IDEALS
ARE LIKE
STARS…
you choose them as your guides,
and following them
you will reach your destiny

Carl Schurz, Boston, 1859

Promotor: **Prof. dr. Daisy Vanrompay**

Laboratory of Immunology and Animal biotechnology

Department of Animal Production

Ghent University

Belgium

Dean: **Prof. dr. ir. Marc Van Meirvenne**

Rector: **Prof. dr. Anne De Paepe**

Sarah VAN LENT

Molecular characterization of the polymorphic membrane proteins of *Chlamydia psittaci*

Thesis submitted in fulfillment of the requirements

for the degree of Doctor (PhD) in Applied Biological Sciences

Nederlandse vertaling titel:

Moleculaire karakterisatie van de polymorfe membraaneiwitten van *Chlamydia psittaci*

Refer to this thesis:

Van Lent, S. (2016). Molecular characterization of the polymorphic membrane proteins of *Chlamydia psittaci*. PhD thesis, Ghent University, Ghent, Belgium

ISBN-nummer: 978-90-5989-909-4

Members of the examination committee

Prof. dr. ir. Monica Höfte (Chairman)
Department of Crop protection
Faculty of Bioscience Engineering
Ghent University

Prof. dr. Andre Skirtach (Secretary)
Department of Molecular Biotechnology
Faculty of Bioscience Engineering
Ghent University

Prof. dr. Daisy Vanrompay (Promotor)
Department of Animal Production
Faculty of Bioscience Engineering
Ghent University

Prof. dr. Katleen Hermans
Department of Pathology, Bacteriology and Avian Diseases
Faculty of Veterinary Medicine
Ghent University

Prof. dr. Els Van Damme
Department of Molecular Biotechnology
Faculty of Bioscience Engineering
Ghent University

Prof. dr. Richard Ducatelle
Department of Pathology, Bacteriology and Avian Diseases
Faculty of Veterinary Medicine
Ghent University

dr. Yvonne Pannekoek
Department of Medical Microbiology
Academic Medical Center
Amsterdam, The Netherlands

Table of Contents

Abbreviations

AA	Amino acid
Abl	Abelson
ANOVA	Analysis of variance
Arf	Adenosinediphosphate ribosylation factor
bp	Base pairs
BFA	Brefeldin-A
C.	*Chlamydia*
cDNA	Copy DNA
CDS	Coding sequence
CERT	Ceramide transfer protein
CL	Cardiolipin
COMC	Chlamydial outer membrane complex
CS	Conserved sequence
Cq	Quantitation cycle
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E.	*Escherichia*
EB	Elementary body
EDTA	Ethyleendiaminetetra-acetic acid
ELISA	Enzym-linked immunosorbent assay
ER	Endoplasmatic reticulum
F	Forward primer
FITC	Fluorescein isothiocyanate
FxxN	Phenylalanine, two random amino acids (x), Asparagine
GC	Guanine Cytosine
GEF	Guanine nucleotide exchange
GGA(I/L/V)	Glycine, Glycine, Alanine (Isoleucine/Leucine or Valine at the 4^{th} position)
GST	Glutathione S-transferase
HeLa	Henriette Lacks
His	Histidine
H&L	Heavy and light antibody chain

HMM	Hidden Markov Model
hpi	hours post infection
HRP	Horseradish peroxidase
Hsp	Heat shock protein
IEM	Immuno-electron microscopy
IF	Immunofluorescence
IFN	Interferron
IFU	Inclusion forming units
Ig	Immunoglobulin
IM	Inner membrane
Inc	Inclusion membrane protein
kDa	kilo Dalton
kdo	3-deoxy-D-mannose-oct-2 ulopyranosonic acids
LD	Lipid droplets
LGV	Lymphogranuloma venereum
LPS	Lipopolysaccharide
MIC	Minimal inhibitory concentration
MOI	Multiplicity of infection
MOMP	Major outer membrane protein
MoPn	Mouse pneumonitis
mRNA	messenger RNA
MTOC	Microtubule organizing center
MVB	Multivesicular body
Mw	Molecular weight
NCBI	National Center for Biotechnology Information
n	Normal culture condition
NIH	National Institute of Health
OD	Optical density
OM	Outer membrane
Omc	Outer membrane complex protein
Omp	Outer membrane protein
OMV	Outer membrane vesicle
ORF	Open reading frame
p	penicillin-induced persistence

pAb	Polyclonal antibody
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PDGFR	Platelet-derived growth factor receptor
PDI	Protein disulfide isomerase
PI	Phosphatidylinositol
Pmp	Polymorphic membrane protein
PorB	Porine B
R	Reverse primer
RB	Reticulate body
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RT-PCR	Reverse transcriptase PCR
RT-qPCR	Reverse transcriptase quantitative PCR
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate – polyacrylamide gelelectroforese
Sec	Secretion signal
SNARE	soluble NSF-sensitive attachment receptor
SNP	Single nucleotide polymorphism
SPG	Sucrose-phosphate-glutamate
Spp.	*Species pluralis*
Tarp	Translocated actin recruitment protein
Tm	Melting temperature
tRNA	transfer RNA
T3S	Type III secretion
USA	United States of America
VS	Variable sequence

Chapter I

Chlamydial infection biology

1. Introduction and taxonomy

Members of the family of the *Chlamydiaceae* are obligate intracellular Gram-negative bacteria that cause a variety of diseases in humans, other mammals and birds. They have a serious impact on both human and animal health and are therefore of major economic importance worldwide. The primary sites of replication are mucosal epithelial cells of the respiratory, urogenital and gastrointestinal tract, the conjunctival epithelium, as well as monocytes and macrophages (Pospischil *et al*., 2010). Currently, eleven *Chlamydia* species have been identified (Table 1.1) and the pylogenetic classification of the *Chlamydiaceae* is shown in Figure 1.1 (Sachse *et al*., 2015).

Table 1.1: The family *Chlamydiaceae*. Adapted from Kerr *et al*. (2005), Longbottom and Livingstone (2006), and Sachse *et al*. (2015).

Species	Natural host	Other hosts	Clinical signs
C. trachomatis	Human		Chronic conjunctivitis and blindness (trachoma), infection of urogenital tract, infertility
C. pneumoniae	Human,horse, koala	Amphibians, reptiles	Pneumonia, bronchitis, encephalomyelitis, laryngitis, atherosclerosis, reactive arthritis
C. abortus [a]	Sheep, goat	Cattle, swine	Reproductive disorders, abortion and bad semen quality
C. psittaci [a]	Birds	Mammals	Respiratory tract infection, rhinitis, conjunctivitis, diarrhoea
C. caviae [b]	Guinea pig	Horse	Ocular and urogenital tract infection
C. felis [b]	Cat		Conjunctivitis, rhinitis and respiratory tract infection
C. muridarum	Rodents		Respiratory and genital tract infection
C. pecorum	Cattle, koala	Sheep, goat, swine	Reproductive disorders, infertility, infection of the urine tract (koala) and abortion, enteritis, polyarthritis, encephomyelitis, metritis, mastitis, conjunctivitis and pneumonia (other animals)
C. suis [b]	Swine	Ruminants	Diarrhea, pneumonia, conjunctivitis, reproductive disorders
C. gallinacea [b]	Chicken	Other poultry	Respiratory tract infection
C. avium [b]	Pigeon, parrot		Enteritis, respiratory tract infection, diarrhea

[a] Zoonotic pathogen, [b] Potential zoonotic pathogen

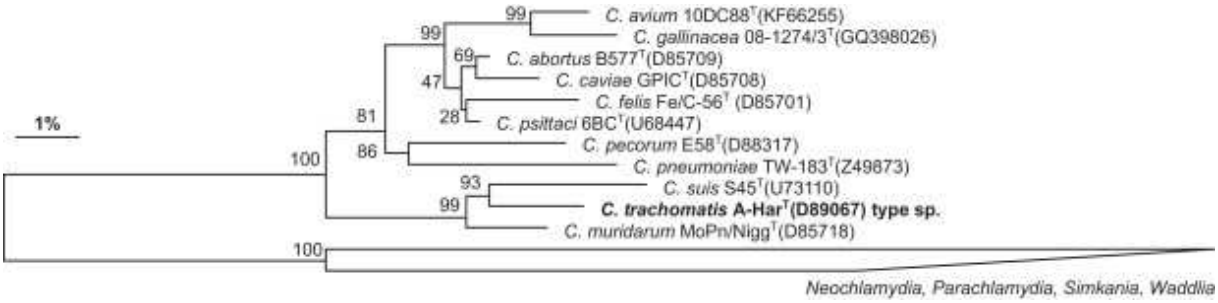


Figure 1.1: Phylogenetic tree of the family of *Chlamydiaceae*. The construction of the tree was based on an alignment of almost complete 16S rRNA genes from the type strains of all established *Chlamydiaceae* species. RaxML generated the tree starting from that alignment. Bootstrap values indicate the stability of the branches based on 100 replicates. The bar indicates 1% sequence divergence (Sachse *et al*., 2015).

2. Chlamydia psittaci

C. psittaci infections have been reported in over 500 species of birds, where infection is either latent or can become systemic and clinically overt in the respiratory tract (Stewardson and Grayson, 2010). The symptoms include conjunctivitis, anorexia, nasal discharge, rhinitis, diarrhea, polyuria, dyspnea and dullness (Vanrompay *et al*., 1995a). *C. psittaci* is an important zoonotic pathogen. In humans, *C. psittaci* infections can vary from mild flu-like symptoms to a life-threatening pneumonia (Beeckman and Vanrompay, 2009; Smith *et al*., 2011). Symptoms commonly reported are high fever, difficulty breathing and a non-productive cough, low pulse, chills, headache, and myalgia. Transmission of *C. psittaci* occurs horizontal via inhalation of infected aerosols of pharyngeal or nasal secretions or dried feces and vertical via the eggshell (Ahmed *et al*., 2015). Sequencing of the *C. psittaci* major outer membrane protein (*ompA*) gene identified 9 genotypes (A to F, E/B, M56, and WC) (Geens *et al*., 2005). The genotypes cluster with host species (Pannekoek *et al*., 2008). Genotype A and B are associated with psittacine birds (cockatoos, parrots, parakeets and lories) and pigeons, respectively. Genotype C has been isolated from ducks and geese, whereas genotype D was found mainly in turkeys. The host range of genotype E is more diverse, since it has been isolated from pigeons, ratites, ducks, turkeys and occasionally humans. Genotype F was isolated from psittacine birds and turkeys. Genotype E/B has been isolated mainly from ducks (Geens *et al*., 2005; Pannekoek *et al*., 2010). Genotypes WC and M56 represent isolates from epizootics in cattle and muskrats, respectively (Spalatin *et al*., 1966; Everett *et al*., 1999).

2.1 Treatment

Chlamydial infections in animals are currently mainly treated by tetracycline and its derivatives (chlortetracycline, oxytetracycline, doxycycline), because it is a cheap, broad spectrum antibiotic with an excellent tissue distribution and low toxicity, which easily resolves the infection (Sandoz and Rockey, 2011). However, the extensive use of the antibiotic also for other bacterial infections in animals, both as therapy and in the past also as a prophylaxis, led to the fast spread of tetracycline resistant Gram-positive and Gram-negative bacteria (Michalova *et al*., 2004). *Chlamydia suis* is the first intracellular bacterium in which a horizontally acquired resistance gene was observed (Dugan *et al*., 2004). Pigs are the natural host of *C. suis*, but also *C. abortus*, *C. pecorum* and *C. psittaci* occur in pigs (Schautteet and Vanrompay, 2011). This might suggest that transfer of the tetracycline resistence, *tet(C)*, gene to other *Chlamydia* species is feasible. However, intuitively we would expect that the *Chlamydia* species should be present at the same site of infection and that the inclusion of two different species should fuse before horizontal gene transfer might occur.

Chlamydiaceae replicate in mucosal epithelial cells of the conjunctivae, the respiratory, urogenital and gastrointestinal tract (Schautteet and Vanrompay, 2011), thus if a host is infected by multiple *Chlamydia* species, the different species can be present simultaneously at the same infection site. However, different fusogenic properties were observed. Multiple *C. trachomatis* inclusions present in the same infected cell mostly fuse (Matsumoto *et al*., 1991; Rockey *et al*., 2002), while many *C. psittaci* strains are non-fusogenic (Rockey *et al*., 1996). The *tet(C)* gene recombined in the *C. trachomatis* genome when cocultured with a tetracycline-resistent *C. suis* strain *in vitro*, while the resistence gene was not succesfully transferred to *C. caviae* (Suchland *et al*., 2009). This might either be due to the non-recombigenic activity of *C. caviae*, or the sequences at potential recombination sites might differ too much between these species or it might be that the *C. suis* inclusion could not fuse with the *C. caviae* inclusions and that this event is essential for recombination. All latter hypotheses are possible, as it is currently unknown how an intracellular bacteria acquired a resistance gene through horizontal gene transfer. Unexpectedly, preliminary results by Suchland *et al*. (unpublished data) suggested that fusion of inclusions is not required for recombination, which favors the other two possible explanations. *C. caviae* clusters phylogenetically together with *C. psittaci*, and therefore *C. psittaci* might show similar results as *C. caviae* in recombination experiments. However, up to now no experiments on the

possible transfer of the *tet(C)* gene from *C. suis* to *C. psittaci* have been performed neither *in vitro* nor *in vivo*. Further research is needed to unravel the horizontal gene transfer mechanism in *Chlamydia* spp., to find out whether or not the *tet(C)* could be transferred to *C. psittaci*. However, the presence of the *tet(C)* gene in *C. suis* is not always associated with a tetracycline-resistant phenotype, so MIC-values for tetracycline should be determined for strains containing the *tet(C)* gene to confirm that the resistant gene is functional (Di Francesco *et al*., 2008).

The occurence of the *tet(C)* gene in one *Chlamydia* species should warn us that the wide use of antibiotics in veterinary medicine creates an environment in which pathogens acquire and maintain antibiotic resistance genes (Dugan *et al*., 2004). Antibiotics are widespread in the poultry industry and spreading of the *tet(C)* gene is possible, as tetracycline resistant *Salmonella* (Kidie *et al*., 2013; Chotinun *et al*., 2014), *Campylobacter jejuni* (Deckert *et al*., 2010; Thibodeau *et al*., 2011) and *Staphylococcus aureus* isolates (Yucel *et al*., 2011; Argudín *et al*., 2013; Nemeghaire *et al*., 2013) have been observed in a high proportion in poultry. These observations, together with the systemic infection capacity of *C. psittaci*, highlight the possiblity that *C. psittaci* might acquire tetracycline resistence. Therefore, vaccination is regarded the best option to prevent chlamydial infections (Longbottom and Livingstone, 2006).

3. Developmental cycle

Chlamydiaceae are obligate intracellular bacteria, which means that they are completely dependent on eukaryotic host cells to replicate. The chlamydial developmental cycle is a unique biphasic developmental cycle, which involves predominantly two distinct morphological forms: the extracellular, infectious elementary bodies (EBs) and the intracellular, non-infectious, metabolically active reticulate bodies (RBs) (Figure 1.2). In addition, intermediate bodies (IBs) can be formed during the maturation from RBs to EBs (Vanrompay *et al*., 1996).

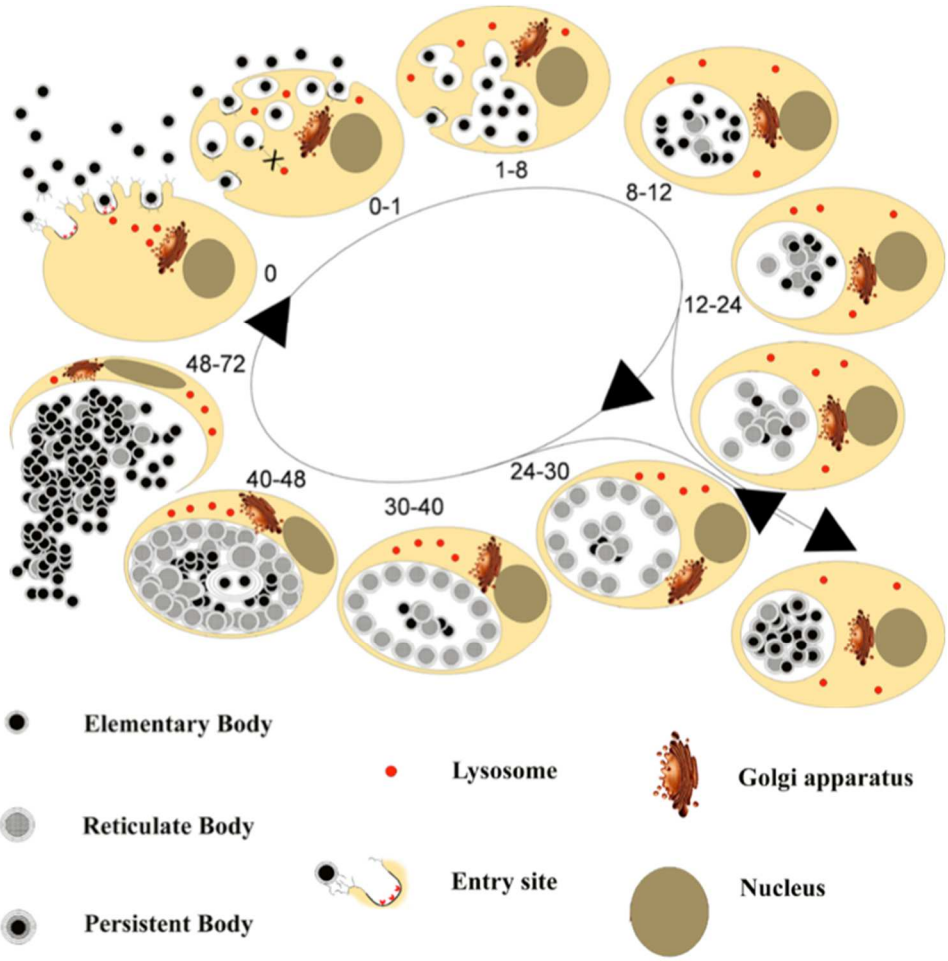


Figure 1.2: Schematic representation of the developmental cycle of the *Chlamydiaceae*. Numbers refer to hours post infection, however, the timing of the different stages varies depending on the chlamydial species and the host cell. The cycle starts when the infectious elementary bodies (EBs) attach to the host cell. Upon binding, EBs are internalized in tight, endocytic vesicles, called inclusions. The EBs differentiate to the metabolically active reticulate bodies (RBs), which divide by binary fission. The normal developmental cycle can be interrupted by different conditions and agents, which lead to persistence. During the persistent infection, no growth of the chlamydial organisms can be observed. Once the stress-induced factor is removed, the normal developmental cycle can be completed. The RBs continue to replicate until they detach from the inclusion membrane and consequently revert into EBs again. The EBs are released from the host cell through lysis or inclusion extrusion (Geens, 2005).

The EBs are small (diameter 0.2-0.3 μm), coccoid shaped particles that have a highly condensed chromosome and an unusual rigid ultrastructure due to disulfide cross-linking of cysteine-rich proteins in the envelope (Raulston, 1995; Hatch, 1996). That cell envelope makes the EBs osmotically stable and poorly permeable and protects the particles to survive up to several months in the hostile extracelullar environment (Longbottom and Coulter, 2003). The EBs initiate infection by attachment to a eukaryotic host cell, by which the EBs are endocytosed and in which they reside within a membrane-bound vacuole, called an inclusion. The EBs convert to the much larger (0.5-1.6 μm) RBs, which are also coccoid shaped, but have a low electron density and their cell envelope is less rigid and more permeable because the disulfide bonds are reduced and some cysteine-rich proteins are absent (Raulston, 1995). In addition, the major outer membrane protein (MOMP) is predominantly present in a monomeric form in RBs, while in EBs the protein has been identified as dimers, trimers and multimeric complexes (Newhall and Jones, 1983; Hatch *et al*., 1984). The RBs divide by binary fission and the inclusion expands (Moulder, 1991). After 20-48h, depending on the species, RBs differentiate asynchronously into EBs (Moulder, 1991; Hatch, 1996), which are then released from the infected host cell through cell lysis or inclusion extrusion, thereby closing the developmental cycle (Hybiske and Stephens, 2007b).

4. Persistence

A recurrent chlamydial infection might either be the result of a repeated infection or of a persistent infection that manifests after an unresolved primary infection (Hogan *et al*., 2004). The detection of chlamydial macromolecules in the absence of cultivability, recurrences that occur when reinfection is unlikely and clinical antibiotic resistance are evidences for persistence *in vivo*. Holland *et al*. (1992) detected *C. trachomatis* by either tissue culture or direct fluorescence cytology for a shorter period after a primary and secondary infection of primates than they detected chlamydial RNA. Dean *et al*. (2000) studied seven women with more than three recurrent infections over two to five years. Four women had identical genotypes at each recurrence and plenty culture-negative samples were positive by ligase chain reaction. All these experiment suggest that chlamydial persistence exists *in vivo*.

Chlamydial persistence has been induced *in vitro* through deviations of conventional cell culture conditions, such as amino acid deprivation (Coles *et al*., 1993), iron depletion (Raulston, 1997), antibiotic treatment (Goellner *et al*., 2006; Hu *et al*., 2015), phage infection (Hsia *et al*., 2000), co-infection with virus (Borel *et al*., 2010) and tryptophan depletion by

IFN-γ treatment (Beatty *et al*., 1994; Goellner *et al*., 2006). Small inclusions, which contain a smaller amount of abnormally enlarged RBs, called aberrant bodies, and loss of infectivity are common characteristics of the different persistent models. The aberrant bodies are formed due to the inability of the RBs to divide, while DNA and protein synthesis continues. Removal of the stressors results in septum formation, RB division and differentation to EBs (Hogan *et al*., 2004). Further research is needed to determine whether the persistent state induced *in vitro* resembles the latent and chronic infections that are observed *in vivo* (Wyrick, 2010).

5. A chlamydial infection from outside to inside

Multiple membranes have a key function during the developmental cycle of the *Chlamydiaceae* (Figure 1.3). In a first step, the EB attaches to the host **plasma membrane** and invades the host cell (Figure 1.3, a). Subsequently, the EB differentiates to RB, which divide inside the inclusion. The **inclusion membrane** is quickly modified by *Chlamydia*-derived proteins (Bastidas *et al*., 2013). It is hypothesized that the modified inclusion membrane is essential to ensure the escape of the inclusion from the endolysosomal pathway, while selective interactions with other cellular compartments are maintained to acquire essential nutrients and allow intracellular survival (Valdivia, 2008; Saka and Valdivia, 2010). The lipids that are needed for the growing membranes (inclusion, RB and EB membranes) are scavenged from the host (Figure 1.3,b; Elwell *et al*., 2011; Scidmore, 2012). While the proteins present in those membranes, are *Chlamydia*-specific (Taraska *et al*., 1996). **EB and RB membrane** compositions differ significantly, which could be expected based on their different role during the developmental cycle (Figure 1.3, c and d; Raulston, 1995; Marques *et al*., 2010; Saka *et al*., 2011). In what follows, the events occuring at the plasma and inclusion membranes as well as the composition of inclusion, EB and RB membranes will be discussed in detail. There are significant species- and strain-specific differences in the way that *Chlamydia* interacts with the host cell, so results can not always be extrapolated and caution should be exercised (Valdivia, 2008).

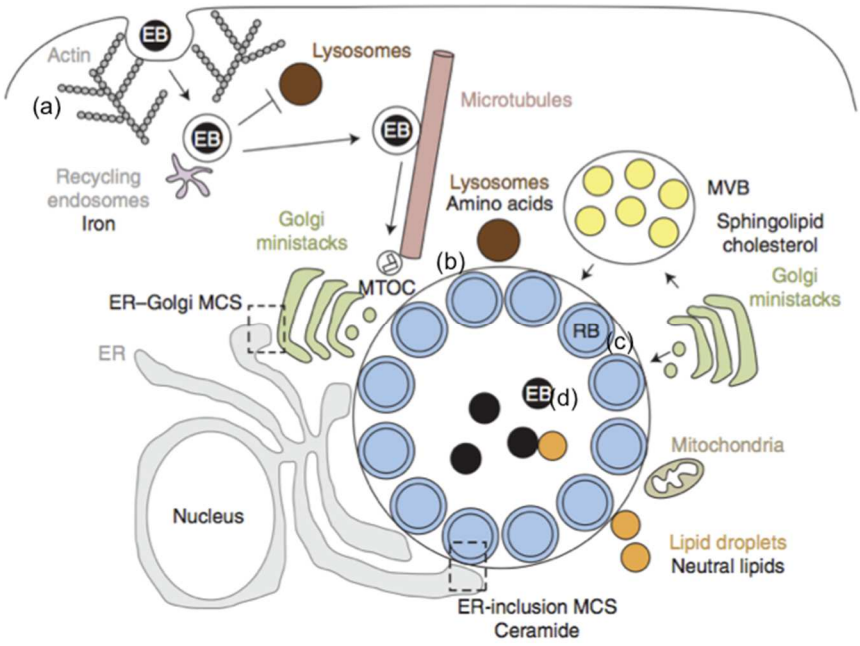


Figure 1.3: Overview of *Chlamydia* and host-cellular interactions. (a) *Chlamydiacea* enter the host cell in an actin-dependent way, that involves both chlamydial and host factors. The EBs are enclosed by a vacuole membrane, named the inclusion membrane. *Chlamydiacea* modify the inclusion membrane by inserting chlamydial proteins and thereby they prevent the fusion with lysosomes. The inclusion migrates across microtubuli toward the microtubule organizing center (MTOC). (b) The EBs differentiate to RBs that divide by binary fission, leading to an expansion of the inclusion. The inclusion interacts with multiple host-cell organelles such as fragmented Golgi ministacks, the ER, lipid droplets and multivesicular bodies (MVBs). (c, d) Eventually, most of the cytoplasmic space is filled by the inclusion, in which RBs differentiate back to EBs, which are released from the host cell and are ready to infect neighbouring host cells. Adapted from Bastidas *et al*. (2013).

5.1 Plasma membrane

5.1.1 Attachment

Bacterial attachment to a host cell is characterized by a two-step process in which primary, reversible, electrostatic interactions (Heckels *et al*., 1976; Hatch *et al*., 1981) are followed by a stronger, more specific binding of adhesins to their cognate receptors present on the surface of the host cell (Boyle and Finlay, 2003; Elwell *et al*., 2008; Lambert and Smith, 2009). The primary interaction of *Chlamydia* species to their host cells is mediated through electrostatic interaction of EBs with host heparan sulfate containing glycosaminoglycans (Zhang and Stephens, 1992; Su *et al*., 1996). Different bacterial adhesins: MOMP (Caldwell and Perry, 1982; Su *et al*., 1996), OmcB (Fadel and Eley, 2007; Moelleken and Hegemann, 2008),

Hsp70 (Mamelak *et al*., 2001), Polymorphic membrane protein 6 (Pmp), Pmp20 and Pmp21 (orthologs of PmpG, PmpB and PmpD of *C.trachomatis*, respectively) of *C. pneumoniae* (Mölleken *et al*., 2010), all Pmps of *C. trachomatis* (Becker and Hegemann, 2014) and host receptors: mannose receptor, mannose 6-phosphate receptor and estrogen receptor (reviewed in Campbell and Kuo, 2006; Cocchiaro and Valdivia, 2011) have been suggested. Protein disulfide isomerase (PDI) is structurally required for EB attachment and PDI-mediated reduction at the cell surface is essential for invasion (Abromaitis and Stephens, 2009). In addition, growth factors and their receptors, such as the platelet-derived growth factor receptor (PDGFR) and Abelson (Abl) kinase are essential host factors for chlamydial binding (Elwell *et al*., 2008). Most likely, adhesion will be mediated by multiple cell surface proteins (Cocchiaro and Valdivia, 2009).

5.1.2 Internalization

Chlamydia species carry a functional type III secretion (T3S) system, through which the pathogen translocates effector proteins directly into the host cell (Peters *et al*., 2007). The translocated actin recruitment protein (Tarp) is an early effector protein that mediates actin remodelling by either direct contact with actin (Jewett *et al*., 2006) or through a Rac1-dependent actin remodelling at the attachment sites (Carabeo *et al*., 2004; Clifton *et al*., 2004). Host Src (Jewett *et al*., 2008), Syk (Mehlitz *et al*., 2008) and Abl (Elwell *et al*., 2008) kinases phosphorylate the N-terminal tyrosine-rich tandem repeats of Tarp from *C. trachomatis*, which leads to the recruitment of guanine nucleotide exhange factors (GEFs), Sos1 and Vav2, which activate Rac1 (Lane *et al*., 2008). The activated Rac1 subsequently leads to the activation of the Arp2/3 complex and actin reorganization (Carabeo *et al*., 2007). Tarp from other *Chlamydia* species cannot be targeted for tyrosine phosphorylation, which highlights the differences in the pathogenesis of different *Chlamydia* species. Clathrin is an additional host factors that contributes to invasion in nonphagocytic cells (Hybiske and Stephens, 2007a). The actin rearrangement induced by Tarp is transient and it is hypothesized that other effectors, such as CT166 (Thalmann *et al*., 2010) and CT694 (Hower *et al*., 2009) in *C. trachomatis* might regulate the actin depolymerization. The EBs are immediately after entry sequestered in a membrane-bound vacuole, called the inclusion. The inclusion quickly dissociates from the endosomal pathway, avoiding lysosomal fusion, through remodelling of the inclusion membrane by insertion of bacterial proteins (Scidmore *et al*., 1996; Scidmore *et al*., 2003). The remodelled membrane subsequently promotes migration of the inclusion along

microtubuli to the MTOC, nearby the peri-Golgi region (Scidmore *et al*., 1996; Grieshaber *et al*., 2003). This transport is dynein-dependent but dynactin-independent. It is suggested that chlamydial effector proteins in the inclusion membrane mimic dynactin (Scidmore, 2011).

5.2 Inclusion membrane

Chlamydiacea acquire most of their metabolic needs from the host cell. These essential factors include nutrients, such as amino acids and iron, and lipids. The arginin transporter ArtJ, the metal transporter zntA, the ATP-ADP transporter Ntp1 and the cytochrome oxidase subunit I CydA may be present in the inclusion membrane (Saka *et al*., 2011). In addition, the inclusion selectively interacts with organelles in the peri-Golgi niche to sequester essential factors for chlamydial development (Figure 1.3, b). Sphingolipids (Hackstadt *et al*., 1996), cholesterol (Carabeo *et al*., 2003) and glycerophospholipids (Wylie *et al*., 1997) are essential eukaryotic lipids for chlamydial development. Chlamydiae interact with various host pathways to acquire those lipids and nutrients, which will be discussed below.

5.2.1 Vesicular pathway

Several endosome and Golgi-related **Rab GTPases**, which regulate organelle identity and vesicular trafficking (Seabra and Wasmeier, 2004), associate with the inclusion membrane (Rzomp *et al*., 2003). Rab proteins were observed in association with the inclusion membrane in both a species-dependent and species-independent manner (Rzomp *et al*., 2003; Scidmore, 2011). Rab1 and 14 are required in *C. trachomatis* development (Elwell *et al*., 2008), while Rab11 is required for *C. caviae* development (Derré *et al*., 2007). Rab1, 4 and 11 are recruited to *C. trachomatis*, *C. pneumoniae* and *C. muridarum* inclusions. Rab6 is associated with the *C. trachomatis* inclusion and not with *C. pneumoniae* and *C. muridarum* inclusions, while for Rab10 the opposite was observed (Rzomp *et al*., 2003). Rab GTPases function in different pathways and the recruitment of different Rabs is suggested to promote selective interaction/fusion with host vesicles containing essential nutrients (Bastidas *et al*., 2013): Rab4, 11 and 14 are endocytic, while Rab6 and 10 are endoplasmatic reticulum (ER) - Golgi related (Rzomp *et al*., 2003). Rab6 and 11 mediate fragmentation of the Golgi into ministacks (Heuer *et al*., 2009; Rejman Lipinski *et al*., 2009) and Rab14 mediates delivery of Golgi-derived sphingomyelin to the inclusion (Capmany and Damiani, 2010). In contrast, Rab4 and 11 may participate in iron acquisition (Ouellette and Carabeo, 2010).

It is suggested that chlamydial proteins which differ between species, may be involved in Rab recruitment (Rzomp *et al*., 2003). The *C. trachomatis* CT229 interacts with Rab4 (Rzomp *et al*., 2006), while the *C. pneumoniae* Cpn0585 interacts with Rab1, 10 and 11 (Cortes *et al*., 2007). CT229 and Cpn0585 are both **inclusion membrane proteins (Inc)**. Incs are identified by a large hydrophobic region, which encodes two transmembrane domains and by a type III secretion signal, that allows the secretion and subsequent insertion of the protein in the inclusion membrane. Bioinformatic studies have predicted that the amount of *inc* genes in chlamydial genomes varies between 50 and 90 genes in *C. trachomatis* and *C. pneumoniae* respectively (Lutter *et al*., 2012). This high amount of genes in the highly reduced chlamydial genomes suggests that Inc proteins mediate an important function in the chlamydial developmental cycle (Moore and Ouellette, 2014). Inc proteins are expressed at different time points during the developmental cycle (early and mid-cycle) (Nicholson *et al*., 2003). In addition to recruiting Rab GTPases, the fusion of vesicles might also be regulated by recruiting host soluble NSF-sensitive attachment receptor (**SNARE) proteins**, which are key components of the intracellular fusion machinery (Südhof and Rothman, 2009). Multiple Inc proteins, such as IncA, CT813 and CT223, contain SNARE-like motifs. IncA interacts with host endocytic SNARE proteins Vamp3, Vamp7 and Vamp8, through its SNARE motif.

The Brefeldin A (BFA)-sensitive vesicular-trafficking pathway is another vesicular pathway that is mediated by *Chlamydiaceae* to intercept cholesterol and sphingomyelin from the Golgi apparatus. *Chlamydiacea* use GBF1, a BFA-sensitive GEF that activates ADP ribosylation factors (Arfs) to acquire sphingomyelin. The activated Arfs recruit namely coat proteins necessary for vesicle formation. The sphingomyelin acquired through this vesicle-mediated pathway is essential for inclusion growth and stability, but not for bacterial replication (Elwell *et al*., 2011).

5.2.2 Non-Vesicular pathway

Host-sphingomyelin is essential for progeny production and inclusion biogenesis (Van Ooij *et al*., 2000; Robertson *et al*., 2009), however, BFA-mediated inhibition of vesicular transport had no effect on the production of infectious progeny (Hackstadt *et al*., 1996). That paradox is solved by the observation that the ceramide transfer protein (CERT) is recruited to the inclusion by *C. trachomatis*, possibly through interaction with IncD (Derré *et al*., 2011). CERT is a cytosolic lipid transfer protein that transports ceramide, the precursor of sphingomyelin, from the ER to the trans-Golgi region, where it is converted to sphingomyelin

by sphingomyelin synthases 1 or 2 (Hanada, 2010). *C. trachomatis* creates a sphingomyelin synthesis factory at the inclusion membrane by recruiting both CERT and at least one sphingomyelin synthase to the inclusion membrane. It is suggested that the CERT-recruitment leads to the formation of an ER-inclusion membrane contact site (Derré *et al.*, 2011). Sphingomyelin acquired through this vesicle-independent pathway is essential for *C. trachomatis* replication. Phosphatidylinositol (PI) and phosphatidylcholine (PC), two eukaryotic glycerophospholipids which are present in purified EBs, are also acquired from the host cell through the non-vesicular transport pathway, which is mediated by ERK and the cytosolic phospholipase A2 (PLA2). *Chlamydia* modifies the sequestered glycerophospholipids by replacing the non-branched chain fatty acids by *Chlamydia*-derived branched chain fatty-acids (Wylie *et al.*, 1997; Su *et al.*, 2004), which is in contrast to cholesterol and sphingomyelin that are not modified (Wylie *et al.*, 1997; Su *et al.*, 2004).

5.2.3 Lipid droplets

Lipid droplets (LD) are ER-derived storage organelles for neutral lipids or long chain fatty acids (Kumar *et al.*, 2006; Cocchiaro *et al.*, 2008). Lipid droplet-associated protein (Lda) 1 and 3 are translocated to the host cytosol and localize to LDs that are adjacent to the inclusion membrane (Kumar *et al.*, 2006). The captured LDs are translocated intact across the inclusion membrane (Cocchiaro *et al.*, 2008). The observations that the LDs do not accumulate in the inclusion lumen and the presence of neutral lipids in RBs, led to the suggestion that the associated lipids are either directly scavenged or metabolized by RBs (Scidmore, 2011). IncA cofractionated with LDs and partially colocalized with intraluminal LDs. Therefore, it was suggested that IncA might mark entry sites for LDs at the inclusion membrane (Cocchiaro *et al.*, 2008).

5.2.4 Multivesicular bodies

Multivescular bodies (MVB) are part of the endolysosomal pathway (Beatty, 2006). MVB are important for the sorting and processing of proteins and lipids that are destined for lysosomal degradation or recycling to the Golgi or plasma membrane exocytosis (Denzer *et al.*, 2000; Piper and Luzio, 2001; Woodman and Futter, 2008). *Chlamydiacea* might use the MVBs as an additional lipid (sphingolipids, phospholipids and cholesterol) source (Beatty, 2006; Gambarte Tudela *et al.*, 2015). MVBs migrate along microtubuli to the inclusion. Different MVB markers, such as CD63 and LBPA, reside within the *C. trachomatis* inclusion lumen.

Rab39a, which labels a subset of late endosomal vesicles – mainly MVB - , participates in the delivery of the MVBs to the inclusion (Gambarte Tudela *et al*., 2015). However, the chlamydial effectors involved in the transport of MVB into the inclusion lumen are unknown (Gambarte Tudela *et al*., 2015; Dumoux and Hayward, 2016).

5.2.5 Mitochondria

Mitochondria were found in close association with *C. psittaci* and *C. caviae* inclusions, but this assocation was not observed in *C. trachomatis* and *C. pneumoniae*-infected cells (Matsumoto *et al*., 1991; Derré *et al*., 2011). The mitochondrial transporter inner/outer membrane (TIM-TOM) complex, which is involved in the recognition and transport of host mitochondrial proteins into the mitochondria, is essential for *C. caviae* inclusion biogenesis and the production of infectious progeny. Consequently, depletion of the complex affected *C. caviae* replication and differentiation, but the *C. trachomatis* development was unaffected (Derré *et al*., 2011). The functional significance of the association of *C. caviae* and *C. psittaci* inclusions with mitochondria is currently unknown (Knittler *et al*., 2014) . It might be related to acquire energy. Although *Chlamydia* species have the capacity to produce ATP (Iliffe-Lee and McClarty, 1999), mimic-ATP/ADP transporters Npt1 and Npt2 are observed in EBs and RBs (Tjaden *et al*., 1999; Saka *et al*., 2011) and the ATP/ADP transporters might also be present in the inclusion membrane (Saka *et al*., 2011), it might be that *C. caviae* and *C. psittaci* have an additional, redundant pathway to acquire energy, as the genes required for ATP production are only transcribed starting from 6 hpi. Therefore, energy needed for the early differentation of EBs to RBs might either come from chlamydial ATP reserves, but also from the host (Iliffe-Lee and McClarty, 1999).

Prevention of apoptosis could be a second reason for the observed association of the inclusion with the mitochondria. The release of mitochondrial cytochrome c into the cytoplasm is essential to induce apoptosis (Yang, 1997). One of the observed effects of chlamydial effectors is the prevention of mitochondrial cytochrome c release into the host cytoplasm, which is normally induded by pro-apoptotic factors (Fan *et al*., 1998).

5.2.6 Lysosomes

Although *Chlamydiaceae* modify the inclusion membrane to prevent fusion with the endolysosomal pathway, lysosomes reside in close approximation to the inclusion membrane.

Ouellette *et al*. (2011) suggested that the lysosomes might be a source of essential amino acids early in the developmental cycle of *C. trachomatis*, while free amino acids are used later in the cycle. However, *C. pneumoniae* requires lysosomal degradation products throughout the developmental cycle (Ouellette *et al*., 2011).

5.3 EB and RB membranes

The functions of the two predominant morphological forms, EBs and RBs, differ significantly (paragraph 3). So it is not surprising that the lipid and protein composition of their membranes differ significantly and knowledge regarding their membrane compositions will give insight in their different functions (Stephens and Lammel, 2001). In what follows, a detailed description of the proteins and lipids present in membranes of both EB and RB will be discussed. If a protein or lipid is specific for one developmental form, it will be mentioned in the text.

Like all Gram-negative bacteria, *Chlamydiaceae* are surrounded by an outer membrane (OM) and a cytoplasmatic inner membrane (IM) (Tamura *et al*., 1971), which is separated by a periplasmatic space (Figure 1.4).The inner and outer membrane differ morphologically and chemically (Glauert and Thornley, 1969). Both membranes contain phospolipids and proteins and the outer membrane contains on top of that also lipopolysaccharides (LPS) (Filip *et al*., 1973). The phospholipids present in the inner and outer membrane of EBs and RBs is a mixture of lipids typically found in prokaryotes (phosphatidylethanolamine [PE], phosphatidylglycerol [PG] and phosphatidylserine [PS] and eukaryotes (PC, PI, sphingomyelin and cholesterol) (Wylie *et al*., 1997). *C. trachomatis* can synthesize the PE, PG and PS *de novo* and the eukaryotic lipids are acquired from the host cell (Wylie *et al*., 1997). The host-derived straight-chain unsaturated fatty acids are replaced by chlamydial branched-chain fatty acids (Wylie *et al*., 1997). Hatch and Mcclarty (1998) suggested that chlamydiae do not regulate the types of phospholipids trafficked and Wylie *et al*. (1997) showed that the host phospholipid synthesis did not alter following an infection with chlamydiae. Another remarkable observation by Wylie *et al*. (1997) is that *C. trachomatis* does not require *de novo* host phospholipids synthesis nor exogenous phospholipids to replicate. However, as mentioned in paragraph 5.2.2, sphingomyelin is essential for chlamydial replication. The ratio of PC to PE differs in EBs and RBs and the number of branched-chained phospholipids is lower in RBs. In general, EBs have a higher phospholipid content and membrane fluidity is greater in RBs (Manire and Tamura, 1967; Raulston, 1995). Chlamydial LPS is composed of a pentasaccharide containing a lipid A core and it contains

multiple cross-reacting epitopes with LPS of enterobacterial Re mutants (Ingalls *et al*., 1995; Vanrompay *et al*., 1995a). However, chlamydial LPS contains also a genus-specific epitope, which is composed of a 3-deoxy-D-mannose-oct-2 ulopyranosonic acids (Kdo) trisaccharide with the sequence αKdo-(2-8)- αKdo-(2-4)-αKdo (Caldwell and Hitchcock, 1984; Brade et al., 1987; Vanrompay et al., 1995a). Chlamydial LPS is immunogenic, however, it is at least 10 times less immunogenic than typical endobacterial LPS, such as *Salmonella* and *Neisseria* (Birkelund *et al.,* 1989; Ingalls *et al.,* 1995; Heine *et al.,* 2003). The strength of the Gram-negative cell wall is usually provided by peptidoglycan in the periplasmic space, which is critical for cell division, maintaining cell shape and hydrostatic pressure (Egan and Vollmer, 2013). However, presence of peptidoglycan in the chlamydial cell envelope was a matter of debate for a long time. Although the chlamydial genomes encode the genes for peptidoglycan biosynthesis (Hesse *et al*., 2003; McCoy *et al*., 2003; McCoy and Maurelli, 2005; McCoy *et al*., 2006; Patin *et al*., 2009; Patin *et al*., 2012) and chlamydial growth is affected by antibiotics targetting peptidoglycan biosynthesis (Moulder *et al*., 1963; Tamura and Manire, 1968), attempts to detect peptidoglycan were unsuccessful. The latter was called 'the chlamydial anomaly' (Moulder, 1993). However, Liechti *et al*. (2014) were able to detect chlamydial peptidoglycan by the use of a new labeling approach, which uses D-amino acid dipeptide probes and click chemistry. So the rigidity of the chlamydial cell wall is mediated both by highly cross-linked proteins and peptidoglycan.

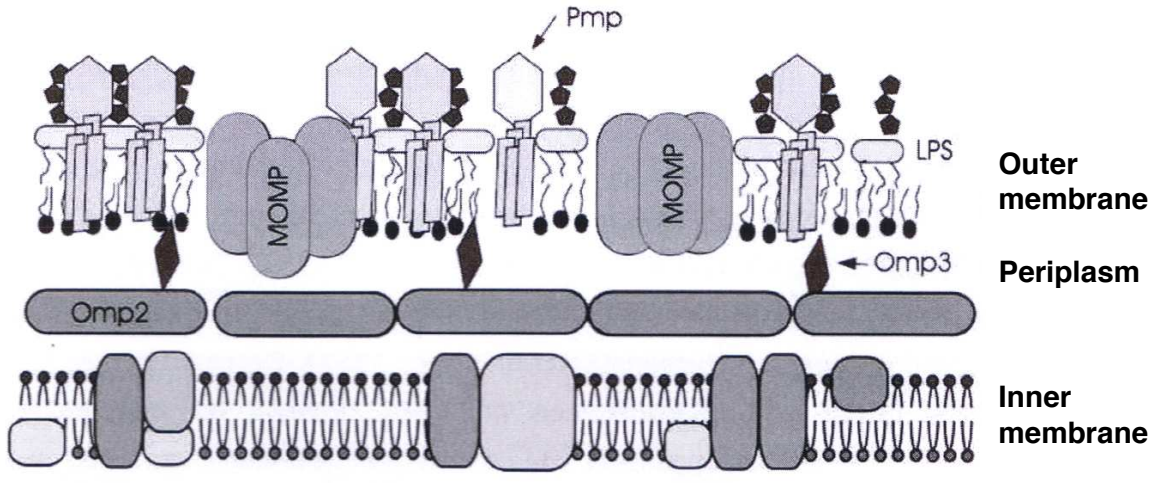


Figure 1.4: Model of the envelope of chlamydial EBs. In the outer membrane, MOMP, Omp3 and Pmps are shown. Omp 2 is the major constituent of the periplasmic space. Peptidoglycan is also a component of the periplasmic space. Specific proteins of the inner membrane are not yet identified. Adapted from Hatch (1996).

5.3.1 Chlamydial outer membrane complex

The location of proteins in the inner or outer membrane in Gram-negative bacteria was previously determined by separation of those membranes on density gradients (Ito *et al*., 1977). However, that method and variations of that method were for unknown reasons not succesfully applied to chlamydiae (Hatch, unpublished data). Therefore, an alternative method has been used to characterize the location of proteins in the chlamydial cell envelope. That method is based on the principle that hydrophobic outer membrane proteins can be separated from many soluble EB proteins by the use of mild detergents, such as Triton X-114 (Everett and Hatch, 1995; Bini *et al*., 1996; Nally *et al*., 2007; Aistleitner *et al*., 2014), Sarkosyl (Caldwell *et al*., 1981; Bavoil *et al*., 1984; Bini *et al*., 1996; Liu *et al*., 2010; Aistleitner *et al*., 2014) and octylglucoside (Bavoil *et al*., 1984; Kawa and Stephens, 2002; Yeung *et al*., 2008). The Sarkosyl-insoluble fraction isolated from purified EBs was called the chlamydial outer membrane complex (COMC) by Caldwell *et al*. (1981). This protein complex consists predominantly of MOMP, two cysteine rich proteins (CRP), which both have been given different names EnvA/OmcA/small CRP/Omp3 and EnvB/OmcB/large CRP/Omp2, and some Pmps (Figure 1.4) (Hatch *et al*., 1984; Stephens *et al*., 1987; Sardinia *et al*., 1988; Liu *et al*., 2010). Other proteins present in the COMC are PorB, Omp85, YscC, CTL0887, CTL0541, CTL0645, OprB and Pal (Liu *et al*., 2010).

5.3.1.1 Major outer membrane protein

Caldwell *et al*. (1981) identified a 39 500-dalton outer membrane protein in the Sarkosyl-insoluble fraction of purified *C. trachomatis* EBs. The insolubility is due to the extensive disulphide cross-linking (Caldwell *et al*., 1981; Newhall and Jones, 1983; Hatch *et al*., 1986). The protein was called MOMP and it accounts for 60% of total envelope proteins in both EBs and RBs (Caldwell *et al*., 1981; Bavoil *et al*., 1984). MOMP is cross-linked to itself in the chlamydial EB and is therefore essential for the structure and morphology of the chlamydial EB (Caldwell *et al*., 1981; Newhall and Jones, 1983), it functions as a porin which is only active in the chlamydial RB (Bavoil *et al*., 1984) and has a potential function as adhesin (Su *et al*., 1990; Su and Caldwell, 1991). The protein consists of five conserved (CS1-CS5) and four variable sequence (VS1-VS4) regions, which are exposed in the periplasmic and extracellular space, respectively (Figure 1.5) (Baehr *et al*., 1988; Kim and DeMars, 2001). MOMP plays an important role in eliciting an immune response. Monoclonal as well as polyclonal antibodies against MOMP, *C. pneumoniae* MOMP is an exception as it is not immunodominant probably

because it is not surface exposed (Campbell *et al*., 1990), are able to neutralize the infectivity of *Chlamydiacea in vitro* and *in vivo* (Caldwell and Perry, 1982; Zhang, Watkins, *et al*., 1987; Zhang *et al*., 1989). MOMP contains genus-, species- and serovar-specific epitopes (Caldwell *et al*., 1981). Protective epitopes locate in VS1 and VS2 (Baehr *et al*., 1988), serovar specific epitopes are also present in those domains and in VS4, while genus- and species specific-epitopes are located in the CSs and some species-specific epitopes are found in the most conserved regions of VS4 (Baehr *et al*., 1988; Yuan *et al*., 1989; Batteiger, 1996). The surface exposure (Baehr *et al*., 1988; Collett *et al*., 1989; Wang *et al*., 2006), high immunogenicity (Caldwell *et al*., 1981), and the observation that MOMP elicits both neutralizing antibodies and T-cell responses (Su and Caldwell, 1992) prompted the suggestion that MOMP would be an ideal vaccine candidate (Stephens *et al*., 1987; Baehr *et al*., 1988). However, experimental vaccines based on native MOMP (Pal *et al*., 2005; Kari *et al*., 2009), purified recombinant MOMP (Tuffrey *et al*., 1992; Shaw *et al*., 2002), synthetic peptides corresponding to B- and T-cell epitopes (Su *et al*., 1995) or DNA based immunogens (Pal *et al*., 1999) have not achieved complete nor consistent protection in none of the various animal models (Shaw *et al*., 2002). Therefore, further research for more efficacious vaccine candidates is necessary (Vasilevsky *et al*., 2016).

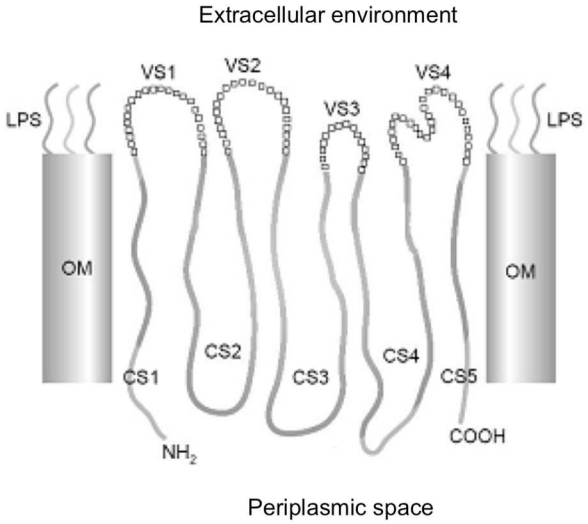


Figure 1.5: Schematic representation of MOMP in the outer membrane of the chlamydial cell wall. Full lines represent the conserved regions (CS1-CS5) and alternating lines represent the variable domains (VS1-VS4). Adapted from Baehr *et al*. (1988) and Kim and DeMars (2001).

5.3.1.2 OmcA and OmcB

OmcA and OmcB, which have previously also been called EnvA/small CRP/Omp3 and EnvB/large CRP/Omp2 respectively, are two other, besides MOMP, abundant proteins present in the COMC (Liu *et al*., 2010). The genes encoding these two proteins are tandemly arranged in a bicistronic operon, that is only transcribed late in the infectious cycle (Lambden *et al*., 1990), while MOMP is located separately and the transcription starts earlier (Raulston, 1995). Both proteins are cysteine-rich and are only present in chlamydial EBs and in RBs when they start to asynchronously differentiate to EBs (around 21-24 hpi) (Hatch *et al*., 1984). The cysteine-rich proteins form disulfide-cross-linked complexes, which are responsible for membrane rigidity. Whether or not the MOMP is linked by disulfide bridges to the cysteine-rich proteins is unknown (Raulston, 1995; Hatch, 1996). The mechanism for crosslinking is also currently unknown. Reduction occurs very rapidly, probably simultaneously with internalization, and it requires chlamydial protein synthesis (Hatch *et al*., 1986). In addition, Hatch *et al*. (1986) observed that MOMP and the two CRPs were intracellularly predominantly observed in the reduced state and the proteins were spontaneously cross-linked after host cell lysis. However, Newhall (1987) observed that cross-linking occured late in the developmental cycle, but before cell lysis and therefore it was suggested disulfide bond formation is enzym-mediated. Membrane-associated and periplasmic protein disulfide isomerases, which are also present in other Gram-negative bacteria (Bardwell, 1994), might be involved in the cross-linking process. OmcB is a possible candidate for this function, as it has a potential sulfydryl-oxidoreductase active site (Hatch, 1996). Similar to MOMP, both proteins possess genus- and species-specific epitopes, in addition only OmcA has serovar-specific epitopes, but none of the epitopes were accessible to antibodies on the native chlamydial cell surface (Batteiger *et al*., 1985; Zhang, Watkins, *et al*., 1987; Watson *et al*., 1994).

OmcA has a molecular mass of 12-15 kDa, is a highly conserved hydrophilic lipoprotein, which is anchored in the outer membrane by its lipid moiety and the proteinaceous portion faces the periplasmic space (Figure 1.4) (Allen *et al*., 1990; Everett *et al*., 1994; Raulston, 1995; Hatch, 1996). OmcA of *C. psittaci* 6BC contains 14 cysteine residues, which is 17% of the total amino acid content (Everett *et al*., 1994).

OmcB has a molecular mass of 57-62 kDa and it is a major immunogen in chlamydial infections (Mygind *et al*., 1998b; Sanchez-Campillo *et al*., 1999). The protein is highly conserved among *Chlamydia* species, with a highly variable N-terminal region (Newhall, 1987; Allen *et al*., 1990; Watson *et al*., 1991), is involved in the conversion of the chlamydial RB to EB (Mygind *et al*., 1998a) and it is believed to be a key structural component to maintain cell-wall rigidity and osmotic stability via disulfide bonding of outer membrane proteins (Newhall, 1987; Allen and Stephens, 1989). The protein migrates as a singlet in the *C. trachomatis* trachoma biovar and as a doublet in *C. psittaci*, *C. caviae* GPIC and the *C. trachomatis* lymphogranuloma venereum (LGV) biovar by SDS-PAGE, which is due to post-translational signal peptide cleavage sites in its amino-terminal region (Batteiger *et al*., 1985; Hatch *et al*., 1986; Allen and Stephens, 1989; Raulston, 1995; Ting *et al*., 1995). The mature form of the larger pair of OmcB of *C. psittaci* 6BC has 37 cysteine residues, which is 7.2% of the total amino acid content (Hatch, 1996), and has a net positive charge, which is in contrast to most chlamydial proteins that are neutral or acidic (Batteiger *et al*., 1985; Allen and Stephens, 1989). Although both members of the doublet were basic in the LGV biovar, the protein has a neutral charge in the trachoma biovar (Batteiger *et al*., 1985). There has been a controversy regarding the OmcB localization. The OmcB protein of *C. psittaci* is hydrophilic, not embedded in a lipid bilayer and susceptible to digestion with trypsin after incubation in Tris–EDTA, which all together suggest a periplasmic localization of the protein in *C. psittaci* (Everett and Hatch, 1995). However, Ting *et al*. (1995) observed that the larger of the CRP doublet proteins of *C. caviae* GPIC was degraded by trypsin, without incubation in Tris-EDTA, to peptides of approximately the same size as the short doublet of the protein. They therefore suggested that at least the N-terminal portion of the larger doublet protein is surface exposed and they observed that the protein plays a role in adhesion of *C. caviae* to host cells. Stephens *et al*. (2001) confirmed that OmcB of *C. trachomatis* LGV is surface exposed and accessible to antibodies and identified a heparin-binding motif in the N-terminal region of OmcB in both *C. trachomatis* LGV and trachoma (serovar B) biovars and in *C. pneumoniae* and *C. psittaci*. Synthetic peptides of this regions in the different *Chlamydia* species and strains bound to heparin (Stephens *et al*., 2001). However, the OmcB of *C. trachomatis* LGV was shown to function as an heparin-dependent adhesin which can be neutralized by anti-OmcB antibodies (Fadel and Eley, 2007), while *C. trachomatis* serovar E (trachoma biovar) was shown not to be dependent on heparin for adhesion (Fadel and Eley, 2008). Further research is needed to elucidate whether the heparin-binding motifs identified by Stephens *et al*. (2001) are surface exposed and functional in different *C. trachomatis* serovars and to decide

whether differences in OmcB binding properties might be related to the different invasiveness observed for those biovars (Allen *et al*., 1990). Moelleken and Hegemann (2008) confirmed that OmcB is a heparin-dependent adhesin in *C. pneumoniae*. No research has been done on adhesion capacities of *C. psittaci* OmcB.

5.3.1.3 Polymorphic membrane proteins

Whole-genome sequencing has revealed the polymorphic membrane protein (Pmp) gene family. This is the largest protein family of *Chlamydia* species and it is a unique feature of the genus (Horn *et al*., 2004; Vandahl *et al*., 2004). The Pmp proteins were first identified in *C. abortus* through their immunogenicity (Longbottom *et al*., 1996; Longbottom *et al*., 1998b) and later it was noticed that the family is present in all currently sequenced chlamydial genomes (Read *et al*., 2000; Read, 2003; Thomson *et al*., 2005; Azuma *et al*., 2006; Voigt *et al*., 2012). The Pmps have been identified as autotransporter (type V secretion system) proteins, based on their cleavable N-terminal signal sequence (type II secretion) for translocation across the inner membrane, a central passenger domain which is responsible for the protein's function and a C-terminal transporter domain that forms a β-barrel and with a phenylalanine at the end, which is suggestive for outer membrane localization, for translocation across the outer membrane (Struyve *et al*., 1991; Henderson and Lam, 2001; Dautin and Bernstein, 2007). Experimental evidence for several Pmps confirmed this in silico prediction (Longbottom *et al*., 1998a; Vandahl *et al*., 2002; Wehrl *et al*., 2004; Kiselev *et al*., 2007; Liu *et al*., 2010). Most autotransported proteins contribute to the virulence of many Gram-negative pathogens (Henderson *et al*., 2004; Dautin and Bernstein, 2007; Tseng *et al*., 2009). Adhesins (e.g. AIDA-I and Ag43 of *Escherichia coli*, Hia of *Haemophilus influenzae*), toxins (e.g. VacA of *Helicobacter pylori*) and proteases (e.g. IgA protease of *Neisseria gonorrheae*) are some functions of the autotransported proteins (Henderson *et al*., 2001; Tseng *et al*., 2009). The autotransported Pmp proteins account for 3.15% and 5.1% of the total coding capacity of *C. trachomatis* and *C. pneumoniae*, respectively (Grimwood and Stephens, 1999). This is a relatively high proportion of the highly reduced genome and therefore it is suggested that the Pmps might play an important function in chlamydial biology.

The size and amino acid sequences of the Pmp proteins are highly variable, but grouping of those proteins in one family is based on the conserved motifs FxxN and GGA (with I, L or V in the 4th position). The *C. trachomatis* and *C. muridarum* genomes encode nine *pmp* genes, named pmpA to pmpI (Stephens, 1998; Read *et al*., 2000). They have been divided in six

phylogenetically related subtypes (PmpA, B/C, D, E/F, G/I and H), which may be able to substitute structurally and functionally for one another (Grimwood and Stephens, 1999). The number of *pmp* genes is variable, ranging from 9 to 16 full length genes in the *Chlamydia* reference strains *C. abortus* S26/3 (Thomson *et al*., 2005), *C. avium* 10DC88 (Sachse *et al*., 2014), *C. caviae* GPIC (Read, 2003), *C. felis* FE/C-56 (Azuma *et al*., 2006), *C. gallinacea* 08-1274/3 (Sachse *et al*., 2014), *C. muridarum* Nigg (Read *et al*., 2000), *C. pecorum* DBDeUG (Bachmann *et al*., 2014), *C. pneumoniae* CWL029 (Kalman *et al*., 1999), *C. psittaci* ATCC VR-125/6BC (Voigt *et al*., 2011) and *C. trachomatis* D/UW-3/Cx (Stephens, 1998) (Figure 1.6). In addition, truncated and frame shifted *pmp* genes have also been observed in several chlamydia genomes.

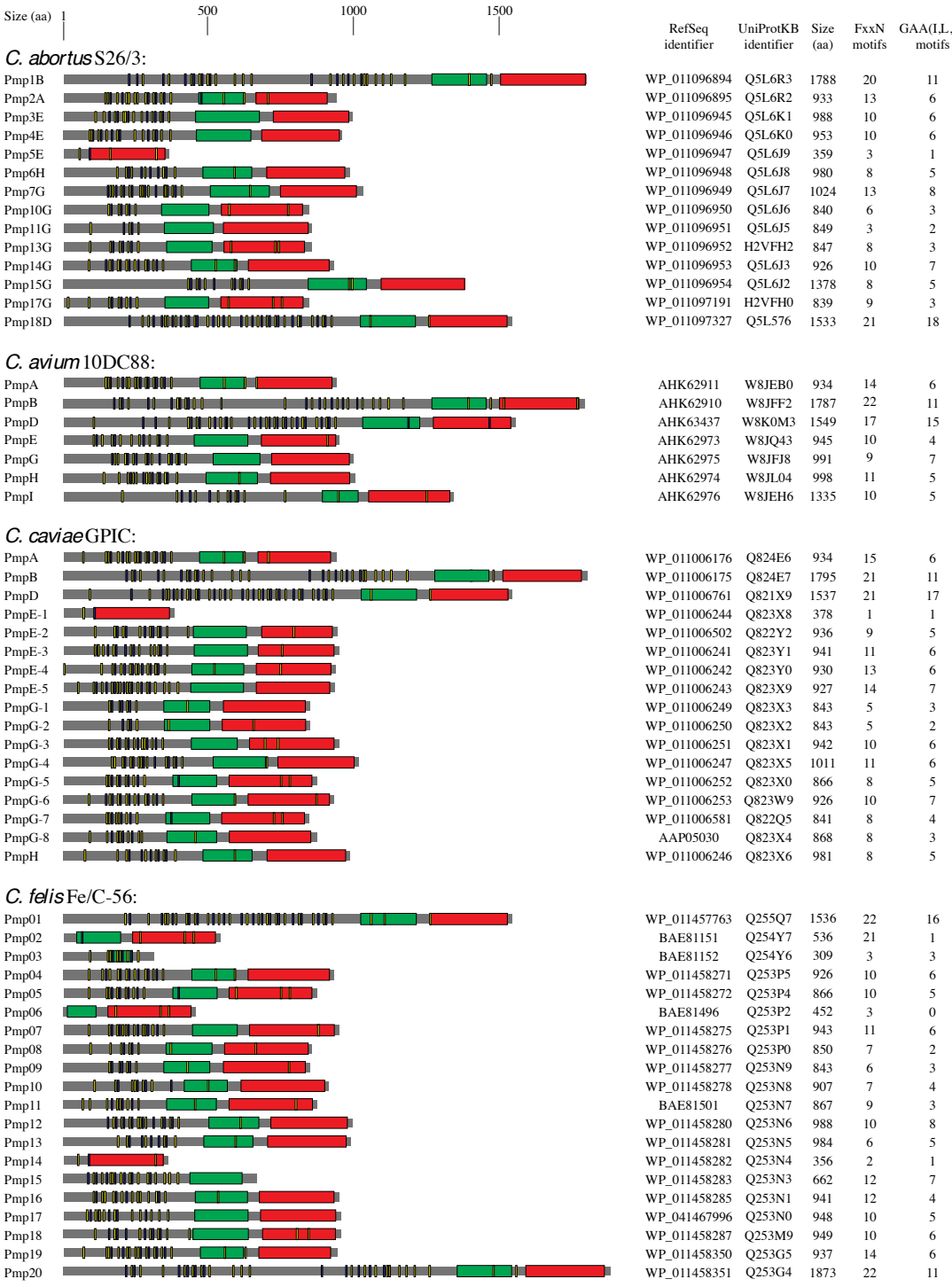
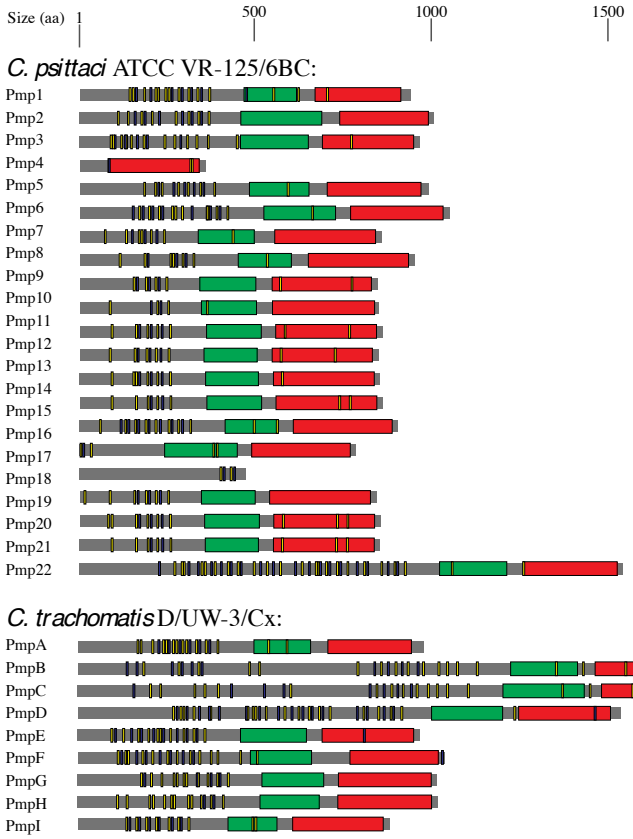


Figure 1.6: Schematic overview of putative Pmp proteins in *Chlamydia* reference strains. FxxN motifs (yellow), GGA (I,L,V) motifs (blue), central PMP_M region (green) and autotransporter domain (red) are shown. The passenger domain is located in between the N-terminal secretion signal (not shown, it is predicted to comprise around 20 amino acids at the N-terminus) and the C-terminal autotransporter domain. The passenger domain is transported across the outer membrane through the autotransporter domain. Frame shifted *pmp* genes are not shown. For each *Chlamydia* species it should be tested experimentally whether the truncated, putative proteins are produced and functional (Vasilevsky *et al*., 2016).

	RefSeq identifier	UniProtKB identifier	Size (aa)	FxxN motifs	GAA(I,L,V) motifs
C. gallinacea 08-1274/3:					
PmpA	WP_021828084	U4T2U0	932	17	6
PmpB	WP_021828083	U4T1L4	1758	23	10
PmpD	WP_021828579	U4T0S3	1553	18	17
PmpE	WP_021828145	U4T134	935	10	4
PmpF	WP_021828146	U4T6L4	655	9	4
PmpG-1	WP_021828148	U4T1G1	976	8	8
PmpG-2	WP_021828149	U4T2P1	1293	12	4
PmpH	WP_021828147	U4T5X0	989	11	5
PmpI	WP_021828236	U4T648	501	10	6
C. muridarum Nigg:					
PmpA	WP_010231239	Q9PJY3	976	15	6
PmpB	WP_010231242	Q9PJY2	1669	17	8
PmpC	WP_010231245	Q9PJY1	1460	15	9
PmpD	WP_010229791	Q9PLB0	1520	20	17
PmpE	WP_010229972	Q9PL47	976	9	8
PmpF	WP_010229975	Q9PL46	1025	14	6
PmpG	WP_010229977	Q9PL45	986	11	4
PmpH	WP_010229979	Q9PL44	980	11	3
PmpI	WP_010229984	Q9PL41	867	12	6
C. pecorum DBDeUG:					
PmpA	WP_024010319	V8TRZ2	1683	19	10
PmpB	WP_024010320	V8TSI8	947	13	8
PmpD	WP_024010164	V8TRS6	1404	17	15
PmpE1	WP_024010358	V8TQL0	1004	15	3
PmpE2	ETF38872	V8TR41	1012	12	6
PmpG1	WP_024010356	V8TSQ9	938	11	6
PmpG2	ETF38862	V8TR31	916	7	4
PmpG3	WP_024010365	V8TRI6	819	7	3
PmpG4	WP_024010366	V8TSQ5	1063	10	6
PmpG5	WP_024010368	V8TQK5	671	4	2
PmpG6	WP_024010369	V8TMN9	932	10	6
PmpG7	WP_024010370	V8TNN9	964	9	5
PmpG8	ETF37859	V8TPW2	1105	11	6
PmpG9	WP_024010559	V8TMX5	946	9	7
PmpH	ETF38870	V8TSB9	969	8	5
C. pneumoniae CWL029:					
Pmp1	NP_224218	Q9Z9G5	922	11	6
Pmp2	NP_224227	Q9Z3A1	841	4	4
Pmp6	NP_224644	Q9Z899	1407	22	16
Pmp7	NP_224645	Q9Z898	936	10	5
Pmp8	NP_224646	Q9Z393	930	9	6
Pmp9	NP_224647	Q9Z398	928	9	6
Pmp11	NP_224649	O86164	928	8	6
Pmp12	NP_224650	Q9Z3D6	514	5	2
Pmp13	NP_224651	Q9Z896	973	12	6
Pmp14	NP_224652	Q9Z895	978	10	3
Pmp15	NP_224664	Q9Z883	938	10	6
Pmp16	NP_224665	Q9Z882	934	11	9
Pmp18	NP_224667	Q9Z880	946	10	8
Pmp19	NP_224735	Q9Z813	947	16	9
Pmp20	NP_224736	Q9Z812	1723	23	11
Pmp21	NP_225156	Q9Z6U5	1609	17	15

Figure 1.6: continued



RefSeq identifier	UniProtKB identifier	Size (aa)	FxxN motifs	GAA(I,L,V) motifs
WP_006342883	F0T396	1797	20	11
WP_006342884	F0T397	933	14	7
WP_006342950	F0T3G0	1000	8	6
WP_006342951	F0T3G1	961	13	5
WP_006342952	F0T3G2	354	2	1
WP_006342953	F0T3G3	984	8	5
WP_006342954	F0T3G4	1043	12	7
WP_013747335	F0T3G5	851	7	3
WP_013747336	F0T3G6	944	7	3
WP_013462619	F0T3G7	840	6	3
WP_006342960	F0T3G8	844	4	2
WP_013747337	F0T3G9	854	7	3
WP_013747338	F0T3H0	843	7	3
WP_013747339	F0T3H1	847	7	3
WP_013747340	F0T3H2	855	7	2
WP_006342966	F0T3H3	898	10	7
WP_013747341	F0T3H4	779	4	1
WP_013747342	F0T3H5	468	2	2
WP_013747378	F0T4E3	837	6	3
WP_013747379	F0T4E4	849	9	3
WP_013747380	F0T4E5	847	8	2
WP_006343467	F0T4W6	1534	23	18
NP_219922	O84417	975	14	6
NP_219923	O84418	1751	17	10
NP_219924	O84419	1770	17	8
NP_220332	O84818	1531	19	17
NP_220391	O84877	964	11	8
NP_219507	O84008	1034	13	8
NP_220393	O84879	1013	11	4
NP_220394	O84880	1016	12	2
NP_220396	O84882	878	9	6

Figure 1.6: continued

Different functions are proposed for members of the Pmp family. The first suggested function was that of **adhesin**, as the conserved motifs GGA (I,L,V) and FxxN were also found in adhesins of *Anaplasma phagocytophilum* (Girard and Mourez, 2006). Mölleken *et al*. (2010) showed that yeast cells, which expressed Pmp6, Pmp20 and Pmp21 (orthologs of PmpG, PmpB and PmpD of *C. trachomatis*, respectively) on their surface, and beads coated with recombinant proteins of these three Pmp proteins adhere to human epithelial cells. The observation that pre-incubation of epithelial cells with these three proteins reduced the binding significantly, confirmed the adhesive capacities of Pmp6, Pmp20 and Pmp21 of *C. pneumoniae*. Mölleken *et al*. (2010) analyzed Pmp21 in more detail and noticed that at least two motifs (FxxN + GGA I/L/V or FxxN + FxxN) are required for adhesion and anti-Pmp21 antibodies can neutralize a *C. pneumoniae* infection *in vitro*. This neutralizing activity of anti-Pmp21 antibodies was previously also observed by Wehrl *et al*. (2004). Moreover, the epidermal growth factor receptor has been identified as the receptor for Pmp21 (Mölleken *et al*., 2013). Crane *et al*. (2006) observed that *C. trachomatis* PmpD is surface localized and that anti-PmpD antibodies neutralize *C. trachomatis*, but not *C. pneumoniae*, *C. muridarum* and *C. caviae* infections, indicating that PmpD is a species-specific antigen. In addition, they

noticed that preexisting anti-MOMP and anti-LPS antibodies blocked significantly the neutralizing activity of anti-PmpD serum *in vitro*. MOMP and LPS are highly abundant and immunodominant antigens on the EB surface (Su *et al*., 1990) and therefore they suggested that MOMP and LPS might act as decoys for the immune system, as binding of neutralizing antibodies to both structures blocks the binding of neutralizing anti-PmpD antibodies (Crane *et al*., 2006). However, anti-MOMP and anti-LPS antibodies do not block anti-PmpD neutralization *in vitro* when anti-PmpD antibodies were preexisting. It is therefore suggested that a vaccine that generates PmpD neutralizing antibodies might be effective (Crane *et al*., 2006). Recently, Becker and Hegemann (2014) showed that all Pmps of *C. trachomatis*, although in a varying degree, mediate adhesion to human epithelial and endothelial cells. In addition, all nine recombinant Pmp proteins can neutralize *C. trachomatis* infections, which suggests that all of the Pmp adhesins are important in the infection process. It was also observed that the Pmp proteins inhibit chlamydial infections in a species-specific manner, as incubation of human epithelial and endothelial cells with *C. trachomatis* Pmp proteins was not effective to block a subsequent *C. pneumoniae* infection and *vice versa*. The latter experiment and the different levels of adhesion of Pmp proteins to different cell types confirmed the previously suggested hypothesis that Pmp proteins are involved in **host and tissue tropism** (Becker and Hegemann, 2014). All nine Pmp proteins of *C. trachomatis* have been shown to be surface localized (Tan *et al*., 2010). A different surface localization of PmpD on EBs and RBs of *C. trachomatis* has been observed by different research groups. Crane *et al*. (2006) and Swanson *et al*. (2009) observed PmpD on EBs, while Kiselev *et al*. (2007, 2009) observed PmpD on RBs but not on EBs. This difference may be attributed to different epitopes that may be recognized by the different anti-sera (Crane *et al*., 2006). Pmp21 (ortholog of PmpD of *C. trachomatis*) was also observed to be surface localized on *C. pneumoniae* EBs and RBs (Vandahl *et al*., 2002; Wehrl *et al*., 2004; Mölleken *et al*., 2010). In addition, Pmp6, Pmp8, Pmp10, Pmp11 (all orthologs of PmpG of *C. trachomatis*) were also observed at the surface of *C. pneumoniae* EBs (Knudsen *et al*., 1999; Vandahl *et al*., 2002). Other Pmp proteins might also be present at the surface, but the epitopes recognized by their antibodies were either unable to be detected, which might be due to destruction of those epitopes by the fixation method used or it might be due to the inaccessibility of the epitopes (Vandahl *et al*., 2002). The 90kDa Pmps (orthologs of pmpG of *C. trachomatis*) were observed at the surface of both *C. abortus* RBs and EBs (Longbottom *et al*., 1998a). At least some Pmp proteins are surface localized antigens on EBs and have been shown to neutralize infectivity *in vitro*. However, vaccine candidates should also be highly immunogenic *in vivo*.

Longbottom *et al*. (1998b) observed that the 90kDa Pmps of *C. abortus* were highly **immunogenic** components of COMC, although they are only minor components of COMC. In addition, Knudsen *et al*. (1999) observed that conformational epitopes of *C. pneumoniae* Pmp10 (PmpG ortholog of *C. trachomatis*) were dominant antigens in experimentally infected mice and Bunk *et al*. (2008) found that Pmp6 (also a PmpG orthologs of *C. trachomatis*) is immunodominant. Up to now, only sera from patients infected with *C. trachomatis* have been tested for their immunogenicity against a panel of recombinant Pmp proteins. Tan *et al*. (2009) observed that patients infected with *C. trachomatis* elicited high titer antibodies against a subset of Pmp proteins, that varies between infected individuals. It is suggested that the variable antibody profiles may reflect the variation in transcription and protein production profiles along the developmental cycle (Grimwood and Olinger, 2001; Vandahl *et al*., 2002; Tan *et al*., 2009; Wheelhouse *et al*., 2009; Carrasco *et al*., 2011; Wheelhouse *et al*., 2012b), which suggests that the Pmp may be involved in **antigenic variation** and contribute to immune evasion in the infected host (Carrasco *et al*., 2011; Tan *et al*., 2009). Other, minor investigated potential functions of the Pmp proteins are: induction of apoptosis in neighbouring uninfected cells and suppression of T-cells (Swanson *et al*., 2009). The Pmps have also been shown to be involved in the induction of cytokine production (Niessner *et al*., 2003) in infected cells and in pelvic inflammatory disease and infertility (Taylor *et al*., 2011).

Previous studies have mainly focused on the Pmp proteins of *C. trachomatis* (Belland *et al*., 2003b; Crane *et al*., 2006; Kiselev *et al*., 2007; Tan *et al*., 2009; Kiselev *et al*., 2009; Swanson *et al*., 2009; Tan *et al*., 2010; Carrasco *et al*., 2011; Saka *et al*., 2011; Humphrys *et al*., 2013; Becker and Hegemann, 2014) and *C. pneumoniae* (Knudsen *et al*., 1999; Vandahl *et al*., 2002; Wehrl *et al*., 2004; Mölleken *et al*., 2010; Mölleken *et al*., 2013), both human pathogens, and on the zoonotic *C. abortus* (Longbottom *et al*., 1998a,b; Wheelhouse *et al*., 2009; Wheelhouse *et al*., 2012a,b; Forsbach-Birk *et al*., 2013). Up to now, the Pmp proteins of *C. psittaci* have not been molecularly analyzed. *C. psittaci* infects pet birds and poultry and infections lead to financial losses, particularly in duck, turkey (Vanrompay *et al*., 1997) and more recently also chicken production (Dickx *et al*., 2010), as they cause mortality, reduced feed conversion, reduced egg production, high expenses for antibiotic treatment and carcass condemnation at slaughter (Vanrompay *et al*., 1997). In addition, *C. psittaci* is the most common zoonotic animal chlamydiosis, which can cause a life-threatening pneumonia (Vanrompay *et al*., 1997; Longbottom and Livingstone, 2006; Grinblat-Huse *et al*., 2011). A thorough molecular characterization of the Pmp proteins of *C. psittaci* is an essential step to

further unravel the host-pathogen interaction. Generated knowledge might indicate whether one or more members of this family might be potential vaccine candidates for *C. psittaci* or not.

5.3.1.4 PorB

PorB, also called OmpB, is a protein of 38 kDa that was not readily detected by biochemical methods, as it has approximately the same molecular weight as well as the same isoelectric point as the abundant MOMP (Kubo and Stephens, 2000). The protein is, although in lower amounts than MOMP, present in the COMC of EBs and is surface accessible by PorB-specific antisera (Sanchez-campillo *et al*., 1999; Kubo and Stephens, 2000; Kubo and Stephens, 2001; Kawa and Stephens, 2002). Anti-PorB antibodies neutralized *C. trachomatis* infectivity *in vitro* (Kawa and Stephens, 2002). The sequence of PorB is highly conserved among *Chlamydia* species (Kubo and Stephens, 2000; Kawa and Stephens, 2002). It has been suggested that PorB is a substrate-specific porin, which is responsible for the diffusion of some specific metabolites like dicarboxylic acids such as 2-oxoglytarate to complete the tricarboxylic adic cycle and as such to provide carbon and energy production intermediates to chlamydiae (Iliffe-Lee and McClarty, 1999; Kubo and Stephens, 2001). This is in contract to MOMP, that functions as a general porin and permits the diffusion of a wide variety of compounds, such as polysaccharides and amino acids (Kubo and Stephens, 2001).

5.3.1.5 Omp85

Omp85 is an outer membrane protein that is conserved in Gram-negative bacteria and organelles of bacterial origin, such as mitochondria (Gentle *et al*., 2005). Omp85 proteins have an N-terminal periplasmic domain and a C-terminal β-barrel domain, which is embedded in the outer membrane (Gentle *et al*., 2005). Omp85 is essential for the assembly of protein in the outer membrane (Voulhoux *et al*., 2003) and it might also be involved in lipid assembly in the outer membrane (Gentle *et al*., 2005). Chlamydial Omp85 has been shown to be part of the *C. trachomatis* COMC (Liu *et al*., 2010). Omp85 is surface-accessible and anti-Omp85 antibodies neutralize chlamydial infectivity *in vitro* (Kubo and Stephens, unpublished data).

5.3.1.6 YscC

YscC is the only T3S system protein that is clearly localized in the outer membrane. For *C. trachomatis* YstC has been shown to be membrane localized (Fields *et al*., 2003) and to be part of the COMC (Liu *et al*., 2010).

5.3.1.7 OprB

OprB is a porin with a carbohydrate-selective porin motif in its C-terminal part. The protein is well conserved in *Chlamydia* species. Anti-OprB antibodies reacted with ring-shaped structures, similar to anti-MOMP stainings, which indicated that the protein is located in a membrane. More specifically, OprB is part of the *C. trachomatis* COMC (Birkelund *et al*., 2009; Liu *et al*., 2010). It is currently unknown whether OprB is surface localized (Birkelund *et al*., 2009).

5.3.1.8 Pal

Peptidoglycan-associated lipoprotein (Pal) is an outer membrane lipoprotein (Parsons *et al*., 2006), which is a component of the *C. trachomatis* outer membrane complex (Liu *et al*., 2010). Orthologs of Pal anchor the outer membrane to peptidoglycan (Parsons *et al*., 2006) and are part of the Tol complex, which connects the inner and outer membranes in other Gram-negative bacteria. Pal is essential for the survival and pathogenesis of certain Gram-negative bacteria (Godlewska *et al*., 2009).

5.3.2 Heat shock proteins

Three chamydial heat shock proteins (Hsp) have been identified: Hsp10, Hsp60 and Hsp70, which are chlamydial homologues of *Escherichia coli* GroES, GroEL and DnaK (Danilition *et al*., 1990; Cerrone *et al*., 1991; LaVerda *et al*., 1999). Hsp are among the most abundant proteins in nature and are highly conserved among both eukaryotes and prokaryotes (Peeling and Mabey, 1999). Hsp are highly conserved chlamydial proteins (>95% amino-acid identity) and share approximately 50% homology with human Hsps (Peeling and Mabey, 1999). The proteins are constitutively expressed throughout the chlamydial developmental cycle and the expression is up-regulated during stress (Peeling and Mabey, 1999). Hsp are suggested to function as chaperones (Zugel and Kaufmann, 1999). Antibodies against Hsp10 and Hsp60 have been shown to contribute to immunopathologic manifestations of the severe upper

genital tract complications of chlamydial disease in women (LaVerda *et al*., 1999). Hsp70 is associated with isolated COMC of *C. trachomatis* (Raulston *et al*., 2002). However, not all proteins associated with COMC are component of the COMC. Liu *et al*. (2010) elucidated which proteins are enriched in the COMC and in the sarkosyl-soluble phase compared to whole EB protein lysates. Hsp70 and Hsp60 are overrepresented in the sarkosyl-soluble fraction, which suggest that they are not components of the COMC (Liu *et al*., 2010). Raulston *et al*. (2002) showed that chlamydial Hsp70 is not surface exposed on purified EBs. The results of their experiments suggested that the structural integrity of the outer membrane of *C. trachomatis* EBs, which is maintained by protein disulfide bonds, is essential for attachment to the host epithelial cell, but after the first initial attachment, reduction of the disulfide-cross-linked outer membrane proteins leads to the exposure of the chlamydial Hsp70 substrate-binding domain, which is suggested to be essential for chlamydial infectivity (Raulston *et al*., 2002).

5.3.3 Nutrient transporters

Saka *et al*. (2011) quantified the proteome of *C. trachomatis* EBs and RBs. Transporters, permeases and translocators account for 7% and 2.5% of the RB and EB proteome of *C. trachomatis*, respectively. Npt1 and Npt2, two integral membrane ATP/ADP antiporters, were more abundant in the RB . Npt1 mediates the import of host cell ATP, through the export of chlamydial ADP. Npt2 transports ATP, CTP, GTP and UTP in a proton-dependent manner (Stephens, 1998; Tjaden *et al*., 1999). Oligopeptide, amino acid and sugar transporters were also more abundant in the RB. Components of the ABC-type oligopeptides transport system, Na^+-linked D-alanine glycine permease DagA_2 and the hexose phosphate transport protein UhpC were more prominent in the RB. YtgA, which is possibly involved in iron transport (Miller *et al*., 2009), was also enriched in the RB. It is suggested that the EB uses glucose to cope with the high demand for energy at the early stage of the infection, while the RB switches to ATP synthesis by generating an ion gradient through eukaryotic like vacuolar (V)-type ATPases (Saka *et al*., 2011). Six predicted V-type ATPase subunits were enriched in the RB. These results suggest that the RB acquires energy both by ATP synthesis and ATP transport from the host. Saka *et al*. (2011) proposed that the ATP is used both by the replicating RB and loaded in the EBs, to fuel early processes such as protein secretion. The above mentioned results are consistent with the functions of RBs, as the actively replicating RB has a high demand for nutrients.

Virulence factors account for 5% and 14% of the RB and EB proteome, respectively. Two components of the basal aparatus of the T3S system, the C-ring component CdsQ and the ATPase CdsN were largely absent in the RB. The relative abscence of these components may either be substituted by additional factors or the RB may have a reduced T3S capacity. This might prevent that an excess of T3S effectors would be secreted, as such excess could harm the host cell, disrupt the development of the inclusion or provide substrates for antigen presentation by the host (Saka *et al*., 2011).

Study objectives

The present study aims to fundamentally characterize the polymorphic membrane protein (Pmp) family of *Chlamydia psittaci*. *C. psittaci* is a Gram negative obligate intracellular pathogen of birds and an important zoonotic agent via inhalation of infected aerosols of pharyngeal or nasal secretions or dried feces (Dickx and Vanrompay, 2011).

In the last decade, the Pmp proteins have been studied intensively, particularly because the *C. trachomatis* and *C. pneumoniae* Pmp families represent a relatively high proportion of the coding capacity (3.15 to 5.1%, respectively) in the highly reduced chlamydial genome. Moreover the occurrence of the *pmp* gene family in all currently sequenced chlamydial genomes (Grimwood and Stephens, 1999) suggests an important function in chlamydial biology. Previous studies have mainly focused on the Pmps of *C. trachomatis* (Belland *et al*., 2003b; Crane *et al*., 2006; Kiselev *et al*., 2007; Tan *et al*., 2009; Kiselev *et al*., 2009; Swanson *et al*., 2009; Tan *et al*., 2010; Carrasco *et al*., 2011; Saka *et al*., 2011; Humphrys *et al*., 2013; Becker and Hegemann, 2014) and *C. pneumoniae* (Vandahl *et al*., 2002; Wehrl *et al*., 2004; Mölleken *et al*., 2010; Mölleken *et al*., 2013), both human pathogens, and on the zoonotic *C. abortus* (Longbottom *et al*., 1998a; Longbottom *et al*., 1998b; Wheelhouse *et al*., 2009; Wheelhouse *et al*., 2012a; Wheelhouse *et al*., 2012b; Forsbach-Birk *et al*., 2013). However, the *pmp* gene family of *C. psittaci* has not been investigated so far.

Whole genome-sequencing of *Chlamydia* spp. has revealed the Pmp family, which is the largest membrane protein family in *Chlamydia* spp. and it is a unique feature of the genus (Horn *et al*., 2004; Vandahl *et al*., 2004). Pmps are grouped into a family based on the conserved repetitive motifs FxxN and GGA (with I, L or V at the 4th position). In *C. trachomatis*, they have been further divided into six phylogenetically related subtypes (PmpA, B/C, D, E/F, G/I, and H) which may be able to substitute structurally and functionally for one another (Grimwood and Stephens, 1999). The passenger domain is responsible for the protein's function (Henderson and Lam, 2001). Pmp6, Pmp20 and Pmp21 of *C. pneumoniae* (orthologs of PmpG, PmpB and PmpD of *C. trachomatis*, respectively) and all Pmp proteins of *C. trachomatis* are proposed to function as adhesins, based on adhesion assays and specific neutralization of the infection by incubation of the host cells with the recombinant Pmp proteins (Crane *et al*., 2006; Mölleken *et al*., 2010; Becker and Hegemann, 2014). Up to now, anti-PmpD and anti-Pmp21 antibodies are the only Pmp-specific antibodies that are tested for

their possible neutralizing capacity. Specific anti-PmpD and anti-Pmp21 antibodies can partially neutralize *C. trachomatis* and *C. pneumoniae* infection, respectively, *in vitro* (Wehrl *et al.*, 2004; Crane *et al.*, 2006; Mölleken *et al.*, 2010).

Patients infected with *C. trachomatis* usually elicit high titer antibodies against a subset of the Pmp proteins, that varies between infected individuals (Tan *et al.*, 2009). The different antibody profiles in patients may reflect different transcription and protein production profiles along the developmental cycle or it may be a result of strain variation or site specificity (Grimwood and Olinger, 2001; Vandahl *et al.*, 2002; Tan *et al.*, 2009; Wheelhouse *et al.*, 2009; Carrasco *et al.*, 2011; Wheelhouse *et al.*, 2012b). An attractive hypothesis is that variation of *pmp* gene expression and the resulting antigenic variation phenotype contributes to immune evasion in the infected host. Finally, Pmp proteins were reported to be involved in host and tissue tropism (Becker and Hegemann, 2014).

The **first objective** of this thesis was to determine the number, organization and size of *pmp* coding sequences (CDSs) in different *C. trachomatis*, *C. pneumoniae*, *C. abortus* and *C. psittaci* genomes. Therefore, the *pmp* CDSs were annotated by a Hidden Markov Model and all CDSs were drawn to scale. Furthermore, conserved proteins are hypothesized to be indispensable for the pathogenesis of an organism. So the level of conservation of the Pmp proteins within and across the above-mentioned 4 *Chlamydia* species was determined.

Quantitative real-time polymerase chain reaction (RT-qPCR) is a major tool to gain insight in the molecular pathogenesis of *C. psittaci* (Vandesompele *et al.*, 2002). However, validated reference genes are needed to avoid biases in RT-qPCR. This normalization step is the most problematic and most neglected part in RT-qPCR. Up to now, stably expressed genes for normalization of RT-qPCR data in *Chlamydia* species have only been determined for *C. trachomatis* (Borges *et al.*, 2010). Therefore, the **second objective** of this study was to validate reference genes for RT-qPCR in *C. psittaci* during the normal developmental cycle, during penicillin-induced persistence and for normal + penicillin conditions. Reference genes validated for normal + penicillin should be used to check whether a certain gene is up- or down-regulated during the persistent state compared to during normal development. The latter could help to further unravel the molecular mechanism of the persistent state and thus by preventing *Chlamydia* spp. to go into the persistent state, this would help to treat chlamydial infections.

Chlamydial infections can easily be resolved by treatment with antibiotics. However, *C. suis* acquired stable tetracycline resistance by horizontal gene transfer (Dugan *et al.*, 2004; Dugan *et al.*, 2007). Pigs are the natural host of *C. suis*, but *C. abortus*, *C. pecorum* and *C. psittaci* also occur in the pig (Schautteet and Vanrompay, 2011). Consequently, spreading of the tetracycline resistance gene is possible. As chlamydial infections are economically devastating (Everett, 2000; Harris *et al.*, 2012; Forsbach-Birk *et al.*, 2013; Kalmar *et al.*, 2015), the need for an effective vaccine is high. Vaccine development is not the aim of this thesis. However, as the Pmp proteins have been hypothesized to be vaccine candidates (Crane *et al.*, 2006; Mölleken *et al.*, 2010), a thorough analysis of these proteins in *C. psittaci* is a first fundamental step to unravel the host-pathogen interactions and generated knowledge can be applied for future vaccine research. As previous studies suggested a unique role in chlamydial pathogenesis for virulence genes expressed during stress (Carrasco *et al.*, 2011), the **third objective** of this study was to determine the expression and production profile of the *pmp* CDSs and Pmp proteins during the normal developmental cycle and during penicillin-induced persistence by RT-qPCR and immunofluorescence (IF) microscopy. In addition, as RT-qPCR determines the expression level in the population and IF in individual inclusions, immuno-electron microscopy (IEM) was used to assess the subcellular localization of the Pmps at late developmental times on individual chlamydiae.

Vaccination of pregnant ewes by the *C. abortus* outer membrane complex conferred protective immunity (Tan *et al.*, 1990). In addition, a multisubunit vaccine encompassing the major outer membrane protein and multiple Pmp proteins conferred better immunity than the single antigens (Yu *et al.*, 2014). The Pmps present in the multisubunit vaccine are overrepresented in the *C. trachomatis* outer membrane complex (Liu *et al.*, 2010). Overall, Pmp proteins present in the chlamydial outer membrane complex are suggested to be potential vaccine candidates. As the composition of the chlamydial outer membrane complex was previously only determined for *C. trachomatis* (Mygind *et al.*, 2000; Birkelund *et al.*, 2009; Liu *et al.*, 2010), *C. pneumoniae* (Knudsen *et al.*, 1999) and *C. abortus* (Tan *et al.*, 1990; Cevenini *et al.*, 1991; McCafferty *et al.*, 1995; Longbottom *et al.*, 1996), the **fourth objective** of this study was to determine which Pmp proteins are present in the *C. psittaci* outer membrane complex by IEM.

Chapter II

The number, organization and size of polymorphic membrane protein coding sequences as well as the most conserved Pmp protein differs within and across *Chlamydia* species

---

Adapted from:

Van Lent S., Piet J.R., Beeckman D., van der Ende A., Van Nieuwerburgh F., Bavoil P., Myers G., Vanrompay D. and Pannekoek Y. (2012). Full Genome Sequences of All Nine *Chlamydia psittaci* Genotype Reference Strains. *Journal of Bacteriology*. 194: 6930-6931.

Van Lent S., Creasy H.H., Myers G.S.A. and Vanrompay D. (2016). The Number, Organization and Size of Polymorphic Membrane Protein Coding Sequences as well as the Most Conserved Pmp Protein Differ within and across *Chlamydia* species. *Molecular Microbiology and Biotechnology*. 26: 333-344.

Abstract

Variation is a central trait of the polymorphic membrane protein (Pmp) family. The number of *pmp* coding sequences differ between *Chlamydia* species, but it is unknown if the number of *pmp* coding sequences is constant within a *Chlamydia* species. The level of conservation of the Pmp proteins has previously only been determined for *C. trachomatis*. As different Pmp proteins might be indispensible for the pathogenesis of different *Chlamydia* species, this study investigated the conservation of the Pmp proteins both within and across *C. trachomatis*, *C. pneumoniae*, *C. abortus* and *C. psittaci*. The *pmp* coding sequences were annotated in 16 *C. trachomatis*, 6 *C. pneumoniae*, 2 *C. abortus* and 16 *C. psittaci* genomes by a Hidden Markov Model. The number and organization of polymorphic membrane coding sequences differed within and across the analyzed *Chlamydia* species. The length of coding sequences of *pmpA*, *pmpB* and *pmpH* was conserved among all analyzed genomes, while the length of the expanded subtypes *pmpE/F* and *pmpG*, and remarkably also of subtype *pmpD* differed among the analyzed genomes. PmpD, PmpA, PmpH and PmpA were the most conserved polymorphic membrane proteins in *C. trachomatis*, *C. pneumoniae*, *C. abortus* and *C. psittaci*, respectively. PmpB was the most conserved polymorphic membrane protein across the four analyzed *Chlamydia* species.

1. Introduction

Members of the *Chlamydiaceae* are well known pathogens, causing a wide variety of infectious diseases in both animals and humans (Longbottom and Coulter, 2003). *Chlamydia trachomatis* and *C. pneumoniae* are the most prevalent species in humans. *C. trachomatis* is the leading cause of preventable blindness and sexually transmitted disease (Schachter and Dawson, 1990), while *C. pneumoniae* causes pneumonia, bronchitis, and sinusitis, with chronic infections contributing to atherosclerosis (Hanh *et al*., 2002; Belland *et al*., 2004). The most common chlamydial species infecting animals are *C. psittaci* (birds), *C. abortus* (sheep and goats), *C. suis* (pigs) and *C. pecorum* (mammals and marsupials). These infections can result in conjunctivitis, cardiovascular or systemic disease, abortion, infertility, enteritis, encephalitis, arthritis or respiratory disease (Everett, 2000). However, the molecular mechanisms behind this observed host tropism has not been unraveled. As variation is a central trait of the members of the polymorphic membrane protein (Pmp) family (Grimwood and Stephens, 1999), these proteins were hypothesized to play a role in host and tissue tropism, which is recently confirmed by Becker *et al*. (2014). Pmp variation is evidenced, for example, by a diversity in the number of Pmp coding sequences (CDSs); for example, the *C. trachomatis* genome encodes only 9 *pmp* CDSs, while the Pmp family is expanded in *C. pneumoniae*, *C. abortus* and *C. psittaci*. The expansion of the Pmp family can be specifically attributed to the expansion of the *pmpE/F* and *pmpG* subtypes. This raises the question whether the number of *pmp* CDSs varies only across species, or also within a species. Therefore, we re-examined the previously published number of *pmp* CDSs of *C. trachomatis*, *C. pneumoniae*, *C. abortus* and *C. psittaci* in 16, 6, 2, and 16 genomes, respectively, as those are the two main human *Chlamydia* pathogens and the two most devastating animal *Chlamydia* species with available genome sequences. More generally, conserved proteins are hypothesized to be indispensable for the pathogenesis of an organism. Consequently, if the Pmp proteins are important for host tropism, it can be hypothesized that different Pmp subtypes may be differentially conserved within different *Chlamydia* species. Until now, the level of conservation of the Pmp proteins had only been determined for *C. trachomatis* (Gomes *et al*., 2006; Carrasco *et al*., 2011). Adding to their possible role in adhesion to different host cells, we hypothesize that some Pmp subtypes may also have a redundant, essential function in different hosts. For this reason, in this study we have determined the level of conservation within and across four key *Chlamydia* species.

2. Materials and methods

2.1 *Chlamydia* species and strains

Genomes of 4 different *Chlamydia* species, namely *C. trachomatis*, *C. pneumoniae*, *C. abortus* and *C. psittaci*, were analyzed. Sixteen complete *C. trachomatis* genomes were selected. Strains belonging to the ocular serovar (A/HAR-13, A/363 and C/TW-3), urogenital serovar (D/13-96, E/150, E/11023, F/1-93, F/6-94, G/9301, G/9768 and J/31-98) and LGV biovar (L1/115, L2/25667R, L2c, L2b/8200/07 and 434/Bu) were included. All *C. pneumoniae* and *C. abortus* genomes available at the moment of analysis were included (B21, AR39, CWL029, J138, LPCoLN, TW-183 and LLG, S26/3 respectively). Sixteen *C. psittaci* genomes were selected, comprising all complete genome sequences that were available at the moment of analysis (01DC11, 02DC15, 08DC60, C19/98, 6BC, 84/55, CP3, GR9, M56, MN, NJ1, VS225, WC and WSRTE30), the previously well-characterized, prototypic *C. psittaci* strain Cal10 (Matsumoto, 1982; Hovis *et al.*, 2013; Mojica *et al.*, 2015) and the first sequenced *C. psittaci* genome, namely strain RD1 (Seth-Smith *et al.*, 2011).

2.2 Bioinformatics analyses

A phylogenetic tree of the above-mentioned *C. trachomatis*, *C. pneumoniae*, *C. abortus* and *C. psittaci* genomes was constructed. Whole genome maximum likelihood trees were constructed as described in Sahl *et al.* (2011). Sequences were aligned with Mugsy v1r2.3 (Angiuoli and Salzberg, 2011) which generates blocks of conserved aligned sequence. Blocks were then joined together and converted to a concatenated multifasta alignment file with the bx-python toolkit (http://bitbucket.org/james_taylor/bx-python/wiki/Home). The alignment file was filtered using mothur filter.seqs (Schloss *et al.*, 2009). A maximum likelihood based phylogenetic tree was inferred using RaxML (raxmlHPC-PTHREADS v8.2.5) (Stamatakis, 2014), using the GTRGAMMA model, with 100 independent runs on distinct starting trees and rapid bootstrap analysis.

We annotated the *pmp* CDSs in the above-mentioned genomes using a newly developed Hidden Markov Model (HMM) and an additional manual search of the NCBI database (Table 2.1). The HMM was developed by individual clustal alignments, which were independently run on each *pmp* subfamily within the seed set, consisting of manually curated *pmp* genes from six sequenced *C. psittaci* genomes (6BC, RD1, 01DC11, 02DC15, 08DC60 and C19/98).

Alignments were reviewed manually and it was determined that no trimming was necessary. The HMMER package (hmmbuild) was used to build a separate model for each *pmp* subfamily. All HMMs were validated using the HMMER package (hmmsearch) against a larger set of *C. psittaci* and *C. caviae* sequences. The protein sequences of each Pmp subtype were aligned with ClustalW2 and the percentage of conserved amino acids was calculated both within and across *Chlamydia* species.

Table 2.1: All *C. trachomatis*, *C. pneumoniae*, *C. abortus* and *C. psittaci* strains that were used for the comparative analysis of the *pmp* coding sequences.

Species	Strain	Length (Mb)	CDS (#)	Protein (#)	Pseudogene	% GC	Reference	Accession number
C. trachomatis	L2c	1.04	949	900	5	41.3	(Somboonna *et al*., 2011)	CP002024.1
	L2/25667R	1.04	948	899	4	41.3	(Harris *et al*., 2012)	HE601954.1
	L2b/8200/07	1.04	949	899	5	41.3	(Harris *et al*., 2012)	HE601795.1
	L1/115	1.04	947	895	7	41.3	(Harris *et al*., 2012)	HE601952.1
	D/13-96	1.04	953	903	6	41.3	(Putman *et al*., 2013)	CP006676.1
	J/31-98	1.04	955	908	3	41.3	(Putman *et al*., 2013)	CP006680.1
	F/1-93	1.04	956	895	17	41.3	(Putman *et al*., 2013)	CP006671.1
	F/6-94	1.04	955	905	6	41.3	(Putman *et al*., 2013)	CP006673.1
	434/Bu	1.04	937	880	11	41.3	(Thomson *et al*., 2008)	AM884176.1
	A/363	1.04	958	905	8	41.3	(Harris *et al*., 2012)	HE601796.2
	A/HAR-13	1.04	958	905	8	41.3	(Harris *et al*., 2012)	CP000051.1
	C/TW-3	1.04	957	897	16	41.3	(Borges *et al*., 2014)	CP006945.1
	E/11023	1.04	953	902	7	41.3	(Jeffrey *et al*., 2010)	CP001890.1
	E/150	1.04	955	904	7	41.3	(Jeffrey *et al*., 2010)	CP001886.1
	G/9301	1.04	953	903	6	41.3	(Jeffrey *et al*., 2010)	CP001930.1
	G/9768	1.04	953	903	6	41.3	(Jeffrey *et al*., 2010)	CP001887.1
C. pneumoniae	B21	1.22	1219	1182	not mentioned	40.5	(Roulis *et al*., 2014)	AZNB01000082.1
	AR39	1.23	1165	1110	not mentioned	40.6	(Read *et al*., 2000)	AE002161.1
	CWL029	1.23	1091	1029	19	40.6	(Kalman *et al*., 1999)	AE001363.1
	J138	1.23	1113	1060	11	40.6	(Shirai, *et al*., 2000a; Shirai *et al*., 2000b)	BA000008.3
	LPCoLN	1.24	1102	1006	54	40.5	(Myers *et al*., 2009)	CP001713.1
	TW-183	1.23	1109	1059	8	40.6	(Geng, *et al*., 2002)	AE009440.1
C. abortus	LLG	1.14	1001	936	23	39.9	(Sait *et al*., 2011)	CM001168.1
	S26/3	1.14	1006	930	34	39.9	(Thomson *et al*., 2005)	CR848038.1

C. psittaci	01DC11	1.17	1019	965	12	39.1	(Schöfl *et al*., 2011)	CP002805.1
	02DC15	1.17	1021	972	7	39.1	(Schöfl *et al*., 2011)	CP002806.1
	08DC60	1.17	1018	963	13	39.1	(Schöfl *et al*., 2011)	CP002807.1
	6BC	1.17	1018	971	5	39.1	(Grinblat-Huse *et al*., 2011)	CP002586.1
	C19/98	1.17	1016	968	6	39.0	(Schöfl *et al*., 2011)	CP002804.1
	Cal10	1.18	1038	982	14	39.0	(Grinblat-Huse *et al*., 2011)	AEZD00000000
	RD1	1.16	1001	943	16	39.1	(Seth-Smith *et al*., 2011)	FQ482149.1
	84/55	1.17	1037	893	102	39.1	(Van Lent *et al*., 2012)	CP003790.1
	CP3	1.17	1121	950	21	39.0	(Van Lent *et al*., 2012)	CP003797.1
	GR9	1.15	1010	949	19	39.1	(Van Lent *et al*., 2012)	CP003791.1
	M56	1.16	1008	946	20	38.8	(Van Lent *et al*., 2012)	CP003795.1
	MN	1.17	1019	956	21	39.1	(Van Lent *et al*., 2012)	CP003792.1
	NJ1	1.16	1015	955	18	39.0	(Van Lent *et al*., 2012)	CP003798.1
	VS225	1.16	1025	898	85	39.0	(Van Lent *et al*., 2012)	CP003793.1
	WC	1.17	1026	963	21	39.1	(Van Lent *et al*., 2012)	CP003796.1
	WSRTE30	1.14	1008	934	32	39.0	(Van Lent *et al*., 2012)	CP003794.1

3. Results

3.1 The number, organization and size of *pmp* coding sequences (CDSs) in the genome varied within and across *Chlamydia* species

A phylogenetic tree of all analyzed genomes was constructed (Figure 2.1). The number of *pmp* CDSs encoded in the genome varied within *Chlamydia* species, ranging from 17 to 28 (including both full length and truncated genes) for *C. pneumoniae*, from 12 to 14 for *C. abortus*, and from 11 to 21 in *C. psittaci* (Figure 2.2 and Tables 2.2 and 2.3). All *C. trachomatis* genomes encoded 9 *pmp* CDSs, except strain F/1-93 which had 8 full length and 1 truncated *pmpE/F* CDS. The number of *pmpE/F* CDSs ranged from 2 to 6 (including both full length and truncated CDSs) in *C. pneumoniae*, from 2 to 3 in *C. abortus* and from 1 to 2 in *C. psittaci* genomes and the number of *pmpG* CDSs ranged from 9 to 18 for *C. pneumoniae*, from 6 to 7 for *C. abortus*, and from 5 to 15 for *C. psittaci*. The above-mentioned ranges included full length and truncated *pmp* CDSs. Frame shifted CDSs were only observed for *pmpE/F* CDSs, *pmpG* CDSs and exceptionally for *pmpB* CDSs in *C. pneumoniae* B21 draft genome.

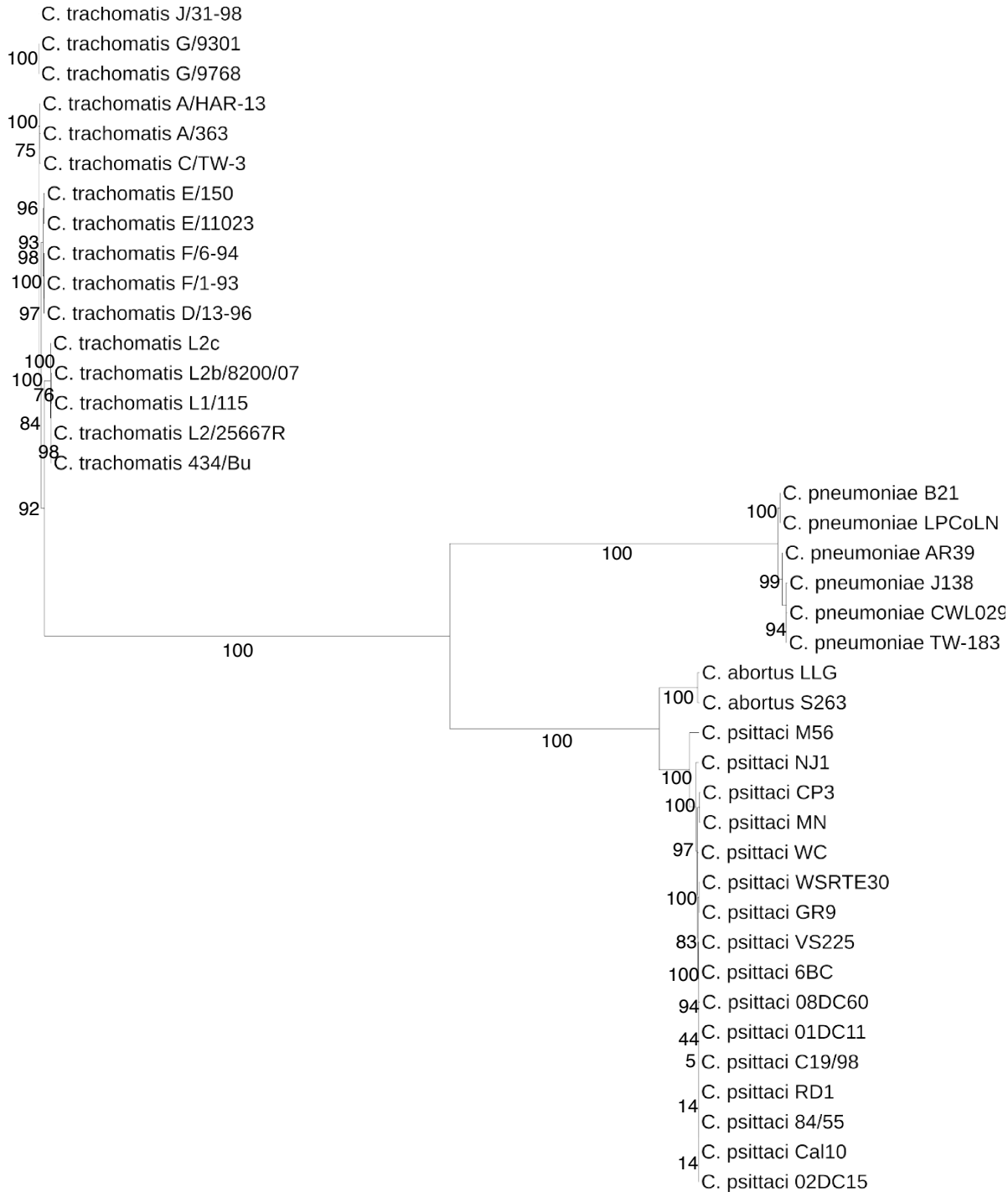


Figure 2.1: A phylogenetic tree of all analyzed *C. trachomatis*, *C. pneumoniae*, *C. abortus* and *C. psittaci* genomes. The genomes clustered by *Chlamydia* species. For *C. trachomatis*, the LGV serovars clustered together and the urogenital and ocular serovars clustered together. Bootstrap values are added on each branch. All branches have high bootstrap values (> 75) except for some *C. psittaci* strains (01DC11, C19/98, RD1, 84/55, Cal10 and 02DC15).

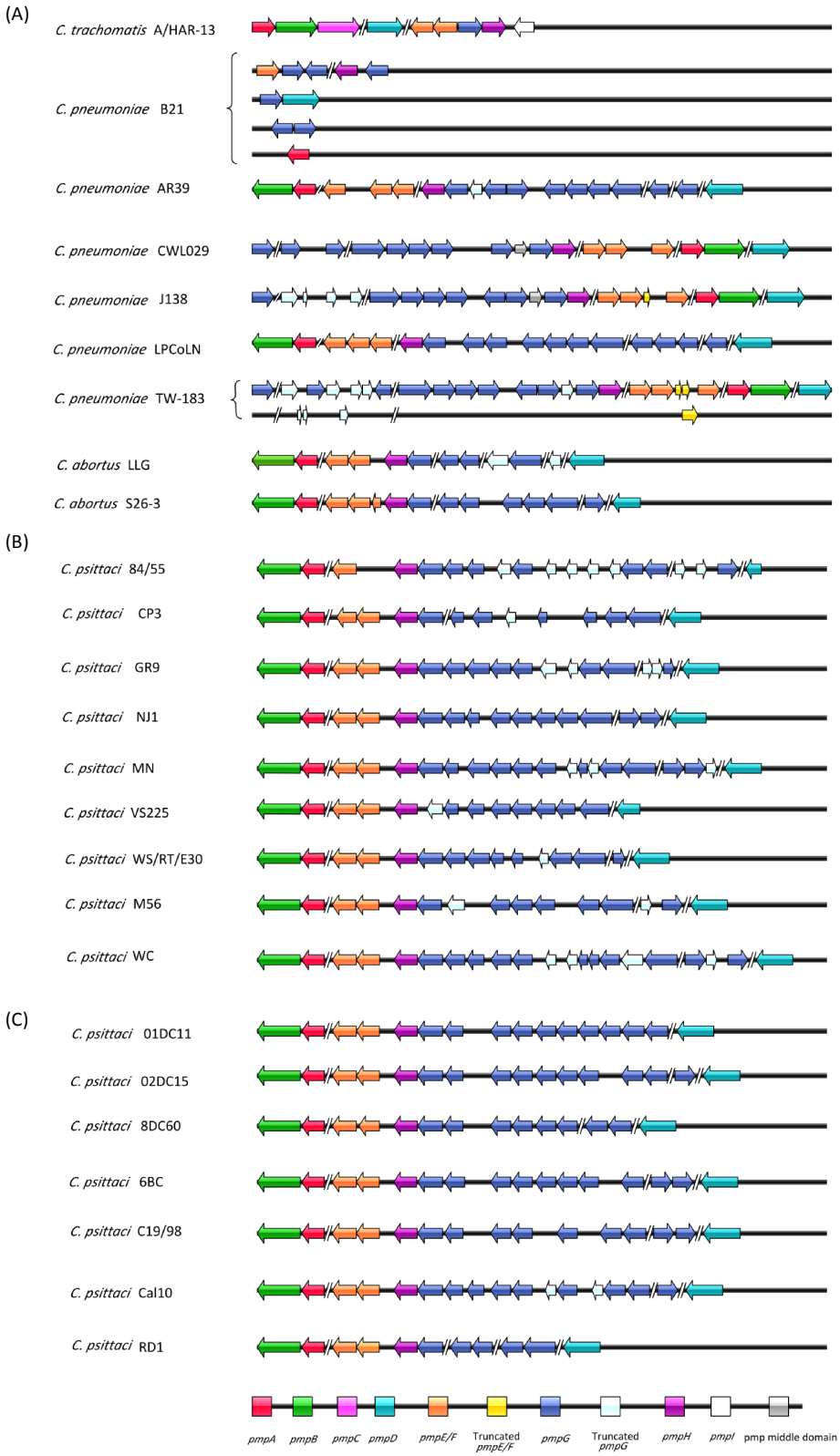


Figure 2.2: Genome organization of *pmp* CDSs in *C. trachomatis*, *C. pneumoniae*, *C. abortus* and *C. psittaci*. All *pmp* CDSs and inter-CDS regions encoded in (A) *C. trachomatis*, *C. pneumoniae* and *C. abortus* genomes, (B) in *C. psittaci* reference strains and (C) in *C. psittaci* strains are drawn to scale. A break (//) is added if the inter-CDS region is bigger than 5000 bp. Both full length and truncated CDSs are shown for all genomes, except for *C. pneumoniae* B21 for which only full length CDS are drawn, as only contigs instead of a complete genome sequence are available and the positions of the *pmp* CDSs are incorrect. If *pmp* CDSs were overlapping, then an additional line was added for that genome.

Table 2.2: The amount of full length, truncated and frame shifted *pmp* CDSs in *C. trachomatis*, *C. pneumoniae*, *C. abortus* and *C. psittaci* genomes.

Species	Strain	Full length *pmp* CDSs	Truncated *pmp* CDSs	Frame shifted *pmp* CDSs
C. trachomatis	A/HAR-13	9	/	/
	F/1-93	8	1	/
C. pneumoniae	B21	10	7	/
	AR39	16	1	4
	CWL029	16	1	8
	J138	15	6	7
	LPCoLN	18	/	1
	TW-183	17	11	/
C. abortus	LLG	10	2	/
	S26/3	14	/	3
C. psittaci	84/55	12	7	/
	CP3	13	1	/
	GR9	14	4	/
	M56	13	2	/
	MN	17	3	/
	NJ1	16	/	/
	VS225	13	1	/
	WC	17	4	/
	WSRTE30	14	1	/
	01DC11	16	/	2
	02DC15	16	/	3
	08DC60	14	/	3
	6BC	16	/	2
	C19/98	15	/	3
	Cal10	15	2	/
	RD1	11	/	1

Table 2.3: The accession numbers of all *pmp* CDSs of *C. trachomatis*, *C. pneumoniae*, *C. abortus* and *C. psittaci*. Full length CDSs are written in plain, black, truncated CDSs in bold and frame shifted CDSs are underlined.

Species	Strain	A	B	C	D	E/F				
C. trachomatis	L2c	ctl2c_703	ctl2c_704	ctl2c_705	ctl2c_203	ctl2c_270	ctl2c_271			
	L2/25667R	L2225667_428	L2225667_429	L2225667_430	L2225667_866	L2225667_933	L2225667_934			
	L2b/8200/07	L2B8200_00426	L2B8200_00427	L2B8200_00428	L2B8200_00863	L2B8200_00929	L2B8200_00930			
	L1/115	L1115_427	L1115_428	L1115_429	L1115_865	L1115_932	L1115_933			
	D/13-96	O176_02240	O176_02245	O176_02250	O176_04535	O176_04865	O176_04870			
	J/31-98	O180_02235	O180_02240	O180_02245	O180_04525	O180_04855	O180_04860			
	F/1-93	O169_02250	O169_02255	O169_02260	O169_04545	O169_04875	O169_04880			
	F/6-94	O172_02235	O172_02240	O172_02245	O172_04530	O172_04860	O172_04865			
	434/Bu	ct434bu_701	ct434bu_702	ct434bu_703	ct434bu_201	ct434bu_268	ct434bu_269			
	A/363	A363_440	A363_441	A363_442	A363_878	A363_943	A363_944			
	A/HAR-13	ctahar_444	ctahar_445	ctahar_446	ctahar_881	ctahar_946	ctahar_947			
	C/TW-3	CTW3_02250	CTW3_02255	CTW3_02260	CTW3_04555	CTW3_04900	CTW3_04905			
	E/11023	cte11023_431	cte11023_432	cte11023_433	cte11023_868	cte11023_933	cte11023_934			
	E/150	cte150_432	cte150_433	cte150_434	cte150_869	cte150_935	cte150_936			
	G/9301	ctg9301_431	ctg9301_432	ctg9301_433	ctg9301_870	ctg9301_935	ctg9301_936			
	G/9768	ctg9768_431	ctg9768_432	ctg9768_433	ctg9768_869	ctg9768_934	ctg9768_935			
C. pneumoniae	B21	gil572024350|gb|ETR80440.1|	**X556_0232; X556_0233**		gil572023388|gb|ETR79739.1|	gil572024253|gb|ETR80366.1|	**gil572024246|gb|ETR80363.1|**			
	AR39	CP_0213	CP_0212		CP_0897	CP_0286	CP_0285	CP_0283	CP_0284	
	CWL029	CPn_0539	CPn_0540		CPn_0963	CPn_0466	CPn_0467	CPn_0471		
	J138	pmp_19	pmp_20		pmp_21	pmp_15	pmp_16	**pmp_17_1**	pmp_17_2	pmp_17_3	pmp_18
	LPCoLN	CPK_ORF01054	CPK_ORF01055		CPK_ORF00378	CPK_ORF00981	CPK_ORF00983	CPK_ORF00982	CPK_ORF00984	
	TW-183	CpB0560	CpB0561		CpB1000	CpB0484	CpB0485	**CpB0486**	**CpB0487**	**CpB0488**	CpB0489
C. abortus	LLG	gil333409995|gb|EGK68982.1|	gil333409994|gb|EGK68981.1|		gil333410522|gb|EGK69509.1|	gil333410058|gb|EGK69045.1|	gil333410059|gb|EGK69046.1|			
	S26/3	CAB201	CAB200		CAB776	CAB265	CAB266	CAB267		
C. psittaci	01DC11	CPS0A_0238	CPS0A_0237		CPS0A_0875	CPS0A_0303	CPS0A_0304			
	02DC15	CPS0B_0234	CPS0B_0233		CPS0B_0862	CPS0B_0299	CPS0B_0300			
	08DC60	CPS0D_0235	CPS0D_0234		CPS0D_0872	CPS0D_0302	CPS0D_0303			
	6BC	G5O_0236	G5O_0235		G5O_0845	G5O_0302	G5O_0303			
	C19/98	CPS0C_0236	CPS0C_0235		CPS0C_0874	CPS0C_0302	CPS0C_0303			
	Cal10	G5Q_0224	G5Q_0223		G5Q_0827	G5Q_0290	G5Q_0291			
	RD1	Cpsi_2191	Cpsi_2181		Cpsi_7911	Cpsi_2811	Cpsi_2821			
	84_55	B595_0240	B595_0239		B595_0921	B595_0307				
	CP3	B711_0240	B711_0239		B711_0922	B711_0310	B711_0311			
	GR9	B598_0235	B598_0234		B598_0860	B598_0301	B598_0302			
	M56	B602_0233	B602_0232		B602_0866	B602_0299	B602_0300			
	MN	B599_0234	B599_0233		B599_0861	B599_0299	B599_0300			
	NJ1	B712_0235	B712_0234		B712_0865	B712_0300	B712_0301			
	VS225	B600_0246	B600_0245		B600_0919	B600_0317	B600_0318			
	WC	B603_0235	B603_0234		B603_0864	B603_0301	B603_0302			
	WSRTE30	B601_0233	B601_0232		B601_0863	B601_0300	B601_0301			

Species	Strain	G									
C. trachomatis	L2c	ctl2c_272									
	L2/25667R	L2225667_935									
	L2b/8200/07	L2B8200_00931									
	L1/115	L1115_934									
	D/13-96	O176_04875									
	J/31-98	O180_04865									
	F/1-93	O169_04885									
	F/6-94	O172_04870									
	434/Bu	ct434bu_270									
	A/363	A363_945									
	A/HAR-13	ctahar_948									
	C/TW-3	CTW3_04910									
	E/11023	cte11023_935									
	E/150	cte150_937									
	G/9301	ctg9301_937									
	G/9768	ctg9768_936									
C. pneumoniae	B21	gil572023612lgblETR79887.1l	gil572024215lgblETR80340.1l	gil572023613lgblETR79888.1l	gil572024213lgblETR80338.1l	gil572024214lgblETR80339.1l	**gil572024223lgblETR80344.1l**	gil572024240lgblETR80359.1l	**gil572023587lgblETR79873.1l**	**gil572023605lgblETR79883.1l**	**gil572024220lgblETR80343.1l**
	AR39	CP_0306	CP_0303	CP_0308	CP_0302	CP_0307	CP_0761	CP_0757	CP_0309	CP_0770	CP_0759
	CWL029	CPn_0447	CPn_0445	CPn_0451	CPn_0446	CPn_0015	CPn_0013	CPn_0444	CPn_0005	CPn_0453	CPn_0449
	J138	pmp_1	**pmp_2_1**	pmp_2_2	**pmp_3_1**	pmp_3_2	**pmp_4_1**	pmp_4_1_2	pmp_4_2	**pmp_5_1**	pmp_5_2
	LPCoLN	CPK_ORF00516	CPK_ORF00960	CPK_ORF00515	CPK_ORF00963	CPK_ORF00965	CPK_ORF00958	CPK_ORF00959	CPK_ORF00514	CPK_ORF00956	CPK_ORF00506
	TW-183	CpB0464	CpB0467	CpB0462	CpB0468	CpB0463	CpB0018	CpB0460	CpB0006	CpB0470	**CpB0015**
C. abortus	LLG	**gil333410066lgblEGK69053.1l**	gil333410063lgblEGK69050.1l	gil333410064lgblEGK69051.1l	gil333410062lgblEGK69049.1l	gil333410067lgblEGK69054.1l	**gil333410355lgblEGK69342.1l**				
	S26/3	CAB281	CAB598	CAB282	CAB277	CAB278	CAB269	CAB283			
C. psittaci	01DC11	CPS0A_0315	CPS0A_0318	CPS0A_0317	CPS0A_0316	CPS0A_0677	CPS0A_0684	CPS0A_0307	CPS0A_0314	CPS0A_0320	CPS0A_0309
	02DC15	CPS0B_0313	CPS0B_0315	CPS0B_0314	CPS0B_0675	CPS0B_0316	CPS0B_0676	CPS0B_0304	CPS0B_0311	CPS0B_0319	CPS0B_0306
	08DC60	CPS0D_0316	CPS0D_0681	CPS0D_0317	CPS0D_0318	CPS0D_0320	CPS0D_0307	CPS0D_0314	CPS0D_0324	CPS0D_0309	CPS0D_0323
	6BC	G5O_0661	G5O_0314	G5O_0316	G5O_0315	G5O_0660	G5O_0657	G5O_0317	G5O_0307	G5O_0313	G5O_0309
	C19/98	CPS0C_0317	CPS0C_0681	CPS0C_0683	CPS0C_0315	CPS0C_0318	CPS0C_0307	CPS0C_0314	CPS0C_0321	CPS0C_0309	CPS0C_0320
	Cal10	G5Q_0648	G5Q_0303	**G5Q_0306**	**G5Q_0302**	G5Q_0294	G5Q_0300	G5Q_0308	G5Q_0296	G5Q_0307	G5Q_0299
	RD1	Cpsi_6121	Cpsi_2851	Cpsi_2891	Cpsi_2911	Cpsi_2901	Cpsi_2881				
	84_55	B595_0726	**B595_0330**	**B595_0722**	**B595_0724**	**B595_0332**	**B595_0328**	**B595_0326**	B595_0316	B595_0324	B595_0334
	CP3	**B711_0330**	B711_0316	B711_0327	B711_0339	B711_0338	B711_0324	B711_0337	B711_0333		
	GR9	**B598_0313**	**B598_0315**	**B598_0667**	**B598_0669**	B598_0306	B598_0310	B598_0318	B598_0307	B598_0317	B598_0309
	M56	B602_0678	B602_0312	**B602_0676**	B602_0303	B602_0311	B602_0316	**B602_0306**	B602_0315	B602_0310	
	MN	B599_0311	B599_0670	B599_0671	**B599_0313**	**B599_0315**	**B599_0672**	B599_0304	B599_0310	B599_0317	B599_0306
	NJ1	B712_0673	B712_0314	B712_0672	B712_0306	B712_0313	B712_0316	B712_0308	B712_0315	B712_0312	B712_0309
	VS225	B600_0332	**B600_0324**	B600_0331	B600_0335	B600_0326	B600_0333	B600_0330	B600_0328		
	WC	B603_0319	B603_0680	B603_0677	B603_0318	**B603_0678**	**B603_0316**	**B603_0314**	B603_0306	B603_0311	B603_0321
	WSRTE30	**B601_0314**	B601_0671	B601_0305	B601_0310	B601_0316	B601_0306	B601_0315	B601_0308	B601_0307	

Species	Strain	G							Middle domain	H	I
C. trachomatis	L2c									ctl2c_273	ctl2c_275
	L2/25667R									L2225667_936	L2225667_938
	L2b/8200/07									L2B8200_00932	L2B8200_00934
	L1/115									L1115_935	L1115_937
	D/13-96									O176_04880	O176_04895
	J/31-98									O180_04870	O180_04885
	F/1-93									O169_04890	O169_04905
	F/6-94									O172_04875	O172_04890
	434/Bu									ct434bu_271	ct434bu_273
	A/363									A363_946	A363_948
	A/HAR-13									ctahar_949	ctahar_951
	C/TW-3									CTW3_04915	CTW3_04930
	E/11023									cte11023_936	cte11023_939
	E/150									cte150_938	cte150_941
	G/9301									ctg9301_938	ctg9301_941
	G/9768									ctg9768_937	ctg9768_940
C. pneumoniae	B21									gi|572024239|gb|ETR80358.1|	
	AR39	CP_0299	**CP_0301**	CP_0760						CP_0298	
	CWL029	CPn_0019	CPn_0016						Cpn_0452	CPn_0454	
	J138	pmp_6	pmp_7	pmp_8	pmp_9	pmp_10	pmp_11	pmp_13	pmp_12	pmp_14	
	LPCoLN	CPK_ORF00967								CPK_ORF00968	
	TW-183	**CpB0023**	**CpB0019**	CpB_0471	CpB_0024	CpB0469				CpB0471	
C. abortus	LLG									gi|333410061|gb|EGK69048.1|	
	S26/3									CAB268	
C. psittaci	01DC11	CPS0A_0319	CPS0A_0313							CPS0A_0306	
	02DC15	CPS0B_0318	CPS0B_0310							CPS0B_0303	
	08DC60	CPS0D_0313								CPS0D_0306	
	6BC	G5O_0320	G5O_0312							G5O_0305	
	C19/98	CPS0C_0313								CPS0C_0306	
	Cal10	G5Q_0297								G5Q_0293	
	RD1									Cpsi_2841	
	84_55	B595_0318	B595_0333	**B595_0323**	B595_0320					B595_0315	
	CP3									B711_0315	
	GR9	B598_0308	B598_0668							B598_0305	
	M56									B602_0302	
	MN	B599_0316	B599_0309	B599_0308	B599_0314					B599_0303	
	NJ1									B712_0305	
	VS225									B600_0322	
	WC	B603_0307	**B603_0320**	B603_0310	B603_0308	B603_0317				B603_0305	
	WSRTE30									B601_0304	

In addition to the different number of *pmp* CDSs between strains of the same *Chlamydia* species, the organization of the *pmp* CDSs varied within and across *Chlamydia* species. Within the species *C. pneumoniae*, most *pmp* CDSs were encoded on the sense strand, while in strains AR39 and LPCoLN most *pmp* CDSs were coded on the antisense strand. However, the order of the CDSs stayed identical. In all analyzed strains of the *C. psittaci* and *C. abortus* species, most *pmp* CDSs were coded on the antisense strand. The CDSs organization was the same in all analyzed *C. trachomatis* strains (only strain A/HAR-13 is shown in Figure 2.2). However, *C. trachomatis* is the only species in which a similar number of *pmp* CDSs were encoded on both strands, and the genome organization of the *pmp* CDSs in *C. trachomatis* is different from the organization observed in the other 3 species, as the *pmpD* CDS is encoded between the *pmpB* and *pmpE/F* CDSs and the *pmpB* and *pmpG* CDSs for *C. trachomatis* and the other 3 species, respectively.

The *pmp* CDSs are drawn to scale in Figure 2.2. The size of *pmpA*, *pmpB* and *pmpH* CDSs was identical in all analyzed genomes, while *C. abortus* S26/3 encoded a shorter *pmpE/F* CDS compared to the other *pmpE/F* CDSs in that genome and all other genomes. The size of the CDSs of the highly expanded *pmpG* subtype varied within and across genomes. The most striking observation was the shorter *pmpD* CDSs in *C. abortus* S26/3 and in three *C. psittaci* strains (84/55, CP3 and VS225). *pmpC* and *pmpI* CDSs were only observed in *C. trachomatis* genomes, while truncated *pmp* middle domain CDSs were only observed in *C. pneumoniae* genomes. Moreover, *C. pneumoniae* is the only species that encoded truncated *pmpE/F* CDSs.

3.2 Different Pmp proteins are highly conserved within and across different *Chlamydia* species

The amino acid sequences of Pmp proteins of the same subtype were aligned and levels of conserved amino acids assessed within and across the *C. trachomatis, C. pneumoniae, C. abortus* and *C. psittaci* genomes (Tables 2.4 and 2.5). PmpD was highly conserved within all analyzed species (= in top 3 of most conserved Pmps), and PmpA and B in three out of four analyzed species. The level of conservation of PmpD in *C. psittaci* was analyzed across 13, rather than 16, PmpD amino acid sequences. Three *C. psittaci* strains (84/55, CP3 and VS225) were excluded from this analysis due to a large deletion within PmpD that skewed conservation analysis. It is striking that PmpH is the most conserved Pmp protein in *C. abortus*, while it is among the least conserved in the other 3 species. As the Pmps were least

conserved in *C. psittaci* (Table 2.4), the most conserved Pmps across *Chlamydia* species were determined both including and excluding *C. psittaci* Pmps. Remarkably, in both cases, PmpB is the most conserved Pmp and PmpD is only the 4th most conserved Pmp (Table 2.5).

Table 2.4: The level of conservation of the amino acid sequence of Pmp proteins in 16 *C. trachomatis*, 6 *C. pneumoniae*, 2 *C. abortus* and 16 *C. psittaci* genomes.

Species	Pmp	% AA conserved	Range (%)
C. trachomatis	D	98.37	83.27-98.37
	A	97.64	
	I	97.61	
	C	95.74	
	B	95.72	
	G	95.36	
	E	91.85	
	H	90.63	
	F	83.27	
C. pneumoniae	A	99.37	0.34-99.37
	B	98.25	
	D	98.20	
	H	96.42	
	E	15.99	
	G	0.34	
C. abortus	H	99.69	3.57-99.69
	D	99.35	
	B	99.16	
	A	99.14	
	E	38.26	
	G	3.57	
C. psittaci	A	94.21	0-94.21
	D	86.13	
	B	82.23	
	H	81.03	
	E	25.81	
	G	0	

Table 2.5: The level of conservation of Pmp proteins across *C. trachomatis*, *C. pneumoniae*, *C. abortus* and (*C. psittaci*).

% AA conserved when Pmp amino acid sequences were included from:	
C. trachomatis*, *C. pneumoniae*, *C. abortus* and *C. psittaci	***C. trachomatis*, *C. pneumoniae* and *C. abortus***
PmpB (25.08%)	PmpB (26.03%)
PmpA (24.27%)	PmpH (25.53%)
PmpH (22.71%)	PmpA (24.77%)
PmpD (21.42%)	PmpD (21.95%)
PmpE (5.72%)	PmpE (7.40%)
PmpG (0%)	PmpG (0.22%)

4. Discussion

As the degree of conservation of the Pmp proteins was until now only determined for *C. trachomatis*, most studies on *C. trachomatis*, *C. pneumoniae* and *C. abortus* Pmp proteins have focused mainly on PmpD (Wehrl *et al.*, 2004; Crane *et al.*, 2006; Kiselev *et al.*, 2009; Wheelhouse *et al.*, 2012a), due to its being highly conserved in *C. trachomatis* (Gomes *et al.*, 2006; Carrasco *et al.*, 2011) and highly immunogenic (Caldwell *et al.*, 1975a,b; Caldwell and Kuo, 1977; Crane *et al.*, 2006; Tan *et al.*, 2009). Gomes *et al.* (2006) reported PmpD as the second most conserved Pmp of *C. trachomatis* at amino acid level, while Carrasco *et al.* (2011) reported it as the third. In this study, we identified PmpD as the most conserved Pmp. This can be explained by the use of genomes of different *C. trachomatis* strains for analyses. However, the two previous studies and the current study have all identified PmpA, PmpD and PmpI as the three most conserved Pmp proteins in *C. trachomatis*, suggesting that these three Pmps are highly conserved in *C. trachomatis*.

Becker *et al.* (2014) recently demonstrated that PmpD is the strongest adhesin of *C. trachomatis* and referred to Tan *et al.* (2010) to document that PmpD was the only Pmp that was expressed in almost all inclusions in *C. trachomatis* infected cells and to Gomes *et al.* (2006) to report that PmpD is the second most conserved Pmp in *C. trachomatis*, thereby arguing for a crucial function of PmpD in the infection cycle of *C. trachomatis*. However, PmpA and PmpI were equally abundantly expressed and equally highly conserved as PmpD (Tan *et al.*, 2010). This might indicate equally important functions for PmpA and PmpI and highlights the need to analyze also Pmp proteins other than PmpD.

As variation is the central theme in the Pmp family, we hypothesize that the Pmp proteins might play a role in the observed difference in host and tissue preferences of different *Chlamydia* species. Consequently, we suggested that different Pmp proteins may be most conserved in different *Chlamydia* species. Indeed, different Pmp proteins were most conserved in each *Chlamydia* species (PmpD, PmpA, PmpH and PmpA in *C. trachomatis*, *C. pneumoniae*, *C. abortus* and *C. psittaci* respectively), with PmpA being most conserved Pmp protein in both *C. pneumoniae* and *C. psittaci*, which is different from what we suggested before. However, multiple proteins likely mediate adhesion and the most conserved Pmp proteins might still play a role for that. The different organization of the *pmp* CDSs within genomes of the same *Chlamydia* species (*C. pneumoniae*) and across *Chlamydia* species, indicate that *pmp* CDSs were located in regions of the genome where high levels of recombination have occurred. The *pmp* CDSs were mainly encoded on the antisense strand of *C. pneumoniae* strain AR39 and LPCoLN. These two genomes also clustered closely together phylogenetically, which may indicate that those genomes were subject to similar recombination events also on loci other than the *pmp* CDSs.

The Pmp proteins were most conserved in *C. abortus*, which can be explained by the availability of only two completed *C. abortus* genome sequences at the time of analyses. Therefore, the level of conservation can only be compared for *C. trachomatis* and *C. psittaci*, as for those species an equal amount of genomes (16) were analyzed. The Pmp proteins were more conserved in *C. trachomatis* compared to *C. psittaci*, which might be explained by the zoonotic nature of *C. psittaci*. *C. psittaci* might have adapted to its numerous different hosts through expression of variation in its Pmps and through expansion of the number of *pmp* genes. However, this does not explain why *C. pneumoniae* encodes the largest number of *pmp* CDSs and why the number of *pmpE/F* and *pmpG* subtype CDSs is highest in *C. pneumoniae*, as it is not a zoonotic bacterium. However, previous comparative genomic analyses of human *C. pneumoniae* strains (AR39, TW-183, J138 and CWL029) and the koala *C. pneumoniae* LPCoLN strain by Myers *et al.* (2009) suggested that *C. pneumoniae* has an animal origin. The observed truncated *pmp* and *inc* CDSs in the human *C. pneumoniae* strains were assigned to a reductive evolution of this species after it adapted to its human host (Myers *et al.*, 2009). The current study supports that hypothesis, as *C. pneumoniae* LPCoLN encodes the largest amount of full length *pmp* CDSs of all analyzed *C. pneumoniae* strains. This is in contrast to *C. psittaci*, where the strains isolated from mammals (*C. psittaci* MN and WC) encode the largest amount of full length *pmp* CDSs. The latter might be explained by the fact that

mammalian *C. psittaci* infection is a zoonosis. Truncated CDSs were mostly observed in *C. pneumoniae* and *C. psittaci*. In *C. pneumoniae* those truncated CDSs might be the result of reductive evolution processes (Myers *et al*., 2009), while for *C. psittaci* we suggest that these truncated *pmp* CDSs might recombine to another region, resulting in a full length, functional *pmp* CDS.

In contrast to the hypothesis that different Pmp proteins might mediate adhesion in different *Chlamydia* species, some Pmp subtypes might have the same redundant, critical function in different hosts. Hence, we determined the level of conservation of the Pmp proteins across different *Chlamydia* species. In our analysis PmpB is the most conserved Pmp protein, with PmpA, PmpH and PmpD being the second, third and fourth most conserved Pmp proteins, respectively.

PmpD is considered to be a vaccine candidate, however, our analysis shows that PmpD is not the most conserved Pmp protein across *Chlamydia* species. It is also not the most conserved Pmp protein within all *Chlamydia* species and in some *C. abortus* and *C. psittaci* strains, the PmpD protein was truncated. This raises the possibility that PmpD is not essential for the pathogenesis of *C. psittaci* and *C. abortus*. Our study highlights that, rather than focusing on PmpD, all Pmp proteins should be examined to accurately determine their utility as vaccine candidates.

Acknowledgements

H. H. Creasy (University of Maryland, School of Medicine, Department of Microbiology and Immunology) is acknowledged for the development of the HMM and the construction of the phylogenetic tree.

Chapter III

Chlamydia psittaci reference genes for normalization of expression data differ depending on the culture conditions and selected time points during the chlamydial replication cycle

---

Adapted from:

Van Lent S. and Vanrompay D. (2016). *Chlamydia psittaci* reference genes for normalization of expression data differ depending on the culture conditions and selected time points during the chlamydial replication cycle. *Journal of Veterinary Research*. Accepted with minor revisions.

Abstract

Chlamydia psittaci is a Gram-negative obligate intracellular pathogen of birds. Infections lead to economic losses in the poultry industry and the infection can be transmitted to humans. No vaccine is available and the bacterium host-cell interaction is not completely understood. The replicating bacteria cause pneumonia, but they can also be present as non-replicating, persistent bacteria inside the cytoplasm of avian cells. Quantitative real-time polymerase chain reaction (RT-qPCR) is a major tool to gain a better insight into the molecular pathogenesis of *C. psittaci* in birds. However, identification of stably expressed reference genes is required to avoid biases in RT-qPCR, when studying active replicating and persistent *C. psittaci*. The expression stability of ten candidate reference genes was investigated for performing gene expression analysis in *C. psittaci* during normal growth and during penicillin-induced persistence, using geNorm. The genes *tyrS*, *gidA*, *radA* and *16S rRNA* ranked among the most stably expressed genes. The final selected reference genes differed according to the bacterial growth status (normal growth versus persistent status), and the time points selected during the duration of a normal chlamydial developmental cycle.

1. Introduction

Quantitative real-time PCR (RT-qPCR) has become a major tool to better understand the molecular pathogenesis of bacterial infections. RT-qPCR is a sensitive, efficient and accurate technique for gene expression studies and it is an established technique for studying bacterium host cell interactions (Vandesompele *et al*., 2002). However, the accuracy and reproducibility of RT-qPCR is influenced by: i) the sample amount, ii) yield of the extraction process, iii) the RNA quality, iv) sample to sample variation and v) reverse transcriptase efficiency (Bustin, 2001). The expression of reference genes is affected by all sources of variation during the experimental workflow, in the same way as the expression of the genes of interest is influenced. Therefore, the use of reference genes is the preferred method to reduce the non-biological variation. However, the normalization step is the most problematic and ignored part of RT-qPCR. A commonly used normalization strategy involves normalization to a single, non-validated bacterial reference gene, such as the *16S rRNA* gene (Mannonen *et al*., 2011), which is generally regarded as the universal bacterial reference gene for data normalization. However, evaluation of RT-qPCR candidate reference genes for expression studies in *Lactobacillus casei* (Zhao *et al*., 2011), *Escherichia coli* (Zhou *et al*., 2011), *Bacillus cereus* (Reiter *et al*., 2011), *Corynebacterium pseudotuberculosis* (Carvalho *et al*., 2014), *Clostridium botulinum* (Kirk *et al*., 2014), *Listeria monocytogenes* (Tasara and Stephan, 2007), and *Staphylococcus aureus* (Valihrach and Demnerova, 2012; Sihto *et al*., 2014) revealed that the *16S rRNA* gene cannot be regarded as a universal reference gene. In fact, the expression stability of candidate reference genes should be validated specifically for each bacterial species and each experimental setting (Vandesompele *et al*., 2002).

RT-qPCR analyses could help to understand the molecular pathogenesis of *C. psittaci*. *C. psittaci* is an obligate intracellular Gram-negative bacterium that is responsible for respiratory disease in birds. A *C. psittaci* infection leads to significant economic losses due to reduced feed conversion, mortality, carcass condemnation at slaughter, reduced egg production and/or the expense of antibiotic treatment (Vanrompay *et al*., 1997). Currently, no vaccine is available. *C. psittaci* is also an important zoonotic agent via inhalation of infected aerosols of pharyngeal or nasal secretions or dried feces. The bacterium replicates by binary fission inside the cytoplasm of host cells but when stressed (iron depletion, exposure to interferon gamma and/or penicillin), the pathogen can go into a non-replicative, persistent status, and once stressors are removed, replication and bacterial excretion starts again.

So far, stably expressed genes, i.e. genes that are expressed at the same level at all analyzed time points and conditions, for normalization of RT-qPCR data in *Chlamydia* have only been determined for the human pathogen *C. trachomatis* (Borges *et al*., 2010). In this study, we present the selection and validation of reference genes for RT-qPCR studies in *C. psittaci* during the normal developmental cycle and during penicillin-induced persistence. Reference genes determined for normal + penicillin should be used to check whether a certain gene is up- or down-regulated during the persistent state compared to during normal development. This is important to further understand the persistent state, which could help to prevent *Chlamydia spp*. to go into the persistent state and therewith this would help to treat chlamydial infections.

2. Material and methods

2.1 *Chlamydia psittaci* strain, cell culture and infection

The previously well-characterized, prototypic strain *C. psittaci* Cal10 (Matsumoto, 1982; Hovis *et al*., 2013; Mojica *et al*., 2015), which was isolated from ferrets inoculated with throat washings from humans with an influenza-like respiratory infection (Francis and Magill, 1938), and with a complete genome sequence available (Grinblat-Huse *et al*., 2011) was used in this study. The bacterium was grown in HeLa 229 cells, the first human cell line established in culture (Gey *et al*., 1952), starting from human cervical cancer cells. The cells were seeded in 100 mm^2 tissue culture dishes for 24h at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Mediatech, Herndon, VA) supplemented with 10% heat inactivated fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), gentamycin (25 μg ml^{-1}; Quality Biological, Gaithersburg, MD) and fungizone (1.25 μg ml^{-1}; Invitrogen, Carlsbad, CA). The medium was aspirated and cells were inoculated with *C. psittaci* Cal10 in SPG (0.25 M sucrose, 10 mM sodium phosphate and 5mM L-glutamic acid) at a multiplicity of infection (MOI) of 1 followed by incubation on a rocking platform for 2 h at 37°C. The unbound organisms were washed away with PBS and the bacterium was grown either under normal conditions using the above-mentioned medium, or by adding penicillin (100U ml^{-1}) to the above-mentioned medium as a way to induce *Chlamydia* persistence (Hu *et al*., 2015). One ml SPG was added to a mock-infected dish. For all cells, addition of medium after rocking and washing marked time 0 hpi of the experiment.

2.2 Total RNA extraction and cDNA synthesis

At 2, 6, 12, 18, 24, 32, and 48 hours post infection (hpi), total RNA was extracted from the monolayers according to the manufacturer's instructions (TRIzol, Invitrogen). Total RNA was quantified (Nanodrop 2000, Thermo Scientific, Wilmington, DE) and the samples were treated with RNase-free amplification grade DNase I (Promega, Madison, WI) following the manufacturer's protocol and confirmed to be DNA-free by PCR (Table 3.1) for the *C. psittaci* Cal10 *16S rRNA* gene. One μg of total RNA was reverse transcribed (Superscript II RT, Invitrogen) with random hexamer primers (Invitrogen) following the manufacturer's protocol. RNA and cDNA samples were stored at -80°C and -20°C, respectively.

Table 3.1: Primers to check the RNase-free DNase I treatment.

Gene	Primer	Primer sequence (5' to 3')	Amplicon size (bp)
16S rRNA	16S-QPCR-F	TGTACAAGGCCCGGGAACGTA	156
	16S-QPCR-R	GGCCAGTACAGAAGGTAGCA	

2.3 Primer design and validation for RT-qPCR

Primers for the candidate reference genes were designed using Primer3 software with the following settings: amplicon size of 100-200 bp, optimal melting temperature of 60°C and a GC content of 50-60% (Table 3.2). For each primer pair, different primer concentrations (100 nM, 150 nM, 200 nM, 300 nM, 400 nM and 500nM) were tested in duplicate. The concentration resulting in the best sigmoid expression curve was chosen (Table 3.2). Melt curve analysis was used to ensure the specificity of the primers. The RT-qPCR efficiency was determined for each gene using slope analysis with a linear regression model. Standard curves were generated with serial dilutions of genomic DNA of purified EBs (1/5 = 8 ng μl^{-1}, 1/25, 1/125, 1/625, 1/3125, 1/15625). The corresponding RT-qPCR efficiencies (E) were calculated according to the equation $E = (10^{(-1/slope)}-1) \times 100$ (Pfaffl, 2001). Primers selected for RT-qPCR displayed an efficiency between 90 and 110% and a coefficient of correlation greater than 0.98.

Table 3.2: Primers used to determine the stability of candidate reference genes.

Gene	Primer	Primer sequence(5'-3')	Amplicon size (bp)	Tm (°C)	GC (%)	Primer concentration (nM)	Efficiency (%)
16S rRNA	16SrRNA-1	TGTACAAGGCCCGGGAACGTA	156	59,9	57,1	200	95,4
	16SrRNA-2	GGCCAGTACAGAAGGTAGCA		58,0	55,0	200	
enoA	enoA-1	AGCCGCAACTTTAGGACGA	187	60,9	52,6	500	90,1
	enoA-2	ATCAGCACCCATACGCACAG		62,1	55,0	500	
gatA	gatA-1	GCGTTAGGTTCCGATACAG	165	55,9	52,6	200	94,9
	gatA-2	GGCGACATCTTCAACAAC		54,9	50,0	200	
hemN	hemN-1	TTTACACATGCGGCCTGAC	170	60,7	52,6	500	101,2
	hemN-2	CAATGGCTTGGTAACCTGCT		60,1	50,0	500	
tyrS	tyrS-1	TGGGACAGGCTTATGGTTTG	169	60,9	50,0	200	96,6
	tyrS-2	CGTGCGACTTTAGGCACTTC		61,0	55,0	200	
fumC	fumC-1	CTTGCATACCGCCAGAGAGT	170	60,4	55,0	200	103,9
	fumC-2	CAACCCAACGCAATGTGA		60,1	50,0	200	
gidA	gidA-1	GATCTCCGGGTTGTTCTTCA	100	60,1	50,0	400	97,9
	gidA-2	GAACGTGGTTTCCCAATCAG		60,4	50,0	400	
hflX	hflX-1	CGTAAGGCTAAAGAG	181	57,3	55,0	500	97,8
	hflX-2	TTGCCCACTAGGAAG		57,2	55,0	500	
radA	radA-1	GTCGCCGCCTAATAGGGTAA	108	61,3	55,0	500	105,6
	radA-2	ACCATAGAGCTGCGAGAGGA		60,1	55,0	500	
map	map-1	AAACGCGTCTGTCAAGCATC	156	61,4	50,0	200	92,2
	map-2	ACCCACACCGTGACCTACAA		61,3	55,0	200	

2.4 Real-time quantitative PCR (RT-qPCR)

The expression level of each candidate reference gene was examined by RT-qPCR on an Rotor-Gene Q Real-Time PCR Detection System (Qiagen, Hilden, Germany). Each reaction mixture contained 1 μl cDNA, the optimal primer concentration for each primer pair (Table 3.2), 10 μl iQ SYBR Green Supermix (Bio-Rad, Richmond, CA) and ddH_2O to a final volume of 20 μl. RT-qPCR reaction conditions were as follows: initial denaturation at 95°C for 3 min, 40 cycles each consisting of 30 s at 95°C and 30 s at 58°C, followed by the melting curve program (95°C for 1 min, 55°C-95°C in steps of 0.5°C each 10 s). Two biological replicates of each sample (normal infection vs. penicillin-induced persistence, each at 7 different time points during the developmental cycle) were tested in duplicate. *C. psittaci* Cal10 genomic DNA was used as a positive control, while HeLa 229 cDNA, non-reverse-transcribed *C. psittaci* Cal10 total RNA, and ddH_2O were used as negative controls. Data analyses were carried out with geNorm software (version 2.4, Biogazelle, Ghent, Belgium) on normal, penicillin and normal + penicillin samples. Expression categories were defined as early (2–6 hpi), middle (12–18 hpi) and late ($\geq$ 24 hpi).

2.5 Selection of reference genes

The expression level of 10 candidate reference genes (*16S rRNA*, *map*, *tyrS*, *hemN*, *hflX*, *gidA*, *gatA*, *fumC*, *radA* and *enoA*) was measured for the two biological replicates of each sample. The Cq-values were used to analyze the expression stability of candidate reference genes by geNorm, implemented in the qBasePLUS software. geNorm is based on the principle that the expression ratio of two ideal reference genes are identical in all samples tested, independent of the experimental conditions. Variation in those ratios indicates the non-stable expression of one or both reference genes. Therefore, geNorm determines the level of pairwise variation for each candidate reference gene with all other candidate reference genes (M-value). Genes with a low M-value are the most stably expressed. Sequential removal of the least stable gene generates a ranking of the candidate reference genes according to their stable expression. geNorm also calculates the pairwise variation $V_{n/n+1}$ to determine the ideal number of reference genes for normalization (Vandesompele *et al.*, 2002). The cut-off value below which the inclusion of an additional control gene was considered not to result in a significant improvement of the normalization, was set at 0.15 (Vandesompele *et al.*, 2002).

3. Results

3.1 Choice and transcript profiles of candidate reference genes

Reference genes for *C. psittaci* during normal and penicillin-induced persistence conditions, at early (2 and 6 hpi), mid (12 and 18 hpi) and late (24, 32 and 48 hpi) time points, were validated in this study. The candidate reference genes were chosen based on the reference genes tested for *C. trachomatis* and the housekeeping genes used for the multi locus sequence typing of *Chlamydiales* (Pannekoek *et al*., 2008; Borges *et al*., 2010). To minimize the risk of co-regulation, ten candidate genes were selected by the following criteria: i) widely spread on the chromosome, ii) involved in different pathways, and iii) not adjacent to putative outer membrane, secreted or hypothetical proteins that might be under diversifying selection (Table 3.3). The gene encoding the *16S rRNA* was the most abundantly expressed (only 3.97 cycles to reach the cycle treshold), while *enoA* and *fumC* were the least abundant transcripts (31.83 and 31.2 cycles respectively) (Figure 3.1). *radA* and *tyrS* transcript levels showed the lowest and *enoA* and *fumC* transcript levels the highest variation in Cq-values (10.67, 10.59, 15.99 and 19.84 cycles respectively). The wide range of transcript levels of the candidate reference genes confirmed that no single candidate reference gene was constantly expressed at the different conditions and time points analyzed. This implicated the need for using multiple reference genes.

Table 3.3: Candidate reference genes.

Gene symbol	Function	Pathways
map	Mitogen-activated protein kinase	Protein phoshorylation
tyrS	Tyrosine-tRNA ligase	Catalysation of the attachment of an amino acid to its cognate tRNA molecule
16S rRNA	16S ribosomal RNA subunit	Translation
hemN	Coproporphyrinogen III oxidase	Coproporphyrinogen III decarboxyation
hflX	GTPase	May have a role during protein synthesis or ribosome biogenesis
radA	DNA recombination/repair protein	DNA repair, homologous recombination
enoA	Component of enolase	Glycolysis
fumC	Fumarase C	Citric acid cycle
gatA	Belongs to the GATA transcription factor family	Transcription
gidA	Glucose-inhibited division protein A	Protein involved in tRNA modification

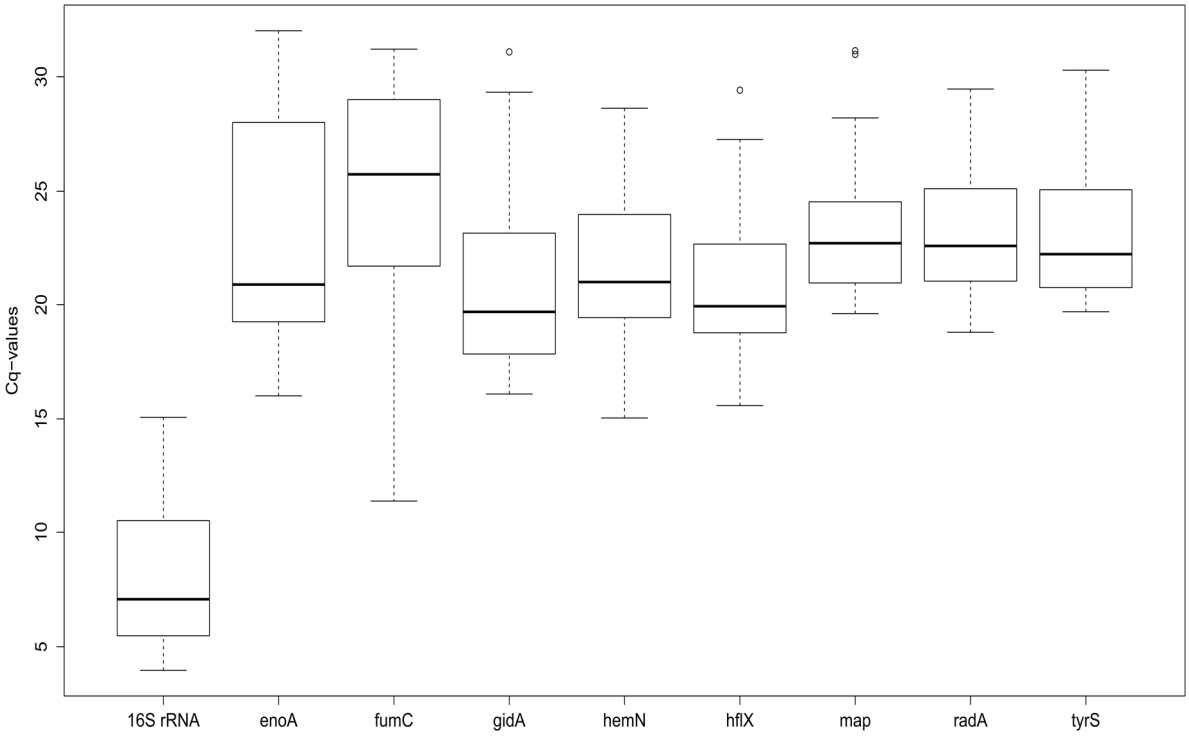


Figure 3.1: Transcript levels of candidate reference genes. The transcript levels for each reference gene in all *C. psittaci* samples (normal + penicillin condition at all time points) are shown. The data are expressed as box plots: the box represents the 25^{th}-75^{th} percentiles, the median is depicted by a bar across the box and the whiskers on each box represent minimum and maximum value (excluding outliers which are depicted as dots).

3.2 Stability of reference genes expression

The stability of the transcript levels of the candidate reference genes was determined using geNorm. The program calculated the average expression stability value (M-value) for each candidate reference gene during normal, penicillin and normal + penicillin conditions (Figure 3.2). For each condition, the stability value for each candidate reference gene was determined for early, mid, late and all time points. In addition to the M-value, geNorm calculated also a $V_{n/n+1}$-value to determine the optimal number of reference genes for accurate normalization for each condition (Figure 3.3). A $V_{n/n+1}$ smaller than 0.15 indicates that an additional reference gene (V_{n+1}) has no significant effect (Vandesompele *et al.*, 2002). If the pairwise variation was bigger than 0.15, than geNorm advised to use the number of reference genes with the lowest $V_{n/n+1}$ value.

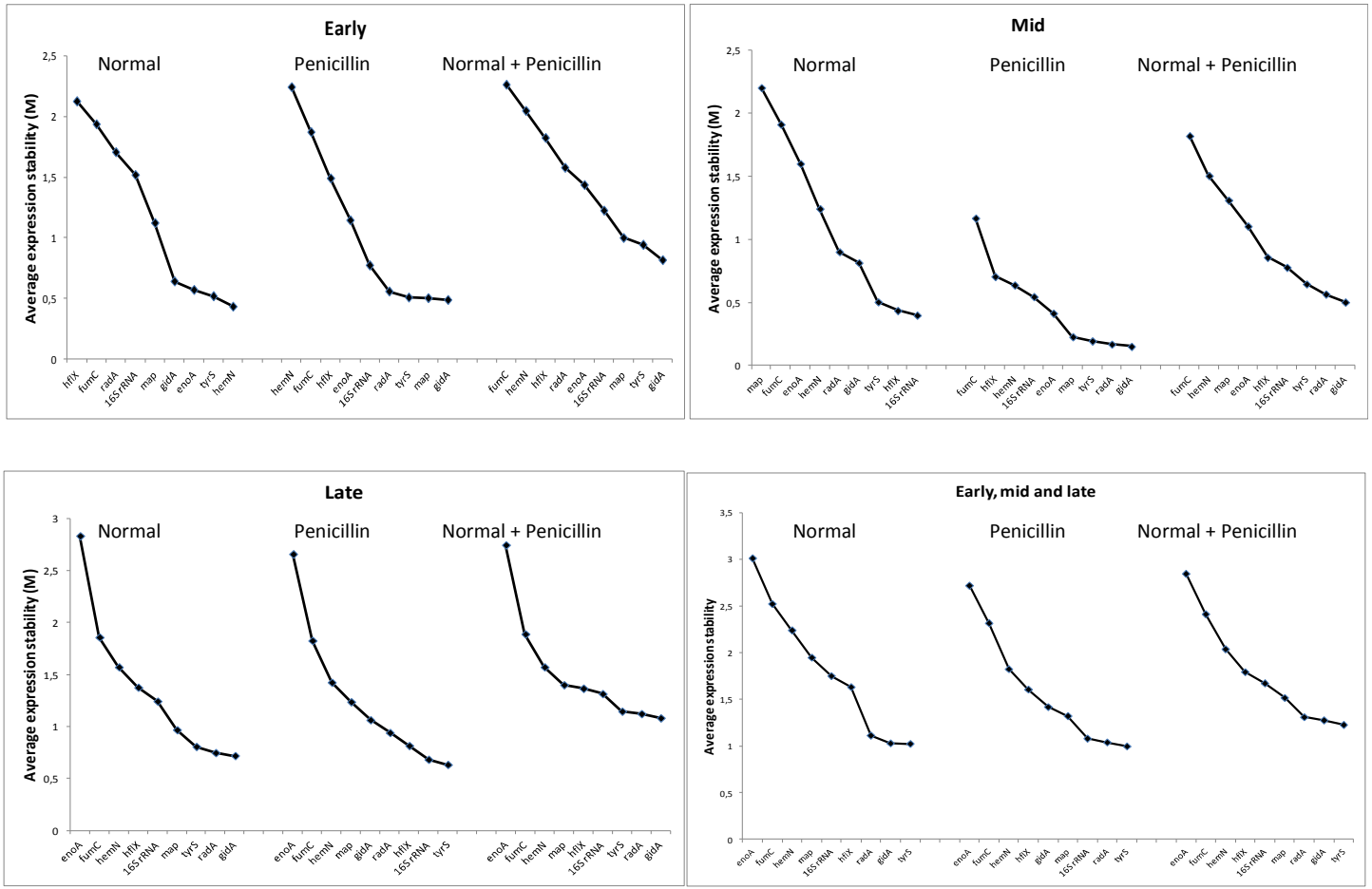


Figure 3.2: Stability ranking of candidate reference genes during normal, penicillin and normal + penicillin conditions by geNorm. Candidate reference genes are ranked from left to right in order of increasing expression stability (decreasing M-value).

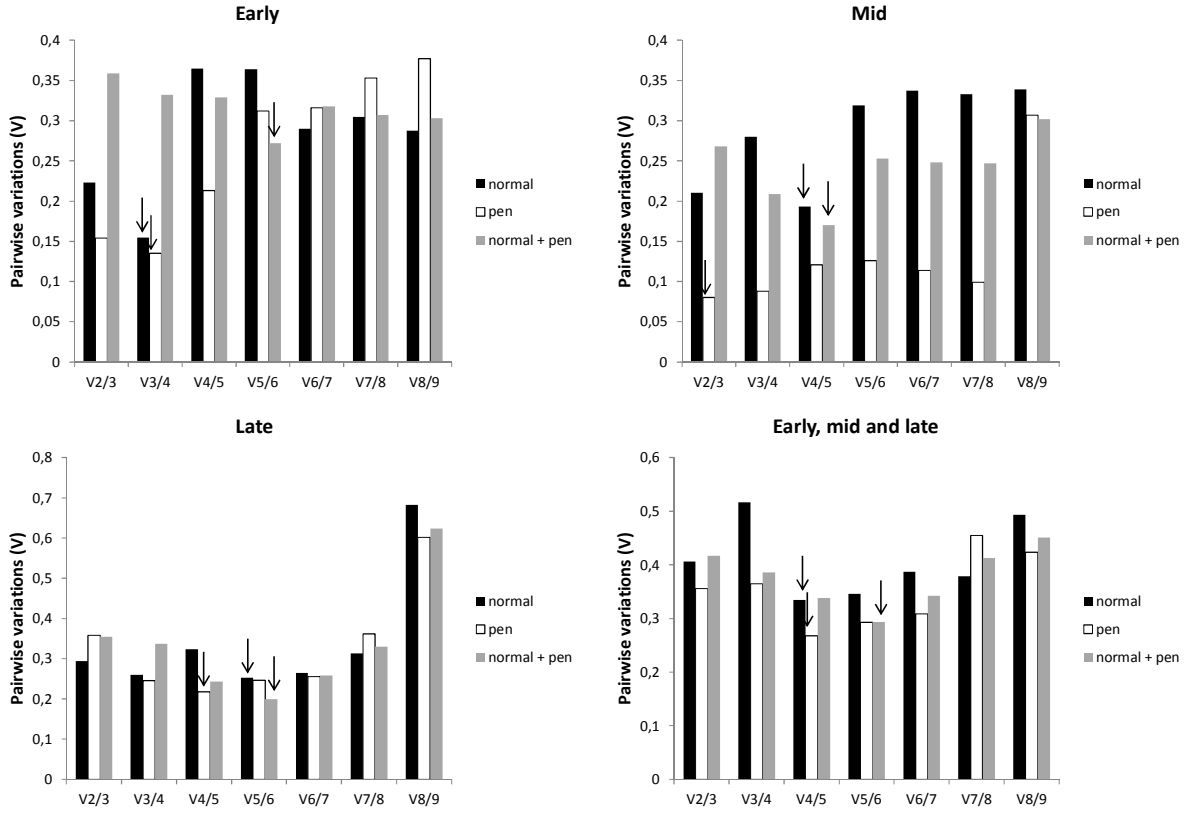


Figure 3.3: Determination of the optimal number of reference genes required for reliable normalization by geNorm. The pairwise variation ($V_{n/n+1}$) was calculated stepwise between normalization factors based on the n and (n+1) most stable expressed reference genes. According to the geNorm developers, a variation of <0.15 indicates no significant contribution of an additional control gene to the normalization factor. If $V_{n/n+1}$ is higher than 0.15, than the lowest $V_{n/n+1}$ is the optimal number. The optimal number of control genes are illustrated by arrows.

The lowest and highest M-value of the reference genes for all the samples (normal + penicillin) and for normal and penicillin conditions separately, as well as which reference genes to use for each condition, are listed in Table 3.4. *gatA* was excluded from the analysis, as it was not transcribed in all samples. In general, *gidA* had the lowest M-value (highest stability) for six conditions, while *enoA* had the highest M-value (lowest stability) for 6 conditions. Genes *tyrS*, *gidA, radA* and *16S rRNA* were among the reference genes suggested to be used in 11, 9, 8 and 8 out of the 12 tested conditions respectively, while genes *map*, *hflX*,

enoA, *hemN* and *fumC* were suggested to be used in 5, 4, 2, 1 and none of the tested conditions. *enoA* and *hemN* were unique reference genes for the normal condition. No gene was unique for the penicillin-induced persistence condition, although in general the reference genes proposed for use during the normal condition at a specific time point differed from the ones proposed for the penicillin-induced persistent condition at the corresponding time point. Including more different samples resulted in less stably expressed reference genes. For example, the lowest M-value of the normal + penicillin condition was higher than the one of normal and penicillin separately for early, mid, late and all time points. In addition, the lowest M-value of normal, penicillin and normal + penicillin was the highest for all time points together compared to early, mid and late time points separately. Late and all time point samples were less stable than early and mid time point samples, as late and all time points showed the highest M-values. Consequently, the more conditions analyzed, the more reference genes were needed to normalize the data accurately (2-5 reference genes for normal and penicillin conditions separately, while 4-5 reference genes for normal + penicillin conditions together).

Table 3.4: Overview of reference genes for three conditions: normal, penicillin and normal + penicillin. The most suitable reference genes for each condition are shown. Reference genes are listed from least to most stable.

Condition	Time point	Lowest M-value	Highest M-value	Reference genes	Vn/n+1 < 0.15?
Normal	**early**	0,432 (*hemN*)	2,126 (*hflX*)	*enoA*, *tyrS*, *hemN*	No
	mid	0,398 (*16S rRNA*)	2,204 (*map*)	*gidA*, *tyrS*, *hflX*, *16S rRNA*	No
	late	0,718 (*gidA*)	2,832 (*enoA*)	*16S rRNA*, *map, tyrS*, *radA*, *gidA*	No
	all	1,024 (*tyrS*)	3,012 (*enoA*)	*hflX*, *radA*, *gidA*, *tyrS*	No
Penicillin	**early**	0,488 (*gidA*)	2,243 (*hemN*)	*tyrS*, *map*, *gidA*	Yes
	mid	0,151(*gidA*)	1,169 (*fumC*)	*radA*, *gidA*	Yes
	late	0,631 (*tyrS*)	2,657 (*enoA*)	*radA*, *hflX*, *16S rRNA*, *tyrS*	No
	all	0,997 (*tyrS*)	2,719 (*enoA*)	*map*, *16S rRNA*, *radA*, *tyrS*	No
Normal + penicillin	**early**	0,817 (*gidA*)	2,263 (*fumC*)	*enoA*, *16S rRNA*, *map*, *tyrS*, *gidA*	No
	mid	0,502 (*gidA*)	1,822 (*fumC*)	*16S rRNA*, *tyrS*, *radA*, *gidA*	No
	late	1,081 (*gidA*)	2,745 (*enoA*)	*hflX*, *16S rRNA*, *tyrS*, *radA*, *gidA*	No
	all	1,226 (*tyrS*)	2,846 (*enoA*)	*16S rRNA*, *map*, *radA*, *gidA*, *tyrS*	No

4. Discussion

RT-qPCR is an accurate and sensitive tool for studying gene expression in bacterial pathogens (Bustin, 2005). Unfortunately, normalization is still the most obstinate problem for real-time quantification. Different normalization methods are available. The use of genomic DNA is less suited to normalize for experimental variations, as the gDNA is determined on DNA samples. So, it cannot be used to correct for differences in RNA extraction and RT-PCR efficiencies (Borges *et al*., 2010). Therefore, the use of reference genes is advised (Vandesompele *et al*., 2002). However, it is essential to validate reference genes for each bacterial species and each specific experiment to be able to control for non-biological variation and therewith obtain accurate and reliable gene expression data. Selection of unstable, unvalidated reference genes can result in miscalculations of gene expression levels and lead to incorrect conclusions (Dheda *et al*., 2005). Several publications underlined the importance to use multiple reference genes, as no single, universal reference gene exists. Nevertheless, many recently published papers still normalized mRNA levels by a single reference gene (Bustin, 2005; Kiselev *et al*., 2009; Almeida *et al*., 2012; Ferreira *et al*., 2013), mostly *16S rRNA*. In fact, none of the previously mentioned studies that used *16S rRNA* as a reference gene, showed data on the stability of the gene in the experimental setting utilized. Other reports justified the choice of the *16S rRNA* as reference gene only by referring to another study, usually performed under different experimental conditions with other strains.

Borges *et al*. (2010), validated reference genes for performing gene expression analyses in *C. trachomatis*, but reference genes have not been validated for gene expression studies in other *Chlamydia* species. Therefore, we investigated the suitability of ten candidate reference genes for future gene expression analysis in *C. psittaci* Cal10. The expression of the *16S rRNA* gene, extensively used as reference gene in *Chlamydia spp*. gene expression studies (Douglas and Hatch, 2000; Mathews *et al*., 2001; Belland *et al*., 2003a; Nicholson *et al*., 2004; Goellner *et al*., 2006; Suchland *et al*., 2008; Ferreira *et al*., 2013), and of nine other *C. psittaci* genes (*map*, *tyrS*, *hemN*, *hflX*, *gidA*, *gatA*, *fumC*, *radA* and *enoA*) was studied during both normal bacterial growth conditions and penicillin-induced persistence.

Our data confirmed the finding that the best-suited reference genes differ among experimental conditions, as the most stably expressed reference gene (lowest M-value) varied for each experimental group. The most striking observation was that the *tyrS* gene was suggested as a

reference gene for all but one conditions, thus not for the mid time point during penicillin-induced persistence. We have no reasonable explanation for the latter observation. *16S rRNA* was suggested as reference gene in only 8 out of the 12 tested conditions. This result is in alignment with an earlier study, in which they found that *16S rRNA* was the most stable reference gene for *C. trachomatis* under normal conditions, but its expression was highly unstable during stress conditions (Borges *et al*., 2010). In addition, the reference genes to be used for *C. psittaci* gene expression analyses differ from those described for *C. trachomatis* gene expression analysis (Borges *et al*., 2010). As also demonstrated by Vandesompele *et al*. (2002), measuring expression levels by using multiple reference genes was more accurate than just using one. The effect of potential regulations of single genes is decreased by the use of multiple reference genes, and improves the reproducibility of relative gene expression analysis.

In conclusion, we successfully identified reference genes, which can be used for *C. psittaci* gene expression analysis during the normal developmental cycle and during penicillin-induced persistence. The importance of proper reference gene evaluation for RT-qPCR data normalization is emphasized by our data and therewith we strongly advise to make systematic validation of reference genes to confirm their stability within the strains and under the conditions selected.

Chapter IV

Characterization of polymorphic membrane proteins in *Chlamydia psittaci*

Adapted from:

Van Lent S., De Vos W.H., Creasy H.H., Marques P.X., Ravel J., Vanrompay D., Bavoil P., Hsia R. Analysis of polymorphic membrane protein expression in cultured cells identifies PmpA and PmpH of *Chlamydia psittaci* as candidate factors in pathogenesis and immunity to infection. *Plos One*. Accepted with minor revisions.

Abstract

Studies of the polymorphic membrane protein (Pmp) paralogous families of *Chlamydia trachomatis*, *Chlamydia pneumoniae* and *Chlamydia abortus* have suggested the potential of these proteins for the development of a *Chlamydia* vaccine. To determine if members of the Pmp family of *C. psittaci* are also vaccine candidates, we analyzed transcription levels, protein production and localization of several Pmps of *C. psittaci* under normal en stress conditions. We found that PmpA was highly produced in all inclusions as early as 12 hpi in all biological replicates. In addition, PmpA and PmpH appeared to be unusually accessible to antibodies as determined by both immunofluorescence and immuno-electron microscopy. These results suggest an important role for these Pmps in the pathogenesis of *C. psittaci*, and make them promising candidates in vaccine development.

1. Introduction

The *Chlamydiaceae* are a family of Gram-negative obligate intracellular bacteria that infect animals and humans, causing diseases with a wide range of symptoms. Among these, a significant species is *C. psittaci* that may cause respiratory disease in poultry and pet birds, and may also cause zoonotic psittacosis in humans. Psittacosis, or parrot fever, is usually characterized by fever chills, headache, dyspnea and cough. Chest X-rays often show an infiltrate (Beeckman and Vanrompay, 2009). However, the disease seems to vary considerably in severity as the clinical features of the infection can range from none to sepsis with multi-organ failure requiring admission in an intensive-care-unit (Heddema *et al*., 2006). People usually contract the infection via inhalation of an aerosol from droppings of infected birds. Epidemics of *C. psittaci* infections in turkeys are economically devastating due to high mortality rates, carcass condemnation at slaughter, reduced egg production and/or the cost of antibiotic treatment to reduce mortality and allow marketing of turkeys (Vanrompay *et al*., 1997). Little is known about the mechanisms by which *Chlamydia* species manipulate host cells and induce disease in different hosts. In spite of diverse infection strategies and symptoms, all *Chlamydia* spp. share a unique, conserved, biphasic developmental cycle. The elementary body (EB) is the infectious, metabolically dormant form of *Chlamydia*, which differentiates into the metabolically active reticulate body (RB) after internalization by the eukaryotic host cell. The developmental cycle takes place entirely inside a vacuole, called the inclusion. After several rounds of exponential growth, the RBs asynchronously differentiate into EBs. The infectious EBs are then released from infected host cell through cell lysis or inclusion extrusion, thereby closing the developmental cycle.

Chlamydial proteins are differentially produced in EBs and RBs (Marques *et al*., 2010; Saka *et al*., 2011). Proteins present on the surface of EBs are of particular interest for vaccine development, as they are putative targets for neutralizing antibodies. For many years, antibodies against polymorphic membrane proteins (Pmps; previously known as 90-kDa protein family) have been detected during natural infections of humans, turkeys and sheep (Campbell *et al*., 1990; Cevenini *et al*., 1991; Souriau *et al*., 1994; Giannikopoulou *et al*., 1997; Longbottom *et al*., 1998b; Knudsen *et al*., 1999) and during experimental infections of specific pathogen-free turkeys (Vanrompay *et al*., 1994; Verminnen *et al*., 2006). Longbottom et al., (1998b), were first to clone and sequence four genes of the 90-kDa gene family. Genome sequencing revealed that the *pmp* genes encode the largest membrane protein family

in *Chlamydia* spp., a unique feature of the genus (Horn *et al*., 2004; Vandahl *et al*., 2004). In the last decade, the Pmps have been studied intensively, particularly because the *Chlamydia trachomatis* and *Chlamydia pneumoniae* Pmp families represent a relatively high proportion of the coding capacity (3.15 to 5.1%, respectively) in the highly reduced chlamydial genome. Moreover the occurrence of the *pmp* gene family in all currently sequenced chlamydial genomes (Grimwood and Stephens, 1999) suggests an important function in chlamydial biology. The observed diversity in the number of alleles, gene and protein sequences, size (90-190 kDa), and expression levels within and across *Chlamydia* spp. also suggests that Pmps may be responsible for differences in the pathogenesis across *Chlamydia* species.

Pmps were classified as autotransported (type V secretion) proteins, based on their N-terminal signal sequence (type II secretion), a central passenger domain and a C-terminal putative transporter domain, predicted to form a β-barrel through which the protein is secreted to the chlamydial surface (Vandahl *et al*., 2001; Henderson and Lam, 2001). This prediction is supported by experimental evidence for several Pmps (Longbottom *et al*., 1998a; Vandahl *et al*., 2002; Wehrl *et al*., 2004; Kiselev *et al*., 2007; Liu *et al*., 2010). Pmps are grouped into a family based on the conserved repetitive motifs FxxN and GGA (with I, L or V at the 4th position). In *C. trachomatis*, they have been further divided into six phylogenetically related subtypes (PmpA, B/C, D, E/F, G/I, and H) which may be able to substitute structurally and functionally for one another (Grimwood and Stephens, 1999). The passenger domain is responsible for the protein's function (Henderson and Lam, 2001). Pmp6, Pmp20 and Pmp21 of *C. pneumoniae* (orthologs of PmpG, PmpB and PmpD of *C. trachomatis*, respectively) and all Pmps of *C. trachomatis* are proposed to function as adhesins, based on adhesion assays and specific neutralization of the infection by incubation of the host cells with the recombinant Pmps (Crane *et al*., 2006; Mölleken *et al*., 2010; Becker and Hegemann, 2014). Up to now, anti-PmpD and anti-Pmp21 antibodies are the only Pmp-specific antibodies that are tested for their possible neutralizing capacity. Specific anti-PmpD and anti-Pmp21 antibodies can partially neutralize *C. trachomatis* and *C. pneumoniae* infection, respectively, *in vitro* (Wehrl *et al*., 2004; Crane *et al*., 2006; Mölleken *et al*., 2010). Patients infected with *C. trachomatis* usually elicit high titer antibodies against a subset of the Pmps, that varies between infected individuals (Tan *et al*., 2009). The different antibody profiles in patients may reflect different transcription and protein production profiles along the developmental cycle or as a result of strain variation or site specificity (Grimwood and Olinger, 2001; Vandahl *et al*., 2002; Tan *et al*., 2009; Wheelhouse *et al*., 2009; Carrasco *et al*., 2011;

Wheelhouse *et al*., 2012b). An attractive hypothesis is that variation of *pmp* gene expression and the resulting antigenic variation phenotype contribute to immune evasion in the infected host. Finally, Pmps were reported to be involved in host and tissue tropism (Becker and Hegemann, 2014). Previous studies have mainly focused on the Pmps of *C. trachomatis* (Belland *et al*., 2003b; Crane *et al*., 2006; Kiselev *et al*., 2007; Tan *et al*., 2009; Kiselev *et al*., 2009; Swanson *et al*., 2009; Tan *et al*., 2010; Carrasco *et al*., 2011; Saka *et al*., 2011; Humphrys *et al*., 2013; Becker and Hegemann, 2014) and *C. pneumoniae* (Vandahl *et al*., 2002; Wehrl *et al*., 2004; Mölleken *et al*., 2010; Mölleken *et al*., 2013), both human pathogens, and on the zoonotic *C. abortus* (Longbottom *et al*., 1998a,b; Wheelhouse *et al*., 2009; Wheelhouse *et al*., 2012a,b; Forsbach-Birk *et al*., 2013). However, the *pmp* gene family of *C. psittaci* has not been investigated so far.

In this study, we hypothesize that Pmps play an important role in *C. psittaci* pathogenesis and in immunity to infection. To test this hypothesis, we studied developmental expression and abundance profiles of different Pmps using quantitative real-time PCR (RT-qPCR) and immunofluorescence microscopy (IF), respectively, for *C. psittaci* strain Cal10. Immuno-electron microscopy (IEM) was used to assess subcellular localization of the Pmps on individual chlamydiae. As previous studies suggested a unique role in chlamydial pathogenesis for virulence genes expressed during stress, RT-qPCR, IF and EM analyses of the *pmp* transcripts and Pmp products of *C. psittaci* were conducted under both normal and stressed conditions (Carrasco *et al*., 2011).

2. Material and methods

2.1 Bioinformatics analyses

We re-annotated the *pmp* genes of the *C. psittaci* Cal10 genome (AEZD00000000.1) using the Hidden Markov Model that was described in paragraph 2.2 of chapter II.

2.2 *Chlamydia psittaci* cell culture conditions

HeLa 229 cells were seeded in 100 mm^2 tissue culture dishes for 24h at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Mediatech, Herndon, VA) supplemented with 10% heat inactivated fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), gentamycin (25 μg ml^{-1}; Quality Biological, Gaithersburg, MD) and fungizone (1.25 μg ml^{-1}; Invitrogen, Carlsbad, CA). The medium was aspirated and cells were inoculated with *C. psittaci* Cal10 in SPG (0.25 M sucrose, 10 mM sodium phosphate and 5mM L-glutamic acid) at a multiplicity of infection (MOI) of 1 followed by incubation on a rocking platform for 2 h at 37°C. The unbound organisms were washed away with PBS and the bacterium was grown either under normal conditions using the above-mentioned medium, or by adding penicillin (100U ml^{-1}) to the above-mentioned medium as a way to induce *Chlamydia* persistence (Hu *et al*., 2015). One ml SPG was added to a mock-infected dish. For all cells, addition of medium after rocking and washing marked time 0 hpi of the experiment.

2.3 Total RNA extraction and cDNA synthesis

At 2, 6, 12, 18, 24, 32, and 48 hours post infection (hpi), total RNA was extracted from the monolayers according to the manufacturer's instructions (TRIzol, Invitrogen). Total RNA was quantified (Nanodrop 2000, Thermo Scientific, Wilmington, DE) and the samples were treated with RNase-free amplification grade DNase I (Promega, Madison, WI) following the manufacturer's protocol and confirmed to be DNA-free by PCR (Table 3.1) for the *C. psittaci* Cal10 *16S rRNA* gene. One μg of total RNA was reverse transcribed (Superscript II RT, Invitrogen) with random hexamer primers (Invitrogen) following the manufacturer's protocol. RNA and cDNA samples were stored at -80°C and -20°C, respectively.

2.4 Primer design and validation for RT-qPCR

Pmp-specific regions were identified by ClustalW2 alignment. Primers for all *pmp* genes were designed using Primer3 software with the following settings: amplicon size of 100-200 bp, optimal melting temperature of 60°C and a GC content of 50-60% (Table 4.1). For each primer pair, different primer concentrations (100 nM, 150 nM and 200 nM) were tested in duplicate. The concentration resulting in the best sigmoid expression curve was chosen (Table 4.1). Melt curve analysis was used to ensure the specificity of the primers. RT-qPCR efficiencies for each gene were determined using slope analysis with a linear regression model. Serial dilutions of genomic DNA of purified EBs (1/5 = 8 ng μl^{-1}, 1/25, 1/ 125, 1/625, 1/3125, 1/15625) were used to generate standard curves. Corresponding RT-qPCR efficiencies (E) were calculated according to the equation $E = (10^{(-1/slope)}-1) \times 100$(Pfaffl, 2001). Ideally, efficiencies should be between 90-110%, but because of the difficulty to find specific regions in the *pmp* genes, primer pairs with efficiencies outside this range were also used (Table 4.1). Obtained Cq-values were corrected for the differences in PCR-efficiencies. Primers that displayed a coefficient of correlation greater than 0.98 were selected for RT-qPCR.

Table 4.1: Primers used for RT-qPCR analysis.

Gene	Primer	Primer sequence(5'-3')	Amplicon size (bp)	Tm (°C)	GC (%)	Primer concentration (nM)	Efficiency (%)
pmpA	pmpA-1	GTCGCCAGAGAAGGTGTTCC	112	62,2	60,0	200	85,6
	pmpA-2	GGGACAAGAAGCACTCAACCT		60,7	52,4	200	
pmpB	pmpB-1	TGCTGCAGCGTTAAGAGTGA	116	56,9	50,0	200	66,9
	pmpB-2	CCCTAGGCGGTAGCATTACA		56,4	55,0	200	
pmpD	pmpD-1	CCAAGACCCTTTGGATTCACC	115	62,9	52,4	200	95,4
	pmpD-2	GGGTATCTTTGCTGGGTCGT		61,3	55,0	200	
pmpE1	pmpE1-1	CGTGGTAGTATCGATGGTGGAA	101	56,2	50,0	200	88,1
	pmpE1-2	GCAGCTGCAACTCGAACAG		57,0	57,9	200	
pmpE2	pmpE2-1	GGGTTGAGTGGAGGGCATT	112	57,6	57,9	200	77,2
	pmpE2-2	CATGGAGACGCACCCAAGT		57,7	57,9	200	
pmpH	pmpH-1	GCAGCTGGATTATCGCCTGT	105	62,6	55,0	200	86,6
	pmpH-2	CGCTAACCTCAATGTCCTTGGT		62,6	50,0	200	
pmpG1a	pmpG1a-1	CCTCCAAATCTGAAGGGACA	119	54,3	50,0	200	69,9
	pmpG1a-2	GGCAAGGTTACCAGCAGTATCA		57,0	50,0	200	
pmpG1b	pmpG1b-1	GATAGCGTGGCTGTTCGAGT	178	60,4	55,0	200	66,8
	pmpG1b-2	ATCCCATACTCTCCGCATCA		60,5	50,0	200	
pmpG1c	pmpG1c-1	AGAAGGTCCCTCTCCGTCAG	174	60,8	60,0	200	63,3
	pmpG1c-2	GGTAGAAGCGATCCCGATGT		61,4	55,0	200	
pmpG1d	pmpG1d-1	TGACTGTTGCAGCACTGTCTCT	128	58,7	50,0	200	73,6
	pmpG1d-2	CCCTACAGGCGGAGCTATTT		56,7	55,0	200	

pmpG2	pmpG2-1	CACCACTAATACGGCGGAAA	108	55,0	50,0	200	81,2
	pmpG2-2	GCTGTGATGGCTTTGGCTAC		56,7	55,0	200	
pmpG3	pmpG3-1	CGTAAGGATAGGCGCTTTGG	156	61,9	55,0	200	68,2
	pmpG3-2	GACATTCGCGGACCTGTAGT		60,1	55,0	200	
pmpG4	pmpG4-1	GAGGGTTGCACGTCGATTAG	171	60,7	55,0	200	68,7
	pmpG4-2	ATCCATACCGAGGGCTTCA		60,4	52,6	200	
pmpG5	pmpG5-1	GCGCCTATATGGCTGATGAA	177	61,1	50,0	200	61,2
	pmpG5-2	TGATCAAGATTCCCGTCCTG		61,0	50,0	200	
pmpG6	pmpG6-1	TAGAACCCGCATAGACGTTTCC	131	62,5	50,0	100	63,3
	pmpG6-2	GGGATATGTGTTAGGAGCCACA		61,1	50,0	100	
pmpG7	pmpG7-1	CCCAGAATCCTTCAGAACACAG	194	61,0	50,0	200	66,5
	pmpG7-2	GGTGACTACTCTTGGCACGAAA		61,6	50,0	200	
pmpG8	pmpG8-1	CTGGAGAGTCCTCCCAGGTT	111	60,6	60,0	200	73,3
	pmpG8-2	TATCCGCTACAGGGCAAGTC		60,2	55,0	200	
tufA	ef-tu-1	ACATAGCTTGCATCGCCTTC	125	60,4	50,0	200	92,2
	ef-tu-2	GATGCCGAGCTTGTAGACTTG		60,0	52,4	200	

2.5 Developmental expression of *pmp* genes (RT-qPCR)

The expression of the *pmp* genes was examined by RT-qPCR on an iQ5 Real-Time PCR Detection System (Bio-Rad, Richmond, CA). Each reaction mixture contained 1 μl cDNA, the optimal primer concentration for each primer pair (Table 4.1), 10 μl iQ SYBR Green Supermix (Bio-Rad) and ddH_2O to a final volume of 20 μl. RT-qPCR reaction conditions were as follows: initial denaturation at 95°C for 3 min, 40 cycles each consisting of 30 s at 95°C and 30 s at 58°C, followed by the melting curve program (95°C for 1 min, 55°C-95°C in steps of 0.5°C each 10 s). Two biological replicates of each sample (normal infection vs. penicillin-induced persistence, each at 7 different time points during the developmental cycle) were tested in duplicate. Genomic DNA of *C. psittaci* Cal10 was used as a positive control. In addition, *tufA* encoding the elongation factor EF-Tu involved in protein synthesis was also included as a positive control, because the gene is constitutively expressed throughout the developmental cycle and it is a reliable indicator of exponential growth (Carrasco et al., 2011). cDNA of HeLa 229 cells, non-reverse-transcribed total RNA of *C. psittaci* Cal10, and ddH_2O were used as negative controls. Data analyses were carried out with qBasePLUS software (version 2.4, Biogazelle, Ghent, Belgium) and validated reference genes (Table 3.4) were used for normalization. Expression categories were defined as early (2–6 hpi), middle (12–18 hpi) and late ($\geq$ 24 hpi).

2.6 RT-PCR

An RT-PCR was performed on cDNA samples (24, 32 and 48 hpi normal condition) to evaluate the putative organization of the *pmp* genes in operons in the *C. psittaci* Cal10 genome. Primers spanning the intergenic regions were designed using Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/) (Table 4.2). *C. psittaci* Cal10 genomic DNA was used as a positive control while cDNA from uninfected HeLa 229 cells, non-reverse-transcribed *C. psittaci* Cal10 total RNA and ddH_2O were used as negative controls.

Table 4.2: Primers spanning the intergenic regions, used for RT-PCR to test the putative organization of *pmp* genes in operons.

Intergenic region	Primer	Primer sequence (5'-3')	Amplicon size (bp)	Tm (°C)	GC (%)
pmpA, pmpB	A-B-F	ACCTACTGCGAATATTCACTGTC	225	58.32	43.5
	A-B-R	GCGACGCTTTTGGTGGTATA		58.64	50
pmpE1, pmpE2	E1-E2-F	CTGAGAAATTGGGCAGGTAAAGA	197	58.66	43.5
	E1-E2-R	TCCAGATCCACATTATGCAACT		57.49	40.9
pmpH, pmpG2	H-G2-F	GCATGCCTCAACCCTATCGT	207	60.18	55
	H-G2-R	CAACGGCGCTAGATGGAAAA		58.92	50
pmpG2, pmpG5	G2-G5-F	TGCTAGACAGGATGCAACAAG	260	58.3	47.6
	G2-G5-R	GGGATCTGGGAAGCCAATTG		58.59	55
pmpG5, pmpG8	G5-G8-F	TTGTCGCCGCTACATTTTGT	561	61.58	45
	G5-G8-R	AAGACATGCTGCACGATTCG		62.35	50
pmpG8, pmpG7	G8-G7-F	ATATAGCCCCGCCGTTATCG	902	59.54	55
	G8-G7-R	GAGGGTTAGCTCCATGGACA		58.8	55
pmpG7, pmpG3	G7-G3-F	TAAATTGCCCGCCTCCTGTA	408	59.09	50
	G7-G3-R	GCTCTGTTGCAAGGATCGAG		58.99	55
pmpG1d, pmpG1b	G1d-G1b-F	TGTTGTTCCTTGAGGTGCAG	366	59.87	50
	G1d-G1b-R	TTGAGCTCCGAGGTTCTTGT		59.99	50
pmpG1c, pmpG6	G1c-G6-F	TTGATCCCCAGCTGTATTCC	419	54.4	50
	G1c-G6-R	GGTTAACCACAGCGACGAAT		55.4	50
pmpG6, pmpG4	G6-G4-F	GCTCCATTTGCATCGAGAAT	299	60.19	45
	G6-G4-R	CGTTGACATAGGAGGCAGGT		60.13	55

2.7 Cloning of *pmp* genes

Fragments of *pmpA*, *B*, *D*, *E1*, *G3* and *H* were amplified from *C. psittaci* Cal10 genomic DNA by PCR using Pfu polymerase (St. Leon-Roth, Germany) and primers flanked with specific restriction sites (Table 4.3) for subsequent cloning. The *pmpA* (aa 309-898) and *pmpH* (aa 420-942) fragments were cloned in pGEX-2T (Amersham Pharmacia Biotech, Piscataway, NJ), while *pmpB* (aa 296-955), *pmpD* (aa 321-1193), *pmpE1* (aa 313-958) and *pmpG3* (aa323-791) fragments were cloned in pET-19b (Novagen, Madison, WI) (Figure 4.1). Clone inserts were completely sequenced to confirm correct in-frame insertion and N-terminal fusion with the GST-tag (pGEX-2T) or His_6-tag (pET-19b).

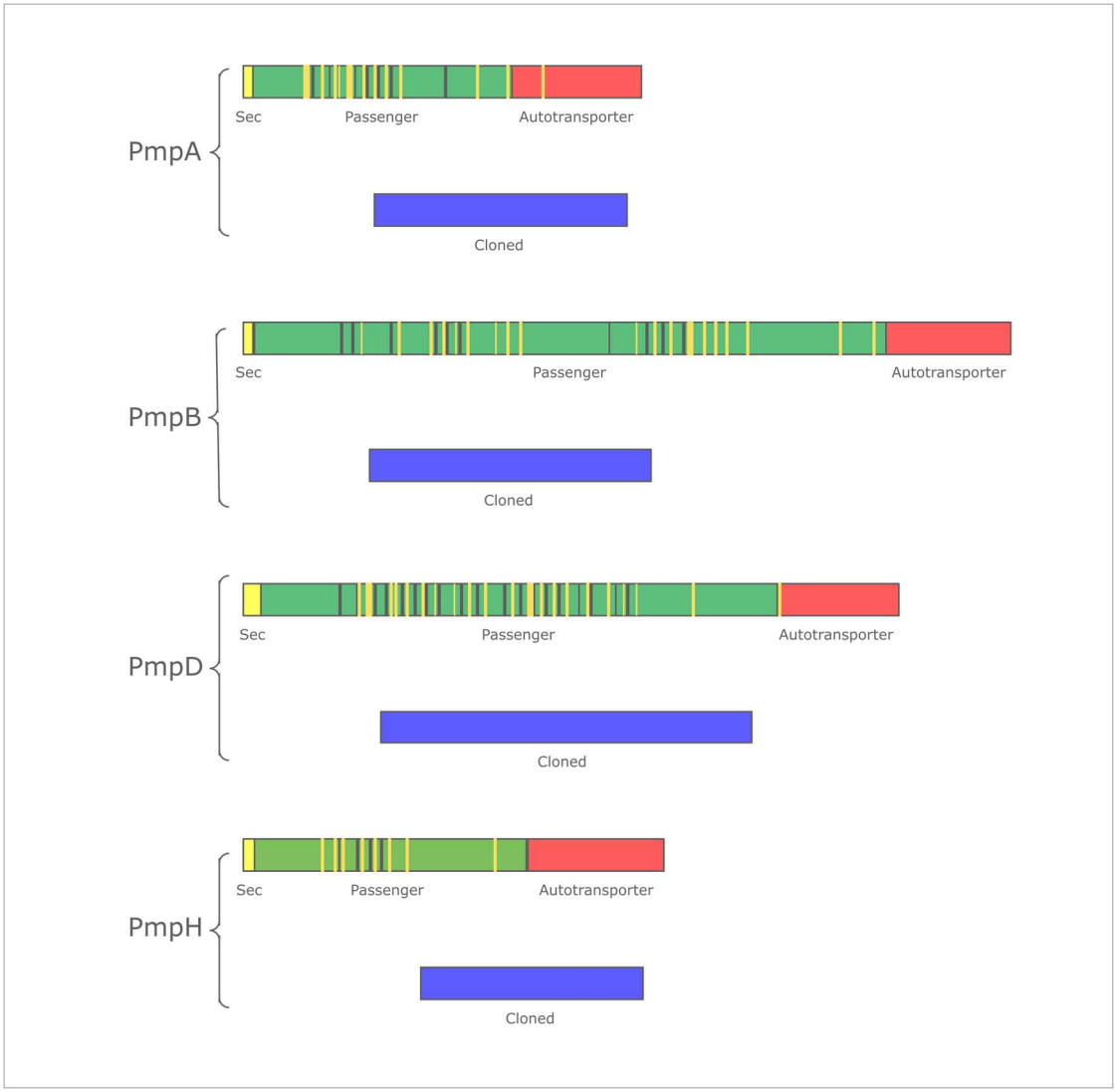


Figure 4.1: Graphical representation of the protein structure of PmpA, PmpB, PmpD and PmpH and the cloned fragment of these proteins. FxxN and GGA (I,L,V) motifs are shown as orange and black vertical lines, respectively.

Table 4.3: Primers and restriction enzymes used for the cloning of the *pmp* genes.

Gene	Primer	Primer sequence (5'-3')	Vector	Restriction enzymes	Molecular mass (kDa)
					Calculated
pmpA	pmpA-F	CCCGGGTGCGGAATTTCAGGTTGAGTGC	pGEX-2T	XmaI	96
	pmpA-R	GGATCCAGCCAAAACTCCGCAGAAGG		BamHI	
pmpB	pmpB-F	GGATCCAGTGTGAAGCTCGTCTTAGC	pET-19b	BamHI	74
	pmpB-R	CTCGAGGCTGAATCTGGAATTGGCGG		XhoI	
pmpD	pmpD-F	CTCGAGCATGCGGATATCCAGTACC	pET-19b	XhoI	95
	pmpD-R	AAGCTTTTAAGACCACGTTCCCATATGTCC		HindIII	
pmpE1	pmpE1-F	AAGCTTTTAAGCATGTCGTGAGTTTGGCG	pET-19b	HindIII	74
	pmpE1-R	CATATGGCAGACCTTAACGGTGGAGC		NdeI	
pmpG3	pmpG3-F	GGATCCAGGGGAGTAGGCCTCCAGA	pET-19b	BamHI	60
	pmpG3-R	CATATGAACTTTTTCATTCATTCCCCTGA		NdeI	
pmpH	pmpH-F	GAATTCTCCATTACGAGCGATATGCGC	pGEX-2T	EcoRI	88
	pmpH-R	GGATCCGGGGATATGGTCTTTATCGGC		BamHI	

2.8 Expression and purification of recombinant Pmps

Escherichia coli BL21 cells were transformed by electroporation and Pmp expression was induced at an OD_{600} of 0.5-0.8 upon the addition of 0.1 mM isopropyl β-D-thiogalactoside for 4 h at 28°C. GST or His_6-tagged protein expressing cells were centrifuged (6 000 x *g* for 15 min at 4°C), resuspended in ice-cold PBS or in 50 mM sodium phosphate, and 300 mM NaCl respectively. Cells were lysed by passing them twice through a French Press cell (American Instrument Co., Urbana, IL) followed by sonication (3 x 30 s). One percent Triton X-100 (Sigma, St Louis, MO) was added and the lysates were placed on ice on a rocking platform for 30 min. Soluble and insoluble proteins were separated by centrifugation (16 000 x *g* for 20 min at 4°C) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Recombinant Pmps were present in the insoluble fractions, which were resuspended in buffer (50 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, and 8 M urea) and placed on a rocking platform (1 h, RT). Refolding of the recombinant Pmps was established by overnight incubation (42°C) in the non-ionic detergent n-octyl-b-D-glucopyranoside (Biosynth, Staad, Switzerland) (McConnell and Pachón, 2011). The samples were dialyzed three times against 1x PBS with 0.1% Triton X-100 and subsequently subjected to affinity chromatography using a Glutathione-Sepharose 4B (GE Healthcare, Little Chalfont, UK) column for GST-tagged proteins, and a TALON metal affinity resin (Clontech, Palo Alto, CA) was used for His_6-tagged proteins according to the manufacturers' instructions. The recovered recombinant protein fractions were subjected to SDS-PAGE and stained by Coomassie blue. Elution fractions containing the recombinant proteins were dialyzed and concentrated with a vacuum concentrator (Spectrum Laboratories, Rancho Dominguez, CA).

2.9 Generation and characterization of Pmp-specific polyclonal antibodies

Polyclonal antibodies (pAbs) against purified recombinant Pmp E1, H and G3 of *C. psittaci* Cal 10 were generated by immunization of guinea pigs. Animal maintenance and experimental treatments were conducted in accordance with the ethical guidelines for animal research established and approved by the institutional Animal Care and Use Committee at University of Arkansas for Medical Sciences, more specifically this study has been approved in protocol number 2975. Two female guinea pigs (strain Hartley) were immunized with each antigen. The guinea pigs were housed individually, fed with Harlan Teklad guinea pig diet, checked twice a day and sacrificed by carbon dioxide. For each animal, 500 μg of immunogen

was mixed with the same volume of Freund's Complete Adjuvant for primary immunization and two subsequent boosters with immunogen and Freund's Incomplete Adjuvant were administered. Previously, pAbs against recombinant PmpA, B and D of *C. caviae* had been generated in the same way (P. Bavoil, unpublished data). All pAbs were characterized by immunoblotting using purified recombinant PmpA, B, D, E1, G3 and H of *C. psittaci* Cal10 as well as density gradient purified EBs of *C. psittaci* Cal10 as antigens. The pAbs were pre-adsorbed with HeLa 229 cells and with inclusion bodies of *E.coli* BL21 expressing an irrelevant antigen (His_6-tagged recombinant β-galactosidase or GST, purified by the same protocol as the recombinant Pmps) to remove any non-specific reactivity. Secondary HRP-rabbit anti-guinea pig antibody (Invitrogen) was used for detection.

2.10 Immunofluorescence microscopy

At 12, 18, 24, 32 and 48 hpi infected HeLa 229 cell cultures (with or without penicillin) were washed with PBS and fixed for 30 min at -20°C with 100% methanol. Monolayers were blocked for 1 h with 5% fetal bovine serum and subsequently double-labelled; Chlamydiae were stained by anti-LPS-FITC (IMAGEN chlamydia test, Novo Nordisk Diagnostics, Cambridge, UK) and Pmps were observed by indirect immunofluorescence after staining with anti-PmpA, anti-PmpB, anti-PmpD or anti-PmpH primary antibodies in combination with Alexa Fluor 568-conjugated goat anti-guinea pig IgG (Invitrogen), and counterstaining with DAPI. Images were acquired and recorded manually via confocal laser scanning microscopy (Nikon A1R, Nikon Instruments Inc., Paris, France), using a 40x Plan Apo oil objective with a numerical aperture of 1.3. DAPI, FITC and AF568 were respectively excited with a 405 nm diode, a 488 nm Ar and a 561 diode laser and their fluorescence emission was respectively detected through a 440/50 nm, 525/50 nm and 595/50 nm bandpass filter. The pinhole was set to 1 Airy unit and acquisition settings (laser power, gain and offset, scan speed) were kept constant throughout the experiment. Image analysis was conducted with ImageJ freeware (Schneider *et al*., 2012) on ten independent experiments by an in-house written colocalization script for Image J to determine the percentage of inclusions expressing PmpA, B, D and H, as described before (Verdoodt *et al*., 2012). In brief, the analyses determined the percentage of inclusions that is positive for specific Pmp proteins (i.e. above an intensity threshold) by calculating the overlap of both binarized channels per object (inclusion). The analysis was benchmarked using a manually curated image data set with varying number of positive inclusions and varying intensity levels. Two different settings were used: setting 1 with both

normal and smaller size inclusions and setting 2 with normal size inclusions only for late developmental times.

2.11 Immuno-electron microscopy

Infected HeLa 229 cell cultures (with or without penicillin) were fixed in 4% paraformaldehyde and 0.1 M PIPES buffer (pH 7.35) at 24 and 48 hpi. Cells were removed with a cell scraper, washed, pelleted, and enrobed in 2.5% low-melting-temperature agarose. Agarose blocks were trimmed into ~1mm^3 size, washed, dehydrated, infiltrated, and embedded in unicryl at -20°C under UV from 24 to 48 h. Ultrathin sections were cut on a Leica UC6 ultramicrotome (Leica Microsystems, Inc., Bannockburn, IL) and collected onto formvar-coated Nickel grids. Immunogold labeling was performed using the guinea pig anti-PmpA, B, D and H specific pAbs followed by a secondary gold conjugated goat anti-guinea pig IgG (H&L) antibody (Electron Microscopy Sciences, Hatfield, PA). Sections were also stained using a rabbit anti-MOMP-specific serum followed by a secondary gold conjugated goat anti-rabbit IgG (H&L) antibody (Electron Microscopy Sciences). Images were acquired using a Tecnai T12 transmission electron microscope (FEI, Hillsboro, OR) at 80 keV and an AMT digital camera (Advanced Microscopy Techniques, Woburn, MA).

2.12 Statistics

Statistical analyses were performed using R (version 3.0.3). The percentage of positive inclusions for PmpA, PmpB, PmpD and PmpH (median for PmpA, B and H and average for PmpD) at different times post-infection were compared based on 10 biological replicates by use of the non-parametric Kruskal-Wallis test (PmpA, B and H) followed by the Mann-Whitney test or the parametric one-way ANOVA followed by Tukey's post hoc analysis (PmpD). For 24, 32 and 48hpi the median percentage of positive inclusions for PmpA, PmpB, PmpD and PmpH, with both normal size and smaller inclusions included was compared with the median percentage of positive inclusions with only normal size inclusions, by the Wilcoxon signed rank test. The abundance of the immunogold labeling for PmpA, PmpH and MOMP was compared on 10 biological replicates by use of the Kruskal-Wallis test followed by the Mann-Whitney test. For all tests, results were considered significantly different if $P < 0.05$.

3. Results

3.1 The *C. psittaci* Cal10 genome encodes 17 predicted *pmp* coding sequences (CDSs)

We developed a Hidden Markov Model for the identification of the *pmp* CDSs in the *C. psittaci* Cal10 genome. Seventeen *pmp* CDS were identified (Table 4.4), representing 4.1% of the genome size at 4 distant genomic loci (Figure 4.2A). All predicted *pmp* CDSs of *C. psittaci* Cal10, except for *pmpG1a*, are encoded on the complementary strand (Figure 4.2B). Eleven *pmpG* alleles are present in the genome, two of which (*pmpG1c* and *G1d*) are predicted to encode truncated products. The *pmpG8* allele codes for a 75.81 kDa protein, therewith reducing the previously determined lower molecular mass boundary (90 kDa) for Pmps (Grimwood and Stephens, 1999).

Table 4.4: *pmp* genes and gene products of *C. psittaci* Cal10

Pmp subtype[a]	Start position	Stop position	Size (bp)	Theoretical pI	Theoretical Mw (kDa)[b]	Locus tag
A	222944	225745	2799	8.82	101.84	G5Q_0224
B	217351	222744	5391	6.04	190.48	G5Q_0223
D	924237	928841	4602	5.22	163.72	G5Q_0827
E1	293884	296886	3000	5.86	109.95	G5Q_0290
E2	296908	299793	2883	6.60	106.57	G5Q_0291
G1a	710413	712956	2541	5.25	91.27	G5Q_0648
G1b	322009	324513	2502	5.81	89.39	G5Q_0303
G1c	326413	327735	1320	5.16	45.85	G5Q_0306
G1d	320559	321878	1317	4.96	45.34	G5Q_0302
G2	304565	307696	3129	7.55	110.88	G5Q_0294
G3	316390	318924	2532	8.01	92.34	G5Q_0300
G4	330783	333605	2820	6.24	100.51	G5Q_0308
G5	307929	310211	2280	7.94	83.90	G5Q_0296
G6	327859	330555	2694	5.79	95.08	G5Q_0307
G7	313712	316234	2520	6.50	90.12	G5Q_0299
G8	310741	312858	2115	6.77	75.81	G5Q_0297
H	301585	304539	2952	7.06	105.05	G5Q_0293

[a] The *Chlamydia psittaci* Cal10 *pmpG* sequences were used to search for *pmpG* sequences across the collection of isolates currently in the NCBI database. All hits were parsed, and protein sequences were aligned using ClustalW. The alignments were used to manually subdivide the pmpG family into subfamilies.

[b] The theoretical Mw includes the signal sequence.

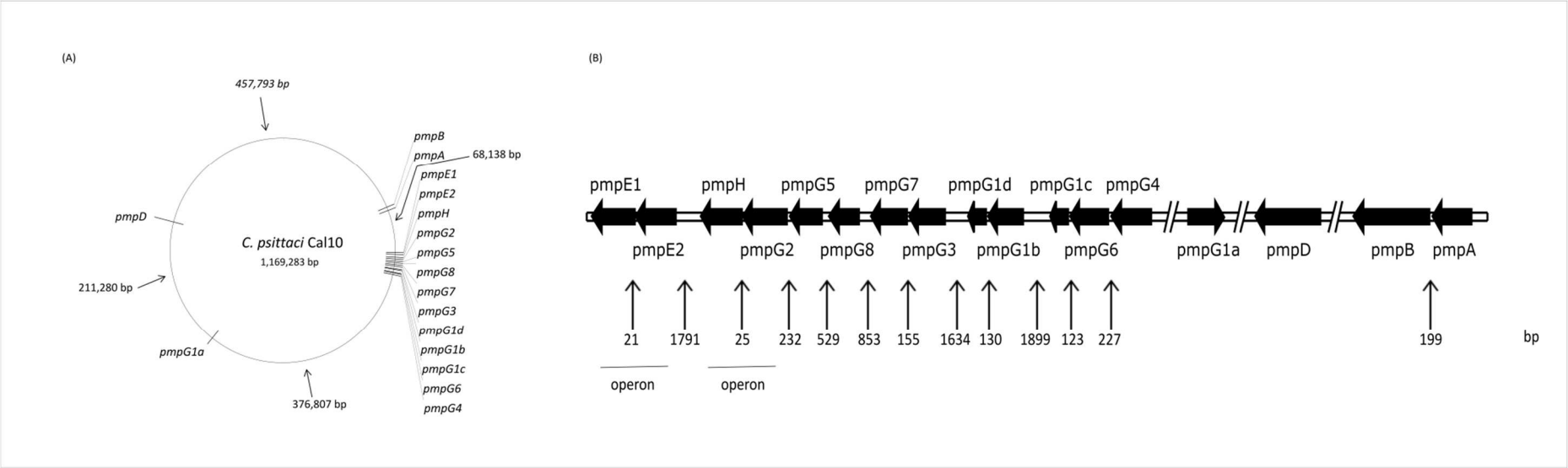


Figure 4.2: *pmp* CDS organization in the *C. psittaci* Cal10 genome. (A) The *pmp* CDSs map to 4 distinct loci on the *C. psittaci* Cal10 genome. Distances (bp) between nearest loci are indicated. (B) Linear representation of the *pmp* loci. CDSs and inter-CDS regions are drawn to scale. Number of bp between 2 nearest CDSs are indicated. A break (//) is added if the inter-CDS region is bigger than 5000 bp.

3.2 Transcription of *pmp* CDSs

To start unravelling Pmp function, we first determined the timing of *pmp* transcription during chlamydial development. To this end, *pmp* transcript levels were measured under normal culture conditions at early (2 and 6 hpi), mid (12 and 18 hpi), and late (24, 32 and 48 hpi) stages of the developmental cycle (Figure 4.3). Analysis at very late developmental times (e.g. 72 hpi) was not possible because of gradual loss of viability and lysis of the host cells. The gene *tufA* (encoding Elongation Factor Tu, EF-Tu) was included for comparative purposes as a house-keeping gene presumed to be expressed throughout the developmental cycle (Carrasco *et al*., 2011). Unexpectedly, transcript levels for *tufA* peaked at 6 hpi, then gradually diminished until 32 hpi, and rose again at 48 hpi. Overall, all *pmp* CDSs were transcribed at a detectable level at some stage of the developmental cycle. All *pmp* genes, were transcribed at very low levels early in the developmental cycle (2 hpi), except *pmpA*, *pmpH*, and *pmpG5*, that were transcribed at or near peak levels. For most *pmp* genes (*pmpE1*, *pmpE2, pmpG1a*, *pmpG1b*, *pmpG1c*, *pmpG1d*, *pmpG2*, *pmpG3*, *pmpG6*, *pmpG8*, and *pmpH*), transcript levels were highest at 24, 32 and 48 hpi, typically peaking at 24 hpi except for *pmpG3* and *pmpG6* that peaked at 32 and 48 hpi respectively, and *pmpG8* that remained high from 24 hpi onward. Surprisingly, *pmpH* transcription was highest at 2-6 hpi, minimal at 12-18 hpi and rose again to similar high level from 24 hpi onward. Somewhat similarly, *pmpA* transcript levels were highest between 2 and 18 hpi, and, although lower, rose again from 24 hpi onward. *pmpB* transcription was highest between 18 and 32 hpi. Transcription of *pmpD* and *pmpG4* peaked at 48 hpi.

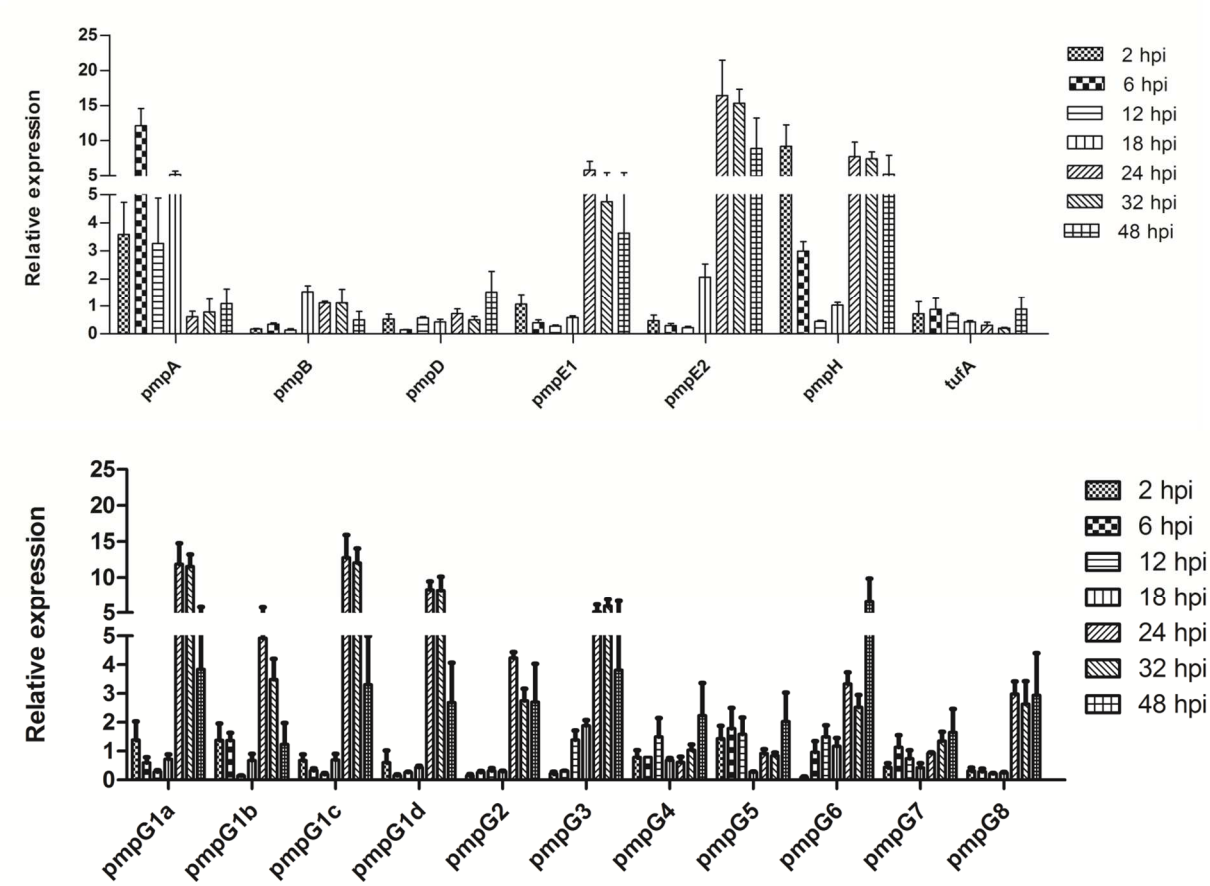


Figure 4.3: Relative expression of the *pmp* genes during normal *C. psittaci* culture conditions. At 2, 6, 12, 18, 24, 32 and 48 hpi, transcript levels were measured by real-time RT-qPCR in *C. psittaci* grown under normal conditions. For each gene, the average expression level of all samples was determined and the expression level of each sample of that gene is represented relative to the average expression level. Therefore, the expression level of a sample can only be compared to the expression level of another sample of the same gene. Two biological and two technical replicates were analyzed for each sample. Error bars are based on the standard error of the mean.

3.3 *pmp* transcription is altered during penicillin-induced stress in *C. psittaci*

Previous studies suggested a unique role for *pmpA, pmpD* and *pmpI* in *C. trachomatis* intracellular pathogenesis as the transcription of these genes remained unaffected during penicillin-induced stress, while the expression of all other *pmp* genes was down-regulated (Carrasco *et al*., 2011). Therefore, we compared transcript levels of all *pmp* genes during normal *C. psittaci* culture conditions and penicillin-induced stress (Figure 4.4). Transcription of *tufA*, *pmpB*, *pmpD*, *pmpG2*, *pmpG4* and *pmpG8* was up-regulated, while that of *pmpA*, the co-regulated *pmpE1-E2*, *pmpH, pmpG1a-d, pmpG3* and *pmpG6* was down-regulated.

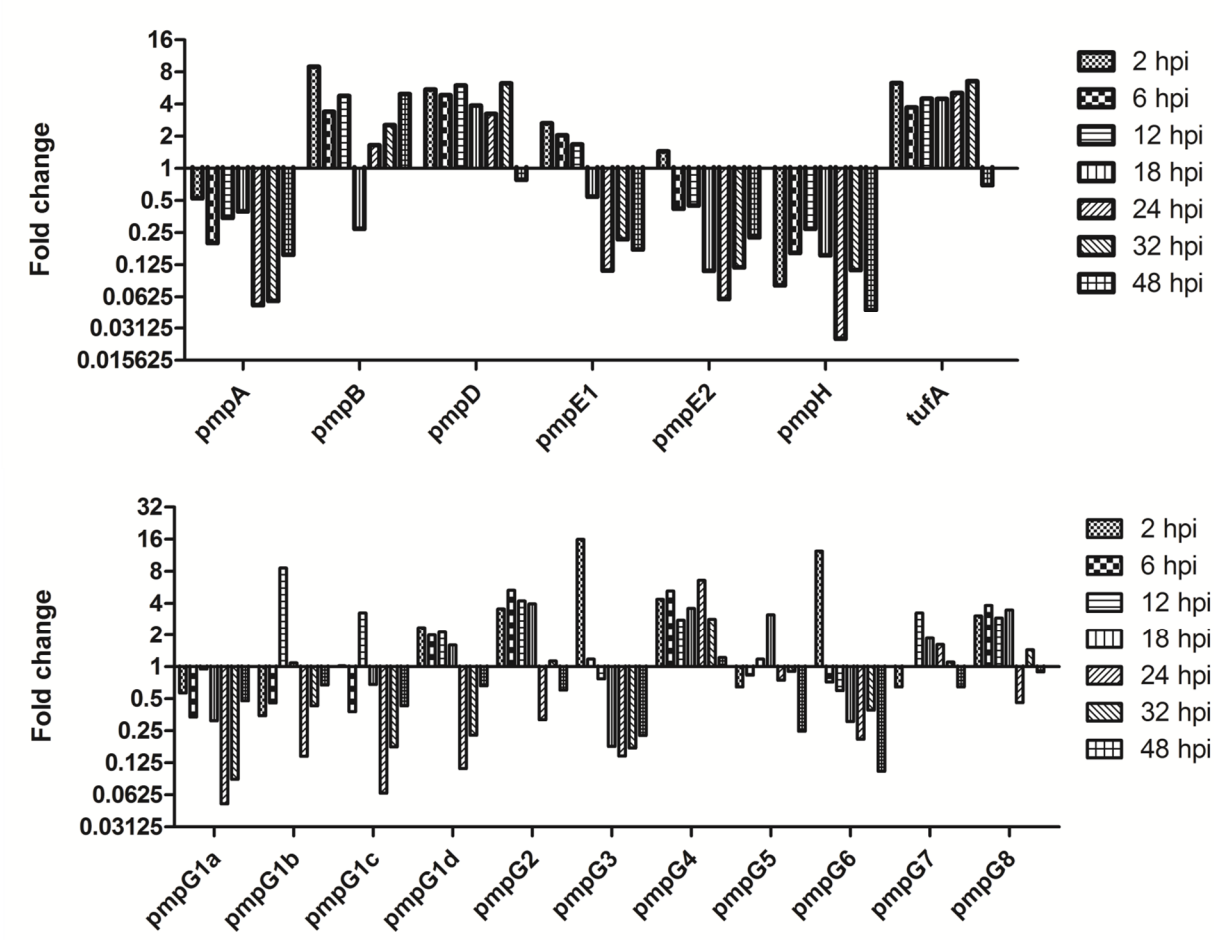


Figure 4.4: Fold change of the *pmp* genes during penicillin-induced stress compared normal *C. psittaci* culture conditions. At 2, 6, 12, 18, 24, 32 and 48 hpi, transcript levels were measured by real-time RT-qPCR in *C. psittaci* grown under normal conditions and in the presence of penicillin. For all samples, the fold change of the penicillin-induced stress condition compared to the normal condition is shown.

3.4 Most *pmp* genes of *C. psittaci* Cal10 are not co-transcribed

The genetic linkage and colinearity of *pmpG4-G6-G1c, pmpG1b-G1d, pmpG3-G7*, *pmpG8-G5-G2-H, pmpE2-E1 and pmpA-B* (Figure 4.2) suggested that these genes might be organized in operons, leading to co-transcription. To partially test this hypothesis, we performed RT-PCR using primers designed to span the relevant intergenic regions (Table 4.3) on cDNA samples generated during normal cell culture conditions at 24, 32 and 48 hpi. The latter time points were selected as the *pmp* genes of *C. trachomatis*, *C. pneumoniae* and *C. abortus* were highly transcribed at mid and late time points (Grimwood and Olinger, 2001; Wheelhouse *et al*., 2009; Carrasco *et al*., 2011). Only intergenic regions between *pmpE1-E2* and *pmpH-G2* were amplified (Figure 4.5), suggesting that *pmpE1-E2* and *pmpG2-H* are arranged in operons, whereas that *pmpG4-G6-G1c*, *pmpG1b-G1d*, *pmpG3-G7*, *pmpG8-G5*, and *pmpA-B* are not.

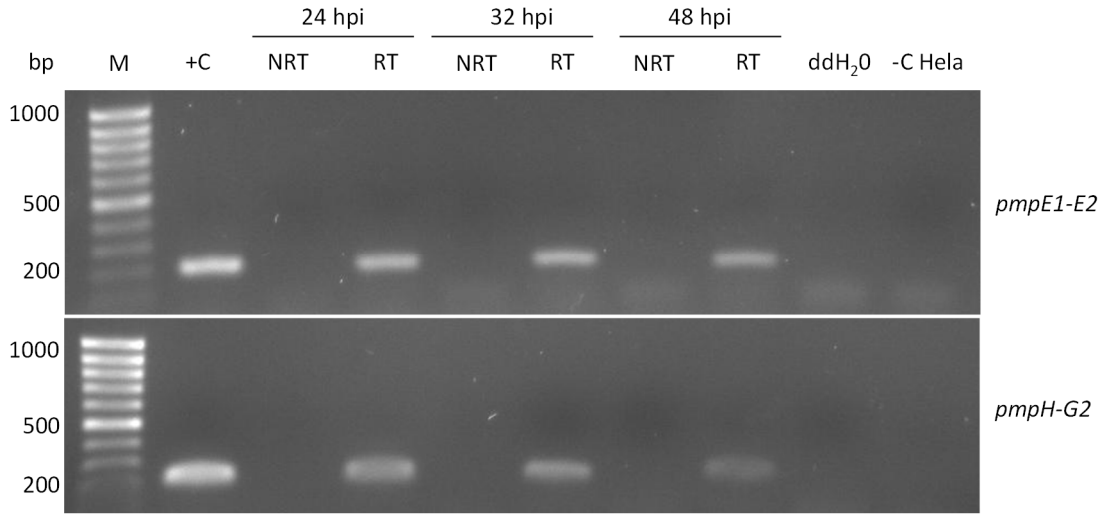


Figure 4.5: *pmpE1-E2* and *pmpH-G2* are organized in operons. Based on co-linearity, 6 putative operons were identified: *pmpE2-E1*, *pmpG8-G5-G2-H*, *pmpG3-G7*, *pmpG1b-G1d* *pmpG4-G6-G1c,* and *pmpA-B*. cDNAs generated at 24, 32 and 48 hpi were amplified by RT-PCR using specific primers spanning the intergenic regions (Table 2). Only results for *pmpE1-E2* and *pmpH-G2* are presented as amplicons were not detected for all other intergenic regions. M: MassRuler Low Range DNA ladder (Thermo Scientific); +C: positive control *C. psittaci* Cal10 genomic DNA; -C HeLa: negative control cDNA from uninfected HeLa cells; RT/NRT: with or without reverse transcriptase.

3.5 Protein production profiles differ between *C. psittaci* Pmps

RT-qPCR profiles provide an indication of when the *pmp* genes are transcribed at the population level. To assess expression at the protein level in individual inclusions, polyclonal, monospecific antibodies against PmpA, PmpB, PmpD, PmpE1, PmpG3, and PmpH were generated in guinea pigs. Analysis of the specificity of the antisera by immunoblot against a panel of recombinant Pmps (rPmps) and gradient-purified *C. psittaci* EBs indicated that PmpA-, PmpB-, PmpD- and PmpH-specific antibodies reacted with high molecular mass bands (Figure 4.6A) in the corresponding immunizing antigen lane, while PmpE1- and PmpG3-specific antibodies cross-reacted with multiple bands in multiple lanes (not shown). Therefore, PmpA-, PmpB-, PmpD- and PmpH-specific antisera were selected for further analysis. The observed molecular masses of rPmpA and rPmpH were smaller than the calculated molecular masses of the corresponding recombinant polypeptides. This may be caused by the instability of the full-length recombinant polypeptides. rPmpH was detected as a triplet of bands of 75, 70 and 60 kDa. However, the 70kDa band was also detected in the rPmpD and rPmpG lanes, suggesting that it is an *Escherichia coli* cross-reactive contaminant. Antibody-reactive bands detected in Western blots against purified EB proteins, were also smaller than the calculated molecular masses of each Pmp (Figure 4.6B), suggesting that proteolytic processing or degradation of these Pmps may have occurred pre- or post-EB purification.

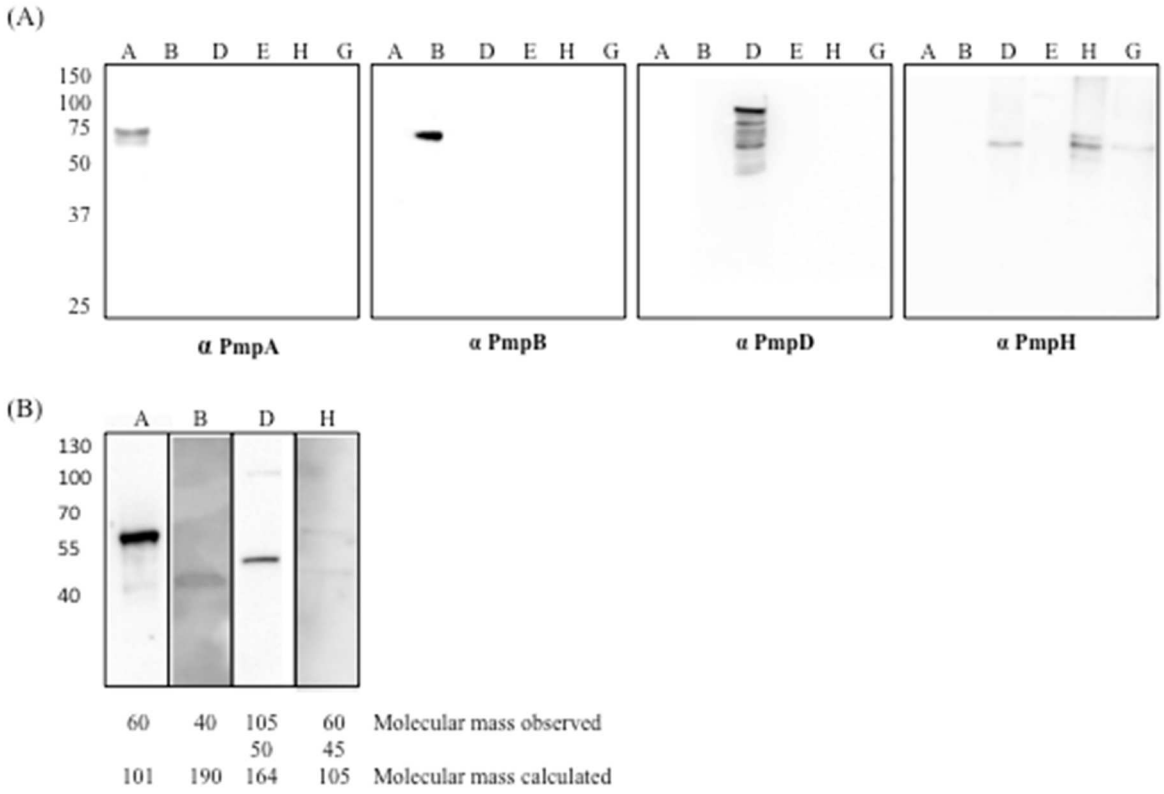


Figure 4.6: Guinea pig polyclonal antibodies against PmpA, B, D and H are specific for their respective immunizing antigens. (A) The specificity of polyclonal antibodies raised against rPmpA (α PmpA), rPmpB (α PmpB), rPmpD (α PmpD) and rPmpH (α PmpH) was verified by immunoblotting using (A) partially purified recombinant PmpA, B, D, E1, G3 and H as well as (B) density gradient purified EBs of *C. psittaci* Cal10. The calculated molecular masses of recombinant PmpA, PmpB, PmpD, PmpE1, PmpH and PmpG3 are 92 kDa, 74 kDa, 95 kDa, 74 kDa, 88 kDa and 60 kDa, respectively. The observed molecular masses were 75 kDa, 74 kDa, 95 kDa, 70 kDa, 75 kDa and 60 kDa, respectively. (B) The calculated and observed molecular masses of the protein bands detected in EBs are shown.

Immunofluorescence microscopy (IF) was used to investigate the PmpA, PmpB, PmpD and PmpH protein production profiles in individual inclusions in 10 biological replicates, and to examine potential post-transcriptional and post-translational regulation. Inclusions positive for a specific Pmp subtype were determined under normal *C. psittaci* culture conditions and during penicillin-induced stress (Figure 4.7). The same time points as used for RT-qPCR were analyzed. At 24 and 48 hpi, both large size and smaller, ovoid and irregularly shaped inclusions were observed during normal culture conditions, possibly the result of the asynchronous start and/or growth of these inclusions.

A macro excluding the smaller inclusions was used to quantify the percentage of inclusions producing PmpA, PmpB, PmpD and PmpH, as the large inclusions are representative for the late developmental times (Figure 4.8). At 12 hpi, under normal *C. psittaci* culture conditions, PmpA was highly produced in all 10 biological replicates, with a median of 90% PmpA positive inclusions (Figure 4.8). At the same developmental time, PmpH was not equally produced in all replicates, such that in 3 replicates, PmpH was detected in 88% of the inclusions, while in 7 replicates, it was only detected with a median of 4% positive inclusions. Thus, PmpH production displayed substantial variation between cultures inoculated with the same seed. Neither PmpB- nor PmpD-positive inclusions were detected at 12 hpi and PmpB-positive inclusions were also not detected at 18 hpi. At 24, 32 and 48 hpi, PmpA and PmpH were highly expressed in all replicates with a median percentage of positive inclusions of 100%. PmpB was first detected at 24 hpi in a median of 67% of the inclusions and was highly expressed in all inclusions at 32 and 48 hpi (median of 100%). Noticeably, the expression of PmpD varied significantly between biological replicates at 18 and 24 hpi, as PmpD was not detectably produced in 1 replicate at both times and between 88% and 99% in the other 9 replicates.

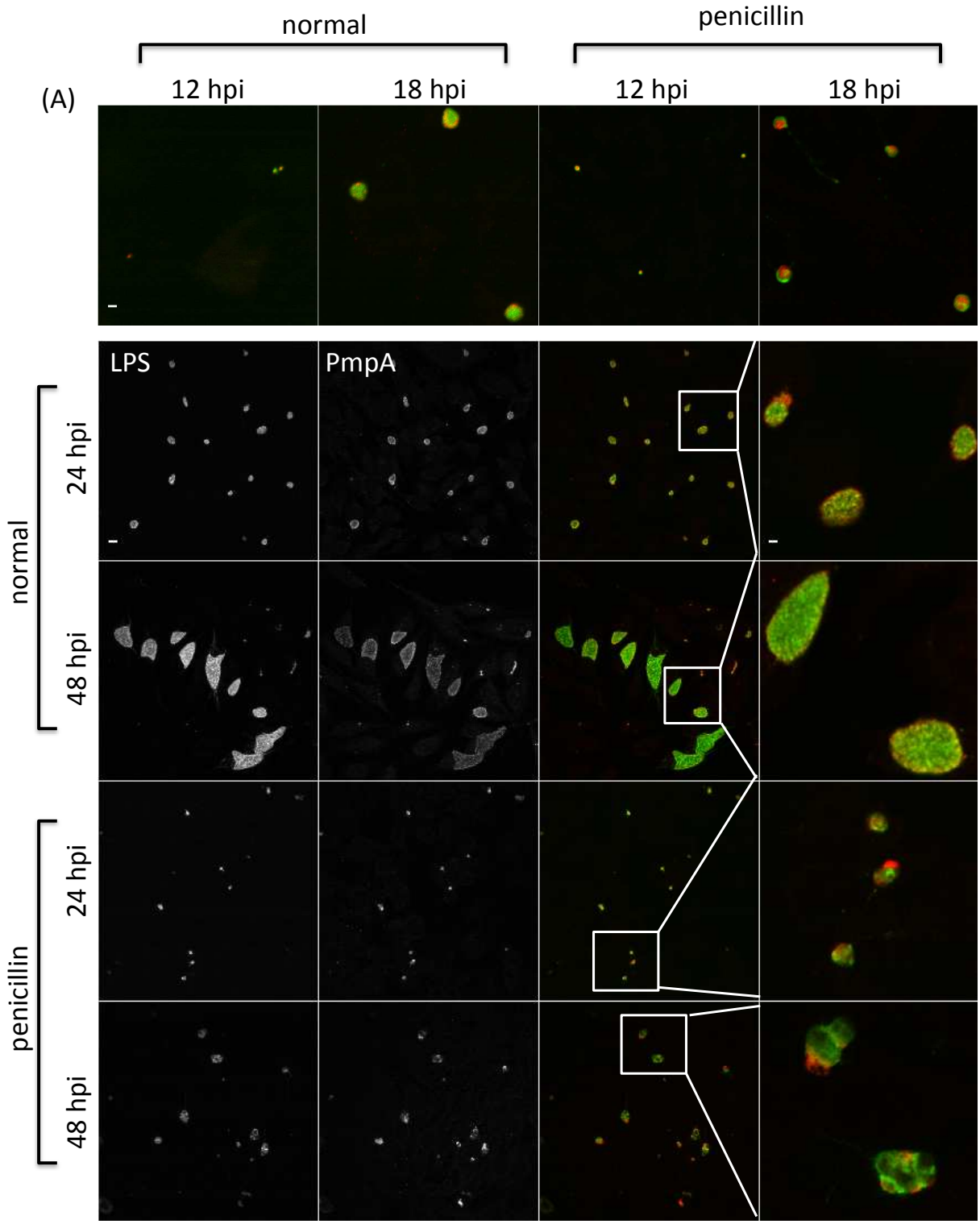
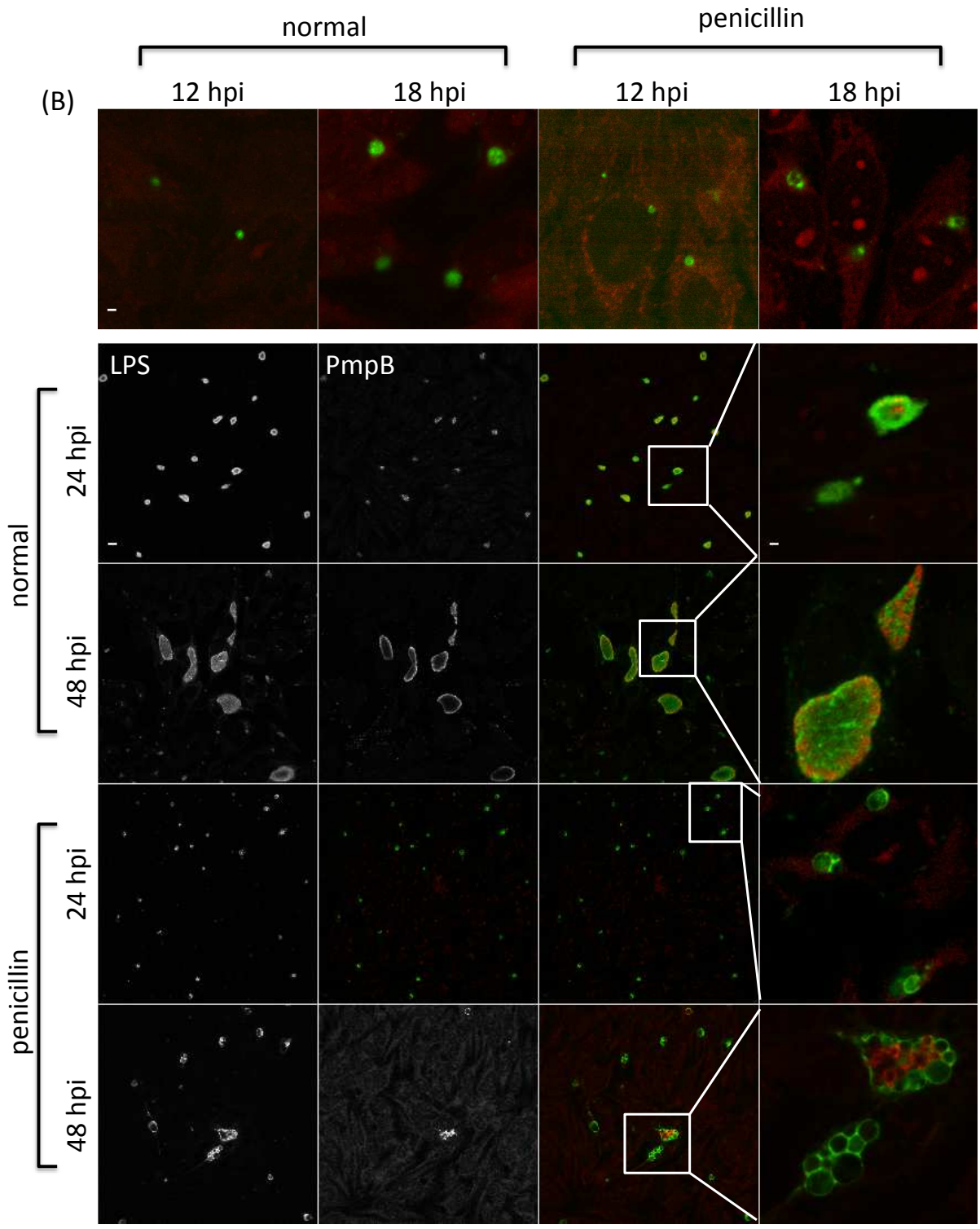
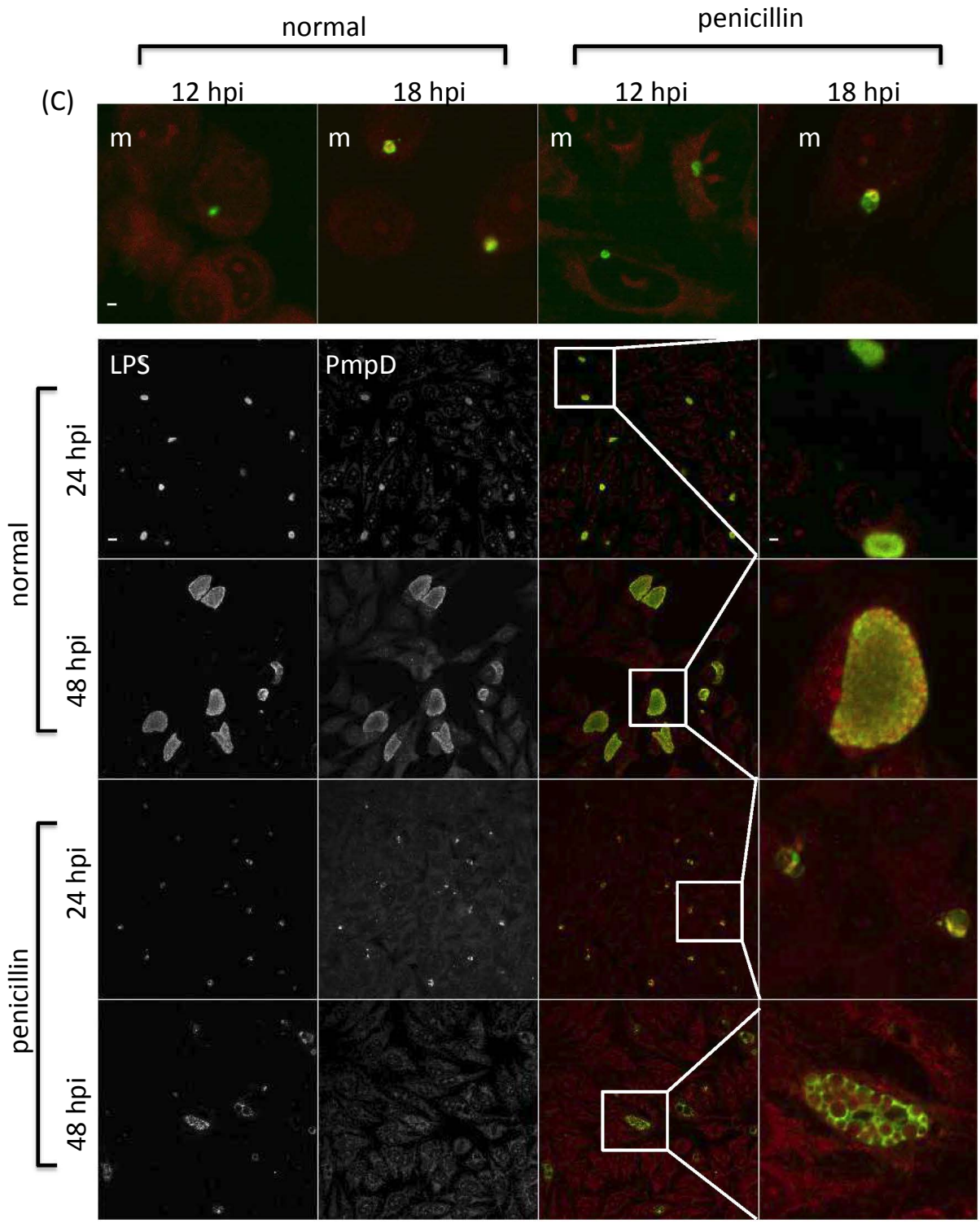
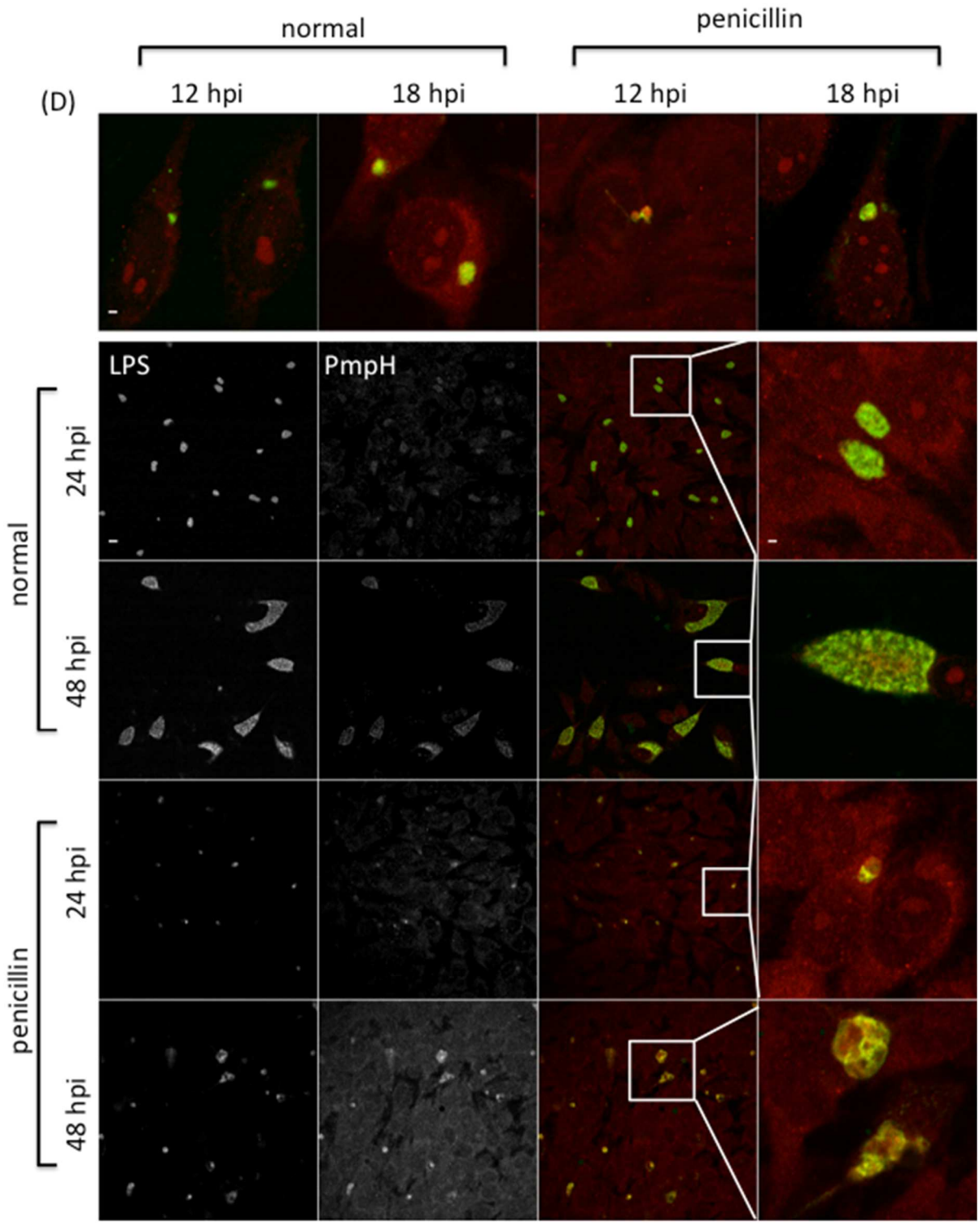


Figure 4.7: The Pmp production profile differs for different Pmp subtypes. *C. psittaci*-infected HeLa cells were fixed at 12, 18, 24, 32 and 48 hpi, and double-stained with chlamydial LPS-specific antibody (FITC-conjugated, green) and Pmp-specific antibody (Alexa Fluor 568-conjugated, red). At 12 and 18 hpi, only colored merged images are shown under normal and penicillin-induced persistence culture conditions (top row). At 24 and 48 hpi, single channel images are shown in black and white (2 left-most columns), while merged images and insets thereof are shown in color (2 right-most columns). Representative micrographs for (A) PmpA, (B) PmpB, (C) PmpD and (D) PmpH are shown. Staining patterns at 32 hpi (not shown) were similar to those at 48 hpi. Bar = 2 μm for the 12 and 18 hpi times, and 24 and 48 hpi insets (i.e. top row and right-most column) and 10 μm for all remaining images.


normal
penicillin
(B)
12 hpi
18 hpi
12 hpi
18 hpi
LPS
PmpB
normal
24 hpi
48 hpi
penicillin
24 hpi
48 hpi


normal
penicillin
(C)
12 hpi
18 hpi
12 hpi
18 hpi
m
m
m
m
LPS
PmpD
normal
24 hpi
48 hpi
penicillin
24 hpi
48 hpi


normal
penicillin
(D)
12 hpi
18 hpi
12 hpi
18 hpi
LPS
PmpH
normal
24 hpi
48 hpi
penicillin
24 hpi
48 hpi

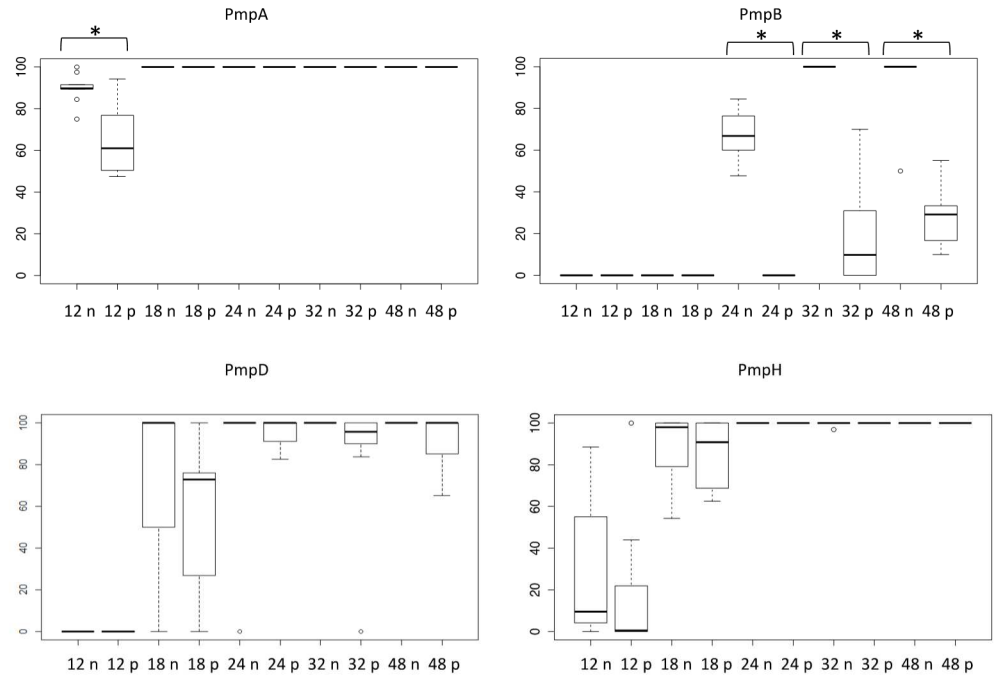


Figure 4.8: PmpA, B, D and H production differs under normal (n) *C. psittaci* and during penicillin-induced stress (p) culture conditions. *C. psittaci* infected HeLa cells were fixed at 12, 18, 24, 32 and 48 hpi, and double-stained with a chlamydial LPS-specific antibody and Pmp-specific antibody. For each Pmp subtype, the percentage of positive inclusions was determined by a macro based on co-localization of the two antigens. The results shown here did not take into account smaller inclusions that are formed at 24, 32 and 48 hpi. The data are expressed as box plots: the box represents the 25^{th}-75^{th} percentiles, the median is depicted by a bar across the box and the whiskers on each box represent minimum and maximum value. Outliers are depicted by dots. Statistically significant differences ($P < 0.05$) are indicated with an asterix.

3.6 Penicillin-induced stress differentially alters protein production profiles of specific *C. psittaci* Pmps

Because of their obligate intracellular life style, the pathogenesis of chlamydiae is intimately linked to their capacity to grow inside cells and on their specific physiologic properties in response to threats such as innate host defenses or nutrient deprivation. To start evaluating potential differential production of Pmp subtypes under different growth conditions, we used penicillin-induced stress as a previously well-defined modulator of *pmp* gene expression in *C. trachomatis* (Carrasco *et al.*, 2011). Under penicillin-induced stress, PmpA was the only detectable Pmp at the early developmental time of 12 hpi (Figures 4.7 & 4.8). PmpA production was observed in all 10 biological replicates, yielding a median of 61% PmpA positive inclusions, which is significantly lower than the 90% observed during the normal

condition (Figure 4.8). At the same developmental time, PmpH was unevenly expressed with 100% of the inclusions staining positive for PmpH in 1 biological replicate, but only 0.3% in the other nine, yielding a median of 0.5% PmpH-positive inclusions (Figure 4.8) Thus, we showed a substantial variation in PmpH production. PmpB and PmpD, which were not expressed at 12-18 hpi, and 12 hpi respectively in unstressed cultures, were also not detected at these times during penicillin-induced stress (Figures 4.7 & 4.8).

At 18, 24, 32 and 48 hpi, PmpA, PmpD and PmpH were expressed in all biological replicates experiencing penicillin-induced stress at levels at or near those of unstressed cultures (Figure 4.8). The median percentage of Pmp positive inclusions was 100% for PmpA at all time points and 91%, 100%, 100% and 100% at 18, 24, 32 and 48 hpi respectively for PmpH. Relatively fewer PmpD-positive inclusions (59%) were observed at 18 hpi, i.e. significantly less than for PmpA and PmpH ($P < 0.05$), while 93%, 84% and 88% of the inclusions were PmpD-positive at 24, 32, 48 hpi, respectively, under penicillin-induced stress. Interestingly, at 18 hpi and 32 hpi, the level of PmpD expression also varied among the biological replicates experiencing penicillin-induced stress, with no expression in 1 replicate, and 65% to 88% respectively in the remaining nine. PmpB expression was significantly down-regulated at all developmental times during penicillin-induced stress (Figure 4.8). It is noteworthy that the median percentages of inclusions producing PmpB, PmpD or PmpH rose significantly ($P < 0.05$) when smaller inclusions were omitted from the analysis. However, this was not the case for PmpA, highlighting the very high level of expression of PmpA at early stages of the *C. psittaci* developmental cycle.

3.7 PmpA, PmpD and PmpH target the chlamydial cell envelope

Immuno-electron microscopy (IEM) was used to assess the subcellular localization of PmpA, PmpB, PmpD and PmpH at the late developmental times under both normal conditions and under penicillin-induced stress. Probably due to compromised antigen recognition or accessibility during dehydration and fixing for IEM, PmpB staining was poor preventing further analysis of ths Pmp subtype by this method. In general, the levels and topology of the three remaining Pmps were grossly similar under both unstressed and stressed conditions. IEM revealed strong PmpA-specific signal mostly localized at the chlamydial cell envelope in both unstressed and stressed cultures (Figure 4.9A). PmpH IEM staining was similar to that of PmpA in localization and relative abundance in both unstressed and stressed cultures (Figure 4.9A). PmpD-specific labeling was also observed at the chlamydial envelope, but less

abundant than PmpA and H (Figure 4.9A). To evaluate relative antigenicity and immuno-accessibility, we compared IEM staining of MOMP, the most abundant chlamydial protein and a strong antigen during infection in other *Chlamydia* spp. (Caldwell *et al*., 1981; Caldwell *et al*., 1987), with that of *C. psittaci* PmpA and PmpH at 24 hpi (Figure 4.9B). Surprisingly, PmpA and PmpH were significantly more labeled at the cell envelope than MOMP ($P < 0.05$, Figure 4.9C). PmpA was also significantly more labeled than PmpH ($P < 0.05$).

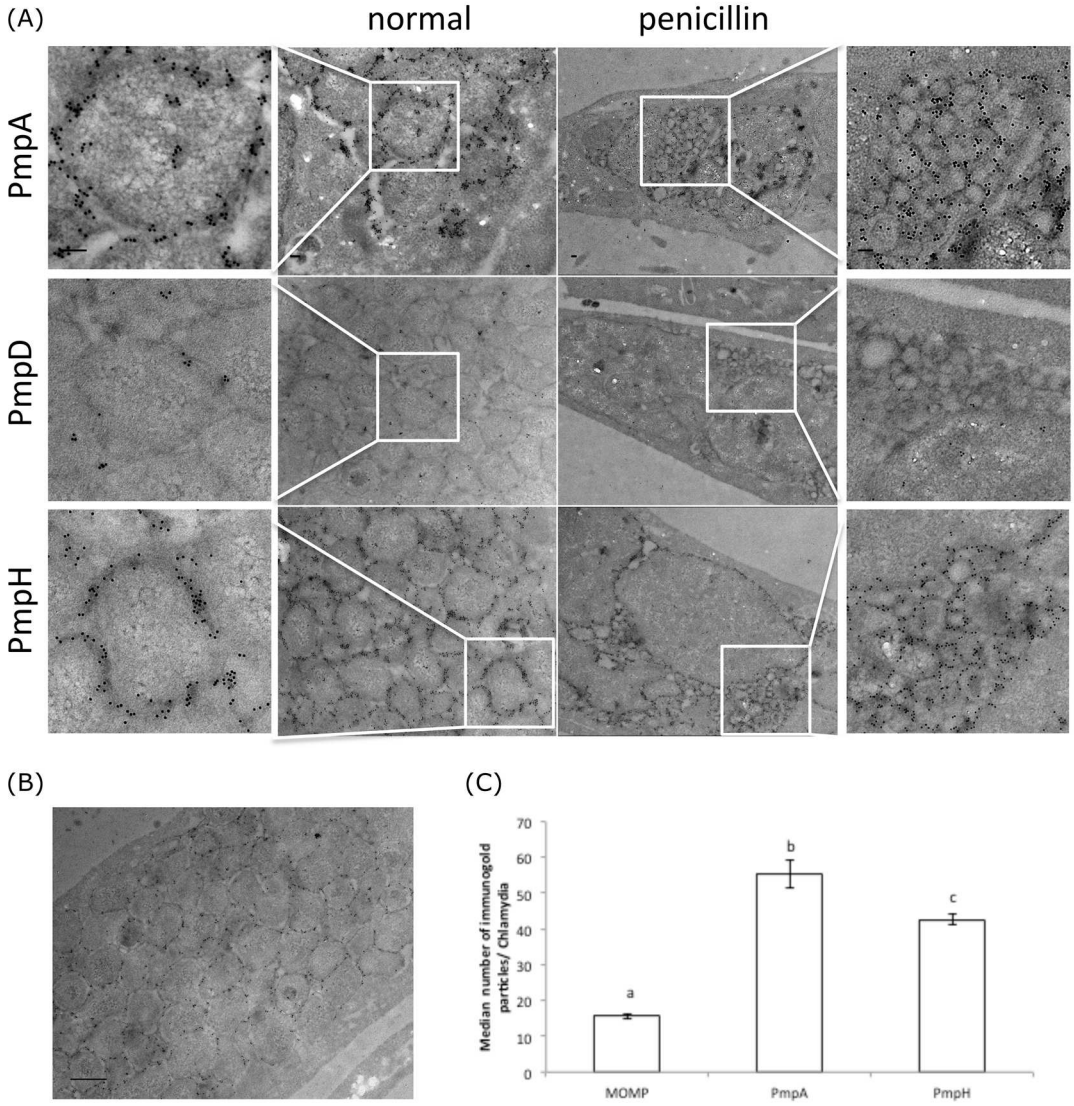


Figure. 4.9: PmpA and PmpH stain more heavily in the chlamydial envelope than MOMP. (A) Immuno-electron microscopic images of *C. psittaci* infected HeLa cells, which were fixed at 24 and 48 hpi both during normal cell culture conditions and during penicillin-induced persistence, and stained with the primary Pmp-specific antibody and the secondary gold conjugated goat anti-guinea pig antibody. Only results for 24 hpi are shown, as the subcellular localization did not change at 48 hpi. (B) Immuno-electron microscopic image at 24 hpi during normal cell culture conditions only, the cells were also labeled with MOMP-specific antibody and the secondary gold conjugated goat anti-rabbit antibody. (C) *C. psittaci* infected HeLa cells were fixed 24 hpi, labeled with MOMP-, PmpA- and PmpH- specific antibodies and secondary gold conjugated goat anti-rabbit (MOMP) or gold conjugated goat anti-guinea pig (PmpA and H) antibody and the number of immunogold particles was counted on 100 chlamydiae. Error bars are based on standard error of the mean. Different letters indicate statistically significant differences ($P < 0.05$). Bars = 0.1 μm.

Detailed examination of higher magnification images revealed inclusion membrane labeling and suggested inner and outer membrane labeling of all analyzed Pmps (Figure 4.10). At 48 hpi, most of the penicillin-stressed aberrant bodies were lysed, probably due to the harsh EM fixation technique. Therefore, only normal culture conditions IEM images were analyzed. As observed at 24hpi, the order of the Pmps in decreasing amount of immunogold labeling was PmpA, PmpH and PmpD. PmpA-, PmpD- and PmpH-specific staining of small vesicles, possibly corresponding to outer membrane vesicles (OMVs) was observed at 48 hpi during normal cell culture conditions (Figure 4.11). Both labeled and unlabeled vesicles were observed.

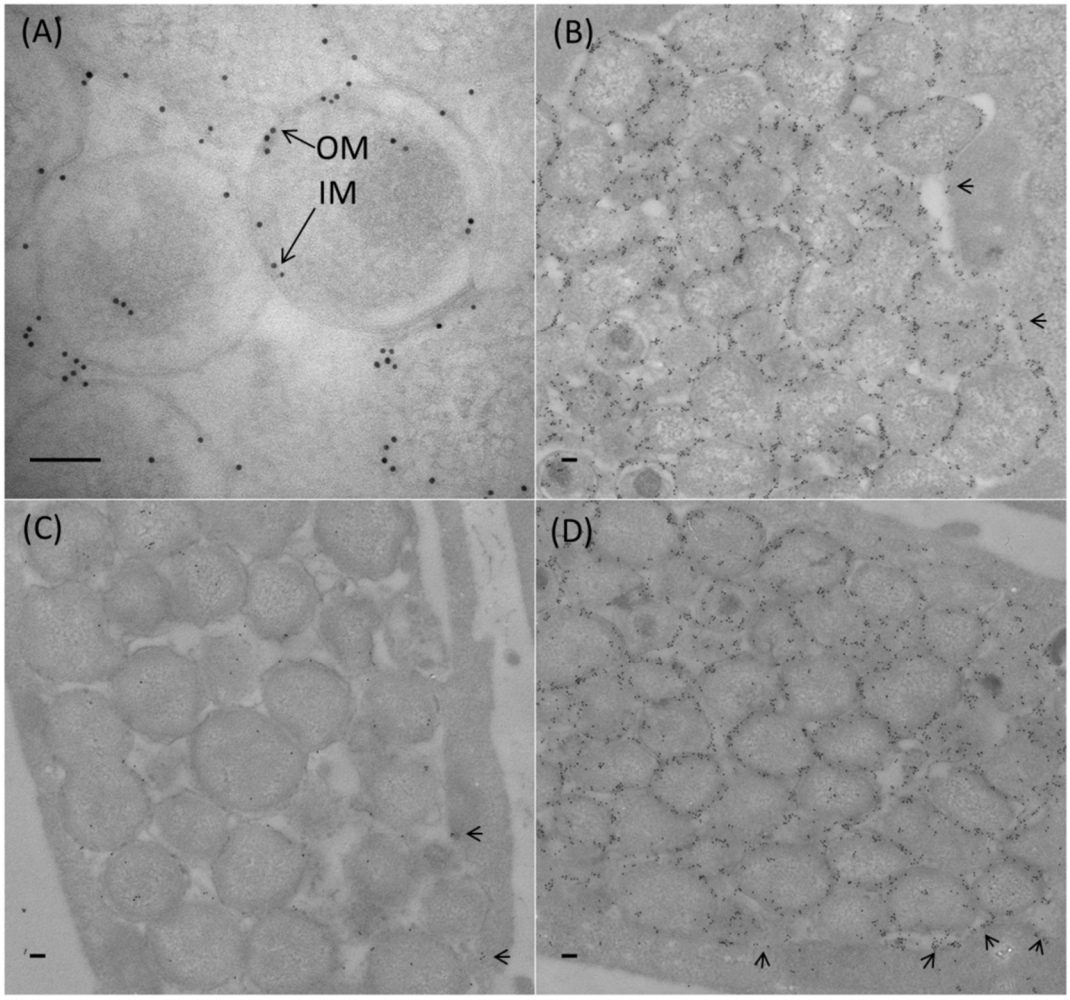


Figure 4.10: PmpA, PmpD and PmpH localize to the inclusion membrane and probably also to the inner membrane (IM), outer membranes (OM). Immuno-electron microscopic images of *C. psittaci* infected HeLa cells, which were fixed at 24 hpi and stained with the primary PmpA-, PmpD- or PmpH- specific antibody and secondary gold conjugated goat anti-guinea pig antibody. The suggested IM and OM localization is shown for PmpA only (A, long arrows). Inclusion membrane labeling (short arrows) is displayed for PmpA, D and H (B, C and D respectively). Bars = 0.1 μm.

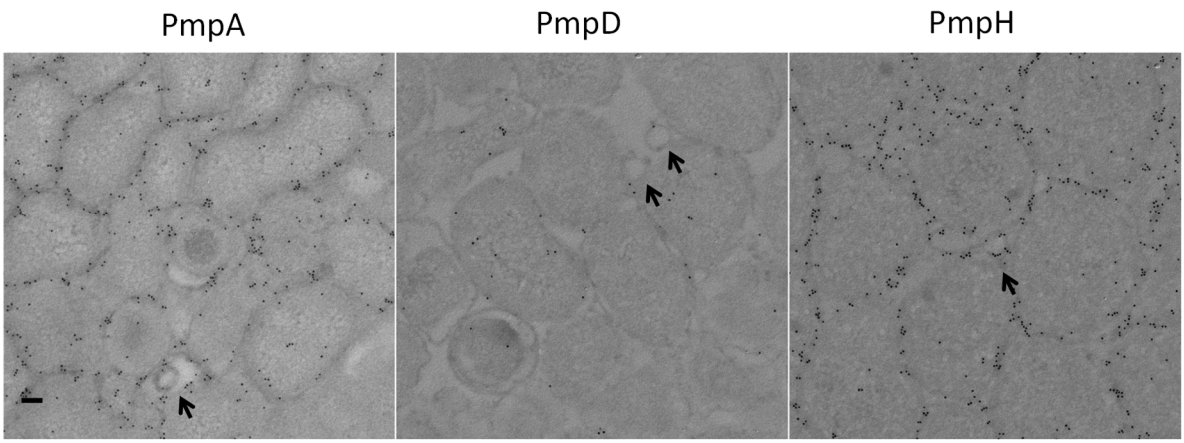


Figure 4.11: PmpA, PmpD and PmpH localize to vesicles observed at 48 hpi. Immuno-electron microscopic images of *C. psittaci* infected HeLa cells, which were fixed at 48 hpi and stained with primary PmpA-, PmpD- or PmpH-specific antibodies and secondary gold conjugated goat anti-guinea pig antibody. Arrows point to the vesicles. Bar = 0.1 μm.

4. Discussion

4.1 Transcription of *pmp* genes

An ultimate assessment of the involvement of the Pmp family in *C. psittaci* virulence and their potential use as vaccine candidates requires the fundamental characterization of the expression and topology properties of these proteins. We used *C. psittaci* Cal10, a previously well-characterized, prototypic strain (Narita *et al*., 1976; Matsumoto, 1982; Hovis *et al*., 2013; Mojica *et al*., 2015), with a complete genome sequence (Grinblat-Huse *et al*., 2011). Consistent with observations of *pmp* gene expression in other *Chlamydia* species (Grimwood and Olinger, 2001; Wheelhouse *et al*., 2009; Carrasco *et al*., 2011), transcripts were detectable for all 17 *C. psittaci pmp* CDSs, although the level of transcription varied over a wide range between *pmps* and with developmental time for each *pmp*. Although nearly all *pmp* transcript levels were high at late developmental times (24-48 hpi; typically with a dip at 48 hpi), *pmpA* transcript levels were high at early-to-mid developmental times (2-18 hpi), similar to the *pmpA* ortholog of *C. trachomatis*, but opposite to the late expressed *pmpA* ortholog of *C. abortus* (Wheelhouse *et al*., 2009; Carrasco *et al*., 2011). Transcript levels for *pmpH* stood out in that they were high at early (2-6 hpi), low at mid (12-18 hpi) and high again at late developmental times (24-32 hpi). This feature appears to be unique for *pmpH* of *C. psittaci* as in all other species, *pmpH* is characteristically transcribed late (Wheelhouse *et al*., 2009; Carrasco *et al*., 2011) and suggests PmpH plays an important role at both ends of the *C. psittaci* developmental cycle. It also indicates that *pmpH* transcript is relatively unstable during mid-cycle development. These observations and the additional observation that *pmpH* is co-transcribed with *pmpG2* suggest that the regulation of *pmpH* expression may follow a complex, multi-level mechanism, and may betray a key role of PmpH in overall Pmp function in *C. psittaci*.

Late transcription of most *pmp* genes is in general agreement with the results of *pmp* transcription analyses for *C. abortus* and *C. trachomatis* where all *pmp* genes, with the exception of *pmp5E* for *C. abortus* and *pmpA* and *I* for *C. trachomatis*, were differentially highly expressed during late development (Wheelhouse *et al*., 2009; Carrasco *et al*., 2011). It is also consistent with transcription of the *pmp* gene family in *C. pneumoniae,* where transcripts could be detected for all known *pmp* genes at 72 hpi (Grimwood and Olinger, 2001). Globally, transcription analyses of *pmp* gene families across several *Chlamydia*

species suggests an important role for these proteins either at late stages of development or, upon storage of late-expressed Pmps in EBs during the early steps of infection. The expression level of a large subgroup of *pmp* genes, including *pmpE* and most *pmpG* alleles parallels that observed for their respective *pmp* orthologs in other *Chlamydia* species (Wheelhouse *et al*., 2009; Carrasco *et al*., 2011). Conversely, differences at the species level (e.g. *pmpA* and *pmpH* of *C. psittaci* Cal10 versus orthologs in *C. trachomatis* and *C. abortus*) may reveal functional differences that are intrinsic to the properties of each species.

Multiple types of stress have been shown to affect chlamydial growth and morphology and to down- or up-regulate various chlamydial genes important in virulence (Hogan *et al*., 2004; Mpiga and Ravaoarinoro, 2006; Goellner *et al*., 2006). We used penicillin-induced stress to comparatively investigate *pmp* transcription and Pmp production in *C. psittaci* inclusions, because the expression of *pmpA, pmpD and pmpI* genes of *C. trachomatis*, was uniquely not affected by penicillin-induced stress and therefore they were previously hypothesized to play a critical role in the pathogenesis of *C. trachomatis* (Carrasco *et al*., 2011). In *C. psittaci* Cal10, the expression of all *pmp* genes was affected by penicillin, as it either resulted in a down- or an up-regulated expression level but unaffected expression was not observed. Adding penicillin resulted in a lower transcript level for all *pmp* genes except for *pmpB*, *pmpD*, *pmpG2*, *pmpG4* and *pmpG8,* as their transcript levels were augmented as for *tufA*. This might be an indication for being even more important in the biology of *C. psittaci* and/or it is a (survival) response to stress, by creating antibiotic resistance, like described for the expression of prokaryotic heat shock proteins in the presence of penicillin in other bacteria, like for instance *Streptococcus pneumoniae*. The major heat shock protein and molecular chaperon Clpl of *S. pneumoniae* play a role in increased resistance to penicillin by augmenting the expression and translocation of the penicillin-binding protein PBP2x to the bacterial cell wall, leading to increased resistance to penicillin (Tran *et al*., 2011). In our study, *C. psittaci* Cal10 remained susceptible to penicillin, but less susceptible than *C. trachomatis*, as more and smaller aberrant bodies were observed for *C. psittaci* compared to *C. trachomatis* (data not shown). This indicates that aberrant RBs of *C. psittaci* can divide in the presence of penicillin and therewith suggest that *C. psittaci* is less susceptible to penicillin. This is confirmed by a minimal inhibitory concentration (MIC) of 0.1 μg/ml and 1 μg/ml for penicillin for *C. trachomatis* and *C. psittaci* respectively (Tamura and Manire, 1968; Kuo *et al*., 1977). The possible involvement of PmpB, PmpD, PmpG2, PmpG4 and PmpG8 in mediating penicillin-resistance should further be examined, for example by inducing stress

through iron depletion or IFN-ɣ, where we do not expect up-regulation of the corresponding *pmp* genes.

To assess transcriptional regulation, we examined the putative organization of the *C. psittaci pmp* CDSs in operons. Remarkably, only the *pmpE2-E1* and *pmpG2-H* intergenic regions were amplified. Thus, it seems like most *pmp* genes of *C. psittaci* Cal10 are not cotranscribed. As the expression of all *pmp* genes at late time points was confirmed by RT-qPCR, the negative result is not due to the chosen time points. Our results differ from the ones obtained for *C. trachomatis*, where the intergenic regions of *pmpA-B-C*, *pmpF-E* and *pmpG-H* were cotranscribed indicating their organization in operons. Although *pmpG2* and *pmpH* are organized in an operon, the two genes have a distinct developmental profile at 2 and 6 hpi, which might be the result of a second promoter in addition to the operon promoter. The latter was also suggested for the *pmpABC* operon of *C. trachomatis* (Carrasco *et al*., 2011).

4.2 Protein production profiles differ for different *C. psittaci* Pmp proteins

The expression level of the *pmp* genes in the population is determined by RT-qPCR. However, different variants may be present in the population (e.g. due to SNPs) (Read *et al*., 2000; Jasper *et al*., 2015). To address this issue, we investigated *pmp* expression at the protein level both during normal and during penicillin-induced stress cultures, using immunofluorescence (IF) and immuno-electron microscopy (IEM) to determine Pmp production respectively at the inclusion and at the chlamydial cell levels, in multiple biological replicates. We will first focus on the normal culture condition. The early high-level *pmpA* transcription was matched by high-level production of PmpA during all stages of development in all biological replicates, indicating the relative stability of the *pmpA* mRNA and PmpA protein and suggesting a relative absence of *pmpA* variation at the genomic level in the study population (the *C. psittaci* culture). PmpB, whose transcript level peaked mid-cycle was detectably produced late in the majority of inclusions in all replicates. Production of PmpD was also detected at developmental times generally corresponding to maximum transcription levels, but was undetectable in one of ten biological replicates at 18 and 24 hpi. The latter was also observed for PmpH, whose transcript exhibited discontinuous high-low-high levels during development, was strongly produced late in all replicates, but only in some of the replicates at mid developmental cycle. This may be loosely comparable to the on-off phase-like variation described for Pmps of *C. trachomatis* (Tan *et al*., 2010).

Next, we focus on the penicillin induced-stress condition. Similar to its ortholog in *C. trachomatis* (Carrasco *et al*., 2011), production of PmpA was not significantly affected by penicillin-induced stress. PmpB production was also similarly down-regulated in *C. psittaci* and *C. trachomatis*. PmpD and PmpH productions however, whose orthologs are down-regulated in *C. trachomatis* (Carrasco *et al*., 2011), were unaffected by penicillin in *C. psittaci*. This may again highlight a yet-to-be-determined species-specific function of PmpD and PmpH in *C. psittaci*. It is noteworthy that transcript and protein levels observed for the Pmps during penicillin-induced stress culture conditions were not always concordant. For example, *pmpA* and *pmpH* transcript levels were lower during stress, but the protein levels showed no significant difference (except 12 hpi for PmpA). Similar findings were observed for *C. trachomatis*, in which *pmpF* transcription was shut down during stress, while the same amount of PmpF protein was present during both normal and stress conditions (Carrasco *et al*., 2011). These results implicate the existence of unknown posttranscriptional and posttranslational mechanisms in *Chlamydia* spp. and suggest the occurrence of multiple transcripts and proteins, which can display a different stability under specific conditions.

The infectious EB was previously suggested to determine the on/off status of each Pmp subtype in an inclusion, as the off frequency did not change over developmental times in *C. trachomatis* (except for *pmpG*) (Carrasco *et al*., 2011). However, the off frequency of *C. psittaci* Pmps did change over time, as was the case for *C. abortus* (Wheelhouse *et al*., 2012b). Furthermore, 10 biological replicates were analyzed in this study and the percentage of PmpD and PmpH positive inclusions ranged from 0% to 100% in some replicates. This is reminiscent of PmpG production in *C. trachomatis* cultures, where the percentage of PmpG-positive inclusions varied between 90 and 99% between experiments (Tan *et al*., 2010). In contrast, the observed high rate of variation of PmpD production at the inclusion level at two developmental times is entirely discordant from that observed for PmpD of *C. trachomatis*, which was the least frequently "off" Pmp subtype in this species (Tan *et al*., 2010). It is unlikely that individual replicates were infected with EBs with PmpD or PmpH in either on- or off- status, as all replicates were infected with the same seed. These results suggest that it is not the incoming EB that determines the on/off status of Pmp production in an inclusion in *C. psittaci*. We postulate that another mechanism determines the on/off status of Pmps and we suggest a mechanism which is both tightly regulated and involves variable factors. For example, frame shifts, SNPs and reversible recombination of *pmp* gene fragments from

transcriptionally silent CDSs into expressed *pmp* genes, might explain the variation between biological replicates.

4.3 PmpA, PmpD and PmpH target the chlamydial cell envelope

IEM was used to assess the subcellular localization of the Pmps. PmpA, D and H localized at the *C. psittaci* cell envelope. This is consistent with the observed surface localization for all nine Pmps of *C. trachomatis* (Crane *et al*., 2006; Swanson *et al*., 2009; Tan *et al*., 2010), Pmp6, 8, 10, 11, 18 and 21 of *C. pneumoniae* (orthologs of PmpGs [Pmp6, 8, 10 and 11], PmpE and PmpD of *C. trachomatis*, respectively) (Vandahl *et al*., 2002; Wehrl *et al*., 2004) and the 90kDa Pmp (ortholog of PmpG of *C. trachomatis*) of *C. abortus* (Longbottom *et al*., 1998a). However, there have not been any thorough investigations to distinguish inner and outer membrane labeling. The IEM images suggested labeling of PmpA, D and H at the inner and outer membrane, but better resolution images are needed to provide conclusive evidence. Remarkably, we observed more abundant antibody labeling of PmpA and H at the chlamydial cell envelope compared to MOMP, the major surface-accessible protein in all *Chlamydia* species. Although this observation may owe to differential properties of the antibodies or the influence of the post-embedding immunolabeling techniques used in our analysis, it may also relate to the unusual antigenicity and immuno-accessibility of these 2 proteins in *C. psittaci*. Similar abundant Pmp labeling at the cell envelope has not been observed for *C. trachomatis* and *C. pneumoniae*, which are phylogenetic relatively distant from *C. psittaci* (Sachse *et al*., 2015). However, the 90kDa Pmp of *C. abortus*, a close phylogenetic relative of *C. psittaci*, was also abundantly labeled at the chlamydial surface (Longbottom *et al*., 1998a). We also detected PmpA-, D- and H-specific immunogold-labeling of small vesicles within the inclusion and of the inclusion membrane itself, which was not observed before for other *Chlamydia* species. We speculate that the observed vesicles may have pinched-off from the outer membranes of RBs and fused to the inclusion membrane. Similarly, Vanrompay *et al*., (1996) observed the small vesicles within the inclusion of four different *C. psittaci* strains and suggested that the fusion of these vesicles with the inclusion membrane could account for its labeling by the polyclonal antibodies used in their study. Similar results were also reported by Taraska et al., (1996), who suggested that the expansion of the inclusion membrane of *C. trachomatis* and *C. psittaci* is driven by fusion with bacterium-derived material. Comparable shedding of vesicular material and the contiguous localization of these vesicles with the vacuolar membrane has also been observed for intracellular protozoan parasites (Sibley *et al*.,

1986; Speer and Whitmire, 1989). More thorough IEM analyses are needed to confirm whether inclusion membrane and small vesicle labeling is unique for *C. psittaci* Pmps and if the remaining *C. psittaci* Pmps are also found at those membranes. Further research is needed to elucidate the function of Pmps present at those membranes.

In conclusion, *C. psittaci* Cal10 has 17 *pmp* genes. Our experiments confirmed that variation is a central requisite of the *C. psittaci* Pmp family as gene transcription profiles and protein expression profiles differed along development and in different biological replicates. We detected PmpA-, D- and H- specific immunogold-labeling of the inclusion membrane and of small vesicles within the inclusion membrane. In addition, the IEM images suggested PmpA-, D- and H- specific immunogold-labeling of the inner and outer membrane of *Chlamydia* particles. PmpA and PmpH of *C. psittaci*, by virtue of their unique expression properties emerge as important players in pathogenesis and their apparent immunoaccessibility/antigenicity suggest their potential in vaccine design.

Acknowledgement

W. De Vos is acknowledged for assisting with confocal laser scanning microscopy. R. Rank and A. Bowlin (Department of Medicine, University of Arkansas for Medical Sciences, USA) are acknowledged for the production of the polyclonal antibodies (Animal maintenance and experimental treatments were conducted in accordance with the ethical guidelines for animal research established and approved by the institutional Animal Care and Use Committee at University of Arkansas for Medical Sciences). P. Bavoil (Department of Microbial Pathogenesis, UMaryland, USA) is acknowledged for his support during Sarah's stay of 8 months in his laboratory and his continuing collaboration during the rest of the experiments of this chapter. R. Hsia (Director of the Core imaging Facility, UMaryland, USA) is acknowledged for the immuno-electron microscopy imaging. H. Shou, J.A. Carrasco, P.X. Marques, V. Grinblat-Huse (Department of Microbial pathogenesis, UMaryland, USA) are acknowledged for their guidance.

Chapter V

Identification of the genetically most conserved polymorphic membrane proteins A, B, D and H in the *Chlamydia psittaci* outer membrane complex

---

Abstract

Chlamydiaceae are very successful pathogens and up to now no vaccine is available for none of the *Chlamydia* species. As the infections lead to significant economic losses in animal production, the need for an effective vaccine is high. Immunity to *Chlamydia* infections is mainly mediated by cellular immune responses. In general, the proteins present in the *Chlamydia* outer membrane complex are expected to be important for vaccine design. The composition of the chlamydial outer membrane complex was previously only determined for *C. trachomatis*, *C. abortus* and *C. pneumoniae*. We therefore determined whether PmpA, PmpB, PmpD and PmpH proteins of *C. psittaci* are present in the *C. psittaci* outer membrane complex by immuno-electron microscopy. Those four Pmp proteins are the most conserved Pmp proteins of *C. psittaci* and we therefore hypothesize that they are essential for the pathogenesis of *C. psittaci*. For all analyzed Pmp proteins specific labeling was observed at the *C. psittaci* outer membrane complex.

1. Introduction

Chlamydia psittaci causes respiratory disease in birds. Birds show symptoms such as conjunctivitis, pneumonia and rhinitis (Vanrompay *et al.*, 1997). The pathogen has a unique biphasic developmental cycle. The infectious elementary body (EB) attaches to the host cell and after the subsequent endocytosis, mainly at clathrin-coated pits, it differentiates to the metabolic active reticulate body (RB) within an inclusion, which is derived from the host cell membrane during the internalization (Vanrompay *et al.*, 1996; Harkinezhad *et al.*, 2009). Infections lead to significant economic losses in the duck and turkey industry due to mortality, reduced egg production and the expense of antibiotic treatment (Vanrompay *et al.*, 1997). Nowadays, *C. psittaci* is also emerging on chicken farms (Dickx *et al.*, 2010; Dickx and Vanrompay, 2011; Yin *et al.*, 2013). An efficacious vaccine is needed to control *C. psittaci* infections. An ideal vaccine should generate long-lasting and sterilizing immunity while avoiding immunopathology. Previous studies showed that vaccines for intracellular bacteria, such as *Chlamydia*, require cell-mediated immune responses (Seder and Hill, 2000). $CD4^+$ T cells are essential to resolve a chlamydial infection (Ramsey and Rank, 1991; Su and Caldwell, 1995; Morrison *et al.*, 2000), while $CD8^+$ T cells and B cells are not necessary to clear a primary infection (Ramsey *et al.*, 1988; Su and Caldwell, 1995; Su *et al.*, 1997), however, they can contribute to clear the infection by the release of gamma interferon (Igietseme *et al.*, 1994) and they play an important role in resistance to chlamydial reinfection (Su *et al.*, 1997; Morrison *et al.*, 2000; Rank and Whittum-Hudson, 2010), respectively.

The polymorphic membrane protein (Pmp) family is the largest membrane protein family in *Chlamydia* species and it is a unique feature of the genus (Horn *et al.*, 2004; Vandahl *et al.*, 2004). The Pmps have been studied intensively because of their relatively high proportion of the coding capacity (3.15 and 5.1% in *C. trachomatis* and *C. pneumoniae*, respectively) and the presence of the protein family in all currently sequenced chlamydial genomes (Grimwood and Stephens, 1999). Some Pmp proteins are highly immunogenic (Caldwell *et al.*, 1975a,b; Kuo *et al.*, 1977; Longbottom *et al.*, 1996; Livingstone *et al.*, 2005; Tan *et al.*, 2010; Marques *et al.*, 2010), all Pmps of *C. trachomatis* are proposed to function as adhesins (Becker and Hegemann, 2014) and anti-PmpD antibodies can partially neutralize a *C. trachomatis* infection (Crane *et al.*, 2006). Therefore, the Pmps are hypothesized to be vaccine candidates (Crane *et al.*, 2006; Mölleken *et al.*, 2010; Carrasco *et al.*, 2011).

Karunakaran *et al*. (2008) identified four Pmp proteins (PmpE, PmpF, PmpG and PmpH) in *C. muridarum*, which is phylogenetically highly related to *C. trachomatis* (Read *et al*., 2000), as $CD4^+$ T cell vaccine candidates by an immunoproteomic approach. Briefly, bone marrow-derived dendritic cells were infected with *C. muridarum*, subsequently MHC–II bound peptides were purified by affinity chromatography using monoclonal antibodies specific to mouse B cells and those peptides were identified by mass spectrometry. Those MHC-II peptides were recognized by $CD4^+$ T cells harvested from immune mice and transfer of dendritic cells pulsed *ex vivo* with those peptides partially protected mice against a chlamydial infection. Yu *et al*. (2014) compared the single antigen (PmpE, PmpF, PmpG and PmpH) vaccines to multisubunit vaccines (PmpE, PmpF, PmpG, PmpH with or without the major outer membrane protein, MOMP). The multisubunit vaccines were more immunogenic and cleared the infection faster than the single antigen vaccines (Yu *et al*., 2014). Remarkably, those four Pmp proteins are overrepresented in the *C. trachomatis* outer membrane complex (COMC), purified from EBs, compared to the Sarkosyl soluble fraction (Liu *et al*., 2010), while PmpD and PmpI were overrepresented in the soluble fraction and PmpA was not detected in the experiment. The absence of PmpA was explained by reports that showed the RB specificity of this protein (Skipp *et al*., 2005; Saka *et al*., 2011) and by the finding that it is not an outer membrane protein in *C. trachomatis* (Grimwood and Stephens, 1999). PmpD and PmpI were also shown to be RB specific proteins by Saka *et al*. (2011). In addition, Tan *et al*. (1990) showed that the *C. abortus* outer membrane complex conferred protective immunity when used in an experimental vaccine. Overall these results suggest that proteins present in the chlamydial outer membrane complex might be potential vaccine candidates. Up to now, the chlamydial outer membrane complex was only studied for *C. trachomatis* (Mygind *et al*., 2000; Birkelund *et al*., 2009; Liu *et al*., 2010), *C. pneumoniae* (Knudsen *et al*., 1999) and *C. abortus* (Tan *et al*., 1990; Cevenini *et al*., 1991; McCafferty *et al*., 1995; Longbottom *et al*., 1996). In this study the presence or absence of PmpA, PmpB, PmpD and PmpH in the *Chlamydia psittaci* outer membrane complex was analyzed by immuno-electron microscopy. Those are the four most conserved Pmp proteins of *C. psittaci* and therefore we hypothesize that they are essential for the pathogenesis of *C. psittaci*.

2. Material and methods

2.1 *Chlamydia psittaci* strain and growth curve

The previously well-characterized, prototypic *C. psittaci* strain Cal10 (Narita *et al.*, 1976; Matsumoto, 1982; Hovis *et al.*, 2013; Mojica *et al.*, 2015), was used in this study. The strain was isolated from ferrets inoculated with throat washings from humans with an influenza-like respiratory infection (Francis and Magill, 1938). The bacterium was grown in HeLa 229 cells, the first human cell line established in culture, starting from human cervical carcinoma cells (Gey *et al.*, 1952). HeLa 229 cells were seeded on a sterile glass coverslip (13mm) at the bottom of Chlamydia Trac Bottles (Bibby Sterilin Ltd., Stone, UK) and grown for 24h at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Merelbeke, Belgium) supplemented with 10% heat inactivated fetal bovine serum (Greiner Bio One, Wemmel, Belgium), gentamycin (25 μg ml^{-1}; Invitrogen) and fungizone (1.25 μg ml^{-1}; Invitrogen). The medium was aspirated and cells were inoculated with *C. psittaci* Cal10 in SPG at an MOI of 1 followed by a centrifugation of 3000 rpm for 30 min at 37°C. The inoculum was removed, the cells were washed with PBS and DMEM was added, which was marked time 0 hpi of the experiment. At 2, 8, 15, 24, 32, 38, 48, 54 and 60 hours post infection (hpi) SPG was added to the Chlamydia Trac Bottle, which was subsequently stored at -80°C. Titration of the EBs present in the supernatant at each time point was performed on HeLa 229 cells by the method of Spearman and Kaerber (Mayr *et al.*, 1974) determining the tissue culture infective dose 50 ($TCID_{50}$) per ml.

2.2 *Chlamydia psittaci* mass production and purification

HeLa 229 cells were seeded in 25 cm^2 culture dishes and incubated for 24h at 37°C with 5% CO_2 in DMEM (Invitrogen) supplemented with 10% heat inactivated fetal bovine serum (Greiner Bio One), gentamycin (25 μg ml^{-1}; Invitrogen) and fungizone (1.25 μg ml^{-1}; Invitrogen). The medium was aspirated and the monolayer was inoculated with *C. psittaci* Cal10 in SPG (0.25 M sucrose, 10 mM sodium phosphate and 5mM L-glutamic acid) at a multiplicity of infection (MOI) of 1 followed by incubation on a rocking platform for 2 h at 37°C. The unbound organisms were washed away with PBS and the above-mentioned medium was added to each tissue culture flask. Elementary bodies were harvested at the developmental time at which most EBs were determined (growth curve result, see paragraphs 2.1 and 3.1) and purified by discontinuous gradient centrifugation following standard

protocols (Caldwell *et al*., 1981), with minor modifications. Briefly, renografin was replaced by Omnipaque 350 (GE Healthcare, Princeton, New Jersey) supplemented with NaCl 160 mM. The gradients were prepared by diluting Omnipaque 350-160 mM NaCl in SPG buffer, such that final concentrations of Omnipaque 350 were 30%, 40%, 44% and 54%. Titration of the EBs was performed on HeLa 229 cells by the method of Spearman and Kaerber (Mayr *et al*., 1974).

2.3 *Chlamydia psittaci* whole cell lysate and isolation of *C. psittaci* outer membrane complex

Two protocols to isolate the *Chlamydia psittaci* outer membrane complex (COMC) were compared. Envelope proteins constitute only 4-16% of the proteome. So a clean extract is expected to produce a distinctive band pattern with very little overlap with that generated by a whole cell extraction (Quan *et al*., 2013). The whole cell extract was prepared by the protocol described by Marques *et al*. (2010) with minor modifications. In short, 2 x 10^9 EBs were centrifuged (16 000 x *g* for 30 min at 4°C), the pellet was washed twice by Tris-EDTA (TE; 10mM Tris-HCl, 1 mM EDTA, pH 8.0) buffer and afterwards resuspended by lysis buffer (7 M urea [Sigma-Aldrich, Bornem, Belgium], 2 M thiourea (Sigma-Aldrich), 1% ASB-14 (Sigma-Aldrich) and protease inhibitor (Roche Diagnostics, Mannheim, Germany)). The sample was incubated at room temperature for 30 min and centrifuged (16 000 x *g* for 30 min at 4°C) to remove insoluble material. The two COMC isolation procedures are the Sarkosyl method (Caldwell *et al*., 1981) and the Tris-sucrose-EDTA method (Quan *et al*., 2013). In brief, during the first method 2 x 10^9 EBs were resuspended in Sarkosyl buffer (2% sodium lauryl sarkosinate (Sarkosyl; Sigma-Aldrich), 1.5 mM EDTA, PBS, pH 8). The samples were sonicated for 2 min and incubated at 37°C for 1 h. The Sarkosyl insoluble fraction was pelleted by centrifugation (16 000 x *g* for 30 min) and the supernatant was collected as the Sarkosyl soluble fraction. The procedure was repeated one more time and the two soluble and insoluble fractions were pooled. The resulting insoluble pellet was washed twice with PBS to remove the detergent and was resuspended in resuspension buffer (0.02 mM sodium phosphate, 10 mM magnesium chloride, 25 μg deoxyribonuclease I [Sigma-Aldrich] and 25 μg ribonuclease [Sigma-Aldrich]) and incubated for 2 h at 37°C. Pellets from these digestions (16 000 *g* for 10 min) were sonicated (10 x 1 min, with intermittent cooling of the samples on ice) and washed with PBS to remove the nucleases (16 000 *g* for 10 min). The pellet, containing the COMC, was resuspended in digestion buffer (50 mM ammonium bicarbonate,

8 M urea, protease inhibitor). During the second method resuspended 2 x 10^9 EBs in Tris-sucrose-EDTA buffer (TSE; 200 mM Tris-HCl, pH 8.0, 500 mM sucrose, 1 mM EDTA, and add protease inhibitor before use). The samples were incubated on ice for 30 min and centrifuged (16 000 x *g* for 30 min at 4°C). The supernatant contained periplasmic and outer membrane proteins, while the pellet contained the inner membrane and cytoplasmic proteins. The fractions were analyzed by SDS-PAGE (4.5 μg protein was loaded of each sample).

2.4 Immuno-electron microscopy

COMC pellets were fixed in 4% paraformaldehyde. Cells were washed, pelleted, and embedded in 2.5% low-melting-temperature agarose. Agarose blocks were trimmed into ~1mm^3 size, washed, dehydrated, infiltrated, and embedded in unicryl at -20°C under UV from 24 to 48 h. Ultrathin sections were cut on a Leica UC6 ultramicrotome (Leica Microsystems, Inc., Bannockburn, IL) and collected onto formvar-coated Nickel grids. Immunogold labeling was performed using the guinea pig anti-PmpA, B, D and H specific polyclonal antibodies (pAbs) (generated by immunization of guinea pigs with the purified recombinant proteins) followed by a secondary gold conjugated goat anti-guinea pig IgG (H&L) antibody (Electron Microscopy Sciences, Hatfield, PA). Sections were also stained using a rabbit anti-MOMP-specific serum followed by a secondary gold conjugated goat anti-rabbit IgG (H&L) antibody (Electron Microscopy Sciences). Images were acquired using a Tecnai T12 transmission electron microscope (FEI, Hillsboro, OR) at 80 keV and an AMT digital camera (Advanced Microscopy Techniques, Woburn, MA).

3. Results

3.1 Choice of time point and isolation method for *Chlamydia psittaci* outer membrane complex analysis

C. psittaci grows asynchronically, so a high amount of EBs is difficult to obtain when using discontinuous gradient centrifugation as such, without determining the optimal cell culture harvest time point. Therefore, the growth curve of *C. psittaci* Cal10 was first determined. Enrichment of EBs was successfully achieved by harvesting *C. psittaci* Cal10 at 54 hpi (Figure 5.1) and subsequent discontinuous gradient centrifugation of the cell culture harvest.

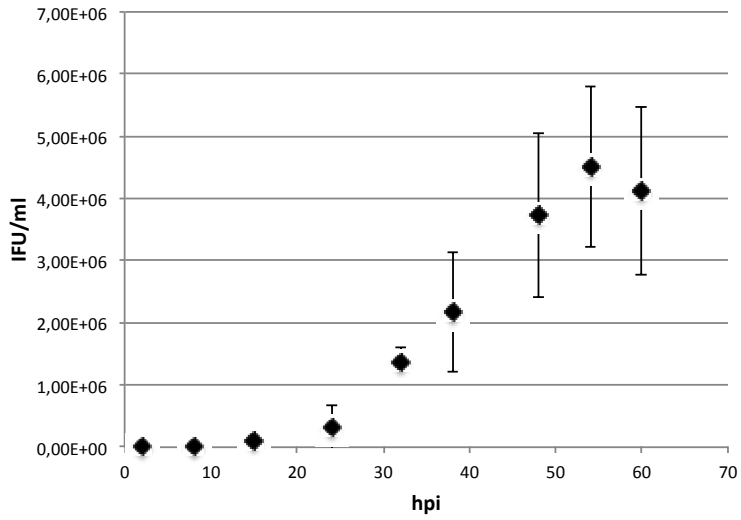


Figure 5.1: Growth curve of *C. psittaci* Cal10. Titration of *C. psittaci* Cal10 grown in HeLa cells was performed at 2, 8, 15, 24, 32, 38, 48, 54 and 60 hpi. Errors are based on standard deviation of the mean.

Two different outer membrane complex extraction methods for Gram-negative bacteria were compared. The method using the detergent Sarkosyl was best to isolate the *C. psittaci* outer membrane complex (Figure 5.2).

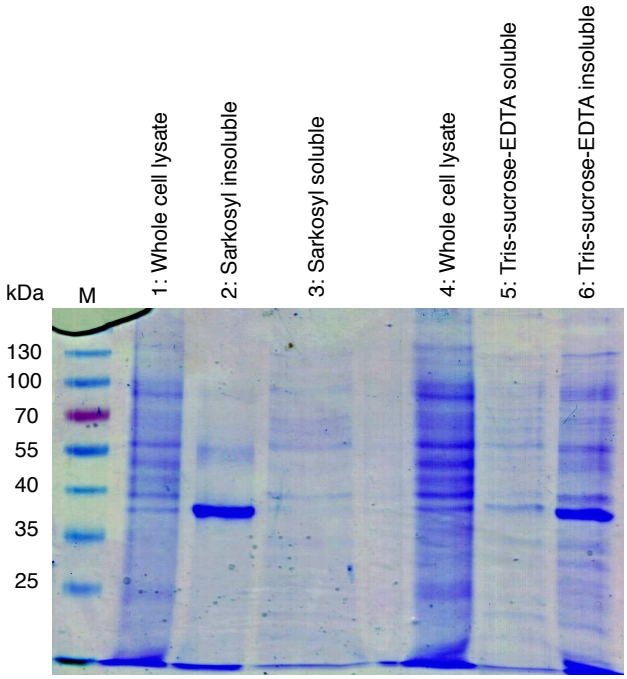


Figure 5.2: Isolation of the *C. psittaci* outer membrane complex (COMC) by two methods using either Sarkosyl detergent or Tris-sucrose-EDTA. The protein profile observed in lane 5 (encompassing the COMC) resembled the profile of the whole cell lysate (lanes 1 and 4), while the protein profile in lane 2 (encompassing the COMC) clearly differed from the whole cell lysate. Three predominant protein bands were observed in the Sarkosyl insoluble lane (~ 38 kDa, ~55 kDa and ~ 90 kDa). The use of Sarkosyl was optimal to extract the *C. psittaci* outer membrane complex.

The SDS-PAGE gel (Figure 5.2) showed a distinctive profile of the outer membrane fraction compared to the whole cell lysate, but the presence of the outer membrane fraction could also successfully be verified by IEM (Figure 5.3).

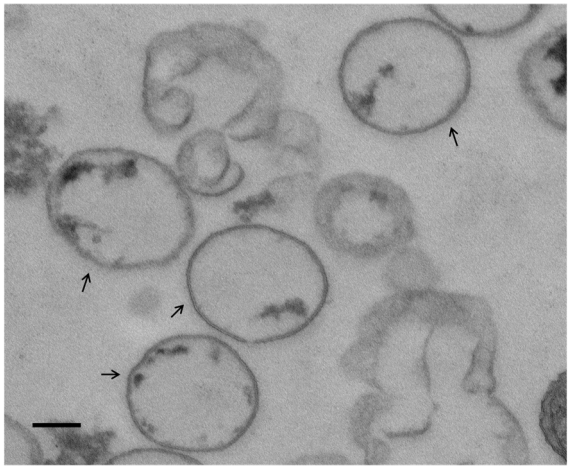


Figure 5.3: The extraction of the *C. psittaci* outer membrane complex (COMC), starting from enriched *C. psittaci* Cal10 EBs and visualized by IEM. Arrows point to the *C. psittaci* outer membrane complexes. Bar = 0.5 μm.

3.2 PmpA, PmpB, PmpD and PmpH localize to the *Chlamydia psittaci* outer membrane complex

The localization of PmpA, PmpB, PmpD and PmpH in the *C. psittaci* outer membrane complex (COMC) was analyzed by IEM (Figure 5.4). PmpA localized most abundantly at the COMC (Figure 5.4 A), while PmpH was present at a lower abundance than PmpA. However, PmpH was observed in all COMCs (Figure 5.4 B), which is different from the other three analyzed proteins, which are only present in a few COMCs.

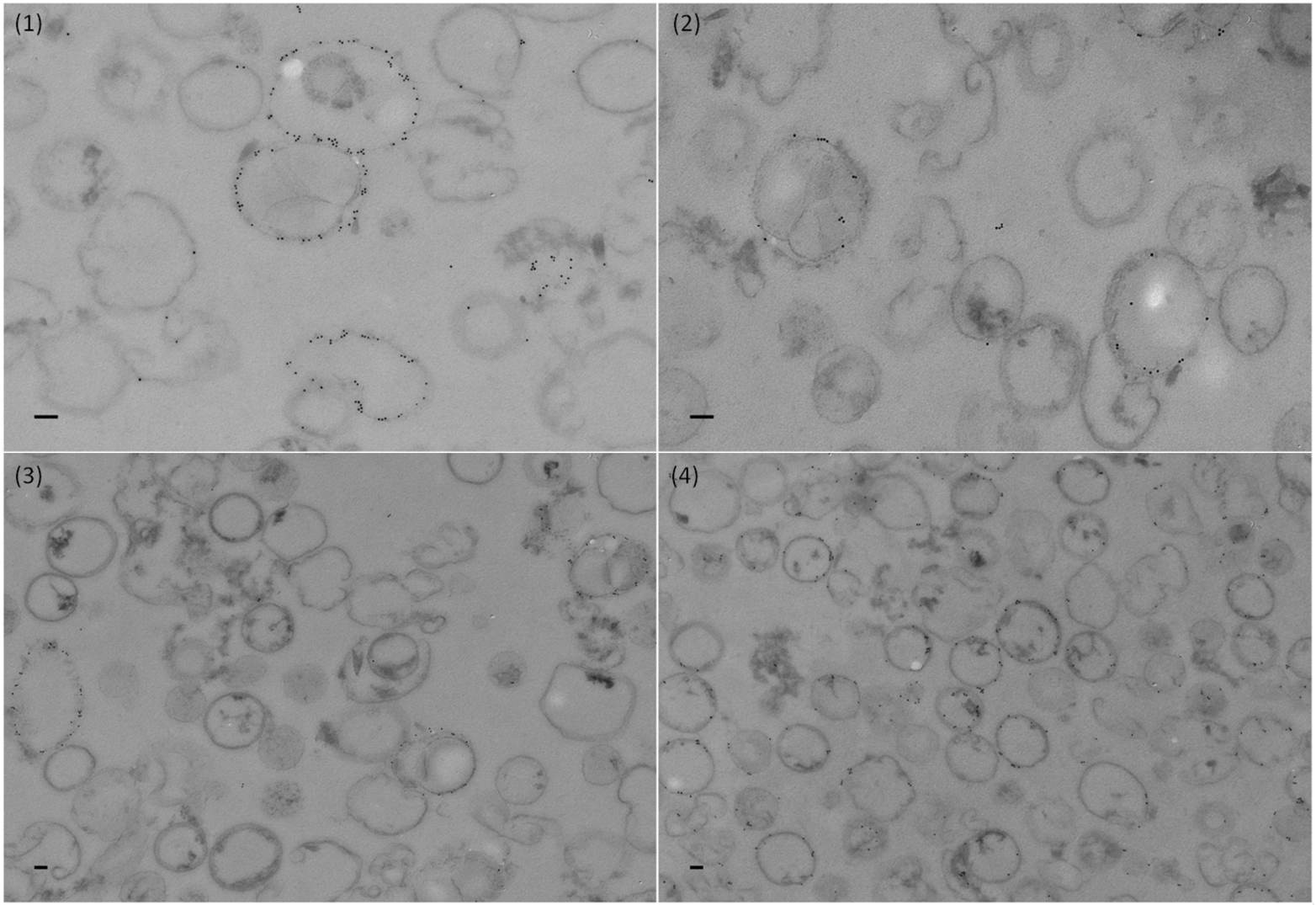


Figure 5.4: PmpA (1), PmpB (2), PmpD (3) and PmpH (4) localize to the *C. psittaci* Cal10 outer membrane complex. Bars = 0.1 μm.

4. Discussion

Chlamydiaceae are very successful intracellular pathogens and up to now, no vaccine is available for none of the different *Chlamydia* species. Liu *et al*. (2010) suggested previously that antibodies to COMC might neutralize *Chlamydia* infections. This was confirmed by vaccination of pregnant ewes by *C. abortus* outer membrane complex, which conferred protective immunity (Tan *et al*., 1990). Yu *et al*. (2014) showed that a multisubunit vaccine comprising the major outer membrane protein and PmpE, PmpF, PmpG and PmpH, which were previously detected in the COMC of *C. trachomatis* (Liu *et al*., 2010), conferred better protection than the single protein antigens. Therefore, knowing the composition of the COMC is relevant for vaccine design. As variation is a central trait of the Pmp family, it was hypothesized that the Pmp proteins might play a role in the observed host and tissue tropism among *Chlamydia* species, which was confirmed by Becker *et al*. (2014). If the proteins present in the COMC determine the host or tissue to which the *Chlamydia* species might adhere and subsequently infect, we expect that the COMC composition would differ between *Chlamydia* species. The composition of the *C. psittaci* outer membrane complex was not determined before, and we hypothesize that other Pmp proteins might be present in the COMC of *C. psittaci*.

In a first step, we compared two different COMC extraction methods to isolate the *C. psittaci* outer membrane complex. Quan *et al*. (2013) compared three different outer membrane extraction methods for Gram-negative bacteria and reported that the Tris–sucrose–EDTA method produced the cleanest extract of periplasmic and outer membrane proteins from *Escherichia coli*. However, we were unable to enrich the outer membrane proteins of *C. psittaci* by the method of Quan *et al*. (2013), as the SDS-PAGE protein profile looked similar to the one of the whole cell lysate of the *C. psittaci* Cal10 strain. The cell wall of *Chlamydiaceae* is different compared to other Gram-negative bacteria, as the peptidoglycan synthesis is minimal early and late in the developmental cycle and it peaks at 18 hpi (Packiam *et al*., 2015). The low amount of peptidoglycan present in the EBs harvested at 54 hpi might contribute to failure of the Tris-sucrose-EDTA method. In contrast, the SDS-PAGE protein pattern obtained by using the Sarkosyl method, was similar to the ones obtained during previous COMC purifications of *C. trachomatis* and *C. abortus* (Caldwell *et al*., 1981; McCafferty *et al*., 1995). Three predominant protein bands were observed, which were previously identified as the major outer membrane protein (MOMP; ~ 38 kDa), the outer

membrane protein 2 (Omp2; ~ 55 kDa) and the polymorphic membrane proteins (Pmps; 90 kDa) (McCafferty *et al*., 1995; Mygind *et al*., 2000; Liu *et al*., 2010). The *C. psittaci* COMC morphology observed in our study by IEM was indistinguishable from the electron microscopic images of the *C. trachomatis* outer membrane complex demonstrated by other researchers (Caldwell *et al*., 1981; Liu *et al*., 2010).

In this study we observed immunogold labeling of PmpA, PmpB, PmpD and PmpH in the *C. psittaci* outer membrane complex. Birkelund *et al*. (2009), detected all Pmps except PmpA and PmpI in the *C. trachomatis* outer membrane complex, while Liu *et al*. (2010) observed all Pmps except PmpA, PmpD and PmpI. Tanzer *et al*. (2001) detected PmpE, PmpG and PmpH and Mygind *et al*. (2000) detected only PmpG and H in the *C. trachomatis* L2 outer membrane complex. Different COMC extraction and analyses methods might explain these differences. Mygind *et al*. (2000), Birkelund *et al*. (2009) and Liu *et al*. (2010) extracted the COMC by the previously described Sarkosyl method, while Tanzer *et al*. (2001), labeled the outer membrane proteins with a photoactivatable lipophilic reagent after which the Sarkosyl extraction method was applied. Tanzer *et al*. (2001) subsequently separated the COMC by SDS-PAGE and protein bands were visualized on the dried gel by phosphorimaging. Mygind *et al*. (2000) stained the SDS-PAGE gel by silver staining. Birkelund *et al*. (2009) applied a combined fractional diagonal chromatography to the COMC, as described by Gevaert *et al*. (2003). In a final step, they all identified the separated proteins by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, while Liu *et al*. (2010) immediately determined the composition of the COMC by liquid chromatography-tandem mass spectrometry without first separating the proteins. Despite the differences, PmpG and PmpH were observed in all studies that examined the *C. trachomatis* outer membrane complex.

Few studies examined the localization of the Pmps in other *Chlamydia* species. In *C. abortus* three 90 kDa Pmp proteins were identified in the COMC (Cevenini *et al*., 1991; McCafferty *et al*., 1995; Longbottom *et al*., 1996), which are orthologs of PmpG of *C. trachomatis*. Knudsen *et al*. (1999) detected Omp4 and Omp5, also referred to as Pmp10 and Pmp11 (both orthologs of PmpG of *C. trachomatis*) in the *C. pneumoniae* outer membrane complex. The labeling of PmpA in the *C. psittaci* outer membrane complex is unique as it was not observed in the above-mentioned studies on other *Chlamydia* species. This might suggest an important function for PmpA in the pathogenesis of *C. psittaci*, as it is present on the surface of *C.*

psittaci EBs while it is RB specific in *C. trachomatis*. It is also remarkable that PmpG was detected in the COMC of *C. trachomatis*, *C. pneumoniae* and *C. abortus*. The presence of PmpG in *C. psittaci* COMC could not be confirmed in this study, as specific pAb for *C. psittaci* PmpG were not available. It might be possible that PmpG has a redundant function in all *Chlamydia* species and that other Pmps have a more species-specific function such as antigenic variation or adhesion to a specific tissue or host. However, the COMC composition of multiple *C. psittaci* strains, including avian strains, should be determined.

Acknowledgement

R. Hsia (Director of the Core imaging Facility, UMaryland, USA) is acknowledged for the (immuno)-electron microscopy imaging.

Chapter VI

General discussion and perspectives

General discussion and perspectives

Members of the family of the *Chlamydiaceae* are important pathogens of both animals and humans. Abortion, carcass condemnation, bad semen quality and reduced egg production are only some of the sequelae of animal chlamydial infections, which lead to significant economic losses (Vanrompay *et al*., 1997; Kerr *et al*., 2005; Longbottom and Livingstone, 2006). Chlamydial infections in animals are currently mainly treated by tetracycline and its derivatives (chlortetracycline, oxytetracycline, doxycycline), because it is a cheap, broad spectrum antibiotic with an excellent tissue distribution and low toxicity, which easily resolves the infection (Sandoz and Rockey, 2011). However, the extensive use of the antibiotic also for other bacterial infections in animals, both as therapy and in the past also as a prophylaxis, led to the fast spread of tetracycline resistant Gram-positive and Gram-negative bacteria (Michalova *et al*., 2004). Tetracycline-resistant *C. suis* isolates have been detected (Dugan *et al*., 2004). The potential transfer of the tetracycline resistence, *tet(C)*, gene to other *Chlamydia* species highlights the need for an effective vaccine.

As both humoral and cellular immune responses are essential for an efficient and long-lasting immunity, the ideal vaccin should contain both B- and T-cell epitopes (Su and Caldwell, 1992; Morrison *et al*., 2000). For decades, MOMP was the focus of vaccination studies, as the protein accounts for 60% of the protein content of the EB envelope (Caldwell *et al*., 1981), it is highly immunogenic (Caldwell *et al*., 1981; Anderson *et al*., 1990; Miettinen *et al*., 1990; Pal *et al*., 2005; Marques *et al*., 2010), anti-MOMP antibodies neutralized the infection *in vitro* and *in vivo* (Caldwell and Perry, 1982; Zhang, Stewart, *et al*., 1987; Zhang *et al*., 1989) and it has both B- and T- cell epitopes (Su and Caldwell, 1992; Batteiger, 1996), which made this protein a very promising vaccine candidate. However, none of the studies, with either DNA-vaccines (Pal *et al*., 1999), recombinant protein (Tuffrey *et al*., 1992; Shaw *et al*., 2002), synthetic peptides corresponding to B- and T-cell epitopes (Su *et al*., 1995) or even native protein (Pal *et al*., 2005; Kari *et al*., 2009), resulted in a complete protection against one or more *Chlamydia* species. Complete protection might not be feasible. However, a significant reduction in the excretion of *Chlamydia* particles might already reduce the infection pressure, which might consequently result in a lower chance that the population get infected. Therefore, the use of additional

candidate vaccine antigens needs to be explored to increase the protection level. The Pmp family has been suggested as vaccine candidate, based on their surface localization (Longbottom *et al*., 1998a; Knudsen *et al*., 1999; Vandahl *et al*., 2002; Wehrl *et al*., 2004; Tan *et al*., 2010; Mölleken *et al*., 2010), immunogenicity (Caldwell and Kuo, 1977; Longbottom *et al*., 1998b; Knudsen *et al*., 1999; Bunk *et al*., 2008; Marques *et al*., 2010; Forsbach-Birk *et al*., 2013), function as adhesins (Mölleken *et al*., 2010; Becker and Hegemann, 2014) and because neutralizing anti-Pmp antibodies were observed (Wehrl *et al*., 2004; Crane *et al*., 2006; Mölleken *et al*., 2010). However, up to now only some Pmp proteins of *C. pneumoniae* and all Pmps of *C. trachomatis* are confirmed to be adhesins, but the Pmp family is subdivided in different subtypes and it is currently unknown whether all subtypes are involved in the same function, whether Pmps of the same subtype have similar or different functions across *Chlamydia* species, whether all Pmps are surface localized in all *Chlamydiaceae*,…

Previously, researchers focused mainly on PmpD. This can be attributed to its high level of conservation (Gomes *et al*., 2006; Carrasco *et al*., 2011) and immunogenicity (Caldwell *et al*., 1975a,b; Caldwell and Kuo, 1977; Crane *et al*., 2006; Tan *et al*., 2009) for *C. trachomatis*. However, although the *Chlamydiaceae* share a unique biphasic developmental cycle, different species manipulate different hosts and tissues which manifest in different symptoms, indicating diverse infection strategies. Hence, results of one species cannot always be extrapolated. Based on the variation observed in the Pmp family, we hypothesized at the beginning of this doctoral thesis that host and tissue preferences of different *Chlamydia* species might be mediated through the Pmp proteins, which was recently confirmed by Becker *et al*. (2014). Pre-incubation of host cells with *C. pneumoniae* Pmp proteins did protect against a subsequent *C. pneumoniae* infection but not against a subsequent *C. trachomatis* infection and *vice versa*, which indicate that Pmp proteins of different *Chlamydia* species use different receptors (Becker and Hegemann, 2014). Consequently, we suggested that if different Pmp proteins might be responsible for the adherence to different hosts and tissues, the most conserved Pmp protein might differ within and among *Chlamydia* species. Therefore, we determined the level of conservation of all Pmp proteins both within and across four *Chlamydia* species in chapter II. As expected, different Pmp proteins were most conserved in the different *Chlamydia* species. PmpD was the most

conserved Pmp protein only in *C. trachomatis* and it was not the most conserved Pmp protein across all analyzed *Chlamydia* species. This confirmed our expectation, Pmp analyses should not be restricted to PmpD analyses. In addition, it also supports our hypothesis that the most conserved Pmp protein might be important for adhesion, as PmpD showed the strongest adhesion capacity of all *C. trachomatis* Pmp proteins (Becker and Hegemann, 2014). PmpA was an exception, as it is the only Pmp protein that was the most conserved Pmp protein in more than one species, namely in both *C. psittaci* and *C. pneumoniae*. Our observation that PmpA is present in the *C. psittaci* outer membrane complex (chapter V) supports our hypothesis that the most conserved Pmp protein, PmpA in *C. psittaci*, might be involved in adhesion. However, PmpA of *C. pneumoniae* did not mediate adhesion *in vitro* (Mölleken *et al*., 2010), anti-PmpA antibodies did not react with any EB protein (Vandahl *et al*., 2002) and PmpA was not observed in the *C. pneumoniae* outer membrane complex (Knudsen *et al*., 1999). These results suggest that PmpA is involved in another, essential function than adhesion in *C. pneumoniae*. However, as transcript and protein levels of Pmp proteins in *C. pneumoniae* have only been tested at 72 hpi (Grimwood and Olinger, 2001) and not along the developmental cycle, we cannot suggest what the function of PmpA might be in *C. pneumoniae*. This further highlights the need to molecularly characterize all the Pmp proteins of each *Chlamydia* species along the developmental cycle, as the latter will give us insight in the function of the Pmp proteins in different *Chlamydia* species. This is also why we sequenced the genomes of all *C. psittaci* genotype reference strains (Van Lent *et al*., 2012).

A first step to gain insight in the function of chlamydial genes is to determine the transcript profile along the developmental cycle, as timing can give a clue about the function. However, before the start of this work, reference genes for the normalization of gene expression data were only determined for *C. trachomatis* (Borges *et al*., 2010). Furthermore, Borges *et al*. (2010) induced stress by D-cycloserine treatment, while the transcript level of *pmp* genes in *C. trachomatis* during normal and stress conditions was previously compared by Carrasco *et al*. (2011), who induced stress by penicillin treatment. There is no general persistence model, as the type of stress (e.g. iron depletion, penicillin treatment and IFN-γ exposure) influences the transcript level of chlamydial genes differently (Mukhopadhyay *et al*., 2006; Goellner *et al*., 2006). It was previously suggested that *pmpA*, *pmpD* and *pmpI*, whose transcript levels are

unaffected during penicillin-induced stress, might play a critical role in the pathogenesis of *C. trachomatis* (Carrasco *et al*., 2011). As we wanted to compare the influence of stress on the transcript level of the *pmp* genes of *C. psittaci* with those of *C. trachomatis*, it was required that we used the same stressor. In chapter III we determined the stability of transcript levels of ten potential reference genes during both normal conditions and penicillin-induced stress. The *16S rRNA* and *opp2_A* gene were the most stably expressed genes during normal and stress conditions, respectively, in *C. trachomatis* (Borges *et al*., 2010). However, the ideal number of reference genes that should be used for normalization was not determined, neither were the most stable reference genes during normal+stress conditions defined. The latter are needed in order to compare the expression level of a gene during normal and stress conditions. We had to use five reference genes (*16S rRNA*, *map*, *radA*, *gidA* and *tyrS*), of which *16S rRNA* and *tyrsS* were the least and most stable reference gene, respectively, to compare the transcript levels of the *pmp* CDSs of *C. psittaci* at different time points (early, mid and late) during normal and penicillin-induced stress conditions. This highlights the need to use multiple, validated reference genes, instead of using a single, unvalidated reference gene such as *16S rRNA* (Vandesompele *et al*., 2002). For each different setting, which can be a different species or a different strain, time point, stressor,… new reference genes should be validated.

We determined the transcript level of the *pmp* CDSs under both normal and penicillin-induced stress conditions in chapter IV. One remarkable finding was that *pmpA* was transcribed early both in *C. psittaci* and *C. trachomatis*, while in *C. abortus pmpA* is transcribed late. This is different from our expectations, as *C. psittaci* is phylogenetically closely related to *C. abortus* and PmpA is highly conserved in both species. This suggests that Pmp subtypes might have different functions in different *Chlamydia* species and that the function does not seem to be similar in more closely related species. The high expression levels of *pmpA* and *pmpH* early, and for *pmpH* late as well, in the normal developmental cycle attracted our attention. However, in contrast to *C. trachomatis*, all *C. psittaci pmp* genes were affected by penicilin, as the transcript levels were either up- or down-regulated. The *pmpA* and *pmpH* genes were down-regulated during the stress condition. However, in chapters II, IV and V we observed that PmpA is the most conserved Pmp protein in *C. psittaci*, and that PmpA and H labeling was abundant at the chlamydial envelope, the inclusion

membrane and in the COMC. Those results suggested that PmpA and PmpH are strong antigenic proteins in *C. psittaci* and that they are potential vaccine candidates. Therefore, we suggest that the genes whose transcript levels are unaffected during stress, might not be most important for the pathogenesis of a *Chlamydia* species, as the transcript level as such does not tell us something about the stability and turn-over of the transcript, the post-transcriptional and post-translational modifications that occur in the cell. Intuitively we would think that Pmp proteins whose production level is unaffected during stress might be most important for the pathogenesis. However, the protein level of *C. trachomatis* PmpD is affected by penicillin, while the protein is immunogenic (Caldwell and Kuo, 1977) and the partially neutralizing capacity of anti-PmpD antibodies (Crane *et al*., 2006) suggest an important function for this Pmp protein in *C. trachomatis*. In general, the result of our RT-qPCR experiment highlights peak transcript levels of most *pmp* genes in different *Chlamydia* species late in the developmental cycle, however, there are also species-specific differences, which might indicate both redundant and different functions throughout the developmental cycle of different Pmp subtypes. However, before we can make that conclusion, more thorough analyses on the production level of Pmp proteins should be performed, as currently only for *C. trachomatis* the production level of all Pmp proteins is determined along the developmental cycle (Carrasco *et al*., 2011), while for *C. psittaci* the production profile along the developmental cycle was only determined for a subset (PmpA, PmpB, PmpD and PmpH) of Pmp proteins. The *C. abortus* Pmp production profile was also only determined for a subset of Pmp proteins (PmpD and 4 PmpG's), and the production level was only determined at late time points (36 hpi – 72 hpi) (Wheelhouse *et al*., 2012b). For *C. pneumoniae* the production of Pmp proteins was only analyzed at 72 hpi (Grimwood and Olinger, 2001). The protein level determined by IF suggest that both *C. trachomatis and C. psittaci* use multiple post-transcriptional and post-translational regulation mechanisms, as the transcript and protein level are not always comparable (Carrasco *et al*., 2011). The protein performs the function in the cell, however, IF is less sensitive than RT-qPCR and not all antibodies have the same affinity for their corresponding antigen and also the immunoaccessibility of different proteins can differ. Consequently, not all antibodies are equally suited for IF and IEM, and a negative staining result does not proof the absence of the protein as it might be due to the limitations of the

microscopy techniques. Therefore, both the transcript and protein results are important.

To further unravel the function of the Pmp proteins, a thorough analyses of all Pmp proteins of different *Chlamydia* species is necessary. Ideally, polyclonal antibodies should be generated against multiple surface localized epitopes, as some epitopes might lead to low affinity antibodies and other epitopes might not be immunoaccessible. Thorough IEM analyses are needed to find out whether inclusion membrane labeling of Pmp proteins is unique for *C. psittaci* and to elucidate the possible function of the Pmp proteins at that location. Proteomic analysis of the inclusion membrane fraction of *C. trachomatis* revealed the presence of PmpD in this fraction. However, that analysis can not distinguish whether PmpD is associated with the inclusion membrane or if it is inserted in the inclusion membrane. We hypothesize that Pmp proteins are only present in the inclusion membrane of more virulent *C. psittaci* strains, as it was previously observed that virulent strains were often found devoid of inclusion membranes scattered throughout the cytoplasm (Vanrompay *et al*., 1996). Therefore, cells infected with more and less virulent *C. psittaci* strains should be analyzed by IEM. In addition, thorough IEM analyses should be performed to determine whether all Pmps of different *Chlamydia* species can be found at both the inner and outer membrane of the cell envelope. Pre- and post-embedding IEM analyses should be compared, as the first one preserves the antigenicity and the last one is better for localization (Longbottom *et al*., 1998a). As IEM is not quantitative, LC/MS-MS analyses should be performed, in addition to IEM analyses, to quantitatively determine the protein composition of EBs, RBs, COMC and the sarkosyl soluble fractions. Saka *et al*. (2011) noticed that PmpA is absent in EBs, which is consistent with the results of Liu *et al*. (2010), who observed that PmpA is absent in the *C. trachomatis* outer membrane complex. These results clarify why PmpA antibodies are rare in *C. trachomatis*-infected patients, while PmpA is produced in nearly all *C. trachomatis*-infected inclusions (off frequency < 1%) (Tan *et al*., 2010). Therefore, we suggest that PmpA does not play a key role in the pathogenesis of *C. trachomatis* and that it is not a vaccine candidate for *C. trachomatis*, which is in contrast to our results for *C. psittaci*.

In conclusion, the main result of this thesis is that PmpA and PmpH are potential vaccine candidates for *C. psittaci*. However, the immunogenicity of these proteins and the neutralizing capacity of the PmpA and PmpH specific antibodies should be tested. This can be done by immunoblotting with sera from *C. psittaci* infected specified pathogen free chickens and by pre-incubating *C. psittaci* EBs with the Pmp antibodies. If the proteins are immunodominant and the specific antibodies neutralize the infectivity by at least 50% *in vitro*, then the vaccine candidate should be tested *in vivo*. The *in vivo* experiment will reveal whether the vaccine candidates reduce/eliminate *C. psittaci* excretion, which should be linked to a strong B- and T-cell response to the corresponding vaccine candidates. The latter should be tested by an antibody ELISA and T-cell proliferation tests, respectively.

Summary

Chlamydiaceae are obligate intracellular Gram-negative bacteria that cause a variety of diseases in humans and animals. This thesis focuses on *Chlamydia psittaci*, which is an avian respiratory pathogen that is able to cause zoonotic disease in human. *Chlamydia psittaci* is mainly spread via inhalation of infected aerosols of pharyngeal or nasal secretions or dried feces. Infections of *C. psittaci* lead to significant economic losses due to reduced feed conversion, carcass condemnation at slaughter, mortality, reduced egg production and/or the expense of antibiotic treatment (Vanrompay *et al*., 1997). Avian psittacosis is often systemic. Symptoms vary from inapparent to severe, including respiratory problems, conjunctivitis, diarrhea and polyuria. The sequelae of a human psittacosis are highly variable, ranging from inapparent to flu-like symptoms or pneumonia (Harkinezhad *et al*., 2009).

Vaccination is the best approach to control the spreading of chlamydial infections, both in animals and humans (Longbottom and Livingstone, 2006). Currently, eleven *Chlamydia* species have been identified (Sachse *et al*., 2015) and for none of them a vaccine is available. Members of the polymorphic membrane protein (Pmp) family have been suggested as vaccine candidates (Vasilevsky *et al*., 2016).

Chapter I gives an overview of the biology of chlamydial infections, with a focus on the composition and events occuring at different membranes that play a key function during the chlamydial developmental cycle.

Conserved proteins are suggested to be essential for the pathogenesis of an organism. Therefore, in **chapter II**, we determined the conservation of the Pmp proteins both within and across *C. trachomatis*, *C. pneumoniae*, *C. abortus* and *C. psittaci*. The first two are the main human *Chlamydia* pathogens and the last two are the most devastating animal *Chlamydia* species. The *pmp* coding sequences were identified in 16 *C. trachomatis*, 6 *C. pneumoniae*, 2 *C. abortus* and 16 *C. psittaci* genomes by a Hidden Markov Model. PmpD, PmpA, PmpH and PmpA were the most conserved Pmp protein in *C. trachomatis*, *C. pneumoniae*, *C. abortus* and *C. psittaci*, respectively. While PmpB was the most conserved Pmp protein across the four analyzed *Chlamydia* species. Previously, researchers focused mainly on PmpD, as it

is the most conserved and also an immunogenic Pmp protein in *C. trachomatis*. However, PmpD is not the most conserved in all analyzed *Chlamydia* species and also not across *Chlamydia* species, which highlights the need to analyze all Pmp proteins to accurately determine their utility as vaccine candidates.

The transcript profile of the *pmp* coding sequences across the chlamydial developmental cycle can give us a first indication about their function. Validated reference genes are necessary to accurately normalize RT-qPCR data. Up to now, reference genes were only validated for *C. trachomatis*. Therefore, in **chapter III**, we validated reference genes for both normal and persistent *C. psittaci* Cal10 infections at early, mid and late time points during the developmental cycle. The reference genes for normalization of expression data differed depending on the culture conditions and interestingly also on the selected time points. We are the first to show that different reference genes should be used along the developmental cycle. Therefore, our results stress the importance to systematically validate reference genes for the specific culture conditions and examined time points of an experiment, instead of using a single, unvalidated reference gene throughout an experiment.

In **chapter IV**, we used the reference genes validated in chapter III to normalize the transcript level of all 17 *C. psittaci pmp* coding sequences during both normal and persistent culture conditions, at early, mid and late time points during the developmental cycle. RT-qPCR profiles provide an indication of when the *pmp* genes are transcribed at the population level. However, different variants may be present in the population (e.g. due to SNPs). Therefore, we determined the Pmp production profile at the inclusion level and at the chlamydial cell level by immunofluorescence microscopy and immuno-electron microscopy, respectively. PmpA and PmpH emerged as important players in *C. psittaci* pathogenesis by virtue of their unique expression properties, both at the transcript and protein level and their apparent immunoaccessiblity and antigenicity suggest their potential in vaccine design.

Proteins present in the chlamydial outer membrane complex (COMC) might be potential vaccine candidates. The composition of the COMC was previously only determined for *C. trachomatis*, *C. abortus* and *C. pneumoniae*. Therefore, in **chapter V**, we determined whether the four most conserved *C. psittaci* Pmp proteins (PmpA, PmpD, PmpB and PmpH, in order of decreasing conservation) localized in the *C.*

psittaci outer membrane complex. Two different outer membrane complex extraction methods for Gram-negative bacteria were compared. The method using the detergent Sarkosyl was best to isolate the *C. psittaci* outer membrane complex All four Pmp proteins localized in the *C. psittaci* outer membrane complex, however, PmpA was previously not determined to be present in the COMC of another *Chlamydia* species and therefore we hypothesize that PmpA is important for adhesion of *C. psittaci*. Further analyses are needed to confirm that.

Finally, in **chapter IV**, we described our conclusions and perspectives for further research. The overall conclusion of this thesis is that PmpA and PmpH are potential vaccine candidates for *C. psittaci*, however, further *in vitro* and *in vivo* experiments are needed to confirm this hypothesis.

Samenvatting

Chlamydiaceae zijn obligaat intracellulaire, Gram-negatieve bacteriën die verscheidene ziekten veroorzaken bij mens en dier. In deze thesis onderzochten we specifiek *Chlamydia psittaci*, een ziekteverwekker die voornamelijk ademhalingsinfecties veroorzaakt bij vogels, maar deze zoönotische bacterie kan ook infecties bij de mens veroorzaken. Infecties met *C. psittaci* worden voornamelijk veroorzaakt door het inademen van aërosols van faryngeale of nasale excreties of van uitwerpselen afkomstig van geïnfecteerde dieren. *C. psittaci* infecties leiden tot grote economische verliezen door een verlaagde voederconversie, de afkeuring van karkassen bij het slachten, een verhoogd sterftecijfer, een verlaagde eierproductie en/of de kosten van antibioticabehandelingen (Vanrompay *et al*., 1997). Psittacosis in vogels leidt vaak tot een systemische infectie waarbij de symptomen sterk variëren van ademhalingsproblemen, conjunctivitis, diarree tot polyurie. Humane psittacosis heeft een zeer divers klinisch beeld dat varieert van onbeduidend tot griep-achtige symptomen of een longontsteking (Harkinezhad *et al*., 2009).

Vaccinatie wordt aanzien als de beste manier om de verspreiding van *Chlamydia* infecties, zowel in dieren als in mensen, te voorkomen (Longbottom and Livingstone, 2006). Momenteel zijn er elf *Chlamydia* soorten geïdentificeerd (Sachse *et al*., 2015), maar voor geen enkele soort is een vaccin beschikbaar. Eiwitten van de polymorfe membraan eiwitfamilie (Pmp) worden gesuggereerd als kandidaat vaccinantigenen (Vasilevsky *et al*., 2016).

Hoofdstuk I van deze thesis geeft een overzicht van de biologie van *Chlamydia* infecties, met focus op de samenstelling van de verschillende bacteriële membranen en de geassocieerde kiem-gastheerinteracties die een sleutelfunctie vervullen in de ontwikkelingscyclus van *Chlamydia*.

Er wordt gedacht dat geconserveerde eiwitten een essentiële rol vervullen in het ziekteproces van een pathogeen. Daarom werd in **hoofdstuk II** de conservering van de Pmp eiwitten zowel in als tussen vier *Chlamydia* soorten bepaald, namelijk C. *trachomatis*, *C. pneumoniae*, *C. abortus* en *C. psittaci*. *C. trachomatis en C. pneumoniae* zijn de belangrijkste humane pathogenen en *C. abortus* en *C. psittaci* zijn

de meest virulente zoönotische *Chlamydia* soorten, die een dier als primaire gastheer hebben. De *pmp* coderende sequenties werden geïdentificeerd in 16 *C. trachomatis*, 6 *C. pneumoniae*, 2 *C. abortus* en 16 *C. psittaci* genomen. PmpD, PmpA, PmpH en PmpA waren het sterkst geconserveerd in *C. trachomatis*, *C. pneumoniae*, *C. abortus* en *C. psittaci*, respectievelijk. PmpB daarentegen was het sterkst geconserveerd tussen de vier geanalyseerde *Chlamydia* soorten. Eerdere studies waren voornamelijk toegespitst op PmpD, omdat dit het meest geconserveerde Pmp eiwit is in *C. trachomatis* en dit een immunogeen eiwit is. PmpD is echter niet het meest geconserveerde Pmp eiwit binnen en tussen de andere geanalyseerde *Chlamydia* soorten. Dit resultaat toont aan dat het noodzakelijk is om alle Pmp eiwitten te bestuderen om te kunnen bepalen of de Pmp eiwitten al dan niet mogelijke kandidaat vaccinantigenen zijn.

Het transcript profiel van de *pmp* coderende sequenties doorheen de chlamydiale ontwikkelingscyclus geeft een eerste indicatie wat betreft hun functionele rol. Het transcriptie profiel kan bepaald worden via een "reverse transcriptase quantitative polymerase chain reaction" (RT-qPCR), maar deze techniek vereist echter gevalideerde referentiegenen – genen die stabiel tot expressie gebracht worden tijdens de vermeningvuldigingscyclus van de bacterie - om accuraat RT-qPCR data te kunnen normaliseren. Voorafgaand aan deze studie waren enkel voor *C. trachomatis* gevalideerde referentiegenen voorhanden. Daarom werden in **hoofdstuk III** referentiegenen gevalideerd voor twee verschillende cultuurcondities, met name een standaard infectie en een persistente *C. psittaci* infectie op vroege, middenste en late tijdstippen van de chlamydiale ontwikkelingscyclus. De referentiegenen die nodig zijn om expressiedata te normaliseren verschillen per conditie en tijdstip. Dit is de eerste studie die aantoont dat verschillende referentiegenen gebruikt moeten worden op verschillende tijdstippen gedurende de vermenigvuldigingscyclus. Deze resultaten bevestigen daarom de noodzaak om steeds referentiegenen te valideren voor de specifieke cultuurcondities en de onderzochte tijdstippen, in plaats van één, niet-gevalideerd gen te gebruiken als referentiegen doorheen de cyclus.

De referentiegenen die gevalideerd werden in hoofdstuk III, werden vervolgens gebruikt in **hoofdstuk IV** om het transcriptniveau van alle 17 *C. psittaci pmp* coderende sequenties tijdens zowel standaard als persistente cultuurcondities op

vroege, middenste en late tijdstippen van de ontwikkelingscyclus te bepalen. De RT-qPCR profielen geven een indicatie van wanneer de *pmp* genen afgeschreven worden op populatieniveau, maar er kunnen echter verschillende varianten voorkomen in de populatie (bijvoorbeeld ten gevolge van mutaties). Daarom werd ook het Pmp productieniveau bepaald, zowel op het niveau van de inclusie als op het niveau van individuele chlamydiale partikels door middel van immunofluorescentiemicroscopie en immuno-electronenmicroscopie, respectievelijk. PmpA en PmpH traden naar voor als mogelijke belangrijke spelers in het ziekteproces van *C. psittaci*. Door hun unieke expressie eigenschappen, zowel op het transcript als op het eiwitniveau en door hun duidelijke immunotoegankelijkheid en antigeniciteit zijn deze twee Pmp eiwitten mogelijke kandidaat vaccinantigenen.

Eiwitten die onderdeel uitmaken van het chlamydiale buitenste membraan complex (COMC) zijn mogelijks kandidaat vaccinantigenen. De samenstelling van het COMC was voordien enkel bepaald voor *C. trachomatis*, *C. abortus* en *C. pneumoniae*. In **hoofdstuk V** werd nagegaan of de vier meest geconserveerde Pmp eiwitten (PmpA, PmpD, PmpB and PmpH, geordend volgens dalende conservering) aanwezig waren in het *C. psittaci* buitenste membraan complex. Eerst en vooral werden twee verschillende buitenste membraan extractiemethoden voor Gram-negatieve bacteriën vergeleken, waarbij de methode die gebruik maakt van het detergent Sarkosyl de beste bleek. Alle vier Pmp eiwitten bevonden zich in het *C. psittaci* buitenste membraan complex. PmpA werd nooit eerder gedetecteerd in het COMC van een andere *Chlamydia* soort en daarom suggereren wij dat PmpA belangrijk is voor de vasthechting van *C. psittaci* aan zijn gastheercel. Verder onderzoek is echter nodig om deze hypothese te bevestigen.

Ten slotte brengt **hoofdstuk VI** de conclusies en toekomstperspectieven. De algemene conclusie van deze thesis is dat PmpA en PmpH potentiële kandidaat vaccinantigenen zijn voor *C. psittaci*, maar er zijn nog aanvullende *in vitro* en *in vivo* experimenten nodig om deze hypothese te bevestigen.

References

Abromaitis, S., and Stephens, R.S. (2009). Attachment and entry of Chlamydia have distinct requirements for host protein disulfide isomerase. *PLoS Pathog* **5**: e1000357.

Ahmed, B., De Boeck, C., Dumont, A., Cox, E., De Reu, K., and Vanrompay, D. (2015). First Experimental Evidence for the Transmission of *Chlamydia psittaci* in Poultry through Eggshell Penetration. *Transbound Emerg* Epub *Dis*: 10.1111.

Aistleitner, K., Anrather, D., Schott, T., Klose, J., Bright, M., Ammerer, G., and Horn, M. (2014). Conserved features and major differences in the outer membrane protein composition of chlamydiae. *Environ Microbiol* **17**: 1397-413.

Allen, J.E., Cerrone, M.C., Beatty, P.R., and Stephens, R.S. (1990). Cysteine-rich outer membrane proteins of *Chlamydia trachomatis* display compensatory sequence changes between biovariants. *Mol Microbiol* **4**: 1543–50.

Allen, J.E., and Stephens, R.S. (1989). Identification by sequence analysis of two-site posttranslational processing of the cysteine-rich outer membrane protein 2 of *Chlamydia trachomatis* serovar L2. *J Bacteriol* **171**: 285–91.

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

Anderson, I.E., Tan, T.W., Jones, G.E., and Herring, A.J. (1990). Efficacy against ovine enzootic abortion of an experimental vaccine containing purified elementary bodies of *Chlamydia psittaci*. *Vet Microbiol* **24**: 21–27.

Angiuoli, S.V., and Salzberg, S.L. (2011). Mugsy: Fast multiple alignment of closely related whole genomes. *Bioinformatics* **27**: 334–342.

Argudín, M.A., Cariou, N., Salandre, O., Le Guennec, J., Nemeghaire, S., and Butaye, P. (2013). Genotyping and antimicrobial resistance of *Staphylococcus aureus* isolates from diseased turkeys. *Avian Pathol* **42**: 572–80.

Azuma, Y., Hirakawa, H., Yamashita, A., Cai, Y., Rahman, M.A., Suzuki, H., *et al*. (2006). Genome sequence of the cat pathogen, *Chlamydophila felis*. *DNA Res* **13**: 15–23.

Bachmann, N.L., Fraser, T.A., Bertelli, C., Jelocnik, M., Gillett, A., Funnell, O., *et al*. (2014). Comparative genomics of koala, cattle and sheep strains of *Chlamydia pecorum*. *BMC Genomics* **15**: 667.

Baehr, W., Zhang, Y.X., Joseph, T., Su, H., Nano, F.E., Everett, K.D., and Caldwell, H.D. (1988). Mapping antigenic domains expressed by *Chlamydia trachomatis* major outer membrane protein genes. *Proc Natl Acad Sci U S A* **85**: 4000–4004.

Bardwell, J. (1994). Building bridges: disulphide bond formation in the cell. *Mol Microbiol* **14**: 199–205.

Bastidas, R.J., Elwell, C.A., Engel, J.N., and Valdivia, R.H. (2013). Chlamydial intracellular survival strategies. *Cold Spring Harb Perspect Med* **3**: a010256.

Batteiger, B.E. (1996). The major outer membrane protein of a single *Chlamydia trachomatis* serovar can possess more than one serovar-specific epitope. *Infect Immun* **64**: 542–7.

Batteiger, B.E., Newhall, W.J.V., and Jones, R.B. (1985). Differences in outer membrane proteins of the lymphogranuloma venereum and trachoma biovars of *Chlamydia trachomatis*. *Infect Immun* **50**: 488–494.

Bavoil, P.M., Ohlin, A., and Schachter, J. (1984). Role of Disulfide Bonding in Outer Membrane Structure and Permeability in *Chlamydia trachomatis*. *Infect Immun* **44**: 479–485.

Beatty, W.L. (2006). Trafficking from CD63-positive late endocytic multivesicular bodies is essential for intracellular development of *Chlamydia trachomatis*. *J Cell Sci* **119**: 350–359.

Beatty, W.L., Morrison, R.P., and Byrne, G.I. (1994). Persistent chlamydiae: from cell culture to a paradigm for chlamydial pathogenesis. *Microbiol Rev* **58**: 686–99.

Becker, E., and Hegemann, J.H. (2014). All subtypes of the Pmp adhesin family are implicated in chlamydial virulence and show species-specific function. *Microbiologyopen* **3**: 544–56.

Beeckman, D.S.A., and Vanrompay, D. (2009). Zoonotic *Chlamydophila psittaci* infections from a clinical perspective. *Clin Microbiol Infect* **15**: 11–17.

Belland, R.J., Nelson, D.E., Virok, D., Crane, D.D., Hogan, D., Sturdevant, D., *et al*. (2003). Transcriptome analysis of chlamydial growth during IFN-gamma-mediated persistence and reactivation. *Proc Natl Acad Sci U S A* **100**: 15971–6.

Belland, R.J., Ouellette, S.P., Gieffers, J., and Byrne, G.I. (2004). *Chlamydia pneumoniae* and atherosclerosis. *Cell Microbiol* **6**: 117–127.

Belland, R.J., Zhong, G., Crane, D.D., Hogan, D., Sturdevant, D., Sharma, J., *et al*. (2003). Genomic transcriptional profiling of the developmental cycle of *Chlamydia trachomatis*. *Proc Natl Acad Sci U S A* **100**: 8478–83.

Bini, L., Sanchez-campillo, M., Magi, B., Marzocchi, B., Comanducci, M., Birkelund, S., *et al*. (1996). Mapping of *Chlamydia trachomatis* proteins by electrophoresis : Spot identification by N-terminal sequencing and immunoblotting. *Electrophoresis* **17**: 185–190.

Birkelund, S., Lundemose, A.G., and Christiansen, G. (1989). Immunoelectron microscopy of lipopolysaccharide in *Chlamydia trachomatis*. *Infect Immun* **57**: 3250–3.

Birkelund, S., Morgan-Fisher, M., Timmerman, E., Gevaert, K., Shaw, A.C., and Christiansen, G. (2009). Analysis of proteins in *Chlamydia trachomatis* L2 outer membrane complex, COMC. *FEMS Immunol Med Microbiol* **55**: 187–95.

Borel, N., Dumrese, C., Ziegler, U., Schifferli, A., Kaiser, C., and Pospischil, A. (2010). Mixed infections with *Chlamydia* and porcine epidemic diarrhea virus - a new in vitro model of chlamydial persistence. *BMC Microbiol* **10**: 201.

Borges, V., Ferreira, R., Nunes, A., Nogueira, P., Borrego, M.J., and Gomes, J.P. (2010). Normalization strategies for real-time expression data in *Chlamydia trachomatis*. *J Microbiol Methods* **82**: 256–64.

Borges, V., Pinheiro, M., Vieira, L., Sampaio, D.A., Nunes, A., Borrego, M.J., and Gomes, P. (2014). Complete Genome Sequence of *Chlamydia trachomatis* Ocular Serovar. *Genome Announc* **2**: 3–4.

Boyle, E.C., and finlay, B.B. (2003). Bacterial pathogenesis: Exploiting cellular adherence. *Curr Opin Cell Biol* **15**: 633–639.

Brade, H., Brade, L., and Nano, F.E. (1987). Chemical and serological investigations on the genus-specific lipopolysaccharide epitope of *Chlamydia*. *Proc Natl Acad Sci U S A* **84**: 2508–12.

Bunk, S., Susnea, I., Rupp, J., Summersgill, J.T., Maass, M., Stegmann, W., *et al*. (2008). Immunoproteomic identification and serological responses to novel *Chlamydia pneumoniae* antigens that are associated with persistent *C. pneumoniae* infections. *J Immunol* **180**: 5490–8.

Bustin, S.A. (2005). Quantitative real-time RT-PCR - a perspective. *J Mol Endocrinol* **34**: 597–601.

Bustin, S.A. (2001). Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *J Mol Endocrinol* **29**: 23–39.

Caldwell, H.D., and Hitchcock, P.J. (1984). Monoclonal antibody against a genus-specific antigen of *Chlamydia* species: location of the epitope on chlamydial lipopolysaccharide. *Infect Immun* **44**: 306–314.

Caldwell, H.D., Kromhout, J., and Schachter, J. (1981). Purification and Partial Characterization of the Major Outer Membrane Protein of *Chlamydia trachomatis*. *Infection* **31**: 1161–1176.

Caldwell, H.D., and Kuo, C. (1977). Purification of a *Chlamydia Trachomatis*-Specific Antigen by Immunoadsorption with Monospecific Antibody. *J Immunol* **118**: 437–441.

Caldwell, H.D., Kuo, C., and Kenny, G.E. (1975a). Antigenic Analysis of Chlamydiae by Two-Dimensional Immunoelectrophoresis : I . Antigenic Heterogeneity Between *C . Trachomatis* and *C . Psittaci*. *J Immunol* **115**: 963-968.

Caldwell, H.D., Kuo, C., and Kenny, G.E. (1975b). Antigenic Analysis of *Chlamydiae* by Two-Dimensional Immunoelectrophoresis : II . A Trachoma-LGV-Specific Antigen. *J Immunol* **115**: 969–975.

Caldwell, H.D., and Perry, L.J. (1982). Neutralization of *Chlamydia trachomatis* infectivity with antibodies to the major outer membrane protein. *Infect Immun* **38**: 745–754.

Caldwell, H.D., Stewart, S., Johnson, S., and Taylor, H. (1987). Tear and serum antibody response to *Chlamydia trachomatis* antigens during acute Chlamydial conjunctivitis in monkeys as determined by immunoblotting. *Infect Immun* **55**: 93–98.

Campbell, L.A., and Kuo, C. (2006). Interactions of *Chlamydia* with the Host Cells that Mediate Attachment and Uptake. In *Chlamydia : genomics and pathogenesis*. Bavoil, P.M., and Wyrick, P.B. (eds). Horizon Bioscience, Wymondham, pp. 505–522.

Campbell, L.A., Kuo, C.C., Wang, S.P., and Grayston, J.T. (1990). Serological response to *Chlamydia pneumoniae* infection. *J Clin Microbiol* **28**: 1261–4.

Campbell, L.A., Kuo, C.C., Wang, S.P., and Grayston, J.T. (1990). Serological response to *Chlamydia pneumoniae* infection. *J Clin Microbiol* **28**: 1261–4.

Capmany, A., and Damiani, M.T. (2010). *Chlamydia trachomatis* intercepts golgi-derived sphingolipids through a rab14-mediated transport required for bacterial development and replication. *PLoS One* **5**:e14084.

Carabeo, R.A., Mead, D.J., and Hackstadt, T. (2003). Golgi-dependent transport of cholesterol to the *Chlamydia trachomatis* inclusion. *Proc Natl Acad Sci U S A* **100**: 6771–6.

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

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

Carrasco, J.A., Tan, C., Rank, R.G., Hsia, R., and Bavoil, P.M. (2011). Altered developmental expression of polymorphic membrane proteins in penicillin-stressed *Chlamydia trachomatis*. *Cell Microbiol* **13**: 1014–25.

Carvalho, D.M., de Sá, P.H., Castro, T.L.P., Carvalho, R.D., Pinto, A., Gil, D.J.P., *et al*. (2014). Reference genes for RT-qPCR studies in *Corynebacterium pseudotuberculosis* identified through analysis of RNA-seq data. *Antonie Van Leeuwenhoek* **106**: 605–614.

Cerrone, M.C., Ma, J.J., and Stephens, R.S. (1991). Cloning and sequence of the gene for heat shock protein 60 from *Chlamydia trachomatis* and immunological reactivity of the protein. *Infect Immun* **59**: 79–90.

Cevenini, R., Donati, M., Broncchi, E., De Simone, F., and La Placa, M. (1991). Partial characterization of an 89-kDa highly immunoreactive protein from *Chlamydia psittaci* A/22 causing ovine abortion. *FEMS Microbiol Lett* **65**: 111–115.

Chotinun, S., Rojanasthien, S., Unger, F., Tadee, P., and Patchanee, P. (2014). Prevalence and antimicrobial resistance of *Salmonella* isolated from carcasses, processing facilities and the environment surrounding small scale poultry slaughterhouses in Thailand. *Southeast Asian J Trop Med Public Heal* **45**: 1392–1400.

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

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

Cocchiaro, J., and Valdivia, R.H. (2009). New insights into Chlamydia intracellular survival mechanisms. *Cell Microbiol* **72**: 181–204.

Coles, M., Reynolds, D., Harper, A., Devitt, A., and Pearce, J. (1993). Low-nutrient induction of abnormal chlamydial development: A novel component of chlamydial pathogenesis? *FEMS Microbiol Lett* **106**: 193–200.

Collett, B.A., Newhall, W.J., Jersild, R.A., and Jones, R.B. (1989). Detection of surface-exposed epitopes on *Chlamydia trachomatis* by immune electron microscopy. *J Gen Microbiol* **135**: 85–94.

Cortes, C., Rzomp, K.A., Tvinnereim, A., Scidmore, M.A., and Wizel, B. (2007). *Chlamydia pneumoniae* inclusion membrane protein Cpn0585 interacts with multiple rab GTPases. *Infect Immun* **75**: 5586–5596.

Crane, D.D., Carlson, J.H., Fischer, E.R., Bavoil, P., Hsia, R., Tan, C., *et al*. (2006). *Chlamydia trachomatis* polymorphic membrane protein D is a species-common pan-neutralizing antigen. *Proc Natl Acad Sci U S A* **103**: 1894–9.

Danilition, S.L., Maclean, I.W., Peeling, R., Winston, S., and Brunham, R.C. (1990). The 75-kilodalton protein of *Chlamydia trachomatis*: A member of the heat shock protein 70 family? *Infect Immun* **58**: 189–196.

Dautin, N., and Bernstein, H.D. (2007). Protein secretion in gram-negative bacteria via the autotransporter pathway. *Annu Rev Microbiol* **61**: 89–112.

Dean, D., Suchland, R.J., and Stamm, W.E. (2000). Evidence for long-term cervical persistence of *Chlamydia trachomatis* by omp1 genotyping. *J Infect Dis* **182**: 909–916.

Deckert, A., Valdivieso-Garcia, A., Reid-Smith, R., Tamblyn, S., Seliske, P., Irwin, R., *et al*. (2010). Prevalence and antimicrobial resistance in *Campylobacter spp*. isolated from retail chicken in two health units in Ontario. *J Food Prot* **73**: 1317–1324.

Denzer, K., Kleijmeer, M.J., Heijnen, H.F., Stoorvogel, W., and Geuze, H.J. (2000). Exosome: from internal vesicle of the multivesicular body to intercellular signaling device. *J Cell Sci* **113 Pt 19**: 3365–3374.

Derré, I., Pypaert, M., Dautry-Varsat, A., and Agaisse, H. (2007). RNAi screen in Drosophila cells reveals the involvement of the tom complex in *Chlamydia* infection. *PLoS Pathog* **3**: 1446–1458.

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

Dheda, K., Huggett, J.F., Chang, J.S., Kim, L.U., Bustin, S.A., Johnson, M.A., *et al*. (2005). The implications of using an inappropriate reference gene for real-time reverse transcription PCR data normalization. *Anal Biochem* **344**: 141–143.

Dickx, V., Geens, T., Deschuyffeleer, T., Tyberghien, L., Harkinezhad, T., Beeckman, D.S.A., *et al*. (2010). *Chlamydophila psittaci* zoonotic risk assessment in a chicken and turkey slaughterhouse. *J Clin Microbiol* **48**: 3244–3250.

Dickx, V., and Vanrompay, D. (2011). Zoonotic transmission of *Chlamydia psittaci* in a chicken and turkey hatchery. *J Med Microbiol* **60**: 775–779.

Di Francesco, A., Donati, M., Rossi, M., Pignanelli, S., Shurdhi, A., Baldelli, R., and Cevenini, R. (2008). Tetracycline-resistant *Chlamydia suis* isolates in Italy. *Vet Rec* **163**: 251–2.

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

Dugan, J., Rockey, D.D., Jones, L., Arthur, A., and Andersen, A.A. (2004). Tetracycline Resistance in *Chlamydia suis* Mediated by Genomic Islands Inserted into the Chlamydial inv -Like Gene Tetracycline Resistance in *Chlamydia suis* Mediated by Genomic Islands Inserted into the Chlamydial inv -Like Gene. *Antimicrob Agents Chemother* **48**: 3989–3995.

Dugan, J., Anderson A.A., Rockey D.D. (2007). Functional characterization of IScs605, an insertion element carried by tetracycline-resistant *Chlamydia suis*. *Microbiology* **153**: 71-79.

Dumoux, M., and Hayward, R.D. (2016). Membrane contact sites between pathogen-containing compartments and host organelles. *Biochim Biophys Acta* **16:** 30017-8.

Egan, A.J.F., and Vollmer, W. (2013). The physiology of bacterial cell division. *Ann N Y Acad Sci* **1277**: 8–28.

Elwell, C.A., Jiang, S., Kim, J.H., Lee, A., Wittmann, T., Hanada, K., *et al*. (2011). *Chlamydia trachomatis* co-opts GBF1 and CERT to acquire host sphingomyelin for distinct roles during intracellular development. *PLoS Pathog* **7**: e1002198.

Elwell, C.A., Ceesay, A., Jung, H.K., Kalman, D., and Engel, J.N. (2008). RNA interference screen identifies Abl kinase and PDGFR signaling in *Chlamydia trachomatis* entry. *PLoS Pathog* **4**: 1–15.

Everett, K.D.E. (2000). *Chlamydia* and *Chlamydiales*: More than meets the eye. *Vet Microbiol* **75**: 109–126.

Everett, K.D., Bush, R.M., and Andersen, A.A. (1999). Emended description of the order *Chlamydiales*,

proposal of *Parachlamydiaceae* fam. nov. and *Simkaniaceae* fam. nov., each containing one monotypic genus, revised taxonomy of the family *Chlamydiaceae*, including a new genus and five new species, and standards. *Int J Syst Bacteriol* **49**: 415–40.

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

Everett, K.D., and Hatch, T.P. (1995). Architecture of the cell envelope of *Chlamydia psittaci* 6BC. *J Bacteriol* **177**: 877–82.

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

Fadel, S., and Eley, A. (2008). Differential glycosaminoglycan binding of *Chlamydia trachomatis* OmcB protein from serovars E and LGV. *J Med Microbiol* **57**: 1058–1061.

Fan, T., Lu, H., Hu, H., Shi, L., McClarty, G.A, Nance, D.M., *et al*. (1998). Inhibition of apoptosis in chlamydia-infected cells: blockade of mitochondrial cytochrome c release and caspase activation. *J Exp Med* **187**: 487–496.

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

Fields, K.A., Mead, D.J., Dooley, C.A., and Hackstadt, T. (2003). *Chlamydia trachomatis* type III secretion: Evidence for a functional apparatus during early-cycle development. *Mol Microbiol* **48**: 671–683.

Filip, C., Fletcher, G., Wulff, J.L., and Earhart, C.F. (1973). Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium-lauryl sarcosinate. *J Bacteriol* **115**: 717–22.

Forsbach-Birk, V., Foddis, C., Simnacher, U., Wilkat, M., Longbottom, D., Walder, G., *et al*. (2013). Profiling Antibody Responses to Infections by *Chlamydia abortus* Enables Identification of Potential Virulence Factors and Candidates for Serodiagnosis. *PLoS One* **8**: e80310.

Francis, T., and Magill, T.P. (1938). An unidentified virus producing acute meningitis and pneumonitis in experimental animals. *J Exp Med* **68**: 147–160.

Gambarte Tudela, J., Capmany, A., Romao, M., Quintero, C., Miserey-Lenkei, S., Raposo, G., *et al*. (2015). The late endocytic Rab39a GTPase regulates multivesicular bodies-chlamydial inclusion interaction and bacterial growth. *J Cell Sci* **11**: 3068–3081.

Geens, T. (2005). Identification and characterization of a type three secretion system in genotypes of *Chlamydophila psittaci*. PhD thesis, Ghent University.

Geens, T., Desplanques, A., Loock, M. Van, Bo, B.M., Kaleta, E.F., Magnino, S., *et al*. (2005). Sequencing of the *Chlamydophila psittaci* ompA Gene Reveals a New Genotype , E/B , and the Need for a Rapid Discriminatory Genotyping Method. *J Clin Microbiol* **43**: 2456–2461.

Gentle, I.E., Burri, L., and Lithgow, T. (2005). Molecular architecture and function of the Omp85 family of proteins. *Mol Microbiol* **58**: 1216–1225.

Gevaert, K., Goethals, M., Martens, L., Damme, J. Van, Staes, A., Thomas, G., and Vandekerckhove, J. (2003). Exploring proteomes and analyzing protein processing by mass spectrometric identification of sorted N-terminal peptides. *Nat Biotechnol* **21**: 566–569.

Gey, G.O., Coffmar, W.D., and Kubicek, M.T. (1952). Tissue culture studies of the proliferative capacity of cervical carcinoma and normal epithelium. *Cancer Res* **12**: 264–265.

Giannikopoulou, P., Bini, L., Simitsek, P.D., Pallini, V., and Vretou, E. (1997). Two-dimensional electrophoretic analysis of the protein family at 90 kDa of abortifacient *Chlamydia psittaci*. *Electrophoresis* **18**: 2104–8.

Girard, V., and Mourez, M. (2006). Adhesion mediated by autotransporters of Gram-negative bacteria: structural and functional features. *Res Microbiol* **157**: 407–16.

Glauert, A., and Thornley, M. (1969). The topology of the bacterial cell wall. *Annu Rev Microbiol* **23**: 159–198.

Godlewska, R., Wiśniewska, K., Pietras, Z., and Jagusztyn-Krynicka, E.K. (2009). Peptidoglycan-associated lipoprotein (Pal) of Gram-negative bacteria: Function, structure, role in pathogenesis and potential application in immunoprophylaxis: Minireview. *FEMS Microbiol Lett* **298**: 1–11.

Goellner, S., Schubert, E., Liebler-Tenorio, E., Hotzel, H., Saluz, H.P., and Sachse, K. (2006). Transcriptional response patterns of *Chlamydophila psittaci* in different in vitro models of persistent infection. *Infect Immun* **74**: 4801–8.

Gomes, P., Nunes, A., Bruno, W.J., Borrego, M.J., Florindo, C., Dean, D., and Acteriol, J.B. (2006). Polymorphisms in the Nine Polymorphic Membrane Proteins of *Chlamydia trachomatis* across All Serovars : Evidence for Serovar Da Recombination and Correlation with Tissue Tropism. *J Bacteriol* **188**: 275–286.

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

Grimwood, J., and Olinger, L. (2001). Expression of *Chlamydia pneumoniae* Polymorphic Membrane Protein Family Genes. *Infect Immun* **69**: 2383–2389.

Grimwood, J., and Stephens, R.S. (1999). Computational analysis of the polymorphic membrane protein superfamily of *Chlamydia trachomatis* and *Chlamydia pneumoniae*. *Microb Comp Genomics* **4**: 187–201.

Grinblat-Huse, V., Drabek, E.F., Creasy, H.H., Daugherty, S.C., Jones, K.M., Santana-Cruz, I., *et al*. (2011). Genome sequences of the zoonotic pathogens *Chlamydia psittaci* 6BC and Cal10. *J Bacteriol* **193**: 4039–40.

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

Hanada, K. (2010). Intracellular trafficking of ceramide by ceramide transfer protein. *Proc Jpn Acad Ser B Phys Biol Sci* **86**: 426–437.

Hanh, D., Azenabor, A., Beatty, W.L., and Byrne, G.I. (2002). *Chlamydia pneumoniae* as a respiratory pathogen. *Front Biosci* **7**: 66–76.

Harkinezhad, T., Geens, T., and Vanrompay, D. (2009). *Chlamydophila psittaci* infections in birds: a review with emphasis on zoonotic consequences. *Vet Microbiol* **135**: 68–77.

Harris, S.R., Clarke, I.N., Seth-Smith, H.M.B., Solomon, A.W., Cutcliffe, L.T., Marsh, P., *et al*. (2012). Whole-genome analysis of diverse *Chlamydia trachomatis* strains identifies phylogenetic relationships masked by current clinical typing. *Nat Genet* **44**: 413–9.

Hatch, T.P. (1996). Disulfide Cross-Linked Envelope Proteins : the Functional Equivalent of Peptidoglycan in Chlamydiae? *J Bacteriol* **178**: 1–5.

Hatch, T.P., Allan, I., and Pearce, J.H. (1984). Structural and polypeptide differences between envelopes of infective and reproductive life cycle forms of *Chlamydia* spp. *J Bacteriol* **157**: 13–20.

Hatch, G.M., and Mcclarty, G. (1998). Phospholipid composition of purified *Chlamydia trachomatis* mimics that of the eucaryotic host cell. *Infect Immun* **66**: 3727–3735.

Hatch, T.P., Miceli, M., and Sublett, J.E. (1986). Synthesis of disulfide-bonded outer membrane proteins during the developmental cycle of *Chlamydia psittaci* and *Chlamydia trachomatis*. *J Bacteriol* **165**: 379–85.

Hatch, T.P., Vance Jr., D.W., and Al-Hossainy, E. (1981). Attachment of *Chlamydia psittaci* to formaldehyde-fixed and unfixed L cells. *J Gen Microbiol* **125**: 273–283.

Heckels, J.E., Blackett, B., Everson, J.S., and Ward, M.E. (1976). The Influence of Surface Charge on the Attachment of *Neisseria gonorrhoeae* to Human Cells. *J Gen Microbiol* **96**: 359–364.

Heddema, E.R., van Hannen, E.J., Duim, B., Vandenbroucke-Grauls, C.M.J.E., and Pannekoek, Y. (2006). Genotyping of *Chlamydophila psittaci* in human samples. *Emerg Infect Dis* **12**: 1989–90.

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

Henderson, I.R., and Lam, A.C. (2001). Polymorphic proteins of *Chlamydia spp*.--autotransporters beyond the Proteobacteria. *Trends Microbiol* **9**: 573–8.

Henderson, I.R., Nataro, J.P., and Henderson, I.R. (2001). Virulence Functions of Autotransporter Proteins MINIREVIEW. *Infect Immun* **69**: 1231-1243.

Henderson, I.R., Navarro-garcia, F., Fernandez, R.C., and Aldeen, D.A. (2004). Type V Protein Secretion Pathway : the Autotransporter Story. *Microbiol Mol Biol Rev* **68**: 692-744.

Hesse, L., Bostock, J., Dementin, S., Blanot, D., Mengin-Lecreulx, D., and Chopra, I. (2003). Functional and Biochemical Analysis of *Chlamydia trachomatis* MurC, an Enzyme Displaying UDP-N-Acetylmuramate:Amino Acid Ligase Activity. *J Bacteriol* **185**: 6507–6512.

Heuer, D., Rejman Lipinski, A., Machuy, N., Karlas, A., Wehrens, A., Siedler, F., *et al*. (2009). *Chlamydia* causes fragmentation of the Golgi compartment to ensure reproduction. *Nature* **457**: 731–735.

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

Holland, S.M., Hudson, A.P., Bobo, L., Whittum-Hudson, J.A., Viscidi, R.P., Quinn, T.C., and Taylor, H.R. (1992). Demonstration of chlamydial RNA and DNA during a culture-negative state. *Infect Immun* **60**: 2040–2047.

Horn, M., Collingro, A., Schmitz-Esser, S., Beier, C.L., Purkhold, U., Fartmann, B., *et al*. (2004). Illuminating the evolutionary history of chlamydiae. *Science* **304**: 728–30.

Hovis, K.M., Mojica, S., McDermott, J.E., Pedersen, L., Simhi, C., Rank, R.G., *et al*. (2013). Genus-optimized strategy for the identification of chlamydial type III secretion substrates. *Pathog Dis* **69**: 213–22.

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

Hsia, R.C., Ohayon, H., Gounon, P., Dautry-Varsat, A., and Bavoil, P.M. (2000). Phage infection of the obligate intracellular bacterium, *Chlamydia psittaci* strain Guinea Pig Inclusion Conjunctivitis. *Microbes Infect* **2**: 761–772.

Hu, Y., Chen, L., Wang, C., Xie, Y., Chen, Z., Liu, L., *et al*. (2015). Transcriptional Analysis of 10 Selected Genes in a Model of Penicillin G Induced Persistence of *Chlamydophila psittaci* in HeLa Cells. *J Microbiol Biotechnol* **25**: 1246–56.

Humphrys, M.S., Creasy, T., Sun, Y., Shetty, A.C., Chibucos, M.C., Drabek, E.F., *et al*. (2013). Simultaneous transcriptional profiling of bacteria and their host cells. *PLoS One* **8**: e80597.

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

Hybiske, K., and Stephens, R.S. (2007). Mechanisms of host cell exit by the intracellular bacterium *Chlamydia*. *Proc Natl Acad Sci U S A* **104**: 11430–5.

Igietseme, J.U., Magee, D.M., Williams, D.M., and Rank, R.G. (1994). Role for CD8+ T cells in antichlamydial immunity defined by chlamydia- specific T-lymphocyte clones. *Infect Immun* **62**: 5195–5197.

Iliffe-Lee, E.R., and McClarty, G. (1999). Glucose metabolism in *Chlamydia trachomatis*: the energy parasite hypothesis revisited. *Mol Microbiol* **33**: 177–187.

Ingalls, R.R., Rice, P.A., Qureshi, N., Takayama, K., Lin, J.S., Golenbock, D.T., and Mmun, I.N.I. (1995). The Inflammatory Cytokine Response to *Chlamydia trachomatis* Infection Is Endotoxin Mediated. *Infect Immun* **63**: 3125–3130.

Ito, K., Sato, T., and Yura, T. (1977). Synthesis and assembly of the membrane proteins in *E. coli* . *Cell* **11**: 551–559.

Jasper, D.K., Sigar, I.M., Schripsema, J.H., Sainvil, C.K., Smith, C.L., Yeruva, L., *et al*. (2015). Genomic variant representation in a Chlamydia population is dynamic and adaptive with dependence on in vitro and in vivo passage. *Pathog Dis* **73**: 1–12.

Jeffrey, B.M., Suchland, R.J., Quinn, K.L., Davidson, J.R., Stamm, W.E., and Rockey, D.D. (2010). Genome sequencing of recent clinical *Chlamydia trachomatis* strains identifies loci associated with tissue tropism and regions of apparent recombination. *Infect Immun* **78**: 2544–2553.

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

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

Kalman, S., Mitchell, W., Marathe, R., Lammel, C., Fan, J., Hyman, R.W., *et al*. (1999). Comparative genomes of *Chlamydia pneumoniae* and *C. trachomatis*. *Nat Genet* **21**: 385–389.

Kalmar, I., Berndt, A., Yin, L., Chiers, K., Sachse, K., and Vanrompay, D. (2015). Host–pathogen interactions in specific pathogen-free chickens following aerogenous infection with *Chlamydia psittaci* and *Chlamydia abortus*. *Vet Immunol Immunopathol* **164**: 30-9.

Kari, L., Whitmire, W.M., Crane, D.D., Reveneau, N., Carlson, J.H., Goheen, M.M., *et al*. (2009).

Chlamydia trachomatis native major outer membrane protein induces partial protection in nonhuman primates: implication for a trachoma transmission-blocking vaccine. *J Immunol* **182**: 8063–8070.

Karunakaran, K.P., Rey-Ladino, J., Stoynov, N., Berg, K., Shen, C., Jiang, X., *et al*. (2008). Immunoproteomic discovery of novel T cell antigens from the obligate intracellular pathogen *Chlamydia*. *J Immunol* **180**: 2459–65.

Kawa, D.E., and Stephens, R.S. (2002). Antigenic topology of chlamydial PorB protein and identification of targets for immune neutralization of infectivity. *J Immunol* **168**: 5184–91.

Kerr, K., Entrican, G., McKeever, D., and Longbottom, D. (2005). Immunopathology of *Chlamydophila abortus* infection in sheep and mice. *Res Vet Sci* **78**: 1–7.

Kidie, D., Bae, D., and Lee, Y. (2013). Prevalence and antimicrobial resistence of *Salmonella* isolated from poultry slaughterhouses in Korea. *J Vet Res* **61**: 129–136.

Kim, S., and DeMars, R. (2001). Epitope clusters in the major outer membrane protein of *Chlamydia trachomatis*. *Curr Opin Immunol* **13**: 429–436.

Kirk, D.G., Palonen, E., Korkeala, H., and Lindström, M. (2014). Evaluation of normalization reference genes for RT-qPCR analysis of spo0A and four sporulation sigma factor genes in *Clostridium botulinum* Group I strain ATCC 3502. *Anaerobe* **26**: 14–19.

Kiselev, A.O., Skinner, M.C., and Lampe, M.F. (2009). Analysis of pmpD expression and PmpD post-translational processing during the life cycle of *Chlamydia trachomatis* serovars A, D, and L2. *PLoS One* **4**: e5191.

Kiselev, A.O., Stamm, W.E., Yates, J.R., and Lampe, M.F. (2007). Expression, processing, and localization of PmpD of *Chlamydia trachomatis* Serovar L2 during the chlamydial developmental cycle. *PLoS One* **2**: e568.

Knittler, M.R., Berndt, A., Böcker, S., Dutow, P., Hänel, F., Heuer, D., *et al*. (2014). *Chlamydia psittaci*: New insights into genomic diversity, clinical pathology, host-pathogen interaction and anti-bacterial immunity. *Int J Med Microbiol*. **304**: 877-93.

Knudsen, K., Madsen, A.S., Mygind, P., Christiansen, G., and Birkelund, S. (1999). Identification of Two Novel Genes Encoding 97- to 99-Kilodalton Outer Membrane Proteins of *Chlamydia pneumoniae*. **67**: 375–383.

Kubo, A., and Stephens, R.S. (2000). Characterization and functional analysis of PorB, a Chlamydia porin and neutralizing target. *Mol Microbiol* **38**: 772–780.

Kubo, A., and Stephens, R.S. (2001). Substrate-specific diffusion of select dicarboxylates through *Chlamydia trachomatis* PorB. *Microbiology* **147**: 3135–3140.

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

Kuo, C.C., Wang, S.P., and Grayston, J.T. (1977). Antimicrobial activity of several antibiotics and a sulfonamide against *Chlamydia trachomatis* organisms in cell culture. *Antimicrob Agents Chemother* **12**: 80–83.

Lambden, P.R., Everson, J.S., Ward, M.E., and Clarke, I.N. (1990). Sulfur-rich proteins of *Chlamydia trachomatis* : developmentally regulated transcription of polycistronic mRNA from tandem promotors. *Gene* **87**: 105–112.

Lambert, M., and Smith, S. (2009). The PagN protein mediates invasion via interaction with

proteoglycan. *FEMS Microbiol Lett* **2**: 209–216.

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

LaVerda, D., Kalayoglu, M.V, and Byrne, G.I. (1999). Chlamydial heat shock proteins and disease pathology: new paradigms for old problems? *Infect Dis Obs Gynecol* **7**: 64–71.

Liechti, G.W., Kuru, E., Hall, E., Kalinda, A., Brun, Y.V., VanNieuwenhze, M., and Maurelli, A.T. (2014). A new metabolic cell-wall labelling method reveals peptidoglycan in *Chlamydia trachomatis*. *Nature* **506**: 507–10.

Liu, X., Afrane, M., Clemmer, D.E., Zhong, G., and Nelson, D.E. (2010). Identification of *Chlamydia trachomatis* outer membrane complex proteins by differential proteomics. *J Bacteriol* **192**: 2852–60.

Livingstone, M., Entrican, G., Wattegedera, S., Buxton, D., McKendrick, I.J., and Longbottom, D. (2005). Antibody Responses to Recombinant Protein Fragments of the Major Outer Membrane Protein and Polymorphic Outer Membrane Protein POMP90 in *Chlamydophila abortus*-Infected Pregnant Sheep. *Clin Diagn Lab Immunol* **12**: 770–777.

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

Longbottom, D., Findlay, J., Vretou, E., and Dunbar, S.M. (1998a). Immunoelectron microscopic localisation of the OMP90 family on the outer membrane surface of *Chlamydia psittaci*. *FEMS Microbiol Lett* **164**: 111–7.

Longbottom, D., and Livingstone, M. (2006). Vaccination against chlamydial infections of man and animals. *Vet J* **171**: 263–275.

Longbottom, D., Russell, M., Dunbar, S.M., Jones, G.E., and Herring, A.J. (1998b). Molecular cloning and characterization of the genes coding for the highly immunogenic cluster of 90-kilodalton envelope proteins from the *Chlamydia psittaci* subtype that causes abortion in sheep. *Infect Immun* **66**: 1317–24.

Longbottom, D., Russell, M., Jones, G.E., Lainson, F.A., and Herring, A.J. (1996). Identification of a multigene family coding for the 90 kDa proteins of the ovine abortion subtype of *Chlamydia psittaci*. *FEMS Microbiol Lett* **142**: 277–281.

Lutter, E.I., Martens, C., and Hackstadt, T. (2012). Evolution and conservation of predicted inclusion membrane proteins in chlamydiae. *Comp Funct Genomics* **2012**: 362104.

Manire, G.P., and Tamura, A. (1967). Preparation and chemical composition of the cell walls of mature infectious dense forms of meningopneumonitis organisms. *J Bacteriol* **94**: 1178–1183.

Mamelak, D., Mylvaganam, M., Whetstone, H., Hartmann, E., Lennarz, W., Wyrick, P.B., *et al*. (2001). Hsp70s contain a specific sulfogalactolipid binding site. Differential aglycone influence on sulfogalactosyl ceramide binding by recombinant prokaryotic and eukaryotic Hsp70 family members. *Biochemistry* **40**: 3572–3582.

Mannonen, L., Markkula, E., and Puolakkainen, M. (2011). Analysis of *Chlamydia pneumoniae* infection in mononuclear cells by reverse transcription-PCR targeted to chlamydial gene transcripts. *Med Microbiol Immunol* **200**: 143–154.

Marques, P.X., Souda, P., O'Donovan, J., Gutierrez, J., Gutierrez, E.J., Worrall, S., *et al*. (2010). Identification of immunologically relevant proteins of *Chlamydophila abortus* using sera from experimentally infected pregnant ewes. *Clin Vaccine Immunol* **17**: 1274–81.

Mathews, S., George, C., Flegg, C., Stenzel, D., and Timms, P. (2001). Differential expression of ompA, ompB, pyk, nlpD and Cpn0585 genes between normal and interferon-gamma treated cultures of *Chlamydia pneumoniae*. *Microb Pathog* **30**: 337–345.

Matsumoto, A. (1982). Electron microscopic observations of surface projections on *Chlamydia psittaci* reticulate bodies. *Infect Immun* **150**: 358–364.

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

Mayr, A., Bachmann, P.A., Bibrack, B., and Wittmann, G. (1974). Quantitative Bestimmung der Virusinfektiositat (Virustitration). In *Virologische Arbeitsmethoden Bd*. I. Jena: Gustav Ficher Verlag, pp. 35-39.

McCafferty, M.C., Herring, A.J., Andersen, A.A., and Jones, G.E. (1995). Electrophoretic analysis of the major outer membrane protein of *Chlamydia psittaci* reveals multimers which are recognized by protective monoclonal antibodies. *Infect Immun* **63**: 2387–2389.

McConnell, M.J., and Pachón, J. (2011). Expression, purification, and refolding of biologically active *Acinetobacter baumannii* OmpA from *Escherichia coli* inclusion bodies. *Protein Expr Purif* **77**: 98–103.

McCoy, A.J., Adams, N.E., Hudson, A.O., Gilvarg, C., Leustek, T., and Maurelli, A.T. (2006). L,L-diaminopimelate aminotransferase, a trans-kingdom enzyme shared by *Chlamydia* and plants for synthesis of diaminopimelate/lysine. *Proc Natl Acad Sci* **103**: 17909–17914.

McCoy, A.J., and Maurelli, A.T. (2005). Characterization of *Chlamydia* MurC-Ddl, a fusion protein exhibiting D-alanyl-D-alanine ligase activity involved in peptidoglycan synthesis and D-cycloserine sensitivity. *Mol Microbiol* **57**: 41–52.

McCoy, A.J., Sandlin, R.C., and Maurelli, A.T. (2003). In Vitro and In Vivo Functional Activity of *Chlamydia* MurA , a UDP- N -Acetylglucosamine Enolpyruvyl Transferase Involved in Peptidoglycan Synthesis and Fosfomycin Resistance. *J Bacteriol* **185**: 1218–1228.

Mehlitz, A., Banhart, S., Hess, S., Selbach, M., and Meyer, T.F. (2008). Complex kinase requirements for *Chlamydia trachomatis* Tarp phosphorylation. *FEMS Microbiol Lett* **289**: 233–240.

Michalova, E., Novotna, P., and Schlegelova, J. (2004). Tetracyclines in veterinary medicine and bacterial resistance to them. A review. *Vet Med* **2004**: 79–100.

Miettinen, A., Heinonen, P.K., Teisala, K., Punnonen, R., and Paavonen, J. (1990). Antigen specific serum antibody response to *Chlamydia trachomatis* in patients with acute pelvic inflammatory disease. *J Clin Pathol* **43**: 758–761.

Miller, J.D., Sal, M.S., Schell, M., Whittimore, J.D., and Raulston, J.E. (2009). *Chlamydia trachomatis* YtgA is an iron-binding periplasmic protein in duced by iron restriction. *Microbiology* **155**: 2884–2894.

Moelleken, K., and Hegemann, J.H. (2008). The Chlamydia outer membrane protein OmcB is required for adhesion and exhibits biovar-specific differences in glycosaminoglycan binding. *Mol Microbiol* **67**: 403–19.

Mojica, S.A., Hovis, K.M., Frieman, M.B., Tran, B., Hsia, R.C., Ravel, J., *et al*. (2015). SINC, a type III secreted protein of *Chlamydia psittaci*, targets the inner nuclear membrane of infected cells and uninfected neighbors. *Mol Biol Cell* **26**: 1918–1934.

Mölleken, K., Becker, E., and Hegemann, J.H. (2013) The *Chlamydia pneumoniae* Invasin Protein

Pmp21 Recruits the EGF Receptor for Host Cell Entry. *PLoS Pathog* **9**: e1003325.

Mölleken, K., Schmidt, E., and Hegemann, J.H. (2010). Members of the Pmp protein family of *Chlamydia pneumoniae* mediate adhesion to human cells via short repetitive peptide motifs. *Mol Microbiol* **78**: 1004–17.

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

Morrison, S.G., Su, H., Caldwell, H.D., and Morrison, R.P. (2000). Immunity to murine *Chlamydia trachomatis* genital tract reinfection involves B cells and CD4+ T cells but not CD8+ T cells. *Infect Immun* **68**: 6979–6987.

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

Moulder, J.W., Novosel, D.L., and Officer, J.E. (1963). Inhibition of the Growth of Agents of the Psittacosis Group By D-Cycloserine and Its Specific Reversal By D-Alanine. *J Bacteriol* **85**: 707–711.

Moulder, J.W. (1993). Why is *Chlamydia* sensitive to penicillin in the absence of peptidoglycan? *Infect Agents Dis* **2**: 87–99.

Mpiga, P., and Ravaoarinoro, M. (2006). *Chlamydia trachomatis* persistence: An update. *Microbiol Res* **161**: 9–19.

Mukhopadhyay, S., Miller, R.D., Sullivan, E.D., Theodoropoulos, C., Mathews, S.A., Timms, P., and Summersgill, J.T. (2006). Protein expression profiles of *Chlamydia pneumoniae* in models of persistence versus those of heat shock stress response. *Infect Immun* **74**: 3853–3863.

Myers, G.S.A., Mathews, S.A., Eppinger, M., Mitchell, C., O'Brien, K.K., White, O.R., *et al*. (2009). Evidence that human *Chlamydia pneumoniae* was zoonotically acquired. *J Bacteriol* **191**: 7225–7233.

Mygind, P., Christiansen, G., and Birkelund, S. (1998). Topological analysis of *Chlamydia trachomatis* L2 outer membrane protein 2. *J Bacteriol* **180**: 5784–7.

Mygind, P., Christiansen, G., Persson, K., and Birkelund, S. (1998). Analysis of the humoral immune response to *Chlamydia* outer membrane protein 2. *Clin Diagn Lab Immunol* **5**: 313–8.

Mygind, P.H., Christiansen, G., Roepstorff, P., and Birkelund, S. (2000). Membrane proteins PmpG and PmpH are major constituents of *Chlamydia trachomatis* L2 outer membrane complex. *FEMS Microbiol Lett* **186**: 163–169.

Nally, J.E., Whitelegge, J.P., Bassilian, S., Blanco, D.R., and Lovett, M.A. (2007). Characterization of the outer membrane proteome of *Leptospira interrogans* expressed during acute lethal infection. *Infect Immun* **75**: 766–773.

Narita, T., Wyrick, P.B., and Manire, G.P. (1976). Effect of Alkali on the Structure of Cell Envelopes of *Chlamydia psittaci* Elementary Bodies. *J Bacteriol* **125**: 300–307.

Nemeghaire, S., Roelandt, S., Argudín, M.A., Haesebrouck, F., and Butaye, P. (2013). Characterization of methicillin-resistant *Staphylococcus aureus* from healthy carrier chickens. *Avian Pathol* **42**: 342–6.

Newhall, W.J. (1987). Biosynthesis and disulfide cross-linking of outer membrane components during the growth cycle of *Chlamydia trachomatis*. *Infect Immun* **55**: 162–8.

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

Nicholson, T.L., Chiu, K., and Stephens, R.S. (2004). *Chlamydia trachomatis* Lacks an Adaptive Response to Changes in Carbon Source Availability. *Society* **72**: 4286–4289.

Nicholson, T.L., Olinger, L., Chong, K., Schoolnik, G., and Stephens, R.S. (2003). Global Stage-Specific Gene Regulation during the Developmental Cycle of *Chlamydia trachomatis*. *J Bacteriol* **185**: 3179–3189.

Niessner, A., Kaun, C., Zorn, G., Speidl, W., Türel, Z., Christiansen, G., *et al*. (2003). Polymorphic membrane protein (PMP) 20 and PMP 21 of *Chlamydia pneumoniae* induce proinflammatory mediators in human endothelial cells in vitro by activation of the nuclear factor-kappaB pathway. *J Infect Dis* **188**: 108–13.

Ouellette, S.P., and Carabeo, R.A. (2010). A functional slow recycling pathway of transferrin is required for growth of Chlamydia. *Front Microbiol* **1**: 1–12.

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

Packiam, M., Weinrick, B., Jacobs, W.R., and Maurelli, A.T. (2015). Structural characterization of muropeptides from *Chlamydia trachomatis* peptidoglycan by mass spectrometry resolves "chlamydial anomaly". *Proc Natl Acad Sci U S A* **112**: 11660–5.

Pal, S., Barnhart, K., Wei, Q., Abai, A., Peterson, E., and De la Maza, L.M. (1999). Vaccination of mice with DNA plasmids coding for the *Chlamydia trachomatis* major outer membrane protein elicits an immune response but fails to protect against a genital challenge. *Vaccine* **17**: 459–465.

Pal, S., Peterson, E.M., Rappuoli, R., Ratti, G., and De La Maza, L.M. (2005). Immunization with the *Chlamydia trachomatis* major outer membrane protein, using adjuvants developed for human vaccines, can induce partial protection in a mouse model against a genital challenge. *Vaccine* **24**: 766–775.

Pannekoek, Y., Dickx, V., Beeckman, D.S.A., Jolley, K.A., Keijzers, W.C., Vretou, E., *et al*. (2010). Multi locus sequence typing of *Chlamydia* reveals an association between *Chlamydia psittaci* genotypes and host species. *PLoS One* **5**: e14179.

Pannekoek, Y., Morelli, G., Kusecek, B., Morré, S.A., Ossewaarde, J.M., Langerak, A.A., van der Ende, A. (2008). Multi locus sequence typing of *Chlamydiales*: clonal groupings within the obligate intracellular bacteria *Chlamydia trachomatis*. *BMC Microbiol* **8**: 42.

Parsons, L.M., Lin, F., and Orban, J. (2006). Peptidoglycan recognition by Pal, an outer membrane lipoprotein. *Biochemistry* **45**: 2122–2128.

Patin, D., Bostock, J., Blanot, D., Mengin-Lecreulx, D., and Chopra, I. (2009). Functional and biochemical analysis of the *Chlamydia trachomatis* ligase MurE. *J Bacteriol* **191**: 7430–7435.

Patin, D., Bostock, J., Chopra, I., Mengin-Lecreulx, D., and Blanot, D. (2012). Biochemical characterisation of the chlamydial MurF ligase, and possible sequence of the chlamydial peptidoglycan pentapeptide stem. *Arch Microbiol* **194**: 505–512.

Peeling, R.W., and Mabey, D.C. (1999). Heat shock protein expression and immunity in chlamydial infections. *Infect Dis Obs Gynecol* **7**: 72–79.

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

Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Res* **29**: e45.

Piper, R.C., and Luzio, J.P. (2001). Late endosomes: sorting and partitioning in multivesicular bodies. *Traffic* **2**: 612–621.

Pospischil, A., Borel, N., and Andersen, A.A. (2010). *Chlamydia*. In *Pathogenesis of Bacterial Infections in Animals*. Gyles, C., Prescott, J., Songer, J., and Thoen, C. (eds). Wiley-Blackwell, pp. 575–588.

Putman, T.E., Suchland, R.J., Ivanovitch, J.D., and Rockey, D.D. (2013). Culture-independent sequence analysis of Chlamydia trachomatis in urogenital specimens identifies regions of recombination and in-patient sequence mutations. *Microbiol (United Kingdom)* **159**: 2109–2117.

Quan, S., Hiniker, A., Collet, J., and Bardwell, J.C.A. (2013). Isolation of Bacteria Envelope Proteins. *In Bacterial Cell Surfaces*. Delcour, A. (ed). Humana Press, pp. 359–366.

Ramsey, K.H., and Rank, R.G. (1991). Resolution of chlamydial genital infection with antigen-specific T-lymphocyte lines. *Infect Immun* **59**: 925–931.

Ramsey, K.H., Soderberg, L.S.F., and Rank, R.G. (1988). Resolution of chlamydial genital infection in B-cell-deficient mice and immunity to reinfection. *Infect Immun* **56**: 1320–1325.

Rank, R.G., and Whittum-Hudson, J.A. (2010). Protective immunity to Chlamydial genital infection: evidence from animal studies. *J Infect Dis* **201**: 168–177.

Raulston, J.E. (1995). Chlamydial envelope components and pathogen-host cell interactions. *Mol Microbiol* **15**: 607–16.

Raulston, J.E. (1997). Response of *Chlamydia trachomatis* serovar E to iron restriction in vitro and evidence for iron-regulated chlamydial proteins. *Infect Immun* **65**: 4539–47.

Raulston, J.E., Davis, C.H., Paul, T.R., Hobbs, J.D., and Wyrick, P.B. (2002). Surface Accessibility of the 70-Kilodalton *Chlamydia trachomatis* Heat Shock Protein following Reduction of Outer Membrane Protein Disulfide Bonds. *Infect Immun* **70**: 535–543.

Read, T.D. (2003). Genome sequence of *Chlamydophila caviae* (*Chlamydia psittaci* GPIC): examining the role of niche-specific genes in the evolution of the *Chlamydiaceae*. *Nucleic Acids Res* **31**: 2134–2147.

Read, T.D., Brunham, R.C., Shen, C., Gill, S.R., Heidelberg, J.F., White, O., *et al*. (2000). Genome sequences of *Chlamydia trachomatis* MoPn and *Chlamydia pneumoniae* AR39. *Nucleic Acids Res* **28**: 1397–406.

Reiter, L., Kolstø, A.B., and Piehler, A.P. (2011). Reference genes for quantitative, reverse-transcription PCR in *Bacillus cereus* group strains throughout the bacterial life cycle. *J Microbiol Methods* **86**: 210–217.

Rejman Lipinski, A., Heymann, J., Meissner, C., Karlas, A., Brinkmann, V., Meyer, T.F., and Heuer, D. (2009). Rab6 and Rab11 regulate *Chlamydia trachomatis* development and golgin-84-dependent Golgi fragmentation. *PLoS Pathog* **5**: e1000615.

Robertson, D.K, Gu, L., Rowe, R.K., and Beatty, W.L. (2009). Inclusion biogenesis and reactivation of persistent *Chlamydia trachomatis* requires host cell sphingolipid biosynthesis. *PLoS Pathog* **5**: e1000664.

Rockey, D.D., Fischer, E.R., and Hackstadt, T. (1996). Temporal analysis of the developing *Chlamydia psittaci* inclusion using flourescent and electron microscopy. *Infect Immun* **64**: 4269–4278.

Rockey, D.D., Scidmore, M.A., Bannantine, J.P., and Brown, W.J. (2002). Proteins in the chlamydial

inclusion membrane. *Microbes Infect* **4**: 333–40.

Roulis, E., Bachmann, N., Polkinghorne, A., Hammerschlag, M., Kohlhoff, S., and Timms, P. (2014). Draft Genome and Plasmid Sequences of *Chlamydia pneumoniae* Strain B21 from an Australian Endangered Marsupial , the Western Barred Bandicoot. *Genome Announc* **2**: 3–4.

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

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

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

Sachse, K., Laroucau, K., Riege, K., Wehner, S., Dilcher, M., Creasy, H.H., *et al*. (2014). Evidence for the existence of two new members of the family *Chlamydiaceae* and proposal of *Chlamydia avium* sp. nov. and *Chlamydia gallinacea* sp. nov. *Syst Appl Microbiol* **37**: 79-88.

Sahl, J.W., Steinsland, H., Redman, J.C., Angiuoli, S. V., Nataro, J.P., Sommerfelt, H., and Rasko, D.A. (2011). A comparative genomic analysis of diverse clonal types of enterotoxigenic *Escherichia coli* reveals pathovar-specific conservation. *Infect Immun* **79**: 950–960.

Sait, M., Clark, E.M., Wheelhouse, N., Livingstone, M., Spalding, L., Siarkou, V.I., *et al*. (2011). Genome sequence of the *Chlamydophila abortus* variant strain LLG. *J Bacteriol* **193**: 4276–4277.

Saka, H.A., Thompson, J.W., Chen, Y.-S., Kumar, Y., Dubois, L.G., Moseley, M.A., and Valdivia, R.H. (2011). Quantitative proteomics reveals metabolic and pathogenic properties of *Chlamydia trachomatis* developmental forms. *Mol Microbiol* **82**: 1185–203.

Saka, H.A., and Valdivia, R.H. (2010). Acquisition of nutrients by Chlamydiae: unique challenges of living in an intracellular compartment. *Curr Opin Microbiol* **13**: 4–10.

Sanchez-Campillo, M., Bini, L., Comanducci, M., Raggiaschi, R., Marzocchi, B., Pallini, V., and Ratti, G. (1999). Identification of immunoreactive proteins of *Chlamydia trachomatis* by Western blot analysis of a two-dimensional electrophoresis map with patient sera. *Electrophoresis* **20**: 2269–2279.

Sandoz, K.M., and Rockey, D.D. (2011). Antibiotic resistance in *Chlamydiae*. *Future Microbiol* **5**: 1427–1442.

Sardinia, L.M., Segal, E., and Ganem, D. (1988). Developmental regulation of the cysteine-rich outer-membrane proteins of murine *Chlamydia trachomatis*. *J Gen Microbiol* **134**: 997–1004.

Schachter, J., and Dawson, C. (1990). The epidemiology of trachoma predicts more blindness in the future. *Scand J INfect Dis Suppl* **69**: 55–62.

Schautteet, K., and Vanrompay, D. (2011). *Chlamydiaceae* infections in pig. *Vet Res* **42**: 29.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., *et al*. (2009). Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* **75**: 7537–7541.

Schneider, C.A., Rasband, W.S., and Eliceiri, K.W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* **9**: 671–675.

Schöfl, G., Voigt, A., Litsche, K., Sachse, K., and Saluz, H.P. (2011). Complete genome sequences of four mammalian isolates of *Chlamydophila psittaci*. *J Bacteriol* **193**: 4258.

Scidmore, M.A. (2011). Recent Advances in *Chlamydia* Subversion of Host Cytoskeletal and Membrane Trafficking Pathways. *Microbes Infect* **13**: 527–535.

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

Scidmore, M.A., Rockey, D.D., Fischer, E.R., Heinzen, R.A., and Hackstadt, T. (1996). Vesicular interactions of the *Chlamydia trachomatis* inclusion are determined by chlamydial early protein synthesis rather than route of entry. *Infect Immun* **64**: 5366–5372.

Seabra, M.C., and Wasmeier, C. (2004). Controlling the location and activation of Rab GTPases. *Curr Opin Cell Biol* **16**: 451–457.

Seder, R., and Hill, A. (2000). Vaccines against intracellular infections requiring cellular immunity. *Nature* **6797**: 793–798.

Seth-Smith, H.M.B., Harris, S.R., Rance, R., West, A.P., Severin, J.A., Ossewaarde, J.M., *et al*. (2011). Genome sequence of the zoonotic pathogen *Chlamydophila psittaci*. *J Bacteriol* **193**: 1282–1283.

Shaw, J., Grund, V., Durling, L., Crane, D., and Caldwell, H.D. (2002). Dendritic cells pulsed with a recombinant chlamydial major outer membrane protein antigen elicit a CD4(+) type 2 rather than type 1 immune response that is not protective. *Infect Immun* **70**: 1097–1105.

Shirai, M., Hirakawa, H., Kimoto, M., Tabuchi, M., Kishi, F., Ouchi, K., *et al*. (2000a). Comparison of whole genome sequences of *Chlamydia pneumoniae* J138 from Japan and CWL029 from USA. *Nucleic Acids Res* **28**: 2311–2314.

Shirai, M., Hirakawa, H., Ouchi, K., Tabuchi, M., Kishi, F., Kimoto, M., *et al*. (2000b). Comparison of outer membrane protein genes omp and pmp in the whole genome sequences of *Chlamydia pneumoniae* isolates from Japan and the United States. *J Infect Dis* **181**: S524–7.

Sibley, L.D., Krahenbuhl, J.L., Adams, G.M.W., and Weidner, E. (1986). Toxoplasma modifies macrophage phagosomes by secretion of a vesicular network rich in surface proteins. *J Cell Biol* **103**: 867–874.

Sihto, H.M., Tasara, T., Stephan, R., and Johler, S. (2014). Validation of reference genes for normalization of qPCR mRNA expression levels in *Staphylococcus aureus* exposed to osmotic and lactic acid stress conditions encountered during food production and preservation. *FEMS Microbiol Lett* **356**: 134–140.

Skipp, P., Robinson, J., O'Connor, C.D., and Clarke, I.N. (2005). Shotgun proteomic analysis of *Chlamydia trachomatis*. *Proteomics* **5**: 1558–73.

Smith, K., Campbell, C., Murphy, J., Stobierski, M., and Tengelsen, L. (2011). Compendium of measures to prevent disease associated with animals in public settings, 2009: National Association of State Public Health Veterinarians, Inc. (NASPHV). *J Exot Pet Med* **20**: 32–45.

Somboonna, N., Wan, R., Ojcius, D.M., Pettengill, M.A., Joseph, S.J., Chang, A., *et al*. (2011). Hypervirulent *Chlamydia trachomatis* Clinical Strain Is a Recombinant between Lymphogranuloma Venereum (L2) and D Lineages. *MBio* **2**: 1–12.

Souriau, A., Salinas, J., De Sa, C., Layachi, K., and Rodolakis, A. (1994). Identification of subspecies- and serotype 1-specific epitopes on the 80- to 90-kilodalton protein region of *Chlamydia psittaci* that may be useful for diagnosis of chlamydial induced abortion. *Am J Vet Res* **55**: 510–4.

Spalatin, J., Fraser, C.E., Connell, R., Hanson, R.P., and Berman, D.T. (1966). Agents of psittacosis-lymphogranuloma venereum group isolated from muskrats and snowshoe hares in Saskatchewan. *Can J Comp Med Vet Sci* **30**: 260–264.

Speer, C.A., and Whitmire, W.M. (1989). Shedding of the immunodominant P20 surface antigen of *Eimeria bovis* sporozoites. *Infect Immun* **57**: 999–1001.

Stamatakis, A. (2014). RAxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* **30**: 1312–1313.

Stephens, R.S. (1998). Genome Sequence of an Obligate Intracellular Pathogen of Humans: *Chlamydia trachomatis*. *Science* **282**: 754–759.

Stephens, R.S., Koshiyama, K., Lewis, E., and Kubo, A. (2001). Heparin-binding outer membrane protein of chlamydiae. *Mol Microbiol* **40**: 691–9.

Stephens, R.S., and Lammel, C.J. (2001). Chlamydia outer membrane protein discovery using genomics. *Curr Opin Microbiol* **4**: 16–20.

Stephens, R.S., Sanchez-Pescador, R., Wagar, E.A., Inouye, C., and Urdea, M.S. (1987). Diversity of *Chlamydia trachomatis* major outer membrane protein genes. *J Bacteriol* **169**: 3879–85.

Stewardson, A.J., and Grayson, M.L. (2010). Psittacosis. *Infect Dis Clin North Am* **24**: 7–25.

Struyve, M., Moons, M., and Tommassen, J. (1991). Carboxy-terminal phenylalanine is essential for the correct assembly of a bacterial outer membrane protein. *J Mol Biol* **218**: 141–148.

Su, H., and Caldwell, H.D. (1991). In vitro neutralization of *Chlamydia trachomatis* by monovalent Fab antibody specific to the major outer membrane protein. *Infect Immun* **59**: 2843–2845.

Su, H., and Caldwell, H.D. (1992). Immunogenicity of a Chimeric Peptide Corresponding to T Helper and B Cell Epitopes of the *Chlamydia trachomatis* Major Outer Membrane Protein. *J Exp Med* **175**: 227–235.

Su, H., and Caldwell, H.D. (1995). CD4+ T Cells Play a Significant Role in Adoptive Immunity to *Chlamydia trachomatis* Infection of the Mouse Genital Tract. *Infect Immun* **63**: 3302–3308.

Su, H., Feilzer, K., Caldwell, H.D., and Morrison, R.P. (1997). *Chlamydia trachomatis* genital tract infection of antibody-deficient gene knockout mice. *Infect Immun* **65**: 1993–1999.

Su, H., McClarty, G., Dong, F., Hatch, G.M., Pan, Z.K., and Zhong, G. (2004). Activation of Raf/MEK/ERK/cPLA2 Signaling Pathway Is Essential for Chlamydial Acquisition of Host Glycerophospholipids. *J Biol Chem* **279**: 9409–9416.

Su, H., Parnell, M., and Caldwell, H.D. (1995). Protective efficacy of a parenterally administered MOMP-derived synthetic oligopeptide vaccine in a murine model of *Chlamydia trachomatis* genital tract infection: serum neutralizing IgG antibodies do not protect against chlamydial genital tract infection. *Vaccine* **13**: 1023–1032.

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

Su, H., Watkins, N.G., Zhang, Y.X., and Caldwell, H.D. (1990). *Chlamydia trachomatis*-host cell interactions: role of the chlamydial major outer membrane protein as an adhesin. *Infect Immun* **58**: 1017–25.

Suchland, R.J., Jeffrey, B.M., Xia, M., Bhatia, A., Chu, H.G., Rockey, D.D., and Stamm, W.E. (2008). Identification of concomitant infection with *Chlamydia trachomatis* IncA-negative mutant and wild-type strains by genomic, transcriptional, and biological characterizations. *Infect Immun* **76**: 5438–5446.

Suchland, R.J., Sandoz, K.M., Jeffrey, B.M., Stamm, W.E., and Rockey, D.D. (2009). Horizontal Transfer of Tetracycline Resistance among *Chlamydia spp*. in Vitro. *Antimicrob Agents Chemother* **53**: 4604–4611.

Südhof, T., and Rothman, J. (2009). Membrane Fusion: Grappling with SNARE and SM Proteins. *Science* **323**: 474–477.

Swanson, K.A., Taylor, L.D., Frank, S.D., Sturdevant, G.L., Fischer, E.R., Carlson, J.H., *et al*. (2009). *Chlamydia trachomatis* polymorphic membrane protein D is an oligomeric autotransporter with a higher-order structure. *Infect Immun* **77**: 508–16.

Tamura, A., Matsumoto, A., Manire, G.P., and Higashi, N. (1971). Electron microscopic observations on the structure of the envelopes of mature elementary bodies and developmental reticulate forms of *Chlamydia psittaci*. *J Bacteriol* **105**: 355–60.

Tamura, A., and Manire, G.P. (1968). Effect of penicillin on the multiplication of meningopneumonitis organisms (*Chlamydia psittaci*). *J Bacteriol* **96**: 875–880.

Tan, C., Hsia, R., Shou, H., Carrasco, J.A., Rank, R.G., and Bavoil, P.M. (2010). Variable expression of surface-exposed polymorphic membrane proteins in in vitro-grown *Chlamydia trachomatis*. *Cell Microbiol* **12**: 174–87.

Tan, C., Hsia, R., Shou, H., Haggerty, C.L., Ness, R.B., Gaydos, C.A., *et al*. (2009). *Chlamydia trachomatis*-Infected Patients Display Variable Antibody Profiles against the Nine-Member Polymorphic Membrane Protein Family. *Cell Microbiol* **77**: 3218–3226.

Tan, T.W., Herring, A.J., Anderson, I.E., and Jones, G.E. (1990). Protection of sheep against *Chlamydia psittaci* infection with a subcellular vaccine containing the major outer membrane protein. *Infect Immun* **58**: 3101–8.

Tanzer, R.J., and Hatch, T.P. (2001). Characterization of Outer Membrane Proteins in *Chlamydia trachomatis* LGV Serovar L2. *J Bacteriol* **183**: 2686–2690.

Taraska, T., Ward, D.M., Ajioka, R.S., Wyrick, P.B., Davis-Kaplan, S.R., Davis, C.H., and Kaplan, J. (1996). The late chlamydial inclusion membrane is not derived from the endocytic pathway and is relatively deficient in host proteins. *Infect Immun* **64**: 3713–3727.

Tasara, T., and Stephan, R. (2007). Evaluation of housekeeping genes in *Listeria monocytogenes* as potential internal control references for normalizing mRNA expression levels in stress adaptation models using real-time PCR. *FEMS Microbiol Lett* **269**: 265–272.

Taylor, B.D., Darville, T., Tan, C., Bavoil, P.M., Ness, R.B., and Haggerty, C.L. (2011). The role of *Chlamydia trachomatis* polymorphic membrane proteins in inflammation and sequelae among women with pelvic inflammatory disease. *Infect Dis Obstet Gynecol* **2011**: 989762.

Thalmann, J., Janik, K., May, M., Sommer, K., Ebeling, J., Hofmann, F., *et al*. (2010). Actin re-organization induced by *Chlamydia trachomatis* serovar D - evidence for a critical role of the effector protein CT166 targeting rac. *PLoS One* **5**: 1–14.

Thibodeau, A., Fravalo, P., Laurent-Lewandowski, S., Guévremont, E., Quessy, S., and Letellier, A. (2011). Presence and characterization of *Campylobacter jejuni* in organically raised chickens in Quebec. *Can J Vet Res* **75**: 298–307.

Thomson, N.R., Holden, M.T.G., Carder, C., Lennard, N., Lockey, S.J., Marsh, P., *et al*. (2008). *Chlamydia trachomatis*: genome sequence analysis of lymphogranuloma venereum isolates. *Genome Res* **18**: 161–71.

Thomson, N.R., Yeats, C., Bell, K., Holden, M.T.G., Bentley, S.D., Livingstone, M., *et al*. (2005). The *Chlamydophila abortus* genome sequence reveals an array of variable proteins that contribute to interspecies variation. *Genome Res* **15**: 629–640.

Ting, L.M., Hsia, R.C., Haidaris, C.G., and Bavoil, P.M. (1995). Interaction of outer envelope proteins of *Chlamydia psittaci* GPIC with the HeLa cell surface. *Infect Immun* **63**: 3600–8.

Tjaden, J., Winkler, H.H., Schwöppe, C., Van Der Laan, M., and Neuhaus, H.E. (1999). Two Nucleotide Transport Proteins in *Chlamydia trachomatis*, One for Net Nucleoside Triphosphate Uptake and the Other for Transport of Energy. *J Bacteriol* **181**: 1196–1202.

Tran, T.D.H., Kwon, H.Y., Kim, E.H., Kim, K.W., Briles, D.E., Pyo, S., and Rhee, D.K. (2011). Decrease in penicillin susceptibility due to heat shock protein ClpL in *Streptococcus pneumoniae*. *Antimicrob Agents Chemother* **55**: 2714–2728.

Tseng, T.T., Tyler, B.M., and Setubal, J.C. (2009). Protein secretion systems in bacterial-host associations, and their description in the Gene Ontology. *BMC Microbiol* **9**: S2.

Tuffrey, M., Alexander, F., Conlan, W., Woods, C., and Ward, M. (1992). Heterotypic protection of mice against chlamydial salpingitis and colonization of the lower genital tract with a human serovar F isolate of *Chlamydia trachomatis* by prior immunization with recombinant serovar L1 major outer-membrane protein. *J Gen Microbiol* **138**: 1707–1715.

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

Valihrach, L., and Demnerova, K. (2012). Impact of normalization method on experimental outcome using RT-qPCR in *Staphylococcus aureus*. *J Microbiol Methods* **90**: 214–216.

Vandahl, B.B., Pedersen, A.S., Gevaert, K., Holm, A., Vandekerckhove, J., Christiansen, G., and Birkelund, S. (2002). The expression, processing and localization of polymorphic membrane proteins in *Chlamydia pneumoniae* strain CWL029. *BMC Microbiol* **2**: 1–12.

Vandahl, B.B., Birkelund, S., and Christiansen, G. (2004). Genome and proteome analysis of *Chlamydia*. *Proteomics* **4**: 2831–42.

Vandahl, B.B., Birkelund, S., Demol, H., Hoorelbeke, B., Christiansen, G., Vandekerckhove, J., and Gevaert, K. (2001). Proteome analysis of the *Chlamydia pneumoniae* elementary body. *Electrophoresis* 1204–1223.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., and Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* **3**: 1-12.

Van Lent, S., Piet, J.R., Beeckman, D., van der Ende, A., Van Nieuwerburgh, F., Bavoil, P., *et al*. (2012). Full Genome Sequences of All Nine *Chlamydia psittaci* Genotype Reference Strains. *J Bacteriol* **194**: 6930–1.

Van Ooij, C., Kalman, L., van IJzendoorn, S., Nishijima, M., Hantada, K., Mostov, K., and Engel, J. (2000). Host-cell derived sphingolipids are required for the intracellular growth of *Chlamydia trachomatis*. *Cell Microbiol* **2**: 627–638.

Vanrompay, D., Butaye, P., Van Nerom, A., Ducatelle, R., and Haesebrouck, F. (1997). The

prevalence of *Chlamydia psittaci* infections in Belgian commercial turkey poults. *Vet Microbiol* **54**: 85–93.

Vanrompay, D., Charlier, G., Ducatelle, R., and Haesebrouck, F. (1996). Ultrastructural changes in avian *Chlamydia psittaci* serovar A-, B-, and D-infected Buffalo Green Monkey cells. *Infect Immun* **64**: 1265–71.

Vanrompay, D., Ducatelle, R., and Haesebrouck, F. (1995a). *Chlamydia psittaci* Infections - a Review with Emphasis on Avian Chlamydiosis. *Vet Microbiol* **45**: 93–119.

Vanrompay, D., Mast, J., Ducatelle, R., Haesebrouck, F., and Goddeeris, B. (1995b). *Chlamydia psittaci* in turkeys: pathogenesis of infections in avian serovars A, B and D. *Vet Microbiol* **47**: 245–56.

Vasilevsky, S., Stojanov, M., Greub, G., Baud, D., *et al*. (2016). Chlamydial polymorphic membrane proteins : regulation , function and potential vaccine candidates. *Virulence* **5594**: 11–22.

Verdoodt, F., Willems, M., Dhondt, I., Houthoofd, W., Bert, W., and Vos, W. De (2012). Measuring S-phase duration of adult stem cells in the flatworm *Macrostomum lignano* by double replication labelling and quantitative colocalization analysis. *Cell Biol Int* **36**: 1251–1259.

Verminnen, K., Van Loock, M., Hafez, H.M., Ducatelle, R., Haesebrouck, F., and Vanrompay, D. (2006). Evaluation of a recombinant enzyme-linked immunosorbent assay for detecting *Chlamydophila psittaci* antibodies in turkey sera. *Vet Res* **37**: 623–32.

Voigt, A., Schöfl, G., Heidrich, A., Sachse, K., and Saluz, H.P. (2011). Full-length de novo sequence of the *Chlamydophila psittaci* type strain, 6BC. *J Bacteriol* **193**: 2662–2663.

Voigt, A., Schöfl, G., and Saluz, H.P. (2012). The *Chlamydia psittaci* genome: a comparative analysis of intracellular pathogens. *PLoS One* **7**: e35097.

Voulhoux, R., Bos, M.P., Geurtsen, J., Mols, M., and Tommassen, J. (2003). Role of a highly conserved bacterial protein in outer membrane protein assembly. *Science* **299**: 262–265.

Wang, Y., Berg, E. a, Feng, X., Shen, L., Smith, T., Costello, C.E., and Zhang, Y. (2006). Identification of surface-exposed components of MOMP of *Chlamydia trachomatis* serovar F. *Protein Sci* **15**: 122–34.

Watson, M.W., Lambden, P.R., and Clarke, I.N. (1991). Genetic diversity and identification of human infection by amplification of the chlamydial 60-kilodalton cysteine-rich outer membrane protein gene. *J Clin Microbiol* **29**: 1188–93.

Watson, M.W., Lambden, P.R., Everson, J.S., and Clarke, I.N. (1994). Immunoreactivity of the 60 kDa cysteine-rich proteins of Chlamydia trachomatis, *Chlamydia psittaci* and *Chlamydia pneumoniae* expressed in *Escherichia coli*. *Microbiology* **140**: 2003–11.

Wehrl, W., Brinkmann, V., Jungblut, P.R., Meyer, T.F., and Szczepek, A.J. (2004). From the inside out-processing of the chlamydial autotransporter PmpD and its role in bacterial adhesion and activation of human host cells. *Mol Microbiol* **51**: 319–34.

Wheelhouse, N., Aitchison, K., Spalding, L., Livingstone, M., and Longbottom, D. (2009). Transcriptional analysis of in vitro expression patterns of *Chlamydophila abortus* polymorphic outer membrane proteins during the chlamydial developmental cycle. *Vet Res* **40**: 47.

Wheelhouse, N.M., Sait, M., Aitchison, K., Livingstone, M., Wright, F., McLean, K., *et al*. (2012a). Processing of *Chlamydia abortus* Polymorphic Membrane Protein 18D during the Chlamydial Developmental Cycle. *PLoS One* **7**: e49190.

Wheelhouse, N.M., Sait, M., Wilson, K., Aitchison, K., McLean, K., Smith, D.G.E., and Longbottom, D. (2012b). Expression patterns of five polymorphic membrane proteins during the *Chlamydia abortus* developmental cycle. *Vet Microbiol* **160**: 525–9.

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

Wylie, J.L., Hatch, G.M., and Mcclarty, G. (1997). Host cell phospholipids are trafficked to and then modified by *Chlamydia trachomatis*. *J Bacteriol* **179**: 7233–7242.

Wyrick, P.B. (2010). *Chlamydia trachomatis* Persistence In Vitro: An Overview. *J Infect Dis* **201**: 88–95.

Yang, J. (1997). Prevention of Apoptosis by Bcl-2: Release of Cytochrome c from Mitochondria Blocked. *Science (80-)* **275**: 1129–1132.

Yeung, Y., Nieves, E., Angeletti, R., and Stanley, E.R. (2008). Removal of detergent from protein digests for mass spectrometry analysis. *Anal Biochem* **382**: 135–137.

Yin, L., Kalmar, I.D., Lagae, S., Vandendriessche, S., Vanderhaeghen, W., Butaye, P., *et al*. (2013). Emerging *Chlamydia psittaci* infections in the chicken industry and pathology of *Chlamydia psittaci* genotype B and D strains in specific pathogen free chickens. *Vet Microbiol* **162**: 740–749

Yu, H., Karunakaran, K.P., Jiang, X., and Brunham, R.C. (2014). Evaluation of multisubunit recombinant polymorphic outer membrane protein T cell vaccine against *Chlamydia muridarum* genital infection in three strains of mice. *Vaccine* **6**: 4672–4680.

Yuan, Y., Zhang, Y.X., Watkins, N.G., and Caldwell, H.D. (1989). Nucleotide and deduced amino acid sequences for the four variable domains of the major outer membrane proteins of the 15 *Chlamydia trachomatis* serovars. *Infect Immun* **57**: 1040–9.

Yucel, N., Citak, S., and Bayhün, S. (2011). Antimicrobial resistance profile of *Staphylococcus aureus* isolated from clinical samples and foods of animal origin. *Foodborne Pathog Dis* **8**: 427–31.

Zhang, Y.X., Morrison, S.G., Caldwell, H.D., and Baehr, W. (1989). Cloning and sequence analysis of the major outer membrane protein genes of two *Chlamydia psittaci* strains. *Infect Immun* **57**: 1621–5.

Zhang, J., and Stephens, R.S. (1992). Mechanism of *C. trachomatis* attachment to eukaryotic host cells. *Cell* **69**: 861–869.

Zhang, Y., Stewart, S.J., and Caldwell, H.D. (1989). Protective Monoclonal Antibodies to *Chlamydia trachomatis* Serovar- and Serogroup-Specific Major Outer Membrane Protein Determinants. *Microbiology* **57**: 636–638.

Zhang, Y.X., Stewart, S., Joseph, T., Taylor, H.R., and Caldwell, H.D. (1987). Protective monoclonal antibodies recognize epitopes located on the major outer membrane protein of *Chlamydia trachomatis*. *J Immunol* **138**: 575–81.

Zhang, Y.X., Watkins, N.G., Stewart, S., and Caldwell, H.D. (1987). The low-molecular-mass, cysteine-rich outer membrane protein of *Chlamydia trachomatis* possesses both biovar- and species-specific epitopes. *Infect Immun* **55**: 2570–3.

Zhao, W., Li, Y., Gao, P., Sun, Z., Sun, T., and Zhang, H. (2011). Validation of reference genes for real-time quantitative PCR studies in gene expression levels of *Lactobacillus casei* Zhang. *J Ind Microbiol Biotechnol* **38**: 1279–1286.

Zhou, K., Zhou, L., Lim Q.E., Zou, R., Stephanopoulos, G., and Too, H.-P. (2011). Novel reference

genes for quantifying transcriptional responses of *Escherichia coli* to protein overexpression by quantitative PCR. *BMC Mol Biol* **12**: 18.

Zugel, U., and Kaufmann, S.H. (1999). Role of heat shock proteins in protection from and pathogenesis of infectious diseases. *ClinMicrobiolRev* **12**: 19–39.

Dankwoord

Ongelooflijk maar waar: we zijn aanbeland bij het laatste stukje van mijn doctoraat. Als ik er nu naar terugkijk, lijkt het een tijd die voorbij gevlogen is. Het was een tijd met heel veel mooie momenten en ervaringen, maar af en toe ging het ook wel wat moeilijker. Daarom wil ik heel graag alle collega's, vrienden en familieleden bedanken die de voorbije jaren steeds paraat stonden om me te helpen, te steunen, om plezier te maken en te zorgen dat er voldoende ontspanning was.

Mijn promotor, **Prof. dr. Daisy Vanrompay** wil ik bedanken omdat ze 5 jaar geleden vertrouwen in mij had en me de kans gaf om een doctoraat te beginnen in haar labo. Daisy, bedankt om mij te laten starten op het boeiende, uitdagende project van de Pmp eiwitten. Ik ben je heel dankbaar dat ik 8 maanden naar het labo van Prof. dr. Patrik Bavoil mocht gaan, want die ervaring is tot de dag van vandaag nog steeds de mooiste ervaring van m'n wetenschappelijke carrière. Verder wil ik je ook bedanken voor de vrijheid die ik steeds kreeg om nieuwe ideeën uit te werken, samen met mij naar mogelijke samenwerkingen met andere labo's te zoeken, de electronen-microscopie beelden samen te analyseren, mij naar verschillende internationale congressen te laten gaan en om mijn artikels en doctoraat te verbeteren.

I would like to thank **Prof. Dr. Patrik Bavoil** and his wife **Prof. Dr. Ru-ching Hsia**. Patrik, I remember the first day in your lab (the 4th of September 2011) like it was yesterday. You were so friendly, asking if I had food and all the furniture I needed at Joshua's place, otherwise you would drive me to a shop. You told me that you didn't care at what time I would start working every day, but if I would be late, you asked me to send an email, as you would be worried otherwise. Your PhD students were like your children. The time in your lab is my best scientific experience up to now. I learned a lot during my stay in your lab, you corrected chapter IV of my thesis, but also very important to me, is that you were always available during the last 4 years, when I was back in Belgium. When we had a chance to meet, you always made some time to discuss my data and give me some new ideas to move on with my research. Also when I had a hard time, you told me you believed in me and that I should hang on and soon I would be able to finish the PhD. Thank you so much! Ru-ching, you did

all the electron-microscopy work and helped me with the analyses. Thank you for your support!

Ik wil ook heel graag alle leden van mijn examencommissie (**Prof. dr. ir. Monica Höfte, Prof. dr. Els Van Damme, dr. Yvonne Pannekoek, Prof. dr. Katleen Hermans, Prof. dr. Richard Ducatelle en Prof. dr. Andre Skirtach**) bedanken om tijd vrij te maken om mijn doctoraatsthesis te lezen en voor het leveren van jullie constructieve opmerkingen. Dankzij jullie is de kwaliteit van mijn thesis er zeker op vooruit gegaan.

Uiteraard wil ik heel graag mijn collega's, de immuno's, bedanken! In het begin was ik maar een maandje bij jullie, toen ik al voor 8 maanden naar Baltimore vertrok. Toch behoorde ik voor jullie al tot de groep en stuurden jullie me een postpakket met leuke boodschappen, dat was fantastisch! Ik geloofde zelfs dat Yin dat Chinees tekstje geschreven had, hilarisch! Ook wanneer ik terug in België was, hadden we een fantastische tijd samen. Altijd was er wel wat te vertellen, de middagpauze's vlogen voorbij, iedereen stond paraat om elkaar te helpen. Ja, ik kan zeker zeggen dat mijn collega's vrienden voor het leven zijn geworden! **Annelien,** als overburen hebben we jaren samen naar het werk gefietst, toen ik ziek was en ik gepland had om een staal te gaan nemen in het weekend stelde jij direct voor om in mijn plaats te gaan, als ik besloot 40 flessen ineens op te kweken stelde jij voor me te helpen bij het oogsten, en ik kan zo nog even doorgaan! Jij stond gewoon altijd paraat, voor een babbel, om te helpen, of met wat eten als ik laat thuis kwam, nooit was er iets teveel! Heel hard bedankt daarvoor! **Stefanie**, jij was de senior van het labo toen ik aan mijn doctoraat startte. Altijd mocht ik je lastigvallen met wat vraagjes. Bovendien was je mijn grote voorbeeld van de 'plantrekker'. Je zorgde mee voor de fantastische sfeer en ik heb je dan ook echt wel gemist toen je een jaartje weg was voor je zwangerschap. Jammer dat je terugkomst van korte duur was, maar ik ben echt heel blij dat je jouw draai in het onderwijs gevonden hebt! Je bent gewoon een geboren lerares! **Leentje, Kristien** en **Evelien**, mijn drie bureaugenootjes. Ondanks dat ik niet tot de 'varkens'groep behoorde, was ik toch heel welkom in jullie bureautje in de kelder. Elke dag kwam ik met plezier naar het werk! Toen jullie 1 voor 1 het bureautje (en immunoland) verlieten miste ik zeker jullie aanwezigheid! We hebben in ons bureautje wat afgelachen en gebabbeld. Ik zal het moment dat Evelien haar kleedje voor de trouw

van haar zus aan ons toonde nooit vergeten ☺. Jullie hebben me allemaal veel geholpen, Leentje o.a. met de microscoop, Kristien o.a. met de Pmps en Evelien o.a. met wat truckjes in Mendeley en excel. Maar ook al verschilden onze projecten zeer hard, we stonden altijd voor elkaar klaar! Merci voor de leuke tijd en de oppeppende gesprekken die ik af en toe eens nodig had! **Julie,** met jouw vrolijk karakter paste je perfect in ons immuno-team! Ik had je graag opgeleid als mede Pmp-onderzoeker, maar begreep jouw keuze om ons labo te verlaten volledig. Onderzoek doen combineren met tussen de koeien zitten, lijkt mij de ideale job voor jou en ik ben dan ook heel blij dat je zo'n job gevonden hebt! **Katelijn**, toen ik terugkwam uit Baltimore werkte je niet meer bij ons, maar gelukkig kwam je ons een tijdje later nog eens vergezellen voor een 6-tal maanden. Je was altijd heel vrolijk en als ik vraagjes had stond je altijd klaar! **Cindy**, bedankt voor de babbeltjes tussendoor. Ik wens je nog veel succes bij jouw doctoraat! **Eamy,** I'll never forget our conversations about REsearch. Good luck with your defense! **Anh** and **Tho**, we had a nice time together in our lab. I want to thank you especially for the nice talks and your delicious food! Tho, I wish you good luck with your PhD! **Joanna** and **Evelien** you both joined the lab when I was finishing my PhD, but I'm sure we would get along very well. Evelien, veel success met je verdediging! **Ine**, ook jij kwam het labo versterken toen ik bijna begon te schrijven. Maar de donderdagen waren steeds heel aangenaam! Met jouw vrolijke, enthousiaste persoonlijkheid pas je zeker bij de immuno's!

Ook naast de immuno's zijn er nog wat collega's van de vakgroep die ik graag wil bedanken! **Lander**, jij bent degene die me op mijn eerste werkdag opving en zei dat mijn bureau niet op het 2de verdiep maar op het gelijkvloers of in de kelder zou zijn. In de jaren die volgden, kwam ik regelmatig wat vertellen aan jouw bureau. Bedankt om altijd zo goed te luisteren en me aan te moedigen! Je was ook een vaste afzetplaats voor mijn baksels ☺. Nog eens dubbel bedankt om me te helpen met mn vele doctoraatsvraagjes de laatste maanden! **Annelies**, het was een fantastische tijd toen jij er was! Als we elkaar tegenkwamen in de gang was het tijd voor een dikke knuffel en een babbeltje!!! Die heb ik zeker wel gemist toen jouw doctoraatsjaren er op zaten! **Lore,** jij werd mn nieuwe glyco-vriendinnetje toen Annelies het schip verliet. We hebben ook ettelijke knuffels gegeven en babbeltjes gedaan! Ik mis jouw vrolijke noot in de bureau nu al! Merci voor de leuke tijd en om altijd paraat te staan! Ik wens je nog heel veel succes bij het afronden van jouw doctoraat! **Sofie**, wij hebben ook wel

wat babbeltjes gedaan! Je hebt me ook veel geholpen met allerlei programma's zoals Inkscape en Illustrator for Biological Sequences. Ik heb ze goed kunnen gebruiken voor mijn doctoraat, merci! Je bent er nu ook bijna vanaf, ik wens je nog veel succes met jouw verdediging! **Katrien**, jou leerde ik kennen toen je plots veel gebruik kwam maken van ons q-PCR toestel. Terwijl je stond te wachten deden we uiteraard een babbeltje! Bedankt om de eerste schrijfsels van mijn eerste artikel na te lezen en me zo op weg te zetten bij het wetenschappelijk schrijven!

Ik wil ook **Sofie** en **Fien** bedanken voor alle administratieve hulp en bestellingen (ook **Annelien** wordt hiervoor bedankt!). Bij jullie langskomen was altijd een plezier, je werd altijd met de glimlach ontvangen en goed geholpen. Een welgemeende dankjewel! **Geert**, jij stond altijd klaar als er iets mis was met mn computer of als er een technisch mankement was in het labo, bedankt! Ik wil ook de andere **collega's van de vakgroep Moleculaire biotechnologie** bedanken voor de aangename tijd! **Winnok** wil ik graag nog in het bijzonder bedanken voor zijn hulp met de microscoop!

Naast alle fantastische collega's wil ik ook nog graag mijn vrienden en familie bedanken. Jullie zorgden er allen samen voor dat ik mijn doctoraat op tijd en stond eens uit mijn hoofd kon zetten. Mijn doctoraat was voor de meesten van jullie totaal niet in jullie branche, toch vroegen jullie regelmatig hoe het er mee ging, luisterden jullie en steunden jullie mij! Eerst en vooral een dikke merci aan **Lot** en **Elien**. Vanaf het begin aan de universiteit waren wij onlosmakelijk verbonden. Op elk chemicafeestje waren we samen te vinden, tijdens de vakanties gingen we samen op reis, naar de Gentse feesten, naar het cactusfestival,… Na onze studententijd zijn we nog steeds beste vriendinnen. Ik wil jullie bedanken voor al jullie steun de voorbije jaren. Zowel tijdens de eerste bachelor als tijdens mn doctoraat had ik wel eens last van faalangst en jullie stonden steeds klaar om me moed in te spreken! Ik wil ook mn vrienden van Chemica bedanken voor de leuke tijd, met in het bijzonder **Lien** en **Jolien**. Bedankt ook aan mijn chirovrienden en in het bijzonder aan **Liesbeth**, **Lieselot**, **Dorien**, **Annik** en **Camille**! Bedankt aan mijn Gentloopt vriendjes en in het bijzonder aan **Tine** en **Bianca**! Ook mn kok- en bakschoolvriendjes zorgden voor de nodige ontspanning, bedankt voor de leuke avonden!

Een heel speciaal woordje van dank gaat uit naar mijn **moeke** en **vake**. Jullie gaven mij alle kansen en steunden mij onvoorwaardelijk. Ik zag het af en toe niet meer zitten,

maar jullie geloofden altijd heel hard in mij! Jullie hebben wel wat afgezien tijdens de examenperiodes aan de universiteit, toen ik weer eens dacht dat ik het niet ging kunnen. Toch bleven jullie altijd zeggen dat het wel zou lukken, en zorgen jullie dat er een thee'tje en wat fruit klaarstond tijdens m'n pauzes. Het eten werd ook steevast klaargemaakt op het exacte moment dat ik een pauze plande. Jullie keken zelfs tv zonder geluid! Toen ik naar Amerika vertrok voor mijn doctoraat kwam moeke 3x op bezoek en zelfs vake, die nog nooit gevlogen had, kwam me bezoeken. Het was heel leuk om met jullie samen New York te ontdekken en ook gewoon om 's avonds bij jullie thuis te komen. Ik weet met zekerheid dat wat ik ook beslis in mn leven, dat jullie altijd als een blok achter mij zullen staan en dat is fantastisch. Dankuwel!!! Ook mn grootouders **moemoe** en **vava** wil ik bedanken. Moemoe, jij verbeterde met veel plezier mn masterthesis. Daaruit concludeerde je dat werken met DNA toch wel heel veel werk is. Altijd waren jullie geïnteresseerd en vroegen jullie hoe het in Gent ging. **Stijn**, **Dries** en **Jelle**, mn drie grote broers, jullie hebben me ook de voorbije jaren onvoorwaardelijk gesteund. Stijn, merci om me te komen bezoeken in Baltimore en zonder problemen reed jij samen met moeke moeke en mij naar de Niagara watervallen in Canada. Jelle, ook jij kwam samen met Eve op bezoek bij mij. Ons tripje naar de Everglades en naar Six Flags was fantastisch! Dries, mijn verblijf in Terhagen was van korte duur, maar samen op het veld werken vorige zomer was heel rustgevend en heeft me veel deugd gedaan! **Mattis**, **Maité**, **Amber**, **Anouk**, **Sam** en **Seppe**, stuk voor stuk zijn jullie mijn hartendieven! Jullie vrolijke kinderlachjes en dikke knuffels zorgden de voorbije jaren meermaals voor de ontspanning die ik nodig had!

Tot slot wil ik m'n vriend **Bart** heel hard bedanken. Ons eerste jaar was zeker geen gemakkelijk jaar. Toen we dan nog eens besloten om de deadline van mijn doctoraat te combineren met een stevige verbouwing, werd het helemaal hectisch. Gelukkig kwamen jouw mama en papa, die ik ook heel hard wil bedanken, veel helpen met de verbouwing! Maar beide deadlines zijn gehaald, nog even volhouden en we kunnen weer volop genieten! Bedankt voor jouw steun, jouw liefde, jouw levensvisie die je graag met me deelt,… Kort samengevat, bedankt om mij zo gelukkig te maken!

Curriculum vitae

Personalia

Van Lent
Sarah
Bommelbekestraat 35
8000 Bruges, Belgium
+32479/501703
sarahvanlent@gmail.com
Belgian
21st June 1988
Drivers license category B

Education

2011 – 2016	PhD Applied Biological Sciences as a Research Assistant of the Research-Foundation-Flanders (FWO – Vlaanderen), Ghent University
2014	Laboratory animal sciences (FELASA C), Ghent University
2013	Advanced Academic English: Writing Skills, Ghent University
2013	Post-academic training: Statistics in R, Ghent University
2012	qPCR experimental design and data-analysis, Biogazelle
2006-2011	Master of Science in Biochemistry and Biotechnology, magna cum laude
2000-2006	Science-Maths, Sint-Norbertusinstituut Duffel

Experience - Research skills

PCR and Real-time PCR, Fluorescence and confocal microscopy, Cell and tissue culture, Bacterial culture, Cloning, Recombinant protein expression (in prokaryotic and eukaryotic expression systems) and purification (His-tag and GST-tag), SDS-PAGE and western blotting, ELISA, Animal experiments, Working in a BSL3 lab, Writing scientific papers and reports, Presentation skills.

Thesis student: **2014-2015**: Astrid Reymer: “Ontwikkeling van een SK-6 celcultuurmodel voor de studie van persistente *Chlamydia suis* infecties”. Masterproef voorgedragen tot het behalen van de graad Master in de bio-ingenieurswetenschappen: cel- en genbiotechnologie

Languages

Dutch	Native language
English	Fluent conversation and writing
French	Basic conversation and writing

Computer skills

- Microsoft Word, Excel and Powerpoint
- qBasePlus (qPCR analysis)
- Vector NTI (sequence database)
- R (statistical software)
- ImageJ (image processing)

Publications

Van Droogenbroeck C, Dossche L, Wauman T, **Van Lent S**, Phan TTT, Beeckman DS a & Vanrompay D (2011) Use of ovotransferrin as an antimicrobial in turkeys naturally infected with Chlamydia psittaci, avian metapneumovirus and Ornithobacterium rhinotracheale. *Veterinary microbiology* **153**: 257–63

Van Lent S, Piet JR, Beeckman D, Van der Ende A, Van Nieuwerburgh F, Bavoil P, Myers G, Vanrompay D & Pannekoek Y (2012) Full Genome Sequences of All Nine Chlamydia psittaci Genotype Reference Strains. *Journal of bacteriology* **194**: 6930–1

Van Lent S, Creasy HH, Myers G & Vanrompay D (2016) The Number, Organization, and Size of Polymorphic Membrane Protein Coding Sequences as well as the Most Conserved Pmp Protein Differ within and across *Chlamydia* Species. *Molecular Microbiology and Biotechnology* **26**: 333-44.

Conferences

- Seventh Meeting of the European Society for Chlamydia Research. Amsterdam, Netherlands, 1 - 6 July 2012. Poster presentation
  "Genome sequences of all Chlamydia psittaci genotype reference strains".
- Sixth Biennal Chlamydia Basic Research Society Meeting. San Antonio, Texas, USA, 19 – 23 March 2013. Poster presentation
  "Transcriptional analysis suggests a critical role of the polymorphic membrane proteins of Chlamydia psittaci in late differentiation and/or early infection".
- Tenth Annual Amsterdam Chlamydia Meeting. Amsterdam, Netherlands, 6 February 2015. Oral presentation (won the price for best oral presentation). *"Transcriptional and translational analysis of in vitro expression profiles of Chlamydia psittaci polymorphic membrane proteins"*.
- Seventh Biennal Chlamydia Basic Research Society Meeting. New Orleans, Los Angeles, USA, 29 March – 1 April 2015. Oral presentation
  "Variable immunogold labeling and subcellular location of polymorphic membrane proteins of two Chlamydia species".

Experience Abroad

Goal: Cloning and purifying one member of each subtype of the polymorphic membrane proteins (Pmp) of *C. psittaci* to obtain polyclonal antibodies. Determine the transcript profile of all *pmp* CDSs of *C. psittaci* by RT-qPCR.

1st September 2011 – 1st May 2012.

Host: Prof. dr. Patrik Bavoil, University of Maryland, School of dentistry, Department of Molecular Pathogenesis, Baltimore, USA

Hobbies

2006-2007: part of the chiro leadership team
2007-2008: sportpraeses Chemica
2008-2009: vice praeses Chemica
Sport: running, snowboarding
Interests: cooking and travelling