

## Microreview

# New insights into *Chlamydia* intracellular survival mechanisms

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### Summary

***Chlamydia* sp. are responsible for a wide range of diseases of significant clinical and public health importance. In this review, we highlight how recent cellular and functional genomic approaches have significantly increased our knowledge of the pathogenic mechanisms used by these genetically intractable bacteria. As the extensive repertoire of chlamydial proteins that are translocated into the mammalian host is identified and characterized, a molecular understanding of how *Chlamydiae* co-opt host cellular functions and block innate immune pathways is beginning to emerge.**

### Introduction

Ocular and genital infections with *Chlamydia trachomatis* represent a significant public health concern because of their association with infectious blindness (trachoma) and adverse effects on female reproductive health (reviewed in Schachter, 1999). Similarly, pulmonary infections with *Chlamydia pneumoniae* are increasingly recognized as a common cause of community acquired pneumonias and a risk factor for chronic obstructive pulmonary disease, asthma and atherosclerosis (reviewed in Blasi *et al.*, 2009). A greater understanding of basic chlamydial biology is clearly important to develop vaccines, and identify new targets for drug intervention.

*Chlamydiae* undergo a distinct developmental cycle, converting between two morphologically and functionally discrete forms, the elementary body (EB) and the reticulate body (RB). The basic cycle follows this sequence: (I) attachment and internalization; (II) EB to RB differentia-

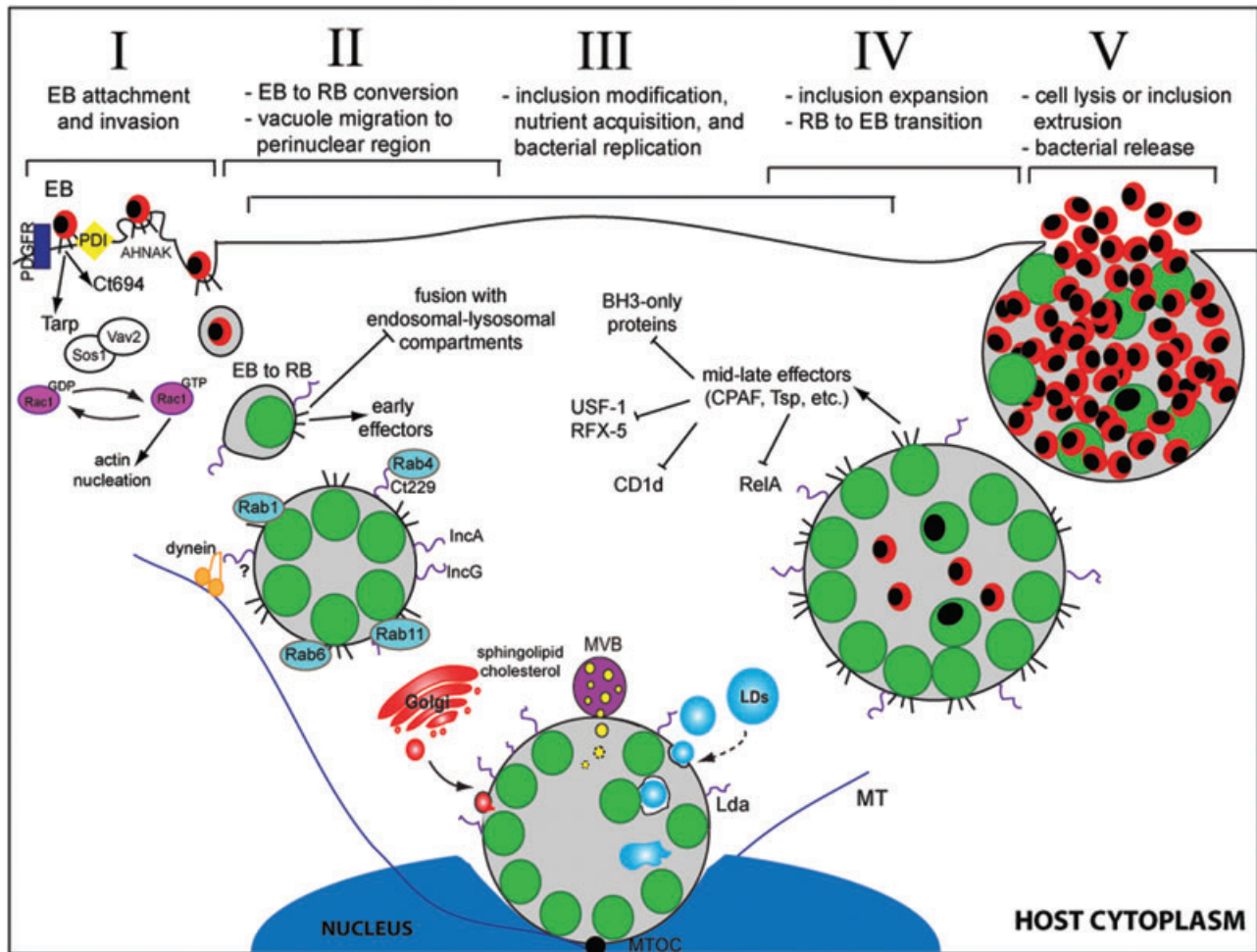
tion; (III) remodelling of the parasitophorous vacuole ('inclusion') and bacterial replication; (IV) inclusion expansion and transition of RB into EB; and (V) release of bacteria from the host cell and infection of new target cells by EBs (Fig. 1).

Studies based on expression analysis (Belland *et al.*, 2003; Nicholson *et al.*, 2003), heterologous type III translocation systems (Fields *et al.*, 2003; Subtil *et al.*, 2005), function-based screens (Sisko *et al.*, 2006) and bioinformatics (Samudrala *et al.*, 2009) have yielded many candidate *Chlamydia* proteins that may participate in the manipulation of host cellular processes. Here we review the basic aspects of *Chlamydia* replication within infected cells and highlight recent findings that have significantly expanded our understanding of chlamydial pathogenesis.

### Attachment and entry

The EB, the infectious bacterial form, attaches to and is internalized by the host cell. Multiple bacterial adhesins and ligands have been proposed, and host receptor utilization depends on both the host cell type and chlamydial species (Dautry-Varsat *et al.*, 2005). Bacterial factors such as glycosaminoglycan (GAG) (Menozzi *et al.*, 2002), major outer membrane (MOMP) (Su *et al.*, 1996), OmcB (Fadel and Eley, 2007) and PmpD (Wehrli *et al.*, 2004) have been proposed as adhesins or ligands for receptor interactions. Previously proposed host receptors include heparan sulfate, mannose receptor, mannose 6-phosphate receptor and estrogen receptor (reviewed in Campbell and Kuo, 2006). Recently, two distinct roles were uncovered for cell surface exposed protein disulfide isomerase (PDI) in internalization: a structural one involved in EB attachment, and an enzymatic one required for bacterial entry (Conant and Stephens, 2007; Abromaitis and Stephens, 2009). PDI is predicted to bind one or more cell surface receptors (Abromaitis and Stephens, 2009) which may account for the severe EB adhesion and invasion defects in PDI-deficient cells (Fudyk *et al.*, 2002). RNAi screening exposed platelet derived growth factor receptor (PDGFR) and Abelson (Abl) kinase as essential host factors for EB entry (Elwell *et al.*, 2008). Multiple cell surface proteins likely act in concert, or redundantly to, initiate EB invasion.

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**Fig. 1.** The *Chlamydia trachomatis* infectious cycle and modulation of host cell functions. *Chlamydia* infection begins when an elementary body (EB) binds to the host cell surface (I). At this stage effector proteins injected into the host via the type III secretion system (T3SS) facilitate bacterial entry. Following endocytosis, EBs transition to reticulate bodies (RBs) (II). New effectors are secreted and the bacterial vacuole is modified by bacterial inclusion membrane proteins (Incs) to limit fusion with the host degradative compartments while promoting contact with other host organelles and factors, including Rab proteins. The inclusion interacts with the Golgi apparatus, multivesicular bodies (MVBs) and lipid droplets (LDs). LDs can be directly translocated into the inclusion lumen for nutrient delivery (III). During infection, host cell death and immune defences are inhibited. Approximately midway through the infectious cycle, bacterial replication becomes asynchronous and RBs re-differentiate into EBs (IV). Late in the cycle, the inclusion is packed with EBs and fills almost the entire cell volume. Eventually, the inclusion and host cell rupture releasing infectious EBs into the extracellular space for re-infection (V).

The exact mechanism of endocytosis remains unclear due to conflicting studies using pharmacological inhibitors and dominant-negative constructs (reviewed in Dautry-Varsat *et al.*, 2005). These results reflect that multiple redundant strategies likely exist to ensure chlamydial entry, and the route is dependent on the *Chlamydia* species or the host cell type being invaded. The unifying feature of EB entry, regardless of cell type or species, is the Rac1-dependent actin remodelling at attachment sites (Carabeo *et al.*, 2004). The early translocated bacterial effector protein Tarp may participate in this process. Phosphorylation of residues in the N-terminal tyrosine-rich tandem repeats of Tarp by host Src (Jewett *et al.*, 2008) and Abl (Elwell *et al.*, 2008) kinases recruits the guanine

nucleotide exchange factors (GEFs), Sos1 and Vav2, which activate Rac1 (Lane *et al.*, 2008). Subsequently, Rac1 recruits WAVE2 and Abi-1 leading to Arp2/3 complex activation and actin reorganization (Carabeo *et al.*, 2007). These findings have been confirmed and extended in two unbiased RNAi screens for host factors required in chlamydial entry and replication (Derre *et al.*, 2007; Elwell *et al.*, 2008). Interestingly, Tarp from other *Chlamydiae* is not phosphorylated, suggesting that Rac GEF recruitment occurs by an alternative pathway in these species or is not essential to control host actin (Clifton *et al.*, 2005). Alternatively, a WH2-like domain in the C-terminal portion of Tarp can directly bind actin subunits and promote nucleation (Jewett *et al.*, 2006). Ct694

is another bacterial protein translocated into the host cytoplasm at *C. trachomatis* attachment sites. Ct694 can bind the host protein AHNAK, a large multidomain actin-binding protein that associates with the plasma membrane (Hower *et al.*, 2009). By analogy to other invasive pathogens, it would not be surprising if multiple chlamydial effectors participate in invasion. Targeted knockdown of endocytic factors also revealed clathrin as a host component important in *C. trachomatis* entry of non-phagocytic cells (Hybiske and Stephens, 2007a).

### **Chlamydia developmental transitions**

Elementary bodies are stable in the extracellular environment by virtue of extensively cross-linked outer-membrane proteins (Newhall and Jones, 1983). These disulfide bonds are reduced during internalization (Hackstadt *et al.*, 1985) followed by nucleoid decondensation and initiation of bacterial transcription. Within 15 min, new bacterial proteins are already being produced and RNA expression can be detected as early as one hour (Plaunt and Hatch, 1988; Belland *et al.*, 2003). RBs replicate by binary fission inside inclusion boundaries. Roughly midway through infection, replication becomes asynchronous as RBs begin differentiating back into EBs. Similar to the early conversion of EBs to RBs, the events involved in RB to EB transition are unknown. Dissociation of the type III secretion apparatus from the inclusion membrane as RBs detach is one proposed trigger for this process (Wilson *et al.*, 2006), but this appealing model remains to be tested.

### **Inclusion modification and nutrient acquisition**

The nascent inclusion membrane initially resembles the host plasma membrane, but these components are promptly lost as bacterially derived proteins are produced and inserted (Scidmore *et al.*, 2003). Because many markers of the endomembrane system do not associate with the inclusion, it was assumed to be disengaged from classical vesicular trafficking pathways (reviewed in Fields and Hackstadt, 2002). New cell biological studies, however, have led to a reassessment of how *Chlamydia* exploits or 'mimics' host machinery to manoeuvre through the host cell and establish interactions with host organelles.

Interestingly, a subset of recycling endosome and Golgi-related Rab GTPases (e.g. Rab1, 4 and 11) – essential host proteins that regulate organelle identity and membrane trafficking (Seabra and Wasmeier, 2004) – associate with the inclusion membrane (Rzomp *et al.*, 2003). Rab6 and Rab10 associate with inclusions in a species-specific manner, suggesting that bacterial proteins which differ between species may be involved in

Rab recruitment (Rzomp *et al.*, 2003). Chlamydial inclusion membrane proteins (Inc) are likely candidates for these bacterial factors. In agreement, the *C. trachomatis* Inc protein Ct229 is a Rab4 binding partner (Rzomp *et al.*, 2006), and the *C. pneumoniae* Inc, Cpn0585, interacts with Rab1, 10 and 11 (Cortes *et al.*, 2007). Ectopic over-expression of Cpn0585 saturates Rab11 binding and inhibits inclusion development indicating a role for Rab–Inc interactions in chlamydial growth (Cortes *et al.*, 2007). SNARE (soluble NSF-sensitive attachment receptor) proteins, which regulate host membrane fusion, are also targeted by *Chlamydia*. The Inc proteins IncA, Ct223 and Ct813 contain SNARE-like motifs, and IncA can bind to a subset of host SNAREs (Vamp3, Vamp7 and Vamp8) *in vitro*. Importantly, SNARE recruitment to the inclusion is impaired in IncA-deficient strains (Delevoye *et al.*, 2008). *Chlamydia* also co-opts the minus-end directed motor protein dynein to facilitate transportation of the inclusion along microtubules to a perinuclear region near the microtubule-organizing centre (MTOC) (Grieshaber *et al.*, 2003).

Once established in this niche, the inclusion can selectively interact with organelles that provide factors essential for chlamydial development such as eukaryotic lipids, including sphingolipids (Hackstadt *et al.*, 1996; Moore *et al.*, 2008), cholesterol (Carabeo *et al.*, 2003) and glycerophospholipids (Wylie *et al.*, 1997). While sphingolipids and cholesterol isolated from bacteria are not modified, fatty acids in the Sn2-position of host glycerophospholipids are exchanged with *Chlamydia*-derived branched chain fatty acids (Wylie *et al.*, 1997; Su *et al.*, 2004). Lipid transport to the inclusion occurs through largely undefined pathways.

The Golgi apparatus is a likely source of lipids for the *Chlamydia* inclusion as it is abundant in the perinuclear region where the inclusion is nestled during development. A portion of *Chlamydia*-acquired sphingolipids are intercepted from exocytic Golgi vesicles (Hackstadt *et al.*, 1996). Changes in Golgi architecture also affect inclusion development. Golgin-84, a component of the structural scaffold that maintains Golgi apparatus morphology, is cleaved during infection resulting in Golgi fragmentation to ministacks (Heuer *et al.*, 2009). Chlamydial replication is significantly reduced when Golgi fragmentation is inhibited by expression of truncated golgin-84. Inversely, RNAi depletion of giantin and GPP130 stimulate Golgi fragmentation and enhance chlamydial replication. Fragmentation is hypothesized to boost delivery of Golgi-derived lipids to the inclusion as ceramide transport is delayed by inhibitors of golgin-84 cleavage (Heuer *et al.*, 2009). Retrograde Golgi traffic during chlamydial infections is further highlighted by the identification of COPI components as important host factors in *C. caviae* infection (Derre *et al.*, 2007).

Multivesicular bodies (MVBs) may represent an alternative lipid transport pathway. MVBs are endocytic organelles where proteins and lipids that are destined for degradation or recycling to the Golgi are sorted and processed (Woodman and Futter, 2008). MVB protein components including CD63, LBPA and MLN64 localize to the lumen of the *Chlamydia* inclusion, and CD63-positive vesicles are observed within the inclusion. Furthermore, pharmacological inhibitors of MVB maturation result in decreased sphingolipid transport to the inclusion and inhibit bacterial replication (Beatty, 2006).

Non-classical transport pathways may also be involved in lipid delivery to the inclusion. Lipid droplets (LDs), eukaryotic neutral lipid storage organelles, proliferate at the inclusion periphery (Kumar *et al.*, 2006). Because LDs are delimited by a phospholipid monolayer, it is unclear how these organelles might interact, fuse and deliver components to the inclusion. Electron and live time-lapse microscopy surprisingly reveal that intact LDs are translocated into the inclusion lumen (Cocchiario *et al.*, 2008). These observations indicate that uptake of entire organelles – and their associated material – by the inclusion may be a general alternative strategy for nutrient acquisition that circumvents the need for membrane fusion.

Physical association of mitochondria with inclusions has long been observed (Matsumoto *et al.*, 1991), but a link between mitochondrial function and *Chlamydia* infection was lacking until recently. RNAi knockdown of the Tim–Tom complex, a mitochondrial protein import system, inhibits *C. caviae* infection, but not *C. trachomatis* (Derre *et al.*, 2007). These findings are another illustration that the requirements for successful bacterial growth and survival differ between chlamydial strains.

Clearly, *Chlamydia* can access multiple sources of lipid precursors and nutrients within its host cell. Given its obligate intracellular lifestyle, any one path is unlikely to be essential for chlamydial survival. It would not be surprising if *Chlamydia* taps into multiple, redundant host membrane trafficking pathways as has been observed in other intracellular pathogens such as *Legionella pneumophila* (reviewed in Isberg *et al.*, 2009).

### Evasion of host defences

*Chlamydiae* manipulate signalling pathways to hinder the activation of innate immune responses that are detrimental to bacterial or host survival. Here we focus on the inhibition of apoptosis and the manipulation of NF- $\kappa$ B-mediated signalling.

#### *Inhibition of apoptosis*

The effect of *Chlamydia* infection on apoptotic signalling programs is complex. *Chlamydia* is postulated to regulate

apoptosis in a temporal manner to prevent the host cell from dying too early in infection and induce host-cell death late in the cycle (reviewed in Byrne and Ojcius, 2004; Ying *et al.*, 2007). The importance of proper temporal regulation of host cell death is illustrated by the fact that chlamydial development is impaired if host cell apoptosis is induced prematurely (Ying *et al.*, 2008).

*Chlamydia* blocks apoptosis primarily by inhibiting mitochondrial cytochrome c (cyt c) release (Fan *et al.*, 1998). The Bcl-2 family of proteins, which includes antiapoptotic Bcl-2 like proteins, pro-apoptotic BH3-only proteins and Bax/Bak, regulates cyt c release (reviewed in Hacker and Weber, 2007). *Chlamydia* induces degradation of BH3-only proteins (Dong *et al.*, 2005; Ying *et al.*, 2005), which likely leads to the observed reduction in Bax and Bak activation (Fischer *et al.*, 2004) and subsequent block in Cyt c release. The secreted chlamydial protease-like activity factor (CPAF) has been implicated in degradation of BH3-only proteins (Pirbhai *et al.*, 2006). However, cleavage of BH3-only proteins in cell lines engineered to express active recombinant CPAF occurs with kinetics distinct from canonical substrates and is prevented by the proteasome-specific inhibitor MG-132, suggesting that degradation occurs via a proteasome-dependent mechanism indirectly influenced by CPAF (Paschen *et al.*, 2008). Interestingly, a recent study did not detect BH3-only protein cleavage during infection (Rajalingam *et al.*, 2008).

Although its anti-apoptotic role is unclear, CPAF is emerging as a central immunoregulatory protein. CPAF downregulates MHC class I and II antigen presentation by degrading the transcription factors USF-1 and RFX-5 (Zhong *et al.*, 2001). Similarly, CPAF also contributes to the degradation of CD1d, a MHC-like protein important in lipid antigen presentation (Kawana *et al.*, 2007).

Since CPAF is synthesized mid-late in the infectious cycle (Belland *et al.*, 2003), it is unlikely to participate in the anti-apoptotic effects observed early in infection. Other potential anti-apoptotic mechanisms include: stabilization of inhibitor of apoptosis (IAP) proteins (Rajalingam *et al.*, 2006), and sequestration of pro-apoptotic phosphorylated BAD and protein kinase C $\delta$  (PKC $\delta$ ) at the inclusion (Tse *et al.*, 2005; Verbeke *et al.*, 2006). Sequestration of phosphorylated BAD is proposed to occur through interaction with 14-3-3 $\beta$ , which is recruited to the inclusion membrane (Scidmore and Hackstadt, 2001; Verbeke *et al.*, 2006). PKC $\delta$  is similarly recruited away from its normal site of action on mitochondria by binding to diacylglycerol-rich membranes at the inclusion periphery (Tse *et al.*, 2005). Increased expression of the anti-apoptotic protein Mcl-1 in infected cells has also been linked to activation of Raf/MEK/ERK (Rajalingam *et al.*, 2008), a signalling cascade that affects inflammatory responses (Buchholz and Stephens, 2007) and chlamydial lipid acquisition (Su *et al.*, 2004). The molecular basis

for these alternative anti-apoptotic pathways remains elusive; however, similar to membrane trafficking modulation, *Chlamydia* appears to have several redundant anti-apoptotic strategies.

#### Modulation of NF- $\kappa$ B signalling

Interference with NF- $\kappa$ B signalling is an emerging theme in chlamydial modulation of host immunity. The NF- $\kappa$ B transcription factor regulates several facets of host innate and adaptive immunity (Hayden *et al.*, 2006). Recent findings present new mechanisms for NF- $\kappa$ B inhibition by chlamydial effectors.

The NF- $\kappa$ B subunits RelA (p65) and p50 form a heterodimeric complex that is translocated into the nucleus and acts as a transcriptional activator (Hayden *et al.*, 2006). During *Chlamydia* infection, RelA is proteolyzed by the *C. trachomatis* Tsp-like protease (Ct441), and NF- $\kappa$ B nuclear translocation is blocked. Furthermore, ectopic expression of Tsp prevents TNF- $\alpha$ -induced NF- $\kappa$ B activation in human cells (Lad *et al.*, 2007).

*Chlamydia* may also block NF- $\kappa$ B activation by regulating ubiquitin-mediated protein degradation. In the canonical NF- $\kappa$ B activation pathway, nuclear translocation is dependent on the degradation of its inhibitor I $\kappa$ B $\alpha$  via ubiquitin-mediated proteolysis (Sun and Ley, 2008). Two *C. trachomatis* proteins, ChlaDub1 and ChlaDub2, are effectors with deubiquitinating and deneddylating activity (Misaghi *et al.*, 2006). Ectopically expressed ChlaDub1 binds to I $\kappa$ B $\alpha$  and inhibits its ubiquitination thereby suppressing degradation and subsequent NF- $\kappa$ B activation (Negrate *et al.*, 2008). Although *C. pneumoniae* lacks ChlaDub1, IL-17-induced NF- $\kappa$ B activation can be suppressed by Inc protein Cp0236 sequestration of NF- $\kappa$ B activator 1 (Act1) (Wolf *et al.*, 2009). As more effector proteins are identified, additional steps in NF- $\kappa$ B activation will likely emerge as targets of inhibition by *Chlamydiae*.

#### Inclusion expansion

The developing inclusion must expand to accommodate increasing bacterial numbers. Inclusion growth is likely fueled by attainment of nutrients and lipid precursors from the host cell. Unlike other intracellular pathogens that remain individually surrounded by tight membrane compartments, the inclusion is a large, relatively spacious organelle. The structural integrity of the inclusion is preserved by a meshwork of host cytoskeletal structures primarily composed of F-actin and intermediate filaments (IFs). The Head domains of IFs surrounding the inclusion are progressively cleaved by CPAF-generating structural changes in the filaments as the inclusion ages. The purpose of inclusion stabilization is unclear but may limit

cytoplasmic exposure of luminal contents. This would benefit the pathogen by reducing activation of innate immune responses via microbial pattern recognition receptors (Kumar and Valdivia, 2008).

#### EBs exit and dissemination

At developmental cycle completion, EBs must exit the cell to initiate subsequent rounds of infection. Egress can occur via two discrete mechanisms. Cell lysis involves the sequential disruption of inclusion and cellular membranes by cysteine proteases, and the host cell is destroyed. Alternatively, the inclusion can remain membrane-bound and be pushed out, or 'extruded', from the host cell. This process is dependent on actin-polymerization and myosin, and the host cell is often left intact (Hybiske and Stephens, 2007b).

One chlamydial effector protein, *Chlamydia* protein associating with death domain, or CADD, can interact with TNF receptor death domains and induce Fas-related apoptosis upon ectopic expression (Stenner-Liewen *et al.*, 2002). Yet, the biological relevance of this is unknown since host cell-death induction at the late developmental stages appears non-apoptotic (Ying *et al.*, 2006). Interestingly, ectopic expression of CPAF in uninfected cells induces morphological changes that mimic phenotypes of *Chlamydia*-induced cell death, suggesting that it may contribute to this process *in vivo* (Paschen *et al.*, 2008).

#### Perspectives

Despite the absence of classical genetic tools and the lack of a cell-free culture system, functional and comparative genomic approaches, combined with high throughput RNAi screens, have shed new light on the biology of these ancient pathogens. With the recent exciting reports of efficient DNA exchange among *Chlamydiae* in experimental systems (DeMars and Weinfurter, 2008) and successful electroporation of recombinant DNA (Binet and Maurelli, 2009), research progress will likely accelerate. Unlike other host-pathogen systems where cellular microbiology had to catch up with genetics, *Chlamydia* investigators have a large set of well-defined phenotypes awaiting the identification of mutants.

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