

Unraveling the basic biology and clinical significance of the chlamydial plasmid

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Chlamydial plasmids are small, highly conserved, nonconjugative, and non-integrative DNA molecules that are nearly ubiquitous in many chlamydial species, including *Chlamydia trachomatis*. There has been significant recent progress in understanding chlamydial plasmid participation in host–microbe interactions, disease, and immune responses. Work in mouse model systems and, very recently, in nonhuman primates demonstrates that plasmid-deficient chlamydial strains function as live attenuated vaccines against genital and ocular infections. Collectively, these studies open new avenues of research into developing vaccines against trachoma and sexually transmitted chlamydial infections.

Human pathogenic chlamydiae are members of a successful and unique lineage of bacteria (Collingro et al., 2011), which infect and cause disease in a wide variety of animals (Longbottom and Coulter, 2003). Although antibiotic chemotherapy is quite effective in treating diagnosed primary uncomplicated ocular and urogenital chlamydial infections, prevention of the serious pathological sequelae, which can develop after infection, and prevention of reinfection are much more difficult tasks (Byrne, 2010). Most chlamydial infections are asymptomatic and thus go undiagnosed and untreated, which can lead to chronic inflammation and irreversible tissue and organ pathology. In some countries and communities, limited access to medical care and infection screening technologies further hampers timely diagnosis and treatment (Centers for Disease Control and Prevention [CDC], 2011). Repeat infections are common, and the risk of developing the serious sequelae of ocular (e.g., blindness) or urogenital infection (e.g., infertility) increases with multiple infections (Darville and Hiltke, 2010).

These obligate intracellular bacteria develop within a membrane-bound vacuole termed the inclusion (Fig. 1), and existence within the inclusion defines much about the biology of the lineage. The challenges to understanding and preventing chlamydial disease are amplified by the difficulties of working with an obligate intracellular bacterial species, in which the development of a practical system of genetic manipulation is only in its infancy. (Binet and Maurelli, 2009; Kari et al., 2011).

The chlamydial plasmid

In most microbial systems, autonomously replicating plasmids function as tools of genetic variation and are built with replicative backbones associated with a variety of functional cassettes encoding niche-specific genes or gene sets. Often these plasmids can be horizontally transferred, which leads to rapid distribution or reassociation of phenotypes among strains (Koonin et al., 2001). The spread of antibiotic resistance is often a function of sharing of plasmids (or other laterally transferred genetic elements) among otherwise susceptible strains. Such plasmid transfer is a major and evolving problem in infectious disease worldwide. Many serious diseases are directly the result of virulence factors carried by plasmids, and variation in plasmid structure is often associated with different disease

syndromes caused by otherwise related strains and species.

Such variability is not found in chlamydia. Several chlamydial species contain one of a homologous set of ~7,500 base pair plasmids with a copy number that is approximately four-fold greater than that of the chromosome (Thomas et al., 1997; Pickett et al., 2005; Fig. 1). Within *C. trachomatis* clinical isolates, the plasmid is virtually ubiquitous. There are occasional studies showing plasmid-negative clinical strains, but little is known about the epidemiology and significance of these relatively rare isolates (Peterson et al., 1990; An et al., 1992; Farencena et al., 1997). Chlamydial plasmids are nonconjugative and nonintegrative. They do not encode antibiotic resistance genes and do not show signs of genetic flexibility. This plasmid and the equally curious chlamydiaophage found in some strains (Storey et al., 1989) represent the entire complement of extrachromosomal elements carried by *Chlamydia spp.* There is a single example of an integrated plasmid in *Chlamydia suis* (Dugan et al., 2004), but this is clearly the exception and not the rule.

Nucleotide sequence identity among plasmids from *Chlamydia spp.* ranges from 69 to 99%, with very high identity within *C. trachomatis* (Thomas et al., 1997; Seth-Smith et al., 2009). Sequence comparisons suggest that the plasmids evolved in parallel with the different chlamydial species. The *C. trachomatis* plasmid carries a set of five open reading frames (ORFs), which share identity with episomal maintenance genes common to other plasmids, and a set of

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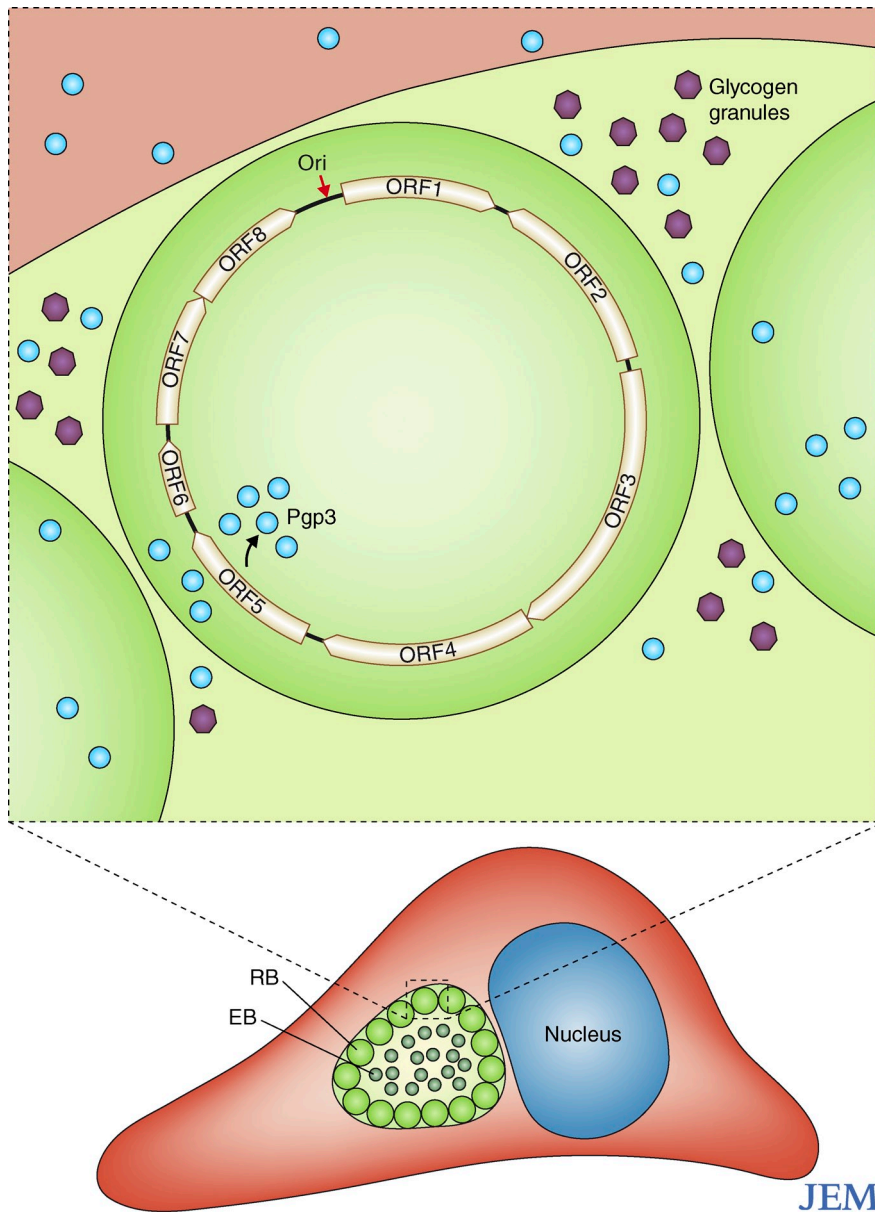


Figure 1. Chlamydial growth and plasmid structure. The obligately intracellular chlamydiae develop within a host vacuole termed the inclusion. The two developmental forms have different functions in growth. Elementary bodies (EB) are infectious but minimally metabolically active, whereas reticulate bodies (RB) grow and divide, but cannot infect. As shown in a magnified reticulate body, the chlamydial plasmid has eight ORFs, which encode plasmid maintenance and chlamydia-specific functions. ORF5 encodes Pgp3, which is secreted from the bacterium and the inclusion and accumulates in the cytosol of the host cell. Pgp3 is immunogenic in many host species. The chlamydial plasmid is also associated with the accumulation of glycogen granules (purple) in the lumen of the inclusion and, as discussed in the text, functions in virulence in animal model systems.

three genes encoding proteins of unknown function, each of which is chlamydia specific. Human strains of *Chlamydomphila pneumoniae* lack plasmid, but equine *C. pneumoniae* strains and other veterinary chlamydial species are plasmid positive.

The chlamydial plasmid also has a practical side. It is a common target for nucleic acid amplification test (NAAT)-based diagnostics of human infections (Fredlund et al., 2004). NAAT-based tests have been, and remain, a valuable aspect of chlamydial disease control.

However, clinical isolates with a deletion in the target sequence used in some diagnostic kits (the Swedish Variant) have been identified. This can lead to false negative test results in patients (Unemo and Clarke, 2011). Studies have reported an apparent clonal expansion of *C. trachomatis* isolates carrying this deletion. It is possible that the inability to detect infections caused by strains carrying the deleted plasmid has contributed to the rapid expansion of the variant strains in patient populations.

As mentioned, the lack of routine genetic tools has been a significant barrier to rapid progress in chlamydial research. Manipulation of the chlamydial plasmid is a logical approach to introduce DNA into these organisms, but such efforts have been frustrating and generally unproductive. However, a very recent study describes successful transformation experiments using hybrid shuttle vectors built with pBR325 and the entire chlamydial plasmid (Wang et al., 2011). The vectors are propagated in both *E. coli* and *C. trachomatis*, and the system was used to generate green fluorescent *C. trachomatis*. This is an exciting and highly significant development in the study of chlamydiae.

The chlamydial plasmid appears to be critical for growth in vivo (Russell et al., 2011), but it is not required for growth in vitro. Multiple laboratories have described phenotypic changes associated with plasmid-deficient strains as compared with closely related wild type strains. Creation of plasmid-deficient isogenic strains began with the careful work of Akira Matsumoto, who developed a plaque-purification technique for chlamydial strains (Matsumoto et al., 1998) and noted that unlike wild-type *C. trachomatis* strains, three of his plaque-purified clones of *C. trachomatis* did not accumulate glycogen within the inclusion. Each glycogen-negative clone was then shown to lack the chlamydial plasmid. Subsequent investigations of plasmid-negative strains (O'Connell and Nicks, 2006; Carlson et al., 2008; O'Connell et al., 2011) confirmed the absence of glycogen and examined transcription across the chlamydial genome relative to wild-type strains.

Although the precise scope and nature of plasmid-chromosome cross-regulation is not fully understood, transcription of a set of chlamydial chromosomal genes is negatively affected by the absence of plasmid. One *C. trachomatis* gene that is down-regulated in the plasmid-negative strains is *glgA*, a glycogen biosynthetic gene. Thus, it is hypothesized that elements encoded by the plasmid regulate, and perhaps directly participate in, the process of glycogen accumulation within chlamydial inclusions.

Linking disease and vaccines to the chlamydial plasmid

Subsequent in vivo studies using plasmid-deficient strains demonstrated that the plasmid is a virulence factor that significantly impacts clinical disease in mouse model systems. For example, although plasmid-negative strains grew similarly to isogenic wild-type strains, they failed to activate Toll-like receptor 2-dependent immune responses either in vivo or in vitro (O'Connell et al., 2011). Other work indicated that plasmid-negative *C. trachomatis* strains had significantly higher 50% infective dose values relative to a wild-type matched isolate (Carlson et al., 2008). The plasmid-deficient strains also functioned as successful attenuated live vaccines in mice, wherein prior infection with plasmid-negative strains limited the pathology associated with a subsequent infection by wild-type strains (O'Connell et al., 2007).

The near universal carriage of this single plasmid by *C. trachomatis* has led to speculation about its importance in human chlamydial infections. Early work demonstrated that chlamydia-infected individuals produce antibodies specific for at least one plasmid-encoded protein, Pgp3, which is encoded by ORF5 (Comanducci et al., 1994). Further work explored the possible utility of Pgp3 as a vaccine candidate (Donati et al., 2003). Recent work has shown that native Pgp3 is a trimeric molecule that is transported to the host cell cytosol during the infectious process (Li et al., 2008; Chen et al., 2010; Fig. 1). Other chlamydial proteins are delivered to the cytosol, where they function to turn the host cell into a "chlamydia factory,"

and it is hypothesized that Pgp3 serves a similar function.

Chlamydial vaccine studies are complicated by the possibility that the debilitating pathology associated with chlamydial ocular and urogenital infections represents collateral damage of innate and/or adaptive immune responses (Rockey et al., 2009; Farris and Morrison, 2011). Protection, in contrast, is thought to be serovar or genovar specific, but neither the mechanisms of pathogenesis nor the mechanisms of protection have been clearly elucidated. Several candidate protective antigens have been explored (Rockey et al., 2009), but no molecule has emerged as a clear and practical choice as a single protective antigen. Work in the mouse system suggests that delivery of antigens, or a combination of antigens, is required to generate a protective response. Indeed, a live attenuated veterinary chlamydial vaccine, generated via chemical mutagenesis, is commercially available and routinely used to reduce the incidence of abortion in sheep (Burall et al., 2009).

In this issue of the *Journal of Experimental Medicine*, Kari et al. describe experiments demonstrating that a plasmid-deficient *C. trachomatis* strain is both avirulent in ocular infections of macaques and functions as a live attenuated vaccine in that system. These fascinating studies conclusively demonstrate that the plasmid functions as a virulence factor in *C. trachomatis* ocular infections, consistent with the work conducted in the genital tract in the murine model system. In addition to the colonization studies, Kari et al. demonstrated that three of the six animals infected with the plasmid-negative strain were completely protected against challenge by an isogenic and virulent plasmid-positive strain. Analysis of the major histocompatibility complex genotypes of each animal demonstrated that all three of the solidly protected animals shared a common M1 haplotype, suggesting a potential role for CD4⁺ T cells in the observed differential protection.

What role does the plasmid play in chlamydial infections and why is it maintained as an extrachromosomal element in this pathogen? Although the latter

question remains unaddressed, it appears that some aspect of chlamydial plasmid biology is strongly selected for during successful infection or transmission of the pathogen within a host population (Russell et al., 2011). It is likely that this competitive advantage tips the scale toward pathogenesis in human infections. In addition, as repeated and/or persistent chlamydial infections are strongly associated with severe pathology, it is likely that inflammatory responses elicited by the bacterium are associated with serious disease sequelae. Thus, the plasmid may encode or control expression of proteins that are responsible for successful bacterial growth and/or of the stimulation of a deleterious immune response. The search for deleterious chlamydial antigens has a long history, and genus-common antigens are thought to be major players in this pathogenetic response. Perhaps a major immunopathogenetic determinant is encoded by the plasmid, or the gene products encoded/controlled by the plasmid allow persistence to a level that facilitates deleterious responses to other chlamydial macromolecules. Under the latter model, absence of the plasmid would allow the bacterium to be cleared before an immunopathogenic response can be generated or amplified. Alternatively, glycogen accumulation might be a metabolic equivalent of a virulence factor, providing energy stores as chlamydiae fight to grow and survive in a hostile host environment.

Although the mechanisms of attenuated virulence and/or of immune protection are not clearly defined by Kari et al., this study is a seminal contribution to the field of chlamydia pathogenesis and forms the basis for continued exploration of the role of the plasmid in the chlamydial infectious process. Future studies, perhaps encouraged by this work, will need to address the challenging issues of antigenic variation and host genetic variation in designing vaccine strategies.

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