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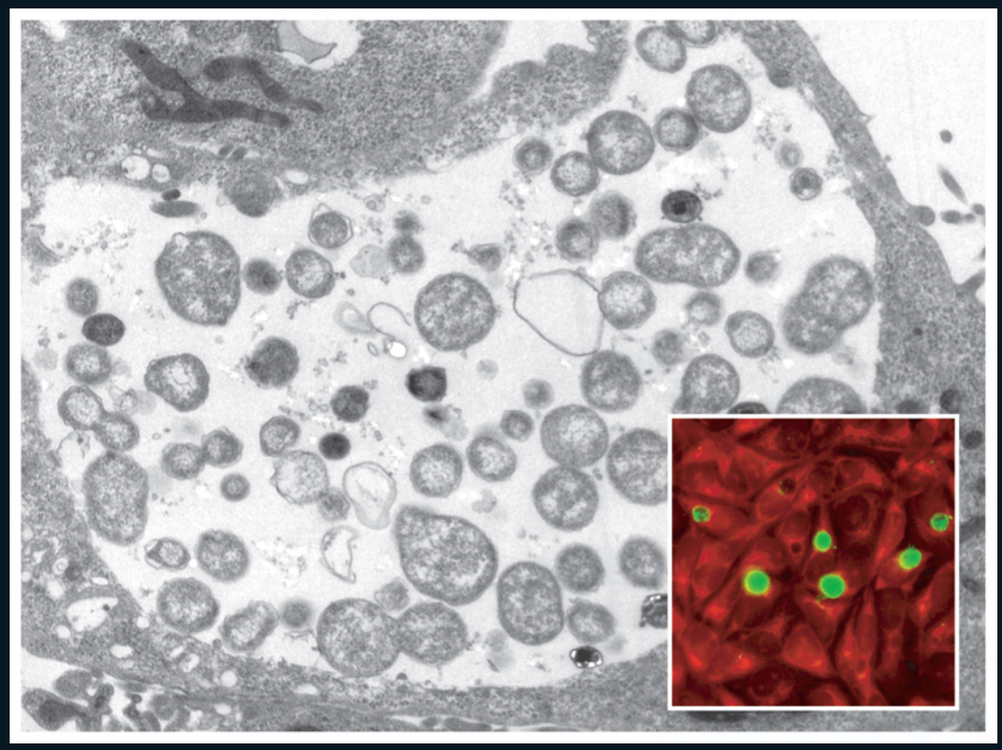

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ABSTRACT

Chlamydiae are obligate intracellular pathogens that cause a variety of diseases with clinical and public health importance. Like many Gram-negative bacteria, *Chlamydiae* employ a type III secretion (T3S) system for invasion and establishment of a protected intracellular niche for successful replication and survival within host cells. Understanding the role of T3S and bacterial effector proteins in *Chlamydia* infection will provide new insights into chlamydial pathogenesis and is important to identify novel therapeutic targets for drug intervention. In this thesis we employed different small molecule inhibitors of T3S activity in *Yersinia*, named INPs, and analyzed their effect on *Chlamydia* development. In addition, we identified and characterized a new family of T3S effector proteins.

In **paper I**, we assessed the effect of INP0400 on *C. trachomatis* development and invasion. INP0400 caused a dose and growth phase-dependent inhibition of RB multiplication at micromolar concentrations. When INP0400 was given at different stages during the infectious cycle, we observed a partial inhibition of *Chlamydia* entry, inhibition of translocation of IncG and IncA and a bacterial detachment from the inclusion membrane during the late stage of infection concomitant with an inhibition of RB to EB conversion causing a marked decrease in infectivity. Our data suggest that INPs impair progression through the infectious cycle suggesting that the T3S system is essential for *Chlamydia* pathogenesis.

In **paper II**, we found that INP0010 displays a strong growth inhibitory effect on *C. pneumoniae* development, affects translocation of the *C. pneumoniae* effector proteins IncB and IncC and leads to down-regulation of T3S associated genes collectively suggesting that INP0010 impairs T3S activity in *C. pneumoniae*.

In **paper III**, we further investigated the effect of INPs on *Chlamydia* invasion. We show that INPs impair *Chlamydia* development after entry into host cells because the efficiency of *C. trachomatis* L2 and *C. caviae* entry into epithelial cells was not altered in the presence of INPs. Moreover, entry appeared normally with recruitment of actin and the small GTPases Rac, Cdc42 and Arf6 to the bacterial entry site.

Finally, in **paper IV** we set out to identify novel T3S effectors in *Chlamydia*. We found a family of chlamydial proteins, represented by a C-terminal domain of unknown function referred to as DUF582 that contains an amino-terminal T3S signal. *C. trachomatis* members of this family were expressed late during the infectious cycle and found to be secreted into the lumen of the inclusion and the cytoplasm of infected cells.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I **Muschiol, S.**, L. Bailey, A. Gylfe, C. Sundin, K. Hultenby, S. Bergström, M. Elofsson, H. Wolf-Watz, S. Normark, and B. Henriques-Normark. 2006. A small-molecule inhibitor of type III secretion inhibits different stages of the infectious cycle of *Chlamydia trachomatis*. Proc Natl Acad Sci USA. 103:14566-14571.

II Bailey, L., A. Gylfe, C. Sundin, **S. Muschiol**, E. Elofsson, P. Nordström, B. Henriques-Normark, R. Lugert, A. Waldenström, H. Wolf-Watz, and S. Bergström. 2007. Small molecular inhibitors of type III secretion in *Yersinia* block the *Chlamydia pneumoniae* infection cycle. FEBS Let. 581:587-595.

III **Muschiol, S.**, S. Normark, B. Henriques-Normark, and A. Subtil. 2009. Small molecule inhibitors of the *Yersinia* type III secretion system impair the development of *Chlamydia* after entry into host cells. BMC Microbiol. 2009. 9:75.

IV **Muschiol, S.**, G. Boncompain, P. Dehoux, S. Normark, B. Henriques-Normark, and A. Subtil. 2009. A new family of secreted proteins in *Chlamydia*. Manuscript.

CONTENTS

LIST OF ABBREVIATIONS

aa	amino acids
CADD	*chlamydia* protein associating with death domains
EB	elementary body
FBS	fetal bovine serum
GAG	glycosaminoglycans
HA	haemagglutinin
IB	intermediate body
LB	luria broth
LGV	lymphogranuloma venereum
LD	lipid droplets
MVBs	multivesicular bodies
PCR	polymerase chain reaction
PMP	polymorphic membrane protein
RB	reticulate body
SDS-PAGE	sodium-dodecyl sulfate-polyacrylamide gel electrophoresis
spp	species
STD	sexually transmitted disease
TARP	translocated actin recruiting phosphoprotein
T3S	type III secretion
T3SS	type III secretion system
TLRs	toll-like receptors
TNF	tumor necrosis factor
TRITC	tetramethyl rhodamine iso-thiocyanate
Vamp	vesicle associated membrane protein
WHO	World Health Organization

1 INTRODUCTION

1.1 *CHLAMYDIA* – BIOLOGY AND PATHOGENESIS

1.1.1 History and taxonomy

The *Chlamydiales* are important intracellular pathogens which cause a wide range of diseases in humans and animals. In 1907, Ludwig Halberstaedter and Stanislaus von Prowazek discovered *Chlamydia trachomatis* within conjunctival scrapings of trachoma patients (102). The organism was named ”chlamydozoa” derived from the Greek word for ”cloak”. *Chlamydiae* were long thought to be viruses due to their intracellular nature. Only 50 years later, the organism was first isolated from chicken embryo yolk sacs that had been inoculated with material from infected human eyes (272, 290). In 1966, *Chlamydiae* were finally recognized as bacteria and the genus *Chlamydia* was established (185). The order *Chlamydiales* was created by Storz and Page in 1971 (255). Since then, numerous chlamydial strains have been isolated from humans and animals. Advances in phylogenetic analyses together with more detailed genetic and phenotypic information led Everett and colleagues to propose a reclassification of the order *Chlamydiales* in 1999 (66). Within the order *Chlamydiales* currently four families are recognized including the *Chlamydiaceae*, *Parachlamydiaceae*, *Waddliaceae* and *Simkaniaceae* (Figure 1). Recently another family has been proposed, the *Rhabdochlamydiaceae* (53, 143).

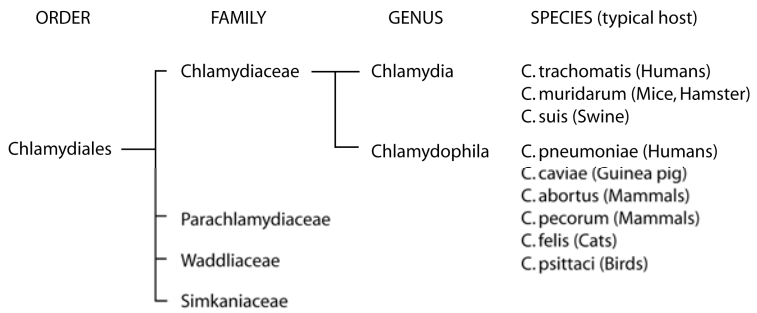

Figure 1. Revised chlamydial taxonomy as proposed by Everett and colleagues in 1999 (66). Adapted from Bush and Everett 2001 (32).

All members of the order *Chlamydiales* share a characteristic developmental cycle and their 16S and 23S ribosomal RNA has at least 80-90% sequence identity to rRNA genes of *Chlamydiaceae* (66). The family of *Chlamydiaceae* originally contained four species, *C. trachomatis, C. pneumoniae, C. pecorum* and *C. psittaci*. Everett et al., based on their phylogenetic analyses of 16S and 23S rRNA genes, separated the *Chlamydiaceae* into two distinct lineages, *Chlamydia* and *Chlamydophila*. The genus *Chlamydia* contains the three species *C. trachomatis, C. suis* and *C. muridarum*. The chlamydial strains *C. pneumoniae, C. psittaci, C. pecorum, C. abortus, C. felis* and *C. caviae* were reorganized into the genus *Chlamydophila*. Researchers in the *Chlamydia* field strongly objected this new chlamydial taxonomy. They argued that there is not enough significant sequence difference to separate the *Chlamydiaceae* into two distinct genera, and that introduction of a new word "Chlamydophila" would make all *Chlamydiae* even more obscure to the general audience (234). In their support, Tanner et al. reported that *C. trachomatis, C. psittaci, C. pecorum* and *C. pneumoniae* represent a taxonomically and phylogenetically coherent grouping into one genus (273). In 2009, the *Chlamydia* Basic Research Society at their meeting in Little Rock, Arkansas, USA agreed on one *Chlamydia* genus within the *Chlamydiaceae* family. Therefore, this classification has been used in this work.

1.1.2 Infection and disease

The different chlamydial species cause diseases in man and in a great variety of animal hosts. *Chlamydiae* are among the most common human pathogens and the three medically significant species include *C. trachomatis, C. pneumoniae* and *C. psittaci*. Infections are usually mild and typical chlamydial sequelae result from chronic inflammation by recurrent infection. A more detailed description of these pathogens and their disease potential will follow below. Within the animal kingdom various chlamydial species occur as pathogens and are commensals in cattle, pigs, horses, cats and mice (242). Infections can result in abortion, pneumonia, conjunctivitis or intestinal syndromes. Most notably *C. abortus* infections causing abortion in cattle represent a major economic burden for farmers of domestic sheep and lambs (258).

Chlamydia trachomatis

C. trachomatis is subdivided into two biovars, the trachoma biovar (serovars A-K) and the lymphogranuloma biovar (serovars L1-L3). Both biovars differ biologically and

clinically. Within the trachoma biovar, serovars A, B, Ba and C infect the conjunctive epithelium and cause **trachoma**, a major preventable eye infection that can lead to blindness. Trachoma is an ancient disease and first descriptions from China and Egypt are dating back several thousand years (3, 167). During the Napoleonic wars trachoma rapidly spread through Europe by infected soldiers and caused a civilian epidemic. In Europe, the disease was controlled by basic hygiene measures and had disappeared by the start of the 20th century. Today, trachoma is no longer a public health problem in the western world but continues to be the leading cause of blindness in developing countries. Endemic trachoma is prevalent in large parts of Africa, Asia, Australia and the Middle East. Active disease is characterized as a chronic, recurrent follicular conjunctivitis mainly affecting children (87, 277). Multiple infections over time and prolonged severe inflammation will lead to inward turning of the eyelid with subsequent scarring of the conjunctiva (trichiasis). Abration of the cornea will ultimately lead to blinding opacification which develops later in life (30-40 years of age). *C. trachomatis*, the etiological agent of trachoma, is transmitted by contact from eye to eye, for example by contaminated fingers, towels or flies (8, 13, 177). Trachoma equally affects male and female children but it is more prevalent in women than men probably due to their closer contact with their children (62, 294). In addition to age and sex, environmental factors like water supply, personal hygiene, flies, cattle, crowding and nutritional deficiencies increase the risk of active trachoma (293). Infections with *C. trachomatis* are treated with azitromycin or tetracycline (240).

According to current estimations, about 8 million people are blind as a result of trachoma and 84 million people suffer from active trachoma infection (31). In 1996, the World Health Organization (WHO) established the Alliance for the Global Elimination of Blinding Trachoma by the year 2020 and endorsed their SAFE strategy. SAFE - surgery for trichiasis, antibiotics for infection, facial cleanliness and environmental improvements - is part of the WHO´s control activities to reduce *C. trachomatis* transmission (7, 148, 174).

C. trachomatis is also the most common cause of **sexually transmitted disease** (STD) in the western world and in developing countries with about 92 million new infections each year (22). The serovars D-K infect the genital epithelium and cause urogenital tract infections. Typing of clinical isolates from the genital tract has shown that serovars D, E and F account for 60-80% of the strains, with E being the most common

(209). In addition, serovars D-K can also be associated with ocular and respiratory infections (201). The major risk group for chlamydial genital infections is sexually active young individuals below 25 years of age, and risk increases with the number of sexual partners. Infections often progress silently as symptoms are usually mild or even absent and about three quarters of infected women and about half of infected men have no symptoms (155). In men, clinical symptoms range from urethritis, proctitis, epididymitis and conjunctivitis. Women infected with *C. trachomatis* can develop urethritis, cervicitis, bartholinitis, salpingitis and conjunctivitis. If untreated, infection can lead to ectopic pregnancy and infertility (201). There is also an association of genital infections by *C. trachomatis* with increased risk for cervical cancer and HIV infection (47, 141). Moreover, both in men and women, genital *Chlamydia* can also cause different types of reactive arthritis (125, 288). The majority of infections is genital and is acquired during sexual intercourse. *Chlamydia* can be transmitted during vaginal, anal, or oral sex and it can also be passed from an infected woman to her baby during delivery. In the latter case, neonates are at risk to develop conjunctivitis or pneumonia (196). Chlamydial genital infections are most commonly treated with a single dose of azithromycin or a week of doxycycline (240). Babies born to infected mothers should be treated with erythromycin. Genital *Chlamydiae* represent a major public health problem as infected women and men are usually asymptomatic and infection spreads unknowingly.

Lymphogranuloma venereum (LGV) is a sexually transmitted disease caused by the L1, L2 and L3 serovars of *C. trachomatis*. In contrast to other *C. trachomatis* serovars, LGV is an invasive biovar and bacteria infect lymphatic and subepithelial tissue. Characteristic for LGV are three clinical stages. It first presents as a self healing papule followed by proctitis, which if untreated develops into lymphedema and anal strictures (166). LGV is most prevalent in tropic and subtropic areas in Africa, Asia and South America and commonly found in men having sex with men (220). The pathogen can be transmitted through any type of sexual intercourse. A number of recent outbreaks in Europe as well as the USA were reported and LGV seems to be rapidly spreading (135). Interestingly, most of these LGV cases have occurred in HIV-positive patients and among homosexual men hence HIV seropositivity is the strongest risk factor for LGV (282). LGV infections are treated with tetracycline or doxycycline (166).

Chlamydia pneumoniae

C. pneumoniae is a respiratory pathogen that causes acute respiratory diseases including **pneumonia**, bronchitis, sinusitis, otitis media and pharyngitis (23, 24). Serologic studies suggest that *C. pneumoniae* accounts for an average of 10% of cases of community-acquired pneumonia and 5% of bronchitis and sinusitis cases (85). The organism was first isolated in 1965 from a child's conjunctiva during a trachoma vaccine trial in Taiwan and became known as TW-183 (149). In 1968, another isolate from the eye of an Iranian child was cultured and later identified as *C. pneumoniae* (64). Despite these two conjunctival sources, only in 1983 the first respiratory isolate (AR-39) was obtained from a university student with pharyngitis (86). The conjunctival isolate (TW-183) and the respiratory isolate (AR-39) later became known as TWAR isolates. Acute infection is most common in children aged >5 years and by the age of 20 approximately 50% of individuals have detectable levels of antibodies to *C. pneumoniae*. Seroprevalence further increases at slower rates with age and peaks at approximately 75% in the elderly (150). The majority of infections cause mild disease or are asymptomatic and therefore often remain unrecognized (179). *C. pneumoniae* is distributed worldwide although the prevalence may vary by region (78). Transmission occurs from person to person via respiratory secretions (67). Patients with *C. pneumoniae* infections are treated with erythromycin, azithromycin or clarithromycin (240). While humans were long thought to be the only reservoir for *C. pneumoniae* it has now been shown that also animals, like horses and koalas, can be infected (254, 291). *C. pneumoniae* has also been associated with a number of chronic diseases including asthma (101), chronic obstructive pulmonary disease (COPD) (100) and neurological disorders such as multiple sclerosis (257) and Alzheimer's disease (9, 256). However, evidence for a role of *C. pneumoniae* in these diseases should be handled with caution. Most importantly, an association between cardiovascular disease resulting from atherosclerosis and *C. pneumoniae* infection has been suggested. In atherosclerosis, the deposition of oxidized low density lipoproteins at the inner lining of the artery causes an inflammatory response which over many years can lead to the formation of atherosclerotic plaques within the vessel wall thereby obstructing the blood flow (227). The progression of this chronic, systemic arterial inflammatory disease is influenced by the individuals´ genetic constitution and various risk factors including age, sex, hyperlipidaemia, hypertension, smoking, diabetes mellitus and abdominal obesity (309). In 1988, Saikku et al. first reported a possible association

between *C. pneumoniae* and atherosclerosis (231). Since then, numerous serological, histopathological and animal studies have investigated the role of *C. pneumoniae* in the pathogenesis of atherosclerosis (reviewed in (292)). Taken together, the evidence we gained during the past 20 years suggests that *C. pneumoniae* is neither sufficient nor necessary to cause atherosclerosis, but that this pathogen likely should be considered as a risk factor (292).

Chlamydia psittaci

C. psittaci is the causative agent of **psittacosis**, also referred to as parrot fever. The bacterium can infect psittacine birds, like parrots, parakeets, canaries or any other avian species and cause upper respiratory infections with nasal or ocular discharge and diarrhea (103). A major concern with *C. psittaci* is its zoonotic potential as the bacterium can be easily spread to humans. In humans, *C. psittaci* infection may be asymptomatic or may manifest as atypical, severe **pneumonia**. The largest psittacosis outbreaks occurred in 1929 and 1930, starting from Latin America rapidly spreading to Europe and affected 750-800 individuals (285). Psittacosis is found worldwide and transmission occurs by inhalation of aerosolized dried avian excreta or respiratory secretions from sick birds. Person to person transmission is possible but believed to be rare (119, 126). Psittacosis is a common occupational disease and individuals working in close contact with birds, like poultry farmers or veterinarians are at risk (157, 279). For the treatment of *C. psittaci* infections the antibiotic of choice is tetracycline or doxycycline (103).

1.1.3 Morphology

Chlamydiae are obligate intracellular pathogens that can exist in two distinct forms, the **elementary body** (EB) and the **reticulate body** (RB), which are characteristic for the unique chlamydial developmental cycle. EBs are small, electron dense, spherical organisms of about 0.3 mm in diameter. They are the metabolically inactive but highly infectious, extracellular form of the bacterium. EBs are osmotically resistant and have extensive disulfide bond cross-linked outer membrane proteins. Their DNA is highly condensed by two histone H1 homologues, Hc1 and Hc2 (94, 207, 274). RBs are the non-infectious but metabolically active form of the organism which are larger in size with about 1-2 mm in diameter. They are sensitive to osmotic lysis and contain dispersed DNA. During *Chlamydia* development a conversion between these two

developmental forms takes place; EBs differentiate into RBs which later differentiate back into EBs. During the maturation from RBs into EBs morphological intermediate forms (IB) can be observed. A characteristic biochemical difference between EBs and RBs is the low ratio of RNA to DNA in EBs.

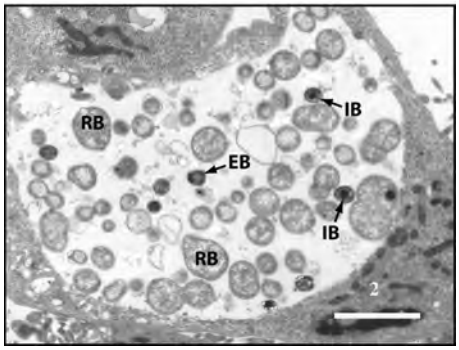

Figure 2. *C. trachomatis* LGV2 inclusion at 30 h post infection consisting of reticulate bodies (RBs), elementary bodies (EBs), and intermediate bodies (IB). The bar represents 2 microns. Electron micrograph adapted from (189).

Chlamydiae share morphological and structural properties of Gram-negative bacteria. The typical *Chlamydia* outer membrane complex contains genus-specific lipopolysaccharide (LPS) (34), the major outer membrane protein (MOMP) (35) and the two cysteine rich outer membrane proteins OmcA and OmcB (181). With the complete genome sequences for *C. trac*homatis and *C. pneumoniae* available, additional outer membrane proteins were described including the family of polymorphic outer membrane proteins (PMPs), porinB, Omp85 and others (summarized in (252)). The major chlamydial envelope components will be discussed in more detail below.

MOMPS

The 40-kDa MOMP is the most abundant surface protein of *Chlamydiae* accounting for 50-60% of the total protein mass in the outer membrane (35). It is present in both EBs and RBs and was first described by Caldwell and colleagues in 1981 for *C. trachomatis* (35). MOMP is extensively cross-linked by disulfide bonds in EBs contributing to the structural integrity of the organism whereas it is in a completely reduced state in RBs (104, 193). Studies by Newhall and others suggest that MOMP is a trimer embedded in

the outer bacterial membrane (193, 269) where it functions as a porin (14, 304, 305). MOMP consists of five constant domains, well preserved in all *Chlamydia* spp., interspersed by four variable domains. Sequence differences in these variable domains account for the different serovars that have been identified for *C. trachomatis* (35, 253). The variable domains tend to be surface exposed and hence are highly immunogenic in all chlamydial strains (6, 37, 76, 299). MOMP has been shown to induce both neutralizing antibodies (29, 263, 313) and T-cell mediated immune responses (139, 198). As quantitatively predominant surface protein of *Chlamydiae*, MOMP has been extensively studied as primary target antigen for the development of a chlamydial vaccine (36, 49, 202). However, most of these studies were of limited success since protective immunity was not generated or was partial at best (55, 261, 311).

OmcA/OmcB

In addition to MOMP, the major proteins in the chlamydial envelope are the cysteine rich proteins, OmcA and OmcB. Extended disulfide cross-linking of these proteins is thought to contribute to cell wall rigidity and osmotic stability of EBs. The 60 kDa protein OmcB was proposed to be found exclusively within the periplasm between the inner and outer bacterial membrane (65, 190). Its cellular localization however is controversial because OmcB can induce a strong antigenic response suggesting a surface exposure (57, 289). Recently, OmcB of different chlamydial species was shown to mediate bacterial adhesion to human epithelial cells, strongly supporting a surface localization (180). OmcA is a lipoprotein of 9 kDa that is presumably anchored to the outer bacterial membrane extending into the periplasm (65).

Pmps

Polymorphic membrane proteins are a family of high molecular weight surface membrane proteins in *Chlamydiae*. These proteins, initially referred to as principal outer membrane proteins (POMPs), were first observed by Campbell et al. (37) and characterized by protein biochemistry (165, 175). Chlamydial genome sequencing later revealed the presence of several *pmp* genes in all *Chlamydia* species. *C. trachomatis* encodes nine *pmp* genes (43, 251) and as many as twenty-one were found in *C. pneumoniae* (134). The *pmp* genes are often clustered together and usually very heterogeneous in sequence and size (93). So far, the function of Pmps remains unknown but there is increasing evidence that Pmps are autotransporters (107, 140). As surface exposed protein, PmpD the longest of the PMPs in *C. pneumoniae* was shown

to be highly immunogenic in human infections and therefore discussed as a potential vaccine candidate (56, 197).

LPS

Like other Gram-negative bacteria, *Chlamydiae* also contain LPS in their outer membrane. Together with MOMP, chlamydial LPS is a major surface component and has been widely used as target molecule for laboratory diagnostics. Structural studies of chlamydial LPS were initially impaired by the inability to produce sufficient amounts of LPS for investigation. These difficulties were later overcome by expression of recombinant chlamydial LPS in *E. coli* which made the production of large amounts of LPS possible (191). In general, chlamydial LPS resembles that of enteric bacteria with two exceptions: (I) the sugar core structure and in particular its linkage varies slightly (27, 142), and (II) LPS of *Chlamydia* shows only weak endotoxic activity (106, 124).

Peptidoglycan

Bacterial peptidoglycan (PG) is a major cell wall component in Gram-negative bacteria which consists of alternating residues of N-acetylglucosamine and N-acetylmuramic acid residues cross-linked by peptides. The existence of chlamydial peptidoglycan has long been debated as *Chlamydiae* lack detectable amounts of this polymer. Yet, *Chlamydiae* are susceptible to cell-wall antibiotics like penicillin which target the PG synthesis (12). This paradox became known as the chlamydial anomaly (186). Although no peptidoglycan has been detected up to date in *Chlamydiae*, it was known for a long time that they do possess penicillin binding proteins that play a crucial role in the final cross-linking of peptidoglycan (12). The full genome sequence of different *Chlamydia* species finally revealed a nearly complete pathway for the synthesis of PG (48, 251). Genomic transcriptional profiling has further shown that genes required for PG synthesis were upregulated at 16-18 h post infection suggesting that peptidoglycan synthesis occurs during intracellular replication of RBs (20, 194). On protein level, some PG synthesis enzymes were detected in EBs (284) and RBs (12) in low amounts which makes it difficult to investigate their role further. In the field of chlamydial PG synthesis numerous questions remain open and further research is needed to clarify how the chlamydial cell wall is build (172).

1.1.4 Development

All *Chlamydia* species share an unique, biphasic developmental cycle alternating between the metabolically inert elementary bodies and the metabolically active reticulate bodies (184). A diverse range of phagocytic (macrophages, monocyte derived cells) and non-phagocytic (epithelial cells, endothelial cells) cells are targeted by *Chlamydia*. Infection typically starts by attachment of EBs to the host cell. The initial interaction with the host cell is believed to be a reversible, electrostatic interaction followed by a temperature-dependent, irreversible binding to an unknown host cell receptor (58). Glycosaminoglycans (GAGs) play an important role in binding of microbial pathogens to host cells (26, 213) and several studies suggested that also *Chlamydia* initially attaches to heparan sulphate-like GAGs (46, 302, 312). A number of *Chlamydia* surface structures including MOMP (262), heat shock protein 70 (219) and OmcB (180, 278) were proposed as bacterial adhesins but little is known about potential host cell receptors. So far, no exclusive receptor-adhesin interaction has been identified to be required during bacterial entry. Instead it is believed that *Chlamydia* relies on multiple strategies to ensure successful invasion.

EB binding to the host cell results in microvillar rearrangements, the formation of pedestal-like structures and the recruitment of small GTPases (40, 267). Moreover, several key players of endocytosis including actin, WAVE2 and components of the Arp2/3 complex are recruited to the bacterial entry site (38). Clifton and colleagues reported that *Chlamydia* induces its own entry by secretion of a bacterial protein upon attachment to the host cells (51). This protein called TARP (translocated actin recruiting phosphoprotein) has actin nucleating activity and triggers a cascade of events which will induce actin rearrangements to facilitate EB internalization (131). TARP is delivered into the host cell cytoplasm by a T3SS, which resembles a molecular syringe used to deliver bacterial proteins into their host to modulate cellular functions. Its structure, function and proteins translocated by this mechanism will be discussed in greater detail in chapter 1.4.

Upon entry, the EBs are surrounded by individual tight vacuoles that are also called inclusions. Throughout their intracellular development *Chlamydia* will remain within this membrane-bound compartment (Figure 3). Soon after internalization of the EBs the primary differentiation takes place and EBs convert into the metabolically active RBs. This stage is characterized by a loss of infectivity, enlargement of the organism and dissociation of the highly condensed EB nucleoid. Bacterial transcription commences

almost immediately in the differentiating RB and newly synthesized proteins can be detected as early as 15 min post infection (214). Upon primary differentiation the RBs start to multiply by binary fission and replicating bacteria acquire amino acids and nucleotides from the host cell. One RB can produce up to 1000 new bacteria (306).

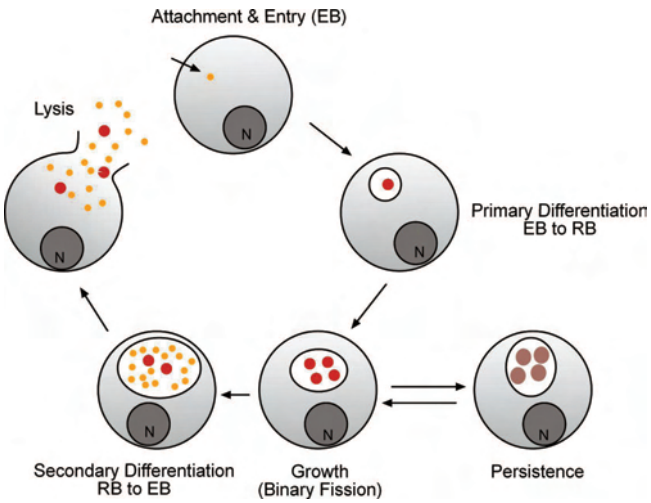

Figure 3. Schematic diagram of the chlamydial developmental cycle.

The early chlamydial inclusion rapidly recruits dynein and migrates along microtubules to the microtubule organizing center in close proximity to the nucleus (91, 236). Furthermore, the inclusion membrane becomes decorated with several Rab proteins, which are central players in membrane trafficking (133, 230). By active modification of the inclusion membrane, the *Chlamydia* containing vacuole will not fuse with lysosomal compartments but remain fusogenic with vesicles derived from other pathways.

With the onset of RB replication the chlamydial inclusion needs to expand to accompany the increasing number of progeny. Hackstadt and colleagues first demonstrated the directed trafficking of Golgi-derived sphingolipids to the chlamydial inclusion (98). Apart from sphingolipids, sterols and glycerophospholipids were found in the inclusion thus providing a lipid source for expansion of the inclusion membrane (41, 237, 260). Despite being capable of *de novo* synthesis of phospholipids *C.*

trachomatis also acquires host derived lipids likely through intimate contact with the inclusion membrane (303). Recently, Beatty identified multivesicular bodies as alternative lipid source for *Chlamydia* (16) and Kumar et al., provided evidence for a direct interaction of *Chlamydia* with lipid droplets (146).

Chlamydial inclusions are morphologically distinct in different species. In cells infected with more than one bacterium, *C. trachomatis* single inclusions will typically fuse to one big inclusion around 10-16 h post infection. Fusion is mediated by the inclusion membrane protein IncA, as discussed in more depth later on (60, 97). In contrast, *C. pneumoniae* multiple infections give rise to several single inclusions which do not fuse. Finally, while *C. trachomatis* and *C. pneumoniae* both form spherical inclusions, *C. caviae* inclusions are extensively lobed (222). Another difference in inclusion morphology concerns the distribution of RBs within the inclusion. *C. trachomatis* RBs are typically found juxtaposed to the inclusion membrane as the inclusion expands, leaving the lumen of the inclusion empty. On the other hand, *C. pneumoniae* RBs seem to be tightly packed throughout the entire volume of the inclusion (298).

As the chlamydial developmental cycle progresses replication becomes asynchronous and RBs will undergo a second differentiation into EBs. Ultrastructural analyses revealed that this second conversion often involves intermediate forms, which eventually become EBs (298).

The signals that trigger this event are largely unknown. Wilson and colleagues proposed a biomathematical model to describe how RB to EB conversion could be initiated (295). According to this model, RBs are in close contact with the inclusion membrane via their T3SS. As the number of RBs increases during the developmental cycle, the contact area for RBs at the inclusion membrane becomes limited; this will in turn lead to detachment of RBs from the inclusion membrane. This physical detachment of RBs, not yet experimentally tested, is believed to trigger the final conversion into EBs thus may represent the signal for late differentiation (15, 95).

Chlamydia can exit the host cell in two different ways, by cellular lysis or a mechanism termed extrusion, reminiscent of exocytosis (121). Lysis occurs in an ordered sequence of membrane permeabilizations including the inclusion membrane, the nuclear membrane and the plasma membrane (121). This release mechanism likely involves chlamydial proteases and will ultimately lead to host cell death. Extrusion describes the packaged release of a part of the bacteria containing inclusion from the host cell. In this process the host cell remains intact and the newly detaching inclusion is surrounded by

plasma membrane (121). The length of the complete chlamydial cycle depends on the species, the type of host cell and environmental conditions but is typically ranging from 48 h for *C. trachomatis* to 96 h for *C. pneumoniae*.

1.1.5 Persistence

In addition to acute infection *Chlamydiae* are associated with a number of chronic infections (as discussed above). Recurrent chlamydial infections might result from repeated infections or persistence of the organism after unresolved infections. Persistence is defined as a long-term association between *Chlamydia* and the host in which the organism remains in a viable but non-cultivable state (18). In cell culture models, the chlamydial developmental cycle can be altered towards persistence by several factors including antibiotic exposure, nutritional deficiencies (amino acid and iron deprivation), continuous infection or IFN-γ exposure (reviewed in (112)).

In vitro persistence is characterized by enlarged aberrant RBs that undergo little or no cell division but continue to accumulate chromosomes. Persistence leads to an arrest in the productive growth cycle as RB interconversion into EBs is impaired. This arrest however can be reversed upon removal of the growth inhibitory factor and RB differentiation into EBs will take place. At the molecular level, Beatty et al. showed that in persistent *C. trachomatis* components of the *Chlamydia* outer membrane complex such as MOMP, LPS and OmcB were greatly reduced thus giving rise to the typical morphology of aberrant RBs (17). DNA replication in persistent RBs has been confirmed by continuous transcription of genes involved in replication (19, 33, 84). The exact molecular mechanisms that lead to the induction of the persistence stage and its reversal remain to be characterized. *In vivo* chlamydial persistence is believed to contribute to disease development with chronic pathologies such as *C. pneumoniae* associated adult-onset asthma or atherosclerosis (24). For *C. trachomatis* it has been reported that infected children living in endemic trachoma areas were disease-free for decades after leaving these areas but later in life developed active trachoma more likely due to activation of persistent bacteria rather than re-infection (276). Further evidence for persistent *Chlamydia in vivo* is the detection of *Chlamydia*-derived molecules at infected sites in the absence of detection of cultivatable organisms (151, 244, 275). Despite abundant experimental evidence of chlamydial persistence it remains difficult to distinguish between real persistence and low-grade productive infection that also fails to be detected by conventional culture methods.

Taken together, persistence plays an important role in chlamydial survival in adaptation to unfavorable growth conditions. From a clinical perspective, persistent chlamydial infections pose a significant problem as they are difficult to treat and therefore might lead to the establishment of long- term chronic infections.

1.1.6 Genomics

Research in the *Chlamydia* field has always been hampered by the lack of tools to genetically modify the bacterium. In 1998, the complete genome sequence of *C. trachomatis* became available and chlamydial research entered a new stage in understanding the complex biology of this human pathogen (251). To date, several *Chlamydia* genomes have been sequenced and are publicly available including the genomes of *C. pneumoniae* and *C. caviae*. The genome data revealed a number of surprising discoveries regarding metabolic pathways, genetic regulation, signal transduction and protein secretion.

The *C. trachomatis* serovar D genome consists of 1,042,519 base-pairs and encodes 894 proteins (251). DNA microarray analysis of different *C. trachomatis* serovars suggests a degree of 99% identity (28, 42). In addition, the genomes of the ocular serovar A and the genital serovar D of *C. trachomatis* share 99,6% identity as shown by genome sequencing (43). Considering this small difference, only a few genes are believed to account for the different tissue tropisms of *C. trachomatis* strains. Among those genes identified is a functional tryptophan-synthase which is absent in oculotropic strains and present in genitotropic strains (43).

In comparison to the *C. trachomatis* genome, the genome of *C. pneumoniae* is slightly larger with 1,230,230 base-pairs and an additional 214 proteins not found in *C. trachomatis* (134). The genes for central metabolic pathways are conserved in both species and *C. pneumoniae*, similar to *C. trachomatis*, encodes proteins for a T3SS, which will be discussed in greater detail below.

The availability of chlamydial genome sequences further allowed detailed transcriptome studies in *Chlamydiae*. First, Shaw et al. identified several temporally expressed genes for *C. trachomatis*. Based on their expression profile during the infectious cycle three major classes were proposed: early genes, mid-cycle genes and late genes (241). In 2003 two comprehensive transcriptional analyses of the chlamydial developmental cycle were published (19, 194). Similar studies have later also been performed for *C. pneumoniae* (171). Taken together, genome sequencing provided

exciting new findings to the chlamydial research field and has greatly advanced our understanding of *Chlamydiae* biology.

1.2 IMMUNITY TO *CHLAMYDIA*

In the absence of an effective vaccine against chlamydial infections the role of innate and adaptive immune players has long been a major focus in *Chlamydia* research. The first human vaccine trials were conducted in the 1960´s (88), with limited success in generating protective immune responses. Since then, however, great progress has been made to further our understanding of immunity to *Chlamydia.* In this paragraph the immune mediated control of *Chlamydia* infections, with a focus on *C. trachomatis*, will be discussed briefly.

1.2.1 Innate immunity

During infection with *Chlamydia*, physical barriers like the mucosal membranes of the genital tract in case of genital *C. trachomatis* infection usually provide the first line of defense. The antimicrobial activity of defensins, lysozyme and complement has been reported to additionally contribute to mucosal innate immunity (162, 215). Upon successful entry of EBs into epithelial cells innate immune effectors provide the second line of defense. Infection of epithelial cells with *C. trachomatis* induces proinflammatory cytokines such as interleukin-1, interleukin-6 and tumor necrosis factor-α (218) and induces secretion of the chemokine interleukin-8 (30) which will lead to the recruitment of macrophages, neutrophils, natural killer cells and dendritic cells to the side of infection. Another important cytokine is interferon-γ, which directly or indirectly plays a crucial role in the inhibition of *Chlamydia* growth in infected cells (summarized in (221, 228)). The production of inflammatory cytokines and interferon-γ is further enhanced by recognition of chlamydial pathogen associated molecular patterns through Toll-like receptors (TLRs) expressed on innate immune cells and epithelial cells. *In vivo* and *in vitro* studies identified TLR2 as the principal TLR in infections with *C. trachomatis* or *C. muridarum*, which is used for genital infections of mice and closely mimics many aspects of *C. trachomatis* genital infections in humans (99).

1.2.2 Adaptive immunity

An effective immune response not only requires innate immunity but is also dependent on adaptive immunity including B-cell and T-cell responses to limit the infection and provide protection in subsequent encounters with *Chlamydia*. It is well reported in the literature that B-cells can produce *Chlamydia* specific antibodies to neutralize *Chlamydia* infectivity and enhance phagocytosis of EBs (182, 206, 211). The contribution of antibodies in controlling *Chlamydia* infections is however debated and B-cell deficient mice were demonstrated to control primary *Chlamydia* genital infections as efficiently as wild-type mice (259). As antibodies are unable to access intracellular *Chlamydia* it is believed that T-cells play a predominant role in protective immune responses to *Chlamydia* infections. The key players of cellular immunity are CD4+ and CD8+ T-cells that recognize antigens presented by the major histocompatibility complex (MHC) (reviewed in (129)). Professional antigen-presenting cells can engulf EBs or infected cells harboring RBs for subsequent presentation of chlamydial antigens via MHC class II molecules to CD4+ T-cells. CD8+ T-cells recognize cytosolic antigens of infected cells which are processed by the proteasome and presented by MHC class I molecules on the host cell surface. T-cell activation will induce further production of effector cytokines and in a complex interplay with other immune cells ultimately lead to *Chlamydia* killing. In summary, innate immune cells, B-cells and T-cells all respond to *Chlamydia* infection. However, in many cases these responses are ineffective in controlling infection. Worse, they can in some cases induce chronic inflammation which can lead to tissue pathology. Better understanding the immune response to *Chlamydia* infection is a field of intensive research, with the ultimate goal of developing an effective vaccine that can generate sterilizing immunity without inducing tissue pathology.

1.3 *CHLAMYDIA* – CELLULAR MICROBIOLOGY

As obligate intracellular pathogen *Chlamydia* employs multiple ways to manipulate host cellular processes to its own benefit. This section will highlight various *Chlamydia*-host interactions including lipid acquisition, cell cycle, apoptosis, immune evasion and cytoskeletal organization.

1.3.1 Lipid acquisition

Early during infection, the chlamydial inclusion separates itself from the endosomal-lysosomal trafficking pathway and instead becomes fusogenic with Golgi-derived exocytic vesicles (96, 236). By this way glycerophospholipids (303), sphingolipids (98) and cholesterol (41) are transported from the Golgi to the inclusion membrane and subsequently to the bacteria within the inclusion. Although no direct interaction between exocytic vesicles and chlamydial proteins is described, it has been suggested that Inc-proteins participate in the rerouting of Golgi-derived lipids to the inclusion membrane (72). Recently, Heuer et al. demonstrated that *Chlamydia* infection causes Golgi fragmentation into Golgi ministacks by proteolytic cleavage of the Golgi-matrix protein golgin-84 thereby enhancing transport of lipids to the chlamydial inclusion (109). In addition, inclusions were shown to acquire host-derived lipids from the late endocytic organelles called multivesicular bodies (MVBs) (16). This study was particularly interesting as it not only demonstrated a novel interaction of *Chlamydia* with MVBs but also challenged the assumption that *Chlamydia* do not interact with endosomal trafficking. Finally, *Chlamydia* can acquire lipids through direct interactions with lipid droplets, neutral lipid storage organelles that can translocate from the host cytoplasm into the inclusion lumen (52). The exact molecular mechanisms of LD capture and translocation remain to be investigated but likely involve the *C. trachomatis* secreted LD associated (Lda)-proteins that bind to cytoplasmic LDs (146).

1.3.2 Cytoskeleton

The cytoskeleton is a dynamic scaffold that consists of three major structural components, microfilaments (actin), intermediate filaments (keratin and keratin-like proteins) and microtubules (tubulin). Given its particular role in maintaining cell shape and regulating vesicular transport, it is perhaps not very surprising that *Chlamydia* as an obligate intracellular bacterium has evolved strategies to manipulate the host cytoskeleton. Chlamydial entry proceeds with local actin rearrangements at the bacterial attachment site, which are mediated at least in part by the chlamydial effector protein TARP (39, 131). Within a few hours after infection the chlamydial inclusion traffics along microtubules to the perinuclear region and remains in close proximity to the Golgi (50). This migration is mediated by the minus-end directed microtubule motor protein dynein (91) and requires chlamydial protein synthesis suggesting that

putative effector proteins, most likely Inc-proteins, are required for the interaction with dynein. With the onset of bacterial replication the inclusion steadily grows and takes up most of the host cell cytoplasm towards the end of the developmental cycle. This process poses huge mechanical stress on the host cytoskeleton and requires a dynamic structural scaffold that can adapt the growing inclusion. Lately, Kumar and Valdivia presented evidence that a scaffold of actin and intermediate filaments encase the expanding inclusion thereby providing structural integrity (147). The authors further showed that *Chlamydia* can actively modulate the host cytoskeleton throughout infection by inducing cleavage of intermediate filaments (147). Finally, actin filaments have been implicated in the release of *Chlamydia* from infected cells (122).

1.3.3 Cell cycle

Several studies indicate that *Chlamydia* targets the host cell cycle presumably to ensure successful intracellular growth and multiplication. In one of these studies, Horoschak and Moulder early noted that the multiplication of mouse fibroblasts infected with *C. psittaci* was twice as slow as of non-infected cells (114).

A more recent study concluded that *C. trachomatis* infection selectively blocks host cell cytokinesis but allows mitosis to progress (90). Recently, evidence was presented that *C. trachomatis* induces cleavage of the mitotic cyclin B1 thereby affecting cell cycle progression (10). Moreover, *Chlamydia* by its tight association with centrosomes was shown to cause supernumerary centrosomes, abnormal spindle poles and chromosomal segregation defects (92).

1.3.4 Apoptosis

Chlamydiae modulate programmed cell death pathways in two opposing directions depending on the stage of the infectious cycle (208). Early during infection *Chlamydiae* are resistant to apoptotic stimuli which may contribute to persistence of *Chlamydia* infection. Chlamydial anti-apoptotic activity was correlated with blockage of mitochondrial cytochrome c release (68), inhibition of activation of pro-apoptotic Bax and Bak (307) and degradation of BH-3 only proteins like BIM, Puma and BAD (75, 212). The phosphorylated pro-apoptotic protein BAD was later shown to bind to 14-3-3ß via IncG at the inclusion membrane thereby preventing the induction of the intrinsic, mitochondrial apoptosis pathway (287). Accordingly, Tse et al. showed that

diacylglycerol accumulates at the chlamydial inclusion and sequesters the pro-apoptotic protein PkCd away from mitochondria (281).

In contrast, towards the end of the developmental cycle, *Chlamydia*-infected cells become pro-apoptotic which likely contributes to the release of the bacteria from the host cell. The chlamydial effector protein CADD might be implicated in this process (see 1.4.3).

1.3.5 Immune evasion

Chlamydia has found different ways to manipulate innate immune responses to ensure its successful intracellular lifestyle. The chlamydial protease CPAF has emerged as a major immune regulator during chlamydial infection. CPAF was demonstrated to down-regulate MHC class I and II antigen presentation by degrading the transcription factors USF-1 and RFX-5. It also participates in the degradation of the MHC-like protein CD1d (137, 314). In addition to its role in *Chlamydia* immune evasion, CPAF was shown to target several other host proteins which will be discussed in more detail in section 1.4.3. In addition, *Chlamydia* actively interferes with NF-κB signaling, which regulates the expression of many host defense genes required for efficient innate and adaptive immunity. NF-κB is a heterodimer composed of two subunits, RelA (p65) and p50 that upon activation translocates into the nucleus and functions as a transcription factor. Recently, Lad et al. showed that the *C. trachomatis* effector CT441 (Tsp) is a chlamydial protease capable of degrading p65 thereby preventing its translocation into the nucleus (152, 153). Likewise, ectopic expression of ChlaDub1, a chlamydial effector protein with deubiquitinating and deneddylating activity (178) prevented ubiquitination and subsequent proteasomal degradation of IκBα, a NF-κB inhibitor (158). Recently, it was further shown that the *C. pneumoniae* Inc-protein Cp0236 sequesters NF-κB activator 1 (Act1) at the inclusion membrane thereby preventing IL-17 induced NF-κB activation (300). Altogether, these results suggest that *Chlamydiae* rely on several redundant mechanisms to interfere with NF-κB mediated signaling.

1.4 *CHLAMYDIA* – TYPE III SECRETION AND EFFECTOR PROTEINS

In recent years, great advances have been made in our understanding of the complex interactions of pathogenic bacteria with their eukaryotic hosts during infection. Within modern microbiology an entire research field, known as cellular microbiology, now strives to elucidate the molecular mechanisms of how pathogens can subvert host cellular functions to their own benefit. In this regard, the bacterial type III secretion system (T3SS), a protein transport device designed for delivery of bacterial proteins into host cells, has attracted a lot of attention. The chlamydial T3SS and associated effector proteins will be central to this thesis. Therefore, this section will focus on the structure and function of the chlamydial T3SS followed by a detailed description of the *Chlamydia* cross-talk with its eukaryotic host.

1.4.1 Type III secretion

T3SSs are exclusively found in a number of Gram-negative animal and plant pathogens, including *Yersinia*, *Pseudomonas*, *Shigella*, *Salmonella*, *E. coli* and *Chlamydia* (118). First identified in the 1990´s, T3SSs are among the most complex protein secretion systems in bacteria used to translocate bacterial virulence proteins, called effectors, into eukaryotic host cells (232). Among the best studied T3SSs are those of *Yersinia*, *Shigella* and *Salmonella*. Despite detailed biochemical and structural characterizations of T3S components, the exact molecular mechanism of translocation has remained elusive.

The T3S apparatus is composed of approximately 25 proteins that assemble into two main structures, the basal body and the needle complex, also referred to as injectisome (183, 308). Embedded in the inner and outer bacterial membrane, a multi-ring structure forms the basal body, which resembles basal bodies of bacterial flagella suggesting a common evolutionary origin. Linked to the basal body is a needle-like projection that protrudes from the bacterial surface. This injectisome is a straight hollow tube, through which T3S effectors are translocated. As this molecular passageway is too narrow for most globular effector proteins, they are believed to traverse through the channel in an unfolded state most likely mediated by several chaperones (2). Assembly of the T3S apparatus proceeds in a stepwise manner beginning with the basal body followed by the needle complex. Upon contact with a host cell so called translocators are secreted. These proteins form a translocation pore in the host cell membrane thereby creating a conduit from the bacteria into the host cell (187).

T3S effector proteins are very diverse and each bacterium endowed with a T3SS possesses its own arsenal of bacterial weapons. As the T3SS constitutes a major bacterial virulence factor it is not surprising that T3S effectors manipulate a vast array of crucial cellular functions (80). Most effectors seem to function by molecular mimicry of eukaryotic proteins involved in signaling pathways, gene expression, cell cycle progression, cytoskeletal dynamics and intracellular trafficking (81). While effector proteins display a great diversity, the overall composition of the T3SS is highly conserved among different pathogens (271). Interestingly, T3S substrates of one bacterium can be recognized by the T3S apparatus of another pathogen (5, 226). This approach has proven to be very useful in the identification of chlamydial T3S effectors because *Chlamydiae* are still intractable for genetic manipulation.

1.4.2 The chlamydial type III secretion system

Chlamydiae are the only known non-proteobacteria with an obligate intracellular life style that possess a T3SS. As *Chlamydiae* diverged from other Gram-negative bacteria ~2 billion years ago, the chlamydial T3SS is probably one of the most ancient T3SSs (113). In support to this hypothesis, genome sequencing revealed that not only animal and human *Chlamydia* species but also environmental species infecting amoeba possess a T3SS. In addition, features of the chlamydial T3SS are remarkably different from those of other proteobacteria endowed with a T3SS. While T3S genes are usually clustered in chromosomal pathogenicity islands or on virulence plasmids, chlamydial T3S genes are dispersed throughout the genome, loosely arranging into four loci (264). Moreover, the G/C content of T3S genes in other Gram-negative pathogens is low with respect to the rest of the genome. *Chlamydia* genomes have a low G/C content overall, and T3S genes have the average G/C content.

Identification

Long before the T3SS was discovered in *Yersinia*, in pioneering work performed by Matsumoto and colleagues, needle-like projections on the surface of *Chlamydia psittaci* were described (169, 170). These peculiar protrusions, present on EBs and RBs albeit in different numbers, seem to be arranged into rosette-like structures. Their function remained elusive for a long time. Shortly before *Chlamydia* genome sequencing, several genes related to T3S were discovered in *Chlamydia* (116) and it was speculated that the needle-like protrusions might correspond to a chlamydial T3SS. Until now, no

biochemical data have confirmed this association. However the chlamydial surface structures described by Matsumoto strongly resemble T3S needles purified from *S. typhimurium* (145), *S. flexneri* (270) and *E. coli* (239).

Structure

The composition of the chlamydial T3SS greatly resembles those of other pathogenic bacteria (Figure 4). Together with a set of proteins that form the actual secretion apparatus *Chlamydia* also possesses cytoplasmic chaperones and translocator proteins. The components of the chlamydial T3SS have been initially assigned based on their homology to proteins present in the *Yersinia* T3SS, so called Ysc-proteins. This nomenclature was later adapted for *Chlamydia* and Ysc proteins are now referred to as Cds-proteins (74), based on the notion of a contact-dependent secretion mechanism proposed by Hsia et al. (116).

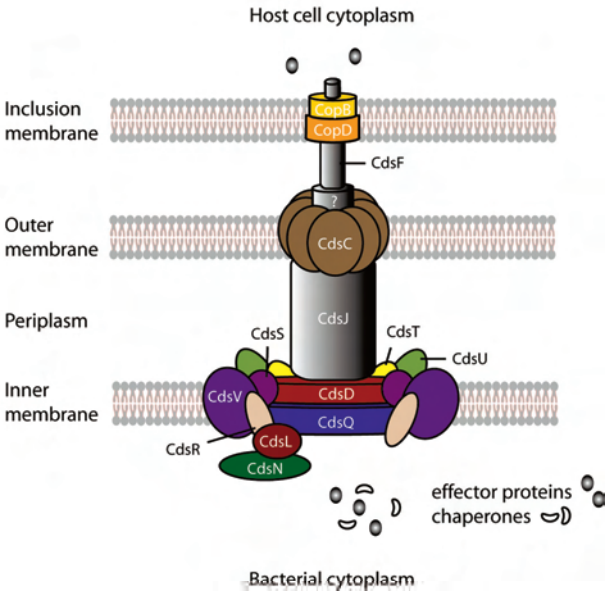

Figure 4. Schematic diagram of the chlamydial T3SS and its composition (adapted from (210)).

The components of the basal apparatus are highly conserved and include the proteins CdsD, CdsQ, CdsR, CdsS, CdsT, CdsU and CdsV, which are believed to assemble in the inner bacterial membrane. CdsL and CdsN both associate with the basal body on the cytoplasmic site. Based on its homology to *Yersinia* YscN, a putative ATPase,

CdsN presumably energizes the chlamydial secretion machinery (296). CdsL might function as a regulator of CdsN as it has been proposed for *Yersinia* (25). The lipoprotein CdsJ spans the bacterial periplasm and associates with CdsC, which forms a secretion pore in the outer bacterial membrane (144). Recently, Betts et al. identified the *Chlamydia* T3SS needle subunit protein CdsF (21). CdsF was able to polymerize into multisubunit structures and found to be present on EB surfaces or localizing to the inclusion membrane during infection (21). Finally, the chlamydial translocators CopB and CopD are predicted to assemble into a secretion pore in the inclusion membrane to facilitate translocation of effector proteins.

In *Chlamydia*, CopB can be detected in the inclusion membrane where it is believed to form the translocation pore. Interestingly, CopB has a paralogue namely CopB2, which was found to localize into the host cell cytosol (71). Given its similarity to YopB, CopB2 most likely also participates in the formation of the translocation pore. However, its exact molecular function awaits investigation. CopN is a chlamydial T3SS substrate and has been shown to be translocated by the heterologous secretion system of *Y. enterocolitica* (73) and *S. typhimurium* (110). The *Yersinia* homologue of CopN, YopN, has been proposed to function as plug for the T3SS that is secreted and released upon contact with the host cell thereby facilitating the secretion of other effectors. CopN is detected in the chlamydial inclusion membrane by immunofluorescence microscopy further strengthening its possible role as T3S regulator (73).

Flagellar T3S genes

Interestingly, all members of the *Chlamydiaceae* contain a set of flagellar genes. *Chlamydia*e have two homologues for the flagellar genes *fliF*, *fliH*, *fliI* and *flhA* and a single homologue for *fliN*, *fliP*, *fliQ*, *fliR* and *flhB* (138). Since *Chlamydiae* are non-motile this observation was surprising. Transcriptional analysis by microarray confirmed that flagellar genes are expressed suggesting that they are not just dwindling evolutionary remains (20). However, their function is unknown. As the flagellar system is thought to be the evolutionary ancestor of T3SSs one could envision a supplementary role of these proteins in T3S. So far, there is no experimental evidence whether flagellar gene products can contribute to the assembly of functional secretion pores or if they are essential for T3S. However, recent data by Spaeth et al. demonstrated that FlhA, the flagellar homologue of CdsV, interacts with CdsQ raising the possibility that

the *Chlamydia* T3S apparatus might be composed of a mixture of flagellar and T3S components (249).

Chaperones

Efficient secretion of T3S substrates frequently requires chaperones. T3S chaperones identified so far are small, acidic, dimeric proteins with no obvious sequence similarities among them (205). Dependent on what kind of substrate they recognize, translocator proteins or effector proteins, they can be distinguished into two categories, class I and class II chaperones (271). Class I chaperones can be associated with one or two effector proteins, whereas class II chaperones assist in the secretion of translocator proteins. Chaperones play a crucial role in recognition and unfolding of T3S substrates (69). Moreover, they have also been implicated to be major players in the regulation of T3S activity as either activators or repressors (205).

In *Chlamydia*, T3S associated chaperones were identified based on their homologies to known chaperones in other T3SSs (73, 74, 116). In this way a family of specific *chlamydia* chaperones, Scc1, Scc2 and Scc3 was uncovered. Scc1 is homologous to the *Yersinia* chaperone SycE, which binds the effector protein YopE. Its substrate in *Chlamydia* remains unknown. Scc2 (LcrH-1) and Scc3 (LcrH-2) chlamydial chaperones are homologous to the *Yersinia* translocator chaperone SycD. In *Yersinia*, SycD facilitates secretion of the translocator proteins YopB and YopD, which form the T3S translocation pore in the eukaryotic membrane. Recently, Fields et al. showed that Scc2 and Scc3 can interact with the *Yersinia* translocator YopD strengthening their potential role in translocator secretion in *Chlamydia* (71). In further analysis, they were able to confirm that Scc2 and Scc3 interact with CopB in *Chlamydia* (71). In addition, Slepenkin et al. showed that Scc3 can interact with CopN, a chlamydial T3S substrate, and therefore may function as its chaperone (245). Lately, Betts et al. described two chaperones, termed CdsE and CdsG, which assist in the translocation of the T3S needle subunit CdsF (21). In an elegant yeast two hybrid study Spaeth et al. recently defined the chlamydial chaperones CT260 and CT700 as hubs of protein-protein interactions linking the *Chlamydia* T3S apparatus to secreted effector proteins (249). While the function of some chlamydial chaperones is beginning to emerge, others await further characterization and yet more are to be discovered.

Expression and regulation of T3S genes in *Chlamydia*

Since the first description of T3S associated genes in *C. caviae* in 1997 (116), numerous studies addressed whether this secretion mechanism is functional in *Chlamydia*. By now, transcriptional analysis has shown that all putative T3S associated genes are expressed both in *C. trachomatis* (19, 194, 241) and in *C. pneumoniae* (247) and proteomic studies indicate that T3S proteins are detectable in EBs and RBs (74, 283, 284). The majority of the genes coding for the apparatus are upregulated relatively late during infection (~18 h post infection), with only little transcription as early as 6-8 h post infection (19, 74, 194, 241).

Transcriptional activity of T3S genes late during the developmental cycle coincides with RB to EB conversion suggesting that infectious EBs are pre-armed with T3SS apparatuses presumably required for successful invasion of host cells. This hypothesis is further supported by the presence of functional T3S pores during early cycle development (74). It has also been shown that *Chlamydia* triggers its own internalization by secretion of the EB-associated effector protein TARP (see below).

Despite detailed knowledge of the temporal expression of T3S associated genes comparatively little is known about their regulation. As mentioned above, chaperones have been implicated in the regulation of T3S activity. In *Chlamydia*, two homologues of the *Yersinia* chaperone LcrH, namely LcrH-1 and LcrH-2, were identified and it was suggested that they might function at different times during infection (264). Ouellette et al. presented evidence that *C. pneumoniae* LcrH-1 and LcrH-2 differentially regulate T3S (199). The *lcrH-1* gene cluster is expressed late in the developmental cycle, when RB to EB conversion occurs and the *lcrH-2* gene cluster was found to be expressed mainly during RB replication. This observation suggests that *lcrH-1* associated gene products are required to pre-arm EBs hence play a crucial role during invasion while proteins of the *lcrH-2* operon likely function in intracellular survival. Recently, Hefty and Stephens presented evidence that T3S associated genes are encoded by six operons that are preceded by σ70-like promoter elements and are differentially expressed during the developmental cycle (105). The presence of a common sigma factor for all T3S associated genes suggests that additional regulatory elements, which remain to be identified, exist.

1.4.3 Effector proteins

Since the discovery of a T3SS in *Chlamydia* attempts to characterize chlamydial effector proteins have been challenging in the absence of a genetic system to modify *Chlamydia*. As bacterial effector proteins are very diverse in pathogens endowed with a T3SS, initial efforts to identify chlamydial effectors, based on sequence and structural similarity with other T3S effectors, were of limited success. In recent years, a number of chlamydial effector proteins were identified by T3S mediated translocation in heterologous secretion systems (73, 110, 265, 266). These assays employ fusion constructs of a chlamydial gene, including the first few codons or in some cases the complete gene, with a reporter molecule which allows for later detection. In this way, chlamydial proteins were demonstrated to be translocated in *Yersinia*, *Shigella* and *Salmonella* therefore likely to be effector proteins in *Chlamydia*. Table 1 gives an overview of *C. trachomatis* T3S associated effector proteins based on their subcellular localization in infected cells and their secretion by a heterologous secretion system. Within the family of inclusion membrane proteins (referred to as Inc-proteins) numerous other members were identified but omitted from the table for clarity (161).

Table 1. *C. trachomatis* T3S associated effector proteins

Demonstrated by heterologous secretion and subcellular localization:

Name	Localization	Function	References
IncA	inclusion membrane	homotypic vesicle fusion	(97, 266)
IncC	inclusion membrane	?	(74, 266)
Tarp	host cell cytosol	actin nucleator involved in EB internalization	(51, 131)
Cap1	inclusion membrane	?	(77, 265)
CT847	host cell cytosol	interacts with cyclin D interacting protein (GCIP)	(44)
CADD	host cell cytosol	involved in apoptosis	(250, 265)
CT694	host cell cytosol	interacts with human AHNAK	(115)

Inc-proteins

The first inclusion membrane proteins were identified using antisera derived from animals infected with *C. caviae* GPIC that labeled the inclusion membrane of GPIC infected HeLa cells but showed no reactivity with either EBs or RBs (224). These sera were then used to screen a bacteriophage library expressing chlamydial genes which led to the identification of *C. psittaci* IncA (223), IncB, and IncC (11). When the first *Chlamydia* genomes were sequenced Inc-proteins were also identified in *C. trachomatis* and *C. pneumoniae*. Since then numerous other Inc-proteins have been described (161, 235) and were also shown to be translocated by the *Shigella* and *Yersinia* T3SS (74, 266). Inc-proteins share only minimal primary aa similarities but a large hydrophobic domain with parts facing the cytoplasmic site of the inclusion is characteristic to all of them (reviewed in (225)). They are in general poorly conserved and many *C. trachomatis* Inc-proteins are not present in *C. pneumoniae*. Inc-proteins possess no conserved secretion signal but based on their hydrophobicity profiles it was suggested that as much as 5% of the chlamydial genome encodes inclusion membrane proteins (225). As Inc-proteins are displayed at the inclusion membrane facing the eukaryotic host cell they are believed to play an integral role in the pathogen-host crosstalk of *Chlamydia*. Functional data for most Inc-proteins is however lacking.

The best characterized inclusion membrane protein is IncA which has been shown to be required for homotypic vesicle fusion of *Chlamydia*-containing inclusions in multiple infected cells (97). Moreover, IncA displays homology to SNARE proteins, key players of membrane fusion events (59, 60). In contrast to other Inc-proteins which are transcribed early during the chlamydial developmental cycle and therefore believed to significantly contribute to early inclusion maturation, *incA* is transcribed around 10-12 h post infection (97). Interestingly, clinical isolates of *C. trachomatis* with a non-fusogenic phenotype lack IncA or possess mutant forms (204, 268). So far, IncA is the only inclusion membrane protein with assigned function.

Other members of the Inc-family include the proteins IncB/C/D/E/F and IncG, which was demonstrated to interact with the mammalian signaling adaptor molecule 14-3-3ß at the inclusion membrane (238). Any downstream signaling events however remain elusive. The *C. trachomatis* gene CT229 belongs to six *inc*-genes expressed within the first few hours after infection (19, 241) and its gene product was demonstrated to interact with Rab4 at the inclusion membrane (229). Rab proteins are key regulators of membrane trafficking and therefore important in the establishment of the intracellular

inclusion. In addition to Rab4, also Rab1, Rab6 and Rab11 are recruited to the *C. trachomatis* inclusion (230). In *C. pneumoniae* Rab1, Rab10 and Rab11 but not Rab6 were demonstrated to interact with the inclusion membrane suggesting both a species-dependent and species-independent recruitment of Rab-GTPases to chlamydial inclusions (230). Recently, the *C. pneumoniae* Inc-protein CPn0585 was shown to interact with multiple Rab proteins including Rab1, Rab10, and Rab11 but not with Rab4 or Rab6 (54). In addition, *C. trachomatis* CT813 was demonstrated to be an inclusion membrane protein (45) capable of interacting with the host SNARE proteins Vamp7 and Vamp8 (60). Taken together, Inc-proteins are a very exciting family of effector proteins that presumably play a crucial role in many interactions of *Chlamydia* with its host cell. Finally, two non-Inc like proteins, CopN and Cap1, that lack the typical bi-lobed hydrophobic domain of classical Inc-proteins, were reported to localize to the chlamydial inclusion membrane (225). CopN is secreted by the *Yersinia* T3SS and believed to function as T3S regulator (73). Similarly, Cap1 is also detected in the inclusion membrane of infected cells and was recognized as CD8+ T cell target (77). Its function is still unknown.

CADD

Stenner-Liewen et al. identified a *C. trachomatis* protein with homologues in other *Chlamydia* species which induced apoptosis when transiently expressed in mammalian cells (250). The authors further showed that this protein was expressed late during the infectious cycle and was capable of binding to death domains of the TNF family receptors hence its name CADD, *chlamydia* protein associating with death domains. CADD localized to the cytoplasm of infected cells and was later shown to be a T3S substrate (265).

TARP

The *C. trachomatis* protein TARP (CT456), translocated actin recruiting phosphoprotein was the first EB-associated effector protein described. TARP is expressed from mid to late cycle during *Chlamydia* development and was shown to be translocated by the *Y. pseudotuberculosis* T3SS. TARP, when secreted upon contact of EBs with the host cell, gets rapidly phosphorylated in the host cytoplasm and induces local actin rearrangements resulting in bacterial internalization (51). Jewett et al. later showed that TARP is an actin nucleator and its tyrosine rich repeat region is

phosphorylated by host cell Src family tyrosine kinases (130, 131). Recently, *C. trachomatis* TARP was demonstrated to interact with two guanidine nucleotide exchange factors in a phosphorylation dependent manner. Their activation initiates a downstream signaling cascade which ultimately results in actin recruitment to the bacterial attachment site (156). Interestingly, phosphorylation of the tyrosine rich repeat region is not required for efficient EB internalization (130). Moreover, TARP present in other *Chlamydia* species lacks those tyrosine rich repeats with no subsequent phosphorylation occuring. These data suggest that alternative signaling events must accompany *Chlamydia* entry.

CT847

By using the heterologous secretion system of *Yersinia*, the *C. trachomatis* protein CT847 was identified as T3S effector protein (44). CT847 is a mid cycle protein that was demonstrated to interact with Grap cyclin D interacting protein (GCIP) by yeast two hybrid studies. GCIP, a protein involved in cell cycle regulation, is degraded during infection with *C. trachomatis* and depletion of GCIP by siRNA enhances *Chlamydia* replication. These results suggest that *Chlamydia* actively interfere with the host cell cycle.

CT694

Recently, the *C. trachomatis* protein CT694 was shown to be a novel T3S effector protein involved in early *Chlamydia* development (115). CT694 is expressed late during the developmental cycle and presumably prepackaged into Ebs. It was secreted by the *Yersinia* T3SS and found, by yeast two hybrid studies, to interact with human AHNAK, a giant phosphoprotein associated with the plasma membrane of epithelial cells. Ectopically expressed in HeLa cells, CT694 localized to the plasma membrane and caused cytoskeletal rearrangements in particular of actin fibers. These data collectively suggest a role of CT694 as modulator of the host cytoskeleton early during infection.

T3S independent effector proteins

Notably, *Chlamydia* also secretes type III independent effector proteins, such as the chlamydial proteases CPAF (314) and Tsp/CT411 (153, 154).

The chlamydial protease-like activity factor CPAF is a type II secretion substrate and is secreted into the lumen of the inclusion before being translocated into the cytoplasm of infected cells (108). CPAF is considered to play a universal role in *Chlamydia* pathogenesis by proteolytically targeting various host proteins. CPAF was first described to contribute to *Chlamydia* immune evasion by degradation of the host transcription factors RFX5 and USF1 required for MHC antigen presentation (314). Similarly, Kawana et al. presented evidence that CPAF is involved in the degradation of the MHC-like protein CD1d during chlamydial infection (137). In addition, CPAF was shown to inhibit host cell apoptosis by degradation of BH-3 only pro-apoptotic proteins (89, 212). Recently, CPAF was even reported to be involved in the modulation of the host cytoskeleton by cleavage of keratin 8, a major component of intermediate filaments (63).

Taken together, CPAF with its diverse substrates is emerging as a central player in the *Chlamydia*-host crosstalk. Similar to CPAF, Tsp/CT411 is predicted to be type II-secreted and cleaves NF-κB upon translocation into the host cytoplasm thus directly modulating host immune responses (153). Other type II secreted proteins remain to be identified and characterized.

1.5 SMALL MOLECULE INHIBITORS

The discovery of antibiotics has revolutionized modern medicine, in particular the treatment of infectious diseases. However, the increasing emergence of antibiotic resistance is a major public health concern and novel strategies to combat bacterial infections are desperately needed. In this section, the classical concept of antibiotics will be discussed and new antimicrobial approaches, including antibodies and small molecules will be highlighted.

1.5.1 Concept of classical antibiotics

At the end of the 19th and the beginning of the 20th century a number of well recognized scientists, like Paul Ehrlich, Robert Koch and Louis Pasteur conducted studies to identify substances against bacterial infections. Paul Ehrlich pioneered the development of antibiotics with his work that led to the identification of a synthetic substance to treat syphilis (reviewed in (83)). However, the discovery of substances derived from environmental bacteria and fungi finally paved the way for a new era in infectious

disease medicine. In 1928, Alexander Fleming discovered penicillin, the first natural antibiotic. When the full therapeutic potential of penicillin was recognized, pharmaceutical companies undertook massive screening programs searching for additional natural substances with antimicrobial activity. Today, most antibiotics are either (I) natural products of microorganisms, (II) semi-synthetically produced from natural products, or (III) chemically synthesized based on the structure of the natural products. The majority of naturally occuring antibiotics was isolated from *Streptomyces* spp. that are common soil bacteria, followed by fungi, including *Penicillium* and *Chephalosporidium*. Taken together, the golden age of antibiotic discovery (1945-1960) yielded most of the chemical classes of antibiotics that are now in clinical use (61).

Antibiotics can be classified in three ways: by target site, by chemical structure or based on whether they are bactericidal (that is they kill bacteria) or bacteriostatic (that is they inhibit bacterial growth). The next paragraph will give a brief overview of the different classes of antibiotics according to their target site.

Inhibitors of cell wall synthesis

The most important inhibitors of bacterial cell wall synthesis are the ß-lactams (penicillin) and glycopeptides (vancomycin). β-lactams act by binding to penicillin binding proteins, like carboxypeptidase, transglycosylase and transpeptidase thereby inhibiting the final stages of cross-linking of the bacterial cell wall. Glycopeptides bind the peptide side chain of N-acetylmuramic acid and interfere with transglycosylation thus preventing peptidoglycan synthesis.

Inhibitors of protein synthesis

Antibiotics of this class commonly bind to different sites of ribosomes which will stall protein translation. Examples of antibiotics that target bacterial protein synthesis include aminoglycosides (kanamycin, gentamycin), tetracycline, macrolides (erythromycine) and chloramphenicol.

Inhibitors of nucleic acid synthesis

There are two classes of nucleic acid synthesis inhibitors: quinolones that inhibit DNA replication and rifampicin that targets the RNA polymerase.

Inhibitors of metabolic pathways

Antimetabolides, like sulfonamides and trimethoprim inhibit bacterial metabolic pathways to synthesize precursors for nucleic acid synthesis.

Inhibitors of plasma membrane function

The most important members of this class are the polymyxins which act by disrupting membranes of Gram-negative bacteria.

Antibiotics are probably the most effective classes of all drugs and their discovery was a major milestone in fighting bacterial infections hence greatly impacting human health. The initial period of antibiotic screenings was followed by many years of chemical refinement of already identified natural substances to improve their pharmacodynamic and pharmacokinetic properties. In the late 1960´s it was believed that the magic weapon to fight bacterial infections was found and the need for new antibiotics was questioned. Thus, pharmaceutical companies turned their attention to other diseases and antibacterial drug discovery dramatically slowed down (200). The reasons for that are very complex as drug development has not only turned into an expensive and time consuming process but is also ruled by many interests including those of manufacturers, scientists, lawyers, clinicians and governmental authorities (217, 243). With the intensive use of antibiotics, an increasing emergence of antibiotic resistant bacteria was noticed all over the world. The term antibiotic resistance describes the ability of microorganisms to continue to grow in the presence of cytotoxic concentrations of antibiotics. Resistance can evolve by multiple means for example through chromosomal mutations or through the acquisition of resistance genes (160). These changes can ultimately lead to altered target sites or altered uptake of antibiotics. Moreover, several bacterial pathogens possess enzymes that directly destroy or inactivate antibacterial substances (301). The drastic increase in number, diversity and range of resistant bacteria has become a huge clinical problem. Nowadays we are not only facing single drug resistant bacteria but also multidrug resistant bacteria such as *Staphylococcus aureus*, enterococci or *Pseudomonas aeruginosa* (4, 163). Since the early 1960´s, only two new classes of antibiotics entered the market, namely daptomycin in 2000 and linezolid in 2003. Clearly, novel antimicrobials are needed to effectively treat infectious diseases and in particular cope with multiple drug resistant pathogens (164).

1.5.2 Next generation antimicrobials

In light of the increasing emergence of bacteria being resistant to antibiotics, new therapeutic strategies are being developed to fight infectious diseases. Over the past two decades significant advances were made in our understanding of the nature of many pathogenic bacteria. The availability of complete bacterial genome sequences together with detailed knowledge about the intimate interplay between pathogenic bacteria and their hosts have led to the idea to combat bacterial infections by specifically targeting bacterial virulence. These newly developed antimicrobials aim to inhibit bacterial virulence traits thereby disarming the pathogen without affecting its growth. In contrast to the concept of conventional antibiotics, this strategy would considerably limit the probability of developing drug resistance.

Virulence factors that are currently in focus as attractive antimicrobial targets include essential regulatory pathways required for virulence gene expression, two component systems, bacterial toxins, adhesins or invasins that promote bacterial entry into host cells and in particular bacterial secretion systems. The approach to target bacterial virulence determinants allows tailoring drugs that are highly selective and pathogen specific without affecting the human normal flora. Antivirulence drugs can be designed to target one specific bacterium or a group of bacteria that all share the same virulence factor. As discussed above, T3SSs of Gram-negative bacteria are complex molecular needle-like injection systems that enable pathogenic bacteria to manipulate diverse host cellular processes hence play an important role for bacterial virulence. Because the overall structure and composition of bacterial T3SSs is quite conserved among different Gram-negative bacteria, T3SSs constitute a promising target for novel antimicrobial substances (188). Examples of two classes of antimicrobials, inactivating antibodies and small molecules that were developed to specifically target the T3SS, will be discussed in more detail below.

Protective antibodies

The Gram-negative, opportunistic pathogen *Pseudomonas aeruginosa* poses a huge problem in hospitalized patients because it is intrinsically resistant to multiple antibiotics. Recently, Frank et al. identified a monoclonal antibody that targets the tip protein PcrV of the *P. aeruginosa* T3SS thereby blocking the translocation of bacterial effectors (79). The antibody was shown to be highly efficient in a murine

chronic *P. aeruginosa* infection model hence possesses promising therapeutic potential to treat infections caused by *P. aeruginosa* (123). In a similar approach, an antibody against LcrV, the PcrV homologue in *Yersinia pestis*, was identified and demonstrated to neutralize bacteria-mediated macrophage cytotoxicity (310).

Small molecules

In recent years, a number of high throughput screens of chemical libraries were performed to identify small organic molecules that are capable of blocking bacterial virulence. One study identified a compound, called virstatin that inhibits the production of cholera toxin by blocking its transcription factor ToxT in *Vibrio cholerae* (120). Lately, several screens have particularly focused on the T3SS as potential antimicrobial drug target since it is present in a wide range of Gram-negative pathogenic bacteria, including *Salmonella*, *Shigella*, *Yersinia* and *Chlamydia*. Kauppi et al. first described T3SS inhibitors in *Yersinia* (136). As these compounds will be the main focus of this thesis, they are discussed in more depth below. Meanwhile, new classes of molecular inhibitors of the *Yersinia* T3SS were identified and characterized (203). In addition to *Yersinia*, Gauthier et al. screened a small molecule library which led to the identification of compounds that affected T3S associated virulence genes in enteropathogenic *E. coli* (82). Felise et al. identified 2-imino-5-arylidene thiazolidinone as potent inhibitor of the *Salmonella* T3SS and could further demonstrate that this compound also targets the T3SS of a wide array of other animal and plant pathogens without affecting bacterial growth (70). Finally, guadinomines produced by a *Streptomyces* spp. were demonstrated to inhibit T3S (1, 127, 128).

Taken together, several studies using protective antibodies and small molecules are holding promising results for the development of novel antimicrobial drugs. In the future, the combination of classical antibiotics and new therapies including highly specific virulence blockers will most likely play an important role in the successful treatment of infectious diseases.

1.5.3 INPs

Small molecule inhibitors of T3S in *Yersinia* were first described by Kauppi et al. and collectively termed INPs (former Innate Pharmaceuticals AB, now Creative Antibiotics AB). These compounds belong to a class of acylated hydrazones of different

salicylaldehydes and have been demonstrated to target T3S in *Yersinia*, *Salmonella*, *Shigella*, *E. coli* and *Chlamydia*. The major findings concerning INPs and their effect on different Gram-negative bacteria will be summarized below.

INPs and *Yersinia*

In an approach to identify small molecule inhibitors of *Yersinia* T3S, a chemical library screen was performed using *Yersinia pseudotuberculosis*. The assay was based on secretion of a luciferase reporter gene under control of the *Yersinia* effector protein YopE and led to the identification of several compounds that target *Yersinia* T3S at different levels (136). The identified compounds, salicylidene acylhydrazides, with no or only modest effect on bacterial growth thus proved to be valuable candidates to chemically attenuate *Yersinia* virulence. In a follow up study, the same authors showed in an elegant way that salicylidene acylhydrazides and related compounds specifically target *Yersinia* T3S by blocking translocation of effector proteins (195). The exact mode of action and the molecular target however remain to be identified.

INPs and *Chlamydia*

In *Chlamydia*, multidrug resistance is not yet considered a major problem and only a few cases of antibiotic resistant *Chlamydia* strains were reported (159, 233, 248). The reason for that might be that intracellular bacteria are in general less prone to genetic exchange, including the acquisition of resistance genes (173) and/or that intracellular bacteria carrying antibiotic resistance genes might have reduced viability (132). Although acute chlamydial infections can still be effectively cured with antibiotics, antibiotic treatment of chronic chlamydial infections is often of limited success thus alternative means of interfering with *Chlamydia* activity are desirable.

The effect of INPs on T3S of *C. trachomatis* and *C. pneumoniae* will be the major part of this thesis and discussed in Chapter 4, papers I-III.

Wolf et al. have used INP0007 (termed C1 in their study) to examine its effect on chlamydial T3S (297). The authors showed that C1 treatment inhibits *C. trachomatis* L2 development in a dose dependent manner. This effect was *Chlamydia*-specific, as treatment of another obligate intracellular bacterium, *Coxiella burnetii*, had no effect on development. Drug-treated *Chlamydiae* remained metabolically active but were

impaired in RB to EB differentiation. Furthermore, C1 inhibited the chlamydial T3SS as shown by accumulation of the effector proteins CADD, IncA and Tarp within the bacteria.

In another study performed by Slepenkin et al. a panel of 25 INPs was analyzed. Consistent with the results of Wolf et al. INPs were found to have a strong effect on chlamydial growth (246). Using RT-PCR, the authors further showed that treatment of *Chlamydia* with INP0341 caused a transcriptional down-regulation of several T3S-associated genes, including those encoding the effector proteins IncA and Tarp as well as *yscC*, *copB* and *copB2*, all encoding structural components of the chlamydial T3SS. Interestingly, the inhibitory effect on growth of INPs was reversible by addition of iron when supplied as ferrous sulfate, ferric chloride or transferrin suggesting that T3S activity might be linked to iron.

Finally, Prantner and Nagarajan investigated the role of T3S on the expression of inflammatory cytokines in *C. muridarum*-infected macrophages (216). The authors demonstrated that T3S inhibition by INP0007 blocked *Chlamydia* growth in macrophages and led to decreased levels of IL-6, IL-1ß and CXCL10. While chlamydial growth in macrophages could be restored by addition of iron, cytokine production was not arguing that the expression of cytokines is dependent on T3S activity.

INPs and *Salmonella*

In *Salmonella*, Hudson et al. showed that INPs affected protein secretion via the *Salmonella* T3SS-1, required for bacterial invasion of epithelial cells, without inhibition of bacterial growth. In further *in vivo* studies using cows, they demonstrated that INPs suppressed *S. enterica* serovar *typhimurium* T3SS-1 induced secretory and inflammatory responses (117). Moreover, Negrea et al. provided additional evidence that INPs inhibit secretion of the *Salmonella* effector protein, SipB, by the *Salmonella* T3SS-1. INPs also inhibited T3SS-2 mediated intracellular replication and flagellum mediated motility (192). Interestingly, they further showed that treatment with INPs led to transcriptional silencing of T3SS-1 associated genes (192).

INPs and *Shigella*

Recently, Veenendaal et al. investigated the effect of INPs on the *Shigella flexneri* T3SS (286). In *Shigella*, INPs were found to inhibit effector protein secretion, T3S-

mediated invasion of epithelial cells and macrophage apoptosis. The authors suggest that INPs function by blocking assembly of the secretion apparatus, as bacteria treated with INPs lacked T3S needles or only had needle structures significantly shorter than those of wild type bacteria.

INPs and *E. coli*

Gauthier et al., using small molecules structurally similar to INPs, demonstrated that these compounds may inhibit the expression of T3S associated genes in enteropathogenic *E. coli* (82). In addition, Tree et al. characterized the effect of four INPs on global gene expression in *E. coli* O157:H7. They confirmed that INPs affect the expression of T3S associated genes, but also of numerous other virulence genes. The authors therefore suggest that INPs act through transcriptional regulators rather than directly affecting the T3S apparatus (280).

2 AIMS

GENERAL AIMS

The general aim of this thesis was to assess the role of T3S in the intracellular development and pathogenesis of *Chlamydia* by using small molecule inhibitors (INPs) that target the T3SS. Moreover, this thesis aimed to identify and characterize novel chlamydial T3S associated effector proteins.

SPECIFIC AIMS

Paper I

To study the effect of INP0400 on *Chlamydia trachomatis* at different stages during the infectious cycle.

Paper II

To assess the effect of INP0010 on the intracellular development of *Chlamydia pneumoniae* and transcription of T3S associated genes.

Paper III

To investigate the effect of INP0400 and INP0341 on *Chlamydia trachomatis* and *Chlamydia caviae* invasion of epithelial cells.

Paper IV

To characterize a new family of chlamydial T3S effector proteins that share a common C-terminal domain of unknown function.

3 METHODOLOGY

This section will briefly summarize the main methods and techniques used in paper I-IV. For more detailed descriptions the reader is referred to every individual paper, in particular the "Material and Methods" sections therein.

3.1 CELL CULTURE AND ORGANISMS

HeLa 229 cells were cultured in Dulbecco´s modified Eagles medium supplemented with 10% (v/v) FBS. The mouse fibroblast cell line McCoy was grown in RPMI 1640 supplemented with 10% (v/v) FBS, 25 mM Hepes, 2 mM L-glutamine and 10 µg/ml gentamycin. Hep2 cells were cultured in RPMI 1640 supplemented with 10% (v/v) FBS, 20 mM Hepes, 2 mM L-glutamine, 1 µg/ml amphotericin B and 8 µg/ml gentamycin. All cell lines were maintained at 37°C in the presence of 5% CO_2. *C. trachomatis* L2 was propagated in HeLa 229 epithelial cells, McCoy cells or Hep-2 cells. *C. caviae* GPIC was grown in HeLa 229 cells and *C. pneumoniae* T45 cultured in Hep-2 cells. The individual chlamydial strains were propagated as described in papers I-IV. *Shigella flexneri* strains *mxiD* and *ipaB* were cultured as previously described (266). The *E. coli* strains DH5α and Bl21 were grown in LB or on LB plates at 37°C.

3.2 SMALL MOLECULE INHIBITORS

In this thesis four different salicylidene acylhydrazides were used: INP0400, INP0010, INP0341 and INP0406. INPs were synthesized from commercially available hydrazides and salicylaldehydes (136, 195) and provided by Creative Antibiotics AB, Umeå, Sweden (former Innate Pharmaceuticals AB). The compounds were dissolved in dimethyl sulfoxide and used at the indicated concentrations.

3.3 DNA TECHNIQUES

C. trachomatis DNA was isolated using the "Qiagen DNeasy Tissue Isolation Kit" according to the manufacturer's instructions. The chlamydial genes of interest were amplified by PCR for subsequent cloning into expression vectors. For more detailed information including a list of primers and restriction sites used please refer to paper IV. Cloning was essentially performed as described (168). Plasmid DNA was transformed into chemically competent *E. coli* DH5α and clones containing the right insert were selected by colony PCR.

3.4 RNA TECHNIQUES

RNA extractions from *C. pneumoniae* infected Hep-2 cells were performed with TRIzol according to the manufacturer's instructions. RNA was further purified using the "Qiagen RNeasy Minikit" and subsequently treated with DNAse.

3.5 PROTEIN EXPRESSION AND PURIFICATION

The *C. trachomatis* proteins of interest were expressed as N-terminal 6xHis-tagged fusion proteins in *E. coli* Bl21. Bacteria were grown in LB until an optical density of 0.5 was reached and protein expression was induced by addition of isopropyl-beta-D-thiogalactoside. Cultures were grown for 3 h at 37°C or over night at room temperature after induction. Bacterial cells were harvested and lysed by French press in buffer containing 5 mM imidazole, 300 mM sodium chloride and 20 mM Tris-HCl. 6xHis-tagged fusion proteins were purified from the soluble fraction by affinity chromatography using "Novagen Ni-NTA His Bind resin" according to the manufacturer's guidelines. To assess the quality of protein after purification, purified proteins were separated on 10% or 12% SDS-PAGE gels and Coomassie-stained for total protein.

3.6 ANTIBODY PRODUCTION AND IMMUNODETECTION

Rabbit polyclonal antiserum specific to IncA was generated by Innovagen (Lund, Sweden) using a peptide sequence corresponding to the amino acids 252-266, CSQIRETLSSPRKSA. For generation of antibodies to CT711, CT620 and CT621 purified proteins were used to immunize New Zealand White rabbits (Agro-Bio, La Ferte Saint-Aubin, France).

Immunodetection of respective proteins was performed with samples of *C. trachomatis* L2 infected HeLa 229 cells. Samples were lysed in 1% sodium dodecyl sulphate, 6 M urea, 150 mM NaCl and 30 mM Tris-HCl and resolved by SDS-PAGE. After transfer to PVDF membranes blots were blocked with 5% skim milk for 1 h followed by incubation with primary antibodies overnight. Membranes were probed with alkaline phosphatase-conjugated secondary antibodies and visualized by Amersham ECL Plus.

3.7 QUANTITATIVE REAL-TIME PCR

C. pneumoniae genome-equivalents in material harvested from infected Hep-2 cells after treatment with INP0010 were measured by quantitative real-time PCR essentially

as described in paper II. Expression of chlamydial T3S genes was evaluated by reverse transcription-PCR using the "iScript one step RT-PCR kit" containing SYBR green (Biorad) according to the manufacturer´s instructions. Detailed information on primers and reaction conditions can be found in paper II.

3.8 MICROSCOPY

3.8.1 Immunofluorescence microscopy

Cells were grown on glass coverslips and infected with *Chlamydia* for the times indicated. Detailed information on individual cell lines and respective chlamydial strains can be found in each paper. When INPs were used, the compounds were added directly to the culture medium at the concentrations and time points indicated. For immunolabelings, coverslips were fixed with paraformaldehyde or ice-cold methanol and dependent on the fixation method different detergents were used to permeabilize the cells (see paper I-IV). All primary and secondary antibodies that have been used in this thesis are listed in table 2. For transfection experiments plasmids were used as indicated and transfection was performed using Fugene transfection reagent (Roche) according to the manufacturer's guidelines.

3.8.2 Electron microscopy

C. trachomatis L2 infected McCoy cells were fixed in 0.1 M sodium cacodylate buffer containing 2% glutaraldehyde, 0.5% paraformaldehyde, 0.1 M sucrose and 3 mM $CaCl_2$ for 30 minutes before being further processed for transmission electron microscopy essentially as described in paper I.

Table 2. Antibodies used in this thesis

Primary antibodies
rabbit anti-IncB antibody (gift from Dr. Lugert, Göttingen, Germany)
rabbit anti-IncC antibody (gift)
mouse anti-*Chlamydia* antibody (Argene)
mouse anti-EfTu antibody (gift from Dr. Zhang, Boston, USA)
rat anti-HA antibody (Roche)
rabbit anti 14-3-3ß (Santa Cruz Biotechnology)
rabbit anti-IncA (paper I)
mouse FITC-conjugated anti-*Chlamydia* LPS antibody (Bio-Rad)
mouse anti-Hsp60 (AffinityBioreagents)
rabbit anti-CT260 (gift from Dr. Valdivia, Durham, USA)
rabbit anti-CT711 (Paper IV)
rabbit anti-CT620 (Paper IV)
rabbit anti-CT621 (Paper IV)
Secondary antibodies
Rhodamine conjugated goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories)
Rhodamine conjugated goat anti-rat antibody (Jackson ImmunoResearch Laboratories)
Cy™-5 conjugated goat anti-mouse antibody (Amersham)
Cy™-3 conjugated goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories)
Alexa488 conjugated goat anti-mouse antibody (Molecular Probes)
Alexa546-phalloidin (Molecular Probes)
Alexa488-phalloidin (Molecular Probes)

4 RESULTS AND DISCUSSION

4.1 PAPER I

In paper I, we employed the *Yersinia* T3S inhibitor INP0400 to examine the role of T3S during *C. trachomatis* development. When INP0400 was given at the time of infection, we observed a dose dependent inhibition of *C. trachomatis* L2 growth at concentrations of 5-25 µM of the drug by immunolabeling, without cytotoxic effect to the eukaryotic host cell. With increasing concentrations of INP0400 chlamydial inclusions became smaller in size, barely visible at 20 µM suggesting a block of replication. To confirm these observations we further analyzed the effect of INP0400 on *C. trachomatis* L2 by electron microscopy. McCoy cells infected with *C. trachomatis* L2 for 30 h showed typical chlamydial inclusions containing RBs and some EBs that had already differentiated from RBs. Infected cells treated with 10 and 20 µM INP0400 showed a dose dependent reduction in inclusion size and a concomitant reduction in the number of bacteria with primarily RBs visible. To assess whether drug-treated bacteria were still viable, a reinfection assay was performed. *C. trachomatis* L2 infected cells treated for 48 h were harvested and used to reinfect a fresh monolayer of McCoy cells. After treatment with 20 µM, we observed a 20-fold reduction in the number of infectious bacteria compared to mock-treated controls and a 3-log reduction when 25 µM were used. These data are in agreement with results from other groups that analyzed the effect of INPs on *Chlamydia* development (246, 297). To further characterize this small molecule inhibitor, the effect of INP0400 on *C. trachomatis* L2 was analyzed when given at different stages during the infectious cycle. First, we assessed the effect on *Chlamydia* entry and observed a 40% reduction of infected cells after treatment with 40 µM INP0400 for the first 3 h of infection suggesting a partial inhibition of entry. Other studies reported that they were unable to inhibit *C. trachomatis* L2 entry into epithelial cells (246, 297). Therefore, the effect of INPs on *Chlamydia* entry was closer examined in paper III. Next we wanted to test whether INP0400 specifically targets the chlamydial T3SS hence blocks the translocation of known effector proteins. We were unable to detect IncG, an early cycle effector protein, in infected cells treated with INP0400 by indirect immunolabeling of 14-3-3β, a protein known to interact with IncG at the inclusion membrane, suggesting that INP0400 impairs IncG secretion. However, we cannot rule out that INPs affected

the transcription of IncG, or that we failed to detect IncG because its abundance was below the detection limit due to the strong inhibitory effect of INP0400 on bacterial replication. We next tested if INP0400 impairs the secretion of another chlamydial effector protein, IncA. IncA is a mid-cycle effector protein and has been shown to mediate homotypic fusions in cells infected with multiple bacteria. McCoy cells were infected at a multiplicity of 5 and treated with 50 µM INP0400 at 8 h post infection for 22 h. While we observed a typical rim-like staining pattern for IncA in mock-treated cultures, IncA was not detectable in drug-treated cultures. Following the same treatment protocol, staining for 14-3-3β in drug-treated cultures revealed multiple single inclusions. This pattern suggests that fusion of inclusions is impaired, which can be attributed to the absence of IncA from the inclusion membrane. These data are in agreement with Wolf et al. who also reported an inhibition of T3S-dependent secretion of IncA upon treatment with another derivative of salicylidene acylhydrazides, while transcription of the gene was not impaired (297). Finally, we analyzed the effect of INP0400 when given late during the infectious cycle. Addition of INP0400 to *C. trachomatis* L2 infected cells at 24 h post infection resulted in a dose-dependent reduction of infectious progeny after 48 h suggesting that INP0400 inhibits RB to EB differentiation. This effect was reversible because cultures treated with the drug after 24 h for 12 h with subsequent removal of the drug contained more infectious bacteria than cultures that were treated after 24 h of infection for additional 24 h. Immunofluorescence labeling further revealed that bacteria treated late during the infectious cycle dissociated from the inclusion membrane and appeared to cluster within the inclusion, leaving an area devoid of bacteria at the inner inclusion membrane. Lately, it was proposed that in *Shigella* INPs act by blocking the assembly of the secretion apparatus as bacteria treated with INPs lacked T3S needles or needle structures were significantly shorter than those of wild type bacteria (286). If that is the case in *Chlamydia*, defective T3S apparatuses might not be capable to establish contact with the inclusion membrane, which could explain the distribution of bacteria within the center of the inclusions. In conclusion, INP0400 has a strong inhibitory effect on growth of *C. trachomatis* L2 and disrupts normal progression through the developmental cycle. If, as our data suggest, INP0400 interferes with T3S activity, this observation implies that a fully functional T3SS is essential for *Chlamydia* pathogenesis.

4.2 PAPER II

In paper II, the effect of the small molecule inhibitor INP0010 was tested on *C. pneumoniae* development. INP0010 was previously identified to block translocation of the *Y. pseudotuberculosis* effector protein YopH into HeLa cells (195). We also included in this study the structurally similar compound INP0406, which is unable to inhibit the T3SS of *Yersinia*. In preliminary experiments, INP0010 was evaluated for any cytotoxic effects using a fluorescence based assay. No cytotoxic effect was observed with Hep-2 cells for concentrations up to 50 µM. When *C. pneumoniae* infected cells were grown in the presence of INP0010 at different concentrations, we observed a dose-dependent inhibition of bacterial replication by immunolabeling and quantitative real-time PCR. In untreated cultures or cultures treated with INP0406 *C. pneumoniae* inclusions developed normally. Pretreatment of host cells or bacteria with INP0010 did not affect *C. pneumoniae* development when grown in the absence of the drug. In *C. trachomatis* L2 infected Hep-2 cells no inhibitory effect on growth was observed in a concentration range of 10-30 µM of INP0010 indicating that *C. trachomatis* is less sensitive to the drug than *C. pneumoniae*. Slepenkin et al. reported that they were able to completely inhibit growth of *C. trachomatis* L2 with 50 µM INP0010 (246). These data suggest that while growth of both organisms is inhibited by INP0010, *C. pneumoniae* responded to lower concentrations. It appears unlikely that the disparity in concentration required to affect bacterial development in both species is caused by structural differences in the T3S apparatus itself but rather due to biological differences. *C. pneumoniae* is in general more difficult to propagate in cell culture and takes longer to grow than *C. trachomatis* strains.

Similarly, we tested INP0400 (189) on *C. trachomatis* L2 and *C. pneumoniae* growth and found that INP0400 affected growth of both *Chlamydia* species at 10 µM. To assess if INP0010 affects the translocation of *C. pneumoniae* effector proteins we analyzed the secretion of *C. pneumoniae* IncB and IncC in the absence and in the presence of the drug. In untreated cells both effectors localized to the inclusion membrane as visualized by immunofluorescence microscopy. When *C. pneumoniae* infected cells were treated with 10 µM INP0010, no specific signal for IncB or IncC was observed, suggesting that INP0010 impairs synthesis and/or translocation of these effectors. To further address this question we analyzed the expression levels of two *C. pneumoniae* T3S apparatus specific genes (*cpn0824* and *cpn0702*) and of the genes encoding the effector proteins IncB and IncC (*cpn0291* and *cpn0292*) in the presence

and in the absence of INP0010. Both T3S apparatus genes, *cpn0824* and *cpn0702*, were down-regulated in drug-treated cultures compared to untreated controls suggesting that INP0010 affects T3S activity by inhibition of transcription of T3S associated genes. Similar observations were made in *Salmonella* (192) and *E. coli* (82, 280), where treatment with INPs was shown to cause transcriptional silencing of T3S genes. The expression levels of *cpn0291*, encoding IncB, increased by two-fold while expression of *cpn0292* remained similar to untreated controls. These data suggest that IncB and IncC expression was not impaired by INP treatment, and that their absence from the inclusion membrane is due to a defect in translocation. However, we cannot entirely rule out that the absence of detectable signal might not be T3S specific, but be a consequence of the strong growth inhibitory effect of INP0010 and concomitant decrease in bacterial numbers that result in low antigen levels.

Taken together, INP0010 displays a strong inhibitory effect on growth of *C. pneumoniae* development, affects translocation of the *C. pneumoniae* effector proteins IncB and IncC and leads to down-regulation of T3S associated genes collectively suggesting that INP0010 impairs T3S activity in *C. pneumoniae*.

4.3 PAPER III

In paper III, we examined the effect of INP0400 and INP0341 on *Chlamydia* invasion of epithelial cells. INP0400 was previously shown to inhibit *Chlamydia trachomatis* L2 growth in a dose-dependent manner from 5 to 25 µM (189). In this study, INP0341 another derivative of salicylidene acylhydrazide, was found to have a strong effect on *C. trachomatis* L2 growth at concentrations of 10 µM, suggesting that INP0341 is an even more potent inhibitor of bacterial replication. We also tested the effect of both drugs on the development of *C. caviae* and found that this chlamydial strain was less susceptible. However, 60 µM of INP0341 resulted in a 99.8% reduction of *C. caviae* infectious progeny with no toxicity on the cells. We therefore chose this concentration to assess the effect of INPs on *C. caviae* entry. HeLa cells were infected with *Chlamydia trachomatis* L2 or *C. caviae*, and intracellular and extracellular bacteria were visualized 2.5 h post infection using a differential immunolabeling approach. We found that none of the INPs had a significant effect on *Chlamydia trachomatis* L2 or *C. caviae* entry into epithelial cells.

Chlamydia attachment to host cells induces local actin rearrangements along with the recruitment of small GTPases to the bacterial entry site (39, 40, 267). To further examine the effect of INPs on bacterial entry we studied some of the molecular events accompanying *Chlamydia* invasion. As these events are very transient during *C. trachomatis* entry and easier to visualize with *C. caviae* we chose to work with the latter. First, we visualized the recruitment of actin in HeLa cells infected with FITC-labeled *C. caviae* in the presence and the absence of INP0341. Characteristic local actin rearrangements were seen in the control and INP0341-treated cells. When we quantified the overall number of actin patches in the control and drug-treated cells no significant difference was observed. Secondly, we studied the recruitment of Rac, Cdc42 and Arf-6 to *C. caviae* entry sites. HeLa cells transfected with the respective constructs were infected with *C. caviae* and labeled for actin at 10 minutes post infection. Rac-GFP, Cdc42-GFP and HA-tagged Arf6 localized to actin patches to the same extend in cells infected in the presence of INPs as in control cells. Thus, INPs do not interfere with the recruitment of actin or small GTPases to *C. caviae* entry sites.

In summary, our data collectively suggest that INPs impair *Chlamydia* development after entry into host cells. We had previously reported that INP0400 partially inhibits *C. trachomatis* L2 entry into epithelial cells because we observed a 40% reduction of infected cells after treatment with 40 µM INP0400 for the first 3 h of infection. Given the results of this study, where we carefully monitored the immediate events of *Chlamydia* invasion in the presence and the absence of INPs a more likely explanation for our initial interpretation is that other early events following *Chlamydia* entry are susceptible to the drug. In particular Inc-proteins, which are T3S substrates detectable in the inclusion as early as 2-4 h post infection, stand as good candidates (74). Because Inc-proteins are considered to be crucial in the establishment of a protected niche for successful *Chlamydia* replication within the inclusion, any interference with their secretion by INPs might drastically affect *Chlamydia* growth. Similar to our results, Wolf et al., and Slepenkin et al. were unable to inhibit *C. trachomatis* L2 entry in the presence of INPs (246, 297). INPs were first identified and characterized as T3S inhibitors in *Yersinia*. The lack of effect of INPs on *Chlamydia* invasion, a process mediated by at least one T3S associated effector protein, TARP, is therefore surprising. So far the molecular target of INPs is unknown and recent studies on the effect of INPs in other Gram-negative pathogens raise the question of their mode of action. While initially described as small molecules that inhibit the translocation of *Yersinia* effector

proteins (195), there is now accumulating evidence that INPs might function at the level of T3S-associated genes (82, 192, 280) or directly interfere with the assembly of the T3S apparatus (286).

In *Chlamydia*, INPs were demonstrated to block secretion of effector proteins by RBs (189, 297) and one could speculate that the molecular target is missing in EBs, which would explain the lack of effect on *Chlamydia* invasion. It was demonstrated that *Chlamydia* has two homologues of the *Yersinia* chaperone LcrH, namely LcrH-1 and LcrH-2 that differentially regulate T3S (264). While the *lcrH-1* gene cluster is expressed late in the developmental cycle, when RB to EB conversion occurs, the *LcrH-2* gene cluster was found to be expressed mainly during RB replication (199). This might suggest that *Chlamydia* possesses functionally different EB and RB associated T3SSs with different sensitivities to INPs which could explain the lack of effect of INPs on *Chlamydia* entry.

Moreover, it was shown that transcription of *lcrH-1* and other T3S associated genes was inhibited by INP0341 whereas transcription of *lcrH-2* was not (246). In line with this observation, recent data from *Salmonella* and *E. coli* support the idea of INPs as transcriptional regulators. In *Salmonella*, treatment with INPs led to transcriptional silencing of T3SS-1 associated genes (192). Gauthier et al. and Tree et al. further demonstrated transcriptional repression of T3S associated genes and numerous other virulence genes in *E. coli* after treatment with INPs or compounds structurally similar to INPs (82, 280). If INPs affect the transcription of T3S substrates in *Chlamydia* they would have no affect on *Chlamydia* invasion because effectors needed for successful bacterial entry are not newly synthesized during entry. Instead EBs are pre-loaded with effectors late during the infectious cycle when RB to EB conversion occurs. This has been shown for TARP which is at least partially stored in RBs to be released from EBs during infection. In contrast, effector proteins like IncG and IncA are newly synthesized during RB proliferation and their secretion was shown to be inhibited by INP0400 (189).

Lately, INPs were proposed to block the assembly of the T3S apparatus in *Shigella flexneri* because bacteria treated with INPs lacked T3S needles or had needle structures significantly shorter than those of wild type bacteria (286). In *Shigella*, INPs were only effective at inhibiting host cell invasion when added during growth, rather than during infection. If INPs act in a similar manner on *Chlamydia*, one would also expect them to function during intracellular growth when new apparatuses are being made rather than

during *Chlamydia* entry. Blocking the assembly of the T3SS might in turn indirectly lead to down-regulation of T3S-associated genes due to feedback inhibition (286). If, in *Chlamydia*, either the transcription of T3S-associated genes or the assembly of the T3S machinery is inhibited, addition of the drugs at the end of one cycle of infection is expected to affect the next round of infection. This is exactly what was observed when looking at the progeny of *C. trachomatis*-infected cells treated with INP0341 24 h post infection. (246). In this experiment, a decrease in the number of infectious progeny was observed although the inclusions formed upon late INP0341 treatment were as abundant as in control cells. These data suggest that EBs formed in the presence of INPs might be defective in their ability to secrete type III effectors. However, due to the asynchronicity of the *Chlamydia* developmental cycle, it cannot definitively be ruled out that the decrease in the formation of infectious EBs is not due to the reduction of RB multiplication upon INP treatment when the drug is added late in the cycle.

Finally, INPs perhaps have a completely different mode of action because the effect of INPs in *Chlamydia* can be fully reversed by the addition of iron (246). However, there are no other published studies that assessed the role of iron on the effect of INPs in other Gram-negative pathogens endowed with a T3SS that would support a different mode of action of INPs than the ones discussed herein.

In conclusion, while INPs have a strong inhibitory effect on *Chlamydia* intracellular development, they do not inhibit *Chlamydia* invasion.

4.4 PAPER IV

In paper IV**,** included in this thesis as a manuscript, we identify and characterize novel T3S effector proteins in *Chlamydia*. We had previously shown that the *C. pneumoniae* protein CPn0853 and its homologues in *C. trachomatis* (CT712) and *C. caviae* (CCA00914) contain amino-terminal secretion signals recognized by the heterologous T3SS of *Shigella flexneri* (265). Interestingly, these proteins possess a well conserved C-terminal domain of unknown function, referred to as DUF582. By using different bioinformatics approaches to further characterize this domain, we identified 4 or 5 DUF582-containing proteins in each *Chlamydia* species. All DUF582 proteins can be grouped into four clusters based on sequence similarity. Given that DUF582 proteins represented in cluster 1 (CPn0853, CT712, CCA00914) possessed a T3S signal we hypothesized that other members of the DUF582 family might also be T3S substrates.

We found that CT621, CPn0852, Cpn0726 and CT619 all possessed an N-terminal secretion signal recognized for T3S in *Shigella flexneri* suggesting that DUF582 proteins indeed constitute a new family of chlamydial T3S effectors.

To further characterize these proteins and their potential role during *Chlamydia* infection, we raised antibodies against three *C. trachomatis* DUF582 proteins, namely CT711, CT620 and CT621. Immunoblotting of these proteins during infection revealed their expression late during the infectious cycle by 25-30 h post infection, with CT621 already detectable at 20 h post infection. Our results are consistent with the described late cycle expression pattern of DUF582 genes by microarray (19, 171) and suggest that DUF582 proteins might be involved late during *Chlamydia* development or during exit from the host cell. When we assessed the expression and localization of *C. trachomatis* DUF582 proteins by indirect immunofluorescence microscopy we found CT620 and CT621 in the host cell cytoplasm, demonstrating that these proteins are secreted. These results are in agreement with a recent study that observed CT621 in the host cell cytoplasm and in the nucleus of infected cells (111). Our antibody against CT711 failed to recognize native protein and was therefore excluded from our immunolocalization studies. Interestingly, CT620 and CT621 were also detected within the chlamydial inclusion, where they did not colocalize with the bacterial marker protein Hsp60. This observation suggests that effector protein secretion can occur in the absence of a fully assembled T3S apparatus. Whether chlamydial effector proteins play a functional role within the inclusion or just represent a form of leakage from the chlamydial T3S apparatus not in contact with the inclusion membrane is unknown. In *Shigella*, it has been shown that there is 4-5% secretion of proteins into the extracellular medium even in the absence of host cells (176). On the other hand, it appears to be possible that effectors fulfill specific functions within the inclusion. For instance, lipid droplets were recently shown to translocate into *Chlamydia* inclusions (146). Bacterial effectors might aid in the lipid acquisition of *Chlamydia* within the inclusion.

After translocation into host cells, effector proteins are likely to be subjected to post-translational modifications and we searched for such modification in the *C. trachomatis* DUF582 proteins. We observed that CT620 and CT711 migration profiles in SDS-PAGE were modified when extracts had been prepared from cells incubated in the presence of proteasome inhibitors. Irrespective of the post-translational modification involved, these data indirectly suggest that CT711 and CT620 represent secreted

proteins that can be degraded by the proteasome once they access the host cell cytoplasm.

In summary, paper IV describes a new family of chlamydial effector proteins, called DUF582 proteins that are secreted by a T3S mechanism during infection. We provide evidence that several DUF582 proteins contain amino-terminal secretion signals and demonstrate that two DUF582 proteins can be detected in the host cell cytoplasm of infected cells. Considering the multiplication of the DUF582 domain during evolution of pathogenic *Chlamydiae*, these proteins are likely to play important roles in the dialog between the bacteria and the host cell. Therefore, this work opens up a new area of investigation, as discussed in the next chapter.

5 CONCLUSIONS AND PERSPECTIVES

The major aim of this thesis was to investigate the role of T3S during *Chlamydia* development by using small molecules that were identified to specifically inhibit T3S activity in *Yersinia*. In papers I-III, we present evidence that INP0400, INP0341 and INP0010 block the developmental cycle of *C. trachomatis*, *C. pneumoniae* and *C. caviae* resulting in a strong inhibitory effect on growth without being cytotoxic to the host cell. INP0400 inhibited the secretion of the *C. trachomatis* effector proteins IncG and IncA and in *C. pneumoniae* INP0010 was found to interfere with translocation of IncB and IncC suggesting that INPs target the chlamydial T3SS. INP0400 was further shown to impair RB to EB conversion in *C. trachomatis* late during the infectious cycle indicating that T3S activity is required for successful terminal differentiation. In addition, we showed that INPs block the development of *Chlamydia* after entry into host cells. In the absence of tools to genetically modify *Chlamydia* we have used INPs to demonstrate that chlamydial T3S activity is required for *Chlamydia* development at different stages throughout the infectious cycle. I am convinced that in the future small molecules will be great tools to answer a number of different biological questions and might be especially valuable to study obligate intracellular pathogens for which conventional genetic approaches are lacking. Although the molecular target of INPs is not identified to date and future research is needed to further characterize these compounds, our findings indicate that INPs constitute powerful drugs with anti-*Chlamydia* activity. While acute chlamydial infections can be effectively treated with antibiotics, persistent infections with *Chlamydia* and their long term sequelae are major public health concerns. The identification of new substances with different modes of action than antibiotics is therefore important for future treatment strategies of chlamydial infections.

In paper IV, we have identified a new family of effector proteins in *Chlamydia*, referred to as DUF582 proteins because all members within this family are characterized by a common C-terminal domain of unknown function. We showed that *C. trachomatis* DUF582 proteins possess an N-terminal T3S signal, are expressed late during the infectious cycle and can be found secreted into the host cytoplasm and the inclusion during infection. Future studies will aim on the functional characterization of DUF582

proteins and their role in *Chlamydia* pathogenesis. With the identification of novel chlamydial effector proteins and their specific functions a clearer picture of the delicate interplay of *Chlamydia* and its host is beginning to emerge and will help us to unravel more mysteries of this fascinating pathogen. I am convinced the future will hold many more exciting findings in the field of *Chlamydia* research and I will follow the literature with great interest.

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