The chlamydial developmental cycle

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Abstract

Intracellular parasitism by bacterial pathogens is a complex, multi-factorial process that has been exploited successfully by a wide variety of organisms. Members of the Order Chlamydiales are obligate intracellular bacteria that are transmitted as metabolically inactive particles and must differentiate, replicate, and re-differentiate within the host cell to carry out their life cycle. Understanding the developmental cycle has been greatly advanced by the availability of complete genome sequences, DNA microarrays, and advanced cell biology techniques. Measuring transcriptional changes throughout the cycle has allowed investigators to determine the nature of the temporal gene expression changes required for bacterial growth and development.

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Keywords: Chlamydia; Prokaryotic development; Microarray analysis

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1. Introduction

The order Chlamydiales encompasses a large group of bacteria characterized by their obligate growth in eukaryotic cells. The last decade has seen a rapid expansion of the number of organisms within the order, including organisms from numerous animal and environmental sources. This has led to the proposed division of the family Chlamydiaceae to include two genera, Chlamydia and Chlamydophila and the inclusion of non-Chlamydiaceae such as Simkaniaceae, Waddliaceae and Parachlamydiaceae [1]. The taxonomy of the Chlamydiaceae is controversial at this time and many feel the genera divisions are unnecessary. Since the taxonomic situation is changing rapidly, and is not the subject of this review, we have chosen to use the terminology described in Schachter et al. [2] i.e. the use of a single genus, Chlamydia.

Chlamydiaceae are the etiological agents of many important human and animal diseases. In humans, the genital serovars of Chlamydia trachomatis are the most prevalent cause of sexually transmitted disease worldwide [3], while the ocular serovars result in blinding trachoma in developing countries [4]. Chlamydia pneumoniae is a widespread respiratory pathogen [5] and chronic infections are associated with an enhanced risk of developing atherosclerotic [6,7], cerebrovascular [8], and chronic lung disease [9]. Pathogenic chlamydial isolates have been characterized from a number of animal hosts including birds, cats, rodents, cattle and pigs (e.g., [10]).

The Chlamydiaceae differ from the other main order of intracellular bacteria, the Rickettsiales, in that growth is associated with a biphasic developmental cycle. In fact, given the diversity associated with host range and disease pathogenesis, the developmental cycle may be seen as the major unifying feature of the group. The purpose of this review is to discuss recent advances that have furthered our understanding of the developmental cycle. For this purpose we have taken a generalist point of view and include findings from one or more species that elucidate relevant features of the developmental cycle, given the caveat that most of these observations have not been shown for all species members.

Chlamydia alternates between two morphological forms, the elementary body (EB) and the reticulate body (RB) [11]. EBs are extra-cellular, metabolically inert forms, responsible for dissemination of infection by their ability to attach to and invade susceptible cells. Upon infection, EBs are internalized in membrane bound vacuoles termed inclusions. EBs differentiate into metabolically active forms, termed RBs, and undergo repeated cycles of binary fission leading to secondary differentiation back to EBs. The host cell then lyases, releasing EBs, that infect neighboring cells. Under stressful growth conditions, imposed by immunological responses, antibiotics, or nutrient deprivation, the developmental cycle is disrupted, resulting in the appearance of large, aberrant RBs (reviewed in [12]). This altered growth scheme appears to be associated with continued expression of genes associated with DNA replication but not with those genes involved with bacterial cell division.

2. Elementary body

2.1. Morphology

The term “elementary body” (EB) refers to the small (ca. 0.3 μm), round, electron dense, infectious form of the organism (e.g., [13,14]). The term, as with other developmental cycle terms, stems from viral nomenclature historically associated with chlamydial research. The EB is metabolically inert and has been likened to a “spore-like” form of the organism. The bacterial nucleoid is highly compacted in EBs due to the condensation of nuclear material by the bacterial histone-like proteins HtA and HtB [15,16]. The nucleoid has an eccentric location in the cell body suggesting an association with the bacterial inner-membrane or cell wall. Chlamydial EBs are unusual in that little or no peptidoglycan is present in the cell wall. Structural rigidity is thought instead to be due to the highly cross-linked nature of the outer-membrane complex. Inter and intramolecular cystine bonds exist between the cysteine rich proteins of the outer envelope including OmpA, OmcB, and OmcA (reviewed in [17]). In addition, a hexagonally arrayed protein layer (predominantly OmcB) at the inner surface of the outer-membrane complex is thought to contribute to cellular stability in EBs.

Electron microscopic examination of EBs using freeze fracture/freezetch etch procedures demonstrated the presence of hexagonally organized surface projections arranged regularly with a center to center spacing of approximately 50 nm [18–20]. These “supramolecular structures” extend approximately 30 nm from the EB surface and have a rotational symmetry corresponding to a 9-subunit composition. Several authors have speculated that these spike-like projections correspond to Type III secretion system (TTSS) “needle” structures, similar to those seen in Salmonella enterica serovar Typhimurium [21]. No conclusive evidence has been shown to confirm these hypotheses but Fields et al. [22] have used an indirect argument that strongly supports the presence of TTSS in EBs. They have shown that inclusion membrane protein genes (inc) are expressed early in the developmental cycle, much earlier than the genes encoding structural components of the TTSS. Furthermore, they showed that secreted Inc protein appeared in the inclusion membrane before TTSS proteins were produced. Since Inc proteins were TTSS substrates in heterologous Yersinia systems, they concluded that EBs must have pre-formed TTSS that func-
tion very rapidly upon the EB-host cell interaction. Further support for the presence and functioning of TTSS in the initial stages of attachment and entry was provided in a subsequent report that will be discussed below [23].

2.2. Attachment and entry

As might be expected for an obligate intracellular parasite, chlamydiae have developed elaborate, and possibly redundant, mechanisms for host cell attachment and entry (reviewed in [24]). The ability to efficiently infect numerous non-phagocytic cell types from diverse animal species indicates that the bacterial adhesins involved must recognize conserved cellular receptors. Despite the central importance of this interaction in bacterial pathogenesis, and the research effort directed towards this problem, no clear consensus exists as to the nature of the adhesin/receptor molecules involved. Bacterial adhesin candidates include OmpA, OmcB, and Hsp70 [24]. The polymorphic membrane protein (Pmp) family of autotransporters has also been suggested to play a role in attachment [25–27]. This family of related proteins (21 members in C. pneumoniae) contains highly repetitive motifs of 4 amino acids (GGAI and FxxN, [27]) that are found in other bacterial adhesins such as rOmpA from the obligate intracellular Rickettsia spp. [25]. Wehrl et al., [28] demonstrated that PmpD of C. pneumoniae was indeed processed as an autotransporter and that antisera raised to the N-terminal passenger domain blocked chlamydial infectivity.

Recent studies indicate the interaction of EBs with host cells occurs in a two-stage process. The initial attachment occurs through electrostatic interactions of the bacteria with heparan sulfate containing glycosaminoglycans [29–32]. This interaction is reversible and has been shown to occur through binding to the surface exposed OmpA protein [31], although clear differences can be found between chlamydial species and serovars. A second, irreversible, binding stage was demonstrated using chemically mutagenized cell lines [33] but the receptor responsible has yet to be identified. Davis et al. [34] have shown that C. trachomatis serovar E is closely associated with protein disulfide isomerase (PDI), a component of the estrogen receptor complex, during attachment and entry of polarized HEC-1B cells. Chlamydial attachment was previously shown to be dramatically enhanced in estrogen dominant primary human endometrial epithelial cells [35] and the receptor complex is located at the apical surface of polarized cells, an important consideration for entry from the luminal surface of the genital epithelium. Furthermore, the authors suggest that the disulfide isomerase activity of PDI may play a role in the reduction of the highly disulfide cross-linked EB OMC, a requirement for productive attachment and entry [36].

Clifton et al. [23] have recently shown that, immediately following the irreversible binding step, a TTSS exported protein is delivered into the host cell. This protein, termed Tarp (Translocated actin-recruiting phosphoprotein, CT456), is rapidly phosphorylated at tyrosine residues and phosphorylation correlates spatially and temporally with actin recruitment (Fig. 1). Further evidence for the direct involvement of Tarp comes from expression studies with a Tarp-EGFP fusion in which the product expressed in the cell cytoplasm is tyrosine-phosphorylated and recruits actin. Tyrosine-phosphorylated Tarp is detected on the cytoplasmic face of the cell membrane while the EB is still extracellular (i.e. prior to chromosome decondensation). These results indicate that chlamydial EBs have a functional TTSS that delivers important signaling molecules to the host cell prior to differentiation. The preformed nature of the TTSS and early effector molecule(s) such as Tarp, suggests that the expression of important proteins for attachment and entry, and their assembly into functional complexes, must be part of the secondary differentiation process in which RBs are converted to EBs.

3. Primary differentiation

The chlamydial developmental cycle has a singular enigmatic stage, the differentiation of the infecting EB to a metabolically active RB. The process of differentiation can be blocked by the addition of antibiotic inhibitors of transcription or translation, suggesting that de novo protein expression is required to begin intracellular growth [37]. This finding is at odds with the generally held understanding that the presence of the bacterial histone-like proteins HctA and HctB render the EB transcriptionally incompetent. This paradox has been at least partially addressed by the findings of Greishaber et al., [38] Chlamydial histone–DNA interactions are disrupted upon germination by a small metabolite in the non-mevalonate pathway (MEP) pathway of isoprenoid biosynthesis. The metabolite is thought to be 2-C-methylerythritol 2,4-cyclodiphosphate and is involved in functional antagonism of HctA. Findings showed that an E. coli HctA expression system was rescued from the lethal effects of HctA by ispE. ispE is an intermediate enzyme in the MEP pathway, stepwise reconstruction of this pathway showed convincing evidence that the aforementioned metabolite is the candidate to be the functional antagonist of HctA. Paradoxically, experimental data adds another question, the E. coli orthologue of ispE could not protect E. coli from the lethal effects of HctA expression. The question arises whether it is special enzyme kinetics attributed to chlamydial IspE or the transcriptional activity of the chlamydial ispE in the presence of HctA, or another unknown function that chlamydial ispE has that helps in the rescue from HctA.
lethality. *Chlamydia* possess another histone-like protein HctB, it is still unknown what antagonizes its function during the differentiation step [38].

### 3.1. Carryover mRNA

The chlamydial EB contains significant quantities of mRNA and ribosomes, despite its lack of metabolic activity. Following chromosome decondensation, the genome rapidly becomes transcriptionally active. During primary differentiation the fate of the so called “carryover mRNA” presents an interesting problem. The bulk of EB mRNA encodes late gene products, a reflection of the transcriptome during the final stages of the RB to EB secondary differentiation. For example, transcript levels for the histone-like protein gene *hctA* are high, much higher than those found for genes expressed early in the cycle [39]. Despite the presence of high levels of mRNA for *hctA*, there is no expression of the HctA protein. Indeed the expression of HctA at the start of the cycle would seem to block new transcriptional activity, in much the same way this occurs late in the cycle. The newly differentiating chlamydial cell therefore appears to have a mechanism to differentiate newly transcribed mRNA from carryover mRNA such that only newly synthesized mRNAs are translationally competent. It is unclear at this point how the cell does this. Speculative hypotheses involve; RNA binding protein(s) that sequester the carryover mRNA from ribosomes, carryover mRNA processing that renders the message translationally inactive (i.e. removal of the 5' end, including translational start signals). Carryover mRNA is rapidly degraded in the bacterial cell and generally drops below detectable levels by ca. 6 h post-infection [39].

### 3.2. Early gene expression

Transcription begins within the differentiating RB almost immediately following internalization. New pro-
tein expression can be detected within 15 min PI using intrinsic labeling procedures [40] and newly synthesized RNA can be detected in “host-free” chlamydia by 1 h PI [41]. Wichlan and Hatch [42] identified the first early gene (euo, Early Upstream ORF) using “host-free” early RNA to select clones from a genomic library. Transcription of euo was confirmed by Northern blotting and primer extension analysis. Euo appears to be specific to chlamydia in that it is highly conserved within the genus but has no homology to other bacterial proteins. Despite its early identification, the function of Euo has not been clearly defined to date.

Following the sequencing of the serovar D genome, a number of studies used RT-PCR to identify genes expressed early in the cycle. Shaw et al. [43], identified a number of inclusion-associated protein genes (inc) as early genes and proposed the generally accepted temporal classes of developmentally expressed genes as (i) early (1–2 h PI), (ii) mid-cycle (3–18 h PI), (iii) late (20–48 h PI). More recently, genome-wide DNA microarrays have been used to determine the transcriptome throughout the cycle. Belland et al. [39] identified 29 genes expressed by C. trachomatis serovar D at 1 h PI in HeLa 229 cells. Seven of these genes had been previously characterized as early genes, including the previously mentioned euo and inc genes [42,43]. Other genes expressed at 1 h PI included genes that encode proteins involved in (i) translocation of metabolites into the bacterial cell (e.g., ADP/ATP translocase, nucleotide phosphate transporter, oligopeptide permease and a D-alanine/glycine permease), (ii) metabolite interconversions (e.g., malate dehydrogenase, nucleoside phosphohydrolase, and a methionine aminopeptidase), (iii) inclusion modification (e.g., inc-like genes CT228 and CT229, and the EE1A-like CT147), and (iv) unknown functions (all genes were conserved among chlamydial species). These studies suggest that genes expressed during primary differentiation serve two general purposes i.e. establishing systems involved in nutrient acquisition and modifying the parasitophorous vacuole (inclusion) to prevent its entry into the endocytic pathway leading to lysosomal fusion.

4. Reticulate body

4.1. Morphology

The “reticulate body” (RB) arises from the internalized EB following primary differentiation, as discussed above. RBs are larger than EBs (ca. 1 μm) and the cytoplasm appears granular with diffuse, fibrillar nucleic acids, in contrast with the highly condensed nucleic acid content of the EB (reviewed in [44]). RBs are non-infectious and are bounded by an inner and outer-membrane, resembling other, Gram-negative, eubacteria. The surface of RBs is covered with projections and rosettes that extend from the bacterial surface through the inclusion membrane (proposed TTSS needle structures), similar to those found on EBs but at a higher density [19]. RBs undergo binary fission throughout the middle part of the developmental cycle. In certain species, the RBs tend to be closely associated with the inner face of the inclusion membrane throughout the period of rapid growth, but this may not apply to all species (i.e. C. pneumoniae appears to completely fill the interior of the inclusion and C. caviae tends to grow in an “articulated” form of the inclusion, without a large internal space [44]).

4.2. Gene expression

Microarray analyses of gene transcription during the developmental cycle have been reported for C. trachomatis serovar L2 [45] and serovar D [39]. While a number of technical and experimental design differences were used for the analyses, both reports indicate that by 6–8 h PI the developing RBs were highly transcriptionally active. Belland et al. [39] classified temporal gene expression groups into early, mid-cycle and late categories with a subgrouping of genes expressed at 1 h PI as immediate-early. While this study focused on the immediate-early and late genes, the complete listing of transcriptional activities ([39] and the accompanying supplementary Table 1) throughout the cycle indicated that the period of intense transcriptional activity (16–24 h PI) correlated with the rapid growth and division of RBs. Furthermore, they reported that virtually every gene in the organism is expressed at some point in the cycle (exceptions being CT496.1 and the repressible trp A and trp B genes), indicating that C. trachomatis has virtually no facultative capacity, as might be expected for an obligate intracellular organism with a “minimal” genome. Delayed expression (16 h PI) of genes involved in certain aspects of cellular metabolism were seen, including cell division and TTSS assembly (discussed in more detail below).

Nicholson et al. [45] used a differential display procedure to classify gene expression data in relation to expression values at 24 h PI, the metabolic and developmental midpoint of the cycle. Expression data were expressed as three developmental stages encompassing 7 temporal clusters, Stages I and II encompassing the RB phase. Stage I genes were expressed by 6 h PI and included the (i) early, (ii) constitutive, and (iii) mid-cycle clusters, according to their expression patterns at later times in the cycle. The largest group was the constitutive cluster which comprised genes that were involved in basic cellular functions including translation (and ribosome assembly), DNA replication, transport-related, energy compound acquisition and membrane energetics. Stage II genes were significantly transcribed by 18 h PI...
and contained a midlate I (remain at 18 h levels through the remainder of the cycle) and midlate II (levels continue to increase through the remainder of the cycle) clusters. The midlate I cluster consisted of genes involved in a number of cellular processes including cell envelope biogenesis, energy metabolism, TTSS, protein folding, and DNA replication and repair. The midlate II cluster is comprised of genes that appear to have dual transcriptional control systems e.g., the tandem promoters of the ompA gene which result in maximal transcription initiation rates at different times in the cycle (giving an additive effect later in the cycle) [45]. Together these stage-specific genes lead to the rapid growth of the RB (Stage III, late genes are discussed below).

4.3. Inclusion modification

Once internalized, chlamydiae actively modify the properties of the nascent vacuole. Modification of the inclusion circumvents normal trafficking through the host endocytic pathway (e.g., [46,47]), effectively dissociating it from late endosomes and lysosomes (reviewed in [24]). The inability to detect endosomal and lysosomal markers in the Chlamydial inclusion membrane demonstrates that the inclusion is non-fusogenic with endosomes or lysosomes [48–51]. Instead, through processes that require the active participation of Chlamydia [37], chlamydial inclusions intersect a subset of vesicles containing sphingomyelin [52] and cholesterol from the Golgi apparatus [53]. These processes occur early in the developmental cycle and are necessary for successful replication of Chlamydia within a host cell. The molecular mechanisms that Chlamydia utilize to control the biogenesis of vacuoles are not known. However, Chlamydial gene expression is required [37], suggesting that Chlamydial proteins that are secreted into the host cell cytoplasm or incorporated into the inclusion membrane are likely to be important mediators of these properties.

Within 2 h after entry into host cells, Chlamydial inclusions are trafficked to the perinuclear region of the host cell and remain in close proximity to the Golgi apparatus, where they begin to fuse with a subset of host vesicles containing sphingomyelin. Chlamydial migration from the cell periphery to the peri-Golgi region resembles host cell vesicular trafficking. Chlamydia moves toward the minus end of microtubules and aggregates at the microtubule-organizing center in a process that is dependent on Chlamydial protein synthesis [54]. Rab GTPases, key regulators of membrane trafficking were seen to be recruited to Chlamydial inclusion membrane, recruitment was species specific, which may function to regulate trafficking or fusogenic properties of the inclusion. The absence of Rab5 and the association of Rab4 and Rab11 with the inclusion membrane demonstrate that inclusions are associated with markers or endosomal domains that are characteristic of late steps in the recycling pathway [55]. Recently it was postulated that chlamydial inclusions intersect the cellular autophagic pathway to acquire nutrients [56]. Using markers for the autophagy pathway (MAP-LC3, calreticulin), autophagic vesicles were proved to be in close proximity to the inclusion, but direct fusion between the two vesicles could not be established. The inclusion of autophagy inhibitors (3-methyl adenine, several amino acids) in the growth medium led to aberrant chlamydial growth [56].

A group of chlamydial proteins (termed Inc) are found in the inclusion membrane and are thought to interact with host proteins. The Inc proteins share very limited amino acid identity among themselves, but share a common bilobular hydrophobic motif that is thought to span the inclusion membrane. Using the characteristic hydrophathy profile, in silico searching for these proteins led to the prediction of 90 candidates in C. pneumoniae J138 and 36 in C. trachomatis serovar D [57]. Inc proteins, were originally identified in C. psittaci, and subsequently found in C. trachomatis [58]. IncA was experimentally proven to be an inclusion membrane protein by intracellular reactivity of the protein following microinjection of fluorescent antibody into infected cells [59]. IncD, IncE and IncF, and IncG were identified in purified inclusion membrane preparations. RT-PCR analysis demonstrated that IncD, E, F, and G, were transcribed within the first 2 h after internalization, making them candidates for chlamydial factors required for the modification of the nascent inclusions [60,61].

C. trachomatis IncA plays a role in homotypic fusion of chlamydial inclusions as microinjection of α-IncA antibodies inhibits homotypic fusion [61]. C. psittaci IncA however did not appear to influence homotypic fusion, rather over-expression of IncA in cells infected with C. psittaci disrupted the development cycle [62]. IncA is also found within fibers that appear as chains of vesicles extending away from the inclusion along distinct routes into the host cytoplasm [63]. The significance and composition of the IncA-laden fibers is unknown. IncA was shown to be phosphorylated at serine and/or threonine residues in host cell by unknown host kinase(s) [59]. Recently, it was shown that IncA has a characteristic signature of the eukaryotic SNARE superfamily, where 6 hepta-repeats (called the SNARE complex) were present in a coiled-coil motif close to the hydrophobic domain of the protein. SNAREs are known to be involved in targeting vesicles. Interestingly, expression of IncA in eukaryotic cells led to retention of the protein inside the ER, and that over-expression of the protein inhibits chlamydial development [64].

C. trachomatis IncG was shown to interact specifically with the eukaryotic protein 14-3-3 β using yeast two-hybrid screening assays and this observation was confirmed by detection of interaction in the inclusion
membrane by immunofluorescence microscopy of infected cells [65]. This was the first demonstration of the interaction of the chlamydial inclusion with a host protein, although its significance has not been determined. Genomic sequences indicated that C. trachomatis inc G had no orthologs in C. caviae or C. pneumoniae, in agreement with the lack of 14-3-3 β association with bacterial inclusions by these organisms [65]. This raises interesting questions related to the species-specific interactions with the host that may influence cellular tropism and disease pathogenesis.

4.4. Type III secretion machinery

Genomic data reveals that all chlamydial species possess a complement of genes encoding type III secretion system (TTSS) [66–68]. C. pneumoniae was shown to possess at least 13 genes that are homologous with other known TTSS systems. TTSS genes in chlamydia show temporal regulation through out the developmental cycle [22,69,70]. In C. pneumoniae for example; (i) early genes include yscC, yscS, yscL, yscJ and lcrH-2, (ii) mid-cycle genes include lcr D, yscN, and yscR, and (iii) late cycle genes include lcrE, yscE, lcrH-1, and yscT [70]. C. pneumoniae may use TTSS to translocate different effectors into the host cell, depending on the phase of the developmental cycle [70]. Several effector proteins were proved to be substrates for TTSS in heterologous secretion systems. It was demonstrated that IncA, IncB and IncC hybrid proteins with cya reporter gene are secreted by TTSS of S. flexneri [71]. In another study to identify bacterial secreted proteins, immunofluorescence assays with antisera raised against Cpn0809 and Cpn1020 showed signals that were distributed within the host cell rather than inside the inclusions, implying that these are proteins secreted by the bacteria to modify host response [72]. The influence of chlamydial infection on the cells apoptotic machinery is probably controlled through secreted effectors. The question of whether infection is pro or anti-apoptotic (or perhaps both at different points in the cycle) is controversial at this point and the complexity of the arguments prevent their coverage here (readers are referred to a recent review [73]).

4.5. Cell division

Cell division takes place during the RB stage. Genome sequencing indicated that chlamydia lack an identifiable ftsZ ortholog, which encodes a protein centrally involved in bacterial cell division and found in all other sequenced eubacteria. Another surprising finding in the genomic analyses was the presence of a complete set of genes for the synthesis of peptidoglycan [66,68,74]. Numerous studies had reported that Chlamydia lacked peptidoglycan, with a single study reporting trace amounts in EBs [75]. Similarly, attempts to identify peptidoglycan in RBs were unsuccessful [76]. Somewhat surprisingly, penicillin and other β-lactams are inhibitory to chlamydial growth (e.g., [77]) and probably target the high molecular weight penicillin binding proteins. This paradoxical situation has been referred to as the “chlamydial anomaly” [78]. RBs may synthesize small amounts of peptidoglycan that play a role in bacterial cell division, perhaps by substituting for the lack of FtsZ in the formation of nascent division septa (e.g., [79]).

McCoy et al. [80] have shown that the chlamydial MurA ortholog (UDP-N-acetylmuramic enopyruvyl transferase), which catalyzes the first committed step in peptidoglycan biosynthesis, is functional in E. coli. The chlamydial MurA was found to encode a fosfomycin-resistant form of the enzyme and this resistance was imparted to E. coli expressing the chlamydial enzyme. Expression studies indicated that chamydia are naturally fosfomycin resistant and that murA is expressed at the point in the developmental cycle immediately preceding cell division [39,80], providing circumstantial evidence for the involvement of peptidoglycan synthesis in cell division.

5. Secondary differentiation

5.1. Late gene expression

Following the period of rapid cell division, RBs begin to redifferentiate to EBs, here termed “secondary differentiation”. The signal for this process is unknown and efforts to identify quorum sensing type pathways are not been supported by genomic analyses. Speculative mechanisms have been proposed that involve the dissociation of dividing RBs from the inclusion membrane as a trigger for secondary differentiation. The removal of the TTSS system needle apparatus from the inner surface of the inclusion membrane has been suggested as a critical event in this process [81]. This hypothetical mechanism has many attractive features, including an explanation for the asynchronous nature of secondary differentiation, but much work remains to provide experimental evidence supporting it.

Expression of a number of late-cycle genes occurs during secondary differentiation (reviewed in [17]), including genes that encode components of the outer-membrane complex (e.g., OmcA and B) and proteins involved in the condensation of the chromosome (e.g., HctA and B). Microarray analyses have extended the number of genes expressed during this stage of development. Belland et al. [39] identified 26 late genes including a number of genes previously characterized as late genes based on other molecular biological studies (e.g., omcAB, hctAB, ltuB, lcrH.1) and a number of new genes that encode proteins with speculative and unknown function. Several newly identified late genes encoded
proteins with functions predicted to be important in the formation of the highly disulfide-cross-linked outer-membrane complex. These include two predicted thioredoxin disulfide isomerases (CT780 and CT783) and two membrane thiol proteases (mtpA and mtpB). The membrane thiol proteases share homology with adenoviral proteases that play a role in the maturation of viral particles [82]. Interestingly, expression of some late cycle genes may be directed towards the arming of EBs with proteins necessary during the early stages of the next infectious cycle (e.g., the TTSS protein LcrH.1), hence “late” genes may encode “early” proteins.

Nicholson et al. [45] identified 70 late genes, classified as Stage III in that they were not expressed until 24 h PI. Two clusters were identified in this stage, the late temporal cluster and the very late cluster, the latter consisting of genes that show increased expression levels between 24 and 36 h PI. The late cluster included previously identified late genes (e.g., omcAB, hctAB, ItuB, lerH.1), genes potentially involved in chromosome condensation (e.g., CT643 and CT660 in addition to hctAB), and a large number of genes (31) with unknown function and found only in chlamydia. The very late temporal cluster consisted of six genes that encode chlamydia-specific proteins of unknown function, including a gene (CT147) identified as an immediate-early gene in the Belland et al. study.

6. Alternate growth modes and persistence

Many chlamydial diseases are associated with a long term or chronic infectious state. In most cases it is difficult to establish whether chronic or recurrent infections arise through the inability of the host to resolve the infection or the occurrence of repeated infections with similar species or genotypes. Despite the unresolved nature of the disease etiology, persistence models of chlamydial infection have been studied to provide insight into the nature of chronic disease (reviewed in [12]). Persistence is defined as a long-term association between Chlamydia and their host cell in which these organisms remain in a viable but culture-negative state. The in vitro persistence systems often share altered Chlamydial growth characteristics for example, many studies have described enlarged, and pleomorphic RBs that neither undergo binary fission, nor differentiate to EBs, but nevertheless continue to replicate their chromosomes. These changes are generally reversible upon removal of the growth inhibitory factor [12]. Persistent in vitro infections have been induced by penicillin treatment [83], amino acid starvation [84], iron deficiency [85], IFN-γ exposure [86], monocyte infection [87], phage infection [88], continuous culture [89]. This subject has been thoroughly reviewed in Hogan et al. [12].

IFN-γ mediated inhibition of intracellular chlamydial replication occurs by depletion of the essential amino acid tryptophan, via the induction of indoleamine-2,3-dioxygenase (IDO) [90]. The effect of IFN-γ on Chlamydia infection could be reversed by addition of tryptophan [91]. Belland et al. have studied the induction of persistence with IFN-γ and the subsequent reactivation, using microarray analysis. Persistent growth, characterized by large aberrant RBs, led to the up-regulation of genes involved in tryptophan utilization, DNA repair and recombination, phospholipid biosynthesis and translation. Up-regulation of the repressible trp BA operon [92] confirms the previous observations that IFN-γ treatment reduces intracellular concentrations of tryptophan. In addition, a number of early genes were up-regulated, particularly the euo gene (30-fold increase) which encodes a DNA-binding protein that has been shown to bind to a late gene promoter region (i.e. omcAB) [93]. Down-regulation of genes involved in RB to EB differentiation (late genes such as hctAB and omcAB), proteinolysis and peptide transport, and cell division were seen during persistent growth. The transcriptional analyses were consistent with the biological properties associated with aberrant RBs in that cells were blocked in cytokinesis and the developmental cycle was arrested at a point preceding late gene expression. Removal of IFN-γ and supplementation with added tryptophan led to a rapid reactivation from persistent growth. During reactivation the expression differences rapidly returned to control levels, i.e. euo expression dropped 20-fold in 12 h. The transcriptional changes in the presence of IFN-γ that result in persistent growth appear to constitute a persistence stimulon. This coordinated biological response appears to have evolved to allow the organism to rapidly respond to immunological pressure in a manner that allows for a period of resistance followed by rapid recovery after the waning of the host response.

7. Concluding remarks

The chlamydial developmental cycle involves a complex interaction between the pathogen and its obligate host. This unique, biphasic life cycle requires the coordinate application of biological functions that involve interactions of protein complexes and membrane structures and is dependent on both physical and metabolic factors. In particular, the temporal patterns of gene expression drive the progression of the cellular infection through the initial stages of primary differentiation, replication, and secondary differentiation. The use of whole genome microarrays has allowed the identification of genes involved in these processes. Functional characterization of the components of the process will require continued molecular and cellular biological studies that should lead to a greater understanding of the role of the cycle in chlamydial disease and stimulate new therapeutic approaches to disease treatment.
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