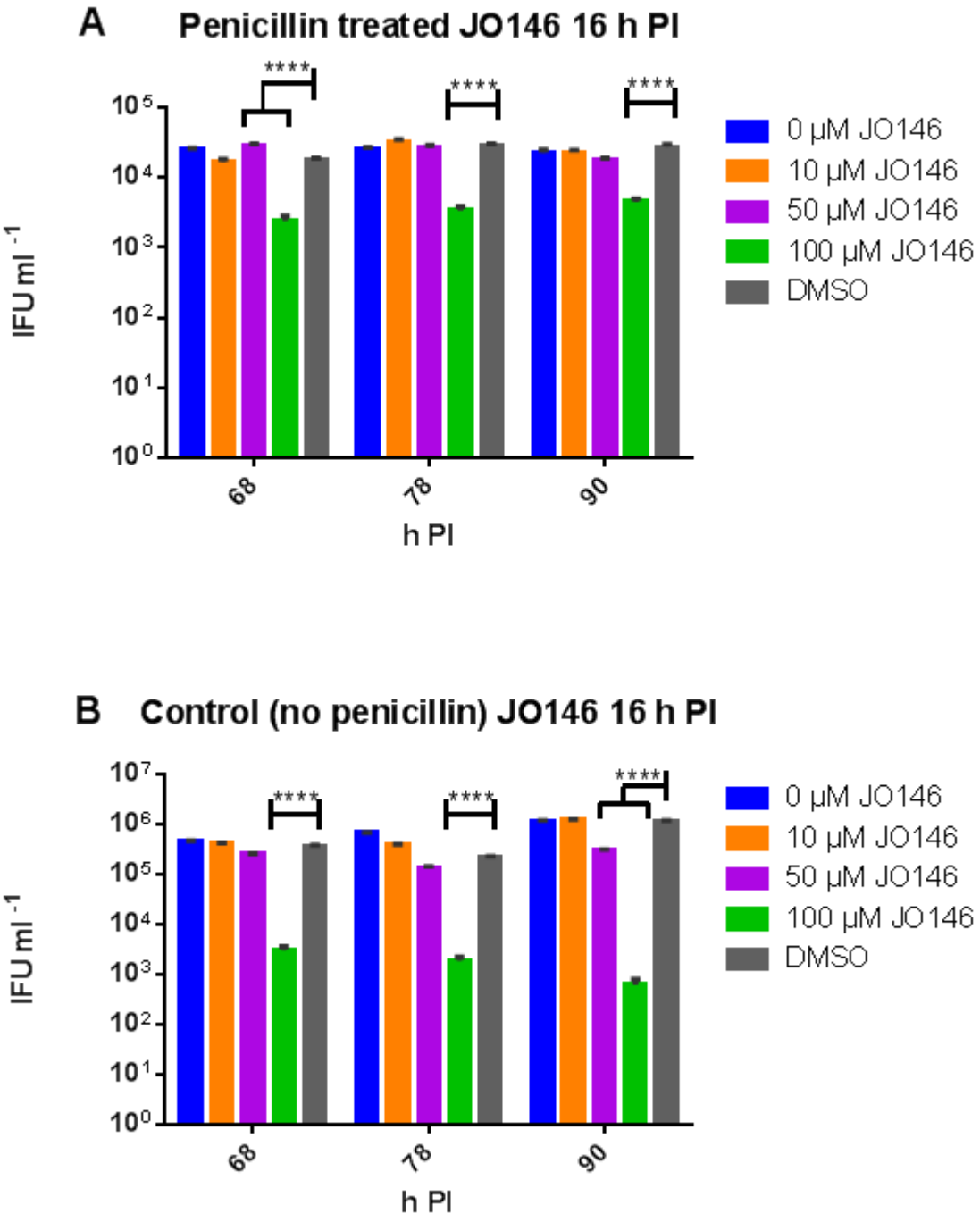


Figure 4.20. Infectious yield of *C. trachomatis* after treatment with JO146 during penicillin persistence

The figure shows IFU ml^{-1} from each culture condition at several time points after reversion was commenced (penicillin removal at 30 h PI). Cultures were treated with penicillin 100 Uml^{-1} at 4 h PI, and with JO146 at 16 h PI (concentrations indicated by the coloured bars, see key to the right of each graph). Penicillin was removed from the cultures at 30 h PI. (A) Infectious yield from penicillin treated and restored cultures. (B) Infectious yield from control cultures which did not have penicillin added. Statistics were conducted using Two-Way ANOVA relative to DMSO controls, n=27. Data are presented as mean ± S.E.M, **** indicates $p < 0.0001$.

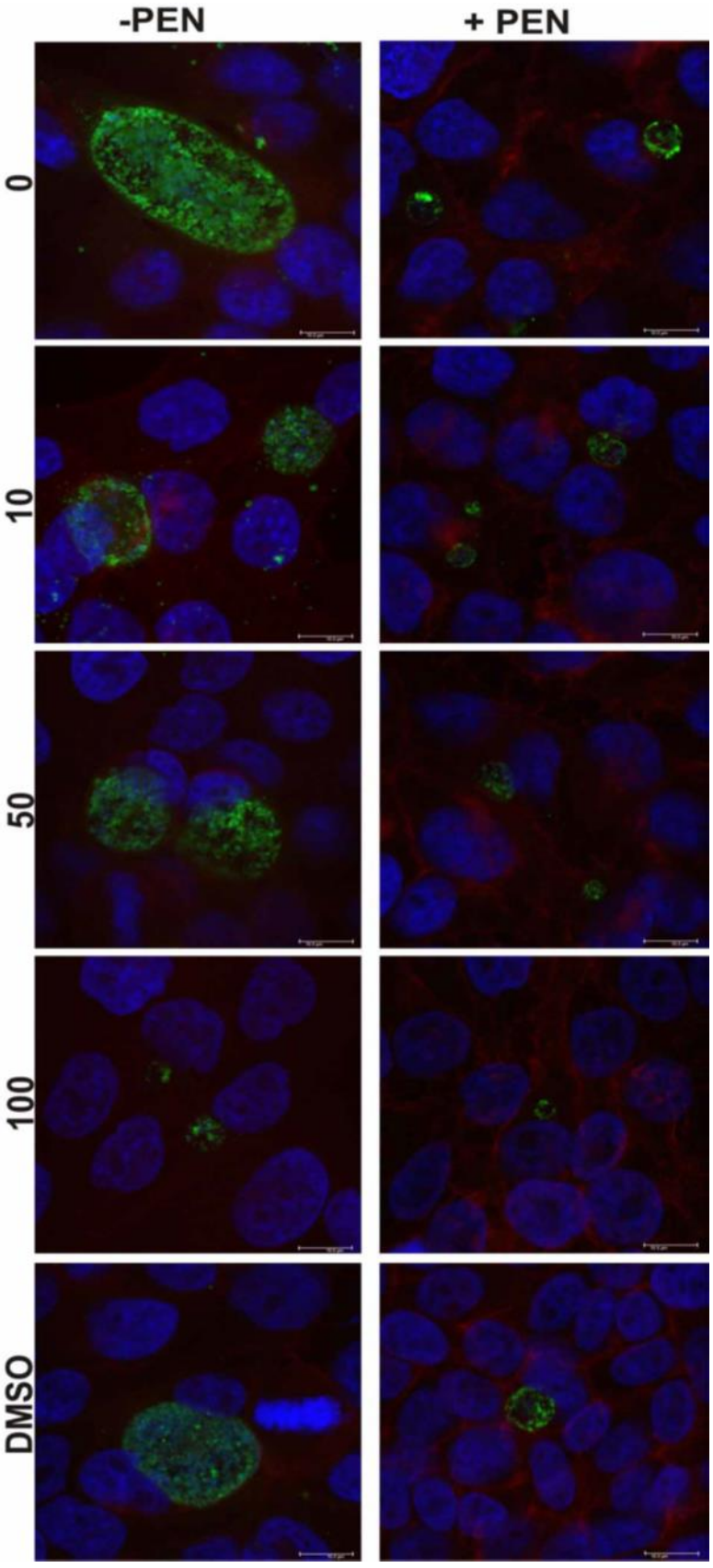


Figure 4.21. Confocal microscopy images of *C. trachomatis* cultures at 68 h PI, or 38 h after penicillin reversion commenced

Cultures were treated with penicillin 100 Uml−1 (right column) at 4 h PI, and with JO146 at 16 h PI (concentrations in µM are indicated to left of the image). Penicillin was removed from the cultures at 30 h PI. The image colours are as follows, green: MOMP (major outer membrane protein) is green, blue: cell nucleus (DAPI), and red indicates β-actin. Scale bar (bottom right) indicates 10 µm.

4.3.8 JO146 addition to *C. trachomatis* HEp2 cultures during reversion from penicillin persistence is lethal.

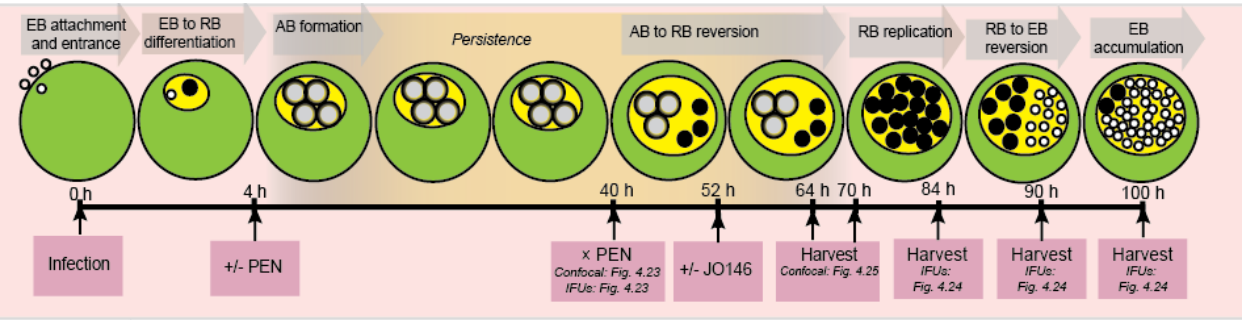


Figure 4.22. Experimental plan to determine the impact of JO146 on *C. trachomatis* reversion from persistence

It was previously determined that JO146 addition at 16 h PI resulted in loss of infectious progeny when cultures were harvested at the end of the developmental cycle (44 h PI) (Figure 4.3), suggesting that JO146 targets RB replication or RB-EB formation or both. Therefore, the impact of JO146 during reversion from penicillin persistence, which involves transitioning of aberrant bodies to infectious EBs, was determined. The mechanism of reversion from penicillin persistence has been described to be very asynchronous; with gradual budding of 'normal' RBs from the aberrant persistent forms in the inclusion over 10 to 20 h after penicillin was removed [158]. Penicillin persistent cultures were established by adding penicillin at 4 h PI and then reversion was commenced by washing and media change at 40 h PI. 12 h (52 h PI) after reversion was commenced, JO146 was added and formation of infectious elementary bodies was monitored at 84, 90 and 100 h PI.

Firstly, the cultures were confirmed to be persistent at 40 h PI by measuring infectious progeny production and examining the morphology of the cultures using confocal laser scanning microscopy. As shown in Figure 4.23 the penicillin treated cultures were not culturable at 40 h PI (Figure 4.23E) and morphologically the inclusions were smaller and appeared to have large cellular forms present inside each

inclusion (Figure 4.23B, D). The penicillin persistent cultures were washed to remove penicillin to commence reversion at 40 h PI. JO146 was added to these cultures 12 h after commencement of reversion (i.e. 52 h PI). Infectious yield was then measured over time from the cultures. As shown in Figure 4.24A no infectious *Chlamydia* were detected at 84 h PI from the persistence reversion cultures (44 h after penicillin was removed), however at 90 and 100 h PI infectious EBs were detected. No chlamydial inclusions were observed at 84 h PI for all the treatments. At 90 and 100 h PI, JO146 treatment dramatically reduced the infectious progeny production of the *Chlamydia.* JO146 at 100 µM was completely lethal to the cultures undergoing reversion at 90 and 100 h PI (Figure 4.24A). JO146 was still completely lethal at 100 h PI when inclusions were recovered in cultures treated with 50 µM JO146. In contrast, the cultures that were not penicillin persistent showed only minor reductions in infectious progeny production due to the addition of JO146 (Figure 4.24B). These control cultures were likely either mainly in elementary body form or early infection stages when JO146 was added (early or very late developmental cycle, or a mix of both), based on the morphological appearance of the inclusions and the expected timing of the chlamydial developmental cycle.

The morphology of the cultures was examined using immunocytochemistry and confocal laser scanning microscopy during the reversion from persistence to monitor the impact of JO146 on inclusion morphology at 64 and 70 h PI (12 and 18 h after JO146 addition). In this case no obvious decrease in the inclusion size relating to JO146 treatment was observed (Figure 4.25, second and forth column), and as expected, the inclusions from the persistent cultures were much smaller than those in the controls (Figure 4.25 controls first and third column).

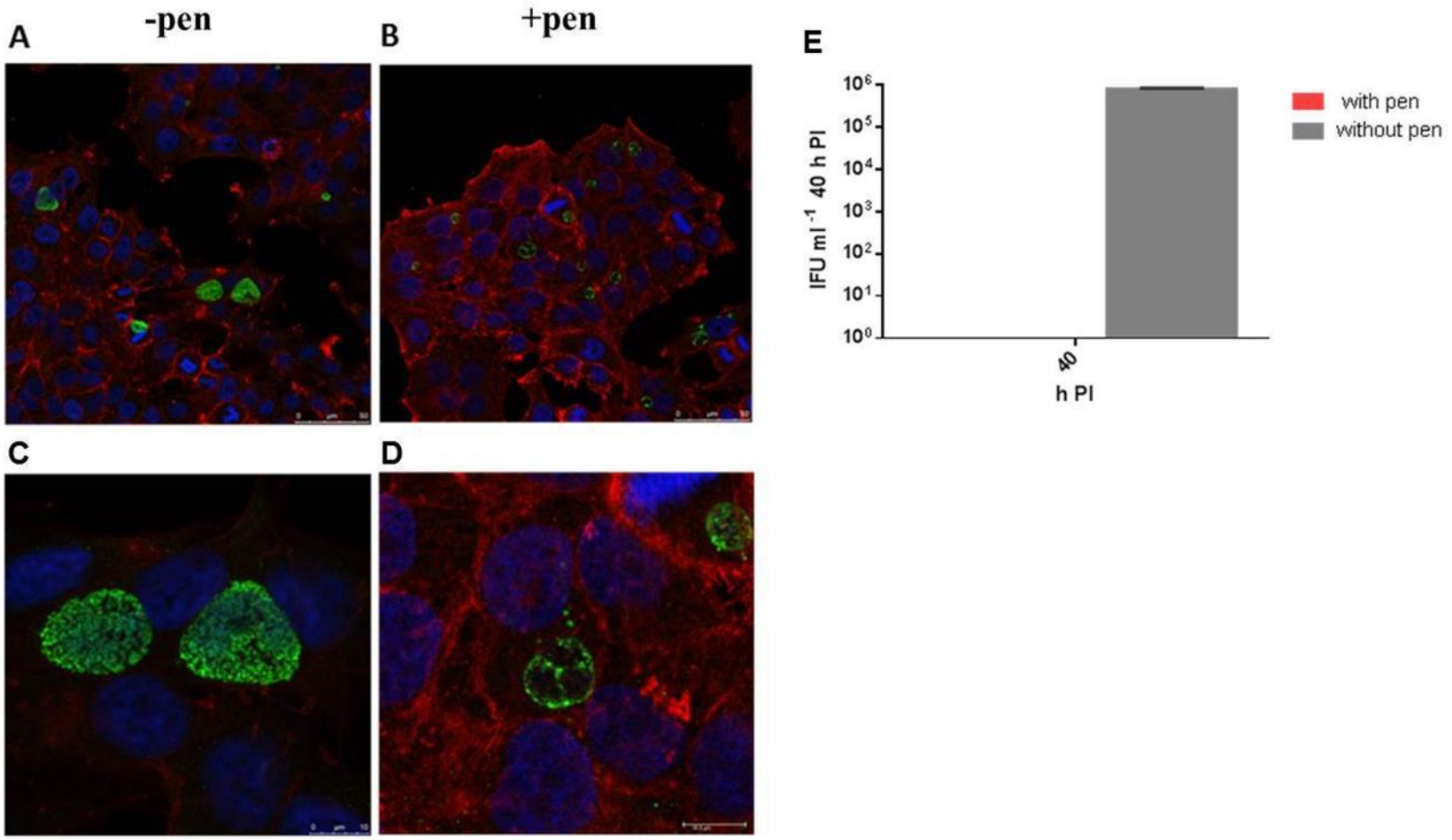


Figure 4.23. Penicillin persistence cultures prior to commencement of reversion were morphologically consistent with persistence

Cultures were treated with penicillin 100 U ml^{-1} at 4 h PI. (A) Confocal microscopy image of cultures at 40 h PI in the absence of penicillin (-PEN) (B) Confocal microscopy image of cultures at 40 h PI in the presence of penicillin (+PEN). The scale bar on A and B indicates 50 µm. (C) Enlarged area of A: confocal microscopy image of cultures at 40 h PI in the absence of penicillin (+PEN). (D) Enlarged area of B: confocal microscopy image of cultures at 40 h PI in the presence of penicillin (−PEN). The contrast has been equally adjusted on C, D to improve the visibility of the morphologies present. The scale bar on C and D indicates 10 µm. (E) Infectious yield of the corresponding cultures (n = 27).

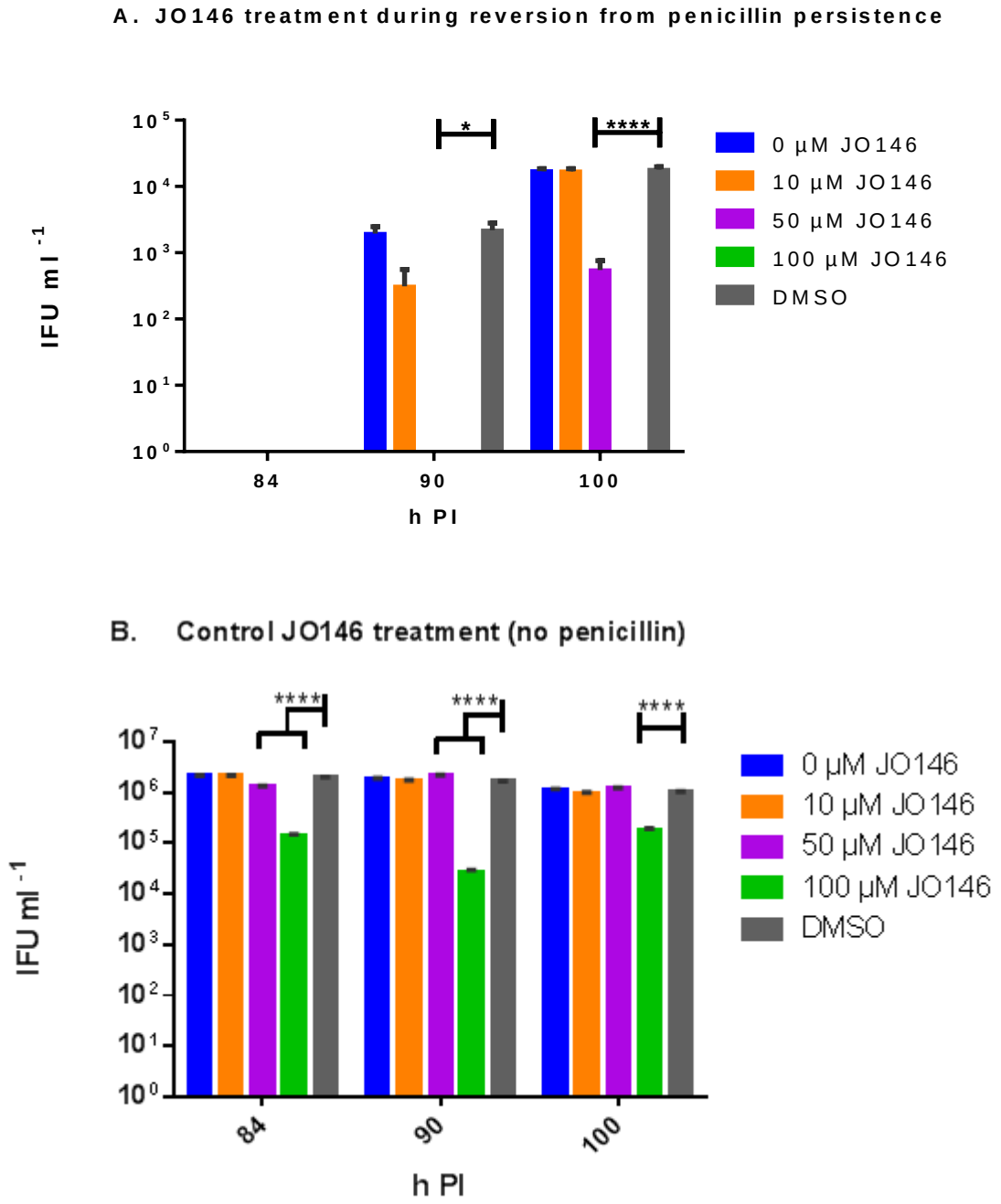


Figure 4.24. Infectious yield when JO146 was added during the reversion from penicillin persistence

Cultures were treated with penicillin 100 Uml^{-1} at 4 h PI. Penicillin was removed at 40 h PI and JO146 treatment was commenced at 52 h PI. (A) JO146 was added 12 h after penicillin persistence reversion was commenced (i.e. at 52 h PI). (B) Control cultures that were not persistent with JO146 treatment also conducted at 52 h PI. The graphs show the inclusion forming units at several time points after reversion from persistence was commenced. Statistics were conducted using Two-Way ANOVA relative to DMSO controls, n=27. Data are presented as mean ± S.E.M, * indicates $p < 0.05$, **** $p < 0.0001$. JO146 concentration is indicated by the coloured bars (legend to the right of each graph).

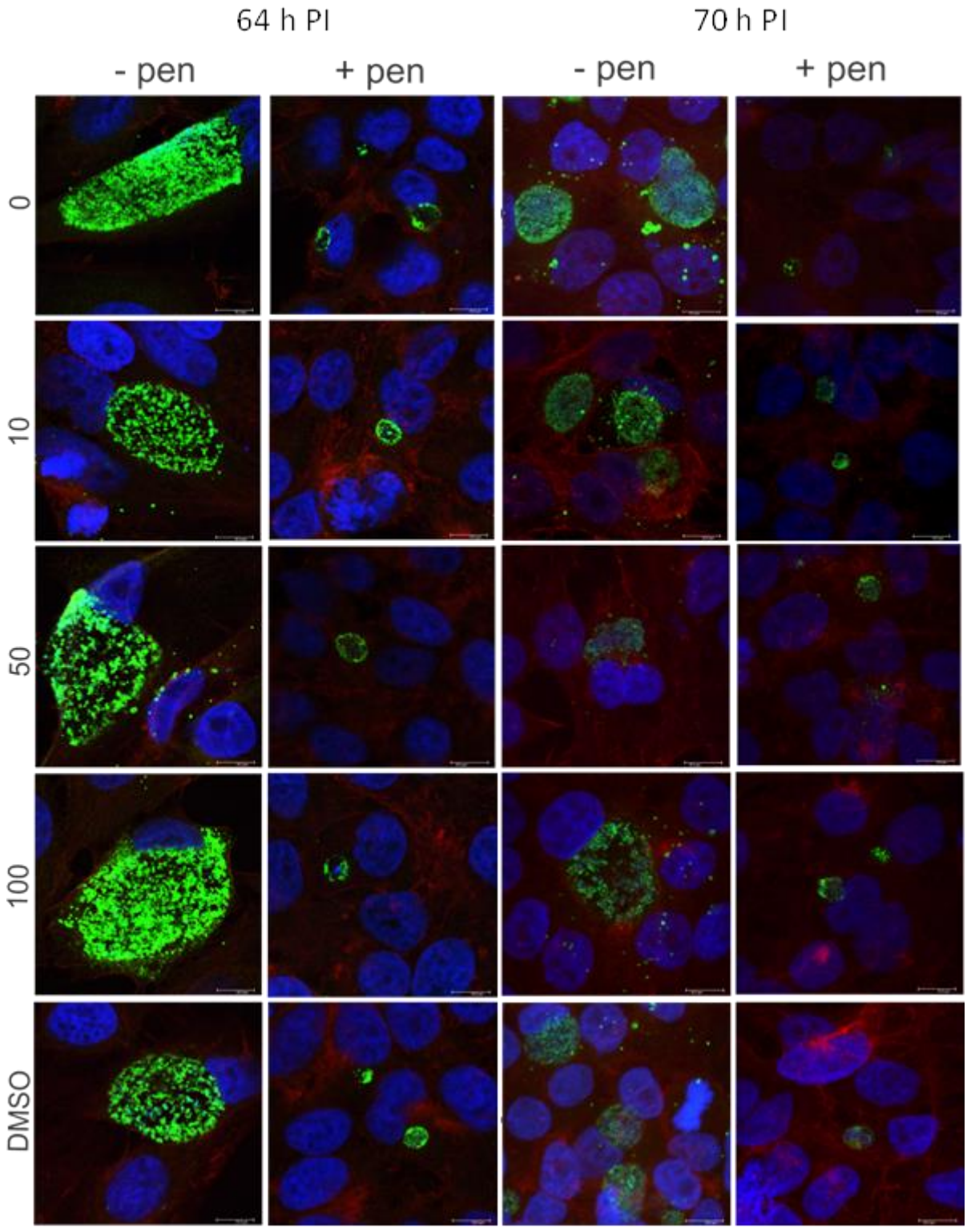


Figure 4.25. Confocal microscopy images of penicillin persistent cultures and controls during reversion from persistence at 64 and 70 h PI

Cultures were treated with penicillin 100 Uml^{-1} at 4 h PI. Penicillin was removed at 40 h PI and JO146 treatment was commenced at 52 h PI. Controls with no penicillin first and third column, penicillin conditions second and fourth columns. JO146 concentrations (in μM) are indicated to the left of the image. The image colours are as follows, green: MOMP (major outer membrane protein) is green, blue: cell nucleus (DAPI), and red: β-actin. Scale bar (bottom right) indicates 10 μm.

4.4 DISCUSSION

The periplasmic chaperone and serine protease HtrA plays important roles in bacterial stress responses and protein quality control (reviewed, [243]). The protein conducts diverse roles in bacteria from outer membrane protein assembly [247] to stress response and survival during stress [281], cleavage of host proteins [9] and intracellular infection survival [282]. Concurrent research conducted in our team used a chemical inhibition strategy to establish that *Chlamydia* HtrA (CtHtrA) was essential during the replicative phase of intracellular development by using the inhibitor compound, JO146. It was demonstrated that JO146 was effective when added at 16 h PI, which was consistent with an effect on the mid-replicative phase, but not when added during earlier or later stages of the chlamydial developmental cycle. Therefore, the present study aimed to determine the duration of treatment required for complete bacterial lethality as well as the mechanism of action of JO146, whether it's bactericidal or bacteriostatic. Given the multi-tasking nature of HtrA already described for many other bacteria, the present study also set out to test the hypothesis that CtHtrA is essential for heat stress conditions and during persistence lab models.

The data presented in this study demonstrate that JO146 addition (100 μM) at 16 h PI resulted in complete bacterial lethality when cells were harvested from the time of treatment up to the end of the chlamydial developmental cycle. While it is known that chlamydial developmental cycle is asynchronous, the completion of replication by binary fission is quite rapid. Miyairi and co-workers [89] comprehensively characterized the parameters of replication and EB formation for a number of serovars and found that for serovar D, logarithmic replication occurs from approximately 12–24 h PI with a marked halt of replication from approximately 24 h PI onwards [89]. At 18-24 h (time point may vary according to *Chlamydia* species and serovars), RB replication becomes asynchronous with some RBs differentiating back to the infectious EB form [189]. EB formation could be detected from approximately 20 h PI onwards and gradually increased until approximately 40 h PI [89]. Thus, EB formation is highly asynchronous; however, replication of the chlamydiae is tightly defined as between 12 and 24 h PI. Therefore, addition of JO146 at the replicative phase (16 hours PI) had significantly inhibited RB replication, and may have led to subsequent decrease on the formation of infectious EBs at completion of *Chlamydia* developmental cycle. Additionally, as transcriptional activity is maintained and completed at 16-24 hours PI [90], treatment of *C. trachomatis* with JO146 at 16 hours PI may have

impacted a crucial stage of the developmental cycle that led to reduction in infectious yield observed in the present study.

Furthermore, it was determined that the inhibitory impact of JO146 and the subsequent chlamydial death and loss of inclusion morphology appeared to relate directly to the observed *Chlamydia* defects and not a host mediated mechanism as indicated by the absence of co-localization between the autophagy and lysosome markers, SQSTM1 and LAMP1, and the chlamydial inclusions during JO146 treatment. The use of LAMP1 and SQSTM1 antibodies monitored the possible involvement of the host cell's autophagy-lysosome system and ubiquitin-proteasome pathway, respectively. Also, since a direct association of p62 with LC3, a specific marker for early autophagic vacuole [283, 284] has been previously documented [285, 286], the use of SQSTM1 antibodies assessed the early step in the autophagic pathway while LAMP1 antibodies monitored the late stages of autophagy. The absence of sequestration of chlamydial inclusions with both LAMP1 and SQSTM1 suggests absence of fusion with autophagosomes particularly at 20 and 24 h PI, indicating that the lethality of JO146 was not mediated by host innate defence or lysosome pathways.

Further experiments were conducted to determine if the inhibitory effect of the compound was mediated by host cell viability. In the present study, although cells from all sample populations were suspended in phosphate buffered saline (PBS) for final processing using the kit, the slight intermediate staining that can be observed on the samples in the second assay (Figure 4.11) could be due to the toxic effect of DMSO, which was used to reconstitute the fluorescent reactive dye provided in the kit. DMSO is an amphipatic molecule and one of the most commonly used solubilizing agents in *in vivo* and *in vitro* studies. A number of previous studies have shown the therapeutic as well as the cytotoxic effect of DMSO, which includes induction of apoptosis in a dose-dependent manner [287-289] as well as enhanced penetration of substances across biologic membranes thus its use as an aid in efficient penetration of drugs [290, reviewed by 291, 292, reviewed by 293]. The low concentrations of DMSO may have enhanced the permeability of the cell membrane thus allowing the reactive dye to react with intracellular amines of the cell. This explains the slight shift of the histogram of the sample populations towards the dead control population. However, a difference of two log values or a complete overlapping of histograms on dead and live control cell populations would readily distinguish live and dead cells. From the results generated in the study, the sample populations appear to be live at all time points whether JO146- or DMSO-treated, indicating that JO146 inhibits chlamydial growth without being

toxic to the host cells. This characteristic highlights the potential application of the CtHtrA inhibitor as an anti-chlamydial agent.

The removal of JO146 at 24 h PI (8 h post-administration) showed a 2.5 log reduction in infectivity indicating that the most effective phase of inhibition was throughout the replicative phase until EB formation. Therefore, these data suggest that JO146 is inhibiting a specific function involved in replication that is essential for *Chlamydia*. Furthermore, the inhibitory effect of JO146 appears to be bacteriostatic since recoverable inclusion forming units were detected at later time points (44 and 54 h PI), during which, a 1.5 log reduction in infectivity was observed.

The present study also demonstrates that CtHtrA is essential during heat stress and recovery from heat stress. The heat stress model is clearly likely to involve extra-cytoplasmic protein stress which will require both the protease activity and chaperone activity of CtHtrA. JO146 treatment in the presence of heat stress in a time frame (4 h) was completely lethal. This is amazingly quick given 4 h is consistent with less than 2 rounds of binary fission for *C. trachomatis* serovar D, which has been identified to take 2.4 h per round of binary fission [89]. This suggests that CtHtrA is essential during certain stress conditions and does not necessarily relate to replication or binary fission. Heat stress is highly likely to be a strong inducer of protein stress and for many bacteria is the main *in vitro* condition during which *htrA*- or *degP*- mutants were lethal [281]. Therefore, perhaps it was not surprising that CtHtrA was essential even in this relatively short time of heat treatment.

As demonstrated in this study, it is also clear that CtHtrA is important, either for the stress, or restoration of protein biogenesis, during the reversion and recovery to EBs from penicillin persistence. Reversion is very asynchronous for this form of persistence. In *C. trachomatis* L2 serovar, which has slightly faster growth kinetics compared to that of the D serovar, reversion has been reported to take 10-20 hours after removal of the persistence inducing agent [158]. Therefore, in the present study, 52 h PI is the most logical choice to target reversion. The mechanism of reversion involves gradual budding of ‘normal’ RBs from the aberrant persistent forms [158]. These budded RBs are then thought to undergo replication by binary fission prior to conversion to the infectious EB form. Due to the asynchronous nature of reversion from persistence, it was not possible to uncouple restoration from persistence and the subsequent replication of the restored RBs. Hence, it is likely that the impact of JO146 in this experiment was on the replication of recovered RBs, the recovery from penicillin persistence to reticulate bodies or both. However, given that infectious

progeny were detectable at 50 h, but not at 44 h after reversion from penicillin persistence was commenced, it is most likely indicative that the impact of JO146 measured in this experiment is largely related to reversion from persistence. The absolute requirement for CtHtrA during recovery from penicillin persistence is an exciting finding, and to our knowledge is the first identification of an essential protein for this transition. Addition of JO146 at 52 h PI on the control, non-persistent cultures resulted to minor reductions in the formation of infectious progeny. Upon addition of JO146, these control cultures were either in the EB form or early infection stage. Concurrent work to this project demonstrated that JO146 was less effective for both of these developmental phases, so these results are consistent with what might be expected [250]. It was previously determined that JO146 was less effective in reducing infectious progeny production when the compound was added either during early or late stages of the developmental cycle. Therefore, these data indicate that during reversion from penicillin persistence and recovery of infectivity, JO146 treatment is completely lethal for *Chlamydia*.

On the other hand, based on the lack of lethality of JO146 when it was added during persistence it seems possible that penicillin persistence does not involve a detrimental level of extra-cytoplasmic protein stress. It is important to note that there is a possibility of some off-target impacts of JO146, however, given the marked phenotypes which correspond with very specific phases and conditions of chlamydial culture observed here, these impacts are likely minor. These data suggests that perhaps penicillin persistence is a strategy to reduce cellular and protein stress which may be an explanation for why CtHtrA was found not to be essential during penicillin persistence.

In summary, the data presented here demonstrate that addition of a CtHtrA inhibitor, JO146, during the replicative phase of *C. trachomatis* disrupts the chlamydial developmental cycle, resulting in the reduction in inclusion vacuole size, and prevented the development of infectious EBs over time without being toxic to the host cells, HEp2. It was also determined that JO146 is lethal for chlamydial recovery from penicillin persistence and for heat stress conditions. These data supports an essential role for CtHtrA during the replicative phase of chlamydial development, and during heat stress and recovery from penicillin persistence.

Chapter 5: **Generation and characterization of *Chlamydia trachomatis* mutants resistant to the CtHtrA inhibitor, JO146**

This work is planned for publication in Molecular Microbiology with the putative title, "Isolation of genetic mutants with resistance to the CtHtrA inhibitor JO146 identified positive selection for mutants in fatty acid related pathways".

Contributor	Statement of contribution
Vanissa A. Ong	Conducted the major experiments, provided feedback on the experimental design, contributed to the intellectual input of the manuscript, will contribute to manuscript drafting.
Bryan Wee	Conducted the bioinformatics analyses for the project, will contribute to manuscript drafting.
Markus Kerr	Conducted high-resolution microscopy for the project, will contribute to manuscript drafting.
Kenneth Beagley	Contributed to the intellectual input of the manuscript, will contribute to manuscript drafting.
John A. Allan	Contributed to the intellectual input of the manuscript, will contribute to

	manuscript drafting.
Wilhelmina M. Huston	Contributed to research plan development and provided feedback on experimental design, data analysis and executions, will contribute to the intellectual input of the manuscript and critically review and approve final version of the manuscript.

5.1 INTRODUCTION

Chlamydia (C.) trachomatis is an obligate intracellular Gram-negative bacterium that is the leading cause of preventable blindness in developing countries [7, 294] and is the most common cause of sexually transmitted bacterial infection in developed countries. Serious sequelae of chlamydial infections include pelvic inflammatory disease, tubal factor infertility and ectopic pregnancies in women [15-17]. The chlamydiae have a unique developmental cycle consisting of two distinct morphological forms; the elementary body (EB) and the reticulate body (RB). The RB replicates by binary fission within a membrane-bound vacuole termed an inclusion. During the replicative phase of development (18-24 h PI, depending on the serovar), RBs asynchronously differentiate back to the infectious EB form, which accumulate in the lumen of the inclusion as the remaining RBs continue to multiply [295]. At the late stage of development, the mature EBs are released through lyses of the host cell or extrusion of the vacuole [86].

Due to the organism's obligate intracellular niche and complex biphasic developmental cycle, the field lacked a system for genetic manipulation for a long time. However, it was recently established that *C. trachomatis* genomes are amenable to genetic manipulation as demonstrated by naturally occurring recombination [296] as well as the application of genetic systems that identify lateral gene transfer among chlamydiae *in vitro* [184, 297]. Previous studies on *Chlamydia* genetics involved the determination of the role of plasmid-encoded components in chlamydial pathogenesis and regulation of chromosomal factors. O'Connell and Nicks [163] described the isolation of plasmid-deficient derivatives of *C. muridarum* strain Nigg using novobiocin as a curing agent. The plasmid-deficient derivatives generated by this method were unable to accumulate glycogen and formed small plaques in culture, and this was observed for all plasmid-deficient *C. trachomatis* strains [163]. Song and co-workers [171] described the use of deletion mutagenesis and chlamydial transformation to genetically characterize the function of chlamydial plasmid open reading frames (ORFs). They identified that the ORF pgp4, a transcriptional regulator of multiple chromosomal genes including the glycogen synthase, *glgA*, plays an important role in chlamydial virulence [171]. Kari and co-workers [176] utilized chemical mutagenesis and a reverse genetic approach to create isogenic *C. trachomatis* mutants. They identified that infectivity of CTD *trpB*- was

not rescued by exogenous indole [176], the substrate used by genital but not ocular *C. trachomatis* serovars to synthesize tryptophan and escape the antimicrobial effect of IFN-γ-induced tryptophan starvation [182]. Recently, Bao and co-workers [183] were able to isolate a mutant, MCR (MoPn with CF0001 resistance) that is resistant to the inhibitor compounds, benzylidene acylhydrazides (CF0001) through continued passage of wild type *C. muridarum* (mouse pneumonitis pathogen, [MoPn]) in the presence of the inhibitor. These studies represent highly significant developments in *Chlamydia* genetics and thus the phrase "genetically intractable" no longer applies to *Chlamydia*.

Small molecule approaches have also been applied to determine specific functions of proteins in bacteria. Through the use of *Yersinia* Type III Secretion system (T3SS) inhibitors, it was identified that T3SS activity and chlamydial development are interlinked processes. Inhibition of T3SS resulted in stalled RB to EB differentiation, disruption of *C. pneumoniae* developmental cycle, prevention of replication of *C. pneumoniae* and *C. trachomatis* in *in vitro* infection models, and failure of the inclusion membrane proteins IncA and IncG to localize in the inclusion membranes [183, 185-188]. Through the use of small molecule inhibitors of LpxC, the enzyme that catalyses the first committed step in the biosynthesis of lipid A, the synthesis of lipooligosaccarides (LOS) in *C. trachomatis* is blocked. In the absence of LOS, *C. trachomatis* remains viable but non-infectious and replicative RBs failed to express selected late-stage proteins and transition to EBs [189]. Engström and co-workers [190] identified the inhibitor compound, 2-pyridone amide KSK120 that targets the glucose-6-phosphate (G-6P) metabolism pathway of *C. trachomatis*. Treatment with KSK120 blocked glycogen accumulation, thus, KSK120 may represent a class of drugs that can specifically inhibit *C. trachomatis* infection [190].

Our group recently identified a unique inhibitor compound, JO146 which is specific for *C. trachomatis* serine protease, High Temperature Requirement protein A (CtHtrA) and is lethal to the organism when added at mid-replicative phase of the chlamydial developmental cycle. Genetic studies targeting essential proteins of *C. trachomatis* particularly the CtHtrA may provide knowledge on the potential role of this serine protease on chlamydial infection and pathogenesis. HtrA has previously been demonstrated to be essential for extra-cytoplasmic bacterial protein folding and stress response [19]. Studies have also found that CtHtrA is secreted into the host cell

[23, 265], suggesting a role in *Chlamydia* interactions with host cells. However, the specific function of HtrA for chlamydial mid-replicative phase is not well elucidated (i.e. the substrates that require HtrA and are critical for mid-replicative phase). Using a combination of chemical mutagenesis, selection, and whole genome sequencing (WGS), the present study aimed to identify the role of CtHtrA. Chlamydial mutants resistant to JO146 were selected and characterized to determine basis of this resistance. Whilst a resistant isolate was not derived, unique mutants that were less susceptible to JO146 were generated and characterized.

5.2 MATERIALS AND METHODS

5.2.1 *Chlamydia* cultures and cells

Chlamydia culture was conducted as described in Materials and Methods section 3.3. Briefly, *C. trachomatis* (serovar D UW-3/Cx) was routinely cultured in HEp2 cells in DMEM supplemented with 5% fetal calf serum, 10 µg ml^{-1} gentamicin, 100 µg ml^{-1} streptomycin sulphate and incubated at 37^{o}C, 5% CO_2. All cultures were conducted at a multiplicity of infection (MOI) of 0.3 unless otherwise stated.

5.2.2 Generation of *C. trachomatis* mutants using EMS mutagenesis

Generation of *C. trachomatis* mutants and estimating the rate of mutations were performed following the procedure of Kari and co-workers [176] with minor modifications. Low-level ethyl methanesulfonate (EMS) was used as a mutagen since it has been shown to introduce C-G to T-A transition mutations. Briefly, a 6-well cell culture plate was seeded with 1.5 x 10^6 McCoy cells $well^{-1}$ in 3 ml DMEM supplemented with 10% FCS, 10 µg ml^{-1} gentamicin and 100 µg ml^{-1} streptomycin. After 24 hours, the cells were infected with *C. trachomatis* D at an MOI of 1.0 by centrifugation at 500 × g for 30 minutes at 25^{o}C. At 22 h PI, infected host cells were exposed for one hour to EMS at 37^{o}C. Preliminary experiment using different concentrations of EMS namely, 2.0, 3.0 and 4.0 mg ml^{-1} was performed to evaluate the right amount of EMS to be used to manage the "percent killing" and mutation

frequency. After treatment with EMS, the media was removed and replaced with fresh media containing 1 µg ml^{-1} cyclohexemide and the cells were incubated at 37°C, 5% CO_2. Untreated (non-mutagenized) *C. trachomatis* D control (CtDpp) was included. The cells were harvested in sucrose phosphate glutamate (SPG) buffer at 44 h PI. In brief, the media was removed from the wells and replaced with 1 ml $well^{-1}$ SPG. The infected monolayers were harvested and stored in aliquots at -80°C. Viable infectious yield and "percent killing" was determined by re-infecting fresh McCoy and HEp2 cells and number of IFUs was determined following the procedures previously described. Wild-type *C. trachomatis* D (UW-3/Cx), designated as CtDpp in this study, was a plaque purified, long term laboratory-passaged strain which has been previously sequenced by the team. CtDpp was neither treated with EMS nor passaged in the presence of JO146 and was included as a control in all of the experiments for this study.

The rate of mutation was estimated by determining the frequency of rifampicin-resistant (Rif^R) mutants which emerged as a result of point mutations in β-subunit of RNA polymerase [181]. To determine the concentration of rifampicin to be used for identifying rifampicin resistant mutants, a preliminary experiment using different concentrations of rifampicin (0.05 µg ml^{-1}, 0.1 µg ml^{-1}, 0.3 µg ml^{-1}) was conducted. This was performed by plaque assay on wild type, laboratory strain of *C. trachomatis* D (UW-3/Cx) as described in Materials and Methods Section 3.6 to confirm which concentration of rifampicin was inhibitory to the wild-type strain. Plates (6-well) seeded with 1.2 x 10^6 McCoy cells $well^{-1}$ were infected with a total of 2 x 10^6 EBs by centrifugation at 500 × g for 30 minutes at 25°C. Rifampicin was added to the non-agarose mixture to achieve the desired rifampicin concentration. The plates were examined for presence of plaques every day for 14 days. Once the concentration of rifampicin (0.1 µg ml^{-1} [176]) for determining mutation frequency was established, the plaque assay was again performed as previously described; however, this time, 2.0 x 10^6 EMS-mutagenized *C. trachomatis* D strains were used to infect the monolayers. The number of plaques was noted and the rate of mutation was calculated.

The *C. trachomatis* genome contains 4.30 x 10^5 C-G base pairs. Transition of five of these C-G pairs results in rifampicin resistance [79, 81, 184, 267-269]. The

rate of mutation was estimated using this formula: [(number of plaques observed/2.0 x 10^6 EBs) x 4.30 x 10^5]/5.

5.2.3 **Calculating for percent killing**

The percent killing corresponding to each EMS treatment was calculated as follows: Percent killing = 100 – [(IFU ml^{-1} in untreated (0 mg ml^{-1} EMS) cultures/ IFU ml^{-1} in EMS treated cultures) x 100]

5.2.4 **Selection and Isolation of JO146 resistant mutants**

Four (4) different selection strategies were conducted in an attempt to isolate JO146 resistant strains. These selection processes utilized either wild-type or EMS-mutated *C. trachomatis* D (UW-3/Cx). Three of the selection processes employed 96-well culture plate format to test small pools of mutants or strains that could be kept and used for a replica culture format/screening process if needed, while the fourth selection process involved continued passage of the chlamydial cells in the presence of JO146 in a T25 flask.

Firstly, a preliminary experiment to determine the dose of the inhibitor compound JO146 most compatible with a high throughput experimental design in 96-well culture plate was performed. The conditions that were varied included host cell density, time post-infection when JO146 was added to the culture, and time post-infection when the cells were harvested in sucrose phosphate glutamate (SPG) buffer. The preliminary experiments with varied conditions are outlined in Table 5.1.

Table 5-1. Screening methods used to determine the conditions in which JO146 was lethal in a 96-well format.

Name of the screening method	Seeding host cell density (cells/well)	JO146 treatment (h PI)	SPG harvest (h PI)
S1A	2.0×10^4	16	44
S1B	4.0×10^4	16	44
S2A	2.0×10^4	20	44
S2B	4.0×10^4	20	44
S3A	2.0×10^4	20	54
S3B	4.0×10^4	20	54

For all experiments, the culture medium was changed at 4 h PI to fresh medium containing 1 µg ml^{-1} cycloheximide. The cultures were harvested for each experiment by replacing the culture medium with 250 µl fresh, cold SPG and dislodging the infected monolayer by vigorously pipetting up and down the material from the wells. Formation of infectious elementary bodies was then determined by reinfecting fresh HEp2 monolayer as described in section 3.4.

Three distinct selection processes were attempted to isolate a JO146 resistant C. trachomatis clone in 96 well format. Briefly, these were: a two-stage (150-150 µM JO146) selection against a library of plaque purified C. trachomatis D (CtDpp) EMS mutants in 96 well plate format (Experiment A); a three-stage selection against a library of non-mutagenized C. trachomatis in a 96 well plate format that involved increasing doses of JO146 over the duration of the experiment (75-75-150 µM JO146, Experiment C); a two-stage (75-150 µM JO146) selection against a library of plaque purified C. trachomatis D (CtDpp) EMS mutants in 96 well plate format (Experiment C).

The fourth selection protocol utilized flask based cultures involving 26 continued passages of wild-type *C. trachomatis* D or a library of EMS mutagenized *C. trachomatis* D in the presence of two-stepped dosing regimen of JO146 (Experiment D). Table 5.2 briefly summarizes the methods for the selection experiments conducted to isolate a JO146 resistant mutant.

Table 5-2. The different selection processes utilized in the present study in an attempt to isolate a JO146 resistant mutant. JO146 regimen corresponds to the sequential rounds of JO146 treatment.

Library format	Name of selection process	JO146 regimen (µM)	Total number of selection cultures	Name of clones with less Susceptibility to JO146
96-well plate	Experiment A	150-150	2	n/a
	Experiment B	75-75-150	3	n/a
	Experiment C	75-150	2	n/a
T25 flask	Experiment D	75 (first 2 cultivations)	26	1A3, 1B3, and 2A3
		125 (for the succeeding cultivations)		

Once the conditions wherein JO146 was lethal and the experiment which was sufficiently high throughput were established, the selection experiments (A, B, and C) were then performed using either wild type or EMS (2 mg ml^{-1}) mutagenized *C. trachomatis* D (UW-3/Cx). A total of 2.0 x 10^6 EBs $well^{-1}$ were subjected to JO146 treatment at the start of each selection process. Each of the experiments was conducted at MOI of 0.3 (6.67 x 10^3 EBs $well^{-1}$). Cultures were exposed to two or three sequential cultivations each in the presence of JO146 with samples from each stage monitored for the formation of infectious progeny and JO146 sensitivity. These sequential cultivations were treated with 150-150 µM JO146 for Experiment A, a three round (75-75-150 µM JO146) of treatments for Experiment B, 75-150 µM JO146 sequential treatments for Experiment C. Controls (wild type *C. trachomatis* D), corresponding to untreated and JO146-treated cells, respectively, were included in the assays.

The other type of selection process performed for this study was generally conducted in T25 culture flasks (Experiment D) (Table 5.1). Selection and isolation of JO146 resistant *C. trachomatis* strains was performed following the method of Bao and co-workers [183]. HEp2 cells were 90% confluent at the time of initial infection with 2.0 x 10^6 EBs in T75 flasks. Either EMS- mutated or wild type *C. trachomatis* D (UW-3/Cx) strains were used to infect the host cell monolayers. At 16 h PI, the cells were treated with JO146. The cells were harvested at 48 h PI and directly used to reinfect fresh HEp2 monolayers. The concentration of JO146 used and the amount of SPG harvest used to reinfect fresh HEp2 monolayers were guided by the apparent multiplicity of infection observed in the cultures. The cells were

cultivated in the presence of JO146 for 26 passages (Experiment D). In the absence of any visible inclusions under the microscope, the culture was harvested and "blind passaging" was performed once or twice without JO146 treatment until inclusions were observed in the cultures.

A total of six T25 flasks were utilized for Experiment D. These flasks were labelled as 1A, 1B, 2A, 2B, 3A and 3B. Two of the flasks, (1A and 1B) were infected with CtDpp EMS mutants, while four (2A, 2B, 3A and 3B) were infected with wild type CtDpp. At the end of the 26th passage, the cultures were expanded three times without the presence of the inhibitor. The resistance of the mutants against the inhibitor was then confirmed by treating the strains with 10, 25, 50, 75, 100 and 125 µM JO146. Treatment with media only (0 µM JO146) and DMSO were used as controls. All JO146 treatments were done in conjunction with wild-type CtDpp control which was not passaged in the presence of JO146.

After confirmation of resistance to JO146, *Chlamydia* from each of the six flasks for Experiment D was plaque purified as described in Materials and Methods Section 3.6. For each unique population, three plaques were picked and passaged in fresh HEp2 cells to obtain a clonal isolate of the JO146-resistant mutants. These mutants were designated according to the initial formats in which they came from, for instance, 1A1, 1A2, 1A3 (for the three plaques from format 1A), 1B1, 1B2, and 1B3 (for the three plaques from format 1B) and so on. The clones were again subjected to JO146 treatments (25 µM, 75 µM and 125 µM) to confirm their resistance to the inhibitor. The three most resistant clones (1A3, 1B3 and 2A3) (Table 1) were selected for downstream processing. These three clones were cultured in T75 flasks three times to obtain high titres. Determination of IFU ml^{-1} was then assessed using the method previously described (Materials and Methods section 3.4).

5.2.5 **Growth analysis of the JO146 resistant mutants**

A growth analysis experiment was conducted to characterise the growth kinetics of the isolated mutants. Cultures conducted in 48-well plates were seeded with 25,000 HEp2 cells well^{-1}. 24 hours after seeding, the cells were infected with the JO146 resistant mutants (1A3, 1B3 and 2A3) at an MOI of 0.3. The cells were harvested in SPG at 24, 30 and 44 h PI. Quantification of *Chlamydia* was performed

as described in Materials and Methods section 3.4. Cultures for immunocytochemistry were conducted on glass coverslips, using the method previously described in Materials and Methods section 3.5. The sizes of the inclusions were measured using the Leica application suite.

5.2.6 Determination of the growth of *C. trachomatis* JO146 resistant mutants under heat stress

To determine the growth of the mutants during heat stress, a heat shock experiment was performed. Culture plates (48-well) were seeded with 25,000 HEp2 cells well^{-1}. 24 hours after seeding, the host cells were then infected with *C. trachomatis* JO146 resistant mutants at an MOI of 0.3. At 20 h PI, the cells were treated with JO146 (0, 50, and 100 µM) or DMSO immediately prior to heat shock at 42^{o}C for 4 hours. At 24 h PI, the compound was removed from the cultures by washing with pre-warmed media three times prior to returning the cultures to 37^{o}C. The cells were harvested in SPG at 44 h PI and *Chlamydia* quantification was performed as described in Materials and Methods section 3.4.

5.2.7 Growth of *C. trachomatis* JO146 resistant mutants during penicillin persistence

A penicillin persistence experiment was conducted to check for recovery of the clones from penicillin-induced persistence. Briefly, 48-well culture plates were seeded with 25,000 HEp2 cells well^{-1}. The host cells were infected with the mutants at an MOI of 0.3. Penicillin (100 U ml^{-1}) was then added to the cultures at 4 h PI. Penicillin was removed from the cultures at 30 h PI by three rounds of washes with pre-warmed media. The cultures were harvested at 68 and 88 h PI in SPG. A separate control culture wherein penicillin was not removed was included. Morphology and sizes of the inclusions were examined by immunocytochemistry and confocal microscopy as previously described in Materials and Methods section 3.5.

5.2.8 *C. trachomatis* genomic DNA extraction

Genomic DNA was extracted from *C. trachomatis* isolates that were purified from cultures as described in Materials and Methods section 3.3 (*Chlamydia* strain propagation). The cell lysates were centrifuged at 18,000 × g for 30 mins at 4^{o}C to

pellet the *Chlamydia*. After centrifugation, the supernatant was removed and the cells were suspended in 100 µl SPG. 100 µl of lysis buffer (Qiagen, Hilden, Germany) and 20 µl of 20 mg ml^{-1} proteinase K (Promega, WI, U.S.A.) were added to 100 µl of the cell lysate. The cells were incubated at 37^{o}C overnight. 1.5 µl of RNAse (Life Technologies, Eugene, OR, U.S.A.) was added to the cell lysate and incubated at 37^{o}C for 1 h. The tubes were then cooled down on ice for 3 mins, after which, the tubes were centrifuged at 18,000 × g for 1 min. The supernatant was transferred to a new 1.5 ml microcentrifuge tube. Equal amount of phenol chloroform isoamyl alcohol solution (Sigma-Aldrich, St. Louis, MO, U.S.A.) was added to the nucleic acid solution and the tubes were inverted until an emulsion formed. The tubes were then centrifuged at 12,000 × g for 5 mins at room temperature. The aqueous phase was transferred to a new tube and equal amount of phenol chloroform isoamyl alcohol solution was then added and this PCI extraction step was done twice. The aqueous phase was then transferred to a new tube and (2.5x the amount of the aqueous phase) 100% cold ethanol and (0.1x the amount of the aqueous phase) 3 M sodium acetate (NaOAc) and 1 µl glycogen solution (Invitrogen, Eugene, OR, U.S.A.) were added to the aqueous phase. The tubes were stored at -20^{o}C overnight. The DNA was pelleted by centrifugation at 18,000 × g for 10 minutes at 4^{o}C. The supernatant was removed and 1 ml 70% ethanol was added. The tubes were centrifuged for 5 mins at 18,000 × g at 4^{o}C and the ethanol wash was repeated once. After the second ethanol wash, ethanol was removed without dislodging the pellet. The pellet was dried and then resuspended in 50 ml nuclease-free water. The tubes were incubated for 30 mins at 65^{o}C to dissolve DNA in the solvent. The total DNA was quantified by measuring the optical density with a NanoDrop Spectrophotometer.

An aliquot of the genomic DNA of each strain was run in 0.8% agarose gels in TBE (Tris-borate-EDTA; 89 mM Tris (ph 7.6), 89 mM boric acid, 2 mM EDTA) buffer at 100 V. The gels were then stained with SYBR® Safe (Invitrogen, Eugene, OR, U.S.A.), viewed and photographed using Gel Doc System (Bio-Rad, CA, U.S.A.).

5.2.9 **Genomic sequence analysis of the clones**

Reads (300 bp paired-end) were generated using Illumina's MiSeq platform. The reads were trimmed and filtered for a minimum average base quality of 28 (Phred+33) across a sliding window of 4 bp. The Nesoni (Victorian Bioinformatics Consortium) pipeline was used for mapping and SNP and indel calling. Sequencing adapters, indexes and primers were trimmed and reads were mapped using Bowtie2 against the reference *Chlamydia trachomatis* D (UW-3/Cx) complete genome (GenBank accession no. AE001273) and the *C. trachomatis* D-EC plasmid pCTDEC1 (GenBank accession no. CP002053).

5.3 RESULTS

5.3.1 ***C. trachomatis* mutants generated by EMS mutagenesis**

C. trachomatis mutants were generated by EMS mutagenesis. Three concentrations of EMS (0, 2 and 3 mg ml^{-1}) were used. Table 5.2 shows the *Chlamydia* IFU ml^{-1} recovered as well as the percent killing that resulted for each of the EMS treatments.

Table 5-3. Infectious progeny recovered and percent killing generated from EMS mutagenesis of *C. trachomatis* D

EMS concentration (mg/ml)	IFU ml^{-1}	Percent killing[a]
0	8.62×10^8	
2	9.797×10^7	88.64%
3	2.04×10^7	97.63%

[a]Controls without EMS treatment were concurrently conducted and this % killing represents the loss relative to control yield.

The ideal EMS concentration to use for the experiments was determined by assessing a suitable mutation frequency rate as well as the final yield of culture (% killing) to ensure adequate numbers of mutants could be generated to be screened. Briefly, 2×10^6 EBs from the EMS library were screened in a plaque formation assay in the presence of rifampicin and 21 plaques were formed for the 2 mg ml^{-1} EMS library. The *C. trachomatis* D genome contains 430,636 C-G base pairs [298] and 5 C-G transitions result in rifampicin resistance. Therefore, the rate of mutation from

this library was calculated to be 0.904 mutations per genome [(21/2,000,000) x 430,636]/5 = 0.904] (88.64% killing) (Table 5.2).

5.3.2 Isolation of JO146 resistant strains was successful using the T25 flask format but not the 96-well library format

The first method of selection conducted to isolate JO146 resistant or less susceptible strains utilized a 96-well culture plate format. Results from preliminary experiments demonstrated that condition S1A (20,000 Hep2 cells well^{-1}, JO146 added at 16 h PI, SPG harvest at 44 h PI) was completely lethal to wild type *C. trachomatis* D. Based on this condition, three distinct selection experiments were conducted in a 96-well format. Serial selection in the presence of JO146 (as described in section 5.2.4) was conducted in order to isolate JO146 resistant clones. The experiments (A, B, and C as outlined in Materials and Methods) resulted in reduction of inclusion forming units (IFU) that were not able to be recovered even after subsequent serial passage in the absence of JO146.

Experiment A was tested for resistant isolates after two rounds of JO146 selection at 150 µM treatments on both occasions. Wells in which inclusions were present (11 out of 32 wells that exhibited formation of IFUs after the first round of JO146 selection) were cultured in the presence of a dose series of JO146 and IFU ml^{-1} determined (Figure 5.1). None of the isolates from this experiment showed a higher level of resistance to JO146 compared to *C. trachomatis* D control (CtDpp) (Figure 5.1), indicating that the selection was not effective. A similar lack of success resulted from approaches in experiments B and C.

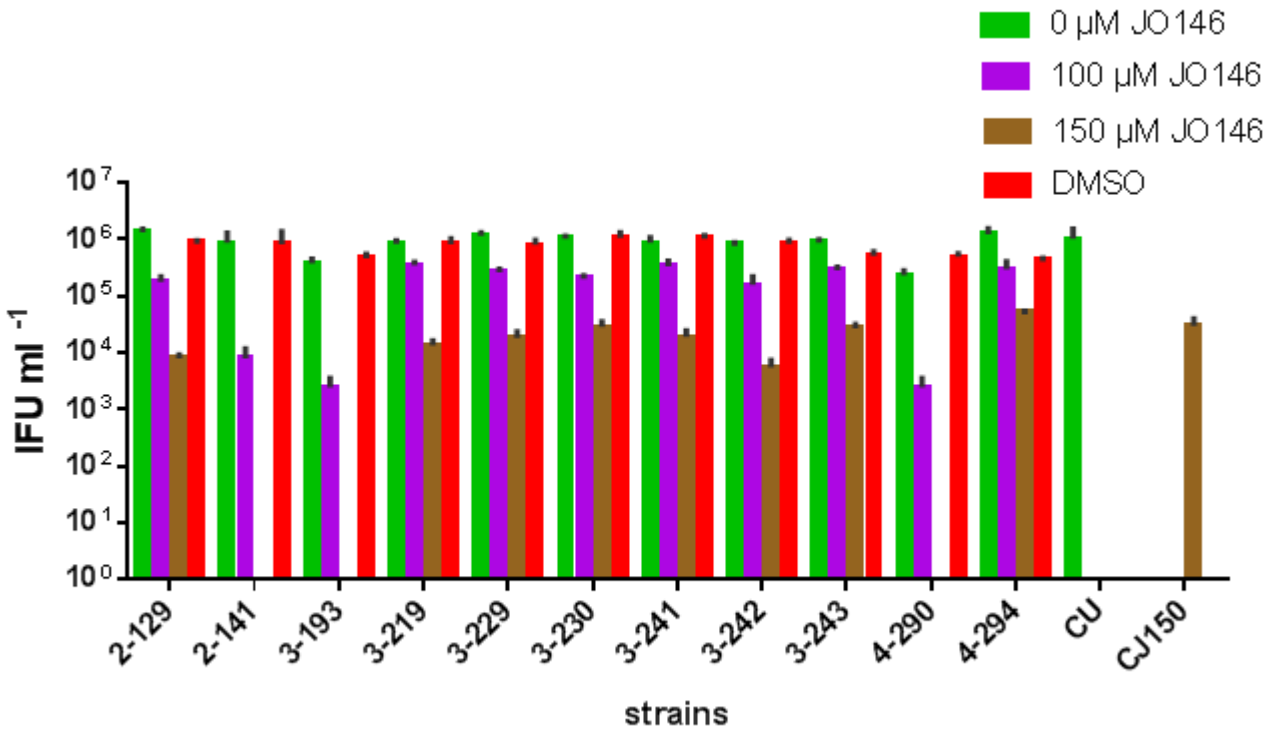


Figure 5.1. Infectious progeny detected after second round of JO146 treatment for Selection A

The names of the strains are indicated on the x-axis and the numbers of infectious progeny (IFU ml^{-1}) are indicated on the y-axis. CU indicates untreated CtDpp, CJ150 indicates CtDpp treated with 150 µM JO146.

The attempt to isolate JO146 resistant mutants using the 96-well culture plate format (experiments A, B, and C) described in this study was unsuccessful. After several rounds of screening for JO146 resistance, no infectious progeny was detected in the cultures in the 96-well library format, thereby indicating that the process either resulted in the absence of JO146 resistance or less susceptible isolates, or a complete loss of any isolates. In contrast, lengthy serial passage of chlamydiae in the presence of JO146 (Experiment D) resulted in the generation of truly resistant strains of the organism. Six unique pools were used. Two (2) of the pools, (1A and 1B) were infected with *C. trachomatis* D EMS mutants, while four (2A, 2B, 3A and 3B) were infected with wild type *C. trachomatis* D. Four of these cultures (1A, 1B, 2A and 2B) were found to be less sensitive to the inhibitor when tested using a dose series after 26 passages in the presence of JO146 (Figure 5.2). 3B did not grow any inclusions after the 26^{th} passage, indicating that the compound had completely inhibited chlamydial growth and infectious progeny production for this culture. Resistance of the mutants to JO146 were identified through the detection of infectious progeny in these cultures even after treatment with 125 µM JO146 (Figure 5.2). No chlamydial growth was observed in cultures infected with control *C. trachomatis* D strain (CtDpp) treated with 125 µM JO146 (Figure 5.2). The number of infectious progeny in cultures treated with DMSO compared to cultures treated with 50, 75, 100, and 125 µM JO146 for all the different populations including the CtDpp control were statistically significant ($p < 0.0001$) (Figure 5.3).

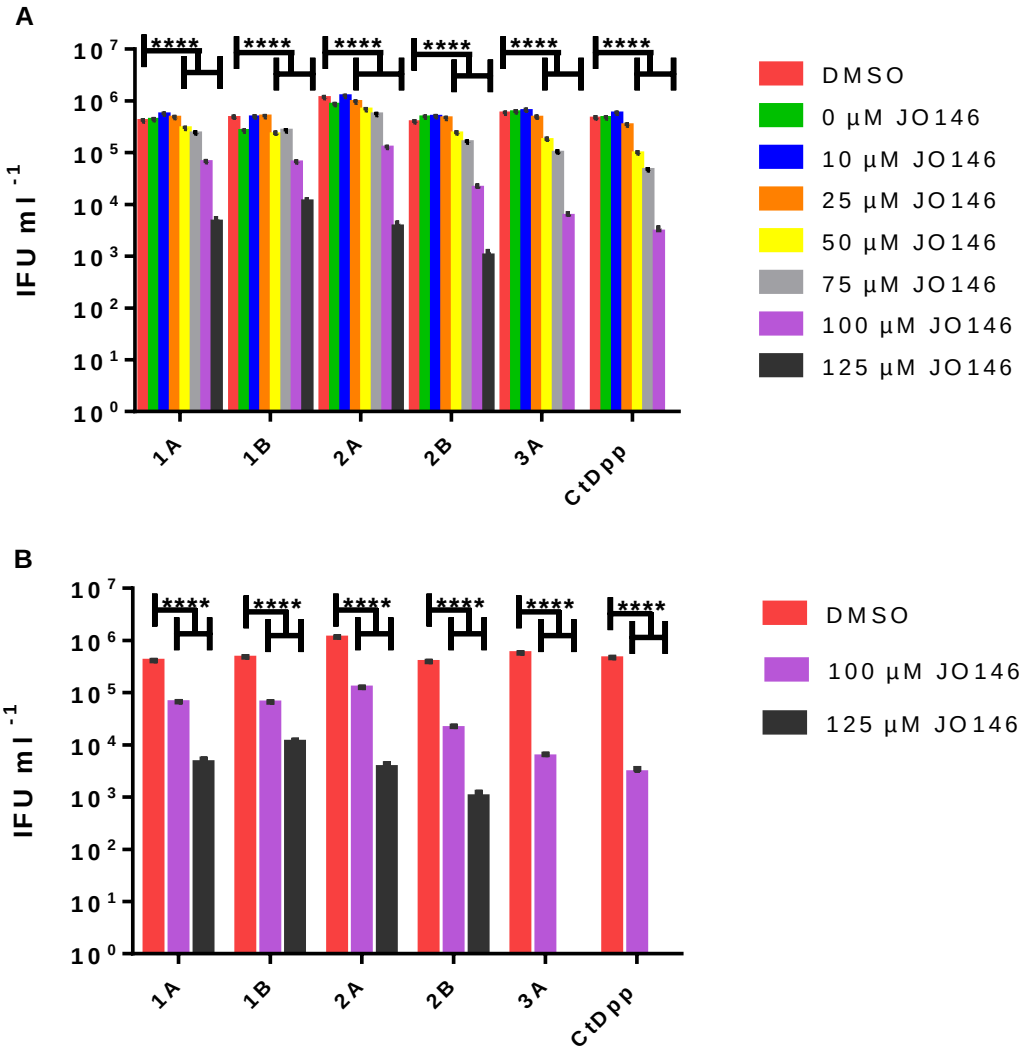


Figure 5.2. Infectious progeny production after JO146 treatment at 16 h PI after continued passage of the cultures in the presence of the inhibitor

A, Infectious progeny production after the mutants and control CtDpp were treated with JO146 at 0 to 125 µM as well as DMSO control; B, Infectious progeny production for cultures treated with 100 and 125 µM JO146 and DMSO control showing the comparative statistics between the susceptibility of the mutants and the parent strain (CtDpp). The names of the strains are indicated on the x-axis and the numbers of infectious progeny (IFU ml^{-1}) are indicated on the y-axis. Statistics were conducted using Two-Way ANOVA relative to DMSO control. Error bars represent the standard error of the mean (n=27), **** indicates $p < 0.0001$.

The three least susceptible populations to JO146 were then selected. These were 1A, 1B and 2A. *Chlamydia* from these selection protocols were then plaque purified and three plaques were picked from each unique protocol and passaged in fresh HEp2 cells to obtain nine clones of JO146-resistant mutants. The JO146

resistant clones were designated as 1A1, 1A2, 1A3, 1B1, 1B2, 1B3, 2A1, 2A2, and 2A3. The mutants were subjected to JO146 (25 µM, 75 µM and 125 µM) treatments to confirm resistance to the inhibitor.

It was observed that there were four less susceptible clones as indicated by the presence of chlamydial growth even after treatment with 125 µM JO146 (Figure 5.3). No infectious progeny was detected in all the other cultures treated with 125 µM JO146. The clones with reduced susceptibility to JO146 were 1A3, 1B2, 1B3 and 2A3 (Figure 5.3). 2A3 had the most reduced susceptibility to the inhibitor, with the number of IFU ml^{-1} in the cultures treated with 125 µM JO146 equal to that of the cultures treated with 25 µM JO146 for this clone (1.35×10^5 IFU ml^{-1}). No infectious progeny was detected in CtDpp treated with 125 µM JO146. The number of infectious progeny in cultures treated with DMSO compared to cultures treated with 75 and 125 µM JO146 for all the clones including the CtDpp control were statistically significant ($p < 0.0001$) (Figure 5.3). The top three clones with least susceptibility to JO146 and from three distinct selection pools, 1A3, 1B3 and 2A3 were then selected for phenotypic and genotypic characterization in order to ensure that the same clone was not isolated multiple times.

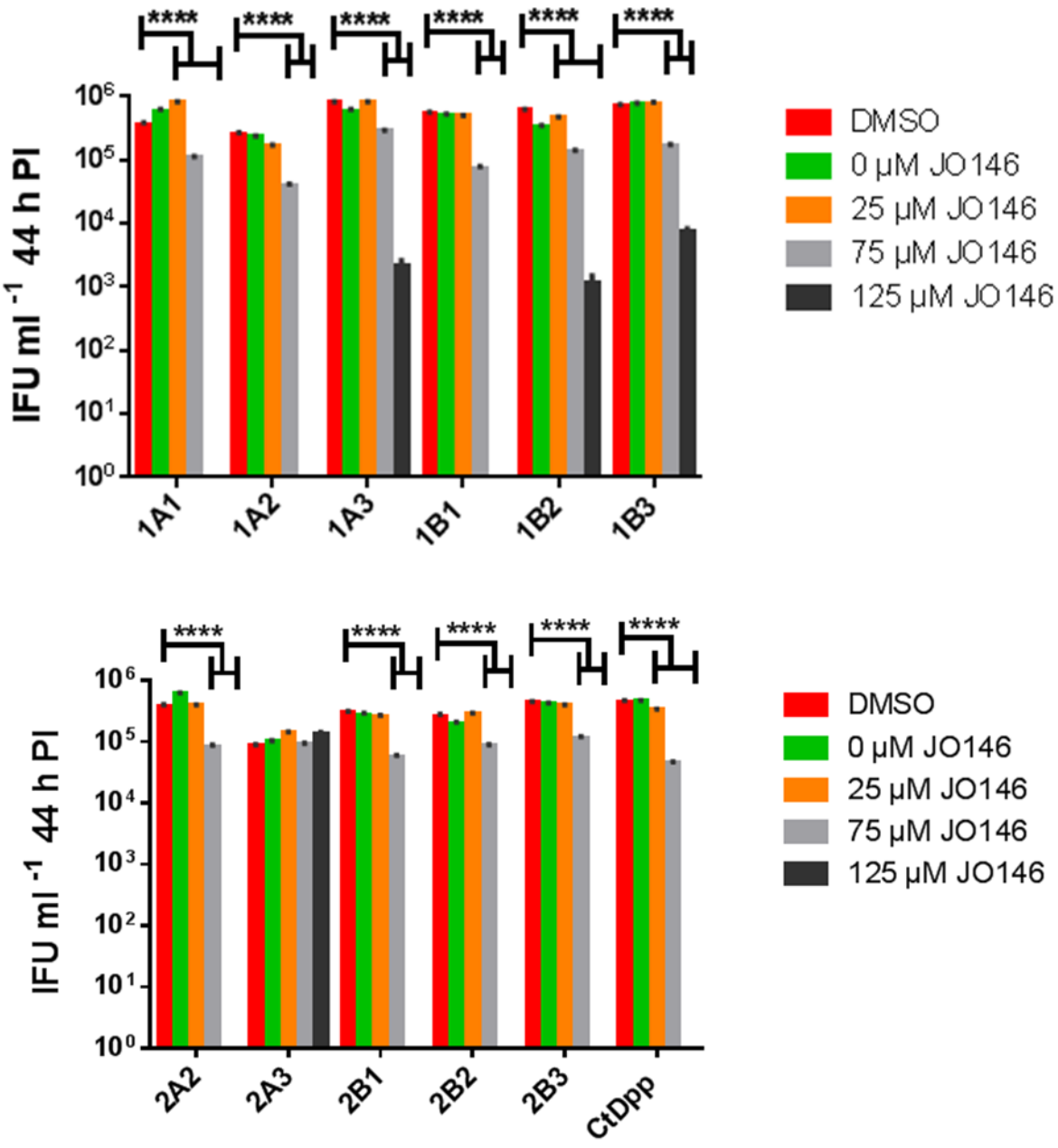


Figure 5.3. Yield of infectious elementary bodies (EBs) after treatment with different concentrations of JO146 for plaque purified resistant mutants

Error bars represent the standard error of the mean (n=27). Names of the plaque purified strains are indicated on the x-axis and IFU ml^{-1} is indicated on the y-axis. Statistics were conducted using Two-Way ANOVA relative to DMSO control. Error bars represent the standard error of the mean (n=27), *** indicates $p < 0.001$.

5.3.3 **A higher yield of infectious progeny was observed in mutants.**

A growth analysis was performed to assess the generation of infectious progeny (Figure 5.4). At 30 h PI, infectious progeny was observed for all the isolates with 2A3 having the highest number of IFU ml^{-1} (8.25 x 10^3 IFU ml^{-1} for 2A3 and 2.92 x 10^3 IFU ml^{-1} for wild type, CtDpp control). At the conclusion of the developmental cycle (44 h PI), there was approximately 1.5 log increase in the number of IFUs for each strain. At this time point, all of the mutant strains generated more infectious EBs compared to CtDpp control. It was observed that all the clones with less susceptibility to JO146, except for 1A3, had higher number of infectious progeny than the wild type strain, CtDpp both at 30 and 44 h PI (Figure 5.4). The number of recoverable EBs for both 1B3 and 2A3 was significantly different compared to the IFUs observed in CtDpp ($p < 0.001$) (Figure 5.4).

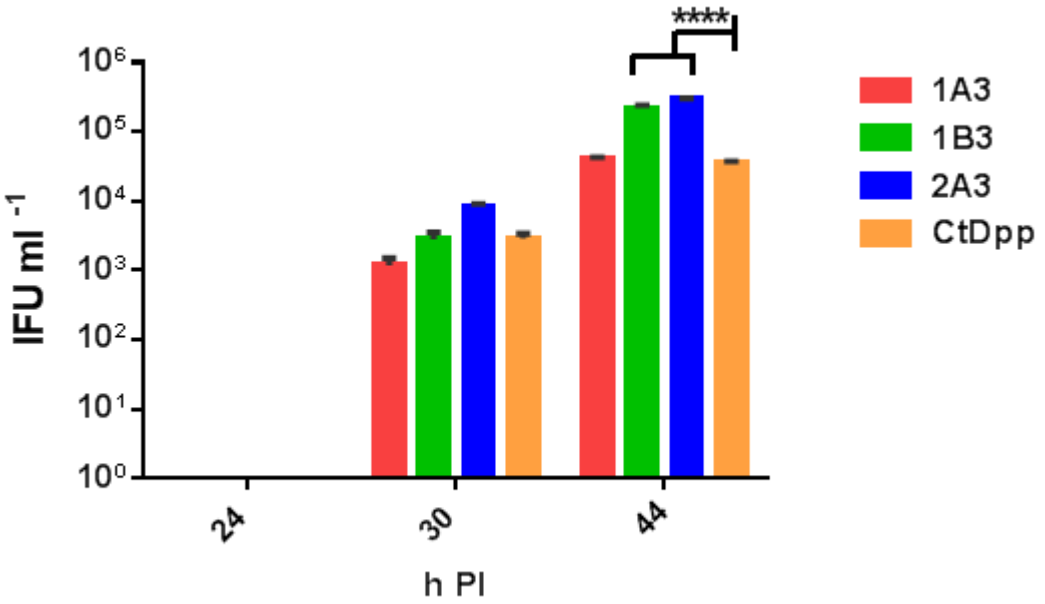


Figure 5.4. Yield of infectious elementary bodies (EBs) throughout the late stage of the *C. trachomatis* developmental cycle for the JO146 resistant strains

The mutant strains are indicated by the coloured bars. The time post-infection (h PI) is indicated on the x-axis and IFU ml^{-1} is indicated on the y-axis. Statistics were conducted using Two-Way ANOVA relative to CtDpp control strain. Error bars represent the standard error of the mean (n=27), *** indicates $p < 0.001$.

Immunocytochemistry was conducted and analysis revealed that the inclusions appeared morphologically the same among the different mutant strains compared to the wild type within the same time point, with the inclusions appearing largest at 40 h PI (Figures 5.5 and 5.6). At 24 h PI, inclusions formed in the cells infected with wild type *C. trachomatis* D (CtDpp) were largest (average inclusion size of 9.39 µm) (Figure 5.6). The inclusions greatly increased in size at 40 h PI. Except for 1B3, the sizes of inclusions formed in the mutants were approximately equal to the sizes of inclusion formed in CtDpp (average inclusion size of 22.53 µm) (Figure 5.6).

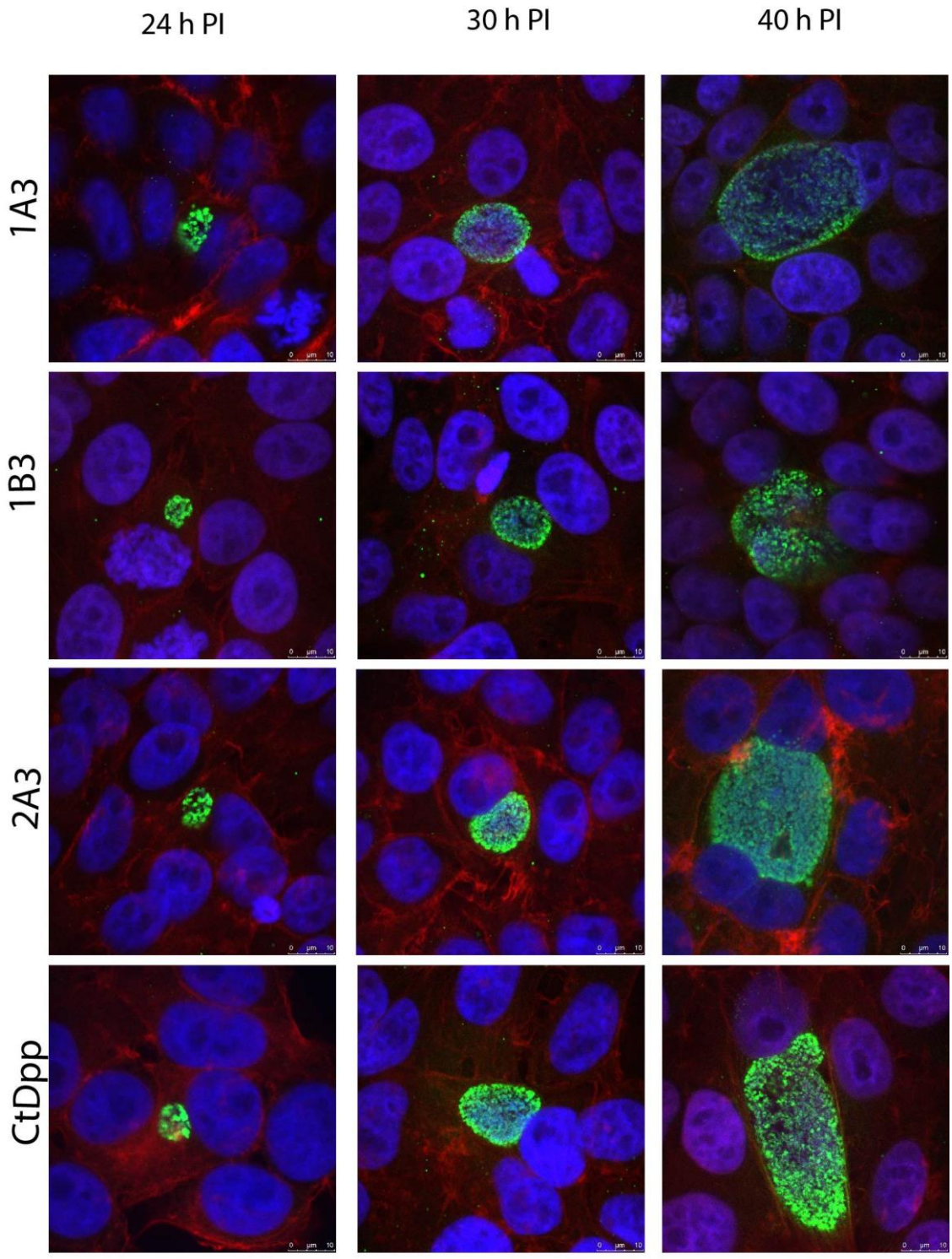


Figure 5.5. Immunofluorescence using anti-MOMP antibodies at 24, 30 and 40 h PI

Analysis of the confocal microscopy images indicates that the chlamydial inclusions increase in size over time for the JO146-resistant mutants as well as the control, wild-type *C. trachomatis* D (CtDpp). Names of the strains are indicated on the left side of the images and corresponding time points are indicated on the top of the panel. Scale bar (bottom right) indicates 10 µm.

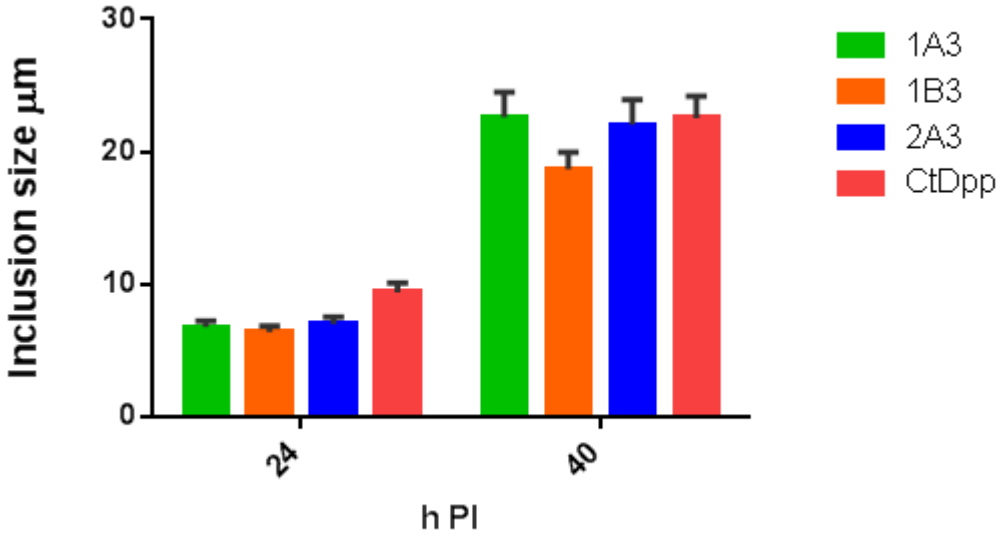


Figure 5.6. Inclusion sizes of JO146 less susceptible strains and wild type *C. trachomatis* D (CtDpp) at 20 and 40 h PI

Inclusion sizes were measured from independent coverslips, n = 30. Time post-infection (h PI) is indicated on the x-axis and inclusion size (µm) is indicated on the y-axis. Statistics were conducted using Two-Way ANOVA relative to CtDpp control strain. Error bars represent the standard error of the mean (n=27). Inclusion sizes of the clones compared to CtDpp were not significantly different for both 24 and 40 h PI.

5.3.4 **JO146 was lethal to mutants when added during heat stress**

The growth of the mutants treated with JO146 during heat stress was determined. Cultures treated with the mutants were exposed to heat stress (42^{o}C) for four hours immediately after addition of JO146 at 20 h PI. The compound was removed at 24 h PI prior to returning the cultures to 37^{o}C. Infectious progeny yield was subsequently measured at 44 h PI. It was observed that JO146 has a dose-dependent inhibitory effect on *C. trachomatis* strains during heat stress. Treatment of infected cells with 100 µM JO146 was lethal. Mutant 1A3 had the least number of recoverable EBs compared to the other mutant strains (1B3 and 2A3) and CtDpp (Figure 5.7) during heat stress condition. There was more than 1 log difference of infectious progeny between 1A3 and both 1B3 and 2A3 during heat stress in the presence of 50 µM JO146. Statistical analysis revealed that the number of infectious progeny observed in cultures treated with 50 and 100 µM JO146 compared to that in cultures treated with DMSO control was significantly different for 1B3, 2A3 and CtDpp ($p < 0.0001$) (Figure 5.7).

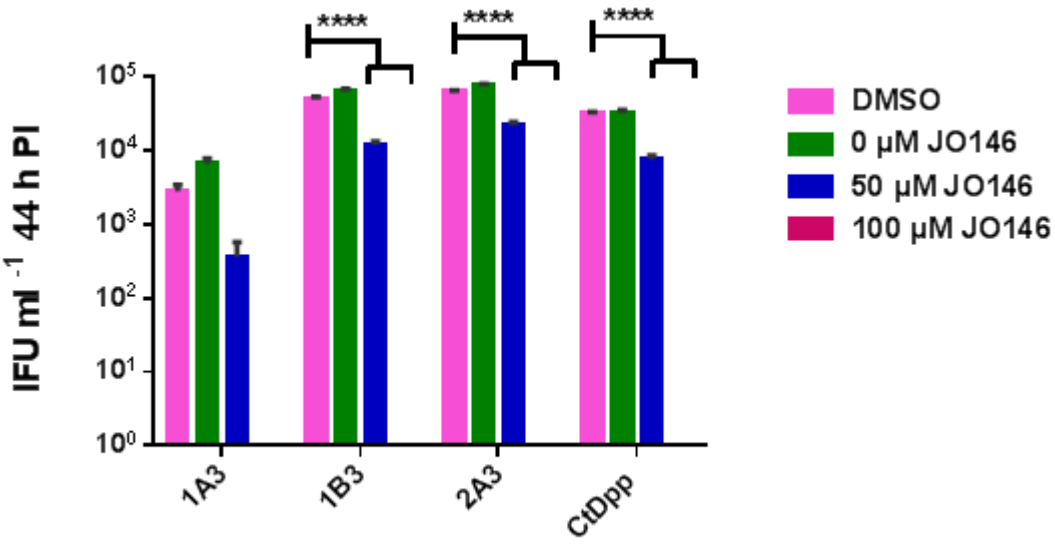


Figure 5.7. Infectious progeny after treatment with JO146 during heat stress (20-24 h PI)

Error bars represent the standard error of the mean (n=27). The name of the strain is indicated on the x-axis and the IFU ml^{-1} is indicated on the y-axis. Statistics were conducted using Two-Way ANOVA relative to DMSO control. Error bars represent the standard error of the mean (n=27), **** indicates $p < 0.0001$.

5.3.5 The mutants did not exhibit impaired recovery of infectious progeny from penicillin persistence.

Penicillin persistence was firstly confirmed by cultivating the mutants and wild type *C. trachomatis* D (CtDpp) in the presence of 100 U ml^{-1} penicillin from 4 h PI until harvest at 44 h PI. No infectious progeny was detected in control cultures wherein penicillin was left in the cultures (data not shown), confirming penicillin-induced persistence for the clones and wild type CtDpp. Confocal microscopy revealed morphologically atypical inclusions in this cultures which were consistent with persistence (Figure 5.9).

Concurrent experimental cultures were grown to assess the recovery of the JO146 less susceptible mutants from penicillin persistence. Penicillin (100 U ml^{-1}; added at 4 h PI) was removed from the cultures at 30 h PI. Growth during reversion from penicillin persistence was assessed for all the mutants and the control, CtDpp. Figure 5.8 shows that at 68 h PI, the less susceptible mutants, 1A3, 1B3 and 2A3 had approximately 1 log more infectious progeny compared to the wild type strain

(CtDpp) (2.5 x 10^4 IFU ml^{-1} for the less susceptible mutants and 2.7 x 10^3 IFU ml^{-1} for the control CtDpp). However, this difference in the infectious yield between the mutants and the CtDpp control at 68 h PI was not statistically significant. The number of IFU ml^{-1} increased at 88 h PI for all the mutants as well as for CtDpp. However, the mutants had more EBs compared to the wild-type strain at 88 h PI. The numbers of infectious progeny recovered at 88 h PI for all the mutants were significantly different ($p < 0.0001$) compared to the number of infectious progeny formed for CtDpp (Figure 5.8). Although all the mutants and the CtDpp control had higher infectious yield at 88 h PI (58 h after removal of the penicillin), it was observed through confocal microscopy that inclusion morphologies more frequently appeared to be atypical (irregularly cell-shaped bodies containing fewer, scattered *Chlamydia*) in mutant clones, characteristic of persistent *Chlamydia*, than the inclusions in CtDpp at this time point (Figure 5.10).

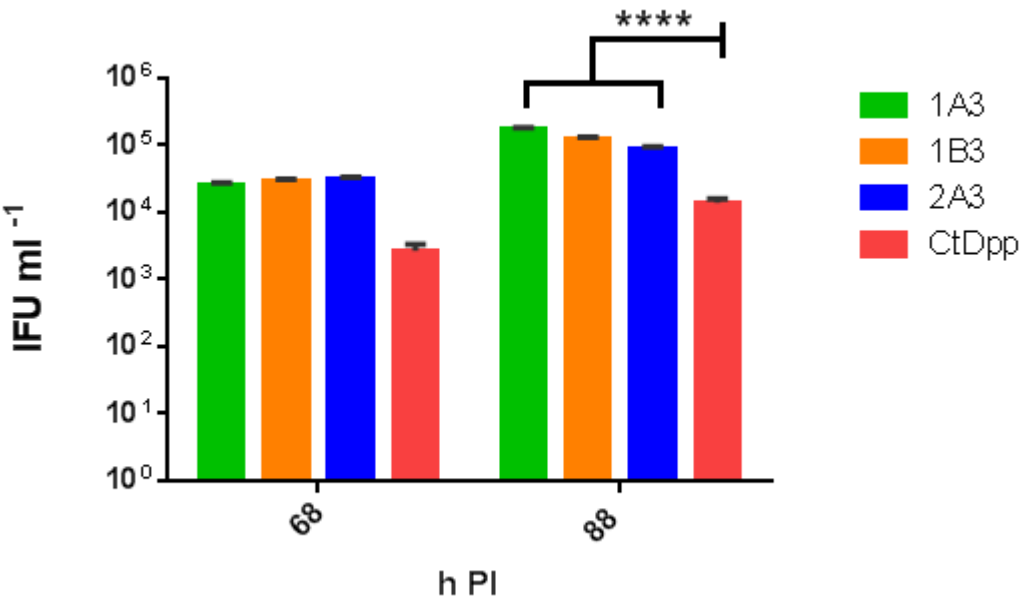


Figure 5.8. Infectious yield of *C. trachomatis* JO146 less susceptible mutants during reversion from penicillin persistence

The figure shows IFU ml^{-1} from each strain at 68 and 88 h PI after reversion from penicillin was commenced (penicillin was removed at 30 h PI). Cultures were treated with penicillin 100 U ml^{-1} at 4 h PI. Penicillin was removed from the cultures at 30 h PI. Statistics were conducted using Two-Way ANOVA relative to CtDpp control strain. Error bars represent the standard error of the mean (n=27), **** indicates $p < 0.0001$.

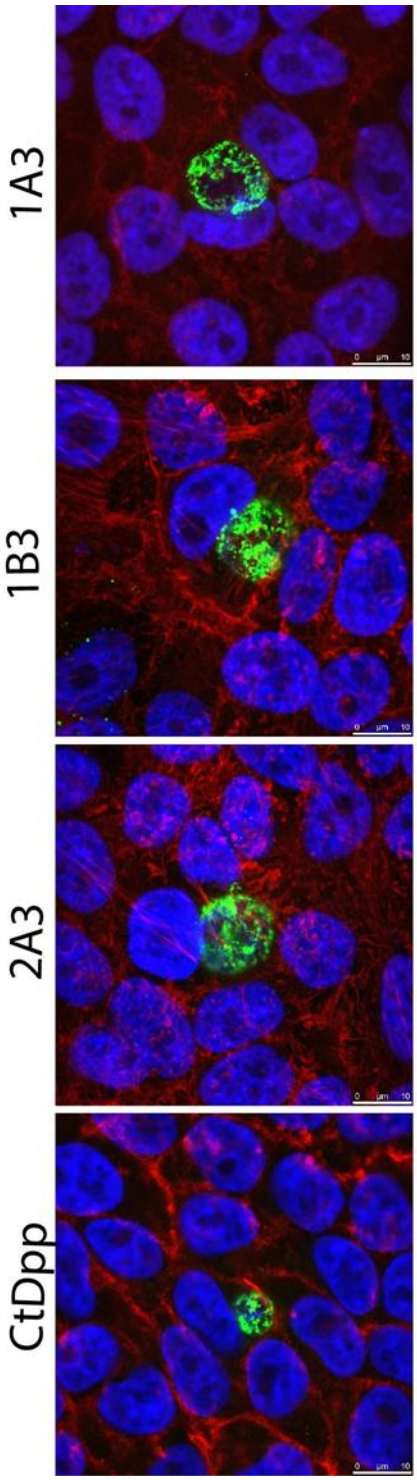


Figure 5.9. Confocal microscopy images of JO146 less susceptible mutants and wild type *C. trachomatis* D (CtDpp) cultures at 44 h PI in the presence of penicillin

The names of the mutants and wild type CtDpp are indicated to the left of the image. Scale bar (bottom right) denotes 10 µm.

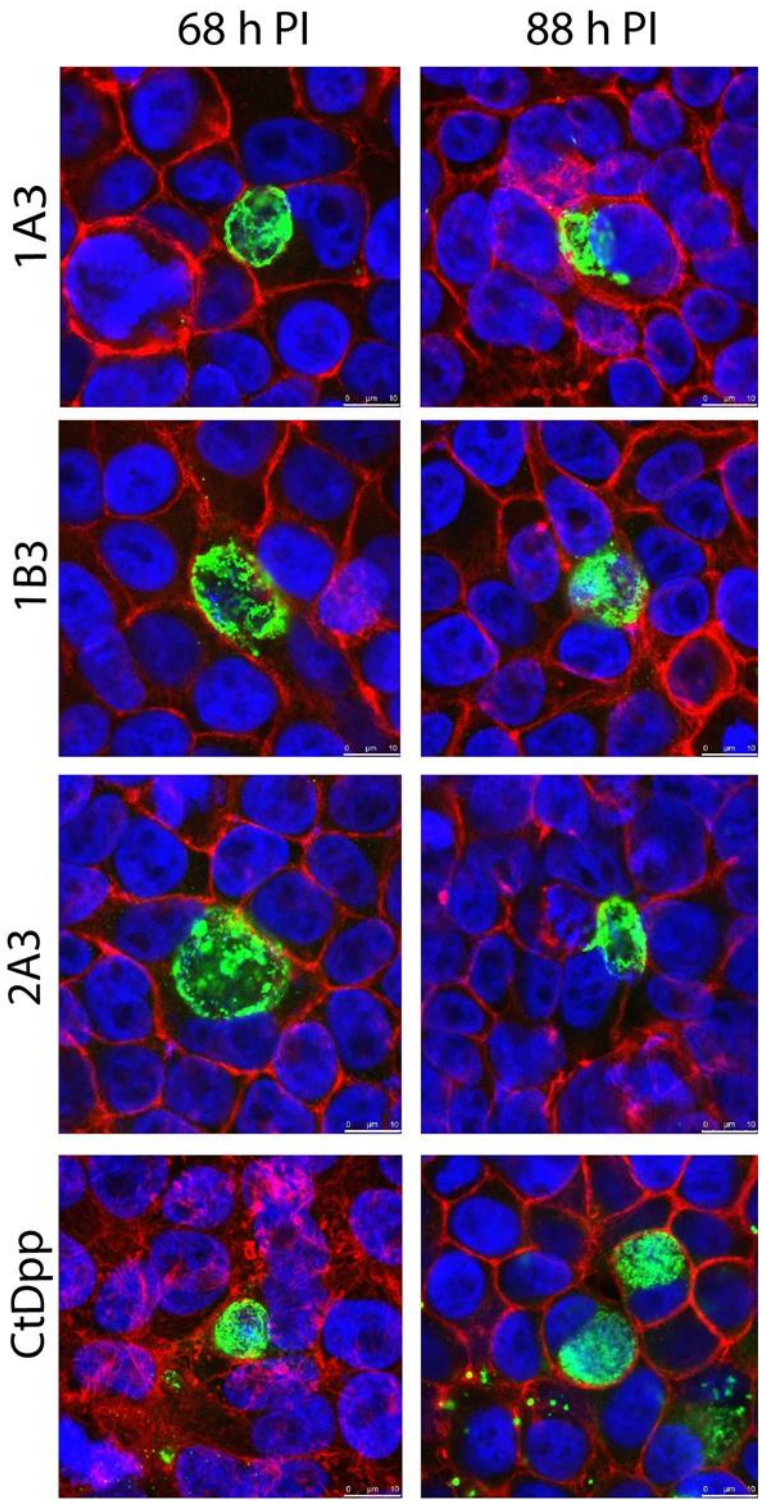


Figure 5.10. Confocal microscopy images of JO146 less susceptible mutants and wild type CtDpp cultures at 68 and 88 h PI after removal of penicillin at 30 h PI

The names of the mutants and wild type CtDpp are indicated to the left of the image. Scale bar (bottom right) denotes 10 µm.

5.3.6 **Whole genome sequence analysis of mutants**

Genomic DNA from the three mutants was isolated and sent for sequencing using a commercial service provider. The DNA concentrations for IA3, 1B3, and 2A3 were 229.04 ng μl^{-1}, 1450.82 ng μl^{-1} and 499.45 ng μl^{-1}, respectively. The whole genome for the plaque cloned, wild type CtDpp used as a control strain for the EMS mutant libraries was previously sequenced. The average depth of coverage across the genome for 1A3, 1B3, and 2A3 were 344x, 471x, and 388x, respectively. Read pairs that were aligned from each clone to the reference strain *C. trachomatis* D (UW-3/Cx) were 1,031,972, 1,386,175, and 1,151,654 for 1A3, 1B3, and 2A3, respectively.

5.3.7 **The mutants acquired unique SNPs in acyltransferases genes**

Table 5.3 shows a summary of the genomic sequence data for the three mutants in comparison with the published reference strain, *C. trachomatis* D (UW-3/Cx) and the laboratory, control strain CtDpp. Genome sequence analysis revealed few single nucleotide variations (SNVs) in the three clones and all were unambiguously supported by read mapping. All SNVs were substitutions of cytosine and guanine bases. No insertion/deletion sites (indels) were observed in the genomes of the clones. Interestingly, no substitutions were observed within, or near, the *htrA* gene. EMS-mutagenized clones, 1A3 and 1B3, each had 2 different SNVs relative to mutant 2A3 which had the most number of SNVs (eight). Both 1B3 and 2A3 had mutations in the gene CT206, which was annotated as an acyltransferase in *C. trachomatis* (L2/434/Bu) (G-to-A substitution at position 232574 for 1B3, and C-to-T substitution at position 232340 for 2A3) (Table 5.3). 1A3 had no mutations in CT206. 1A3 had synonymous mutation at position 952 when mapped against the *C. trachomatis* D-EC plasmid. 2A3 had synonymous mutations (C-to-T transitions) in the genes CT390 and CT404, which code for aspC aspartate aminotransferase, and SAM dependent methyltransferase respectively (Table 5.3). Clone 2A3 had non-synonymous C-to-T transition mutations in CT414 (A to V) and CT474 (G to R), which were annotated as pmpC putative outer membrane protein C and a hypothetical protein, respectively (Table 5.3). Both 1A3 and 2A3 had non-coding sequence mutations at positions 611146 and 368480, respectively. 2A3 had a non-synonymous mutation (A-to-T) at CT664, an adenylate cyclase-like protein (Table

5.3). It is noteworthy that all the three clones, 1A3, 1B3, and 2A3, had mutations in gene CT776, which encode for acylglycerophosphoethanolamine acyltransferase (aas). Each clone had a unique SNV in this gene. Overall, sequence analysis revealed that the clones were mutated in two loci namely, CT206 and CT776, which relate to fatty acid synthesis. There were different single nucleotide variations (SNVs) in these two loci (CT206 and CT776), indicating that they were strongly selected by JO146 (Table 5.3).

Figure 5.12 shows schematic diagrams of the locations of the SNVs in CT206 and CT776 for clones 1A, 1B3 and 2A3. The SNVs in the two most frequently identified loci, CT206 and CT776, were located in unique sites for each of the clones. Mutations in CT206 for 1B3 was in codon 275, which is in the C- terminus of the protein while for 2A3, the SNV was in position 197 residue of the protein which corresponds to the alpha-beta hydrolase (abhydrolase) domain (Figure 5.12). SNVs in CT776 were located in semi-conserved (codon 200) and well-conserved (codon 434) active sites of the AMP-binding domain of the *aas* locus for clones 1A3 and 1B3, respectively, while for 2A3, the SNV was not in a conserved site (codon 500) (Figure 5.12). Conserved domain means the protein has the same function. The SNVs in CT776 for clones 1A3 and 1B3 resulted in changes of amino acids to ones with different properties. The G-to-A substitution in codon 200 of gene CT776 for 1A3 resulted in a change from glycine (G, polar) to glutamic acid (E, acidic) (Figure 5.12). A C-to-T transition in the 434 residue of the protein resulted in arginine (R, basic) to cysteine (C, polar neutral) amino acid change. Although the SNV (codon 500) in CT776 for 2A3, resulting in serine (S, polar neutral) to asparagine (N, polar neutral) change, was not located in a conserved site, its SNV for CT206 resulted in a change from glutamine (Q, polar neutral) to a stop codon (Figure 5.12).

Table 5-4. Summary of the single nucleotide variations (SNVs) observed in the mutants 1A3, 1B3 and 2A3 in comparison with the published reference strain *C. trachomatis* D (UW-3/Cx) and the laboratory plaque purified *C. trachomatis* D strain (CtDpp) as well as the *C. trachomatis* D-EC plasmid.

The bases in green signify the SNVs observed in the mutants. The blue bar (*) denotes a nonsense mutation (i.e. a stop codon). The positions where the SNVs were observed, the SNV effects (the resulting amino acid) and their corresponding annotations are listed in the table.

		Published Reference		Mutants			Lab reference	SNP effect			
Position	Change type	Ct D (UW-3/Cx)	Ct D-EC plasmid	1A3	1B3	2A3	CtDpp	1A3	1B3	2A3	Annotations
232340	substitution	C		C	C	T	C			Q=>*	CT_206 predicted acyltransferase family protein
232574	substitution	G		G	A	G	G		G=>S		CT_206 predicted acyltransferase family protein
368480	substitution	C		C	C	T	C			noncoding	
444970	substitution	C		C	C	T	C			synonymous	CT_390 aspC Aspartate Aminotransferase
463177	substitution	C		C	C	T	C			synonymous	CT_404 SAM dependent methyltransferase
481998	substitution	C		C	C	T	C			A=>V	CT_414 pmpC Putative outer membrane protein C
548301	substitution	C		C	C	T	C			G=>R	CT_474 hypothetical protein
611146	substitution	G		A	G	G	G	noncoding			
762369	substitution	G		G	G	A	G			A=>T	CT_664 adenylate cyclase-like protein
910317	substitution	G		A	G	G	G	G=>E			CT_776 aas Acylglycerophosphoethanolamine Acyltransferase
911018	substitution	C		C	T	C	C		R=>C		CT_776 aas Acylglycerophosphoethanolamine Acyltransferase
911217	substitution	G		G	G	A	G			S=>N	CT_776 aas Acylglycerophosphoethanolamine Acyltransferase
952	substitution		C	T	C	C	C	synonymous			CTDEC_p001 Virulence plasmid integrase pGP7-D

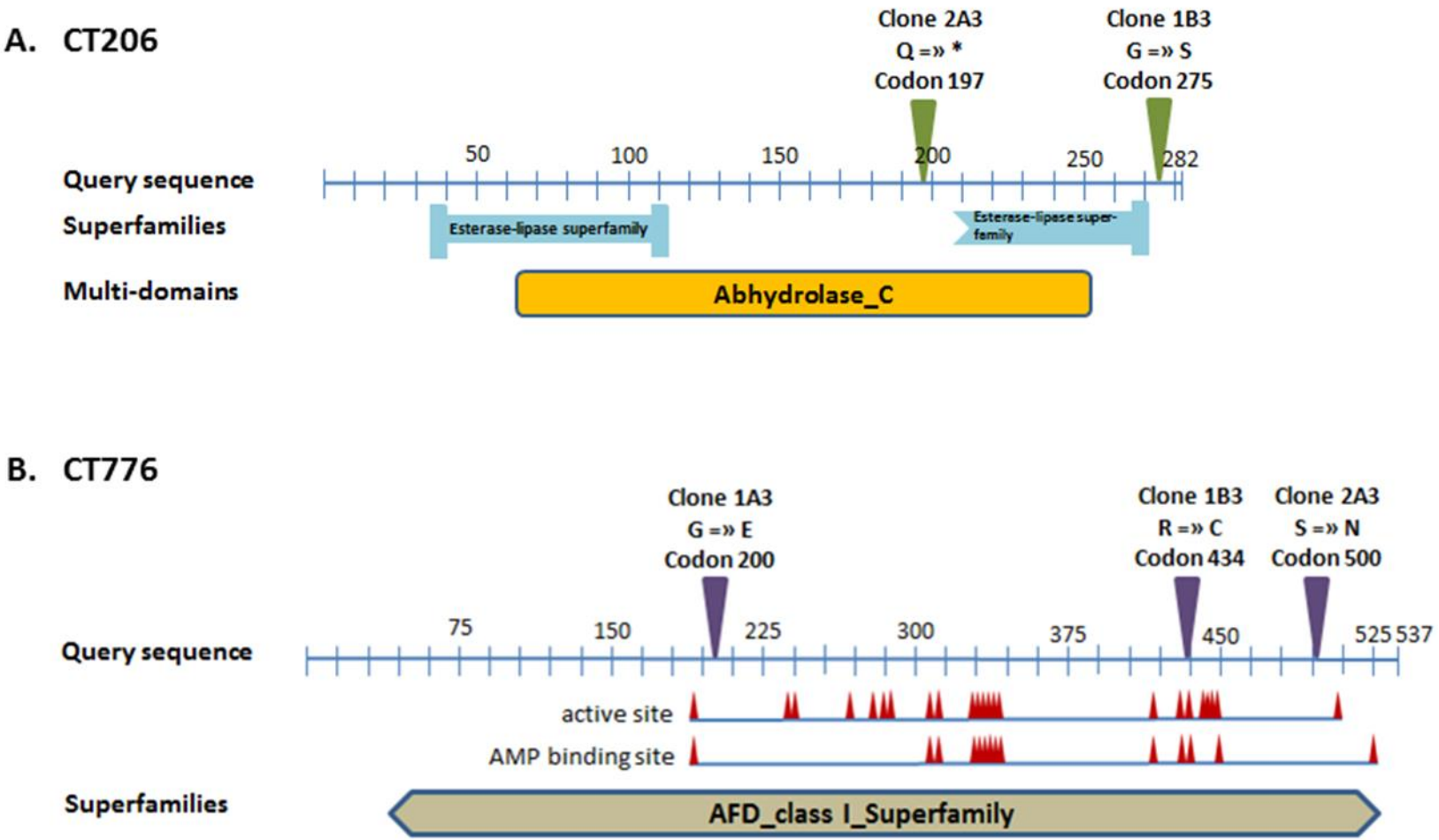


Figure 5.11. Schematic diagrams showing the locations of the single nucleotide variations (SNVs) in the genes CT206 (A) and CT776 (B) for clones 1A3, 1B3, and 2A3.

The codon number (indicated by the green and violet pointed arrows for CT206 and CT776, respectively) where the SNVs were observed as well as the change in amino acids brought about by the SNVs for each of the genes for each clone are indicated in the diagrams. The superfamilies and domains wherein the SNVs were observed are also shown. *, stop codon.

5.4 DISCUSSION

It was previously demonstrated (as outlined in Chapter 4) that CtHtrA inhibition during mid-replicative phase resulted in significantly smaller inclusion vacuole size and loss of infectious progeny production in *C. trachomatis*. To identify and further understand the functions of CtHtrA for chlamydial development, the present study aimed to isolate mutants resistant to JO146.

Two different general selection protocols were conducted to generate and isolate *C. trachomatis* JO146 resistant mutants. The size of the library that would need to be screened to identify JO146 resistant mutants was determined based on an estimated rate of 1 mutation per genome. The *C. trachomatis* genome contains 430,636 G-C base pairs and the present study aimed to accomplish four times coverage of the genome, hence the starting library of 2.0×10^6 EBs. The present study failed to isolate JO146 resistant or less susceptible strains through the utilization of the 96-well library format. Different factors such as host cell density and the surface area of the cell culture system might have contributed to the failure to isolate resistant mutants using this protocol.

However, a multiple passage selection process which involved continued cultivation of EMS mutated or non-mutated *C. trachomatis* D in the presence of the inhibitor JO146 resulted in the successful selection of mutants. Both EMS mutagenized and non-mutagenized stocks of plaque cloned *C. trachomatis* D (UW-3/Cx) were used in the selection process. The top three least susceptible clones, 1A3 and 1B3 (EMS mutagenized) and 2A3 (non-mutagenized), were further phenotypically and genotypically characterized. The method of isolating mutants resistant to an inhibitor utilized in the present study was similar to that previously conducted by Bao and co-workers [183] wherein they were able to isolate *C. muridarum* (mouse pneumonitis pathogen, MoPn) resistant to benzylidene acylhydrazides, a novel class of antichlamydials that act by inhibiting the *Chlamydia* T3SS virulence system.

In the present study, the higher number of recoverable IFUs in the cultures infected with the mutants compared to that of the cultures infected with CtDpp at both 30 and 44 h PI may be a consequence of the mutation and explains why these

clones were less susceptible to JO146. Previous experiments in our laboratory determined that JO146 was more effective at a lower multiplicity of infection (MOI) [250]. Therefore, it can be speculated that the higher infectious progeny generated by the clones made them less sensitive to JO146 inhibition, and that the mutations might be indirectly conferring the resistance via this manner. However, surely other loci that can enhance growth kinetics would have been selected for if this was the mechanism of resistance. As the less susceptible clones were isolated after sequential treatments with JO146 under normal growth conditions, it can be hypothesized that the continued exposure to the compound likely conferred a positive selective pressure on these mutants, particularly on the genes that might be indirectly involved in the CtHtrA function such as the acyltransferase genes wherein unique SNPs where observed in the clones. The transcript for CtHtrA is first detected at 8 h PI and accumulates to its highest levels toward the end of the developmental cycle [90]. The findings in the present study signify the possibility that continued cultivation while adding JO146 at 16 h PI for every passage may have positively enhanced the cellular functions of CtHtrA-related genes and the stability of *Chlamydia* over time. Furthermore, the growth analysis of the three clones demonstrated that none of the SNVs observed in the clones affected an essential gene, as revealed by the high infectious yield generated in these cultures. In addition, any mutants that were resistant to the inhibitor could have been thought to have competitive disadvantage over wild-type and other strains during growth in the absence of the compound. However, the mutants did not exhibit growth disadvantage as implicated by the higher yields of infectious progeny.

Genomic DNA sequence analysis indicated that as expected, the three mutants were genetically unique since they came from different pools. Among the three clones, 2A3 had the most number of SNVs. It was particularly striking because acquiring eight SNVs in non-EMS mutagenized clone 2A3, after 26 serial cultivations in the presence of JO146 corresponds to a high rate of mutation. *C. trachomatis* L2/434/Bu has a rate of mutation per base per replication of 0.0011 (i.e. 0.11% of the new clones generated after one single replication in a bacterial population will carry a mutation) [299]. However, it has been proposed that mutation rates in microbial populations can increase physiologically through different ways [300]. Ninio [301] suggested that errors of transcription, translation and molecular

segregation could lead to production of mutators which would immensely contribute to multiple mutations per genome per replication. In the present study, it can be speculated that the continuous serial transfer of pool 2A in cultures with JO146, a process that selects for, or against certain mutators, may have mediated the bacteria that were recently selected for a mutation to be under selective pressure for a second mutation, and so on and so forth. This process may explain why 2A3 acquired a high number of SNPs compared to other clones after 26 continued cultivations in the presence of the inhibitor. However, it was observed that different clones from different pools exhibited varying susceptibility to JO146. Non-resistant clones were isolated from pools that were resistant to JO146. For instance, none of the clones 2B1, 2B2 and 2B3 were resistant to JO146 despite the fact that the "parent pool", 2B, was resistant or less susceptible to JO146 at 125 µM. This indicates that after 26 continued cultivations in the presence of JO146, the pools were heterogeneous (i.e. the mutant alleles were not yet evolutionarily "fixed" in the populations).

Unique single base substitutions were observed in the CT206 gene for both 1B3 and 2A3. All of the clones had unique SNPs in the gene CT776. Both CT206 and CT776 encode for acyltransferases. CT206 is annotated as a predicted acyltransferase family protein while CT776 is annotated as Acyl-acyl carrier protein synthase (Aas). Aas functions as 2-Acylglycerophosphoethanolamine (2-Acyl-GPE) acyltransferases *in vivo*. In *E. coli*, 2-acyl-GPE acyltransferases and acyl-acyl carrier protein (acyl-ACP) synthase, both encoded by *aas* gene, mediate the acyl-CoA-independent incorporation of exogenous fatty acids and 2-acyllysophospholipids into the cell [302]. Mutation in *aas* resulted in the inactivation of the two activities, leading to lack of exogenous lysophospholipid uptake and acylation [302, 303]. In the present study, single base substitutions in the active sites of *aas* gene, as observed for 1A3 and 1B3, as well in the non-active site of *aas*, in the case for 2A3, did not have any negative effects on the growth characteristics of the mutants. This was indicated by the high infectious progeny yield during normal development and during stress conditions for all the three mutants. The change in the amino acid to one with different properties (e.g. basic to polar), brought about by single nucleotide changes, may have altered the tertiary structure of the encoded protein. However, it can be speculated that these mutations did not change the protein functions of *aas* but rather compensated for the inhibition of CtHtrA functions by JO146, although not

affecting CtHtrA per se. HtrA in other bacteria have been found to play essential roles in membrane integrity and outer membrane protein biogenesis [304-306].

It was previously demonstrated that CtHtrA was upregulated during heat stress [19] and this project (outlined in Chapter 4) demonstrated that inhibition of CtHtrA during heat stress was lethal to *C. trachomatis*. The number of infectious progeny formed after JO146 treatment during heat stress indicated that isolate 1A3 had a lesser number of recoverable EBs compared to the other mutants, 1B3 and 2A3. Both 1B3 and 2A3 had mutations in the gene CT206. The absence of mutation in gene CT206 for 1A3 can be hypothesized to have led to more binding capacity of JO146 to CtHtrA. This may have resulted to the increased susceptibility of 1A3 to heat stress in the presence of JO146. Therefore, it can be speculated that changes in CT206 might have allowed *Chlamydia* to survive heat stress in the presence of JO146, as observed for clones 1B3 and 2A3. The key function of HtrA proteins is heat stress response, most likely for the proteolytic degradation of damaged or misfolded proteins that are likely to form aggregates during this stress condition. Mutation in *E. coli degP*, a homologue of HtrA, led to the absence of growth at elevated temperatures [307], suggesting that HtrA is essential for heat stress response. Furthermore, *cthtrA* heterologously complemented the lethal high temperature phenotype of *E. coli htrA*- [19]. CT206 might be one of the loci involved in heat stress functions for CtHtrA. Although CT206 was annotated as an acyltransferase in *C. trachomatis* L2, conserved domain analysis suggests hydrolase, lipase, peptidase and esterase functions. The hydrolase, lipase, and peptidase functions of CT206 may facilitate the proper hydrolysis of lipids in the membrane, as well as the proteolytic cleavage and proper protein folding of OMPs when *Chlamydia* is challenged by heat stress. Previous investigation in this project (outlined in Chapter 4) has determined that addition of JO146 during heat stress for 4 hours led to more than 2 log less infectious progeny. In the present study, treatment with 50 µM JO146 during heat stress for the mutants except for strain 1A3, led to less than 1 log reduction in the number of infectious progeny compared to DMSO control cultures, indicating that even during heat stress these mutants were less susceptible to JO146. This is interesting as the selection process was conducted during normal development, not during heat stress, indicating important functions for chlamydial development especially during the replicative phase.

Assessment of the production of infectious progeny during recovery from penicillin persistence demonstrated that the mutant clones produced a higher yield of EBs than wild type CtDpp. CtHtrA levels are upregulated during penicillin persistence but not during IFN-γ persistence [19, 21, 22]. Previous experiments (outlined in Chapter 4) for this project demonstrated that addition of JO146 during reversion from penicillin persistence was lethal to *Chlamydia*. The control cultures (treated with 0 μM JO146, media only) for this experiment had a lower number of recoverable EBs (1.71×10^4 IFU ml^{-1}) for 100 h PI or 60 h after removal of penicillin from the cultures compared to the mutant strains analysed in the present study. The mutants 1A3, 1B3 and 2A3 had $\sim 10^5$ IFU ml^{-1} (1.69×10^5 IFU ml^{-1} for 1A3) recovered EBs at 88 h PI or 58 h after removal of penicillin from the cultures, indicating that the mutants have a higher number of infectious progeny during reversion from penicillin persistence than wild type.

Persistence is hypothesized to provide *Chlamydia* with a survival mechanism during adverse conditions such as iron limitation, amino acid deprivation, immune pressure, co-infection with other intracellular pathogens, and the presence of antibiotics such as penicillin [160, 271-273]. Penicillin typically acts by binding to penicillin-binding proteins (PBPs), blocking the synthesis of peptidoglycan (PG) [150, 151]. Although the mechanism is poorly understood, *C. trachomatis* persistent infection induces reactive synovial arthritis to a limited number of patients [308]. CT776, which codes for 2-Acyl-GPE acyltransferase, was identified to be one of *C. trachomatis* genes orthologous to the genes upregulated during persistence in *Mycobacterium tuberculosis* [308]. CT776 was expressed generally the same levels in the patient synovial biopsy fluid sample as the *in vitro* monocyte model of persistence [308]. In the present study, CT776 and another acyltransferase protein, CT206, were found to be mutated in the clones. All of the three clones, 1A3, 1B3 and 2A3 had mutations in CT776, however, despite these mutations, they recovered more infectious yield than the wild type CtDpp during recovery from penicillin persistence.

Acyltransferases transfer acyl thioesters to a variety of different substrates [309]. The long-chain-length hydrophobic acyl residues, one of the compounds transferred by acyltransferases, play important, versatile roles in a suite of biological processes, such as building the hydrophobic layers of biological membranes,

modification of protein properties, as well as acting as membrane anchors [309]. Acyltransferases are also involved in the metabolism of fatty acids. *C. trachomatis* encodes all the genes that are important for bacterial type II fatty acid synthesis (FAS II) and for the synthesis of phospholipids that are the main component of bacterial membranes namely, phosphatidylethanolamine (PE), phosphatidylglycerol (abbreviated in the present study as PTG) and cardiolipin (CL) [310]. Yao and co-workers [311] identified that during RB replication, the genes that are required for FAS II, a process that depends on Fab1 (enoyl-acyl carrier protein reductase), were induced while CL genes were upregulated during RB to EB differentiation phase. FAS II was essential for the replication of *C. trachomatis* and inhibition of CtFab1 blocks *C. trachomatis* replication [311]. Recovery from penicillin persistence involves replication of active RBs after they asynchronously revert back from aberrant bodies (ABs) [136]. In the present study, mutations in the acyltransferases CT206 and CT776 in the clones 1A3, 1B3 and 2A3, may have positively, rather than negatively, enhanced these genes' pivotal functions for the lipid metabolism and membrane biogenesis of RBs that were replicating during penicillin persistence reversion. Although 2A3 had a null mutation for CT776, the absence of this gene's function can be speculated to be compensated by the presence of a beneficial mutation in gene CT206 for this mutant. For all the mutants, the high number of RBs in turn, led to higher number of infectious EBs observed for the clones during recovery from penicillin persistence. It is noteworthy that these clones generated high infectious yield during normal development. Therefore, it is highly likely that their ability to recover more infectious progeny during normal growth condition which would have been brought about by beneficial mutations in CT776, conferred them an advantage over the wild type CtDpp even during recovery from penicillin persistence.

Confocal microscopy revealed that the inclusions appeared atypical from all the resistant mutants as well as the control wild type CtDpp. It was observed through confocal microscopy that inclusions of the mutant clones appeared to contain aberrant reticulate bodies, characteristic of persistent *Chlamydia*, than the inclusions in CtDpp at 88 h PI (58 h after removal of the penicillin) although all of these strains had higher yields of infectious EBs at this time.

In conclusion, the present study demonstrated that mutations in CT206 and CT776 loci yielded mutants that were capable of generating high infectious progeny yield during normal development, heat stress in the presence of JO146 as well as during recovery from penicillin persistence. The isolated mutants that were less susceptible to JO146 had specific phenotypes that may be associated with alterations in acyltranseferase genes that may have resulted in cell membrane integrity especially during conditions that were detrimental to the organism such as heat stress and penicillin persistence. The less susceptible clones had higher recoverable EBs during the late stages of the chlamydial developmental cycle, indicating that the specific mutations observed through sequence analysis, particularly the gene CT776, might have enhanced the growth kinetics of the clones. Furthermore, the clones were less susceptible to the CtHtrA inhibitor even during heat stress and were demonstrated to have higher yields of infectious progeny compared to wild-type *C. trachomatis* D (CtDpp) during reversion from penicillin persistence. Comparative analysis of genomic sequences between the clones and the wild-type CtDpp identified that acyltransferases CT206 and CT776 could be indirectly involved in membrane protein biogenesis function of CtHtrA. Mutations in these two genes could have led to downstream effect on CtHtrA, outer membrane assembly, or lipid biosynthesis. It is highly likely that these mutations were compensating for the inhibition of CtHtrA by JO146, rather than directly impacting CtHtrA. It can be that base substitutions in these two loci, particularly CT776 as it was present in all the clones, were somehow responsible for the resistance phenotype of the mutants.

Although a truly resistant variant was not generated in the present study, the isolation of mutants that were less susceptible to JO146 signifies a challenging feat to *Chlamydia*, which for a long period of time has been thought to be genetically unamenable. In summary, phenotypic characterization of the mutants with reduced susceptibility to JO146 demonstrated no significant growth defect and higher recovery of infectious particles during stress conditions compared to wild-type CtDpp. These data indicate that microevolutionary acquisition of mutations in the genes involved in fatty acid synthesis, CT206 and CT776 might have conferred better growth kinetics and higher infectious yield during normal growth conditions as well as during stress conditions compared to wild type CtDpp. All of the three clones had mutations in CT776, indicating that CT776 may have been the major target site

of mutation in the clones during serial passages *in vitro* in the presence of JO146. CT776 has also been previously demonstrated to be involved in the recycling of phosphoethanolamine, a major component of the bacterial membrane, during lipoprotein turnover [311]. Therefore, CT776 may have bacterial membrane biogenesis/maintenance functions that are highly related to the functions of CtHtrA especially during heat stress and recovery from penicillin persistence.

Chapter 6: *In vitro* susceptibility of recent *Chlamydia trachomatis* clinical isolates to the HtrA inhibitor JO146

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STATEMENT OF JOINT AUTHORSHIP

The authors listed below have certified that:

1. they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
2. they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. there are no other authors of the publication according to these criteria;
4. potential conflicts of interest have been disclosed to (a) granting bodies, and (b) the editor or publisher of **Microbes and Infection**, and;
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Contributor	Statement of contribution
Vanissa A. Ong (candidate) Signature  Date 14/7/15	Contributed to the design and implementation of overall project, conducted the majority of the laboratory work in the project, conducted the analysis and interpretation of the data and contributed to writing of the manuscript
Amba Lawrence	Conducted some of the experiments, contributed to the intellectual input of the manuscript, contributed to manuscript drafting
Lenka A. Vodstrcil	Provided feedback on experimental design, contributed to manuscript drafting
Peter Timms	Provided feedback on experimental design, contributed to the intellectual input of the manuscript
Sepehr N. Tabrizi	Provided feedback on experimental design, contributed to the intellectual input of the manuscript

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John A. Allan	Provided feedback on experimental design, contributed to the intellectual input of the manuscript
Jane S. Hocking	Provided feedback on experimental design, contributed to the intellectual input of the manuscript
Wilhelmina M. Huston	Contributed to research plan development and provided feedback on experimental design, data analysis and executions; contributed to the intellectual input of the manuscript, critically reviewed manuscript and approved final version of manuscript

Principal Supervisor Confirmation

I have sighted email or other correspondence from all Co-authors confirming their certifying authorship.

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Name	Signature	Date

6.1 INTRODUCTION

Chlamydia trachomatis is an obligate intracellular bacterial pathogen and is the most commonly reported bacterial sexually transmitted pathogen worldwide. In the United States, over 1.4 million cases of *C. trachomatis* infection were reported in 2012, the highest number of cases ever reported to CDC for any condition [38]. Due to the asymptomatic nature of the disease [1-3], many infections are neither diagnosed, nor treated and therefore the actual burden of disease is likely underestimated. *C. trachomatis* can have serious sequelae including pelvic inflammatory disease, infertility and ectopic pregnancy as well as chlamydial conjunctivitis and pneumonia in neonates passing through the birth canal of an infected woman [18].

Currently, the recommended first line of treatment for uncomplicated genital *C. trachomatis* infections is a single 1.0 g oral dose of the macrolide antibiotic azithromycin [71]. However, a number of recently published studies challenge the efficacy of azithromycin therapy for chlamydial infections [75-78, 312]. Batteiger and co-workers [78] conducted a cohort study among adolescent women and used a classification algorithm to characterize treatment outcomes among the study subjects after directly observed azithromycin treatment. Among women with paired episodes of chlamydial infections, 8% were probable treatment failures. A partner treatment study conducted by Golden and co-workers [312] reported that 8% (22 of 289 originally treated for *Chlamydia)* of cases treated reported no sexual intercourse after treatment and were classified as treatment failures. These studies suggest the possible future need for new anti-chlamydial therapies.

The *C. trachomatis* developmental cycle consists of an infectious extracellular form called the elementary body (EB) and the intracellular replicative form termed the reticulate body (RB) that divides by binary fission prior to converting back to EBs that can initiate further rounds of infection (reviewed [85]). In the search for new treatment options for infectious diseases, small molecule approaches have been previously described for many bacteria [183, 262, 313, 314]. Through the use of *Yersinia* type three secretion inhibitor, Wolf and co-workers [188] identified that the chlamydial type III secretion system plays an important role in the progression of *C. trachomatis* developmental cycle

and could be a therapeutic target. Our group identified a serine protease inhibitor, JO146, specific for the *C. trachomatis* High Temperature Requirement A (CtHtrA) [19, 250, 254, 257]. JO146 was lethal to *C. trachomatis* D (UW-3/Cx) when added at the mid-replicative stage of the chlamydial developmental cycle [250]. The addition of JO146 was lethal during reversion or recovery from penicillin persistence and during heat stress [315]. In enteropathogenic organisms such as *Escherichia coli*, *Campylobacter jejuni*, and *Shigella flexneri*, HtrA was exported and cleaved E-cadherin on host cells disrupting the gut epithelial barrier [10]. HtrA contributes to the virulence of *Clostridium difficile* [316]. Therefore, HtrA is a key virulence factor for many pathogens and could be a good target for development of new therapeutics.

Laboratory strains of *C. trachomatis* that are commonly used for biological experiments may not reflect the isolates currently infecting men and women [317]. Differences in genome dynamics, and virulence attributes and infectivity [210, 299, 318] may result in varying sensitivities to JO146 between recent clinical isolates and the type strains of *C. trachomatis* used for investigations to date. Therefore, the present study aimed to validate that CtHtrA is a clinically relevant target for potential future therapeutic development by testing the efficacy of the inhibitor JO146 against recent clinical isolates from women.

6.2 MATERIALS AND METHODS

6.2.1 Clinical isolates, *Chlamydia* culture and J0146 treatment conditions

Six *C. trachomatis* clinical isolates were obtained and cultured from separate women enrolled in the Australian *Chlamydia* Treatment Study (ACTS) [319] (The Alfred Human Research Ethics Approval number 223/12). The isolates were designated as: 1-017(13) (serovar K), 1-079(1) (serovar G), 1-019(1) (serovar D), 1-048(1) (serovar E), 1-028(1) (serovar E), and 1-020(1) (serovar D). The isolates were cultured in McCoy B cells grown in Dulbecco's minimal essential medium (DMEM, Life Technologies, Eugene, OR, U.S.A.)

supplemented with 5% fetal calf serum (Lonza), 10 µg ml^{-1} gentamicin (Invitrogen, Eugene, OR, U.S.A), 100 µg ml^{-1} streptomycin sulphate (Sigma-Aldrich, St. Louis, MO, U.S.A.), incubated at 37°C, 5% CO_2.

The impact of JO146 on *Chlamydia* was determined in McCoy B cells infected at a multiplicity of infection (MOI) of 0.3 by centrifugation for 30 minutes at 500 × g at 28°C. At 16 hours post infection (h PI), the cells were treated with JO146 (0, 10, 50 and 100 µM) and DMSO (solvent) control (all experiments were done in triplicate, on at least two separate occasions, with one representative occasion shown). At the completion of experiment (44 h PI unless otherwise stated) *Chlamydia* were harvested into storage medium (sucrose phosphate glutamate (SPG): 10 mM sodium phosphate, 250 mM sucrose, and 5 mM L-glutamine for subsequent determination of infectious yield (IFU ml^{-1} as described in Materials and Methods section 3.4).

The bacteriostatic or bactericidal effect or duration of treatment required for JO146 effect was determined by removal of the compound from the cultures after 8 h treatment. At 16 h PI *C. trachomatis* cultures were treated with either 0 µM (media only), 100 µM JO146, or DMSO. At 24 h PI (i.e. 8 hours after treatment) treatments were removed by three sequential washes with pre-warmed media and the cultures continued until harvest into SPG media at 44 and 68 h PI.

Inclusion forming units (IFU ml^{-1}) were determined by subsequent passage culture on McCoy B cells and enumerated through immunocytochemistry and confocal microscopy as described in Materials and Methods section 3.4.

6.2.2 **Confocal microscopy**

McCoy B cells infected with *C. trachomatis* cultured on coverslips were used for confocal microscopy. At nominated time points the cells were fixed and immunocytochemistry conducted as previously described in Materials and Methods section 3.5. Confocal images were obtained using an Olympus FV1200 confocal laser scanning microscope (FluoView® FV1200, Olympus Corporation, Japan). Sizes of 30 independent inclusions for each treatment and time point were measured manually through the use of NIS-Elements Basic Research 3.2 software.

6.2.3 **Western blot**

Cultures of T25 flasks of McCoy B cells infected with *C. trachomatis* were harvested for western blot analysis of the major outer membrane protein (MOMP) and host β-actin after JO146 treatment at 16 h PI and harvested at 24 h PI. Western blots were conducted as previously described [22]. Briefly, cells were lysed with SDS PAGE buffer (50 Mm Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 12.5 mM EDTA, 0.02% bromophenol blue), followed by electrophoresis and immunoblotting using a standard protocol. The blots were probed with sheep anti-*C. muridarum* MOMP (Southern Biotech, Alabama, U.S.A.), followed by rabbit anti-sheep-BIOT (Southern Biotech, Alabama, U.S.A.), then streptavidin-HRP conjugates (Southern Biotech, Alabama, U.S.A.). Duplicate blots were probed with anti β-actin polyclonal antibody (Abcam, Australia) followed by an anti-rabbit secondary antibody conjugated to HRP (Southern Biotech, Alabama, U.S.A). The reaction was developed with Pierce ECL Western Blotting Substrate (Thermo Scientific, Australia).

6.2.4 **Statistical analysis**

Statistical analysis of all results was performed using PRISM (GraphPad Software Inc., V7.0). Statistical significance was defined as $p < 0.05$. Statistical tests used and number of samples are indicated with each figure.

6.3 RESULTS

6.3.1 **HtrA inhibition using JO146 during McCoy B culture of chlamydial clinical isolates prevents chlamydial inclusion vacuole size development**

Our previous work using HEp-2 cells and *C. trachomatis* D (UW-3/Cx) demonstrated that the inclusions decreased in size and were lost from the host cells (Chapter 4) [250]. Therefore, the present study examined the morphology of the McCoy B cultures of the clinical isolates at 20, 24, and 40 h PI after JO146 treatment (16 h PI) using immunocytochemistry and confocal laser scanning

microscopy. JO146 treatment resulted in smaller inclusion vacuole sizes than the DMSO treated control cultures (Figure 6.1). Inclusions were visible up to 40 h PI in all cultures after JO146 treatment and there were no visible differences in the intensity of staining or appearance of the chlamydial cells inside the inclusions at any time point compared to DMSO controls (Figure 6.1), only that the size of the inclusions did not increase to the same size as controls.

Inclusions sizes were measured to quantify these observations. This difference in inclusion size was apparent at 24 h PI although only four clinical isolates (1-079(1), 1-079(13), 1-019(1), and 1-028(1)) showed statistically significant differences ($p < 0.01$) in the size of inclusions compared to DMSO control at this time point (Figure 6.2). At 40 h PI, *Chlamydia* inclusions in the presence of JO146 were smaller than those formed in the control (DMSO-treated cells) in all clinical isolates as well as for the *C. trachomatis* D (UW-3/Cx) a laboratory isolate ($p < 0.0001$) (Figure 6.2).

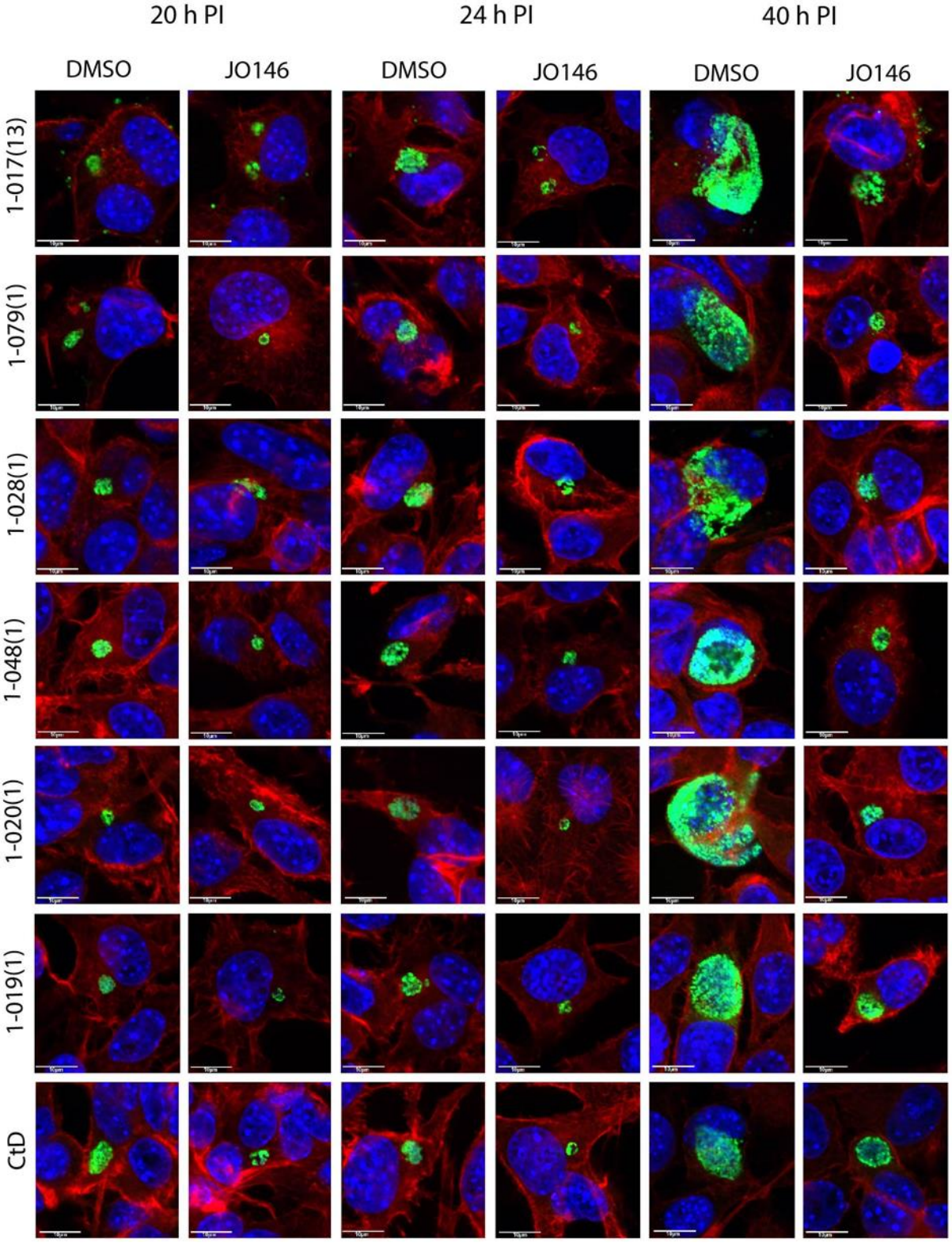

Figure 6.1. Confocal microscopy images of *C. trachomatis* clinical isolates treated with JO146 or DMSO at 16 h PI and examined at 20, 24, and 40 h PI

Representative images of control (DMSO treated) cultures are shown on the left panel while representative images of cultures treated with JO146 are shown on the right for each time point (time points indicated above figure) (strain identity indicated to left of the figure). The image colours are as follows, green; MOMP (major outer membrane protein); blue: host cell nucleus (stained by DAPI); red: β actin (stained by phalloidin 594). Scale bar (bottom left) indicates 10 µm.

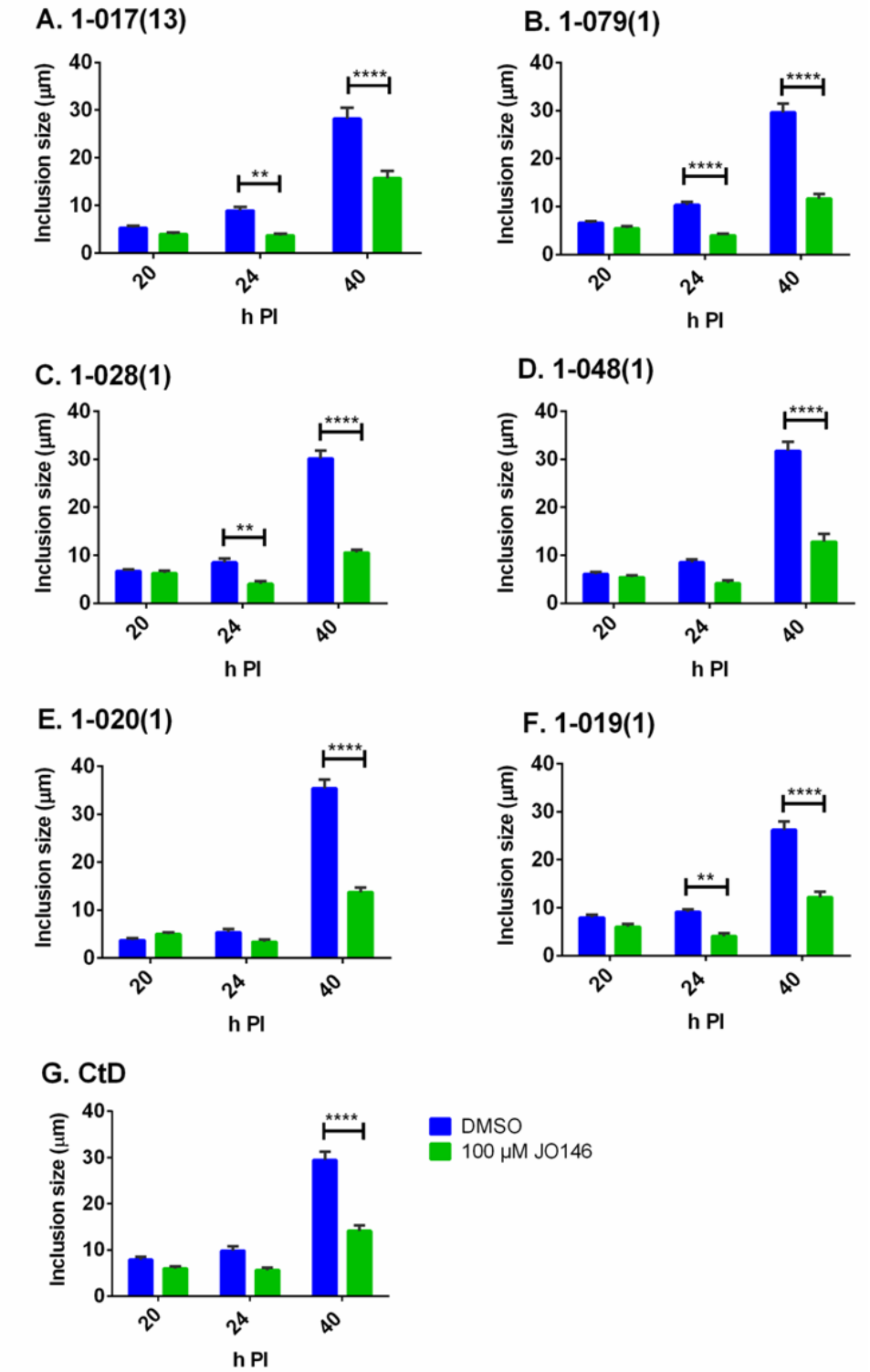

Figure 6.2. Inclusion sizes at 20, 24 and 40 h PI for DMSO and JO146 treated isolates of *C. trachomatis* grown in McCoy B cells are shown in the graphs

DMSO and 100 µM JO146 treatments are shown in blue and green columns, respectively. Each isolate is shown in a separate graph: A. 1-017(13), B. 1-079(1), C. 1-028(1), D. 1-048(1), E. 1-020(1), F. 1-019(1), G. *C. trachomatis* D (UW-3/Cx). Statistical analysis was conducted using Two-Way ANOVA with Bonferroni's multiple comparison tests. The bar colours represent treatment conditions; blue: DMSO, and green: 100 µM

JO146. Data are presented as mean ± S.E.M (n=30), ** indicates $p < 0.01$, ****$p < 0.0001$.

6.3.2 JO146 treatment during mid-replicative phase of chlamydial development in McCoy B cells leads to loss of infectious progeny for clinical isolates

The effect of 16 h PI JO146 treatment on the development of infectious progeny was tested. No infectious progeny (44 h PI) were observed for all clinical isolates treated with 50 and 100 µM JO146 except for isolate 1-017(13) (Figure 6.3). For isolate 1-017(13) treatment with 50 µM JO146 resulted in approximately 1 log less infectious yield compared to DMSO and media controls ($p < 0.001$) and 100 µM was lethal as consistent with the other clinical isolates. Treatment of the type strain *C. trachomatis* D with 50 and 100 µM JO146 both resulted in ~1.5 log less infectious progeny compared to that observed in the DMSO control ($p < 0.0001$) (Figure 6.3).

The reduction in infectious progeny and reduced inclusion sizes (Figure 6.2) should correlate with a decrease in the detection of chlamydial protein. A western blot to detect levels of MOMP at 24 h PI, 8 h after JO146 treatment at 16 h PI, shows a clear reduction on MOMP levels compared to matched DMSO controls and relative to the host protein β-actin (Figure 6.4).

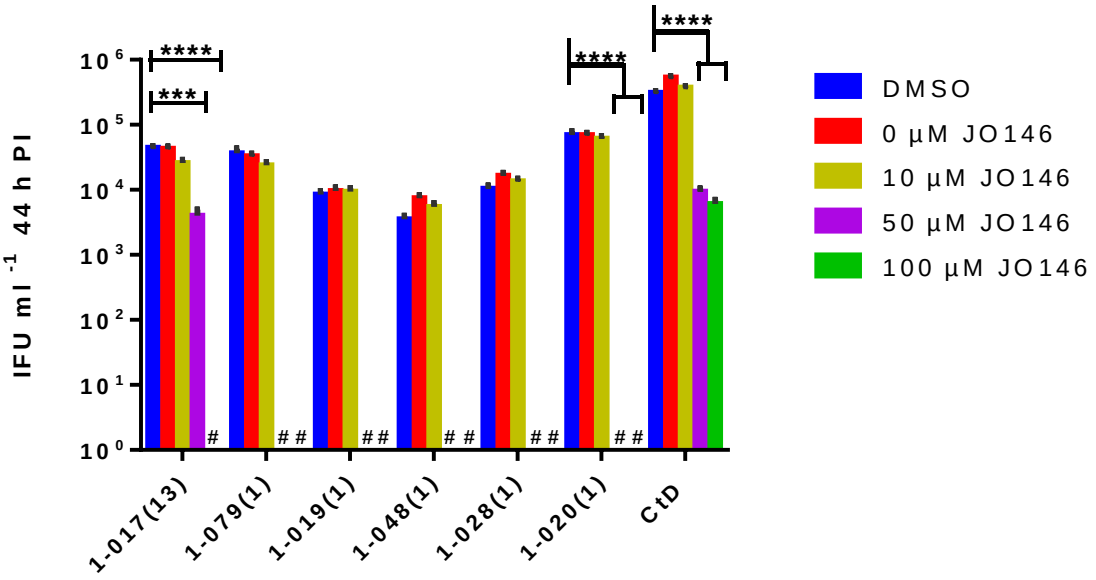

Figure 6.3. Inclusion forming units after treatment with JO146 at 16 h PI for each of the clinical isolate and control CtD

The bars are in colours (as indicated on the right) to represent the different concentrations of JO146. The identity of each of the isolates is indicated on the x-axis and the IFU ml-1 are indicated on the y-axis in log scale. Statistical analysis was conducted using Two-Way ANOVA with a post hoc Bonferroni multiple comparison test relative to the DMSO control. Error bars represent the standard error of the mean (n=27) (non-logarithmic data), *** indicates $p < 0.001$, ****$p < 0.0001$. # indicates no detectable inclusion forming units.

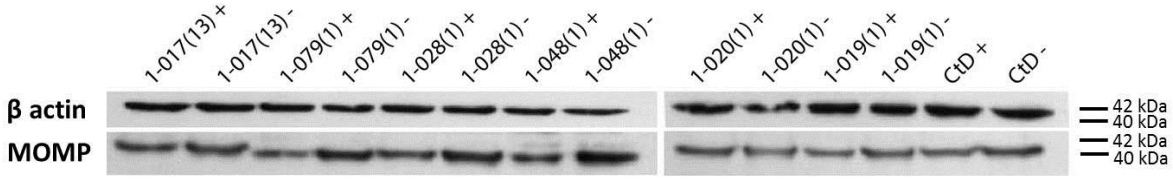

Figure 6.4. Western blot for MOMP in cell lysates of JO146-treated and DMSO-treated *C. trachomatis* clinical isolates in McCoy B cells.

JO146 or DMSO were added at 16 h PI and cells were harvested at 24 h PI (i.e. 8 hours after treatment). Treatments are indicated above each lane at the top of each isolate name. "+" denotes treatment with JO146 and "-" denotes treatment with DMSO control. Laboratory strain C. trachomatis D (UW-3/Cx) (CtD) was included as a control strain. The size of relevant molecular weight markers are indicated to the right of the figure and the western blot identity (i.e. MOMP or β-actin) are indicated to the left.

6.3.3 **JO146 requires long treatment times to be effective against *C. trachomatis* in McCoy B cultures**

In order to determine if JO146 activity against chlamydial clinical isolates is effective with a short duration of treatment, a reduced time of treatment with JO146 was conducted. Removal of JO146 from the cultures at 24 h PI (8 h after addition) resulted in recovery of infectious progeny at 44 and 68 h PI in all clinical isolates (Figure 6.5) in contrast to complete bacterial lethality when the compound was left in the cultures (Figure 6.3). In *C. trachomatis* clinical isolates 1-028(1) and 1-019(1), there were fewer infectious EBs in cells treated with 100 μM JO146 compared to the cells treated with 0 μM JO146 (media only) and DMSO controls either at 44 or 68 h PI (Figure 6.5). A non-significant increase was observed in the number of infectious progeny at 68 h PI compared to 44 h PI with 100 μM JO146 for isolates 1-017(13), 1-079(1), 1-048(1) and 1-020(1) (Figure 6.5). Overall, an 8 h JO146 treatment of the cultures resulted in a minor loss of infectious progeny compared to when the compound was left in the cultures until the end of the developmental cycle (Figure 6.3), suggesting that in McCoy B cultures there is a need for longer treatment duration for efficacy.

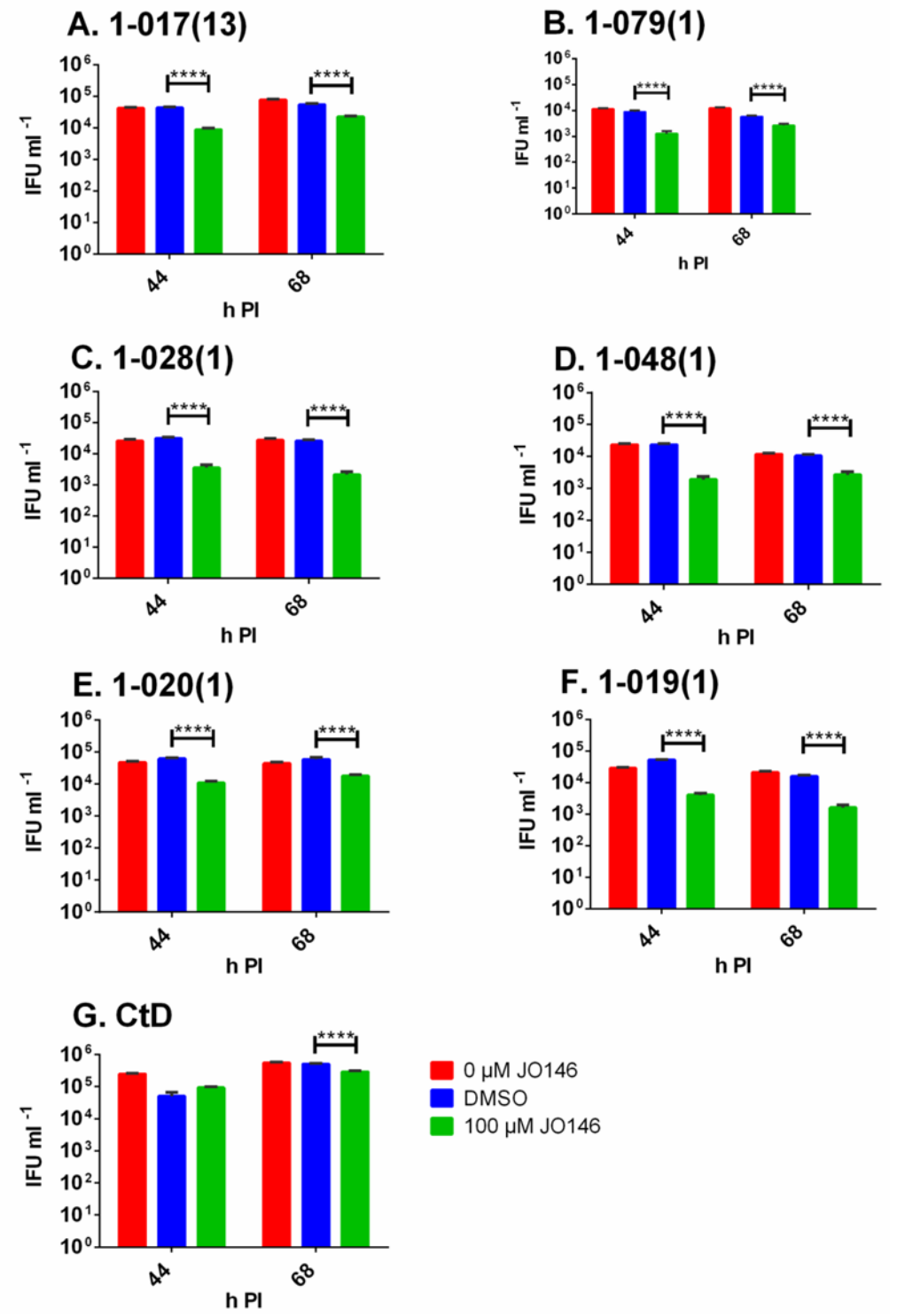

Figure 6.5. Inclusion forming units ml-1 at 44 and 68 h PI after JO146 addition at 16 h PI and removal after 8 h (at 24 h PI).

Conditions are represented by the coloured bars on the right (red: media only (0 µM JO146), blue: DMSO, green: 100 µM JO146). Each isolate is shown in a separate graph: A. 1-017(13), B. 1-079(1), C. 1-028(1), D. 1-048(1), E. 1-020(1), F. 1-019(1), G. *C. trachomatis* D (UW-3/Cx). The IFU ml^{-1} is indicated on the y-axis and the two time points (44 and 68 h PI) are indicated on the x-axis. Statistical analysis was conducted using Two-Way ANOVA with a post hoc Bonferroni multiple comparison test relative to the DMSO control. The error bars represent the standard error of the mean (n=27) (error bars are for the non-logarithmic scale), ****$p < 0.0001$.

6.4 DISCUSSION

The present study determined if the previous observations of a critical function for CtHtrA during chlamydial replicative phase was also relevant for recent clinical isolates of *C. trachomatis*. The addition of JO146 during mid-replicative phase was completely lethal if the compound was left in the cultures until the completion of the developmental cycle, providing preliminary supporting that JO146 is effective against clinical isolates. These experiments were conducted in McCoy B cells. This was consistent with other previously reported studies on *C. trachomatis* clinical isolates that all mainly used McCoy B cells (mouse fibroblasts) as host cells [320-322] due to difficulty in cultivating the isolates. As the effect of JO146 on *Chlamydia* grown on McCoy B cells has not been extensively studied, *C. trachomatis* D was included as a control in all experiments.

It had already been demonstrated that treatment of HEp2 cultures with the CtHtrA inhibitor JO146 resulted in diminishing chlamydial inclusion size, eventual loss of the inclusions, and loss of infectious progeny without being toxic to the host cells [250]. CtHtrA was found to be essential for the reversion and recovery to viability from penicillin persistence and during heat stress [299]. The critical role that CtHtrA plays during the replicative phase of the chlamydial developmental cycle was also demonstrated to be conserved among other *C. trachomatis* strains and other *Chlamydia* species such as *C. pecorum*, *C. suis*, and *C. caviae* [256]. HtrA has also been found to have a pivotal contribution to pathogenesis of other bacterial infections. Mutation in the *htrA* gene in *Bacillus anthracis* resulted in increased sensitivity to stress-related conditions such as reactive oxygen species, as well as heat and osmotic stress [278] and *htrA2* mutants in *Mycobacterium tuberculosis* were attenuated in mice [323].

The data presented here demonstrated that treatment with JO146 at mid-replicative phase resulted in chlamydial inclusions failing to increase in size and loss of infectious progeny for recent clinical isolates of *C. trachomatis* in McCoy B cells. The lack of lethality of 100 µM JO146 on type strain *C. trachomatis* D grown in McCoy B cells contrasts the previous result in HEp2 cells, in which complete bacterial lethality for this same strain was observed. These results

suggest that there could be differences in the pharmacokinetics of JO146 between mouse fibroblast (McCoy B) and human epithelial (HEp2) cell lines or in the chlamydial susceptibility during growth in these different cell lines. Previous studies have demonstrated different pharmacokinetics and bioactivity of drugs such as erythromycin in different mammalian cell lines [324, 325].

In the present study, the chlamydial inclusions were not completely lost from the host cells after JO146 treatment (in contrast to complete inclusion loss previously observed in HEp2 cells), however these inclusions did not contain infectious progeny (Figures 6.1 and 6.3) [250]. Infectious progeny (with comparatively minor loss compared to controls) were observed for all strains tested when the cultures were treated with JO146 for 8 h at the replicative phase whereas there was lethality when the inhibitor was left in the cultures until the end of the developmental cycle. This result indicates that the inhibitory effect of JO146 was reversible by removal of the compound from the cultures and may be bacteriostatic, or may require longer than 8 h treatment to be effective in McCoy B cells. The data presented here are not adequate to fully conclude the process by which JO146 has a lethal effect, or as measured here a loss of infectious progeny. In some models the inclusions are lost from the cells, although the mechanism does not appear to involve the lysosome or autophagy [22]. Here in McCoy B cells the inclusions failed to increase in size and did not contain infectious progeny. This difference in the underlying process leading to loss of infectious progeny could be explained by several reasons; in these fibroblast-like cells perhaps the dosing was reduced due to cellular processes or once the *Chlamydia* in the inclusion have been inhibited by the JO146 treatment in HEp2 a distinct host process than that of these fibroblasts was able to target the vacuoles.

The growth curves of these isolates have not been completed in full, but we do know from previous work in the team that maximal effectiveness of JO146 required dosing at mid-replicative phase, however, the impact did result in loss of infectious progeny. Although, previous growth curves analysis of different *C. trachomatis* strains show relatively minor differences in the timing of the replicative phase [89], hence we suspect the duration and timing of treatment in these experiments is within the replicative phase. Shorter exposure time to the compound were much less effective (Figure 6.5), with limited differences when

extended recovery times were permitted. This suggests that even though inclusions were present these cannot be rescued by extended growth (Figure 6.5). Nonetheless it is clear that CtHtrA is a valid possible target should future therapeutics need to be developed against *Chlamydia*, based on the effectiveness against these clinical isolates.

The subtle differences in JO146 efficacy observed between the isolates and the type strain *C. trachomatis* D is not able to be explained by differences in CtHtrA amino acid sequence. The CtHtrA sequence is highly conserved with at most 4 amino acids different in the 647 amino acid sequence across the published *C. trachomatis* genomes to date. The *C. trachomatis* genomes cluster into three predominant clades (LGV, T1, T2) [280]. The CtHtrA sequences that we have determined so far from our clinical isolates are consistent with the T2 clade (1-017(13), 1-017(1), 1-028(1), 1-048(1) (sequences will be published elsewhere). The amino acid variation is not near the residues that form active site where JO146 binds to the protein and is not likely to explain the variation.

In summary, this preliminary study supports that JO146 is effective against recent clinical isolates of *C. trachomatis*. The data could indicate the compound acts by a bacteriostatic mechanism of activity in McCoy B cells and is lethal when added mid-replicative phase and maintained until the conclusion of developmental cycle. Alternatively, the data may indicate that extended exposure throughout the developmental cycle in McCoy B cells is needed for the maximum impact of JO146. Together, our study indicates that *in vitro* application of an inhibitor compound that targets CtHtrA during the replicative phase of recent clinical isolates of *C. trachomatis* is lethal. CtHtrA therefore, could be a potential target for future drug development for *C. trachomatis* and that CtHtrA inhibition should be further investigated using additional cell lines and clinical isolates.

Chapter 7: **General Discussion**

In the present study, a critical role of *Chlamydia trachomatis* High Temperature Requirement protein A (CtHtrA) for the replicative phase of the developmental cycle, for heat stress, and recovery from penicillin induced persistence were demonstrated using a chemical inhibitor, JO146. Genome sequence analysis of mutants with less susceptibility to JO146 identified that two genes, which code for acyltransferases, acquired single nucleotide variants (SNVs), indicating that these genes might be involved in CtHtrA function. JO146 was also found to be effective against currently circulating clinical isolates of *C. trachomatis* representing different serovars.

In the present study, addition of the CtHtrA-specific inhibitor, JO146, at 16 h PI (mid-replicative phase) completely inhibited the formation of infectious EBs as demonstrated by the complete loss of infectious yield when the cells were examined at the end of the chlamydial developmental cycle. This data indicated that the compound significantly inhibited RB replication. Treatment with JO146 at mid-replicative phase also led to loss of chlamydial cellular morphology and reduction in inclusion vacuole size when JO146-treated cells were examined for *Chlamydia* HtrA and major outer membrane protein (MOMP). The inclusions formed in JO146-treated cells were significantly smaller than the inclusions formed in the control, DMSO-treated cultures. These effects occurred without inducing measurable host cell cytotoxicity and were not mediated by host cell autophagy pathways. There was no significant difference in the number of dead cells between JO146-treated and DMSO-treated HEp2 cells. There was no sequestration between autophagy and lysosome markers, SQSTM1 and LAMP1 and the *Chlamydia* inclusions during JO146 treatment, indicating that the inhibitory effect of JO146 was not mediated by the host defence system or lysosome pathways. The lethality of JO146 was only partially reversible by removal of the compound, possibly indicating a bacteriostatic mechanism. The removal of the compound eight hours after it was administered led to recovery of infectious progeny at later time points compared to longer duration treatments, indicating that the most effective phase of inhibition was throughout the replicative phase until EB formation.

The complex developmental cycle and obligate intracellular lifestyle of chlamydiae has impeded efforts to establish a genetic manipulation system for the

organism for a long time. Chemical biology approaches such as the use of small molecule inhibitors are a strategy used for the investigation of the pathophysiology of bacteria and other organisms. The use of small molecule inhibitors that target specific virulence factors has been reported previously for many bacteria. Small molecule inhibitors designated as INP0010 and INP0400, a class of acylated hydrazones which were initially shown to inhibit *Yersinia pseudotuberculosis* T3SS [185], were also found to block *Chlamydia* T3SS mainly due to the homology between T3SS of *Yersinia* and *Chlamydia*. INP0010 and INP0400 inhibited *C. pneumoniae* developmental cycle and prevented replication of *C. pneumoniae* and *C. trachomatis* in *ex vivo* infection models respectively, thereby indicating an important role for T3SS in the chlamydial developmental cycle [186, 187]. INP0400 inhibition varied depending on the time of compound addition. Treatment of *C. trachomatis* cultures with INP0400 at mid-replicative phase blocked homotypic vesicular fusion, whilst adding the compound during the late-phase of the chlamydial developmental led to a marked decrease in infectious progeny production [187]. Therefore, it can be deduced that T3SS activity and the chlamydial development are interlinked processes and inhibition of T3SS resulted in stalled RB to EB differentiation [183, 188]. The inhibitor compound, 2-pyridone amide KSK120 that targets the glucose-6-phosphate (G-6P) metabolism pathway of *C. trachomatis* blocked glycogen accumulation in *C. trachomatis* [190]. Small molecule approaches have also been employed to determine the role of lipooligosaccharides (LOS) in *C. trachomatis*. Through the use of small molecule inhibitors of LpxC, the enzyme that catalyses the first committed step in the biosynthesis of lipid A, the synthesis of lipooligosaccarides (LOS) in *C. trachomatis* was blocked [189]. In the absence of LOS, *C. trachomatis* remained viable but non-infectious and replicative RBs failed to express selected late-stage proteins and transition to EBs [189].

The present study utilized the inhibitor, JO146, which was found to be specific to *C. trachomatis* and lethal to the organism when added at mid-replicative stage of the developmental cycle, to characterize and identify the role of CtHtrA in chlamydial development. HtrA in other bacterial pathogens have been found to be vital in host cell interaction through specific mechanisms. HtrA chaperone activity, which may be involved in folding of outer membrane

adhesins, facilitated the binding of *Campylobacter jejuni* to both epithelial cells and macrophages [8]. It is also well established that HtrA is important for survival of most bacteria during stress because HtrA degrades and prevents accumulation of misfolded periplasmic proteins during stress [241, 326, 327]. In *E. coli*, three periplasmic chaperones, DegP, Skp and SurA have a role in the proper folding and insertion of proteins into the outer membrane [247]. The major protease, DegP, a homologue of HtrA, is responsible for the degradation of misfolded envelope proteins especially at high temperature and is important for the rapid turnover of abnormal and normal (i.e. native and functional) proteins [241, 281]. Disruption of these processes causes growth defects and eventual cellular death due to prevention of proteolytic modifications to produce mature, specific proteins and failure to destroy potentially harmful polypeptide aggregates. In this respect, HtrA exhibits a rather indirect but very crucial effect for bacterial viability. Mutation in *degP(htrA)* and *surA* had bactericidal effect for *E. coli*, mainly due to loss of chaperone activity as the lethal phenotype can be complemented by *degP* lacking protease activity [328]. The role of Skp and DegP was amplified in the absence of SurA [329]. DegP/Skp function in one pathway whilst SurA acts on a different, parallel pathway [328] and DegP/Skp function to rescue off-pathway intermediates that would have accumulated in the absence of SurA [329, 330].

HtrA has also been described to be a secreted virulence factor in many bacteria. HtrA cleaves the ectodomain of the cell adhesion protein E-cadherin during infection with *Helicobacter pylori* [9, 10], an important pathogen that colonizes the gastric epithelium. The same HtrA-mediated E-cadherin cleavage mechanism was subsequently detected in other enteropathogenic organisms such as *Escherichia coli*, *Shigella flexneri*, and *Campylobacter jejuni* [10, 331]. HtrA plays an important role in *S. flexneri* as it was found that DegP, a homologue of HtrA, assembles the major virulence factor, IcsA on the bacterial outer membrane [247]. Purdy and co-workers [306] demonstrated that *S. flexneri degP* mutant SM1100 formed smaller plaques than those formed in the wild-type and failed to localize IcsA to the bacterial pole, a process required for actin polymerization into actin "tails", resulting in failure of the SM1100 bacteria to spread within and between epithelial cells. IcsA is a member of the autotransporter family of outer membrane proteins [332]. IcsA is thought to be transported across the inner

membrane in a Sec-dependent mechanism and translocates itself across the outer membrane [248, 333]. The chaperone activity of DegP may be involved in the efficient delivery of IcsA to the bacterial surface by facilitating the proper folding of IcsA in the periplasm or the rapid transport of IcsA to the outer membrane [306]. HtrA has also been implicated to play crucial roles for the virulence of other bacterial pathogens, including *Salmonella enterica* serovar Typhimurium, *Streptococcus pneumoniae*, *Legionella pneumophila*, and *Listeria monocytogenes* [11-13, 282].

The present study clearly indicated that CtHtrA is important for growth and development of *C. trachomatis*. A number of chaperones and proteases have been previously demonstrated to be important for virulence and stress resistance in a variety of bacteria [334]. CtHtrA has been previously demonstrated to be important for bacterial protein folding and stress response [19]. However, the role of CtHtrA for infection and its potential role in the chlamydial developmental cycle are poorly understood.

The role of HtrA in the replication of bacteria has been previously described. DegP in *E. coli* strain called adherent and invasive *E. coli* (AIEC) isolated from patients with Crohn's disease was important for the intracellular replication of the bacterium within J774-1A macrophages [335]. The LF82 – *ΔhtrA* isogenic mutant showed absence of bacterial replication at 24 h PI and exhibited small inclusions similar to those observed at 1 h PI. Complementation of LF82 *–ΔhtrA* with a cloned *htrA* gene led to restoration of the replication of the bacterium similar to that of wild type LF82 [335]. LF82 *–ΔhtrA* was also found to be sensitive to oxidative stress and this may explain why LF82 *–ΔhtrA* mutant was unable to replicate within macrophages and evade killing by phagosomes [335].

During phagocytosis, bacteria are taken up by phagosomes, and fusion of the phagosomes with lysosomes involves an oxygen-dependent mechanism that subsequently produces H_2O_2 and reactive oxygen species. However, this increased susceptibility to oxidative stress was not observed in non-pathogenic *E. coli* harbouring a mutation in the *htrA* gene [336] suggesting differences in the regulation of the *htrA* gene between non-pathogenic and pathogenic strains of *E. coli*. HtrA was also found to be important for *Legionella pneumophila* intracellular replication within mammalian macrophages and alveolar epithelial

cells and for intrapulmonary replication in A/J mice, yet dispensable for the intracellular infection of protozoa such as *Acanthamoeba polyphaga* [282]. Overall, infection with some facultative intracellular bacteria such as *Yersinia* is initiated by macrophage phagocytosis, to which the bacteria respond by expression of several stress response proteins, such as HtrA, which can be induced by heat stress and oxidative stress, as in the case of the pathogenic *E. coli* strain, LF82. Therefore, HtrA clearly has essential roles for infection and subsequent intracellular replication of a number of pathogens.

Concurrent work in the team has identified that CHtrA has a conserved critical function during the replicative phase [256]. HtrA inhibition with JO146 resulted in dramatic loss of infectious progeny in a number of *Chlamydia sp*. capable of infecting a wide variety of hosts: (*C. trachomatis*: human, *C. suis*: pig, *C. pecorum*: cattle/sheep, and *C. caviae*: guinea pig) [256]. This signifies that HtrA is a good target for antibacterial therapeutics not only for *C. trachomatis* but also for the aforementioned species of *Chlamydia* and other bacterial pathogens.

The present study clearly demonstrated that inhibition of CtHtrA using the specific inhibitor, JO146, during the replicative phase led to disruption of the developmental cycle of *C. trachomatis* D (UW-3/Cx). The current study has also provided evidence for the important role of CtHtrA during detrimental conditions such as heat stress and penicillin-induced persistence. Selection and genome sequence analysis of mutants with less susceptibility to JO146 suggested that CtHtrA function is important for membrane maintenance and outer membrane protein biogenesis as mutations were identified in two genes that code for fatty acid acyltransferases, one of which is in involved in outer membrane recycling and the other one with unknown function.

Under stressful growth conditions imposed by immunological responses, iron limitation, amino acid deprivation, co-infection with other intracellular pathogens and the presence of antibiotics [137, 138, 144, 271], the developmental cycle is disrupted and chlamydiae enter a viable but non-culturable phase called persistence [137, 153, 160, 270], resulting in a long-term relationship with the infected host [138]. Persistence favours chronic chlamydial infections which are proposed to be a possible mechanism that results in sequelae such as infertility [146]. It has also been reported that penicillin-induced persistent *Chlamydia* are

phenotypically resistant to azithromycin [274]. An exciting and one of the major findings in the present study was the key role that CtHtrA plays in penicillin persistence laboratory models. It was previously reported that CtHtrA levels were up-regulated during penicillin persistence but down-regulated during IFNγ-induced persistence [19, 21, 22]. The present study demonstrated that treatment with JO146 during penicillin persistence resulted in reduction in levels of infectious progeny, and JO146 treatment during recovery from penicillin persistence was lethal to *Chlamydia*. These data indicate a functional role for CtHtrA during reversion from penicillin persistence and recovery to infectivity. The lack of bacterial lethality when JO146 was added during penicillin persistence suggests that penicillin persistence may not involve a high level of extra-cytoplasmic protein stress. Although the morphological changes appear similar regardless of the persistence-inducing agent, transcriptomic and proteomic analysis of persistent *Chlamydia* revealed different transcriptional profiles associated with the persistence-stimulating growth factor. Therefore, penicillin persistence may be a defence strategy of bacteria to escape cellular protein stress, and does not always initiate CtHtrA functions which may explain why CtHtrA was not found to be important during this stress condition.

Penicillin is a β-lactam antibiotic that acts by blocking peptidoglycan (PG) biosynthesis. In *Chlamydia*, PG synthesis occurs during the early stages of EB to RB transition [21, 155], suggesting that PG is crucial for RB replication, stability of the cell envelope, and provides an anchor to outer membrane proteins (OMPs). Penicillin persistence inhibits RB replication and PG biosynthesis, possibly compromising the stability of the PG-anchored OMPs. It can be proposed that the loss of PG-OMP crosslinks leads to disruption of outer membrane stability and leakage of membrane and periplasmic proteins as described in other bacteria [337-340]. Due to the lethality of JO146 when added during recovery from penicillin persistence as observed in the present study, it can be deduced that CtHtrA may be essential for restoration of outer membrane integrity and stability of PG-OMP during recovery from penicillin persistence and subsequent reversion of aberrant RBs to infectious EBs.

The bacterial envelope, being in direct contact with the external milieu, is the initial target of various stresses that may change envelope components. These

stresses include heat stress, cold stress, oxidative stress, pH change, overexpression of OMPs, exposure to ethanol and detergents, and pathogenesis [334]. Bacteria employ multiple stress signalling systems to respond to these stimuli that challenge the integrity of cell envelope. One of the factors that trigger cellular imbalances is oxidative stress, to which the bacteria respond by inducing the genes encoding antioxidant enzymes and mechanisms to repair oxidative damage to cellular components [341]. HtrA was found to be vital for the pathogenesis and survival in oxidative stress for *Salmonella typhimurium* [246, 342]. *S. typhimurium htrA* mutants were highly sensitive to killing by reactive oxygen species (ROS) and exhibited diminished capacity to survive and/or replicate within macrophages [246, 342, 343]. In *E. coli*, accumulation of specific OMPs within the periplasm triggers a series of events called regulated intramembrane proteolysis (RIP) that leads to the degradation of the inner membrane protein, RseA [344-346]. This is initiated by DegS, a member of the HtrA family of proteases, which cleaves RseA that normally binds and prevents the regulatory activity of σ^E. ·

The sigmaE (σ^E) is the extra-cytoplasmic function (ECF) factor which controls the expression of genes that encode proteins in the extra-cytoplasmic localisation that are involved in the folding of polypeptides, such as proteases and stress response. Walsh [345] demonstrated that peptides ending with OMP-like C-terminal sequences bind to the PDZ domain of DegS thereby activating cleavage of RseA by DegS, and subsequent release of σ^E into the cytoplasm and induction of σ^E-dependent transcription. The SigmaE (σ^E), together with other transduction pathways, namely, the phage shock protein (Psp) pathways, Cpx (conjugated plasmid expression), and Bae (bacterial adaptive response [347]) pathways constitute the main elements of the extracytoplasmic stress response in *E. coli* [334].

HtrA (DegP) is also a member of the Cpx regulon in *E. coli*, which controls the expression of proteins activated in response to certain envelope stress conditions [334]. In *E. coli*, Cpx is a two-component signal transduction system composed of a sensor histidine kinase (CpxA) and a cytoplasmic response regulator (CpxR) [348]. [348]. The Cpx is activated by a number of external disturbances such as pH changes and altered membrane composition. One of the

mechanisms employed by the Cpx system in combatting extra-cytoplasmic protein stress that challenges the integrity of the bacterial cell envelope is by upregulating DegP synthesis [349-352]. Therefore, both the Cpx and σ^E pathways are activated by the expression of misfolded protein and protein aggregates in the envelope, and upon perceiving these environmental cues, these two envelope stress responses induce the increased synthesis of DegP [353]. It is therefore tempting to speculate that in *Chlamydia*, CtHtrA proteolytic activity facilitates the removal of damaged proteins during reversion from penicillin-induced persistence when the integrity of the cell envelope is compromised. To our knowledge, this is the first experiment that demonstrated the essential role that CtHtrA plays during such conditions that would have otherwise been unfavourable to the bacterium.

It is commonly reported that HtrA is required for bacterial survival under high temperature, hence the name of the protein. CtHtrA has been found to be upregulated during heat stress while expression of the major outer membrane protein (MOMP) decreased under the same condition [19]. HtrA (DegP) is essential for survival at temperature above 42^{o}C for *E. coli* [354] and the lethal phenotype brought about by a *E. coli htrA*- can be heterologously complemented and protected by *cthtrA* against the lethal effects of heat stress [19]. During high temperature and other stress conditions, aggregation of the misfolded proteins can rapidly compromise the bacterial metabolic balance and is harmful to the organism. The present study demonstrated that addition of JO146 immediately prior to, and during, heat stress was lethal to *Chlamydia*.

Most bacteria respond to elevated temperatures by transiently upregulating the synthesis of a conserved set of heat shock proteins (HSPs). The most widely studied genes and proteins that are upregulated in response to heat shock in bacteria are the chaperones, HSP60, encoded by the gene *groEL* [355], and HSP70, encoded by the *dnaK* gene [356]. The molecular mechanisms underlying heat stress response had been extensively studied in *E. coli*. The heat shock response in *E. coli* is mediated by the alternative sigma factor, σ^H (σ^{32}) that acts as a transcriptional regulator [357-359]. The bacterium monitors the free pools of cytoplasmic chaperones DnaK and DnaJ, which in the presence of heat shock, interacts with denatured proteins. During normal conditions, DnaK and DnaJ form a stable complex with σ^{32} *in vitro*, however, in the presence of heat shock, these

chaperones, by sequestering to the denatured proteins, allow σ^{32} to associate with RNA polymerase and activate transcription of heat shock promoters [360]. DegP (HtrA), another protein that is upregulated in *E. coli* during growth at elevated temperature, encodes a periplasmic protease. Like most HSPs, the synthesis of *degP* is regulated at the transcriptional level, however, this induction is not mediated by σ^{32} but by a homologous promoter to one of the promoters of the σ^{H}-encoding gene *rpoH* [307], the P3 promoter which is recognized by σ^{E} [307]. Therefore, the role of HtrA as a heat stress protein is indispensable, most likely for the proteolytic degradation of damaged or misfolded proteins that accumulate during heat stress. The present study indicated that JO146 was effective in inhibiting chlamydial growth and viability when the compound was added at mid-replicative phase, which relates to the stage wherein RBs are asynchronously replicating and/or differentiating back to infectious EBs. Previous reports identified that prolonged exposure to heat shock (42^{o}C) stagnates *Chlamydia* in the RB stage [20], while the present study demonstrated that JO146 treatment in the presence at 42^{o}C for a short period of time (4 h) was lethal to *Chlamydia*. These findings indicate that CtHtrA is vital during detrimental conditions such as heat stress and does not necessarily relate to replication or binary fission.

To gain further insights into the potential pathways targeted by JO146, which may therefore be involved in CtHtrA function, *C. trachomatis* mutants that are resistant to the inhibitor were aimed to be isolated. Although a resistant mutant was not derived, unique mutants that were less susceptible to JO146 were generated and characterized phenotypically. The inability to isolate highly JO146-resistant mutants despite extensive efforts and the utilization of different screening formats suggests the possibility that interplay between multiple gene defects is required for resistance. Isolation of JO146 resistant mutants using the 96-well library format with two to three sequential rounds of compound treatment was unsuccessful. However, serial cultivation of wild-type and EMS-mutagenized *C. trachomatis* D in the presence of JO146 in T25 flasks resulted in isolation of mutants with reduced susceptibility to the compound. The use of different protocols and the resulting isolation, or lack thereof, of mutants with resistance to JO146 indicated that different factors such host cell density, the surface area of the cell culture system as well as the concentration of JO146 contributed to the

success or failure of isolating these mutants. After growing EMS-mutagenized and non-mutagenized *C. trachomatis* in the presence of JO146, the genomes of three plaque purified mutants with less susceptibility were sequenced and analysed. The isolation of mutants that were less susceptible to JO146 is an exciting feat for *Chlamydia* genetics. *Chlamydia* has been intractable to routine genetic manipulation due to its complex, obligate intracellular developmental cycle. However, several major advances in the study of *Chlamydia* genetics have been recently reported. Many of these genetic approaches have centred on construction of mutants through chemical mutagenesis [176, 180], *C. trachomatis* transformation and recombination studies as demonstrated by horizontal gene transfer with antibiotic resistant mutants [184, 296, 297], as well as analysis of naturally-occurring variants. Major breakthroughs in *Chlamydia* genetics also involved the determination of the role of *C. trachomatis* plasmid and plasmid-encoded components to virulence [163, 171]. These abovementioned studies, together with the present study, which utilized chemical mutagenesis coupled with whole genome sequencing to determine the specific genes that might be involved in CtHtrA function, indicate that genetic capabilities for *Chlamydia* continue to develop and expand.

Whole genome sequencing of the mutants and comparative sequence analysis with our laboratory wild-type *C. trachomatis* D provided knowledge about which genes contribute to the basis of the lesser susceptibility phenotype. Single nucleotide variants (SNVs) were present in the genes CT776 and CT206 which code for 2-acylglycerophosphoethanolamine (2-Acyl-GPE) acyltransferase and a predicted acyltransferase protein, respectively. All of the clones had mutations in gene CT776 although mutant 2A3 had a null mutation and thus the normal function of the gene could be completely lacking. Two of the three mutants had unique mutations in CT206. The clones that had mutations in CT206 recovered more infectious progeny during heat stress in the presence of JO146. Therefore, it can be speculated that changes in CT206 might have enabled *Chlamydia* to survive heat stress in the presence of JO146. The null mutation in CT776 for mutant 2A3 might have been compensated by the presence of nucleotide changes in CT206. It is therefore likely that single base substitutions in the genes CT206 and CT776 might have conferred beneficial mutations for these

clones as demonstrated by the high infectious yield during normal development and during heat stress in the presence of JO146 compared to the wild-type strain. These mutants were generated by continued cultivation in the presence of JO146 for a total of 26 passages. JO146 was a selective inhibitor towards CtHtrA, and CT206 and CT776, which code for acyltransferases, were the two most frequently identified loci in the generation of mutants with reduced susceptibility to JO146, suggesting that these two genes were strongly affected by continuous JO146 treatment.

Acyltransferases play pivotal functions in the metabolism of fatty acids. *C. trachomatis* encodes fatty acid synthesis (FAS II) genes that are required for the synthesis of phosphoethanolamine, phosphatidylglycerol and cardiolipin which constitute the main components of the bacterial membrane. FAS II is involved in the generation of 3-hydroxy fatty acids for lipopolysaccharide (LPS) synthesis in Gram-negative bacteria [361] and in *Chlamydia*, it was found to be essential for RB replication [311]. Lipid A (endotoxin), the hydrophobic moiety of LPS makes up the outer monolayer of the outer membrane of Gram-negative bacteria [362, 363]. The biosynthesis of lipid A is catalysed by the enzyme LpxC. Inhibition of LpxC led to downstream effect of blocking lipooligosaccharides (LOS), which in turn prevented *Chlamydia* from generating infectious EBs [189]. Therefore, the function of FAS II in *Chlamydia* would be to provide 3-hydroxy fatty acids for LOS synthesis, and that the acyltransferase CT776 (2-acyl-GPE acyltransferase) was essential for recycling PE during lipoprotein turnover [311]. Thus, CT776 may have bacterial membrane anabolic/membrane maintenance and outer membrane protein biogenesis functions that are highly associated to the essential role of CtHtrA especially during stress conditions.

It was demonstrated in the present study that JO146 was effective in inhibiting CtHtrA functions in currently circulating *C. trachomatis* clinical isolates representing different serovars. Treatment with the CtHtrA inhibitor JO146 had the same significant and reproducible detrimental effect on *C. trachomatis* clinical isolates with that observed on *C. trachomatis* laboratory strain. *C. trachomatis* infections are typically resolved by treatment with antibiotics that are able to be absorbed by the lipophilic plasma membrane to reach the RBs [146]. The drugs of choice for chlamydial infections include

cyclines (doxycycline and tetracycline), quinolones (ofloxacin and levoflaxin), and macrolides (erythromycin and azithromycin) [146]. Although these regimens were thought to be both safe and efficacious [72-74], recurrent exacerbating infections are reported. Treatment failure with azithromycin, the most commonly used drug for uncomplicated chlamydial infections, has also been reported [75-77, 312, 364]. Batteiger and co-workers [78] conducted a longitudinal cohort study among adolescent women and identified that 8% of women with paired episodes of chlamydial infections were probable treatment failures. A partner treatment study conducted by Golden and co-workers [312] demonstrated that 8% of originally treated cases had chlamydial infections after follow up despite denials of re-exposure, and were classified as treatment failures. There is likely to be a future need for development of new anti-chlamydial therapeutics. In the present study, treatment with the CtHtrA inhibitor, JO146, was effective against currently circulating clinical isolates of *C. trachomatis*, indicating that CtHtrA is a virulence factor that can be targeted by anti-chlamydial drugs. In addition, inhibition of CtHtrA in the mid-replicative phase wherein RBs are asynchronously actively replicating and reverting back to infectious EBs is a good developmental phase to inhibit chlamydial growth since this is the stage in which RBs are metabolically active and thus most susceptible to treatment. Direct examination of cultures treated with JO146 by immunocytochemistry and confocal microscopy indicated that the decrease in inclusion size and the lack of recoverable EBs did not reflect a deficiency in accumulated RBs to retransform back to EBs but was instead due to inhibition of growth. It can therefore be proposed that the effect of JO146 on chlamydial development and CtHtrA function was probably not via induction of characterized persistent state of growth. This finding is of particular relevance because a good inhibition strategy would mean that the compound or drug does not induce the *Chlamydia* to enter into persistence, a condition which results in a lasting association between the pathogen and its host.

As laboratory strains may not reflect the strains currently infecting men and women, *C. trachomatis* clinical isolates have been used to assess the *in vitro* antibacterial properties of different inhibitors of *Chlamydia* virulence factors. One of these inhibitors whose potency was tested against clinical isolates of *C. trachomatis* was Iclaprim. Iclaprim, a novel dihydrofolate reductase (DHFR)

inhibitors, act through inhibition of thymidylate synthase and therefore nucleic acid synthesis [365]. When tested on *C. trachomatis* clinical endocervical isolates, Iclaprim exhibited anti-chlamydial activity comparable to those of both azithromycin and levoflaxin [365]. Clinical isolates of *C. trachomatis* have also been used to test the *in vitro* activity of different antibiotics such as clarithromycin, azithromycin, roxithromycin, erythromycin, doxycycline and tetracycline [322]. The *in vitro* anti-chlamydial property of the macrolides was found to be better than that of the tetracycline's [322]. Clinical strains have also been used to study horizontal gene transfer conferring resistance to different antibiotics among different *Chlamydia* species [297]. It was identified that resistance to tetracycline can be transferred to recent clinical isolates of *C. trachomatis*, raising public health concerns regarding human pathogens acquiring resistance to tetracycline [297].

Experiments for the present study used McCoy B cells as consistent with previous studies on clinical isolates of *C. trachomatis* that all mainly used this cell line. For the present study, the clinical isolates did not grow well in HEp2 cells, a human epithelial cell line, but thrived in McCoy B cells. There are no explanations in the literature why *C. trachomatis* clinical isolates grow better in the mouse macrophage cell line, McCoy B cells, even though previous studies on *C. trachomatis* clinical isolates used this cell line [320-322]. However, it can be speculated that infection of the human epithelial cell line, HEp2, with recent clinical isolates of *C. trachomatis* might have triggered the production of host defence factors against the *Chlamydia* which could explain the inability of *C. trachomatis* clinical isolates to grow in HEp2 as observed in the present study.

Overall, the data presented in this study could be interpreted to the conclusion that CtHtrA inhibition through JO146 treatment is lethal to *C. trachomatis* due to the various functions that CtHtrA plays in the different stages of the chlamydial developmental cycle. Previous studies utilizing biochemical, transcriptomic, proteomic, antibody labelling and cytosolic localization analyses revealed that CtHtrA is present in the envelope and has been found in the lumen of inclusions, surface of EBs and cytosol of the infected cells [19, 254, 257, 265]. Furthermore, the chaperone activity of CtHtrA potentially mediates the outer membrane assembly of extracytoplasmic proteins including the polymorphic

membrane proteins (Pmps). *C. trachomatis* Pmps have been implicated to increase genital tract inflammation and may play a role in virulence [366], denoting that CtHtrA has essential roles for the pathogenicity of *Chlamydia* and may serve as a substrate for other virulence factors of the bacterium. CtHtrA is expressed throughout much of the developmental cycle [19] with up-regulation during the replicative phase (8–24 h PI). The high expression of CtHtrA can be due to the fact that hydrolysis of penicillin-binding proteins and other PG factors during this phase result in the instability and increased permeability of the outer membrane. As a molecular chaperone that recognizes and interacts with the surface-exposed hydrophobic residues in non-native proteins, CtHtrA is likely to regulate protein homeostasis in extracytosolic compartments by degrading misfolded or unstable, non-functional proteins in the replicative phase, allowing RB replication to ensue. Significant findings that emerged from the present study were the loss of chlamydial inclusion morphology, diminishing inclusion size, and complete loss of infectious progeny production when the CtHtrA inhibitor was added to the cultures at mid-replicative phase of the developmental cycle.

7.1 CONCLUSION

The findings presented in this study support our hypothesis that CtHtrA is essential for *C. trachomatis* viability in human cell culture models. CtHtrA is also critical for the viability of *C. trachomatis* especially during heat stress and during penicillin persistence recovery. This study has therefore provided evidence that JO146 inhibits *C. trachomatis* development and CtHtrA activity and presents a model in which progression of the *C. trachomatis* developmental cycle requires a fully functional CtHtrA. Whole genome sequence analysis of mutants that were less susceptible to JO146 indicated that two loci, CT776 and CT206, which code for 2-Acyl-GPE acyltransferase and a predicted acyltransferase protein, respectively, acquired single base substitutions for all the mutants. This data signify that these two acyltransferases were mainly targeted by JO146 and may be indirectly involved in CtHtrA functions. An increasing number of studies question the reliability of azithromycin therapy and there are no currently available

vaccines for *C. trachomatis* infections and infection does not provide immunity against reinfection. Therefore, there is likely to be a need for development of new anti-chlamydial drugs. Results generated in the present study strongly support the concept that CtHtrA is a good target for drug development and may form a basis for developing a new class of anti-chlamydial agents.

7.2 FUTURE DIRECTIONS

There are a range of approaches that could be used to further understand the functions of CtHtrA. *C. trachomatis* infects a diverse portion of the female reproductive tract including the endocervix [367] and the endometrium [368] and can also ascend and infect the upper genital tract including the fallopian tube [140]. Therefore, the next logical approach would be to test the compound, JO146 on *C. trachomatis* grown in primary human cell culture models of *Chlamydia* infection such as the fallopian tube, endometrium, and endocervix [369, 370]. The inhibitory effect of JO146 on *Chlamydia* grown in human immune cell lines such as primary human monocyte-derived macrophages can also be investigated. The absorption capacity of JO146 might be different in these cell lines than in HEp2 and McCoy B cells which were mainly used in the present study. The effects of JO146 on these primary human cells would more closely emulate the inhibitory action of the compound *in vivo* compared to using HEp2 and McCoy B cells. Additional experiments can also be performed to further understand the functions of CtHtrA. Transcriptome analysis on an *in vitro* model of CtHtrA inhibition would help identify expressed genes, targeted transcripts and transcriptional changes in *C. trachomatis* upon treatment with JO146 particularly at the mid-replicative phase.

The mutants generated in the present study may serve as useful model organisms for investigation of the function and regulation of proteins affected by the SNVs and how these proteins mediate reduced susceptibility to JO146. Transmission electron microscopy can also be performed to determine if the mutations alter the cellular membrane morphology of the mutants. Since interplay between multiple gene defects might be required for resistance, recombination

between the less susceptible strains might lead to isolation and characterization of a JO146 truly resistant *C. trachomatis* variant that can further be studied for the specific roles of CtHtrA particularly for chlamydial development during normal as well as during stress conditions. Complementation of the mutants with CT776 and CT206 and determination of the resulting JO146 binding capacity of the complemented strains expressing these two genes, in comparison to their mutant forms clearly deserves further study. Identifying expression profiles through Western blot analysis of HtrA and MOMP as well as that of acyltransferases CT776 and CT206, in relation to host cell β-actin of cultures treated with the mutants can shed light on the impact of these mutations on these chlamydial proteins. Real-time (rt) or quantitative PCR (qPCR) can also be performed to determine the doubling time of *Chlamydia* DNA for these mutants. The two loci, CT776 and CT206, which are both involved in fatty acid synthesis, were clearly targeted by JO146 since the three mutants had unique base substitutions in these genes. Therefore, the roles of the genes mutated in the clones can be further confirmed by fatty acid metabolomics. Additionally, transcriptome profiling of these mutants will determine the expression of CT776 and CT206 throughout the chlamydial developmental cycle which can therefore be associated with the replicative phase impact of JO146. Loss-of-function mutations in CT776 and CT206 and determining the phenotypic effect on *Chlamydia* would help determine the specific functions of these genes particularly in the chlamydial developmental cycle during normal and stress conditions. Identifying the other genes that might be upregulated when *htrA* is compromised is also a good avenue for further research.

One of the limitations of this study is the solubility of the compound JO146. Biochemical and structural alterations of the compound are currently underway in partnership with collaborators to make the compound more soluble at higher concentrations. Concurrent experiments from the group also identified that JO146 effectivity is modulated by the type of host (epithelial such as Hep-2 and mouse fibroblasts such as McCoy cells) and the host cell density. Understanding the pharmacokinetics (absorption, distribution, metabolism and excretion) [371] of JO146 will further elucidate the mechanisms responsible for the differences of JO146 effectivity in different cell lines. Transcriptional profiles of different host

cell lines mostly used in *Chlamydia* culture studies will also elucidate the different sensitivities of the organism to anti-chlamydial compounds such as JO146. This technique would shed knowledge on the expression profiles of different host enzymes that are involved in the compound's bioavailability and whether these enzymes could be concomitantly affected by the use of other drugs. The resulting metabolites that may be contributing to the pharmacological action of the compound also deserves further study (i.e. metabolite profiling).

In addition, an attractive feature of JO146 and its derivatives that would need to be explored is its inhibition capacity against members of the vaginal microbiome such as the lactobacilli. The low pH resulting from fermentation of carbohydrates to lactic acid by *Lactobacillus* bacteria is toxic to many pathogenic microbes. Current anti-chlamydials also kill lactobacilli, resulting in yeast vaginitis [372]. Therefore, potential, alternative anti-chlamydial treatment strategies may include inhibitor compounds that target specific virulence factors without disrupting the normal bacterial flora. An example of a compound that kills a sexually transmitted pathogen but does not disturb the normal vaginal microflora of women is chlorhexidine which is a biguanide broad-spectrum disinfectant that eradicates Gram-positive and Gram-negative bacteria and has been used in skin and mouth washes and as a preservative in some vaginal lubricants [373]. Rabe and Hillier [373] demonstrated that chlorhexidine may be an appropriate microbicide against *Neisseria gonorrhoea* but not for the protozoan, *Trichomonas vaginalis*, and was more effective at a higher pH and in the absence of blood. Shubair and co-workers [374] identified that chlorhexidine gluconate (CHG) did not affect the normal microflora of the vagina which consists of aerobic bacteria such as *Lactobacillus spp., Gardnerella vaginalis, and Staphylococcus epidermidis* and anerobic bacteria such as *Bacteroides spp*. The same bacterial composition was observed 30 days after treatment as the pre-treatment flora [374]. This finding was corroborated by the study of Patton and co-workers [375] who demonstrated that CHG had only small effects on the vaginal microflora of pig-tailed macaques, which was remarkably similar to that of humans, compared to the spermicidal contraceptive, benzalkonium chloride (BZK). Likewise, development of novel anti-chlamydial compound that does not

affect the normal vaginal microflora is of utmost consideration and warrants further investigation.

This project demonstrated an essential role of CtHtrA for the replicative phase of chlamydial development. CtHtrA inhibition during the replicative phase resulted in decreased inclusion vacuole size and prevented the formation of infectious progeny. This study also demonstrated that CtHtrA is essential for heat stress during mid-replicative phase. CtHtrA was important for recovery from penicillin persistence. This is the first time that an essential protein for this phase has been uncovered. Phenotypic and genotypic characterization of mutants that were less susceptible to JO146 demonstrated that the genes CT776 and CT206, which both code for acyltransferases genes that are involved in cell envelope integrity, signify that these two loci are targeted by JO146. Single base substitutions in these genes conferred beneficial mutation for these mutants as indicated by the high infectious progeny yield during normal development, heat stress in the presence of JO146 as well as during recovery from penicillin persistence compared to wild-type *C. trachomatis* D. The genes CT776 and CT206 may therefore have an indirect contribution to the membrane biogenesis or maintenance functions of CtHtrA. This is the first report that unveils the specific chlamydial genes that might have downstream effect on CtHtrA functions. JO146 was also effective against currently circulating *C. trachomatis* clinical isolates representing different serovars. Therefore, CtHrA inhibition through the use of the compound, JO146 provides proof of concept that this strategy can be further applied in the future development of new anti-chlamydial drugs.

Chapter 8: **Bibliography**

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