

Chlamydial Intracellular Survival Strategies

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Chlamydia trachomatis is the most common sexually transmitted bacterial pathogen and the causative agent of blinding trachoma. Although *Chlamydia* is protected from humoral immune responses by residing within remodeled intracellular vacuoles, it still must contend with multilayered intracellular innate immune defenses deployed by its host while scavenging for nutrients. Here we provide an overview of *Chlamydia* biology and highlight recent findings detailing how this vacuole-bound pathogen manipulates host–cellular functions to invade host cells and maintain a replicative niche.

Chlamydiae are Gram-negative obligate intracellular bacterial pathogens responsible for a range of diseases of clinical and public health importance. For instance, distinct serovariants of *Chlamydia trachomatis* cause a gamut of diseases, including blinding trachoma (serovars A–C), urogenital tract infections leading to urethritis, cervicitis, and proctitis (serovars D–K), and systemic lymphogranuloma venereum (LGV) disease (serovars L1–L3) (Schachter 1999). In genital tract infections, *C. trachomatis* primarily replicates within the epithelium of the urethra of men and endocervix of women causing inflammation, edema, and mucosal discharge. Ascending uterine infections can lead to pelvic inflammatory disease (PID), tubal scarring, ectopic pregnancies, and infertility (re-

viewed in Bebear and de Barbeyrac 2009; Haggerty et al. 2010).

Chlamydia displays a biphasic developmental cycle (Fig. 1). The elementary body (EB) form of the bacteria attaches and invades host-epithelial cells. The nascent phagocytic compartment is rapidly modified by *Chlamydia*-derived proteins to generate a parasitophorous vacuole termed an inclusion. Within the inclusion, the EB differentiates into a reticulate body (RB), the metabolically active and replicative form of the pathogen. RBs divide by binary fission as the inclusion expands and midway through the infectious cycle begin to asynchronously differentiate back into the EB form. Newly formed EBs are eventually released by cell lysis and/or extrusion to initiate new rounds

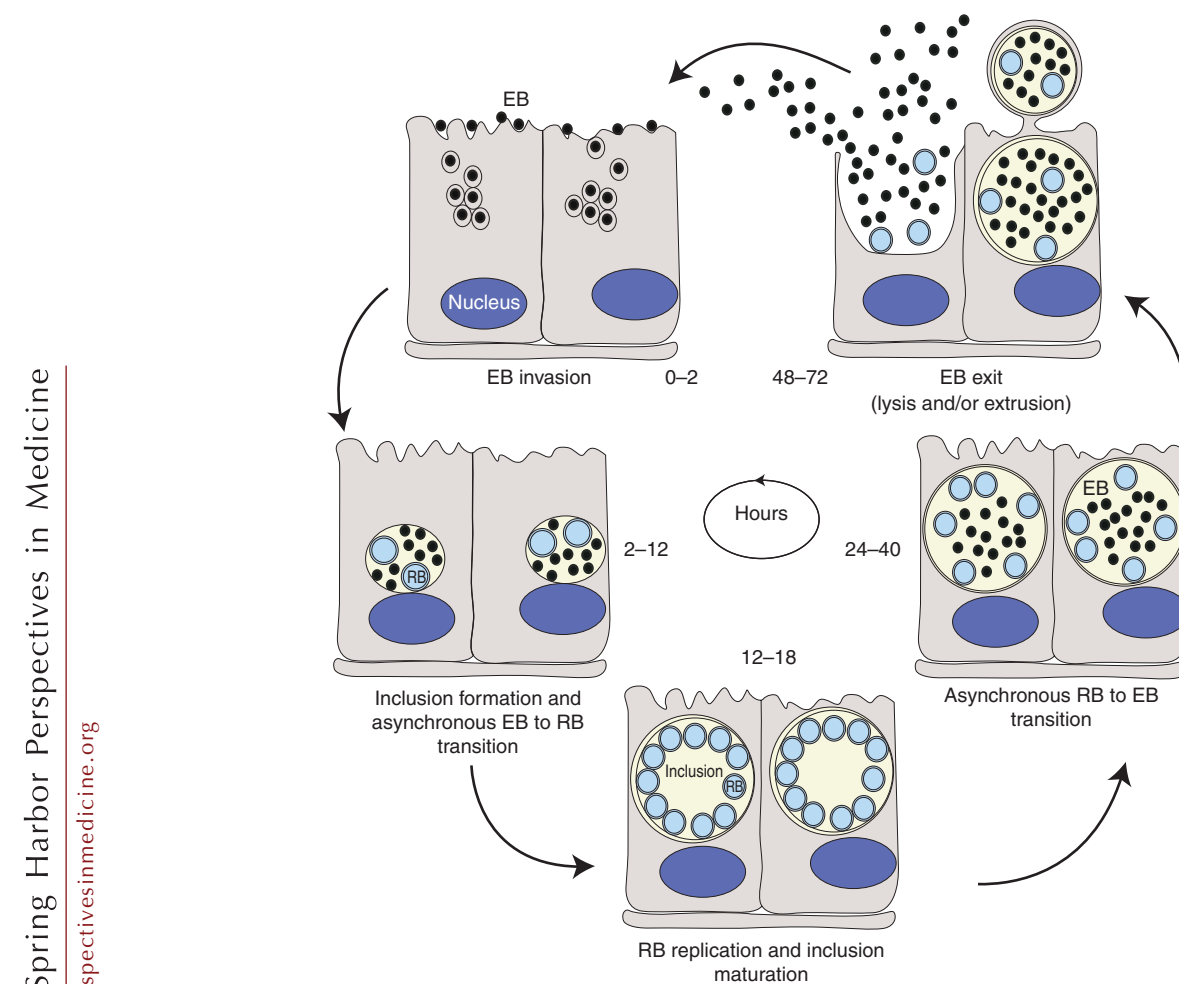
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R.J. Bastidas et al.



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Figure 1. Life cycle of *Chlamydia*. Within the first 2 h following internalization into cells, elementary bodies (EBs) fuse to form a nascent inclusion. Between 2 and 6 h postinternalization, EBs begin to differentiate into reticulate bodies (RBs). By 12 h postinfection (hpi) RBs can be observed dividing by binary fission and by 18–24 hpi they peak in numbers. Increasing numbers of RBs differentiate back to EBs around 24 hpi and continue differentiating until lysis or release occurs ~48–72 hpi depending on the chlamydial species (Hatch 1999).

of infection (reviewed in Dautry-Varsat et al. 2005; Hybiske and Stephens 2007b).

At all stages of infection, *Chlamydia* translocates “effector” proteins directly into host membranes and cytoplasm for manipulation of host–cellular functions, including signal transduction pathways (Valdivia 2008). Here we discuss novel strategies used by *Chlamydia* to generate an environment conducive for intracellular replication and emerging avenues for dissecting chlamydial pathogenic strategies.

CELLULAR BASIS FOR THE INTERACTION OF *Chlamydia* AND HOST CELLS

Mechanisms of *Chlamydia* Invasion of Epithelial Cells

Chlamydiae can invade most cultured cells, suggesting that the receptor(s) facilitating invasion are ubiquitous or that multiple receptors can be used. Binding is thought to be a two-step process for some species, involving an initial reversible interaction between the EB and the host



cell mediated by heparan sulfate proteoglycans (HSPGs), followed by high-affinity irreversible binding to a secondary receptor (Dautry-Varsat et al. 2005). In addition to heparan sulfate (Chen and Stephens 1994, 1997; Chen et al. 1996; Wuppermann et al. 2001), the mannose receptor, the mannose 6-phosphate receptor, and the estrogen receptor have been proposed to act as host receptors for *Chlamydia* entry (reviewed in Cocchiario and Valdivia 2009). Cell surface-exposed protein disulfide isomerase (PDI) has also been shown to play an important role in EB attachment and entry (Abromaitis and Stephens 2009).

Recent studies have revealed an unexpected role for growth factors and their receptors in *Chlamydia* binding (Elwell et al. 2008; Kim et al. 2011). A genome-wide loss-of-function RNAi screen in *Drosophila* S2 cells identified a potential role for the platelet-derived growth factor receptor (PDGFR), a transmembrane tyrosine kinase, in *Chlamydia* binding. In mammalian cells, depletion of PDGFR β or inhibition with PDGFR β -neutralizing antibodies reduces bacterial binding (Elwell et al. 2008). In subsequent studies, fibroblast growth factor 2 (FGF2) was shown to bind to EBs, where it appears to function as an HSPG-dependent bridging molecule that enhances interactions between EBs and the FGF receptor (FGFR) (Kim et al. 2011). Remarkably, bacterial binding stimulates production and release of FGF2 through a pathway that is independent of FGFR stimulation although dependent on bacterial protein synthesis and activation of the ERK1/2 signaling pathway (Kim et al. 2011). These events establish a positive-feedback loop that may further propagate bacterial binding and spreading to surrounding cells.

Multiple bacterial adhesins and ligands have been proposed to mediate invasion and their use may vary depending on both the host-cell type and the chlamydial species (Cocchiario and Valdivia 2009). These include glycosaminoglycan (Menozzi et al. 2002), the major outer membrane protein (MOMP) (Su et al. 1996), OmcB (Fadel and Eley 2007), and PmpD (Mölleken et al. 2010). OmcB from *Chlamydia pneumoniae* but not from *C. trachomatis* has an identifiable

heparan sulfate-binding domain that, when expressed on the surface of yeast, is necessary for OmcB binding to HSPGs (Moelleken and Hegemann 2008). In the absence of a system for targeted inactivation of *Chlamydia* genes, it has been difficult to confirm the relative contribution of these putative ligands to *Chlamydia* attachment and entry.

Chlamydiae entry into nonphagocytic cells is mediated by small GTPase-dependent reorganization of the actin cytoskeleton (reviewed in Hackstadt 2000; Dautry-Varsat et al. 2005; Cocchiario and Valdivia 2009; Carabeo 2011; Scidmore 2011). Rac1 is required for *C. trachomatis* entry (Carabeo et al. 2004), whereas Cdc42 and Arf6 are also required for *Chlamydomydia caviae* internalization (Subtil et al. 2004; Balana et al. 2005). Rac1 activation results in the recruitment of the actin regulators WAVE2, Abi-1, and Arp2/3, which are necessary for *C. trachomatis*-induced actin reorganization (Carabeo et al. 2007). Both chlamydial and host proteins may function synergistically to promote invasion. In the EB, the type III secretion system (a multi-protein needlelike delivery system) (reviewed in Beeckman and Vanrompay 2010), is poised for immediate discharge of effectors on contact with the host cell. At least one early secreted effector, TARP, contributes to bacterial internalization by its ability to directly nucleate actin polymerization through a WH2 actin-binding domain mimic (Jewett et al. 2006) and by recruiting the guanine nucleotide exchange factors (GEFs) Sos1 and Vav2, which activate Rac1 and signal to the actin machinery (Carabeo et al. 2007; Lane et al. 2008). This latter mechanism is only relevant in chlamydial species in which the polymorphic TARP gene (Clifton et al. 2005) can be targeted for tyrosine phosphorylation by Abl, Src, and Syk kinases (Elwell et al. 2008; Jewett et al. 2008; Lane et al. 2008; Mehltitz et al. 2008, 2010). Microinjection of cells with antibodies directed to the actin-binding domain of TARP before infection significantly reduces bacterial invasion, providing the most conclusive support for a direct role of TARP in mediating bacterial entry (Jewett et al. 2010). In addition, several host-tyrosine kinases, including PDGFR, FGFR, and Abl kinase are phosphorylated upon infection,

R.J. Bastidas et al.

recruited to the site of bacterial attachment, and may function redundantly in entry (Elwell et al. 2008; Kim et al. 2011). Therefore, the entry process is complex, requiring a concerted activation of growth factor receptors, cytoplasmic tyrosine kinases, small GTPases, and TARP to synergistically remodel the actin cytoskeleton and promote bacterial uptake. The actin rearrangements that occur during entry are transient and may be terminated by secreted chlamydial effectors such as CT166, which glucosylates Rac1 (Thalmann et al. 2010), or CT694, which interacts and colocalizes with AHNAK, an actin-binding protein (Hower et al. 2009). Additional host factors that contribute to uptake into nonphagocytic cells include clathrin (Boleti et al. 1999; Hybiske and Stephens 2007a) and cholesterol-rich microdomains (Norkin et al. 2001; Jutras et al. 2003; Stuart et al. 2003; Gabel et al. 2004).

Creating a Unique Niche

Following entry, EBs are sequestered within a membrane-bound compartment, termed the inclusion. The inclusion then quickly dissociates from the endolysosomal pathway, traffics along microtubules to a peri-Golgi location, and avoids fusion with lysosomes (reviewed in Hackstadt 2000). Early studies failed to identify host proteins on the inclusion membrane, leading to a model wherein the inclusion was viewed as an isolated compartment with little to no interactions with other host-cell organelles (reviewed in Fields and Hackstadt 2002). However, recent studies reveal a complex set of interactions between the chlamydial inclusion and host-cell trafficking pathways to facilitate acquisition of essential host-derived nutrients, such as amino acids, lipids, and iron, while limiting detection by the innate immune system. The list of host proteins that associate with the inclusion is rapidly growing; however, the mechanism by which these proteins are recruited to the inclusion has only been elucidated in a few cases.

Transport to the Peri-Golgi Region

Soon after entry, nascent inclusions are transported along microtubules to the microtubule

organizing center (MTOC) in a dynein-dependent but dynactin-independent manner (Claussen et al. 1997; Grieshaber et al. 2003; Grieshaber et al. 2006), suggesting that one or more unknown bacterial effectors mimic the cargo-binding activity of dynactin and tether the inclusion to dynein and/or to centrosomes. Src family kinases are required in human-adapted *Chlamydia* strains for inclusions to migrate to the MTOC and for intracellular growth, even though they are dispensable for binding and entry (Mital and Hackstadt 2011). Recent studies indicate that the inclusion membrane is not homogenous and that microdomains consisting of a subset of inclusion proteins (Incs) (IncB, Inc101, Inc222, and Inc850), active Src family kinases, and cholesterol, associate with centrosomes and dynein (Mital et al. 2010; Mital and Hackstadt 2011). One or more of these Incs may recruit active Src family kinases to the inclusion to promote microtubule nucleation, dynein-dependent movement, and sphingomyelin acquisition (Mital et al. 2010; Mital and Hackstadt 2011). The inclusion maintains a dynein-dependent association with the centrosome throughout the cell cycle, resulting in supernumerary centrosomes, abnormal spindle poles, and segregation defects (Grieshaber et al. 2006; Johnson et al. 2009). These findings are intriguing in light of the association of *Chlamydia* and human papilloma virus-associated cervical cancer (Wallin et al. 2002).

Interactions with Regulators of Membrane-Trafficking Pathways

The inclusion interacts with a number of host-cell molecules that are involved in membrane trafficking, fusion, and organelle identity. A subset of these are Rab GTPases including Rabs 4, 11, and 14, which are associated with recycling endosomes, and Rabs 1, 6, and 10, which are associated with ER-Golgi traffic (Rzomp et al. 2006; Brumell and Scidmore 2007; Moorhead et al. 2007, 2010; Capmany and Damiani 2010). Rabs 6 and 10 are recruited to inclusions in a species-specific manner, whereas recruitment of Rab1, 4, and 11 appear to be ubiquitous to all *Chlamydiae* (Rzomp et al. 2003). The



C. trachomatis Inc protein CT229 has been identified as a Rab4-interacting protein, although it is not conserved in other strains or serovars that recruit Rab4, suggesting redundancy in Inc protein function (Rzomp et al. 2006). In the case of *C. pneumoniae*, Cpn0585 is thought to be responsible for the selective recruitment of Rab1, 10, and 11 (Cortes et al. 2007). In some instances, Rab-binding proteins are directly recruited to the inclusion membrane. Bicaudal D1 (BICD1), a Rab6-interacting protein, is recruited in a Rab6-independent manner, suggesting that an Inc protein may directly recruit BICD1 (Moorhead et al. 2007). The recruitment of Rabs and their associated proteins likely promotes selective interaction/fusion with host vesicles containing essential nutrients. Rab6, 11, and 14 play a role in the acquisition of sphingomyelin by the inclusion (Rejman Lipinski et al. 2009; Capmany and Damiani 2010), whereas Rab4 and Rab11 participate in iron acquisition by regulating a slow transferrin recycling pathway intercepted by the inclusion (Rzomp et al. 2003, 2006; Ouellette and Carabeo 2010). Rab6 and Rab11 facilitate sphingomyelin transport to the inclusion by regulating fragmentation of the Golgi into ministacks during infection (Heuer et al. 2009; Rejman Lipinski et al. 2009).

Phosphoinositides are also key determinants of membrane identity and vesicle fusion (Poccia and Larijani 2009), and several proteins associated with phosphoinositol-4-phosphate (PI4P) metabolism are recruited to the inclusion. These include oculocerebrorenal syndrome of Lowe protein 1 (OCRL1), phosphatidylinositol 4-kinase (PI4KII α), and Arf1 (a GTPase that recruits PI4K enzymes and PI4P-binding proteins to the Golgi) (Moorhead et al. 2010; Elwell et al. 2011). Depletion of OCRL1, PI4KII α , and Arf1 reduces inclusion formation and the production of infectious progeny, suggesting that generation of PI4P at the inclusion plays an important role in infection (Moorhead et al. 2010). Recruitment of Arf1 is intriguing, as it also regulates actin dynamics and vesicle trafficking at the Golgi (D'Souza-Schorey and Chavrier 2006), and thus may play multiple roles during infection.

In addition to recruiting Rab GTPases, *Chlamydia* may regulate fusion with host vesicles by recruiting host SNAREs, which are key components of intracellular fusion machinery (Sudhof and Rothman 2009). In support of this notion, at least two Golgi-specific SNAREs, including Syntaxin 6 and GS15 (Moore et al. 2011; Pokrovskaya et al. 2012), as well as vesicles containing the endocytic SNAREs, Vamp3, Vamp7, and Vamp8, are recruited to the inclusion (Delevoe et al. 2008; Paumet et al. 2009). Interestingly, a subset of Incs contains motifs that display similarities to eukaryotic SNAREs, including Inca, CT813, and CT223 (Delevoe et al. 2008; Paumet et al. 2009). It is speculated that these SNARE-like domains pair with host-SNARE proteins on target vesicles to assemble a four-helix bundle that provides the energy to promote fusion of opposing membranes. Indeed, Inca, along with CT813, binds to host SNAREs, Vamp3, 7, and 8, suggesting that host SNAREs may work in concert with Inca to regulate membrane fusion (Delevoe et al. 2004, 2008; Paumet et al. 2009). However, in vitro liposome fusion assays indicate that instead of promoting fusion with endocytic compartments, Inca acts as an inhibitory SNARE to limit fusion with these compartments (Paumet et al. 2009). Inca has also been shown to participate in homotypic fusion of individual inclusions, as naturally occurring Inca-deficient variants or infected cells microinjected with anti-Inca antibodies display an aberrant multilobed inclusion structure (Hackstadt et al. 1999; Delevoe et al. 2004; Xia et al. 2005).

Interactions with Host-Cell Organelles for Acquisition of Host Nutrients

Early studies established a close association of the *Chlamydia* inclusion with the Golgi apparatus from which it intercepts sphingomyelin and cholesterol-containing exocytic vesicles by a Brefeldin A (BFA)-sensitive vesicular-trafficking pathway (Hackstadt et al. 1996; Carabeo et al. 2003). BFA inhibits activation of Arf1 (a key player in vesicle formation) by targeting its activators, the GEFs GBF1 and BIG1/2 (Claude et al. 1999; Mansour et al. 1999; Yamaji et al.

R.J. Bastidas et al.

2000; Kawamoto et al. 2002), which are localized to the *cis*- and *trans*-Golgi, respectively, and have distinct functions at the Golgi (Manolea et al. 2008). Recent studies reveal that *Chlamydia* uses GBF1 (but not BIG1/2) to acquire sphingomyelin, which is necessary for inclusion membrane growth and stability but not bacterial replication (Elwell et al. 2011). Nonvesicular trafficking may also play a major role in lipid acquisition by *Chlamydia* (Derre et al. 2011; Elwell et al. 2011). Ceramide transfer protein (CERT), a cytosolic protein that transports ceramide from the ER to the *trans*-Golgi (Hanada 2010), is recruited to the inclusion membrane, possibly by interaction with the inclusion membrane protein IncD (Derre et al. 2011), and is involved in sphingomyelin acquisition (Elwell et al. 2011). Depletion of CERT significantly reduces the production of infectious progeny (Derre et al. 2011; Elwell et al. 2011). It is speculated that CERT mediates the transfer of ceramide from the ER to the inclusion, perhaps at ER-inclusion membrane contact sites, to generate specialized metabolic and/or signaling platforms (Derre et al. 2011). In addition, at least one of the two sphingomyelin synthase enzymes, which converts ceramide to sphingomyelin (SM) (Huitema et al. 2004), is recruited to the inclusion membrane (Elwell et al. 2011), raising the possibility that ceramide may also be converted to SM at the inclusion membrane, thereby creating a localized SM biosynthetic factory at the inclusion membrane. These observations shed some light on the paradox that although host-sphingomyelin biosynthesis is required for progeny production and inclusion biogenesis (van Ooij et al. 2000; Robertson et al. 2009), blocking vesicular transport with BFA has no inhibitory effect on the production of infectious progeny (Hackstadt et al. 1996). Acquisition of sphingomyelin by BFA-sensitive vesicular and nonvesicular pathways may play distinct functional roles, with BFA-sensitive vesicular transport participating in inclusion biogenesis and nonvesicular transport playing a key role in bacterial replication (Elwell et al. 2011). Host glycerophospholipids, such as phosphatidylinositol and phosphatidylcholine, are also acquired through a nonvesicular trans-

port pathway by a process that requires activation of ERK and cytosolic phospholipase A2 (Wylie et al. 1997; Hatch and McClarty 1998; Su et al. 2004).

In addition to the Golgi, the inclusion interacts with other host-cell organelles, including (1) multivesicular bodies (MVBs) (Beatty 2006, 2008; Robertson et al. 2009), which may also serve as a source of sphingolipids and cholesterol, (2) lipid droplets, which are translocated into the inclusion lumen following their capture by the chlamydial proteins Lda1 and/or Lda3 and which may serve as a source of neutral lipids (Kumar et al. 2006; Cocchiario et al. 2008), (3) mitochondria (Matsumoto et al. 1991; Derre et al. 2007), and (4) lysosomes, which may be a source of essential amino acids derived from host-protein degradation (Ouellette et al. 2011). Together, these interactions (summarized in Fig. 2) likely facilitate acquisition of nutrients for bacterial replication as well as for inclusion membrane stability and expansion.

Exiting the Host Cell

At the end of the developmental cycle, EBs are released by two mutually exclusive mechanisms: cell lysis and extrusion (Hybiske and Stephens 2007b). Cell lysis involves the sequential disruption of the inclusion and cellular membranes by cysteine proteases (Hybiske and Stephens 2007b). In contrast, the extrusion of inclusions requires actin polymerization, myosin, the Rho family GTPase RhoA, and leaves the host cell intact (Hybiske and Stephens 2007b). EB exit from *C. trachomatis* serovar E-infected cells is accompanied by lysosome-mediated repair of the plasma membrane (Beatty 2007).

MODIFYING THE HOST RESPONSE

Detection of *Chlamydia* by the Host

As with most bacteria, *Chlamydia* infections are detected by host pattern recognition receptors (PRRs) that recognize chlamydial LPS via TLR4 (Ingalls et al. 1995; Prebeck et al. 2001, 2003; Heine et al. 2003) and heat shock protein Hsp60 through TLR2 and TLR4 (Kol et al. 1999,

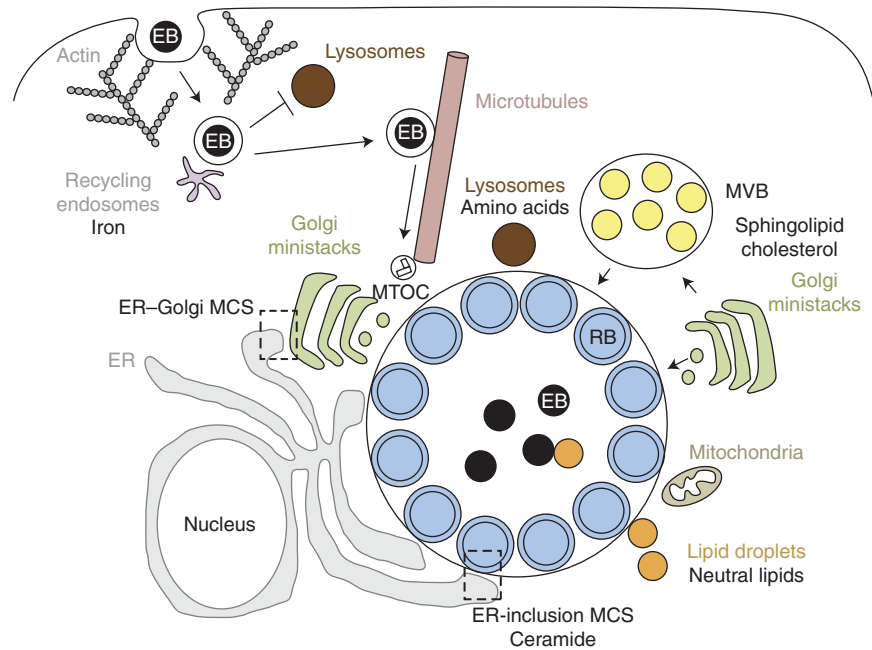


Figure 2. *Chlamydia* and host–cellular interactions. *Chlamydia* enters host cells by an actin-dependent mechanism that involves both host and bacterial factors. The EBs are enclosed within a membrane-bound inclusion, which becomes modified by insertion of bacterial inclusion proteins (Incs) that prevent fusion with lysosomes and promote interactions with recycling endosomes for iron acquisition. The nascent inclusion is transported along microtubules to the MTOC and EBs transition into RBs. The inclusion expands to accommodate the replicating RBs. During this time, the inclusion interacts, likely via bacterial effectors, with multiple host-cell organelles, including fragmented Golgi ministacks, the ER, lipid droplets, MVBs, and the mitochondria. Delivery of essential host lipids to the inclusion involves vesicular trafficking from Golgi ministacks and MVBs as well as nonvesicular trafficking from lipid droplets and membrane contact sites (MCS) formed between the ER and the inclusion. Although the inclusion does not fuse with lysosomes, *Chlamydia* may import essential amino acids derived from host-protein degradation within lysosomes.

2000; Vabulas et al. 2001; Bulut et al. 2002; Costa et al. 2002; Bulut et al. 2009). TLR2 appears to be the predominant receptor required for an inflammatory response to infection (Prebeck et al. 2001; Darville et al. 2003; O’Connell et al. 2006). Interestingly, TLR2 and its adaptor MyD88 localize to the periphery of the chlamydial inclusion during active infection, suggesting that TLR2 may signal intracellularly during infection (Mackern-Oberti et al. 2006; O’Connell et al. 2006).

In addition to TLRs, cytosolic PRRs recognize chlamydial PAMPs. RNAi-mediated knockdown of NOD1, or its downstream adaptor RIP2, in infected epithelial and endothelial cells ablates expression of inflammatory cyto-

kines (Opitz et al. 2005; Welter-Stahl et al. 2006; Buchholz and Stephens 2008). Similarly, *Nod1*^{-/-} and *Rip2*^{-/-} mice show significantly delayed clearance of *C. pneumoniae* from their lungs (Shimada et al. 2009). Presumably, *Nod1* is activated by trace amounts of peptidoglycans (PGNs), and even though PGN components such as *N*-acetylmuramic acid have not been detected in *Chlamydiae* above trace levels, the full complement of genes required for PGN biosynthesis and assembly are present in *Chlamydiae* genomes (Stephens et al. 1998; Kalman et al. 1999; Read et al. 2000). Interestingly, in place of an abundant layer of PGNs, the cell envelope of EBs is comprised of a highly cross-linked latticework of outer membrane proteins

R.J. Bastidas et al.

(MOMP, OmcA, OmcB, and others) (reviewed in Hatch 1996), many of which are highly immunogenic (Wang et al. 2010) and could possibly be recognized as PAMPs by cytosolic receptors. In addition, infected cells induce type I IFNs, which can activate cell-autonomous resistance mechanisms to *Chlamydia* (Bernstein-Hanley et al. 2006). The ligands responsible for inducing the expression of type I IFNs are unknown, but the recently reported role for STING in IFN expression (Prantner et al. 2010) during *Chlamydia* infection point to a role for bacterial nucleic acids as potential ligands.

Antimicrobial molecules such as reactive oxygen species are also synthesized in response to infections (reviewed in D'Autreaux and Toledano 2007), and K^+ efflux triggered by *Chlamydia* infection activates reactive oxygen species (ROS) production (Abdul-Sater et al. 2009; Boncompain et al. 2010; He et al. 2010). ROS production also leads to activation of caspase 1 through the NLRP3-ASC inflammasome and processing of the proinflammatory cytokines IL-1 β and IL-18 in *Chlamydia*-infected cells (Rothermel et al. 1989; Entrican et al. 1999; Lu et al. 2000; Gervassi et al. 2004; Abdul-Sater et al. 2009; He et al. 2010).

Initiating Innate Immune Responses

Early during *Chlamydia* infection there is an acute localized inflammatory response largely mediated by polymorphonuclear leukocytes (PMNs) and mononuclear leukocytes, recruited by cytokines and chemokines released by infected epithelial cells (Kelly and Rank 1997; Rank et al. 2000, 2008). Proinflammatory cytokines, chemokines, and interferons such as IL-1 α , IL-1 β , GM-CSF, IL-8, and IFN- α and γ are secreted by *Chlamydia*-infected cells in in vitro culture systems (Table 1). Activation of MEK-ERK (independent from the p38 and Jun amino-terminal MAPK pathways) and Nod1 signaling pathways, as well as nuclear IL-1 α , contribute to cytokine production by infected epithelial cells (Buchholz and Stephens 2007, 2008; Cheng et al. 2008).

Cytokine/chemokine profiles have also been measured in vivo using a mouse model of geni-

tal tract infections with *Chlamydia muridarum* (Table 1). As early as 3 h postinfection, genes encoding chemokines (CCL20, CCL3, and CCL24) that recruit immature dendritic cells (DCs) are up-regulated, as well as those encoding for TNF- α and C3 (alternative complement). Therefore, early responses prime the site of infection for immature DC infiltration, which is an important step of the adaptive response (Rank et al. 2010). By 12 h postinfection, expression of chemokines that are chemotactic for natural killer (NK) cells (CXCL9-11, CCL2-4, CCL7, CCL8, and CCL12) is significantly induced, which is in agreement with the observed recruitment of NK cells to the site of infection (Tseng and Rank 1998; Rank et al. 2010). Not surprisingly, the proinflammatory cytokines IL-1 α , IL-1 β , IL-1F6, and IL-1F8 are also expressed as are PMN-recruiting chemokines (CXCL1, CXCL5, and CXCL15) (Rank et al. 2010).

The recruitment of immune cells that mediate innate and adaptive immune responses to *Chlamydia* infections are, under most circumstances, sufficient for bacterial clearance during primary infections, which may explain why most infections are asymptomatic. However, inflammatory responses resulting from recurring infections or when bacteria access sterile sites (i.e., the upper genital tract) contribute to the detrimental scarring and pathology observed in some infected individuals (Stephens 2003; Darville and Hiltke 2010).

Subversion of Host-Innate Immune Response

Despite the inflammatory response generated by the host during primary *Chlamydia* infection, in some individuals *C. trachomatis* infections can persist asymptotically for months before being spontaneously cleared (Parks et al. 1997; Golden et al. 2000). How infection can persist for so long is not well understood. *Chlamydia* uses a wide range of strategies that may potentially subvert the host-immune responses, including interfering with the function of the transcription factor NF- κ B (p65/RelA), a central regulator of immune responses. NF- κ B functions downstream from PPRs and proinflammatory cytokine receptors such as the

Table 1. Chemokines and cytokines produced during chlamydial infection

	Mouse genital tract			Cell types			
	<i>Chlamydia muridarum</i>			<i>C.trachomatis</i>		<i>C.pneumoniae</i>	
	3 hpi	12 hpi	24 hpi	HeLa	PBMC	PBMC	BMM
Chemokine receptors							
CCR1							
CCR2							
CCR6							
Chemokines							
CCL2 (MCP-1)							
CCL3 (MIP-1 α)							
CCL4 (MIP-1 β)							
CCL5 (RANTES)							
CCL20 (MIP-3 α)							
CCL24							
CXCL1 (GRO- α)							
CXCL4 and 5							
CXCL8 (IL8 [h], MIP-2 [m])							
CXCL9 (MIG)							
CXCL10 (IP-10)							
CXCL11 (I-TAC)							
Cytokines							
IFN- α							
IFN- γ							
IL-1 α							
IL-1 β							
IL-6							
IL-10	NE	NE	NE				
IL-11							
IL-12							
IL-13							
IL-18	NE	NE	NE				
TGF- β 1							
TNF- α							
GM-CSF							

Induced chemokines and cytokines are represented as gray boxes.

Data adapted from Kaukoranta-Tolvanen et al. 1996; Rasmussen et al. 1997; Dessus-Babus et al. 2000; Geng et al. 2000; Netea et al. 2004; Rothfuchs et al. 2004; Rank et al. 2010.

PBMC, peripheral blood mononuclear cell; BMM, murine bone marrow-derived macrophage; IL-8 (h), human CXCL8 homolog; MIP-2 (m), murine CXCL8 homolog; NE, not expressed.

TLR/IL-1R and tumor necrosis factor (TNF) family of receptors. Signaling is initiated by adaptor proteins belonging to a family of TNF receptor-associated factors (TRAF) such as TRAF2 and TRAF6. In the canonical pathway, a family of I κ B proteins maintains NF- κ B homo- or heterodimers sequestered in the cytoplasm. Activation of the pathway triggers phos-

phorylation of I κ B and subsequent ubiquitination and proteasome-dependent degradation of I κ B proteins culminating in NF- κ B release, nuclear translocation, and expression of hundreds of genes, including innate immune response factors (reviewed in Chen 2005).

Chlamydia blocks degradation of the NF- κ B retention factor I κ B α and nuclear translocation



R.J. Bastidas et al.

of NF- κ B (Lad et al. 2007a). This activity has been attributed to *Chlamydia* proteins possessing deubiquitinating (DUB) and deneddylating activity, ChlaDub1 and ChlaDub2, which may potentially interfere with host-ubiquitination pathways (Misaghi et al. 2006). Ectopic expression of ChlaDub1 in HeLa cells impaired ubiquitination and degradation of I κ B α on stimulation with a variety of NF- κ B agonists including TNF- α and IL-1 β (Le Negrate et al. 2008). ChlaDub1 did not affect ubiquitination of TRAF2 or TRAF6, or I κ B α phosphorylation, indicating that ChlaDub1 activity does not interfere with upstream components of the pathway. However, ChlaDub1 coprecipitates with I κ B α suggesting that ChlaDub1 activity may very well be directed at I κ B α (Le Negrate et al. 2008).

Chlamydia can also block NF- κ B activation directly through the proteolysis of the p65/RelA subunit of NF- κ B (Lad et al. 2007a). Initially this activity was attributed to the chlamydial tail-specific (TSP) endoprotease CT441 (Lad et al. 2007a,b). Although CT441 shows robust human p65 proteolytic activity, it is unclear whether CT441 can access the cytoplasm of the host cell where it could engage its target. Instead, a chlamydial protease named CPAF (chlamydial protease-like activity factor) has been recently implicated in the degradation of p65 during infection (Christian et al. 2010). Although CPAF is an extensively characterized protease with numerous potential substrates of importance to innate immunity (reviewed in Zhong 2011), a recent report suggests that several proteins targeted by CPAF, including the p65/RelA subunit of NF- κ B, RFX5, Bim, and Puma (discussed below), may not be bona fide CPAF substrates in in vivo settings (Chen et al. 2012).

Chlamydia has also evolved a mechanism to limit the recognition of PAMPs by ensuring the stability of the inclusion during the intracellular stage of infections. This is achieved by reorganizing actin and intermediate filaments at the periphery of the inclusion (Kumar and Valdivia 2008). Disruption of these cytoskeletal elements results in inclusion membrane instability and leakage of inclusion luminal contents into the

host-cell cytoplasm. As a result, there is an increase in IL-8 gene transcription (Kumar and Valdivia 2008).

CPAF may also play a role in limiting the presentation to immune cells of chlamydial antigens by major histocompatibility complexes (MHCs). MHC class I and II complexes present bacterial antigens to CD8⁺ and CD4⁺ T lymphocytes, respectively (reviewed in Germain 1994). To limit detection by CD8⁺ and CD4⁺ T cells, *Chlamydia* promotes CPAF-dependent degradation of the transcription factors RFX5 and USF-1, two upstream transcriptional regulators of IFN- γ -induced MHC I and II genes (Zhong et al. 1999, 2000). CPAF also stimulates degradation of CD1d, an MHC-like protein complex that stimulates NK and NKT immune cells (Kawana et al. 2007, 2008). In parallel, CPAF also cleaves a subset of chlamydial secreted effectors and inclusion membrane proteins, potentially curtailing their availability as substrates for antigen presentation (Jorgensen et al. 2011). These multiple potential mechanisms used by *Chlamydia* to dampen immune responses are summarized in Figure 3.

Maintaining the Host Cell Alive

Cell death on stimulation by death-inducing ligands secreted by immune cells or in response to cellular cytopathic signals is a common cell-autonomous response to infection (Ying et al. 2006). *Chlamydia* interferes with multiple proapoptotic pathways as well as potential necrotic cell death to guarantee survival within host cells (Fan et al. 1998; Fischer et al. 2001; Rajalingam et al. 2001; Yu et al. 2010). A prominent mechanism for preventing cell death includes CPAF-mediated degradation of the proapoptotic BH3-only proteins Bad, Bim, and Puma (Fischer et al. 2004; Dong et al. 2005; Ying et al. 2005; Pirbhai et al. 2006). This activity is proposed to be responsible for the reduced activation of Bax and Bak and subsequent block in cytochrome *c* release observed during infection (Fan et al. 1998; Fischer et al. 2004; Xiao et al. 2004). However, new observations indicate that Bim and Puma may not be targeted for degradation during infection (Chen et al. 2012). These differences will

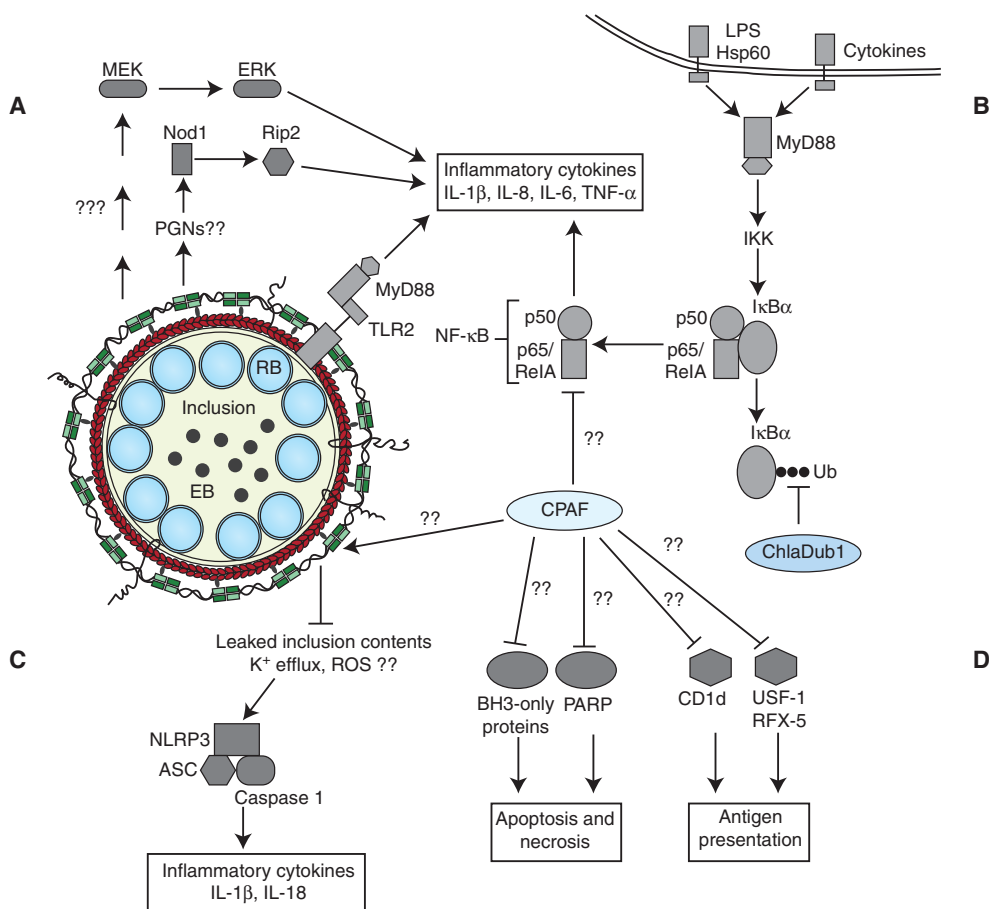


Figure 3. Current model of chlamydial effector interactions with host-innate immune signaling pathways. (A) TLR2 and MyD88 are trafficked to the inclusion by unknown mechanisms and promote expression of inflammatory cytokines. Nod1 and MEK-ERK MAPK signaling pathways also stimulate inflammatory cytokine gene expression. (B) Chlamydial microbial products and cytokines secreted by recruited immune cells and neighboring infected cells activate Toll-like and cytokine receptors. Receptor signaling activates NF- κ B signaling and expression of proinflammatory cytokines/chemokines. Established inclusions impede NF- κ B signaling by reversing I κ B α ubiquitination through the activity of secreted ChlaDub1 and possibly by CPAF-mediated degradation of p65/RelA. (C) A cytoskeleton cage comprised of actin (red) and intermediate filaments (green) envelops the established inclusion. Remodeling of the cage curtails inclusion contents from activating cytosolic PRRs, possibly the NLRP3-ASC-caspase 1 inflammasome. However, unknown chlamydial agonists can activate the NLRP3-ASC inflammasome and caspase 1-dependent processing of inflammatory cytokines. (D) Additional mechanisms facilitating host survival may include the degradation of BH3-only proteins and PARP (Yu et al. 2010) and blocking death-inducing signaling pathways presumably originating from the TNF family of receptors and cytokine receptors. CPAF activity may also be deployed by *Chlamydia* to subvert antigen presentation.

need to be reconciled in the future. In parallel, infection also leads to stabilization of IAP2 (inhibitor of apoptosis protein 2) and up-regulation of the prosurvival factor Mcl-1 (myeloid cell leukemia) (Rajalingam et al. 2006, 2008; Sharma et al. 2011). Other mechanisms poten-

tially include sequestration of Bad at the inclusion via 14-3-3 β , and of protein kinase C- δ (PKC δ) through binding to diacylglycerol-enriched membranes in the vicinity of the inclusion (Scidmore and Hackstadt 2001; Tse et al. 2005; Verbeke et al. 2006).

R.J. Bastidas et al.



In addition, *Chlamydia* contends with the antimicrobial function of IFN- γ , which is secreted by infiltrating immune cells. IFN- γ induces tryptophan depletion by increasing host-indoleamine 2,3-dioxygenase (IDO) expression (reviewed in Taylor and Feng 1991). Tryptophan depletion is detrimental to *Chlamydia* because most strains are tryptophan auxotrophs. In response to this stress, *Chlamydia* transitions into quiescent noninfectious aberrant structures termed the “persistent” form (Beatty et al. 1994; Schoborg 2011). Interestingly, cells infected with these chlamydial forms are still resistant to various forms of apoptosis, using unknown mechanisms that are largely independent of CPAF (Dean and Powers 2001; Rodel et al. 2011).

Infection activates the MAPK and PI3K pro-survival signaling pathways. The ERK MAPK signaling pathway appears to be a central regulator for bacterial nutrient acquisition, expression of antiapoptotic factors, and synthesis of proinflammatory cytokines (Su et al. 2004; Buchholz and Stephens 2007; Rajalingam et al. 2008). Epithelial cells display sustained activation of upstream components of the ERK signaling module including RAS and RAF (Su et al. 2004; Gurumurthy et al. 2010; Vignola et al. 2010). However, this activation of ERK is uncoupled from activated RAS and RAF during infection (Gurumurthy et al. 2010; Vignola et al. 2010), suggesting multiple signaling pathways are engaged to further *Chlamydia* replication and intracellular survival. Avoidance of cell death and promoting self-survival are complex and very likely temporally regulated strategies deployed during infection and reinfection.

GENETICS AND GENOMICS OF *Chlamydia*

The genomes of a wide range of *Chlamydiae* serovars and species have been sequenced. Chlamydial genomes are small (<2 Mb), with few repetitive elements and pseudogenes, reflecting their long-term coadaptation to life within eukaryotic hosts (Collingro et al. 2011). Indeed, many of the genes required for the biosynthesis of key metabolites have been lost, presumably as these are now directly acquired from the host. The dearth of genes encoding DNA compe-

tence, conjugation, phages, or DNA modification systems in pathogenic *Chlamydiae* is consistent with that of an organism undergoing very limited genetic exchange (Stephens et al. 1998). Surprisingly, recent observations indicate that *Chlamydia* can exchange DNA efficiently with other strains. A genomic analysis of clinical *Chlamydia* isolates provided the first evidence of chimeric strains (Gomes et al. 2007). Such in vivo recombinant strains can have significant clinical implications. For instance, a hypervirulent *C. trachomatis* strain (L_{2c}) isolated from a patient with severe hemorrhagic proctitis represents a recombination event between serovar L2 and serovar D strains (Somboonna et al. 2011).

In landmark experiments, Robert DeMars provided evidence of DNA exchange among *Chlamydiae* in the laboratory (Demars et al. 2007; DeMars and Weinfurter 2008). In cells coinfecting with *C. trachomatis* serovars that were naturally resistant to ofloxacin or to rifampin, doubly drug-resistant recombinant *Chlamydia* arose at $\sim 10^4$ times higher frequency than that of spontaneous mutations, and the genomes of these recombinants were chimeric recombinants of the parental strains. Whole genome sequencing of recombinants from additional crosses provided evidence that recombination events ranged from 40 kb to 790 kb and that the sizes of recombination sites range from a short 11-bp sequence to a 366-bp stretch (Suchland et al. 2009). The mechanisms underlying the release of DNA, uptake, and recombination are largely unknown.

Introduction of recombinant DNA into *Chlamydia* has been of limited success until very recently. Early efforts achieved transient transformation of chimeric *C. trachomatis* (pCT) plasmids (Tam et al. 1994). In later experiments, *Chlamydia psittaci* was transformed with allelic exchange vectors that targeted the 16S rRNA gene, and the investigators were able to show successful replacement of 16S rRNA loci with the introduced DNA (Binet and Maurelli 2009). Very recently, Clarke and colleagues reported successful stable transformation of *C. trachomatis* with a pCT shuttle plasmid by calcium chloride-mediated transformation of EBs

and selection for resistance to β lactams (Wang et al. 2011). These stable transformants can express a variety of recombinant genes including the green fluorescent protein. Although in its present form the transformation and selection procedures are not very efficient, these experiments represent a significant breakthrough in our ability to experimentally manipulate these organisms.

Despite the lack of molecular genetic tools to manipulate *Chlamydiae*, significant advances have been made to experimentally address the role of individual genes in pathogenesis. This includes gain-of-function approaches, such as expressing *Chlamydia* genes in heterologous systems to identify proteins that modulate host functions (Sisko et al. 2006) or to identify proteins that can be secreted by surrogate type III secretion systems (Subtil et al. 2001). Loss-of-function studies have also recently become possible with the implementation of targeting-induced local lesions in genomes (TILLING), an approach used extensively in plant and zebrafish to screen for mutants in genes of interest (reviewed in Stemple 2004). In this method, PCR fragments that span the gene in which one wishes to identify mutations are generated from wild-type and mutagenized strains. DNA heteroduplexes are then generated by annealing these fragments, and if the gene amplified from the mutants strains harbors a nucleotide variant in the target gene, the annealed PCR products will generate a mismatch that can be recognized and cleaved by the endonuclease CEL I (reviewed in Stemple 2004). Kari and colleagues applied TILLING in EMS-mutagenized *Chlamydia* to identify mutations, including null alleles, in the tryptophan synthase operon *trpBA* (Kari et al. 2011). Such approaches enable the implementation of reverse genetic approaches by identifying strains with specific mutations. In a complementary approach, a system that uses the concept of “phenotype sequencing” (Harper et al. 2011) has been recently implemented in *Chlamydia* to perform forward genetic screens. In this approach, the genomes of EMS-derived mutants with similar phenotypes (“granular” plaque morphologies) were sequenced and a common gene (*glgB*-encoding glycogen

branching enzyme) was found to be mutated in a majority of strains (Nguyen and Valdivia 2012). By taking advantage of genetic exchange among *Chlamydia* strains during coinfections, recombinant strains were generated in which a *glgB* mutation was the only gene lesion inherited from the parental mutant strain, thus unambiguously linking this mutation to a glycogen granule accumulation plaque morphotype. The ability to screen and select for mutants, mapping the underlying genetic lesions and generating congenic strains by recombination now opens up a broad range of possibilities for the genetic analysis of *Chlamydia* virulence.

CONCLUDING REMARKS

The obligate intracellular lifestyle of *Chlamydia* offers a unique opportunity to understand how the host-immune system contends with invading pathogens. *Chlamydia* species translocate a staggering number of protein effectors into the mammalian host cell to coopt cellular processes. Elucidating the function of these effectors will lead to new insights as to how pathogens subvert their hosts and on the function of the cellular processes they target. Despite the absence of classical genetic tools to study these ancient organisms, recent advances in deep sequencing technologies, the implementation of high-throughput RNAi screens, and comparative genomics approaches have broadened our understanding of the biology of these pathogens. Observations of widespread DNA exchange among *Chlamydia* organisms in experimental settings coupled with exciting new reports of *Chlamydia* plasmid transformation techniques will likely lead to major advances in our molecular understanding of chlamydial pathogenesis in the near future.

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R.J. Bastidas et al.

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R.J. Bastidas et al.



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Chlamydial Intracellular Survival Strategies

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