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Genome and proteome analysis of *Chlamydia**

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It has been difficult to study the molecular biology of the obligate intracellular bacterium *Chlamydia* due to lack of genetic transformation systems. Therefore, genome sequencing has greatly expanded the information concerning the biology of these pathogens. Comparing the genomes of seven sequenced *Chlamydia* genomes has provided information of the common gene content and gene variation. In addition, the genome sequences have enabled global investigation of both transcript and protein content during the developmental cycle of chlamydiae. During this cycle *Chlamydia* alternates between an infectious extracellular form and an intracellular dividing form surrounded by a phagosome membrane termed the chlamydial inclusion. Proteins secreted from the chlamydial inclusion into the host cell may interact with host cell proteins and modify the host cell's response to infection. However, identification of such proteins has been difficult because the host cell cytoplasm of *Chlamydia* infected cells cannot be purified. This problem has been circumvented by comparative proteomics.

1.1
Introduction

Chlamydia is an obligate intracellular bacterium comprising a number of important animal and human pathogens causing infections with serious sequelae. *Chlamydia trachomatis* is a cause of ocular and genital infections. *Chlamydophila pneumoniae* (previously *Chlamydia pneumoniae*) causes respiratory diseases and has been associated with asthma and atherosclerosis. Sequelae are primarily due to an inflammatory response, which may be sustained by bacteria persisting in the infected organism due to a special intracellular nonreplicative state [1] but delayed-type hypersensitivity may also be involved.

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Molecular biological studies of *Chlamydia* have been hampered by the lack of genetic transformation systems. Therefore, sequencing of the genomes of several *Chlamydia* species has been especially important for chlamydial research. The *C. trachomatis* serovar D genome was published in 1998 [2] and in 1999 the first *C. pneumoniae* genome followed [3]. Today *Chlamydia* is one of the most extensively sequenced microorganisms with seven published genomes including four from different isolates of *C. pneumoniae* (http://www.ncbi.nlm.nih.gov:80/PMGifs/ Genomes/org.html). Besides direct analysis of genome sequences (genomics), global investigation of transcripts (transcriptomics) and protein content (proteomics) are developed based on the genome sequences.

1.1.1
Chlamydia biology

Traditionally *Chlamydia* was the only genus in the family of *Chlamydiacae* which was the only family in the order of *Chlamydiales*. Since the introduction of *C. pneumoniae* in 1989 [4] there were four species, distinguished mainly by serology: *C. pneumoniae*, *C. trachomatis*, *C. pecorum* and *C. psittaci*. In 1999 a new taxonomy was suggested [5], introducing more genera and species based on phylogenetic relationships with requirement of $>$ 95% 16S rRNA identity within a genus. The suggested taxonomy placed *C. trachomatis* in the genus *Chlamydia* and divided the *C. trachomatis* into three species. The remaining *Chlamydia* species were placed in the new genus *Chlamydophila,* and *C. psittaci* was divided into a number of different species. Similar developmental biology, similar genome size and genome organization of *C. trachomatis* (1.0 Mb) and *C. pneumoniae* (1.2 Mb) [3], representing *Chlamydia* and *Chlamydophila,* respectively, indicate basic similarities but differences are also found [6]. In the present review the new taxonomy will be followed with respect to species names, but *Chlamydia* will be used as a unifying term describing both of the suggested genera *Chlamydophila* and *Chlamydia*.

1.1.1.1 Diseases

The main human pathogenic chlamydiae are *C. trachomatis* and *C. pneumoniae*, but also bird pathogenic *C. psittaci* can cause severe pneumonia, psittacosis, if transferred to humans [7]. *C. trachomatis* is divided into three groups of serovars: (i) serovars A–C are endemic in developing countries and the cause of trachoma, which may lead to blindness by scarring of the cornea [7]; (ii) serovars D–K are sexually transmitted and cause urethritis, cervisitis and salpingitis. It is the most widespread sexually transmitted bacterial disease and infections are often asymptomatic. The infection may cause sterility and increased risk for ectopic pregnancy by scarring of the fallopian tubes if it spreads from the cervix [7]; (iii) serovars L1–L3 are also sexually transmitted but cause lymphogranuloma venereum (LGV). LGV is a more severe infection as it readily spreads to the lymphatic system and becomes systemic [7]. Serovars A–K are known as the trachoma biovar and L1–3 as the LGV biovar.

C. pneumoniae is a respiratory pathogen that causes acute and chronic respiratory diseases. Most infections are asymptomatic, but about 30% cause more severe pneumonia, bronchitis or other upper airway illness [8]. About 10% of the cases of community acquired pneumonia in adults and about 5% of the cases of bronchitis and sinusitis are caused by *C. pneumoniae* [9]. Persistent infections have been described [10] and there are indications that treatment may not eliminate the organism [11]. *C. pneumoniae* has been associated with chronic lung diseases [8] and as a possible risk factor for the development of atherosclerosis [12]. *C. pneumoniae* has been detected in atherosclerotic lesions [13] and studies have shown that atheromatous plaques are commonly infected with *C. pneumoniae*. Animal studies suggest that *C. pneumoniae* can accelerate atherosclerosis-like disease [14, 15]. However, other studies fail to detect *C. pneumoniae* in plaques and many studies find no significant association by serology [16, 17]. At present it is not clear whether there is an increased risk of coronary artery disease due to *C. pneumoniae* infection and if there is, the increase may be small.

1.1.1.2 **The developmental cycle**

Chlamydia is a Gram-negative, obligate intracellular bacterium, characterized by a biphasic developmental cycle. The developmental cycle (Fig. 1) in which the bacteria alternate between an infectious, extracellular form, the elementary body (EB) and a noninfectious intracellular replicating form, the reticulate body (RB) is unique for chlamydiae [18–20]. EBs are small rigid bodies of about 300 nm in diameter that are traditionally described as being metabolically inactive with their DNA packed by histone-like proteins [21, 22]. They are adapted for extracellular survival with a heavily disulfide cross-linked outer membrane, that provides osmotic stability. RBs are about 1 μm in diameter with an outer membrane that is permeable for transport of host cell nutrients and the DNA is unpacked as in other bacteria.

Infectious EBs attach to a susceptible host cell by which they are phagocytosed. The exact mechanism is not known but the uptake is thought to be induced by the bacteria. Inside the phagosome, named the inclusion, the EBs develop into RBs, which divide by binary fission. This includes unpacking of the DNA and reduction of the disulfide bridges of the outer membrane [23], but it is not known what triggers these events. After multiple divisions, the RBs begin conversion into EBs, including packing of the DNA and synthesis of late outer membraneproteins that are disulfide bridged. Ultimately, a new generation of infectious EBs is released upon disruption of the host cell. The bacteria stay inside the inclusion throughout the intracellular stage, which lasts for 72–96 h for *C. pneumoniae* grown in cell culture. The inclusion membrane grows by the acquisition of lipids derived from the host cell [24–26]. It is modified by the insertion of chlamydial proteins, the so-called inclusion membrane proteins (incs), and prevented from fusion with lysosomes [27, 28].

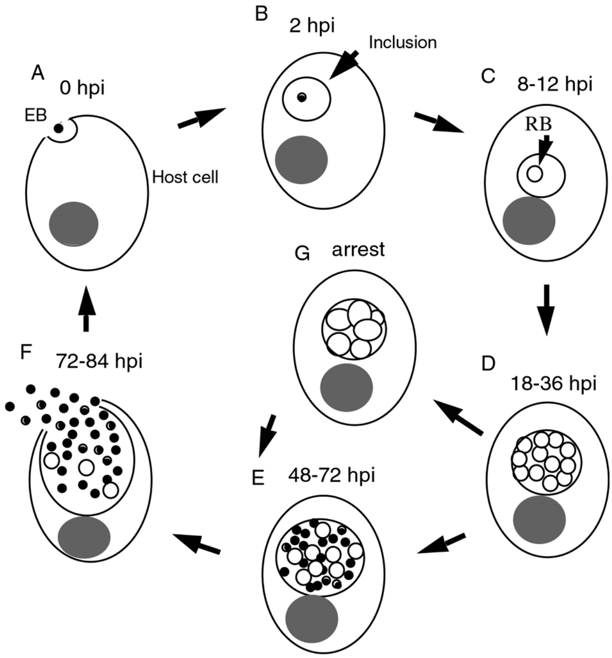

Fig. 1 The developmental cycle of *Chlamydia*. Hours post infection (hpi) are listed for *C. pneumoniae* in cell culture. A, the infectious EB adheres to a host cell and is taken up by endocytosis. B, *Chlamydia* modifies the phagosome, the chlamydial inclusion, to escape the endocytic pathway. C, the EB develops into the metabolically active RB. D, the RBs divide by binary fission and the inclusion grows by incorporation of host cell derived lipids. E, after multiple divisions, the RBs reorganize into EBs. F, ultimately, a new generation of infectious EBs is released by lysis of the host cell. G, low nutrient availability, IFN-γ mediated tryptophan starvation or other stressful conditions can trigger a persistent state with abnormal nondividing RBs. These RBs can be reactivated to enter the developmental cycle when the conditions are again suited for growth. Redrawn from [8].

The developmental cycle of *C. pneumoniae* can be arrested by interferon-gamma (IFN-γ)-induced tryptophan catabolism of the host cell [29]. Tryptophan starvation leads to a nonproductive infection in which enlarged aberrant RBs evolve. These abnormal RBs do not divide and do not mature into EBs, but the developmental cycle can be reactivated [30, 31]. Also *C. trachomatis* can enter a persistent state [32] and in addition to cytokines, limited nutrient availability [33] and treatment with antibiotics that fail to eradicate the infection have been shown to trigger this state [34, 35].

1.2
Chlamydia genomes

1.2.1
Sequenced *Chlamydia* genomes

The first *Chlamydia* genome sequences of *C. trachomatis* [2] and *C. pneumoniae* [3] are from the *Chlamydia* Genome Project (CGP) (http://chlamydia-www.berkeley.edu:4231/). The sequenced genomes provide insight into genome organization and metabolic pathways of *Chlamydia* and form a basis for further research in gene regulation and protein expression [36]. Genome sequences of *C. muridarum* (previously *C. trachomatis* MoPn) [37], three other isolates of *C. pneumoniae* [37–39] and most recently that of *C. caviae* (previously *C. psittaci* GPIC) [40] have been published. An overview of the sequenced genomes is given in Tab. 1 where the number of predicted protein encoding open reading frames (ORFs) is the number given in the respective references. The number of ORFs is dependent on what sequence length is considered minimum for an expected protein and the cut-off varies slightly between sequencing projects.

C. trachomatis serovar D and *C. muridarum* contain a plasmid, and in *C. caviae* and *C. pneumoniae* AR39 a bacteriophage was found. The genomes of *C. trachomatis* D and *C. muridarum* (human and mouse genital pathogens, respectively), are very similar with an average of about 10% difference between orthologous genes [37]. Most differences between these genomes were found in the replication termination region (RTR) [40] including those in *C. trachomatis* D genes involved in tryptophan synthesis, which are missing in *C. muridarum*.

The *C. pneumoniae* genomes are more than 99.9% identical and the few differences are mainly found in *pmp* [37] and *ppp* genes [41, 42]. A double-stranded circular DNA, the replicative form of a bacteriophage was found upon sequencing the *C. pneumoniae* AR-39 genome [37]. The phage of *C. pneumoniae* AR-39 was suggested as contributing to pathogenicity [43], and a similar phage was identified in *C. abortus* [6].

Tab. 1 Genome size and number of ORFs

Genome	Reference	Base pairs	ORFs	Plasmid/Phage
C. trachomatis D	[2]	1,042,519	894	7493 bp plasmid
C. pneumoniae, CWL029	[3]	1,230,230	1073	–
C. pneumoniae, AR39	[37]	1,229,853	1052	4524 bp phage
C. pneumoniae, J138	[38]	1,226,565	1072	–
C. muridarum	[37]	1,069,412	924	7501 bp plasmid
C. caviae (GPIC)	[40]	1,173,390	1009	7966 bp phage

C. pneumoniae TW-183 has also been sequenced [39] but is not contained in this table as no paper has yet been published on the results

1.2.2
Chlamydial genes

The environment of *Chlamydia* can be considered hostile, since the host cell will attempt to eradicate the bacteria, or friendly, since the bacteria have access to nutrients form the host cell. Analyzing the genome sequences of *Chlamydia* by comparing metabolic pathways and energy systems to those of free-living bacteria reveal many consequences of the availability of nutrients. However, the defense systems implicated by the intracellular nature do not appear as readily from the genome sequences since these may be unique for *Chlamydia*.

No genes are found that encode proteins involved in *de novo* purine and pyrimidine synthesis and the ability to synthesize amino acids is greatly reduced. Correspondingly, a large number of genes encoding different transport proteins have been identified, including many ABC transporters which are primarily involved in transport of smaller peptides and amino acids [2]. Also in good agreement with the intracellular and thus isolated nature of *Chlamydia*, no genes involved in DNA uptake were identified and no insertion sequences were found [2].

Chlamydiae have traditionally been described as energy parasites obtaining ATP from their host cells [19, 44], and the genomes of *C. trachomatis* and *C. pneumoniae* confirmed the presence of two genes *CT065/Cpn0351* and *CT495/Cpn0614* (CT*XXX* and Cpn*XXXX* refer to *C. trachomatis* and *C. pneumoniae* gene numbers, introduced by the CGP [2, 3]), homologous to genes encoding ATP transporting proteins from *Rickettsia prowazekii* [45]. The orthologs from *C. trachomatis* L2 were cloned and used to express functional nucleoside phosphate transporters (npt) in *Escherichia coli*, one (CT065) exchanging ADP for ATP, the other (CT495) transporting all four ribonucleoside triphosphats [46]. Surprisingly, also genes encoding a wide range of ATPases as well as phosphoglycerate kinase, pyruvate kinase, and succinate thiokinase were identified, suggesting the capability of *Chlamydia* to produce ATP itself [2]. This ability may be important in the early and late stages of the developmental cycle where *Chlamydia* supposedly cannot obtain ATP form the host cell [44]. Genes encoding the proteins of an intact glycolytic pathway (although it is questionable whether an enzyme with fructose-1,6-diphosphate aldolase activity is present or this is circumvented), a partial TCA cycle, a complete glycogen synthesis and degradation system, and genes involved in aerobic respiration were also found [2]. Furthermore, proteins encoded by many of these genes were shown to be present in EBs [47] and pyruvate kinase, phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase and glucose-6-phosphate dehydrogenase were shown to complement *E. coli* mutants when expressed recombinantly [48]. During the intracellular stage, *Chlamydia* may store glycogen that is used to fuel the chlamydiae in the beginning and the end of the developmental cycle together with stored pools of ATP [48].

Four groups of chlamydial proteins have been indicated as especially interesting and important results of the genome project [49]. These groups were (i) peptidoglycan synthesis proteins; (ii) type III secretion proteins; (iii) inclusion membrane proteins (incs); and (iv) polymorphic membrane proteins (pmps).

The presence of a nearly full set of genes involved in peptidoglycan synthesis was unexpected since a peptidoglycan layer is not detected in EBs. However, *Chlamydia* is sensitive to beta-lactam antibiotics and peptidoglycan has been suggested to play a role in the division of RBs [50] supported by the finding of three amidases with probable peptidoglycan degradating activity.

The finding of type III secretion system genes was expected as such genes had earlier been found in *C. caviae* (*C. psittaci* GPIC) [51]. The type III secretion system is known from other Gram-negative bacteria to facilitate the transport of molecules from the bacterial cytosol into a future host cell by penetration of the host cell membrane with a surface protrusion that is thought to function as a channel. Surface projections of both EBs and RBs observed in electron microscopy [52, 53] thought to be involved in nutrient uptake, were speculated to be such type III needles when type III genes in *Chlamydia* were found [54, 55].

Inclusion membrane proteins are chlamydial proteins that are inserted into the inclusion membrane. Such proteins were first identified in *C. caviae* and termed incA, B and C [56, 57]. Homologs of these were found in the genomes of all sequenced chlamydiae but have not been found in any other organism. Several additional incs have since been identified and all of these share a characteristic bilobed hydrophobic region, even though no sequence motif is apparent [58]. Thirty-three genes encoding proteins with this hydrophobicity pattern have been identified in the *C. trachomatis* genome and 93 in the *C. pneumoniae* CWL029 genome [59].

Another group of *Chlamydia* specific proteins found in the genome was the family of polymorphic membrane proteins (pmps). These were initially identified in *C. abortus* (ovine abortion subtype of *C. psittaci*) being immunogenic proteins present in the outer membrane [60]. Nine *pmp* genes were identified in *C. trachomatis*, 17 in *C. caviae* and 21 in *C. pneumoniae*. The pmps are defined by being predicted outer membrane proteins containing repeated sequences of GGAI and FxxN [61] and by protein structure analysis they are predicted to be autotransporters [47, 62]. Incs and pmps are likely to be pivotal for *Chlamydia* biology indicated by the fact that 37.4% of the *Chlamydia* specific coding sequence of *C. pneumoniae* is constituted by *inc* and *pmp* genes (18.9% and 17.5%, respectively) [49].

1.2.3
Genome comparison

Genome sequences are thus available for *C. trachomatis* serovar D, *C. muridarum, C. caviae* and four isolates of *C. pneumoniae* (CWL029, AR39, J138 and TW-183), all of these share the unique developmental cycle but they are diverse in tissue tropism; *C. trachomatis* serovar D infects the genital tract of humans, *C. pneumoniae* infects the human respiratory tract; *C. caviae* the conjuctiva of guinea pigs and *C. muridarum* is a mouse pathogen. Hence, genome comparisons may reveal differences that are important for pathogenicity and tissue specificity.

Comparison of the *C. caviae* genome [40] to those of *C. pneumoniae* and *C. muridarum* showed that only 68/1009 *C. caviae* genes were not found in any of the other *Chlamydia* genomes, but differential expression of genes shared by the different organisms may contribute to pathogenicity differences. Seven hundred and ninety-eight genes were found in all genomes and may be the minimum set of genes required for the basic growth and development of *Chlamydia*. Out of the 798 shared genes, 183 could not be found in any other of 70 published microbial genomes in the TIGR database [40]. Investigation of these genes, which include the *inc* and *pmp* genes, may elucidate functions that are specifically related to the intracellular characteristics of *Chlamydia* and its developmental cycle.

The most prominent *C. caviae* specific genes compared to *C. pneumoniae* are the genes required for tryptophan synthesis found in the RTR. *C. caviae* appears to be able to synthesize tryptophan from anthranilate, which is a very early precursor [40]. *C. trachomatis* possess a more limited set of tryptophan synthesis genes [2] and the genital and LGV serovars can produce tryptophan from the intermediate precursor indole, whereas the ocular serovars A and C have a truncated TrpA and serovar B lacks the *trpA* operon [63] similar to that which is found for *C. pneumoniae* [3]. A tox gene similar to cytotoxic genes from enterobacteria has been found in *C. caviae* and *C. muridarum*, the product of which may be secreted by the type III secretion system in order to inhibit actin polymerization [40]. In addition, a gene with homology to an invasin/intimin family protein was identified but the gene is interrupted by two frame shifts [40]. Specific genes found in *C. pneumoniae* that are absent from *C. caviae* include a uridine kinase, two 3-deoxy-D-manno-octulosonic acid (KDO) transferases, and two genes involved in biotin synthesis. In addition, 168 genes with unknown function are present in *C. pneumoniae* but not in any other *Chlamydia* [40].

Comparing *C. pneumoniae* to *C. trachomatis*, 80% of the predicted protein encoding genes have an ortholog in *C. trachomatis* [3]. From the 214 genes found in *C. pneumoniae* but not in *C. trachomatis*, most have no known function, but those that have include genes for purine and pyrimidine salvage pathways and completion of the biotin synthase pathway. A prominent difference is the expansion of the *pmp* gene family from nine members in *C. trachomatis* to 21 members in *C. pneumoniae* [61]. The *C. trachomatis pmp* genes are located in two clusters *pmpA-C* and *pmpE–H* except for one gene, *pmpD*. Most of the difference between *C. pneumoniae* and *C. trachomatis* is accounted for by expansion of *pmpG* to 13 *pmps* (*pmp1–13*) in *C. pneumoniae* [61]. The amino acid identity between pmp1–13 is 34–55%.

The *C. pneumoniae* genomes elucidated that several *pmp* genes contain frame shifts, and these vary between isolates, as listed in Tab. 2. Furthermore, at least pmp10 was shown to be differentially expressed between chlamydiae within the same cell, and this is likely due to a polyG tract that varies in length [64]. Based on the relatively high variability in the *pmp* gene family, considering the otherwise very conserved sequences between isolates, it has been speculated that the pmps may function in surface variation of *Chlamydia* as seen in other pathogenic bacteria [65].

Another gene family in *C. pneumoniae* that shows remarkable variation is the recently identified Cpn1054 family or *C. pneumoniae* polymorphic protein (ppp) family [42]. *Cpn1054* was initially identified as one of eleven paralogous genes located in four hyper-variable regions in *C. pneumoniae* CWL029, one of which is situated between *pmp1* and *pmp2* [66]. The genes were predicted to encode inc

Tab. 2 Variation in *C. pneumoniae* polymorphic membrane protein (*pmp*) genes

pmp	**CWL029**	**AR39**	**J138**
2			frame shift
3	frame shift	frame shift	frame shift
4	frame shift	frame shift	+1 frame shift
5	frame shift	frame shift	frame shift
6		393 bp del.	393 bp del.
10	frame shift		
12	truncation	truncation	truncation
17	frame shift	frame shift	frame shift

proteins by the presence of the characteristic bilobed hydrophobic motif. Many of the genes contain stop mutations that differ between sequenced strains and as in *pmp10*, a poly-G tract was identified in the 5' end of *cpn1054* [66]. Recently, poly-G tracts present in seven of eleven 1054 family members were analyzed by sequencing of a number of clinical isolates [67]. Five out of seven were found to vary in all investigated isolates, and functional analysis of protein products from this gene family will be interesting.

1.3 Proteome analysis of *Chlamydia*

The genome sequence reveals the coding capacity of an organism and thus what proteins it theoretically can produce. The coding capacity is informative, but does not reveal information about when, where and in what quantities the genes are transcribed and whether the possibly resultant proteins are modified or secreted. The direct investigation of proteins in their post-translationally modified and processed form present in a given biological compartment at a specific time and in a defined environment is the task of proteomics.

Proteomics is used to describe any large-scale investigation of proteins and can be approached in many ways but in principle it involves two steps: separation of the proteins in a sample and subsequent identification of these proteins. The perfect proteome study would provide a quantitative measure of every single protein present in the investigated sample. Unfortunately, such a study is so far not possible. Novel quantitative mass spectrometric techniques come close, but these are still in the development phase. Today 2-D gels as a separation tool coupled to mass spectrometry protein identification provides the most comprehensive way of analyzing complex protein mixtures [68].

1.3.1
Early *Chlamydia* proteome studies

In 1985, 2-DE was used to compare the protein content of outer membrane preparations from *C. trachomatis* serovars L2 and F [69]. Chlamydiae were selectively radiolabeled by [^{35}S]methionine incorporation in the presence of the inhibitor of eukaryotic ribosomes, cycloheximide. EBs were purified and *Chlamydia* outer membrane complex (COMC) was prepared by sarkosyl extraction [70]. The COMC was solubilized in a 2-D buffer based on urea with NP-40 as detergent and mercaptoethanol as reducing agent and subjected to 2-D PAGE where the first dimension was carried out in tube gels in which the pH gradient was established during focusing. Three proteins, major outer membrane protein (MOMP), a 60 kDa protein and a 12 kDa protein were observed for *C. trachomatis* F, whereas the 60 kDa protein was missing for *C. trachomatis* L2. However, by NEPHGE it could be concluded that the 60 kDa protein was also present in *C. trachomatis* L2, but migrating more basic than in serovar F [69].

Improvements in 2-D PAGE, including IPG strips for the first dimension, means that today more proteins can be resolved in 2-D gels of COMC [71]. Lambden *et al*. [72] identified the 60 kDa large cysteine-rich outer membrane protein, OmcB (Omp2), and the 12 kDa small cysteine-rich protein, OmcA (Omp3) to be developmentally regulated and transcribed as a polycistronic mRNA late in the developmental cycle. A model of the COMC architecture has been proposed [73] in which Omp2 is localized in the periplasmic space, disulfide cross-linked to Omp3, which is suggested to be anchored in the outer membrane by its lipid moiety. Comparing COMC from different *C. trachomatis* serovar to *C. pneumoniae* and *C. caviae* [74] showed that Omp2 from *C. trachomatis* L2 was resolved in the gels, but migrated one pH unit more basic than Omp2 of *C. trachomatis* F and two pH units more basic than *C. trachomatis* D. No additional proteins were identified for any of the species even though high molecular bands were observed by 1-D SDS gels for *C. trachomatis* serovar D [74].

1.3.2
C. trachomatis proteome studies

The first proteome study on whole *Chlamydia* aimed at identifying early proteins in *C. trachomatis* L2 by pulse labeling with [^{35}S]methionine at 2–4 h post-infection (hpi), 8–10 hpi, 14–16 hpi and 28–30 hpi [75]. Seven proteins were detected earlier than MOMP, four of which were labeled at 2–4 hpi. Three of these were identified by colocalization with proteins detected by immunoblotting with known antibodies. These were the heat shock proteins DnaK and GroEL and the ribosomal protein S1. The remaining four proteins were not identified. Early transcription of the *groEL* gene has recently been confirmed by transcript analysis [76, 77], but *dnaK* was designated a late gene in [78]. However, the designation “late” was based on lower transcription at earlier points in time than 24 hpi and higher transcription at later points in time and this does not exclude early transcription.

A second global study [79] aimed at providing a basis for the development of a protein database of *C. trachomatis* proteins. This was the first *Chlamydia* study to use IPG strips. Approximately 600 spots were separated in the area from pH 4–9 and 10–120 kDa in silver-stained gels. The very good resolution compared to earlier studies can be ascribed to the use of IPGs but also the substitution of mercaptoethanol with dithioerythritol (DTE), and NP-40 with CHAPS may have contributed to the superior results. Mercaptoethanol will more readily migrate out of the first dimension gel than DTE due to its charge, and removal of reducing agent will cause reoxidation and precipitation of proteins. A combination of immunoblotting with known antibodies and *N*-terminal sequencing was used to identify nine known proteins [79]. Seven sequences were obtained from yet uncharacterized proteins distributed in different areas of a 2-DE map and even though the gels showed very good resolution, the study like all other pregenomic proteome studies, suffered from the lack of identification methods for unknown proteins.

In the pregenomic area, 2-DE was most appropriate in studies where antibodies were available for identification of the proteins. One such study demonstrated the superiority of 2-DE in comparison to 1-DE with respect to the resolution of different isoelectric isoforms [80]. A family of high molecular weight *C. abortus* proteins detected by post-abortion sera from sheep were shown to be identical to immunogenic putative outer membrane proteins (POMPs). As the proteins had similar molecular weight, they could not have been distinguished in 1-D gels.

Western blotting of 2-D gels has also been applied to identify immunogenic proteins in *C. trachomatis* using sera from 17 patients suffering from genital inflammatory disease [81]. Fifty-five immunogenic proteins were detected with frequencies varying from 17 to 1. Eight proteins could be identified by colocalization with previously determined proteins. In addition, *N*-terminal sequences were obtained for nine proteins from which six could be identified in the genome sequence. Omp2, GroEL, MOMP and DnaK were the most frequently recognized proteins. These are known antigens, but also previously unknown antigens were detected such as elongation factor TU and ribosomal proteins.

1.3.3
***C. pneumoniae* proteome studies**

The first comprehensive proteome map of *Chlamydia* in the postgenomic area was that of *C. pneumoniae* [47] (Fig. 2). Like Bini *et al.* [79] this study used IPGs in the first dimension and thiourea was incorporated into the 2-DE buffer to obtain the best possible recovery of hydrophobic proteins. Mass spectrometry was used to identify 263 protein spots representing 167 different genes and all identifications were published on the internet at http://www.gram.au.dk in a searchable form. Data for pH 4–7 (Fig. 2) was also included in the bacterial proteome database at the Max Planck Institute for Infection Biology at http://www.mpiib-berlin.mpg.de/2D-PAGE/. The proteome map can thus serve as a reference for 2-D PAGE studies performed in other laboratories. A good agreement between predicted and observed number of proteins was observed in the acidic region, whereas recovery in

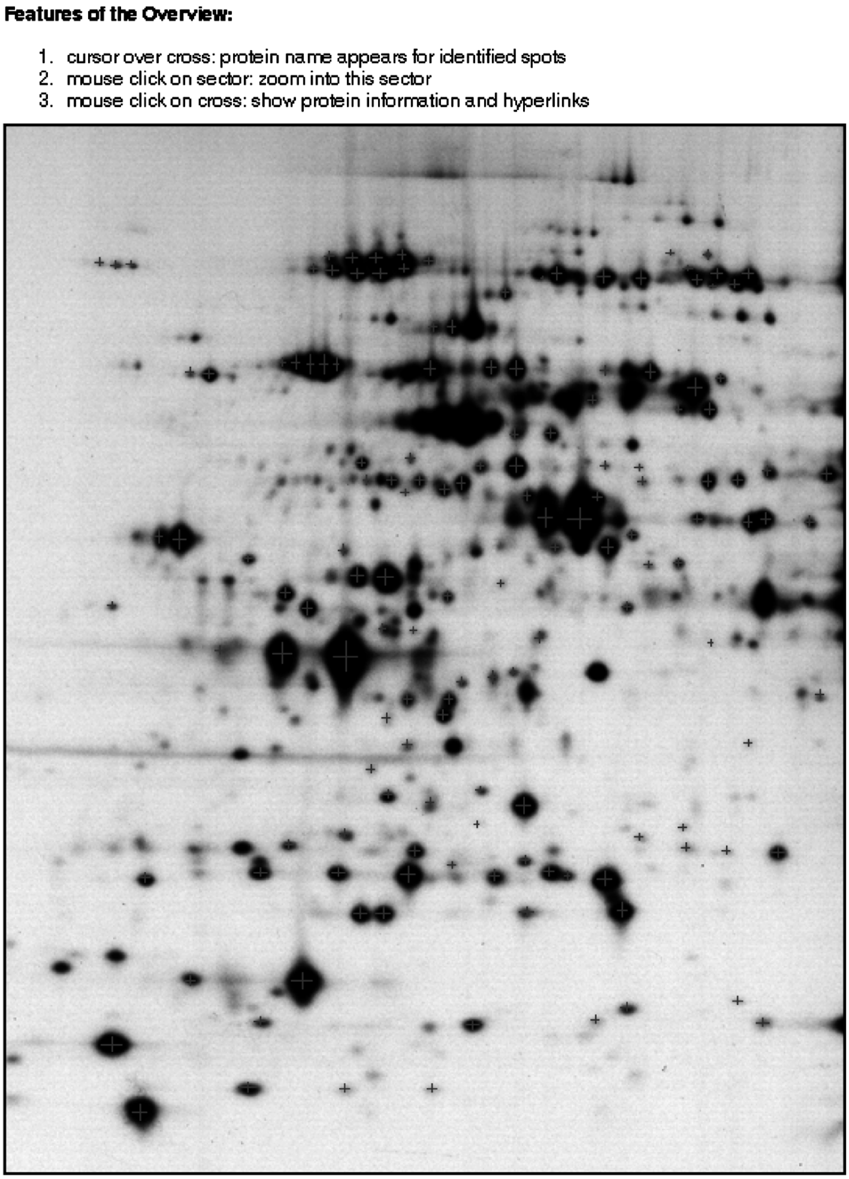

Fig. 2 Screen dump of the clickable IPG4-7 proteome map of *C. pneumoniae* available at www.mpiib-berlin.mpg.de/2D-PAGE/EBP-PAGE/index.html. Crosses represent identified proteins.

the basic region was poor. The use of basic strips (pH 6–11) did not significantly improve the number of resolved protein spots, but gave a better spatial distribution which is important when proteins are to be excised for further analysis.

To cover the highest possible number of radiolabeled EB proteins in one gel, samples were pooled after labeling with [^{35}S]methionine/cysteine at different points in time during cultivation [47]. Therefore proteins synthesized at different stages of the developmental cycle were labeled, but in consequence the actual protein content of EBs was not reflected by the spot volumes. Radiolabeling was chosen rather than staining to avoid the *Chlamydia* purification step in future experiments. Furthermore, as autoradiography is a more sensitive method for visualization than staining, the protein load can be reduced, which gives better resolution in the gels.

The study provided a reference map [47] but in addition, there were important findings: eight pmps were found to be highly abundant; type III secretion proteins were found in EBs for the first time indicating that this secretion system is present in EBs; the presence of a high number of energy-metabolizing proteins and proteins involved in transcription and translation suggested that EBs are "good to go" when they enter a host cell [47]. Furthermore, a high number of 'hypothetical' proteins were present (31/167) and many of these were abundant. One of the most abundant hypothetical proteins was Cpn0808. This was suggested to be loaded in EBs, ready for secretion by type III secretion upon contact with a future host cell based on its genomic location close to *lcrH1*, which is homologous to a gene encoding a type III secretion chaperone in *Yersinia* [47]. This hypothesis has been further supported by the recent finding of Cpn0809 in the cytoplasm of *C. pneumoniae* infected cells [82].

1.3.4
Identification of secreted proteins by comparative proteomics

Secreted *Chlamydia* proteins may carry out important functions in relation to interaction with the host cell. However, the identification of secreted proteins has been hampered by the fragility of the inclusion and RBs, making it impossible to isolate host cell cytoplasm from infected cells. This problem was circumvented by a 2-D gel comparison approach using the difference between 2-D protein profiles of infected cells and purified chlamydiae as a measure of which *Chlamydia* proteins are found outside the bacteria [83]. The method is outlined in Fig. 3. Proteins present in purified bacteria were subtracted from the protein content of whole lysates of infected cells and the differing proteins identified by MS. Chlamydial proteins were distinguished from eukaryotic by radiolabeling in the presence of cycloheximide. The idea of analyzing chlamydial components present in the infected cell but not in the chlamydiae themselves is somehow parallel to the approach leading to the identification of the first incs by comparing proteins reacting with convalescent sera to those reacting with sera obtained by immunization with inactivated bacteria [56].

The comparative proteomics approach resulted in the identification of CT858 of *C. trachomatis* and Cpn1016 of *C. pneumoniae* as secreted proteins [84] (Fig. 4). These proteins were orthologs and known as a *Chlamydia* protease-like activity factor (CPAF) [85]. CPAF was originally identified by its property to down-regulate host cell transcription factors required for MHC class I and II presentation and subsequently confirmed to be secreted [85]. The expression characteristics of CPAF

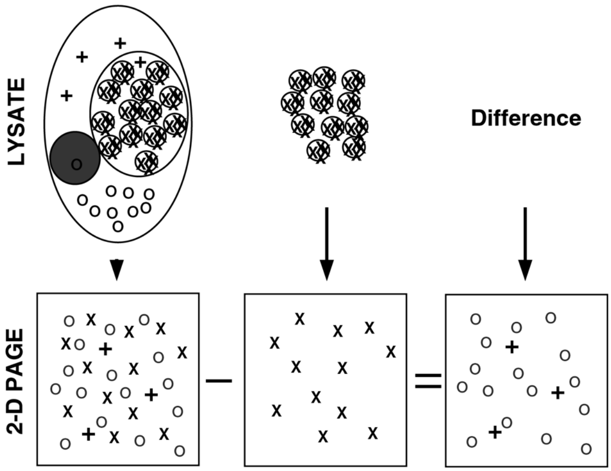

Fig. 3 Schematic drawing of the strategy used in subtractive proteomics. The protein content of whole lysate of infected cells and that of purified bacteria are separated by 2-D PAGE. The infected cells are radioactively labeled and the protein synthesis of the eukaryotic host cell is stopped by cycloheximide. Only chlamydial proteins are labeled. O indicates unlabeled eukaryotic cell proteins; X indicates labeled chlamydial proteins found in EBs; and + indicates labeled, potentially secreted proteins found in the host cell cytoplasm but not in purified bacteria.

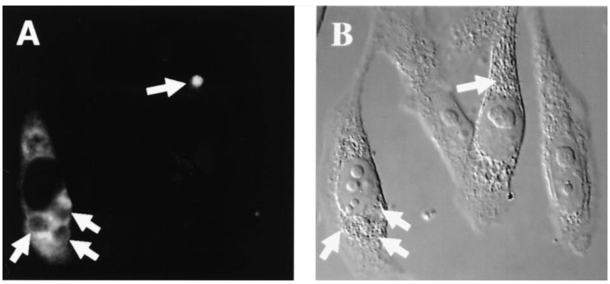

Fig. 4 HEp-2 cells infected with *C. pneumoniae* CWL029 and fixed 54 h postinfection. A, immunofluorescence microscopy with an antibody against the secreted protein Cpn1016 detected by a FITC conjugated secondary antibody. B, Nomarsky image of the same section. Arrows point at *Chlamydia* inclusions. Note that Cpn1016 is detected in the cytoplasm of infected cells but not in the cytoplasm of uninfected cells.

in *C. trachomatis* A, D and L2 as well as in *C. pneumoniae* were further characterized by 2-D PAGE [84]. The study demonstrated how proteome comparison of different biological compartments can lead to the identification of supposedly important molecules, and that genome analysis must be supplemented by further experiments as CPAF was predicted to be an outer membrane protein [86].

1.3.5
Proteome studies of comc

COMC, the sarkosyl-insoluble fraction of EB, is the only separable part of EBs [70]. The proteome analysis of COMC identified several proteins including known outer membrane proteins and the predicted membrane component of the type III secretion apparatus, YscC, indicating that the apparatus is assembled in EBs and that YscC is the membrane component in *Chlamydia* [71]. Other type III secretion proteins identified in the reference map of *C. pneumoniae* [47] were not found in the membrane fraction. Major constituents of the COMC were the pmps, which were characterized by 2-DE with respect to expression in the study by Vandahl *et al.* [87]. Of the 21 pmp genes, 16 were of full length in *C. pneumoniae* CWL029. Proteins encoded by seven of the 16 full length genes were found in COMC. The structure of the pmp proteins has similarities to that of autotransporter proteins [62] with a *C*-terminal part predicted to form a beta-barrel and an *N*-terminal passenger domain. Pmps are heavily up-regulated at the time of conversion of RB to EB, and at least ten pmps are present in EBs. Due to their reaction in formalin fixation it is likely that pmp6, 8, 10, 11 and 21 are surface-exposed [87]. Identified cleavage sites of pmp6 and pmp21 are in agreement with the theory that pmps are autotransporters [87], and this theory has recently been further confirmed by studies by Wehrl *et al.* [88].

1.3.6
Proteome comparison of *S. trachomatis* serovars

2-D reference maps for *C. trachomatis* A, D and L2 EBs were published in 2002 [89]. The general findings for these serovars were very similar to those for *C. pneumoniae*, with many hypothetical proteins (including large amounts of CT579, the ortholog of Cpn0808), type III secretion proteins, highly abundant pmps and many proteins involved in transcription, translation and energy metabolism. Protein products were identified from a total of 134, 133 and 127 different genes in the three serovars, respectively, thereby providing well covered reference maps for further studies. From 144 protein species (including different post-translational variants) identified in all serovars, 55 migrated differently in serovars D and L2, 52 differed between A and L2 whereas only 26 differed between A and D. This reflects the greater similarity between the trachoma serovars A and D than between the LGV serovar L2 and A/D. Most differences are probably caused by substitution of charged amino acid with noncharged (or *vice versa*) and do not have biological implications. Significant differences included a much higher abundance of malate dehydrogenase in L2 than in serovars A and D and the absence of fumarate hydratase (FumC) in L2. The *fumC* gene was confirmed to be truncated in L2 by *in vitro* translation [89]. It was speculated that higher amounts of malate dehydrogenase may be required in L2, if fumarate hydratase is impaired and malate thus must be obtained from the environment.

1.3.7
Proteome analysis of growth conditions

Proteome analysis has also been used to analyze the effect of different growth conditions on protein synthesis. A prominent example is the investigation of the effect of IFN-γ treatment of different *C. trachomatis* serovars [90]. This study reported on up-regulation of tryptophan synthetase in *C. trachomatis* serovar D in response to IFN-γ. Both TrpA and TrpB were found to be up-regulated in serovars A, D and L2, but TrpA was found at a lower molecular weight in serovar A and upon sequencing the gene it was found to be truncated in serovar A. Also, serovar C was found to have a truncated *trpA* gene and in serovar B the gene is missing. The fact that all ocular serovars have impaired *trpB* genes may indicate a role for this process in the development of trachoma [63]. The up-regulation of Trp proteins was confirmed at the transcriptional level by RNA chip analysis [91]. MOMP and other proteins were found to be down-regulated in serovar A but not in serovars D and L2. This is in contrast to the findings of transcript analysis of serovar D, where mRNA encoding MOMP and several other proteins were found to be down-regulated [91]. Also *C. pneumoniae,* which lacks the entire *trp* operon is inhibited by IFN-γ. Several *C. pneumoniae* proteins including MOMP were found up-regulated by IFN-γ and none were found to be down-regulated [92], though the effect was small for all of the proteins.

1.3.8
Considerations in proteomics

Comparison of experimental results is an essential issue in proteome analysis. In transcription analysis, normalization to genome copy numbers can be used to standardize samples [93], and this approach could to some degree be adopted in proteome analysis using parallel samples. As a minimum, gel loading of radiolabeled samples should be adjusted according to scintillation counts and quantified protein levels should be expressed relative to the total amount of a defined set of spots or specific constitutive proteins.

Just as proteome analysis can supplement genomics by elucidating truncations and post-translational modifications it can assist genome annotation by the detection of proteins from unrecognized ORFs. By searching MS/MS sequence tags of small proteins against whole genomes translated in all reading frames, genes may be recognized which were not annotated automatically. A novel *C. trachomatis* D protein of 7 kDa specific for RBs was identified by proteome analysis as a product from a previously unrecognized gene of 204 base pairs, located between *ct804* and *ct805* [94]. Proteome analysis can be specifically designed to identify small genes by casting gels with high resolution in the low molecular weight area and analyzing the protein products by MS/MS.

Compared to proteomics, RNA analysis has the advantage that amplification can be performed and that every gene can be studied at a given time. Thus, transcript analysis is well suited for studying global regulations upon environmental changes.

In such studies, transcript measurements relative to standard amounts of genomic DNA [93] seem better than measurements relative to RNA levels at standard conditions [78]. To minimize RNA degradation total RNA should be extracted from infected cells and bacterial RNA then purified, rather than purifying the bacteria first [95]. Although *Chlamydia* has been the subject of both global transcriptome studies [93, 81] and large-scale proteome studies [47, 89] no comparative studies have been made. By transcript analysis, information can be obtained on the regulation of genes encoding proteins that are too low in abundance to be quantified by proteomic approaches. Proteomics suffers from the demand of protein solubility and low abundant proteins may be hard to detect. Still, small regulations of abundant proteins are better studied and quantified by proteomics, and post-translational modifications can only be studied by proteomics.

1.4
Concluding remarks

Methods for generation of genome, transcriptome and proteome data are now available and such data will be valuable for further investigations. In recent years, many chlamydial proteins have been identified that are at the interface of interaction with the host cell. These include novel surface exposed proteins, inclusion membrane proteins and secreted proteins. Many of the proteins were identified by approaches made possible only by the availability of the *Chlamydia* genomes. In addition, the structure and function of the individual proteins must be further investigated. Proteome analysis has identified proteins of COMC that should be analyzed for surface exposure. This may provide evidence for surface variation due to changes in the expression of interchangeable surface proteins [64].

As more chlamydial proteins that interact with the host cell are identified and characterized, we will learn more about *Chlamydia* developmental biology, the infection and ultimately even more about the eukaryotic cell. The humane genome sequence will aid the identification of interaction partners for chlamydial proteins.

1.5
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