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Title: The Role of Chlamydial Inclusion Membrane Proteins in Host-Pathogen Interaction and the Development of Novel Methods for Studying Chlamydial Biology

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Daniel D. Rockey

The majority of our modern understanding of bacterial pathogenesis is based on the strategy that involves screening bacterial genomes for the presence of the genes encoding pathogenic factors, and analysis of these genes via forward and reverse genetics. Chlamydiae represent a unique group of pathogenic bacteria in which it is not feasible to apply genetic approaches that are currently used for other organisms. The obligate intracellular nature of these organisms also makes transfection with foreign DNA and subsequent selection for potentially successful transformants very challenging.

This thesis explores techniques that address the study of these organisms in the absence of a workable genetic system. The first goal of this study was to examine the role of a collection of unique chlamydial proteins, known as the Inc-proteins, in the host-pathogen interactions. Inc proteins are localized on the inclusion membrane,

and are exposed to the cytosolic surface of this membrane. Such localization brings Inc proteins into direct contact with the cytoplasm of the host cell. To study the influence of Inc proteins on host cell biology we used the method of transient expression of genes from a eukaryotic expression plasmid, followed by the analysis of the resulting host cell phenotype. These experiments were conducted in both infected and uninfected cells.

It was demonstrated that expression of *Chlamydophila* (*Chlamydia*) *caviae* IncA protects host cells from infection with *C. caviae* by blocking the development of chlamydiae inside of the host cells. This effect of *C. caviae* IncA was specific, because the expression of the homologous IncA protein from *Chlamydia trachomatis* has no effect on the development of *C. trachomatis* or *C. caviae*.

Using the same approach it was shown that Inc proteins CT223, CT224 and CT225 from *C. trachomatis* each were capable of affecting the cell cycle of the host cells by blocking cytokinesis. This is significant, as other investigators have reported that infection with *C. trachomatis* leads to a similar phenotype. Transient expression of CT223, CT224, and CT225 affect host cell cytokinesis on the same manner as *C. trachomatis* infection. A statistically significant proportion of cells producing any of these three Inc proteins had a multinuclear phenotype and multiple centrosomes, indicating that cell division was blocked late in the cell cycle. This effect was specific for these particular Inc proteins and was observed both in murine and human cultured cells.

Many scientists are trying to develop methods for chlamydial mutagenesis and transformation. However, these attempts have not yet been successful, and the

delivery of DNA into chlamydiae is not the only obstacle. Even if chlamydiae are transformed, the successful transformants have to be effectively selected and segregated. To address the challenge of isolating individual candidate chlamydial transformants, we developed a novel technique for the rapid and productive separation of microbiological clones of chlamydiae. This was accomplished using a new method that is based on a unique feature of live chlamydiae to accumulate and retain the fluorescent dye C_6-NBD-ceramide. This labeling results in clearly identifiable chlamydial inclusions, and thus, clearly identifiable infected cells. These labeled, infected, cells can then be separated from uninfected cells on a fluorescence activated cell sorter. The approach was used to isolate clonal populations of prototype strains from *Chlamydia trachomatis, Chlamydia caviae,* and *Chlamydia suis*. Recent clinical isolates were also successfully cloned. The procedure is simple and rapid, with single cloning cycles being completed 24h post-culture of a sample. As will be discussed, this technique has applications in both research and clinical settings.

The Role of Chlamydial Inclusion Membrane Proteins in Host Pathogen Interaction and the Development of Novel Methods for Studying Chlamydial Biology

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LIST OF ABBREVIATIONS

Abbreviation	
EB	Elementary Body of chlamydiae
RB	Reticulate Body of chlamydiae
MOMP	Major outer membrane protein of chlamydiae
TTSS	Type III secretion system
ER	endoplasmic reticulum
GFP	green fluorescent protein
PI	phosphatidylinositol
PC	phosphatidylcholine
LGV	Lymphogranuloma venerum
mRNA	matrix RNA
RT-PCR	reverse transcription-PCR
PI	post infection
sRNA	small RNA
FACS	Fluorescence Activated Cell Sorter
MOI	multiplicity of infection
RT	room temperature

Chapter 1

Introduction and literature review

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1.1. Chlamydial developmental cycle

1.1.1. Overview

Every intracellular pathogen must be capable of entering host cells, escaping host defense mechanisms and establishing an intracellular niche for further multiplication. Then, those parasites have to exit the host cell and survive extracellularly prior to a new round of infection. The chlamydiae, as obligate intracellular pathogenic bacteria meet all of these challenges.

Chlamydiae cause various diseases in humans and animals (37, 89, 94, 136, 157). Chlamydiae have their own order, termed Chlamydiales, and many new organisms from animals and environmental sources have been added to this order over the last decade. The family Chlamydiaceae has also been recently divided into two genera, *Chlamydia* and *Chlamydophila.* Non-chlamydiaceae such as Parachlamydiaceae, Waddliaceae and Simkaniaceae were also included in this family (42). However, the taxonomy of chlamydia remains controversial. Division into two genera is considered to be unnecessary by many chlamydiologists and it is likely that this system will be subject to changes in a future. In this thesis, the terminology that considers only a single genus *Chlamydia* will be used (123).

1.1.2. Two morphological forms of the microorganism

The chlamydial developmental cycle, as we know it today, was first described by Bedson and Bland in 1932 (15). At that time they used light microscopy and considered chlamydial infection as viral. This classification remained intact the early 1960's. The presence within the infectious form of DNA and RNA, a cell wall, ribosomes, and sensitivity to antibiotics such as tetracycline, led to the conclusion that the organisms were bacteria.

The modern point of view on chlamydial development describes two distinct morphological and functional forms of the microorganism. The first form is the elementary body (EB) and the second form is the reticulate body (RB). The life cycle of chlamydiae is a transition from EB into RB, RB into RB, and finally, RB into EB (Fig.1). The developmental cycle occurs within a membrane-bound vacuole, called

"inclusion". Metabolically inert, EB is adapted for extracellular survival and can be transmitted from one host to another. It is a small (0.3µm), round, electron-dense, "spore-like" infectious form of the organism, whereas RB are larger (1µm) then EB, have less condensed structure, un-infectious and metabolically active. (38, 98). Chromosomal material in EBs is condensed in the nucleoid by bacterial histone-like proteins HctA and HctB (11, 19). Chlamydial EBs do not have a detectable peptidoglycan layer in the cell wall. The structure of EB is supported by a cross-linked outer-membrane complex (OMC). This complex is composed of cysteine rich proteins such as OmpA, OmcB and OmcA. Proteins inside of the OMC are connected by cysteine bonds. Stability of EBs depends on disulfide-linked outer membrane proteins (12, 74, 76, 107).

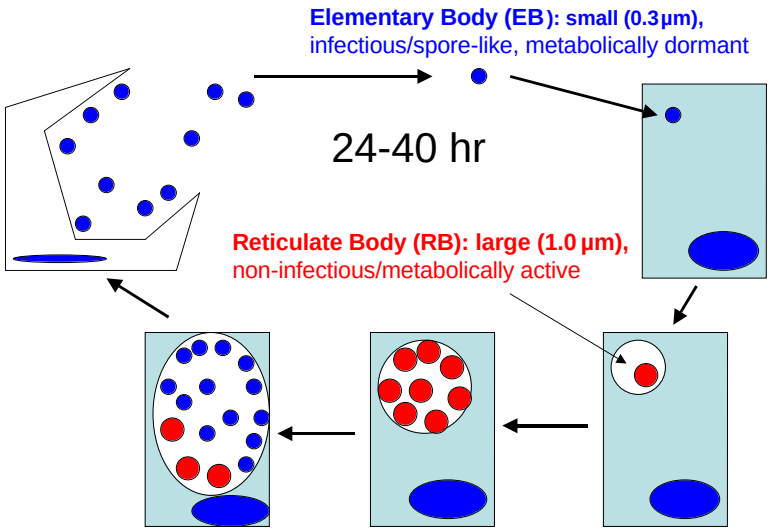

Fig. 1.1. Schematic representation of the developmental cycle of chlamydiae
Chlamydiae have two developmental forms. The elementary body (EB) is infectious, metabolically inactive and small (0.2-0.3 µm). The reticulate body (RB) is larger (1.0 µm), non-infectious and metabolically active. The whole cycle takes between 24 – 40 hr depending on species.

Electron microscopy revealed unique structural appendages, termed "projections" extending approximately 30 nm from the surface of EBs (97, 99, 108). These structures are observed in at least three of the four *Chlamydia* species, including *C. pneumoniae* (103). One report documented their presence *in vivo* (134).

Approximately 15-30 projections are arranged in a hexagonal array on the EB surface. These projections become randomly redistributed when EB transforms into the metabolically active RB. The projections originate in the inner, cytoplasmic membrane and then emerge from the outer membrane through a hole surrounded by a "rosette". By electron microscopy it was shown that these projections pierce the inclusion membrane and extend into the host cell cytoplasm. It was suggested that the projections are somehow involved in interactions between RB and the host cell. The projections had some resemblance to flagella and it was supported by findings of flagella gene orthologues in *C. trachomatis* serovar D genome (139). When sequencing results of large genome fragments of the guinea pig strain of *C. psittaci* were analyzed, an operon containing four genes encoding products homologous to structural and regulatory components of the type III secretion system was discovered (81). Then it was reasoned that the chlamydial projections may serve as the type III secretion machinery in chlamydiae (12). The projections may play an important role in the development of the parasitic relationship between intracellular chlamydiae and host cells. Similar surface structures that anchored in both the inner and the outer membrane, have been observed in several bacterial pathogens possessing type III secretion systems (57, 102, 109, 121).

Upon infection, EBs became internalized in inclusions. Almost nothing is known about the biochemical and kinetical processes that take place during the transition from infectious EB to non-infectious but metabolically active RB. Signals that trigger these changes are also mostly unknown.

1.2. Stages of infection process

1.2.1. Attachment and entry

Studying the mechanisms that are used by chlamydia to attach and enter the host cell is a subject of research for a long time (87). Chlamydiae are capable of attaching to and infecting non-phagocytic cells from various animal species. Protein Disulfide Isomerase (PDI), which is a component of the estrogen receptor complex, is responsible for the attachment of *C. trachomatis* serovar E to human epithelial cells

(32). Chlamydial EB also depend on polysaccharides and heparin sulfate for attachment (135, 162, 166). These components can be helpful in establishing strong bonds between host cells and bacteria (36).

As *Chlamydiae* are obligate intracellular pathogens, entry into host epithelial cells is absolutely essential for their survival. Internalization of EBs by eukaryotic cells is a very efficient process. To accomplish this goal, chlamydiae have to use any possible mechanism available for entry, including pinocytosis (114), receptor-mediated endocytosis (165), and phagocytosis (20, 159). Data on several adhesins that regulate receptor-mediated endocytosis and pinocytosis of chlamydiae remains controversial. There is no evidence for macropinocytosis of chlamydia. Until recently it was believed that chlamydiae do not enter host cells like many other bacterial pathogens that can use "triggered" entry (49) by inducing actin activation or membrane ruffling, as described for *Salmonella typhimurium* (51). It is also unknown if chlamydiae can cause a major upheaval of host apical membrane, such as microvillus effacement described for enteropathogenic *Escherichia coli* (EPEC) (83). However, interesting results were obtained for the role of the type III secretion system (TTSS) in chlamydial internalization. Immediately after irreversible binding, TTSS exports Tarp protein (Translocated actin-recruiting phosphoprotein, CT456) into the host cell (25). Inside the host cell, Tarp is phosphorylated at tyrosine residues. Expression in the cell cytoplasm of the recombinant Tarp-EGFP revealed that the product becomes tyrosine-phosphorylated and recruits actin. Tyrosine-phosphorylated Tarp was detected on the cytoplasmic face of the cell membrane while the EB was still extracellular and the bacterial chromosome was still at the condensed state. All of these findings support the idea that chlamydial EB contain functional TTSS that is capable of delivering signals inside the host cell prior to differentiation of EB into metabolically active RB. It is logical to suggest that assembly of TTSS into functional complexes and expression of early effector molecules such as Tarp must be accomplished during differentiation of RB into EB. This may supply metabolically inert EBs with molecules that are active upon EB attachment and important for invasion into the host cell.

The difficulties in studying the interactions between receptors and ligands involved in chlamydial invasion can be described by several factors. First, various chlamydial strains and species have been studied on various cell types under different experimental conditions. The second reason because this is difficult is the absence of the genetic system to mutate chlamydial genes and identify chlamydial proteins involved in the attachment. The obligate intracellular life cycle of chlamydiae is another obstacle for identification of molecules involved in attachment. However, even if genome manipulations were possible, the disruption of the ability to attach to the host cell would prevent any further development of the infection. It is also very possible that chlamydiae use multiple mechanisms of attachment and disruption of one of them will not change the course of the infection dramatically. Different serovars may have different mechanisms of attachment.

The key question is the identification of chlamydial ligands responsible for the attachment to host cells. Several chlamydial proteins are exposed at the surface and that makes them potential candidates for the role of adhesin proteins. Among these proteins are OmcB, OmpA and HSP70 (MOMP) (68). Cysteine rich protein OmcB is conserved among different chlamydia species. OmcB from *C. caviae* was proposed as an adhesin candidate (154). Later it was also demonstrated in *C. trachomatis* that denatured OmcB could bond heparin (140). It also was shown that OmcB-specific peptides designed to have the heparin-binding domain are capable of binding heparin *in vitro*. Treatment of *C. trachomatis* EB with antisera specific to these peptides revealed a specific binding to the bacterial surface. Chlamydial surface MOMP protein is the major serovariant antigen of both *C. trachomatis* and *C. psittaci* species with high serovariant specificity (21, 77). However, it is conserved among different isolates of *C. pneumoniae.* Treatment of EBs with antibodies specific to MOMP blocks attachment (21, 44, 90, 144). Purified recombinant *C. trachomatis* MOMP can bind heparin similarly to the mode of interaction with EB (143).

Members of the *Chlamydiales* have a family of polymorphic membrane proteins (Pmp), which is unique (58) to this order. Pmp proteins are also suggested to be involved in attachment (43, 63, 64). Pmp proteins all contain a repetitive motif specific for adhesins from other intracellular pathogens such as *Rickettsia spp.* (43,

64). For surface-exposed PmpD protein it was shown that treatment of EBs with neutralizing antibodiesspecific to PmpD resulted in a 50% reduction in inclusion forming units (IFU) for various serovars of *C. trachomatis* (29). The process of chlamydial attachment has a specific term known as " parasite-specific endocytosis" (20). According to the contemporary hypothesis, the process of interaction of EBs with host cells has two stages. The first stage is the initial attachment, which is reversible. This event occurs through electrostatic interactions between bacterial OmpA protein, exposed on the surface of EB (143), and heparan sulfate containing glycosaminoglycans (GAGs) (140, 142, 143, 150).

The second binding stage is irreversible. It was identified on mutagenized host cell lines (22). However, the receptor responsible for this interaction was not identified. Some data support the hypothesis that association with PDI may be involved in attachment and entry of chlamydia (32, 96). This phenomenon may be explained by the fact that activity of PDI may reduce disulfide cross-linked OMB of EBs and may be important for attachment and entry (115).

1.2.2. Intracellular development

For any given intracellular pathogen the development inside a host cell is a significant challenge (105). Intracellular parasites have evolved various strategies to escape lysosomal killing. One strategy for surviving inside is occupying one of three distinct compartments of the host cell. Such pathogens like *Shigella, Listeria* or *Rickettsia* replicate in the cytoplasm (69, 70, 95). Others like *Leishmania* or *Coxiella* even survive harsh acidic conditions inside the lysosome to support their own metabolism (75, 106). Chlamydia is a member of another group of intracellular parasites that use unique vesicles that do not fuse with lysosomes. For most cases, the biogenesis of these vacuoles is not well understood. This strategy of forming non-lysosomal vacuoles for surviving inside host organisms is another survival technique used by intracellular parasites (53, 116, 160).

Another specific feature of the chlamydial inclusion is the lack of acidification. It was demonstrated that, in cells that phagocytose both yeast and chlamydiae, the vacuoles containing chlamydia are protected from phagolysosomal

fusion (39). It was also shown that early vacuoles with EB are prevented from maturation into phagolysosomes (40). It was demonstrated that inhibition of chlamydial protein synthesis leads to fusion with lysosomes (128). The formation of specialized vacuoles happens in close proximity of the endosomal/lysosomal pathway and it is possible that parasites are capable of alterning the development of vesicles at the stage that is favorable for them (8, 141).

Contact of EB with the epithelial surface of the host cell programs both chlamydia and host cell for productive infection. During the first four hours of intracellular development many events occur. At first, the EB endosome pH drops to 6.2 and then stabilizes at 6.6 (124). At this point, the endosome containing EB escapes fusion with lysosomes. By two hours after infection the EB-containing vesicle is devoid of markers that distinguish early and late endosomes or lysosomes. Phosphorylation of epithelial proteins triggered by the EB receptor results in rearrangement of the host cell cytoskeleton (18, 45). Local accumulation of F-actin and clathrin helps to redistribute endosome containing EB to the perinuclear region (92). This translocation also depends on dynein motor movement of the EB vacuoles on microtubules (24, 125). If the intracellular concentration of calcium remains at homeostatic level, EB-containing endosomes can fuse with one another, but not with lysosomes (91). Early chlamydial gene expression results in vacuole modification and EB trafficking is changed from the endocytic to the exocytic pathway (73). It is possible that early chlamydial development is dependent on chlamydial-specific transcription and translation. The exposure of infected cells to rifampin and chloramphenicol leads to the dispersion of EB vesicles throughout the cytoplasm and accumulation of lysosomal markers (126). Approximately six hours after internalization, the transition of EBs into RBs is complete and metabolically active RBs enter the logarithmic growth stage, with a generation time of 2-2.5 hours, which continues until 24-40 hours after infection. Very little is known about the function and composition of the inclusion membrane that forms the chlamydial inclusion. As bacteria grow and proceed through their developmental cycle, the inclusion also grows and expands. This process is not dependent on host protein synthesis because the chlamydial inclusion develops normally in cells treated with the inhibitor

cycloheximide. It was demonstrated that chlamydial inclusion can intercept vesicles released from the trans-Golgi (73). Chlamydiae also acquire phosphatidylinositol (PI), and phosphatidylcholine (PC), components that are normally produced in the endoplasmic reticulum (ER). The inclusion is also capable of intercepting cardiolipin (CL) that is contained in mitochondria and cholesterol (163). Chlamydiae also produce a number of specific proteins termed Inc-proteins that also incorporate into the inclusion membrane (5, 117, 118, 120, 129).

It was shown that treatment of host cells with Gamma interferon induces the depletion of tryptophan. This amino acid is required for some chlamydia, and absence of tryptophan results in abnormal RB unable to mature into EB (14). The inclusion membrane is not likely permeable for small compounds. Microinjection of fluorescent tracer molecules as small as 520 Da does not result in accumulation inside inclusions (79). The level of parasitism is dependent on species. In the case with *C. trachomatis* LGV, bacteria can produce their own ATP and posses biologically active, energy-producing enzymes and have all the components for a functional electron transport chain. However, *C. psittaci* have ATP/ADP translocase and has to take ATP from the host. It also has inclusions tightly associated with mitochondria and MOMP with functions of porin that allow the passage of ATP (164). Above all, chlamydiae are unable to produce nucleotides. They also lack genes involved in amino acid transport and compensate for that shortage with a complement of amino acid transporters (101).

The differentiation of the infectious EB to a metabolically active RB is one of the least-understood stages of the chlamydial developmental cycle. For instance, if EBs are metabolically inactive, which is supported by the fact that DNA in EBs binds to the histone-like proteins HctA and HctB, why does the addition of antibiotics that block transcription or translation prevents transition of EB to RB (128). Recent findings that histone-DNA interactions in chlamydia are degraded by the time of germination (61) did not solve this contradiction. Disruption of interactions between DNA and histones depends on a metabolite in non-mevalonate pathway (MEP) of isoprenoid biosynthesis. This metabolite, encoded by chlamydial gene CT804, a homolog of *ispE*, acts as an antagonist of HctA. It was demonstrated that the lethal

effect of expression of HctA in *E.coli* is prevented by co-expression of CT804 (*ispE*). This rescue experiment revealed that *ispE* is an intermediate enzyme of the MEP pathway. However, the *E. coli* orthologue of *ispE* did not rescue *E. coli* from the lethal effect of HctA expression. The mechanism that makes chlamydial IspE specific against HctA is still not clear. Chlamydiae also encode another histone-like protein HctB. For now it remains unsolved what prevents it from functioning during the differentiation from EBs to RBs (61).

Another interesting feature of EBs is the presence of a significant amount of mRNA ("carryover mRNA') and ribosomes. It is especially intriguing because the EB is believed to be metabolically inactive. One hypothesis describes, upon the chromosome decondensation, the pathogen has a genome that is immediately transcriptionally active. The composition of mRNA inside EBs has some specific features. The significant fraction of this "carryover mRNA" is represented by mRNAs encoding late gene products. This may be explained by the fact that these mRNA were packed into EBs during the final stages of RB to EB differentiation (17). However, the presence of the late stage mRNAs do not lead to expression of their specific products at the early stages of a new infection process. This fact supports the hypothesis that chlamydia have a mechanism to differentiate between carryover mRNA and newly transcribed mRNAs. For now this existing hypothesis may be explained by two possible mechanisms.

First, RNA binding protein(s) prevent carryover mRNAs from interaction with ribosomes. The second mechanism could be the processing of carryover mRNAs in such a way that translational start signals on 5' end becomes degraded. During further development of infection, carryover mRNA rapidly became degraded (17). Active gene expression starts almost immediately after internalization of chlamydia (30, 113, 161).

Chlamydial histone-like proteins Hc1 and Hc2 regulate a nucleoid structure and down-regulate gene expression at the stage of differentiation RB back into EB (62). It was shown that co-expression of small RNA (sRNA) with *hctA*, the gene that encodes Hc1, in *E. coli* inhibited Hc1 translation, but did not act on *hctA* mRNA stability or transcription. It also was demonstrated that IhtA, which inhibits translation of *hctA*

mRNA, is present only in RB, while Hc1 was detectable in only in EB. During infection IhtA was downregulated while Hc1 was upregulated upon differentiation of RB into EB. IthA could be part of a global regulatory system that governs transition of RB to EB during the chlamydial developmental cycle.

Sequencing of the whole genome of *C. trachomatis* serovar D and usage of the RT-PCR technique helped to identify multiply genes that are expressed in different stages during the developmental cycle. Early expressing genes were identified (130). Temporal stages of expression based on different times post infection (PI) were classified as early (1- 2 h PI), mid-cycle (3-18 h PI), and late (19-48 h PI). Usage of genome-wide micro-arrays revealed various stage specific genes (17).

Many inclusion membrane protein genes (*inc*) were identified as early expressed genes (130). Despite the fact that functions of most of inclusion membrane proteins were not yet established, these proteins are considered as promising candidates for the role of interactors with the host cell (2, 127). It is logical to speculate that, in general, all proteins translated at the early stage of chlamydial life cycle serve two major purposes. The first is the effective establishment of nutrient delivery to developing bacteria and the second is the protection of the inclusion from fusion with lysosomes.

1.2.3. Exit from the host cell

Escape into the environment is the final step of the chlamydial life cycle. Despite the apparent simplicity of the exiting process, the real mechanisms that direct the end of the developmental cycle and trigger transition of RB back to infectious EB are almost completely unknown. This process may involve multiple events. A decrease in host nutrients may signal detachment of RB from the inclusion membrane and activation of histone proteins. Histone proteins can regulate stage-specific expression of genes and initiate DNA condensation (11, 149).

The fact that different species of chlamydiae exit host cells in different ways supports the idea of complexity of this final stage of the developmental cycle. Some chlamydiae lyse the host cell when exiting. Others exit in a less aggressive manner. For example, *C. trachomatis* serovar D (UW3 isolate) exit cells by exocytosis. The

inclusion moves along the exocytic pathway to the epithelial surface for fusion with the plasma membrane. During this process the inclusion exvaginates at the plasma membrane and the host cell remains viable (155). Other serovars of *C. trachomatis*, such as LGV strains, destroy the host cell when exiting. It was reported that chlamydiae encode a cytotoxin with un unknown role (16). It is possible that this toxin is involved in the process of releasing bacteria from the cell.

1.3. Chlamydia and human health

1.3.1. Chlamydial infection as a world wide problem

The genus *Chlamydia* contains three species that infect humans and cause various diseases. These species are *Chlamydia trachomatis, C. psittaci*, and *C. pneumoniae* (formerly TWAR agent). Genital serovars of *Chlamydia trachomatis* are the most common cause of sexually transmitted diseases (33, 54, 55), while the ocular serovars cause blinding trachoma in people living in developing countries (122). These infections are found in about 400 million people in the world today and every year about 90 million new cases of *C. trachomatis* infection occurr worldwide. Approximately 4 million cases occur annually in the USA, leading to billions of dollars in medical expenses.

C. pneumoniae species were described as respiratory pathogens that infect many humans in all areas of the world (59, 86). Seroprevalence rates of 60 to 70% are common among adults. *C. pneumoniae* primarily infect children and young adults, but are also capable of causing recurrent infections in older people. There is some evidence that

C. pneumoniae is responsible for atherosclerotic cardiovascular disease and perhaps for asthma and sarcoidosis (26, 31, 85, 88). It appears to be an obligatory human pathogen because no animal reservoir has been found. It spreads from one person to another via the respiratory route through close personal contact. All strains of *C. pneumoniae* are serologically homologous.

C. psittaci is widely distributed in nature. It affects many mammalian and avian species causing genital, intestinal, conjunctival, or respiratory infections.

Genital infections with *C. psittaci* have been well characterized and can cause abortion and infertility. Although mammalian strains of *C. psittaci* are not known to infect humans, avian strains occasionally can cause infection in humans. Infection with *C. psittaci* leads to pneumonia and the systemic disease known as *psittacosis.* In general, chlamydial infection causes mild diseases with persistent infections and "poor immunity". This mode of infection makes recurrent infections possible. Only at the late stages of the pathogenic process serious consequences can occur.

The first attempts to describe cytoplasmic inclusion bodies were made in 1907 by Halberstaedler and von Prowazek. They studied epithelial cells from orangutans infected with material obtained from humans with trachoma. At that time they considered the new pathogen as protozoan and named inclusions "chlamydozoa" or " mantle bodies" because of specific mantle or cloak appearance. These "chlamydozoa" had reddish particles embedded in a blue mantle. Shortly after this discovery, similar "chlamydozoa" inclusions were detected in infants with blenorrhea and in cervical samples from their mothers. The same "chlamydozoa" structures were found in men with non-gonococcal uretritis. The first attempt to bring all facts together was made by Linder, in a review written in 1911. It was noticed that similarities in inclusion formation were detected in the genital tract, in the conjunctivae of babies, and in trachoma cases. These results were confirmed when ocular and genital materials obtained from humans were used for infecting monkeys.

The unique developmental cycle of chlamydia was first described for the agent causing psittacosis in the early 1930's (15). Despite the fact that agents causing inclusion conjunctivitis and trachoma had not yet been isolated, the similarity of the morphologic features to psittacosis led to recognition that the same cycle is specific for all of these diseases (152). An agent still hadn't been isolated when it was shown that infection might be controlled by treatment with sulfonamides. T'ang and his collegues were the first to isolate the trachoma agent in 1957 (148). Since that time multiple attempts to develop a vaccine against trachoma were made. Despite all attempts, an effective vaccine was not developed because immunity was short-lived and possibly led to more serious diseases (34).

1.3.2. Infections with *Chlamydia trachomatis*

The disease that now known as "trachoma" was first mentioned more then two thousand years ago. At that time trachoma was described as "roughness of the conjunctiva." However, it is very likely that the disease was known from much earlier times. In the middle ages, trachoma became epidemic among the Crusaders. In Napoleon's army it was known as "military or Egyptian ophthalmia." During the last hundred years trachoma mostly disappeared from western countries because of the improvements in the standards of living. However, in developing countries it is still a major heath problem.

Modern medical research describes trachoma as a chronic conjunctivitis that is associated with infection caused by *C. trachomatis* strains A, B, Ba and C. Inclusion conjunctivitis is an acute ocular infection in adults exposed to infected genital secretions and usually caused by *C. trachomatis* strains D and K. Children can also be affected. It is especially a concern in newborns of infected mothers. Moreover, in areas endemic for trachoma the main reservoir of infection is children suffering from chlamydial conjunctivitis (84).

Trachoma is the disease of poor hygienic condition and low living standards. In modern times it is endemic only in tropical and subtropical regions such as North and sub-Saharan Africa, the Middle East, Latin America and India (153). The worldwide morbidity of trachoma is estimated as 600 million cases per year. No more than ten percent of cases will lead to severe visual impairment and thus, there occur about 6 million cases of blinding trachoma (153). Infection spreads from one person to another through direct contact via hands, towels, and even insects such as flies. Infection commonly occurs during the first year of life and may remain in a persistent state if proper treatment is not available. In areas with a moderate rate of cases, infection and disease occur later and often the first contact happens after entering school or a day care center. For adults a similar epidemiological pattern can be applied and re-infection has to be taken to in consideration as well.

The first response in both cases is conjunctivitis characterized by small lymphoid follicles in the conjunctiva. Congestion and edema are followed by follicle formation. The rapture of follicles leaves characteristic scars named Herbert's pits.

Another specific sign of trachoma is hyper-vascularization (pannus) of the superior limbus. During further development the cornea becomes involved. As a result, punctate erosions of the epithelium with infiltrates of the corneal epithelium and anterior stroma (keratitis) will develop. The most damaging effect of trachoma is the sequelae of infection. Scars on the conjunctiva produce a distortion of the lids. In a combination with physical damage to the cornea that causes blindness, this usually happens decades after the acute infection.

In the natural progress of trachoma, the affected individuals may be infected multiple times. As a result, a hypersensitivity reaction to specific *Chlamydia* heat shock protein may be responsible for the inflammation during reinfection (151). Moreover, antibodies against heat shock protein 60 (Hsp60) were shown to be associated with scar formation (111). Immunity is an important factor of trachoma pathogenesis and attributed to active inflammatory reactions during reinfection. Infection with *C. trachomatis* produces strong local and circulatory immune responses but doesn't lead to any protection against subsequent infection.

The clinical diagnosis of trachoma is based on the presence at least two of the following signs: presence of lymphoid follicles on the conjunctiva; typical scarring of conjunctiva; vascular pannus is present; presence of Herbert's pits (limbal follicles). Isolation of *C. trachomatis* from site of infection and using of Giemsa or immunofluorescent staining are also important in diagnostics. However, Giemsa staining of smears obtained from infected peoples revealed intracellular chlamydial inclusions only 10 to 60% samples. PCR is a very effective diagnostic tool and it is especially useful when smears and cultures are not effective. The lifestyle of humans in the areas of the world where trachoma remains endemic is not likely be changed in the future. Because of this fact, control of trachoma in general based on a strategy known as SAFE (surgery to repair damaged lids, antibiotic treatment, face washing and environmental improvements). All of these principals have to be applied together (153).

C. trachomatis is also the most common bacterial STD agent in the world. In the United States infections due to *C. trachomatis* have been reportable since 1985. Like all other genital pathogens, *C. trachomatis* has been found in larger proportions

among individuals with lower social and financial income status. These people more often are young, nonwhite, unmarried and unemployed. Prevalence of chlamydial infection varies significantly geographically, with the highest rates in the southeast part of the country. All sites of the human body that are covered with superficial columnar epithelial cells can be affected by *C. trachomatis,* however most infections do not penetrate deeper tissues. Infection with *C. trachomatis* can lead to a very broad spectrum of diseases such as salpingitis, endometritis, male and female uretritis, epididimitis, adult inclusion conjunctivitis, cervicitis, proctitis and arthritis (Reiter's syndrome). Acute infection induces an inflammatory response typical for all chlamydial infections. However the most important epidemiologic aspect is that infection may be asymptomatic or "latent" for long periods of time. This makes the detection and treatment of chlamydial infection very difficult. In the absence of antibiotic treatment infection can be persistent for months or years and sequelae of re-infection in epithelial sites lead to morphological changes of tissues. It is typical for *C. trachomatis* infection to leave scars in sites of infection. All of these factors makes genital chlamydial infection especially threatening for women. Undiagnosed and untreated infection in women usually leads to sterility due to occlusion of the fallopian tubes. The percentage of *C. trachomatis* infection in salpingitis or PID can reach up to 40% (93).

Another serious disease that is caused by *C. trachomatis* is Lymphogranuloma venerum (LGV). LGV is a sexually transmitted disease caused by invasive strains of *C.trachomatis* (serovars L1, L2, and L3) that can infect lymphatic tissues. It is less prevalent then other STDs. Acute LGV is characterized by transient primary genital or rectal lesion followed by regional lymphadenopathy (appears in 1 to 6 weeks after primary lesion) and is almost always associated with systemic symptoms that include fever, headache, malaise and other symptoms common in most inflammatory reactions. Usually inguinal or femoral lymph nodes are involved. Without proper antibiotic treatment, serious long-term problems can be associated with regional lymphatic stasis and fistulas of the rectum, urethra and penis. Massive pelvic lymphadenopathy may lead to the life-threatening surgical procedures. The reservoir of infection is the cervix of the asyptomatic female and rectal mucosa of both male

and female patients. In the United States LGV has a rate of 0.1 per 100,000 people and is present more often in the male homosexual population.

1.3.3. Infection with *Chlamydia psittaci*

Psittacosis is a primary an infectious disease of avian species and mammals that is caused by *C. psittaci.* The source of psittacosis in humans is infected birds. Not only psittacine birds, such as parrots, can harbor *C. psittaci,* almost any avian species can be a source of infection. More then a hundred strains of *C. psittaci* can potentially be infectious for humans. Psittacosis is a rare disease in the U.S. Infection in humans results in a febrile illness with pneumonitis and systemic symptoms. It is likely that many cases of human psittacosis go undetected because diagnostics involve special tests and many clinicians do not recognize the necessity of these exams. Often psittacosis is considered simply as atypical pneumonia and treatment is not adequate. In some cases infection can be life threatening.

During the last few decades psittacosis has been considered mostly like an occupational disease of people working with birds or products that come from such activities. Infection is almost always acquired through the respiratory tract and symptoms can vary significantly. In general, after an incubation period of 7 to 14 days the disease starts with chills and fever, with temperatures ranging as high as 105°F (40.5°C). Other symptoms include prominent headache, dry and nonproductive cough, and chest pain. Generalized myalgia with prominent malaise is also common. More serious and often life-threatening symptoms are developed when internal organs are involved. This can consist of dyspnea with notable cyanosis, hart complications, and central neural system involvement. Treatment with antibiotics is very effective. Without such treatment recovery takes up to one month or longer. The mortality rate now is not high since antibiotics are available but in pre-antibiotic eras the mortality rate was no less then 20% in young people and up to 50% in elderly patients. Prevention involves control of the avian sources of infection. Presently it is especially important to control infection in the poultry industry. There is no specific vaccine available for humans or birds and, because of the infrequent occurrence of the disease a vaccine is not likely to be developed.

1.3.4. Infection with *Chlamydia pneumoniae*

Approximately three decades ago *C. pneumoniae* was described as a third chlamydial species that causes disease in humans. At the time it was distinguished from the other two species on the basis of EB morphology and differences in DNA hybridization. Although *C. pneumoniae* can be grown in many types of cell cultures, it is more difficult to culture than other chlamydiae.

Epidemiological studies on *C. pneumoniae* shown that the pathogen is very common worldwide and 60-70% of adults are seropositive. The pathogen is a cause of a broad spectrum of respiratory tract infections with a tendency to become chronic. Reinfection is a common event. The most serious outcome of respiratory tract infection is pneumonia. Transmission appears to be from person to person. Clinical manifestations include a wide spectrum of diseases such as bronchitis, pneumonitis, sinusitis, and acute pharyngitis. Cases of primary infection are more severe and prolonged than those of reinfection. Clinical studies have shown an association between serologic evidence of *C. pneumoniae* infection and atherosclerosis of the coronary arteries. The pathogen has been also identified in atherosclerotic plaques by DNA hybridization, immunocytochemistry and electron microscopy. *C. pneumoniae* is responsive to antibiotics of both the erythromycin and tetracycline groups at maximum dosages and with a prolonged course of treatment. Diagnosis is based on serological tests performed in parallel with PCR and culture tests. Culture tests are not available for routine clinical use and non-culture tests play a more important role.

1.4. Model systems for studying intracellular pathogens.

1.4.1. Challenges in chlamydia research and alternative approaches in studying intracellular pathogens

In the case of chlamydiae, both the lack of a genetic system to manipulate the genome and the unique developmental cycle of the pathogen, makes research on chlamydia virulence factors very challenging.

In nature, horizontal gene transfer in chlamydia recently was reported (37). Resistance to tetracycline in *C. suis* is dependent on the transposable element IScs605 that is closely related to *IS605* of human pathogen *Helicobacter pylori*. These data demonstrate that chlamydiae are capable of accepting DNA horizontally. However, introduction DNA into chlamydiae is only one part of the story. The next challenge is the control of successful transformation. As mentioned before, chlamydiae grow only on cell cultures. Hundreds, if not thousands, of individual bacterial cells live inside of the inclusion, which is also located inside of host cells.

Alternative methods for obtaining microbiological clones of chlamydiae are complicated and involve long terms of culturing and multiple dilution steps (56, 100, 147). In this thesis, the new method of isolation of chlamydial clones using Fluorescent Activated Cell Sorting (FACS) will be discussed in detail.

The absence of the genetic tools to manipulate the chlamydial genome can be addressed by using other approaches. Very interesting research on pathogenic factors of bacterial pathogens in general, and chlamydiae in particular, has been conducted via modeling of functions of bacterial virulence genes in yeast (131, 156). Another alternative is a genetic screen by heterologous expression of chlamydial genes in model bacterial organisms. Identification of chlamydial Type III secretion components and proteins involved in histone-DNA interaction was done by this approach (47, 48, 61, 145, 146). Methods of transient expression of chlamydial proteins inside of the host cell were used for studying changes in host cell phenotypes (2).

1.4.2. Modeling of functions of bacterial virulence factors in yeast

Understanding of the molecular mechanisms of bacterial pathogenesis is mainly based on data obtained from genetic screens. Usually the design of the experiments is based on a strategy where the gene (or genes) of interest is affected by transposon mutagenesis (50, 78). Then mutants have to be screened for phenotypic changes. However, understanding of the pathogenic factors in microorganisms involves studying not only the pathogen itself, but the host as well. There are different host model systems that are simple enough and genetically tractable (137). For

example, organisms such as *Drosophila melanogaster* and *Caenorhabditis elegans* can be infected with various pathogens and are capable of modeling multiple processes specific for mammalian diseases (1, 35). Single-cell models such as *Acanthamoeba* and *Dictyostelium spp.* are also popular (104, 132).

Many cellular mechanisms that are affected by bacterial pathogens are conserved among of yeast and mammals (4, 9, 41, 65-67, 138). A significant volume of information about yeast genetics is now available (23). However, studying pathogenicity in yeast has some specific moments and *S. cerevisiae* differs significantly from all other model systems mentioned. Only some DNA viruses and bacterium *Pseudomonas aeruginosa* are capable of invading yeast (167, 168).

Instead of modeling interactions between the host and whole pathogen it is possible to analyze the functions of individual virulence factors by expressing them in yeast. As an example of successful application of the modeling of bacterial virulence factors in yeast, the identification of toxin YopE from *Yersinia enterocolitica* was done (83, 158). YopE is a protein that is injected by bacterium into the cytoplasm of infected cells and is involved in local disruption of the actin cytoskeleton (3).

This yeast expression system is especially useful for studying pathogenesis of intracellular parasites. For instance it was effectively used to identify virulence factors in chlamydiae (131). Potential virulence factors were identified by analysis of phenotypes induced in yeast. The design of yeast-based expression assay can be explained as several sequential steps. Chlamydial genome libraries containing all ORFs of unknown function were translated in yeast. For expression in yeast, 236 different *C. trachomatis* serovar D-specific or hypothetical ORFs of unknown function were targeted. Among Chlamydia-specific proteins were 33 inclusion membrane proteins (Inc proteins) that have a specific region also known as the signature hydrophobic domain (5). This hydrophobic domain was removed to prevent potential incorrect folding of heterologously expressed proteins. In addition, 34 ORFs encoding proteins homologous to known virulence factors in other bacteria were analyzed. Chlamydial ORFs were cloned into yeast expression vectors via homologous recombination. Phenotypic changes were analyzed in strains expressing chlamydial proteins by culturing them in liquid media containing a tetrazolium-based

dye. The goal was to identify bacterial proteins that disrupt yeast cellular functions. Analysis was performed by examining the reduction of dye in liquid media with an automated plate reader. Plating of serial dilutions on galactose-supplemented agar was performed too. Colony formation was used for detecting differences in growth dynamics. Conditions of growth were adjusted in such way that various culture temperatures, and osmotic and salt concentrations of media mimic various stress factors. The goal was to test the effect of chlamydial ORFs expression on yeast growth at different conditions. As a result 32 chlamydial ORFs from the total 236 ORFs analyzed were identified as capable of impacting yeast growth significantly.

Yeast two-hybrid analysis also was used for studying chlamydial interactions with the host. By using this approach the first eukaryotic protein was found to interact with the chlamydial inclusions. The usage of the yeast two hybrid assay helped to reveal that *C. trachomatis* IncG protein interacts with host protein 14-3-3β (127). This interaction between IncG and 14-3-3β was confirmed in infected HeLa cells by indirect immunofluorescence microscopy and interaction with GFP-14-3-3β fusion protein. 14-3-3β is a phosphoserine binding protein and it was demonstrated that IncG is phosphorylated in both chlamydia-infected HeLa cells and in yeast expressing IncG. Mutation of the predicted phosphorylation site of 14-3-3β revealed that IncG binds to 14-3-3β via a specific and conserved motif. It was also shown that interaction of 14-3-3β is specific for *C. trachomatis* inclusions only but not *C. psittac*i or *C. pneumoniae.*

1.4.3. Heterologous expression of chlamydial genes in model bacterial organisms

Various bacteria were used as a model system for expression of chlamydial genes. In contrast to chlamydiae genomes, these model organisms can be genetically modified. Expression of the homologous genes of chlamydiae inside of mutated model organisms can restore lost functions and directly confirm roles of chlamydial proteins.

Research on the chlamydial Type III secretion apparatus is an excellent example of working around the lack of genetic tools for the direct knocking out of

chlamydial pathogenic factors. Type III secretion systems have been found in various animal pathogenic bacteria such as *Yersinia, Shigella, Salmonella,* enteropathogenic *E. coli*, and *Pseudomonas*, and plant pathogens *Ervinia, Ralstonia, Rhizobium* and *Xanthomonas* (52, 82). The role of this apparatus is the delivery of specific proteins from the bacterial cytoplasm into the host cell (52). Analysis of the *C. trachomatis* genome has confirmed that chlamydiae have genes that may encode a TTSS apparatus. This makes identification of the corresponding genes in chlamydial genomes possible. The goal was to identify a functional TTSS in *C. trachomatis.* The chlamydia type III secretion system plays a central role in host-pathogen interactions also (13). Components of the TTSS apparatus are very conserved between chlamydiae and other bacteria. The enteropathogen *Yesrsinia enterocolitica* was used as a model system for study of mechanisms of TTSS in chlamydiae. In *Yersinia* spp. secretion of anti-host proteins known as Yops proteins depend on a virulence plasmid that encodes the Yop secretion apparatus (Ysc) (27, 28).

Previously it was demonstrated that *C. psittaci* possess a locus of four genes that are predicted to be similar to TTSS gene components of *Yersinia* (81). One of these chlamydial genes was *cop*N or *C*hlamydia outer protein. It was shown that chlamydiae can translocate this CopN, which is homologous to the TTSS protein YopN of *Yersinia sp.*, into the inclusion membrane (47). Active expression of other TTSS-specific genes in chlamydia was confirmed by RT-PCR. It was also shown that chlamydial TTSS proteins SCC-1 and CopN are present in EB, RB and cell culture extracts from infected cells. The presence of CopN in the inclusion membrane was confirmed by immunofluorescense microscopy. Based on these findings, the CopN protein of *C. trachomatis*, was chosen as a target for study. Expression of His-tagged CopN and chlamydial cytoplasmic control protein NrdB was performed in *Y. enterocolitica* strain containing or lacking the pYV virulence plasmid. It was demonstrated that CopN but not NrdB was specifically secreted by *Y. enterocolitica* into the host cell. Results confirm that putative TTSS machinery of *C. trachomatis* is expressed and CopN is secreted to the inclusion membrane. It was also observed that CopN can be secreted by the TTSS apparatus of *Yersinia.* An example of using model microorganisms for confirming the presence of functional TTSS in chlamydiae

is the report that *C. trachomatis* can secrete Inc-proteins during early stages of development (48). Chlamydial early expression IncC protein was expressed in *Yersinia pseudotuberculosis* and secreted via *Yersinia* TTSS. The similar approach of using heterologous TTSS was used for demonstration that tyrosine phosphorylated protein CT 456 of *C. trachomatis* is secreted by TTSS (25).

Another model microorganism used for studying functions of chlamydial proteins was *Shigella flexneri* (145, 146). It was demonstrated that Inc proteins (5-7) can be secreted by TTSS of *S. flexneri* (146). The N-terminal parts of Inc proteins from *C. pneumonia* were fused with a Cya reporter that is a protein of *Bordetella pertussis,* and these recombinant proteins were expressed in various strains of *S. flexneri.* Incs were secreted by TTSS of *S. flexneri*, and this was evidence that the same proteins may be secreted by TTSS in chlamydiae. The same approach was applied for a broader screen for chlamydial proteins that can be secreted by TTSS of *S. flexneri* (145). The same secretion assay, that is based on the recognition of chlamydial TTSS signals in
S. flexneri, was performed. Chlamydial Inc proteins were used as a control for effective secretion. Various proteins that did not belong to Inc family were also found to be secreted via TTSS. These proteins are conserved between three species of chlamydia: *C. caviae, C. pneumonia,* and *C. trachomatis.* Genes of proteins of interest were amplified by PCR and cloned into pUC19cya vector. The transport of effector proteins of one bacterial species by TTSS machinery of another pathogen has been documented for various species, including chlamydiae (47, 146). Secretion signals recognized by TTSS are universal for all species and proteins. Mechanisms by which secreted proteins are recognized by the TTSS machinery have not been identified.

Another example of the use of a model microorganism for studying TTSS is a demonstration that *C. trachomatis* CopD, Pkn5 and CopN proteins can be translocated into the host cell cytosol by *Salmonella enterica* (80). The functions of more and more chlamydial proteins become characterized by this alternative approach. Using heterologous expression of *C. trachomatis* chaperones proteins Scc2 and Scc3 inside of *Y. enterocolitica* demonstrate that chlamydial chaperones are

involved in TTSS translocation of other *C. trachomatis* proteins CopB and CopB2 (46).

Putative TTSS proteins of *C. pneumoniae*, whose function in other bacterial TTSS was determined as chaperones, were used to identify interactions between chlamydial proteins. A bacterial two-hybrid system was used for this (133). It was shown that gene lcrH-2, when used as "bait", interacts with a product of *lcrE* (*copN*) gene. Interactions were confirmed by pull-down immuno-precipitation experiments and on homologous genes from *C. trachomatis* and *C. psittaci*. LcrH-2 from *C. pneumoniae* interacted with LcrE from three other species of *Chlamydiaceae*.

Research on the role of the chlamydial histone homologs is also an example of using model microorganisms as an alternative for direct genome screening. The DNA of chlamydial EBs is condensed by the action of two proteins that are homologous to histone H1. These histone homologs are Hc1 and Hc2 (19, 71, 72, 112, 149). Genes *hctA* and *hctB,* which encode Hc1 and Hc2 respectively, are both transcribed late in the developmental cycle (17, 130). Expression of both chlamydial histone homologs in *E. coli* results in the formation of a condensed nucleoid similar to the condensed nucleoid of chlamydial EBs (11, 19). This event is also accomplished by total down-regulation of transcription and translation which is similar to differentiation of RBs to EBs. The expression of Hc1 in *E. coli* is lethal (10, 110). This lethal effect could be explained by lack of the means to release the histone-DNA complex in *E. coli*. By using a heterologous genetic screen the possible mechanism of the release of chlamydial chromatin from Hc1 was solved. The genome locus on the chlamydial genome that encodes IspE (CT804) was identified. IspE is an intermediate enzyme of the non-mevalonate methylerythritol phosphate (MEP) pathway of isoprenoid biosynthesis, and was selected as a candidate. When EspE and Hc1 were expressed together in *E. coli* the lethal effect of Hc1 expression was suppressed. Inhibition of the MEP pathway rescued Hc1 from the IspE effect and the lethal effect of histone was restored (61). The role of the small regulatory RNA (sRNA) encoding region of the chlamydial genome in Hc1 regulation was also confirmed by co-expression of the sRNA gene with *hctA* in *E. coli* (62). When appropriate sRNA and *hctA* genes were coexpressed the translation of Hc1 was inhibited.

1.4.4. Transient expression of chlamydial proteins inside of host cells and other alternatives

Previously we discussed various approaches that were used for studying the functions of chlamydial genes when the system for genetically transforming chlamydiae is not available. However, all of these methods are useful for proteins that have homologs in other microorganisms.

A different strategy has to be used when functions of chlamydiae specific genes are studied. The family of Inc-proteins is a good example of such proteins (6, 7, 117, 119). These proteins are unique for chlamydiae and have no homologs.

Expression of Inc proteins encoded by plasmids transformed into the host cells was used for characterization of *C. caviae* and *C. trachomatis* IncA (2) and the group of Inc proteins from the CT223 operon (Alzhanov & Rockey, Unpublished data). To study the action of Inc proteins on host cell phenotype, the plasmids encoding different Inc proteins were transfected into various host cell lines. The resulting effects on cellular morphology were examined using fluorescence microscopy. It was shown that plasmid-driven expression of *C. caviae* IncA prevents host cells from infection with *C. caviae.* Marked reduction in the number of inclusions formed within cells was detected and the very few inclusions that can be identified in cells expressing *C. caviae* IncA were morphologically aberrant (2).

The same approach was used for studying functions of *C. trachomatis* Inc proteins IncA, IncC, and all proteins encoded by the CT223 operon. Expression of CT223 results into inhibition of cell growth by blocking cytokinesis. Other proteins encoded by the CT223 operon had significantly less effect or no effect at all on cytokinesis of mammalian cells. A smilar effect on cytokinesis was detected in different types of host cells infected with various strains of *C. trachomatis* (60). Multiple nuclei were detected in the infected cells. Cytokinesis was arrested while mitosis was not affected. It was also shown that this effect depends on chlamydial protein synthesis. The exact mechanism or bacterial factors responsible for this blockage were not determined. Using a plasmid-driven expression of chlamydial

proteins inside the host cells, it was possible to find candidate proteins that could be involved in the disruption of cytokinesis.

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Chapter 2

Chlamydial development is blocked in host cells transfected with *Chlamydophila caviae incA*

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2.1. Abstract

Chlamydiae produce a set of proteins, termed Inc proteins, that are localized to the inclusion membrane and exposed to the host cell cytosol. Little information exists regarding the interaction of Inc proteins with the eukaryotic cell. To examine these interactions, Vaccinia virus vectors and mammalian plasmid-based systems were used to express *inc* genes in mammalian cells. Cells transfected with plasmids expressing *Chlamydophila caviae incA* were not productively infected by *C. caviae*. Expression of *C. caviae incA* also reduced inclusion formation by *Chlamydia trachomatis*, but not to the degree seen for *C. caviae*. *Chlamydia trachomatis incA* did not block development of either *C. trachomatis* or *C. caviae*. Deletion mutagenesis was used to demonstrate that plasmids encoding either the amino or carboxy-terminal regions of the protein, as well as the changing of a single amino acid within IncA (serine 17) could not block *C. caviae* infection. Immunoblot analysis of truncated IncA in a Vaccinia virus system provided evidence that serine 17 of *C. caviae* IncA is a target for phosphorylation.

These experiments provide insight into the interaction of Inc proteins with the host cell and introduce a model system where these interactions can be explored further.

2.2. Introduction

The obligatory intracellular chlamydiae occupy a nonacidified vacuole (the inclusion) as they grow and divide within infected cells (9). These bacteria produce a set of inclusion proteins (Inc proteins) that are secreted and localized to the inclusion membrane(2) (3, 13, 15, 18). At least ten Inc proteins have been identified and it is likely that many more Inc proteins are produced by chlamydiae (1).

The analysis of Inc protein structure and function is hindered by the absence of a system for genetically transforming chlamydiae. However, some progress has been made recently using alternate approaches. Microinjection studies demonstrated that Inc proteins contact the host cell cytosol, and that selected Inc proteins are phosphorylated, likely by host cell protein phosphokinases (13, 17). Two-hybrid

analysis demonstrated that *C. trachomatis* IncA dimerizes, and microinjection of antibody directed at this protein blocks fusion of inclusions (10). Two-hybrid analysis also showed that IncG interacts with a host cell protein (14-3-3β), and that phosphorylation of IncG is required to facilitate this interaction (17). These results support a hypothesis that places Inc proteins at a central point in the interaction between the host cell and the intracellular, intravacuolar chlamydial developmental forms.

In this study we examined the effects on host cells of plasmid- or virus- encoded IncA or IncA-fragments (Fig. 1), either in the presence or absence of a chlamydial infection. We demonstrate that expression of full-length *C. caviae incA* completely blocks the development of *C. caviae* and partially blocks development of *C. trachomatis*.

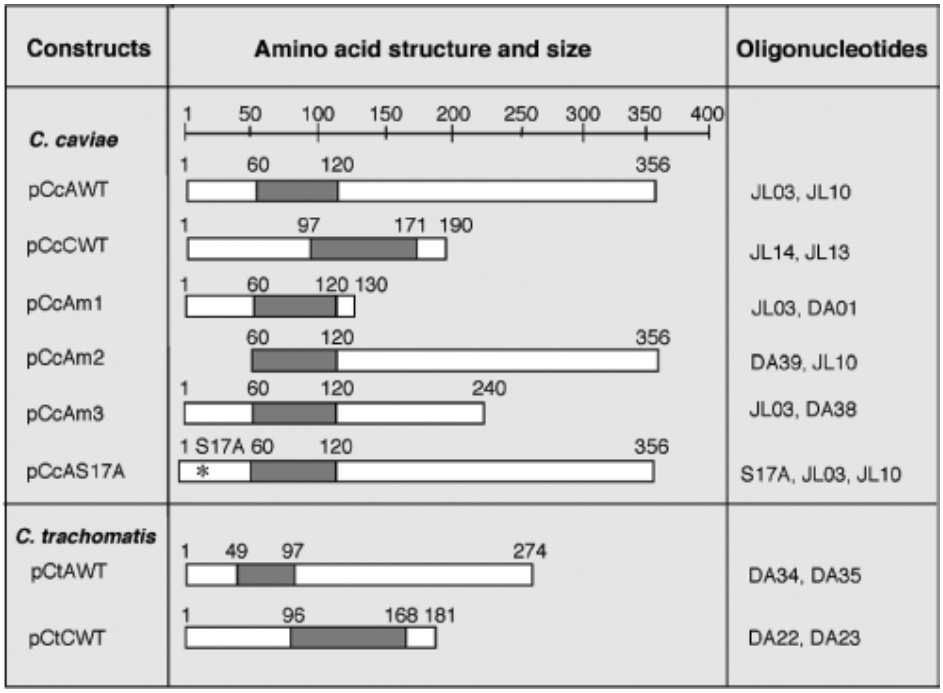

Fig. 2.1. Schematic representation of *inc* gene constructs used in this study
The plasmid name (left) and a schematic of the predicted protein structure (center), and oligonucleotides used in construction (right; Table 1) are indicated for each plasmid. The scale bar at top indicates the length in amino acids of each predicted gene product. All pcDNA4/HisMax C constructs encode a polyhistidine tag at the amino terminus. *Chlamydophila caviae* and *C. trachomatis incA* were also expressed via pcDNA3.1(+), in constructs that do not encode protein with a polyhistidine tag. A large region of predicted hydrophobicity within each predicted protein is indicated for each construct (darker bar)

2.3. Materials and methods

2.3.1 Chlamydial strains, host cell lines, and chlamydial infections

Chlamydia trachomatis strain 434/Bu (serovar L2) and *C. caviae* strain GPIC elementary bodies were purified from infected monolayers by passage over a 30% Hypaque density gradient. Purified chlamydiae were stored in 0.25 M sucrose, 10 mM sodium phosphate, 5 mM L-glutamic acid (SPG) at -80°C prior to use (6). HeLa and ChoK1 cells (ATCC) were cultured in Minimal Essential Medium supplemented with 10% fetal bovine serum (Gibco) and 10 $\mu g ml^{-1}$ gentamicin and grown at 37°C in 5% CO_2. Elementary bodies diluted in SPG were added to the cells at multiplicities of infection (MOI) as indicated. Infected cells were centrifuged at 500 *g* for 1 h at room temperature (RT), after which the inocula were removed, the cells were washed with Hank's Balanced Salt Solution (Gibco) and medium was added.

Cells to be used for microscopic analysis were cultured on sterile glass coverslips at 50 to 80% confluency. After 30–35 h, infected cells were fixed in 100% methanol for 10 min and washed with phosphate-buffered saline (PBS). Cells were then subjected to fluorescent antibody staining or stored at 4°C.

2.3.2. Vaccinia virus recombinants

Full length *C. caviae incA* and a truncated *incA* fragment (nucleotides 1–459) were amplified using *Pwo* polymerase (Boehringer). Terminal oligonucleotide primers (Table 1) were designed to yield restriction enzyme sites for cloning into plasmid pRB21, a shuttle vector that allows simple selection of VV recombinants (13). Alanine substitutions of serine or threonine residues in IncA were produced using a three-primer technique (12). All amplification products were confirmed by DNA sequencing. Recombinant Vaccinia virus diluted in PBS were inoculated onto HeLa cells and incubated at room temperature for 1 h prior to addition of MEM-10 and incubation at 37°C (13). Production of IncA and the identification of different IncA isoforms were monitored by immunoblot analysis of VV-infected HeLa cell lysates collected 16 hours post-infection (13).

Primer	Sequence
JL03	AGCAGAATTCATGACAGTATTCACAGACAA
JL10	AGCAGATATCACTTAACTATCTTTATGCTC
JL13	AGCAGATATCTAAATGTCCGGTAGGCCTAG
JL14	AGCAGAATTCATGACCTCTGTAAGAACCGA
DA01	AGCAGATATCTTAACGGACCACTTGAGACGGTGT
DA38	AGCAGATATCCAAATTGGTTCTTAACTCAGT
DA39	AGCAGAATTCCAAAGGATTTGCTATCTC
S17A	AGAGCGTCCGGACCTACT
DA34	AGCAGATTTCATGACAACGCCTACTCTA
DA35	AGCAGATATCCTAGGAGCTTTTTGTAGA
DA22	AGCAGAATTCGGCAACGTTATGACGTACTC
DA23	AGCAGATATCTTAGCTTACATATAAAGTTTG

Table 1.1. Oligonucleotides used in the construction of plasmid and Vaccinia virus recombinants

2.3.3. Construction of plasmids used in transfection experiments

Plasmids pcDNA3.1(+) and pcDNA4/HisMax C (Invitrogen) were used as vectors in all experiments (Fig. 1). Plasmid pcDNA4/HisMax C vector encodes a polyhistidine tag that was fused to the amino terminus of each recombinant polypeptide. Intact or truncated coding regions of *C. caviae incA*, or *incC*, and *C. trachomatis incA*, were amplified using primers indicated in Table 1. Restriction sites for *Eco*RI and *Eco*RV were introduced into the forward and reverse primers, respectively, for each amplification product to be cloned. Reactions were carried out using *Pwo* polymerase and chlamydial genomic DNA as template. Plasmids from positive clones were purified (Qiagen) and confirmed by sequence analysis.

2.3.4. Antibodies and Immunofluorescence Microscopy

Monoclonal antibody A57B9, directed at a genus common epitope on chlamydial GroEL (HSP60), was used to label chlamydial developmental forms(22). Polyclonal anti-IncA was produced as described(14). Monoclonal antibodies to *C. trachomatis* IncA (mAb 3H7) or to *C. caviae* IncA (mAb 17F12) have been previously described (13, 16). Monoclonal antibody to polyhistidine was purchased from Clontech. Fixed monolayers were incubated in 2% bovine serum albumin in PBS (BSA-PBS), as a blocking reagent, for 20 min. Primary antibodies diluted in BSA-PBS were then incubated on cells for 1 h. Cells were washed three times with PBS and then incubated in the dark with the appropriate secondary antibodies for 1 h. Secondary antibodies were purchased from Southern Biotechnology Associates. The cells were washed three times in PBS and coverslips were inverted onto 3 µl of Vectashield (Vectors Laboratories) containing the DNA-specific fluorochrome 4', 6'-diamino-2-phenylindole (DAPI; Sigma; 2 $\mu g ml^{-1}$). Labeled monolayers were examined at 1000× magnification using a Leica fluorescence microscope and images were collected with a SPOT digital camera system (Diagnostic Instruments). Images were processed in Photoshop 5.0 (Adobe Software) and Canvas 6 (Deneba Software).

2.3.5. Transfections and transfection/infection experiments

HeLa or ChoK1 cells grown on sterile glass coverslips were transfected with the indicated plasmid constructs using Lipofectamine 2000 (Gibco) according to manufacturer's instructions. All quantitative infection/transfection analyses were conducted in cells with MOI of 1. Transfected cells were incubated 24 h post transfection and then either fixed with methanol or infected with chlamydiae. Infected cells were incubated 30 h prior to methanol fixation, unless indicated. The efficiency of infection of transfected cells was determined by labeling inclusions with anti-polyhistidine (to detect the product of the transgene) and anti-HSP60 (to detect the chlamydiae). Following labeling, coverslips were removed and the infection rates determined by counting infected and transfected cells, and total numbers of transfected cells, under 1000× magnification.

In experiments where HeLa cells were doubly transfected with plasmids expressing *incA* and *incC*, a pcDNA3.1(+) construct encoding IncA in the absence of the 6-his tag was used in combination with pcDNA4/HisMax C encoding IncC. Cells were transfected as described and examined by fluorescence microscopy, using anti-IncA and anti-6-His reagents to detect IncA and IncC, respectively.

2.3.6. Statistical analysis

Standard deviations were calculated for each combination of plasmid and chlamydial strain from the average of two or three wells in the same experiment. The number of transfected cells that were infected by chlamydiae in a particular experiment was evaluated in at least three independent experiments for each plasmid construct tested. A total of at least 300 individual transfected cells were counted for each tested plasmid construct. In each case, the significance of the differences between means were evaluated using both the Student's T test and the Kruskall-Wallis test, as calculated using the Instat software program (GraphPad Software). Reported P values represent calculations based on the Student's T test.

2.4. Results

2.4.1. Cytosolic expression of *C. caviae incA* blocks *C. caviae* development

Plasmids encoding *C. caviae* and *C. trachomatis* IncA were transfected into HeLa cells and the resulting effects on cellular morphology were examined using fluorescence microscopy. IncA was distributed to discrete regions throughout the cytosol but the cell morphology was not affected (Fig. 2a). However, cells expressing *C. caviae incA* were refractory to productive infection by *C. caviae* (Fig. 2b, Fig. 3). These data were expressed quantitatively by examining the percentage of transfected cells that contained chlamydial inclusions (Fig. 4). Plasmid-driven expression of *C. caviae incA* led to a marked reduction in the number of inclusions formed within cells ($P < 0.01$ against any other tested plasmid), and the very few inclusions that can be identified in these transfected cells are morphologically aberrant (Fig. 3).

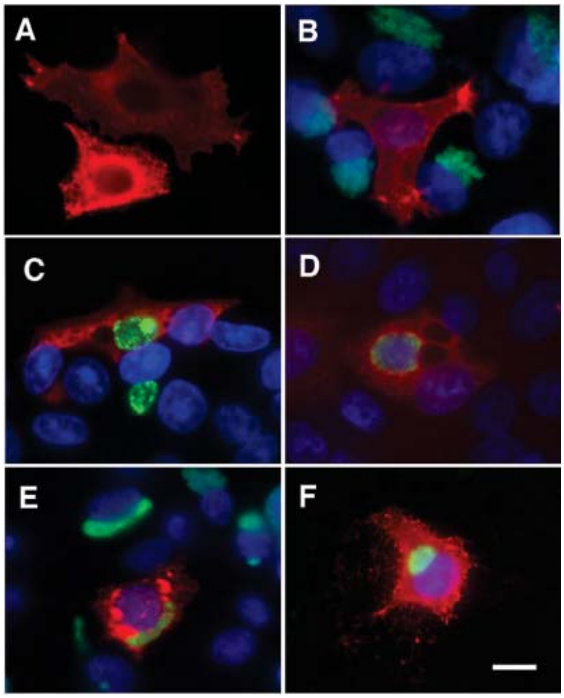

Fig.2.2. Effect of cytosolic expression of *incA* and *incC* from *C. caviae* or *C. trachomatis*
The vector pcDNA4/HisMaxC was used in each construct examined in this figure. Expressed IncA was detected by using specific monoclonal antibodies and stained with red in each panel. Distribution of His/IncC fusion protein in the cytoplasm was detected indirectly by using anti polyhistidine tag monoclonal antibodies (Clontech) and also labeled in red. In panels B-F, the nuclei are stained with blue (DAPI) and chlamydiae are labeled with anti-HSP60 antibodies and stained with. Panel A; HeLa cell transfected with pCcAWT (*C. caviae incA*). Panel B; HeLa cell transfected with pCcAWT in a monolayer subsequently infected at an MOI of 1.0 with *C. caviae.* Panel C; HeLa cell transfected with pCcAWT in a monolayer subsequently infected at an MOI of 0.25 with *C. trachomatis.* Panel D; HeLa cell transfected with pCtAWT and subsequently infected at an MOI 0.25 with *C. trachomatis.* Panel E; HeLa cell transfected with pCtAWT and subsequently infected at an MOI 0.25 with *C. caviae*. Panel F; HeLa cell transfected with pCcCWT (*C. caviae incC*) and infected with C. caviae at an MOI 1.0. The scale bar in F indicates 8 microns for each panel.

This aberrancy is manifested as larger than normal reticulate bodies, with single chlamydial developmental forms occupying individual vacuoles within cells (4). This is in contrast to inclusions formed in nontransfected cells or cells transfected with any other plasmid produced for this work (Fig. 2,3).

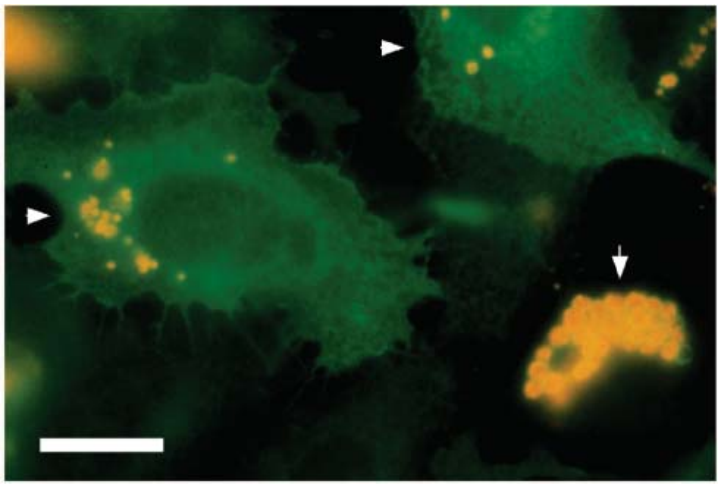

Fig. 2.3. Examples of aberrant *C. caviae* inclusions formed in cells transfected with pcDNA3.1(+) encoding *C. caviae incA*
Cells expressing *C. caviae* IncA are stained with green and chlamydial HSP60 is stained with red. Aberrant inclusions within transfected HeLa cells are shown with white arrowheads. A typical *C. caviae* inclusion within a nontransfected cell is shown in the lower right corner of the figure (arrow). Cells were infected at an MOI of 0.5 and fixed with methanol 30 h post infection. The scale bar indicates 10 microns.

Plasmid-driven expression of *C. caviae incA* also caused a statistically significant reduction in inclusion formation by *C. trachomatis* ($P < 0.05$: Fig. 4), but did not lead to morphological aberrancy of the *C. trachomatis* inclusions and developmental forms (Fig. 2c). The developmental block is specific to transfection with plasmids expressing *C. caviae incA*, as transfected cells producing *C. trachomatis* IncA showed no qualitative (Fig. 2d,2e) or quantitative (Fig. 4) difference in inclusion formation by either species.

The developmental block observed for *C. caviae incA* was not host-cell type specific. Each of the transfection/infection experiments was repeated in ChoK1 cells with similar results (not shown).

Plasmid pcDNA4/HisMaxC encoding chlamydial IncC were also used in the transfection/infection experiments. Expression of *incC* from *C. caviae* (Fig. 2f) or *C. trachomatis* (not shown) did not block chlamydial development but this expression led to the formation of an extensive network of plasma membrane extensions at the surface of transfected cells.

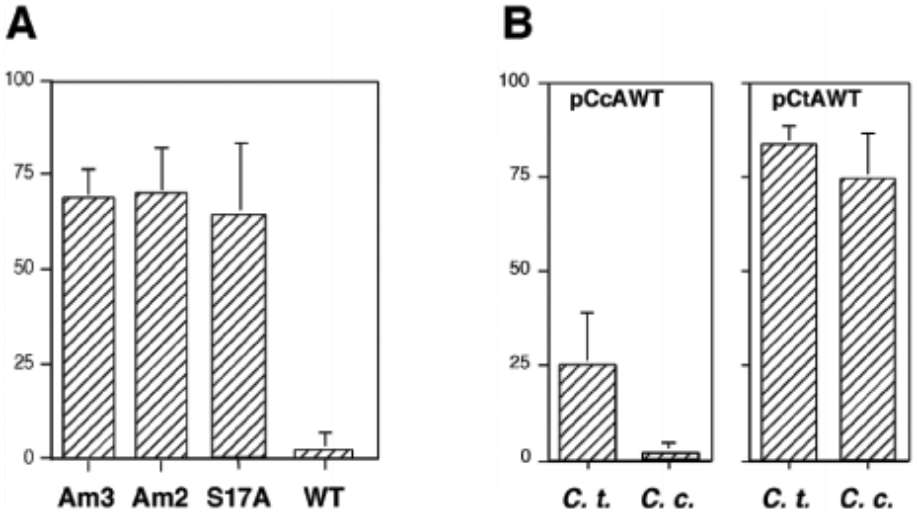

Fig. 2.4. Quantitative analysis of transfection/infection experiments
The data shows the percentage of the infected cells among of one hundred transfected cells in the population. In all cases, cells to be used for quantification were infected at an MOI of 1 and were fixed for microscopy at 24 h post infection. The error bars indicate standart deviations. Panel A; The relative infection efficiency of cells transfected with plasmids carring each of the truncated or S17A mutated genes of *C. caviae* shown in Fig.1 and Fig.5, as compared to transfection with a plasmid expressing wild type (WT) of *C. caviae incA.* Panel B; Percent of cells infected with *C. trachomatis* (shown as C.t) and *C. caviae* (shown as C.c) after transfection with plasmids expressing either *C. caviae* of *C. trachomatis* incA (pCcAWT and pCtAWT, respectively).

2.4.2. Analysis of *C. caviae* IncA regions important for developmental block

Analyses were then conducted to identify regions of *C. caviae* IncA that might be important in facilitating the developmental block within these cells. Expression plasmids were constructed that encoded amino- and carboxy-terminal deletions of IncA (Fig. 1). None of the truncated forms of *C. caviae* IncA blocks development of *C. caviae* (Fig. 5b,5c Fig. 4a: P values greater than 0.2). A final construct was examined that encoded full length *C. caviae* IncA with a single changed amino acid residue, a serine to alanine mutation at position 17 (pCcAS17A). Transfection with pCcAS17A also failed to block development of *C. caviae* (Fig. 5a) and led to similar numbers of infected/transfected cells, as did controls (Fig. 4a). Therefore changes as small as a single amino acid abrogated the ability of IncA to block subsequent *C. caviae* development.

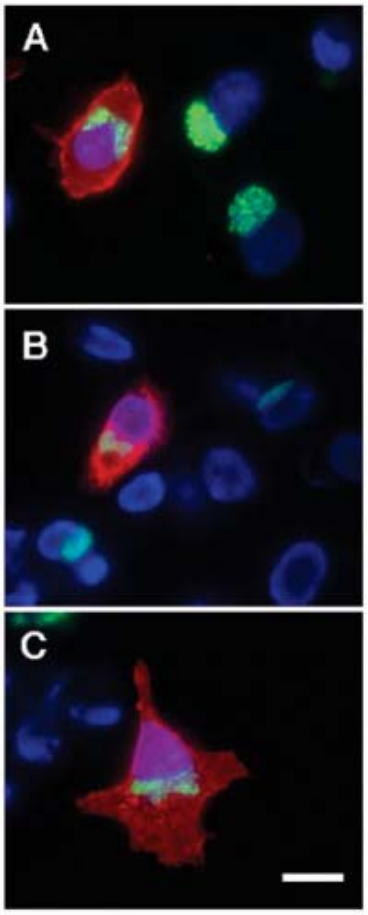

Fig. 2.5. Effect of cytosolic expression of deleted or mutated *C. caviae incA* sequence
Cells were transfected with either pCcAS17A, which encodes a protein differing from wild type at only a single amino acid (panel A), pCcAm2, which encodes a protein lacking the amino terminal 60 amino acids of IncA, (panel B), or pCcAm3, which encodes a protein lacking the carboxyl terminal 116 amino acids of IncA (panel C). The fluorescence images are labeled with anti-polyhistidine monoclonal antibody (red), and anti-chlamydial HSP60 (green). The nuclei are labeled with DAPI (blue). The scale bar in C indicates 8 microns for each panel.

2.4.3. The role of phosphorylation in *C. caviae* IncA blockage effect

In previous work we showed that phosphorylation of IncA was associated with an electrophoretic mobility shift resulting in two additional protein species at a higher apparent molecular mass in polyacrylamide gels (13). For our initial analyses of the site of phosphorylation in IncA, a truncated *incA* sequence was engineered into Vaccinia virus (VV) and the encoded protein was examined by immunoblotting of infected cell lysates. These experiments demonstrated that a polypeptide containing the amino terminal 153 amino acids of *C. caviae* IncA was modified in HeLa cells similarly to that seen with the full-length protein (Fig. 6). Site-directed mutagenesis of serine or threonine codons in the truncated *incA* were conducted to examine the effect of individual amino acid changes on the migration of the protein. Altering serine 17 resulted in the elimination of the highest of the three electrophoretic species seen in immunoblots of VV-infected cells (Fig. 6).

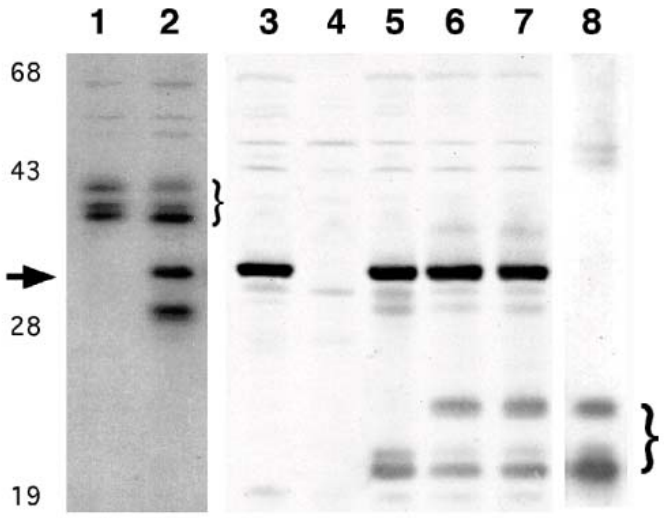

Fig. 2.6. Immunoblots of lysates of HeLa cells infected with different Vaccinia virus (VV) recombinants
Full length (lanes 1 and 2) and truncated (lanes 5-8) IncA bands are indicated with brackets. Control VV-infected cells are in line 3 and uninfected cells are in lane 4. As previously reported [4,8], full length *C. caviae* IncA migrates as three isoforms when produced during chlamydial infection (lane 1) or when expressed via a VV vector (lane 2). Lane 8 shows that truncated wild type IncA (residues 1 to 153) encoded by VV also is represented as three similar isoforms. Mutation of serines or threonines in the truncated *incA* are represented in lanes 5 (S17A), lane 6 (S121A and lane 7 (T145A). Molecular mass standards are indicated in kilodaltons. The arrow indicates a background VV band recognized by the anti-incA antisera in some samples.

These results were consistent in three independent constructs that altered either serine 17 alone or serine 16 and 17 in combination (data not shown). Changes at any other serine or threonine in the amino terminal 153 amino acids of *C. caviae* IncA did not alter on the migration pattern of the protein in this assay. These results suggest that *C. caviae* IncA is phosphorylated at serine 17 in the VV expression system.

2.5. Discussion

Although several Inc proteins have been identified in each of three different chlamydial species, their role in chlamydial biology is poorly understood. Inc proteins are present at the cytoplasmic face of the inclusion and likely are critical for direct

interactions with proteins in the host cell cytosol (10, 13, 17). The study of Inc proteins is complicated by their overall lack of sequence identity with proteins in the sequence databases and the absence of a system for directed mutagenesis in the chlamydiae. We used plasmid- or virus-driven expression systems to localize chlamydial Inc proteins into the cytosol of mammalian cells, and to examine the resulting phenotypes either in the presence or absence of a chlamydial infection. Similar approaches have been used to examine proteins secreted by other pathogens into the cytosol of infected cells (11, 21).

The primary focus of this work was to examine the utility of plasmid-driven expression of chlamydial *inc* genes to deliver Inc proteins to the cytosol of host cells. These experiments were initially designed to determine if IncA could be localized to the inclusion membrane after production in the host cell cytosol. It should be noted that we were unable to deliver an Inc protein to the inclusion membrane using this technique (not shown). However, these experiments allowed the analysis of cellular phenotypes associated with the cytosolic expression of *incA* or *incC*. First, we determined that cells transfected with *C. caviae incA* were refractory to productive infection by *C. caviae*. These results were demonstrated qualitatively and quantitatively, and the experiments were independently repeated with two plasmid constructs. The observed developmental block was specific to *C. caviae incA*; *C. trachomatis incA* had no effect in this system. The differences in effect by these two genes is not surprising, as their predicted protein sequences share only 20% identity (3, 14, 20).

All chlamydial Inc proteins contain a large hydrophobic motif within the coding sequence. As high level expression of foreign hydrophobic proteins can be toxic to cells we included a second Inc protein (IncC) as a control in these experiments. Although production of IncC from *C. caviae* (Fig. 2b) or *C. trachomatis* (not shown) altered cell structure significantly, expression of *incC* in this system had no effect on chlamydial development. Therefore the described block in *C. caviae* and *C. trachomatis* development was specifically a function of the production of *C. caviae* IncA.

We observed that, in the few transfected cells that contained inclusions, development of *C. caviae* was universally aberrant. These results suggest that the reduction in titer is a result of interfering with development, as opposed to interfering with attachment. If the block was associated with attachment, it is likely that some normal *C. caviae* development would have been observed in the few inclusions within C. *caviae inc*A-transfected cells.

Deletion mutagenesis was used to examine the region of *C. caviae* IncA responsible for blocking *C. caviae* development. No truncated fragment of IncA affected the subsequent ability of *C. caviae* to infect transfected cells. The abrogation of the ability of *incA* expression to block *C. caviae* development was observed with changes to IncA sequence at the single amino acid level. A modification of serine 17 was sufficient to remove the developmental block observed with the wild type gene. The mechanism of this block is not known, but we hypothesize that IncA encoded by the transfected plasmid serves as a competitive inhibitor for some host cell molecule critical for *C. caviae* development. We also hypothesize that the proper phosphorylation state of IncA is required to facilitate this interaction. Through the use of the VV expression system, we provide data suggesting that *C. caviae* IncA is phosphorylated at that residue, but further experimentation in this area will be required. The importance of the phosphorylation state of an inclusion membrane protein has previously been demonstrated for the interaction of *C. trachomatis* IncG with host cell 14-3-3β (17).

The results we observed with the *C. caviae* transgene are analogous to the data in which the effects on chlamydial growth of a dominant negative dynamin transgene were examined (5). Dynamin participates in both endocytosis and vesicle trafficking within cells (19), and it is possible that IncA and dynamin affect single process that leads to the lack of productive development by *C. caviae* (8).

Many intracellular microbes use diverse mechanisms to parasitize the host cell (7). The intracellular, intravacuolar parasites, including the chlamydiae, interact with the cytosolic compartment at many levels. The localization of Inc proteins at the surface of the inclusion supports a hypothesis that these proteins are important in the cell biology of host cell-chlamydia interactions. We anticipate that the model systems

utilized in this work, in concert with other approaches, will be useful for the elucidation of the intracellular biology associated with chlamydial development. Three conclusions can be made based on obtained results. Plasmid-driven expression of *C. caviae incA* in mammalian cells blocked productive *C. caviae* infection. *C. caviae incA* also reduced the ability of *C. trachomatis* to infect cells. *C. trachomatis incA* had no effect on *C. caviae* or *C. trachomatis* infection. Alterations of *C. caviae incA* sequence as small as a single amino acid abrogated the blocking effect.

2.5. Acknowledgements

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Chapter 3

Cytokinesis is blocked in host cells transfected with *Chlamydia trachomatis* gene CT223

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3.1. Abstract

Chlamydial inclusion membrane proteins (Incs) are localized to the inclusion membrane and exposed to the cytosol of the host cell. The function of most Inc proteins remains unclear. In this study we determined that some Inc proteins are responsible for blocking cytokinesis in host cells. Previously it was known that infection with various strains of *Chlamydia trachomatis* disrupts host cell cycle at the stage of cytokinesis. Multiple nuclei were detected in the infected cells. However, the mechanism of that blockage or effector molecules responsible for this effect were not established. Cells were transfected with plasmid constructs encoding genes of various Incs. Proteins of interest were expressed inside of different lines of host cells and changes in phenotype were analyzed by fluorescence microscopy. Three Inc proteins, encoded by a single operon, were found responsible for the polynuclear phenotype when expressed inside host cells.

3.2. Introduction

Chlamydiae are obligate intracellular bacterial pathogens with a unique developmental cycle. They replicate inside a cytoplasmic vacuole termed inclusion that develops inside of eukaryotic cell (10). *Chlamydia trachomatis* is a member of chlamydiae, consisting of more then 15 various serotypes. These serotypes are capable of causing various diseases in humans. Serovars A-C all cause trachoma (17) and serovars D-K are responsible for sexually transmitted diseases (14). Persistence of chlamydial infection in host cells is responsible for the development of chlamydia-induced diseases (5). Findings that *C. trachomatis* alone or together with human papilloma virus (HPV) are associated with increased incidence of cervical cancer (7), suggesting that chlamydia may be capable of interfering with the normal host cell cycle.

The fact that chlamydial infection can affect proliferation of host cells was noted decades ago. The comparison of cell numbers between chlamydia-infected and uninfected cultures show that host cell proliferation is suppressed by chlamydial

infection (4). However, it was also demonstrated that infection with *C. trachomatis* did not alter the generation time of host cells, and infected and uninfected cells had a similar rate of DNA synthesis (2). These data were clarified when it was shown that although the number of cells in the infected culture is lower than that in the control culture, the total number of nuclei is similar in both cases (3). It was also reported that infection with various *Chlamydia trachomatis* strains inhibits host cell growth by blocking cytokinesis. Multiple nuclei were detected in the infected cells, and the effect was dependent on chlamydial protein synthesis (3).

There is a group of specific chlamydial proteins named Inc proteins that localized to the chlamydial inclusion membrane and exposed to the cytosol of the host cell (12). These distinct proteins do not share significant identity with one another or with any genes within the sequence database (11, 12). The function of Inc proteins and their role in interaction with the eukaryotic host cell is not well understood. Immunofluorescence microscopy and statistical analysis were used to study plasmid expression of *inc* genes within uninfected cells to explore the biology of chlamydial Inc proteins (1). During these studies we found that expression of CT223, and to a lesser extent, other *inc* genes adjacent to CT223, led to a block in host cell cytokinesis. Cells transfected with plasmids expressing *C. trachomatis* CT223 resulted in an inhibition of cytokinesis that was similar to that seen in *C. trachomatis* infected cells. Different alleles of CT223 yielded similar inhibition of cytokinesis, consistent with the inhibitory effect of all tested *C. trachomatis* serovars (3). Deletion mutagenesis was used to identify the region of CT223 important for inhibition of cytokinesis.

3.3. Materials and Methods

3.3.1. Chlamydial strains, Inc genes and host cell lines

Elementary bodies (EB) of *Chlamydia trachomatis* strains D UW3, J 9235, J(s)1980, J(s)6686 and L2 and *C. caviae* were used for preparation of genomic DNA samples that were used as PCR templates (6, 13, 15, 16, 18). Genomic DNA was prepared by boiling EB suspensions in a water bath for 10 minutes. Inc protein genes

of *C. trachomatis* CT223, CT224, CT225, CT226, CT227, CT228, CT229, *incA, incC*, *C. caviae incA, incB*, *incC*, and GFP gene were tested. HeLa or McCoy cells (ATCC) were cultured in Minimal Essential Medium supplemented with 10% fetal bovine serum and 10μg/ml^{-1} gentamicin and grown at 37˚C in 5% CO_2.

3.3.2. Construction of plasmids and oligonucleotides used in transfection experiments

Plasmid pcDNA4/HisMaxC (Invitrogen) was used for cloning and expression of intact or truncated coding regions of *C. trachomatis* CT223, CT224, CT225, CT226, CT227, CT228, CT229, *incA, incC*, C. *caviae incA, incB*, *incC*, and GFP genes. This vector encodes a polyhistidine tag that was fused to the amino terminus of each recombinant polypeptide tested. Oligonucleotides were designed to include appropriate restriction sites for cloning. Names, sequences and targets of oligonucleotides used in this study are listed in Table 1. PCR reactions were carried out using *Pwo* polymerase and chlamydial genomic DNA as template, and the entire coding sequence as predicted from the serovar D genome sequence was used in the constructs. Constructs were confirmed by nucleotide sequence analysis.

3.3.3. Transfection experiments, antibodies and Immunofluorescence microscopy

HeLa or McCoy cells grown on sterile glass coverslips were transfected with the indicated plasmid constructs using Lipofectamine 2000 (Gibco) according to the manufacturer's instructions. Transfected cells were incubated 36 hours post transfection, and then fixed with methanol. The efficiency of transfection was determined by labeling with monoclonal anti 6xHis antibodies (Clontech) and secondary FITC or TRITC fluorescent antibodies (Southern Biotechnology Associates) to detect the product of the gene. Cells expressing GFP were analyzed without labeling. Coverslips were examined under 1000x magnification using a Leica fluorescence microscope and images were collected a with SPOT digital camera system. The rates of cells with a polynuclear phenotype were determined by counting transfected cells with two or more nuclei among of total population of transfected cells.

#	Name/ Site	Sequence	Target gene
1	DA71 EcoRI	agcaGAATTCttgagatctagaaaaatagaagc	*CT223C C. trachomatis*
2	DA97 KpnI	agcaGGTACCaatggtgagtttagcattagg	*CT223 C. trachomatis*
3	DA116 EcoRV	agcaGATATCctacacccgagagccgtaattg	*CT223C C. trachomatis*
4	DA119 EcoRV	agcaGATATCctaattagccgtttttagcagatt	*CT223 2/3 C. trachomatis*
5	DA121 EcoRV	agcaGATATCctactcttctatctgtttctcttt	*CT223C L1/2 C. trachomatis*
6	DA122 EcoRI	agcaGAATTCatggagcttaaagctatgttagag	*CT223C R1/2 C. trachomatis*
7	DA98 BamHI	agcaGGATCCatgagtactactattagcgg	*CT228 C. trachomatis*
8	DA99 KpnI	agcaGGTACCaatgagctgttctaatattaa	*CT229 C. trachomatis*
9	DA74 PstI	agcaCTGCAGctaagaagcttggttagcgtc	*CT228 C. trachomatis*
10	DA131 EcoRI	agcaGAATTCatgtcttatctttttgttcc	*CT227 C. trachomatis*
11	DA132 EcoRV	agcaGATATCtcatgagacacttatagtcac	*CT227 C. trachomatis*
12	DA129 EcoRI	agcaGAATTCatgttggcctttttttgcga	*CT226 C. trachomatis*
13	DA130 EcoRV	agcaGATATCttatatcagactttcttccaa	*CT226 C. trachomatis*
14	DA127 EcoRI	agcaGAATTCatggtggctaacaactcctttatt	*CT225 C. trachomatis*
15	DA128 EcoRV	agcaGATATCttaatcccacccatgaaattt	*CT225 C. trachomatis*
16	DA125 EcoRI	agcaGAATTCatgagttttgttggagatagt	*CT224 C. trachomatis*
17	DA126 XhoI	agcaCTCGAGctaatcattgggaaaaattga	*CT224 C. trachomatis*
18	DA34 EcoRI	agcaGAATTCatgacaacgcctactctaatc	*incA C. trachomatis*
19	DA21 EcoRV	agcaGATATCctaggagcttttgtagaggg	*incA C. trachomatis*
20	DA22 EcoRI	agcaGAATTCggcaacgttatgacgtactc	*incC C. trachomatis*
21	DA23 EcoRV	agcaGATATCttagcttacatataaagtttg	*incC C. trachomatis*
22	JL003 EcoRI	agcaGAATTCatgacagtatccacagacaa	*incA C. caviae*
23	JL010 EcoRV	agcaGATATCacttaactatctttatgctc	*incA C. caviae*
24	JL007 EcoRI	agcaGAATTCatgtcaacaacaccagcatc	*incB C. caviae*
25	JL006 EcoRV	agcaGATATCttaagattctgtttgaaaat	*incB C. caviae*
26	JL014 EcoRI	agcaGAATTCatgacctctgtaagaaccga	*incC C. caviae*
27	JL013 EcoRV	agcaGATATCtaaatgtccggtaggcctag	*incC C. caviae*
28	DA114 EcoRI	agcaGAATTCatggtgagcaagggcgaggа	GFP
29	DA115 EcoRV	agcaGATATC ctacttgtacagctcgtccatg	GFP
30	DA76 BamHI	agcaGGATCCttatttttacgacgggatgc	*CT229 C. trachomatis*

Table 1. Oligonucleotides used for amplification of inc genes by PCR
Sequences of the restriction sites used in oligonucleotides are shown in capital letters.

3.3.4. Statistical analysis

Standard deviations were calculated for every construct analyzed in this study. The number of transfected cells having polynuclear phenotype was evaluated in at least three independent experiments for each plasmid construct tested. A total of at least 500 individual transfected cells were counted for each tested plasmid construct.

In each case, the significance between means was evaluated using the Krunskall-Wallis test, as calculated using the Instat software program (GraphPad Software).

3.4. Results

3.4.1. Cytosolic expression of *C. trachomatis* CT223 and CT223 COOH changes host cell phenotype

Plasmids encoding CT223 from *C. trachomatis* strains D UW3, J 9235, J(s)1980, J(s)6686 and truncated gene encoding CT223 COOH from strain D UW3 were transfected into McCoy or HeLa. The resulting effects on cellular cytokinesis were observed using fluorescent microscopy (Fig.3.1 A and B)

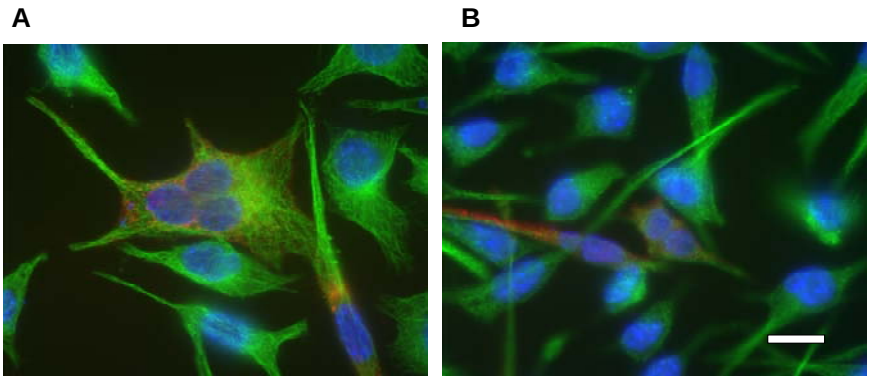

Fig. 3.1. The effect of cytosolic expression of CT223 and CT223 COOH form *C. trachomatis* serovar D UW3
The vector pcDNA4/HisMaxC was used in each construct. CT223 and CT223 COOH were detected by monoclonal specific antibodies and stained with red in each panel. Structures of α-tubulin were detected by labeling with specific anti-tubulin antibodies and stain green in each panel. The nuclei are labeled with DAPI (blue). Panel A; McCoy cell transfected with pcDNA4/HisMaxC encoding CT223. Three nuclei are localized inside of a single cell expressing CT223. Panel B; McCoy cells transfected with pcDNA4/HisMaxC encoding CT223 COOH. Two nuclei are localized inside of a single cell expressing CT223 COOH. The scale bar in B indicates 10 microns for each panel.

Plasmid-driven expression of *C. trachomatis* CT223 and truncated constructs of this protein revealed that the last 56 amino acids from the C-terminal part of CT223 is important for altering host cell phenotype. Expression of constructs missing this

stretch of CT223 resulted in a significantly decreased number of cells with a multinuclear phenotype. Schematic representations of truncated constructs of CT223 protein and the percentage of multinuclear cells expressing these constructs are shown (Fig. 3.2 A and B). These data were expressed quantitatively by examining the percentage of transfected cells that contained multiple nuclei ($P<0.01$). Five independent experiments were performed.

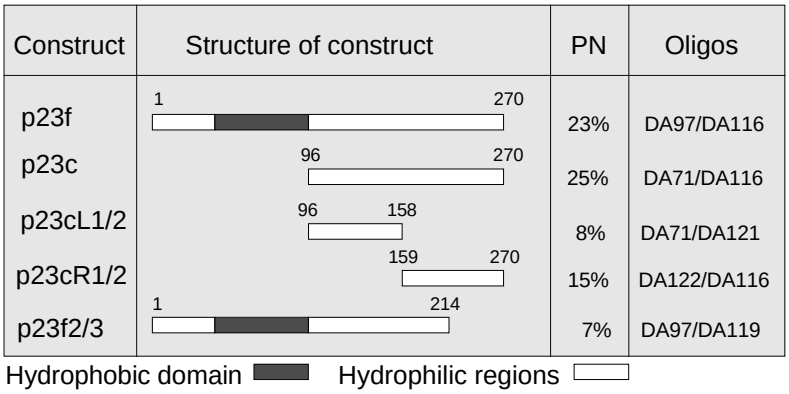

Fig. 3.2. Expression of truncated constructs of CT223 in McCoy cells

Cells were transfected with pcDNA4HisMaxC encoding truncated fragments of the CT223 gene of *C. trachomatis* D UW3. Panel A; Schematic pictures of used CT223 constructs. Amino acid positions are shown by numbers and the names of constructs are given in the left column. The percentage of polynuclear (PN) cells is shown in the PN column. Names of oligonucleotides used for amplification are given in the right column.

3.4.2. Production of other Incs from *C. trachomatis* in comparison with CT223 and CT223 COOH expression

The same approach was used for testing the effects on cell cytokinesis of other Inc proteins. HeLa or McCoy cells transfected with plasmids encoding genes of *C. trachomatis* CT224, CT225, CT226, CT227, CT228, CT229, IncA and IncC from *C. trachomatis* D UW3 and *C. caviae* IncA, IncB and IncC were compared with cells expressing CT223 and CT223 COOH. Cells also were transfected with plasmid encoding green fluorescent protein (GFP) or plasmid lacking any additional construct (empty plasmid). We also tested cells when all manipulations needed for transfection were performed without adding plasmid DNA (mock transfected cells). Cells without

any special treatment (intact cells) were also analyzed. Data were presented quantitatively by the examining the percentage of multinuclear cells among tested groups (Fig.3.3). Expression of CT224 and CT225 genes had less prominent effect on cytokinesis than expression of CT223 or CT223 COOH. However, the percentage of multinuclear cells was significantly higher among of cells expressing CT224 and CT225 in comparison to cells that express any other Inc proteins.

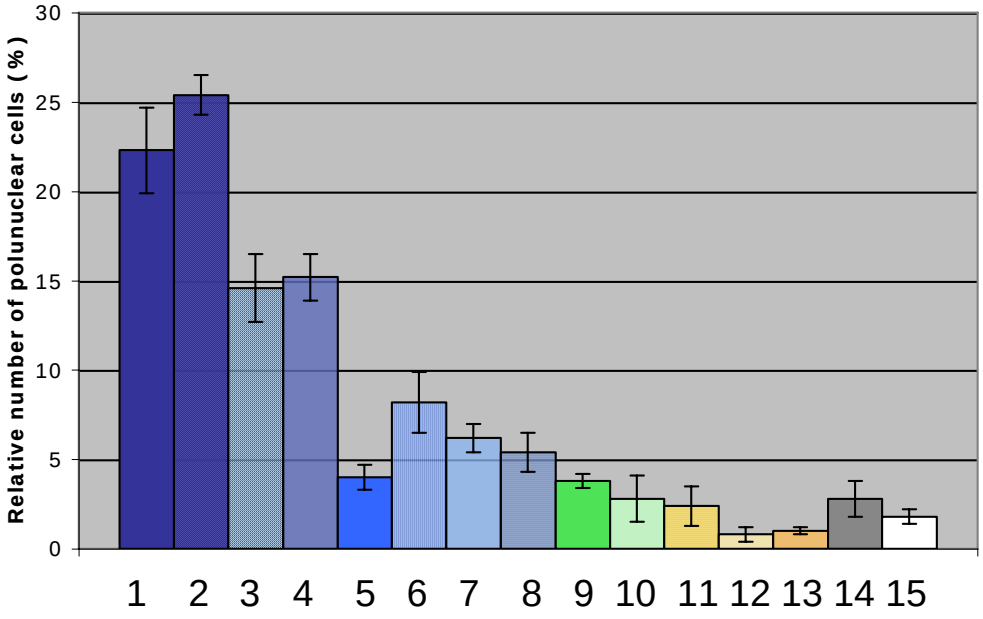

Fig. 3.3. The effect of the expression of different Inc proteins on host cell cytokinesis

Graph represents percentage of polynuclear cells among McCoy cells expressing different Inc proteins of *C. trachomatis* and *C. caviae*. Each indicated protein was delivered into cells in a pcDNA4/HisMaxC plasmid. The tested genes include genes ORFs encoded by CT223 operon of *C. trachomatis* serovar D UW3, *incA* and *incC C. trachomatis* serovar L2, *incA, incB* and *incC C. caviae, gfp* and an empty pcDNA4/HisMaxC vector. The vertical axis indicates the percent of transfected cells that had two or more nuclei. Numbers on X-axis stand for: 1- *C. trachomatis* D UW3 CT223; 2- *C. trachomatis* D UW3 CT223COOH; 3- *C. trachomatis* D UW3 CT224; 4- *C. trachomatis* D UW3 CT225; 5- *C. trachomatis* D UW3 CT226; 6- *C. trachomatis* D UW3 CT227; 7- *C. trachomatis* D UW3 CT228; 8-*C. trachomatis* D UW3 CT229; 9- *C. trachomatis* D UW3 IncA; 10- *C. trachomatis* D UW3 IncC; 11- *C. caviae* IncA; 12- *C. caviae* IncB; 13- *C. caviae* IncC; 14- GFP; 15- Empty pcDNA4HisMaxC;

The percentage of multinuclear cells expressing *C. trachomatis* Inc proteins CT226, CT227, CT228, CT228, IncA and IncC and *C. caviae* IncA, IncB and IncC was comparable to control groups expressing GFP or transfected with empty plasmid. These cells had the number of multinuclear cells similar to mock transfected and intact cells.

The effect was not host-cell specific and both HeLa and McCoy cells demonstrated similar results. Obtained data were expressed quantitatively by examining the percentage of transfected cells that contained multiple nuclei ($P<0.01$). Five independent experiments were performed. Based on obtained results, we can propose that *C.trachomatis* D UW3 CT223, CT224 and CT225 proteins, encoded in one operon, can be involved in specific mechanisms for blocking host cell cytokinesis without disrupting mitosis.

3. 4. 3. Cells producing Inc proteins CT223 of *C. trachomatis* and its truncated mutant CT223 COOH both have multiple centrosomes

To confirm that the multinuclear appearance of cells expressing CT223 is not the result of cell fusion but specific blockage of host cell cytokinesis, cells expressing CT223 were labeled with antibodies specific against γ-tubulin. While α- and β-tubulins are both components of microtubules, another type of tubulin, known as γ-tubulin, has an alternate role in the host cell. γ-tubulin is responsible for the nucleation of microtubule growth in all eukaryotes (8, 9). The microtubular organizing center, or centrosome, contains γ-tubulin. There is only a single centrosome in non-dividing cells, but the centrosome duplicates during interphase. Following division, each daughter cell carries a single centrosome. In our experiments, multinuclear cells expressing *C. trachomatis* D UW3 CT223 or CT223 COOH also have multiple centrosomes (Fig.3.4). These results confirm that cells expressing CT223 or CT223 COOH are altered at the stage of the cytokinesis.

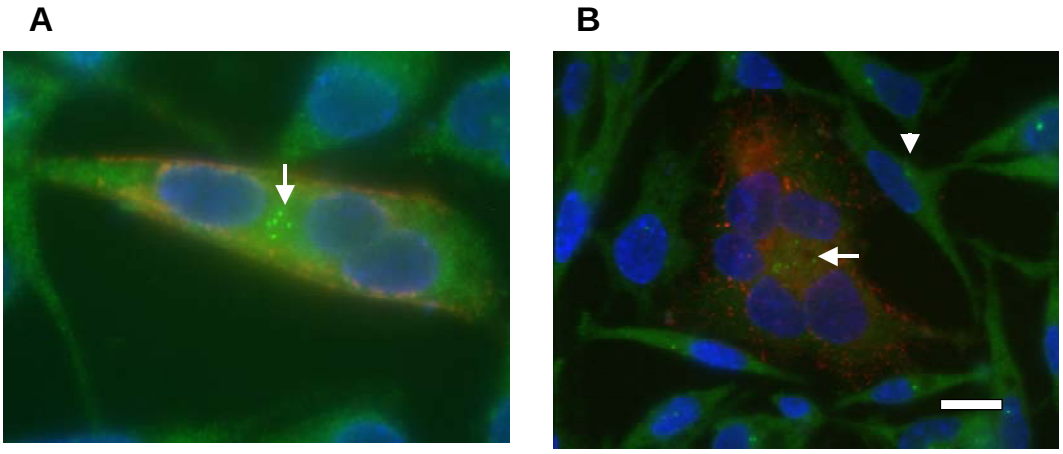

Fig. 3.4. Confirmation of the specificity of the effect of CT223 and CT223 COOH from *C. trachomatis* serovar D UW3 on host cell cytokinesis
The vector pcDNA4/HisMaxC was used in each construct. CT223 and CT223 COOH were detected with monoclonal specific antibodies and labeled in red. Structures of γ-tubulin were detected by labeling with anti γ-tubulin antibodies and stained in green. The nuclei are labeled with DAPI (blue). Panel A; McCoy cell transfected with pcDNA4/HisMaxC encoding CT223. Three nuclei are localized inside of a single cell expressing CT223. Multiple centrosomes are shown with arrow. Panel B; McCoy cells transfected with pcDNA4/HisMaxC encoding CT223 COOH. Five nuclei are localized inside of a single cell expressing CT223 COOH. Multiple centrosomes are shown by arrow. Note that cells that do not express CT223 COOH contain a single centrosomes (shown by arrow head). The scale bar in B indicates 10 microns for each panel.

3.5. Discussion

It was reported that infection with different strains of *C. trachomatis* has an effect on the cell cycle of various types of host cells by blocking cytokinesis (3). However, any chlamydial protein(s) responsible for this phenomenon were not identified. Our study describes *C. trachomatis* Inc protein CT223 as a potential candidate involved in blocking cytokinesis.

There are differences in DNA sequences among of CT223 from different *C. trachomatis* serovars (13). Expression of allelic CT223 genes from *C. trachomatis* strains D UW3, L2, J(s)1980, J(s)6686, J9235 in various mammalian cell lines confirmed their specific effect on cytokinesis. Analysis of truncated CT223 constructs revealed that the last one third of the C-terminal part of this protein is responsible for cytokinesis blockage. Two other Inc proteins from the same operon, CT224 and CT225, also affected host cell cytokinesis. However, expression of CT224 or CT225 had less intensive effects on the host cell phenotype. It has to be mentioned that gene cluster of CT223-CT227 was not found in any other chlamydiae. Despite of the fact that all of these genes share very little homology, it is likely that they all derived from one ancestor gene due to duplication events. The fact that most of them have common function and alter cytokinesis when produced inside of host cells supports this hypothesis.

The mechanisms of action of CT223 and others on the cell cycle of host cells are not known. We can suppose that they act at the M-phase stage of the cell cycle, which includes two distinctive events, mitosis and cytokinesis. Mitosis is always precedes cytokinesis. We know that the entire cell cycle is under control of a family of protein kinases known as Cyclin-dependent kinases (Cdks). The activity of Cdks rises and falls during the progression of the particular cell through the cycle and depends on interaction with regulator proteins known as cyclins. Different cyclins regulate the activity of few Cdks. In mammals M-phase is controlled by Cdk1 kinase in cooperation with cyclin B. There are two hypothesis how cyclin-Cdks works. It can be direct phosphorylation of structural or regulatory proteins that affect the cell cycle or by activating other protein kinases (target proteins) that will perform certain functions and alter the cell cycle too. It is logical to propose that CT223 and other Inc proteins somehow disrupt Cdks system and prevent it from cell cycle control. It can be a direct contact with Cdks that prevent binding with cyclins and formation of Cdk-cyclin complexes. It also can be binding to cyclins with similar disruption of Cdks-cyclin contact. Finally CT223 and others Incs can somehow affect target proteins and prevent them from getting command from Cdk-cyclin complexes (Fig. 3.5).

The process of centrosome duplication and separation, also known as the centrosome cycle, is happening during interphase. The mechanism of centrosome duplication is mainly unknown and the fact that cells expressing CT223 and CT223 COOH have multiple centrosomes suggests that the cell cycle can be disrupted not only at cytokinesis stage. Other phases of the cell cycle can be affected also.

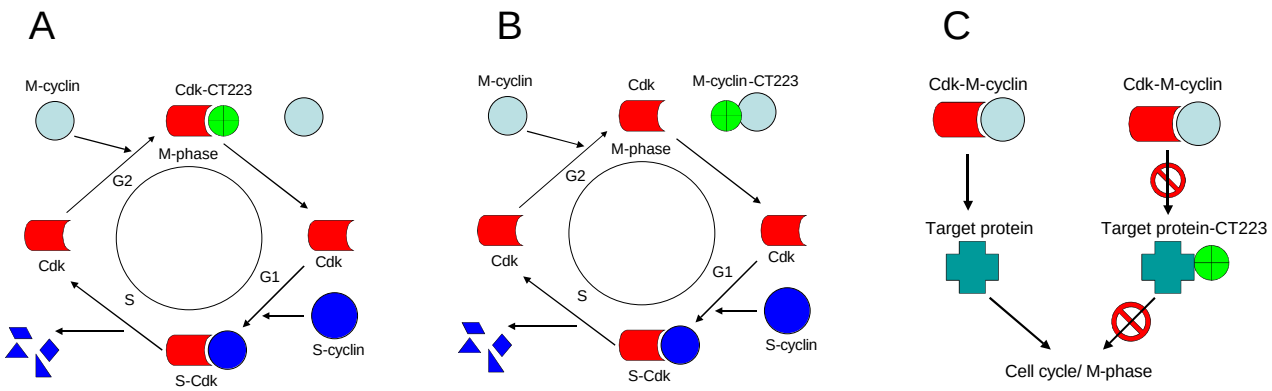

Fig. 3.5. Schematic representation of the hypothesis for CT223 action on host cell cytokinesis

Panel A; CT223 binds Cdk and prevents it from interaction with a cylin. Panel B; CT223 binds cyclin and prevents it from interaction with Cdk. In both cases cyclin-Cdk complex cannot be formed. Panel C; CT223 binds target protein and makes it unable to respond to cyclin-Cdk complex.

Based on the proposed hypothesis the future research has to be focused on finding partners (or targets) for CT223 and other Inc proteins among of the host cell proteins. Various methods, such as yeast or bacterial two-hybrid analysis, immunoprecipitation or pull-down assays with subsequent mass-spectrometry can be useful for accomplishing this goal. Identification of host proteins interacting with chlamydial Inc proteins will help to revealed mechanisms of their action. Knowing this we can understand how chlamydiae affect the cell cycle of the host cells. All of

these are important for studying potential carcinogenic role of chlamydia infection in humans.

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Chapter 4

Clonal Isolation of Chlamydiae Using Flow Cytometry of Chlamydia-Infected Cells.

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Running title: Chlamydial cloning by FACS

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4.1. Abstract

This manuscript describes a new technique for the microbiological cloning of chlamydiae using a Fluorescence Activated Cell Sorter (FACS). The approach exploits chlamydial acquisition of the fluorescent, Golgi-specific, stain (N-[7-(4-nitrobenzo-2-oxa-1,3-diazole)]) aminocaproylsphingosine. This fluorescent lipid is delivered from the Golgi Apparatus to the chlamydial inclusion membrane and then to the developmental forms within the inclusion in living, infected, cells. This labeling results in easily identifiable chlamydial inclusions that can then be analyzed and sorted by FACS. This technique was used successfully to sort individual chlamydia-infected cells into individual wells of a culture dish, leading to the production of cloned chlamydial isolates. The approach was used to isolate clonal populations of prototype strains from *Chlamydia trachomatis, Chlamydia caviae,* and *Chlamydia suis*. Recent clinical isolates were also successfully cloned using FACS. The procedure is simple and rapid, with single cloning cycles being completed 24h post-culture of a sample. It is anticipated that FACS-based sorting of live Chlamydia-infected cells will be a significant technical tool for the isolation of clonal populations of any chlamydial strain.

4.2. Introduction

Chlamydia are obligate intracellular bacterial pathogens that cause a broad spectrum of diseases within a variety of hosts (2, 8, 16, 19). The study of these genetically intractable, obligate intracellular bacteria pose many unique challenges. One of these is the challenge of microbiological cloning of isolates. Although infection by multiple strains is a relatively common event in chlamydial infection and disease (17), it is not common to work with clonal isolates of any chlamydiae in the diagnostic or research laboratory. This is changing however, as limiting dilution (18) and focus-forming assays have been developed for producing microbiological clones (3, 9). These techniques are very effective research tools, but each requires extended culture and significant labor prior to generation of a clone.

Hackstadt et al., (5) demonstrated that treatment of *C. trachomatis*-infected monolayers of cells with the fluorescent Golgi-specific probe, (N-[7-(4-nitrobenzo-2-oxa-1,3-diazole)]) aminocaproylsphingosine (C_6-NBD-cer) leads to accumulation of the fluorescent label within the chlamydial inclusion. This label has routinely been used to examine vesicle trafficking events associated with the Golgi Apparatus in mammalian cells (7, 10). Within infected cells, chlamydiae acquire C_6-NBD-cer from the Golgi apparatus within 30 minutes following treatment and retain the label for the entire developmental cycle. In contrast, uninfected cells lose label via transport from the Golgi apparatus to the plasma membrane. Subsequent analyses demonstrated that *Chlamydia spp*. acquire C_6-NBD-sphingomyelin, endogenously synthesized from C_6-NBD-cer, and transported to the vesicle (inclusion) in which chlamydia multiply (4, 5). This label appears green when observed in the fluorescence microscope (14) against a background of uninfected, weakly fluorescent cells. The relative abundance of label in the cell varies directly with the number of developing reticulate bodies (RB), and thus the fluorescence within the inclusion increases in intensity until RBs are extensively differentiated back to elementary bodies (EBs). All tested chlamydia accumulate and retain C_6-NBD-cer, indicating that the host cell processes highlighted by this label are common among *Chlamydia spp*. (4-6, 11, 15, 20).

In this report the utility of C_6-NBD-cer for the rapid isolation of clonal populations of chlamydia within infected cells is explored. Isolates of different chlamydia species were successfully cloned using this approach, and single cycles of the procedure were completed within 24 h of initial culture.

4.3. Materials and methods

4.3.1. Chlamydial strains, host cell lines, and chlamydial infections

Elementary bodies of *C. trachomatis* [D UW/3 and J(s)893], *C. suis* S45, and *C. caviae* GPIC were purified from infected monolayers by passage over a 30% Hypaque density gradient (1). Purified chlamydia were stored in 0.25 M sucrose, 10

mM sodium phosphate, and 5 mM L-glutamic acid (SPG) at -80°C prior to use. Eight random primary clinical isolates were collected from stored isolation swabs collected from patients seen at King County Public Health Clinics. For Chlamydia culture, McCoy cells were grown in Minimal Essential Medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 10 μg/ml gentamicin (MEM-10) and incubated at 37°C in 5% CO_2. Elementary bodies diluted in SPG were added to cells at multiplicities of infection (MOI) of 0.1 and the chlamydiae were centrifuged onto cells at 500 x *g* for 1 h at room temperature (RT). Inocula were then removed, the cells were washed with Hank's Balanced Salts Solution (HBSS; Gibco, Carlsbad, CA) and MEM-10 was added to the wells.

4.3.2. Labeling infected cells with C_6-NBD-cer

Monolayers of McCoy cells were grown on 12 mm-diameter glass cover slips in 24-well plates at 50% to 80% confluency. Cells were infected with *Chlamydia spp.* and, after approximately 20 h of incubation, MEM-10 was replaced with HBSS containing 1μg/ml of C_6- NBD-cer (Molecular Probes, Eugene, OR). These cells were incubated for 20 minutes at 37°C followed by removal of label and incubation in MEM-10. Cells were then cultured for 2-3 h to allow transfer of the label to chlamydia within infected cells, and exocytosis of label from uninfected cells (5). Cultures were rinsed with HBSS before preparation for analysis and FACS sorting.

4.3.3. Analysis of Chlamydiae via FACS

Host cells to be used for FACS analysis were treated with trypsin for 20 minutes at 37°C. Cells were then suspended in an equal volume of MEM-10 and examined via FACS. All sorting procedures were performed on a Becton Dickinson FACS Vantage SE instrument with DiVa software (BD Biosciences, San Jose, CA). It was critical to sort using a nozzle with a 130 μm aperture, and to reduce the sorting pressure to 10 psi during sorting. Sorting with smaller nozzles or higher psi led to lysis of infected cells (not shown). Chlamydia-infected cells were sorted into sterile

wells of a 24 or 96-well tray containing MEM-10 at either 1, 10, or 100 cells per well. Experiments designed to establish appropriate sorting conditions and the efficiency of sorting were conducted in wells lacking an existing monolayer. For these initial experiments, sorted cells were analyzed by immunofluorescence microscopy following methanol-fixation 16 h after sorting. Actual cloning of chlamydial strains was conducted using wells containing a monolayer of uninfected McCoy cells.

4.3.4. Antibodies and immunofluorescence microscopy

Monoclonal antibody A57B9, directed at a genus-common epitope on chlamydial GroEL, was used to label chlamydial developmental forms (13, 21). Monoclonal antibodies specific for different Inc proteins were used for confirming separation of individual clones from cell population (12-14). These antibodies were used for identifying different chlamydial strains in the separations of mixed populations of chlamydiae. Monoclonal antibodies to polyhistidine were purchased from Clontech (Mountain View, CA). Methanol- fixed monolayers (13) were incubated in 2% bovine serum albumin in PBS (BSA-PBS) for 20 min. Primary antibodies diluted in BSA-PBS were then incubated on cells for 1 h. Cells were washed three times with PBS and then incubated in the dark with the appropriate secondary antibodies (Southern Biotechechnology Associates, Birmingham, AL) for 1 h. After three more washes in PBS, the coverslips were inverted onto 3 μl of mounting medium containing the DNA-specific fluorochrome 4', 6'-diamino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). Labeled monolayers were examined at 100X magnification using a Leica fluorescence microscope and images were collected with a SPOT digital camera system from Diagnostic Instruments (Sterling Heights, MI). Images were processed in Photoshop CS from Adobe Software (San Jose, CA) and Canvas 6 from Deneba Software (Miami, FL).

4.4. Results

4.4.1. C_6-NBD-cer labeling of cells infected with *C. trachomatis, C. caviae* and *C. suis*

Fluorescence microscopy demonstrated that McCoy cells infected with all tested laboratory strains were capable of accumulating C_6-NBD- cer (Fig. 1 A-D). Eight primary isolates taken directly from patient samples were also tested and each was positive for C_6- NBD-cer uptake (Fig. 1 E). These results confirm observations of many investigators (4-6, 11, 15, 20) and add the observation that clinical isolates taken directly from patients also accumulate C_6-NBD-cer during primary culture.

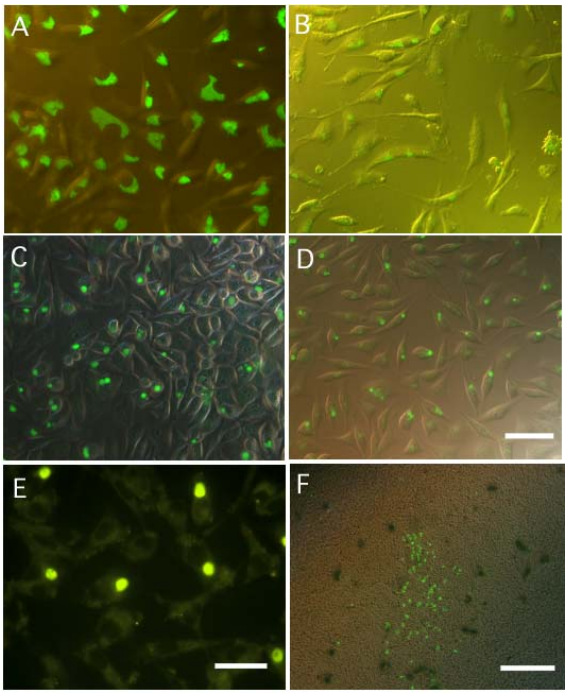

Fig. 4.1. Labeling of selected *Chlamydia spp*. by C_6-NBD-cer
All panels show the fluorescence images of live infected cells and have C_6-NBD-cer labeling shown in green, against a Differential Interference Contrast image of the monolayer. Panels A-E: McCoy cells infected with *C. caviae* GPIC (A), *C. suis* S45 (B), *C. trachomatis* D UW/3 (C), *C. trachomatis* J(s)893 (D), and a primary cervical isolate (E). These cells were incubated 20 h prior to labeling with C_6-NBD-cer, and were viewed using the 100X objective and oil immersion. Panel F: A live, C_6-NBD-cer-labeled, focus of a *C. suis* S45 clone observed under 20X magnification. This focus was observed 48 h post sorting. Scale bar in panel D represents 50 microns for panels A - D. Scale bars in panels E and F represent 25 and 150 microns respectively.

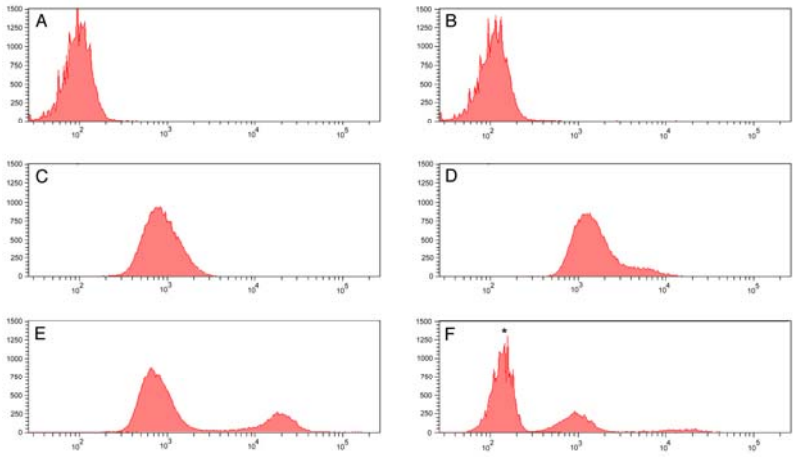

Fig. 4. 2. Analysis of FACS sorting for measuring the retention of C_6-NBD-cer by infected and uninfected cells

McCoy cells were sorted to examine the ability of the FACS to discriminate between infected and uninfected cells. All infections were conducted at an MOI of 0.1. All C_6-NBD-cer-treated cells were labeled 20 h post infection, and were processed through the FACS at 3 h (panels C-E) or 8 h (panel F) post labeling. Cell numbers are shown on the vertical axis and fluorescence intensity is represented on the horizontal axis for each panel. Populations of uninfected (A) and *C. caviae*-infected cells (B) that were not labeled with C_6-NBD-cer have identical fluorescence profiles. Uninfected, C_6-NBD-cer-treated wells (C) show a shift in baseline fluorescence that reflects the baseline level of C_6-NBD-cer retention by both infected and uninfected cells. *Chlamydia trachomatis*-infected cells (D) are present as a shoulder of fluorescence at the right edge of the total cell peak. This is also the profile observed with *C. suis*- infected cells (not shown). Cells infected with *C. caviae* (E and F) sort as a discrete peak to the right of the uninfected labeled cells. Labeling of *C. caviae*-infected cells was examined both 3 h (E) and 8 h (F) post-labeling. Note that in panel F a population of uninfected, unlabeled cells (under the *) was mixed with the *C. caviae*-infected, labeled, cells just prior to sorting.

4.4.2. FACS measurement of fluorescence activity of Chlamydia-infected cells

Flow cytometry was explored as a means for visualizing and quantifying the accumulation of C_6-NBD-cer within infected cells. McCoy cell cultures were infected with *C. caviae*, *C. suis* and *C. trachomatis* at an MOI of 0.1. Twenty hours post infection, cells were treated with C_6-NBD-cer, trypsinized, and analyzed via

FACS. Uninfected and infected cells not treated with C_6-NBD-cer exhibited a basal level of fluorescence (Fig. 2 A, B), while treatment of uninfected cells resulted in a shift of fluorescence within the population (Fig. 2 C).

C_6-NBD-cer-treatment of Chlamydia- infected cells (Fig. 2 D-F) led to a FACS profile that was different from the uninfected, C_6-NBD-cer treated cells (Fig. 2 C). Flow cytometry of infected cells resulted in either a shoulder of more highly fluorescent cells (Fig. 2 D) or a unique peak of fluorescence that was well separated from the peak for the uninfected cells (Fig. 2 E). These results consistently demonstrated that *C. caviae*- infected cells (Fig. 2 E) acquire a higher fluorescence profile than do *C. trachomatis* infected cells (Fig 2 D). FACS analysis of *C. suis*-infected cells resulted in *C. trachomatis*-like fluorescence profiles (data not shown). Temporal analysis of the retention of C_6- NBD-cer demonstrated that a *C. caviae* -specific fluorescence peak was identifiable in labeled infected cells a least eight hours post-labeling (Fig. 2 F).

Two procedures were used to quantitatively assess the survival of cells. First, a monolayer of infected cells were labeled with C_6- NBD-cer and then fluorescence-positive cells were collected into a tube on the flow cytometer. These cells were then immediately resorted, and the total numbers of fluorescence-positive cells in the second sort were compared with those in the first sort. In these experiments, approximately 25-40% of positive cells from the primary sort were accounted for in the secondary sort. A second approach involved the quantification of individual sorted cells that resulted in culturable Chlamydia-infected cells. For this, infected monolayers were trypsynized and sorted into wells of a tissue culture tray at a rate of 1, 10, or 100 positive cells per well. Cells were then fixed with methanol and subjected to fluorescent antibody analysis to quantify the number of infected cells in each well. In these experiments, populations sorted at a rate of 10 or 100 fluorescence-positive events per well resulted in between 25 and 60% survival (Fig. 3). In these same experiments, sorting of cells at 1 event per well resulted in a single positive cell in approximately 50% of the culture wells. The data from these experiments demonstrated that there is significant cell death or false positive sorting

during the FACS procedure, but that at least 25% of cells identified as positive by FACS led to Chlamydia-positive cells in subsequent culture.

4.4.3. Microbiological cloning of single chlamydial strains using FACS

We next used the developed technique to produce clonal populations of three different chlamydial strains. For these experiments, infected cells were labeled with C_6- NBD-cer and then sorted at a rate of one positive event per well. Inoculated wells were then incubated for an additional 16 h to allow the inclusions to increase in size, prior to fixation and labeling with anti-HSP60 antibody. These experiments were conducted independently with *C. caviae*, *C. trachomatis* D UW/3 and *C. trachomatis* J(s)893, and the results of the cytometry and the quantification of infected wells are presented in Fig. 4. While there were many wells that contained no Chlamydia-infected cells in each case culture wells containing single foci of infection were identified. Wells with two or more foci were also identified in these experiments. These data demonstrated that the flow cytometer was useful for generating clonal populations of single chlamydiae following sorting of infected cells.

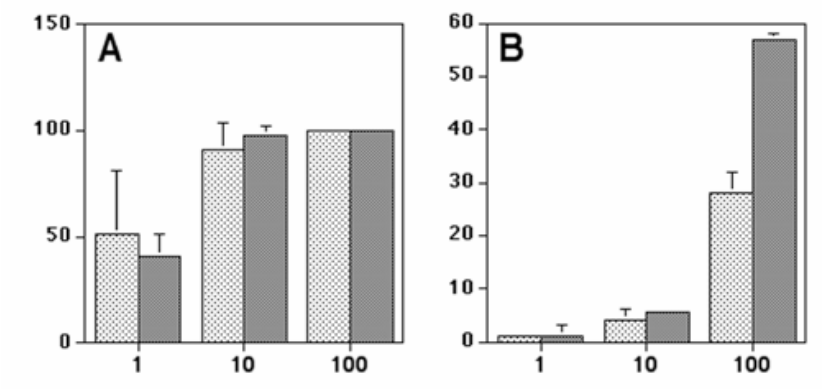

Fig. 4.3. Survival rates for *C. caviae* (black bars) and *C. trachomatis* (white bars) sorted to wells at different rates
Cells infected at an MOI of 0.1 were treated with C_6-NBD-cer and prepared for flow cytometry. Cells were then sorted to individual wells at 1, 10, or 100 infected cells per well. The horizontal axis for each panel indicates the number of infected cells, as measured via flow cytometry, that were placed in each well of a 24 well tray. Panel A shows the percentage of infected wells per tray at each of the three tested sort rates. Panel B shows the average number of infected cells per positive well at the different numbers of sorted cells per well. Experiments were conducted at least three times and the error bars indicate the standard error of each value.

4.4.4. Efficiency of sorting mixed chlamydial populations

Because it is relatively common to have patients with mixed chlamydial infections, we assessed the ability of FACS-based cloning to isolate individual chlamydial strains from mixed populations. For these experiments, monolayers infected with *C. caviae* GPIC, *C. trachomatis* DUW/3, J(s)893 or *C. suis* S45 were combined in pairs and then sorted at a rate of a single C_6-NBD-cer positive cell per well (Fig. 5). All tested strains are antigenically distinct and thus were differentiated via fluorescence microscopy.

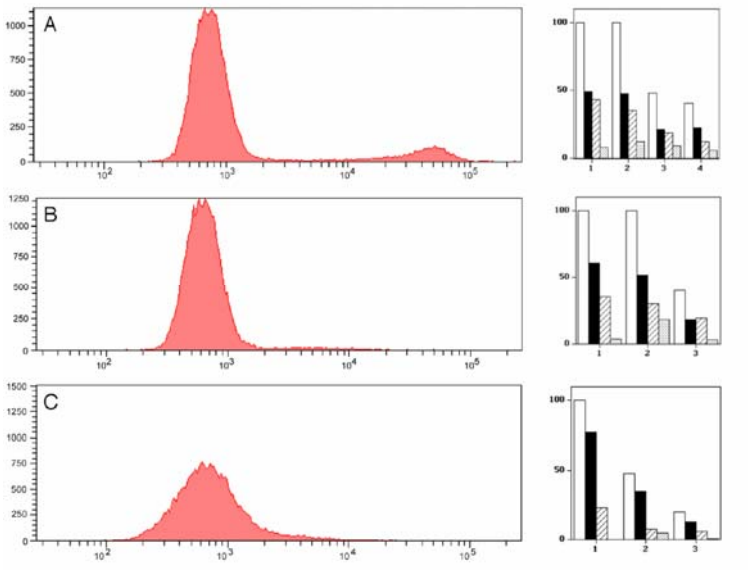

Fig. 4. 4. FACS-based cloning of single chlamydial strains
Sorting of McCoy cells infected with *C. caviae* (A), *C. trachomatis* D UW/3 (B), and *C. trachomatis* J(s)893 (C), all at MOI 0.1. For this experiment, single fluorescent cells were sorted to individual wells. Data were analyzed by fluorescence microscopy at 12 h post sorting, using anti-HSP60 to label chlamydiae within methanol-fixed, infected cells. Plots at the left side of all panels represent fluorescence intensity of infected and uninfected cells in the sorted suspension. Cell numbers are shown on the vertical axes and fluorescence intensity is represented on the horizontal axes. The horizontal line in each FACS profile represents the window of fluorescence that was collected for sorting to culture wells. To the right of each FACS panel is a quantitative analysis of the number of wells with no infected cells (black bars), a single infected cell (thin-hatched bars), and cells with more than one infected cell (thick-hatched bars). The open bars indicate the total number of wells examined for each experiment. The number of independent experiments are indicated on the horizontal axis for each bar graph.

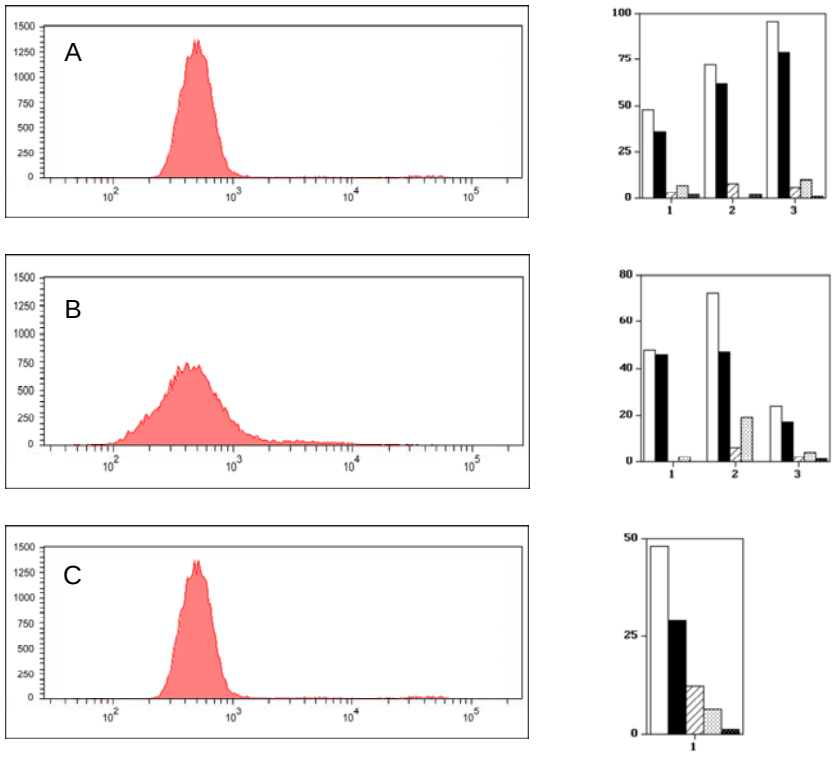

Fig. 4. 5. Separation of individual chlamydial isolates from a cell population containing a mixture of two species
Suspensions of cells infected with different chlamydial species (MOI = 0.1) were sorted to yield 1 infected cell per tissue culture well. The efficiency of sorting was then tested by fluorescence microscopy of methanol-fixed cells with antibodies specific for the particular chlamydial species or strain that was tested. The graph to the right of each FACS profile shows the quantification of the efficiency of the sorting process. The numbers of independent experiments are indicated on the horizontal axis for each plot. The total number of wells analyzed in each experiment is indicated with the white bars. The black bars indicate the number of wells with no infected cell. The thin-hatched and thick-hatched bars indicate wells with individual clones of one strain, with the first listed strain shown as the thin-hatched bar and the second listed strain the thick-hatched bar. The bars with vertical hatch marks (the last bar for each experiment) indicate wells with mixtures of the two chlamydiae.
The mixtures used for each experiment were: panel A) *C. trachomatis* D UW/3 and *C. caviae* ; panel B) *C. trachomatis* D UW/3 and *C. trachomatis* J(s)893; and panel C) *C. suis* and *C. caviae*.

On occasion, the sorting of mixed populations did not result in the acquisition of clones of each strain (Fig. 5B, experiment 1). There were also common examples of wells containing both strains used in the sorting process. However, pure cultures

of each chlamydial strain were recovered from each combination of tested organisms. This was true for populations of cells infected with two species (Fig. 5 A, C) or with different serovars of a single species (Fig. 5 B). Therefore, the data demonstrated that FACS-based sorting of infected cells can be used to separate mixed populations of Chlamydia into clonal populations of single strains.

4.4.5. Isolation of live, clonal populations of *Chlamydia spp*

The next goal was to demonstrate that this approach can be used for acquiring viable chlamydiae from a chlamydial isolate that was sorted using FACS. For this, suspensions of McCoy cells infected with different chlamydia were segregated into individual cell culture wells that contained an existing monolayer of McCoy cells. After segregation, the cell culture plates containing the sorted cells were incubated for an additional 48 h. Chlamydial development was allowed to proceed and the infected cells lysed at the end of the cycle, leading to additional cycles of infection within the monolayer of cells. As a result, foci of infected cells were formed. Live monolayers containing these foci were identified via low magnification fluorescence microscopy using C_6-NBD-cer labeling, and wells with single foci were cultured and expanded (Fig. 1 F). These isolates were then collected by water-mediated lysis of the cell monolayer and stored at –80°C. Aliquots of frozen samples were thawed and inoculated onto cells, and the success of this was measured via fluorescence microscopy. Each of 24 clonally isolated strains from the sorting of mixed populations (Fig. 4) were successfully propagated and recovered using this technique. The data from these experiments demonstrated that FACS-based sorting is an effective method for isolating and recovering chlamydial clones from mixed chlamydial populations.

4.5. Discussion

The present work was undertaken to address the challenge of rapidly acquiring clonal chlamydial strains from clinical isolates. The experiments describe the development of a system for cloning chlamydial strains using the flow cytometer

(summarized in Fig. 6). The assay is based on the fact that all tested chlamydiae become fluorescent after treatment with C_6-NBD-cer, including primary isolates that were cultured directly from patient swabs. A single cloning cycle using the described system can be completed within 24 h of the initial culture. The procedure is rapid, straightforward, reproducible, and requires minimal chlamydial expertise to produce a clonal population. A drawback to this techinque is that specific tissue culture phenotypes visible in cultured foci cannot be selectively isolated, as can be conducted with other successful clonal isolation procedures (3, 9). It is likely that both approaches – focus formation and FACS sorting – will be useful to chlamydiologists wishing to create microbiological clones from laboratory and clinical isolates.

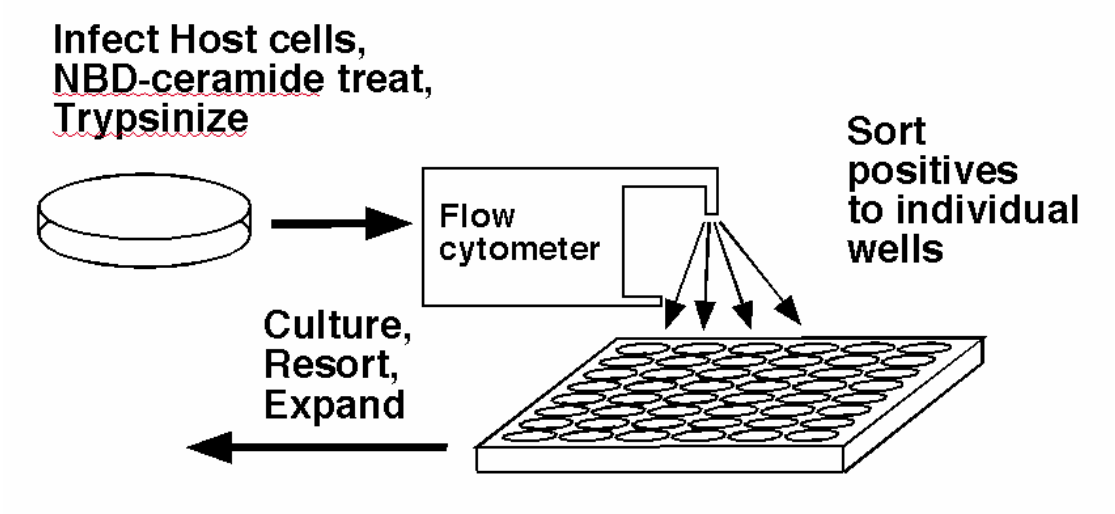

Fig. 4.6. Schematic representation of the clonal isolation process
The optimized sorting procedure is outlined in this cartoon. First, a monolayer of host cells is infected with chlamydia at an MOI of 0.1 and treated with C_6-NBD-cer 16-18 h post infection. At 2-3 h post-treatment, infected cells are trypsinized and collected to a tube for sorting. FACS-based cloning is conducted using a nozzle with a 130 μm aperture, at a sorting pressure of 10 psi. Individual fluorescence positive events are sorted into individual wells of a tissue culture tray. Each well contains a monolayer of host cells to support further growth of clones after lysis of the sorted, infected, cell. The sorting can then be evaluated using C_6-NBD-cer and positives can be expanded or possibly resorted to conduct a second round of cloning.

While all tested *Chlamydia spp.* acquire C_6-NBD-cer following treatment of an infected monolayer, the cytometry data suggest that different chlamydiae acquire or retain the label at different rates. Our data demonstrated that *C. caviae*- infected

cells manifest a brighter fluorescence profile relative to cells infected with other *Chlamydia spp.* (Fig. 2). However, all tested species presented an identifiable peak or shoulder of fluorescence that was successfully targeted in the sorting. We also demonstrated that labeled Chlamydia-infected cells can be sorted between 3 and 8 h post labeling, allowing flexibility in the timing for the cytometry. For our purposes, sorting cells 3 h post-labeling was optimal for generating clonal populations.

The major technical challenges of this procedure involved establishing the conditions for sorting. Initial experiments using high flow rates and pressures were universally unsuccessful, likely because infected cells were all lysed in the procedure. Even under optimal conditions, our data demonstrated that approximately 50-80% of sorted cells do not survive the process (Figs. 3 and 4). Careful attention was also given to sterile technique, as flow cytometers are not routinely placed within a biosafety cabinet. However, although problems with contamination were anticipated prior to these experiments, we observed no examples of contamination in any wells used in the FACS-based sorting. It was also important to sort during the period that the intracellular chlamydiae were predominately RB, as labeling at later developmental stages led to fluorescence signals that were less clear and the cells were more fragile (not shown). Also, as chlamydial RB are not infectious, working with cells carrying only RB reduced the biosafety concerns.

We also explored whether flow cytometry can be used to isolate individual chlamydial strains from mixed populations, which are relatively common in clinical isolations (16, 17). The data in Fig. 1 demonstrated that primary chlamydial cultures are capable of accumulating C_6-NBD-cer, and, therefore, sorting can be used during primary isolation of strains. We used three different combinations of chlamydiae to examine the utility of this system to separate mixed chlamydial populations. While there were differences in the results of individual sorting experiments, we were successful in isolating each species or strain from one another using FACS. This confirmed that FACS may be used for the rapid microbiological cloning of chlamydiae directly from patients.

It is often not considered a standard procedure to work with microbiological clones in chlamydial research. The technical limitations of generating clones are

significant barriers to isolating clones, a procedure that is routine in the study of most other bacteria. It is anticipated that the use of FACS sorting will complement existing cloning procedures and allow researchers to work with clonal isolates in an increasing number of experimental protocols.

4.6. Acknowledgements

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Chapter 5

Conclusion

5.1. Conclusion

The major objective of this work was to develop methods for studying the biology of the intracellular bacterial parasites, chlamydiae. As it was mentioned above, the major problem of the chlamydial research is that at the present time there is no established system for transformation and mutagenesis of the chlamydial genome. Various alternative approaches that are currently used in order to overcome this significant obstacle (discussed in Chapter 1) do not apply to all groups of chlamydial genes and do not solve the problem completely.

This work describes use of the method of transient expression of chlamydial proteins as one of such approaches. Plasmid- or virus-driven expression was used for studying functions of virulence factors in other pathogens (7, 11, 12). Inc proteins are unique to chlamydiae (5, 8, 9) and are exposed to the outer inclusion membrane surface. This distinctive localization of the proteins suggests that they may play an important role in host-pathogen interactions. Transient expression of Inc proteins in uninfected and chlamydia infected host cells revealed potential functions of the proteins and contributed to our understanding of their role in chlamydial developmental cycle and host-pathogen interactions in particular. Here it was demonstrated that transiently expressed Inc proteins might block chlamydial infection (IncA *C. caviae*, Chapter 2), alter host cell phenotype (IncC *C. caviae* and *C. trachomatis* Chapter 2) and interrupt the host cell cycle (CT223 *C. trachomatis*, Chapter 3).

We determined that cells transfected with *C. caviae incA* were unable to support a productive infection by *C. caviae*. Production of *C. caviae* IncA due to a plasmid expression blocked the ability of *C. caviae* to form normal inclusions within the cell. Instead, in very few cases aberrant inclusions were observed in cells expressing *C. caviae* IncA from plasmid expression system. Expression *C. caviae* IncA was also

effective in blockage of *C. trachomatis* infection, but expression of *C. trachomatis* IncA did not block infection *C. caviae* or *C. trachomatis*. The observed developmental block was specific to *C. caviae incA*; *C. trachomatis incA* had no affect in this system.

Deletion mutagenesis confirmed that changes as small as a single amino acid that disrupts a phosphorylation of IncA eliminated the block induced by expression of IncA *C. caviae.*

The mechanism of this block is not known, but we proposed the hypothesize that IncA encoded by the transfected plasmid serves as a competitive inhibitor for some host cell molecule critical for *C. caviae* development.

Another function of some Inc proteins, that was found and described using the method of transient expression, is the ability of *C. trachomatis* Inc proteins CT223, CT224 and CT225 to block the cell cycle of the host cells. We demonstrated that different types of host cells, transiently expressing CT223, CT224 and CT225, became multinuclear. In this work we confirmed that changes in the host cells phenotype is the result of the disruption of cytokinesis. Cells expressing CT223 or CT223 COOH had several centrosomes. It was known before that infection with *C. trachomatis* blocks cytokinesis in host cells (2). However, the effecting molecules were not found and our work gave the first description of chlamydial proteins that can be involved in blocking cytokinesis inside of the host cells.

The development of the genetic system for transforming chlamydiae needs a fast, simple and highly productive technique for obtaining and screening clones of chlamydial isolates. One of the important aspects of chlamydial transformation is the screening and separation of the transformants. All existing methods of clonal isolation of chlamydiae are time-consuming and not very efficient (6, 10). Here we propose a novel and highly productive technique for isolation of microbiological clones of various chlamydial species based on the ability of the chlamydial inclusion to uptake and store fluorescent dye C_6-NBD-cer (3, 4). It was demonstrated that using FACS can facilitate in obtaining clonal isolates of chlamydiae (Chapter 4). This method provides researchers with an instrument to separate different chlamydial strains or species from a suspension of host cells into the individual cell culture trays. Sorting

with FACS also can be used for obtaining chlamydial clones from samples taken directly from patients. This will provide researchers with chlamydial clones that have not been cultured for a long time outside of the host organism. This technique is not only important in studies involving serological typing of chlamydiae but also in rapidly growing sector of genome sequencing research.

The strategy of future research has two aspects. The first goal would be to find partners for Inc proteins among the host and chlamydial proteins. To fulfill this goal yeast two-hybrid analysis (9), and various methods of immunoprecipitation (13, 14) with subsequent mass spectrometry analysis have to be applied. The second goal is to use the FACS based clonal isolation technique in experiments to transform the chlamydial genome. The studies of how to transform and mutate the chlamydial genome are undergoing in several different directions, two of which seem to be the most promising. The first one involves use of the chlamydial transposable element (1). The second approach involves using chlamydial bacteriaphage as a delivery system (personal communications). Combination of one of these approaches and the technique of clonal isolation of chlamydial transformants based on FACS will allow us to solve the major problem in chlamydial research: mutagenesis of the chlamydial genome.

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