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Chlamydial Plasmid-Dependent Pathogenicity

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Abstract

Most *Chlamydia* species carry a 7.5kb plasmid encoding 8 open reading frames conventionally called plasmid glycoproteins 1–8 or pGP1–8. Although the plasmid is not critical for chlamydial growth *in vitro*, its role in chlamydial pathogenesis is clearly demonstrated in the genital tracts of mice infected with *Chlamydia muridarum*, a model for investigating the human pathogen *Chlamydia trachomatis.* Plasmid-free *C. trachomatis* is also attenuated in both the mouse genital tract and nonhuman primate ocular tissue. Deficiency in pGP3 alone, which is regulated by pGP4, largely reproduced the *in vivo* but not *in vitro* phenotypes of the plasmid-free organisms, suggesting that pGP3 is a key *in vivo* virulence factor. The positive and negative regulations of some chromosomal genes by pGP4 and pGP5 respectively may allow the plasmid to promote chlamydial adaptation to varied animal tissue environments. The focus of this review is to summarize the progress on the pathogenic functions of the plasmid-encoded open reading frames, which may motivate further investigation of the molecular mechanisms of chlamydial pathogenicity and development of medical utility of the chlamydial plasmid system.

Introduction of the Chlamydial Plasmid

Chlamydiaceae [1], a family of obligate intracellular bacteria, consist of multiple species for colonizing different host species, including *Chlamydia trachomatis* and *Chlamydia pneumoniae* for humans, *Chlamydia suis* for pigs, *Chlamydia muridarum* for mice, *Chlamydia caviae* for guinea pigs and *Chlamydia felis* for cats while the species *Chlamydia psittaci*, *Chlamydia pecorum* and *Chlamydia abortus* can infect a range of animals from chicken to cattle and sheep. The *Chlamydia psittaci* organisms can also be transmitted to human respiratory tract to cause severe pneumonia. However, the most significant medical concern for humans is caused by *Chlamydia trachomatis*, particularly via the sexually transmitted infection (http://www.cdc.gov/std/tg2015/chlamydia.htm). Although the initial infection occurs in the lower genital tract, if not treated appropriately, *Chlamydia trachomatis* can ascend to the upper genital tract to induce inflammatory pathology, which may result in tubal fibrosis and hydrosalpinx (accumulation of fluids in the fallopian tube), leading to ectopic pregnancy or infertility [2]. Despite the extensive research on chlamydial

biology, it remains unknown how chlamydial organisms cause pathologies and infertility in humans. Since most chlamydial organisms isolated from humans contain a highly conserved plasmid, the chlamydial plasmid is thought to contribute to chlamydial pathogenicity in humans.

More than 35 years ago, a 7.5kb cryptic plasmid was described for *Chlamydia* [3]. Since then, extensive efforts have been made to characterize the plasmid. The plasmid has been identified in many chlamydial species and strains [4], such as pCTA, pCTT1, pCHL1, pSW2, pLVG440/pL1 and pLGV2/pL2 from *Chlamydia trachomatis* serovars A [5], B [6], D [7], E [8], L1 [9] and L2 [10] respectively, pMoPn/pCM from *Chlamydia muridarum* Nigg strain [4, 11], pCpnE1 from *Chlamydia pneumoniae* N16 strain [12], pCpA1 from *Chlamydia psittaci* avian strain N352 [4], pCfe1 from *Chlamydia felis* Fe/C-56 strain [13] and pCpGP1 from *Chlamydia caviae* GPIC strain [14]. These plasmids, although isolated from chlamydial species that infect different animal host species, are highly conserved and all code for 8 putative open reading frames (ORFs) designated as pORF1 to 8 [4]. The pORF immediately downstream of the plasmid origin of replication was designated as pORF1 or pCDS1. However, there has been some confusion about the pORF annotation in the literature. pORF1 is also referred to as pCT7 (for *C. trachomatis* plasmid) or pGP7 [15] and as a result, pORF5 or pCDS5 is also pCT3 or pGP3 [4].

Based on sequence homology, pORF1 & 2 (pGP7 & 8) encode proteins probably involved in plasmid replication while pORF3 (pGP1) is homologous to a helicase involved in unwinding double stranded DNA during replication. The function of the protein encoded by pORF4 (pGP2) is unknown, but is 345-254 amino acids in length (depending on host strain). pORF5 (pGP3) encodes a 28 kDa protein [16]. pORF6 (pGP4) encodes a protein of 101–102 amino acids with unknown function while pORF7 & 8 (pGP5 & 6) could encode proteins involved in regulating partitioning and copy number [4].

By using deletion and introducing premature termination mutation analyses, it is now known that pGP1, 2 & 6 are important for plasmid maintenance while pGP3, 4, 5, 7 & 8 are not [17–19]. pGP4 is a master positive regulator of both plasmid-encoded and some chromosomal genes [17, 18] while pGP5 as a negative regulator [19]. pGP3, 7 or 8 has minimal impact on other gene expression [17–19]. pGP3 deficiency largely phenocopied plasmid-deficiency when infecting animals [20, 21], indicating a key role of pGP3 in plasmid-dependent pathogenicity.

In addition, the plasmid also encodes two small antisense RNAs, sRNA-2 (sRNA-2, anti-sense to ORF2/*pgp8*) and sRNA-7 (anti-sense to ORF7/*pgp5*) [22, 23]. sRNA-2 was found to be most abundantly expressed among all the *C. trachomatis* plasmid pORFs [22]. Plasmid depleted of *por*f2/*pgp8* gene, lack of both pGP8 protein and sRNA-2, was no longer recoverable [18], suggesting an important role of either pGP8 or sRNA-2 or both in plasmid maintenance. It will be interesting to directly evaluate whether plasmids with loss of function mutation in either sRNA-2 or -7 but without affecting the expression of the pORFs are viable. Viable mutants can be further evaluated their roles in promoting chlamydial colonization in animal tissues.

Finally, the plasmid is known to regulate more than a dozen of chromosomal genes including the genes involved in glycogen synthesis [24], which explains the lack of glycogen accumulation in plasmid-free chlamydial inclusions [17–19]. The plasmid transcriptional regulation capacity was further mapped to *pgp4* [17, 18] and *pgp5* [19] respectively.

While the plasmid may encode and regulate many virulence properties for *Chlamydia*, this review will mainly discuss the pathogenic roles of the plasmid-encoded pORFs that are dispensable for plasmid maintenance.

The Plasmid Contributes to Chlamydial Pathogenicity

The facts that the cryptic plasmid is widely distributed among many different chlamydial strains and that most clinical isolates contain the plasmid suggest that the plasmid offers advantages to the plasmid-laden chlamydial organisms. Furthermore, the plasmid sequence homology largely parallels with the chlamydial genome sequence homology [4, 8, 25], suggesting that the plasmid has been selected to adapt to the different chlamydial hosts. All 8 plasmid transcripts [26] are translated into proteins during chlamydial infection [27], although the precise functions of these proteins have not yet been clearly defined, suggesting that the plasmid may be maintained for fulfilling functions required for promoting chlamydial infection. However, when a plasmid-deficient *C. trachomatis* L2 isolated from a proctocolitis patient was compared with a wild type L2 strain for their *in vitro* growth in cultured cells, no apparent difference was noted [28], suggesting that the plasmid is at least not required for chlamydial growth *in vitro*. This conclusion was validated in a subsequent study in which a *C. trachomatis* urethral isolate (a serovar B variant) lacking the plasmid was successfully cultured and recovered [29]. Using a plaque-cloning assay [30], Miyashita *et al.* isolated plasmid-free *C. trachomatis* and found that the *C. trachomatis* organisms with or without the plasmid displayed a similar susceptibility to various chemotherapeutic agents [31, 32], indicating that the plasmid does not encode drug resistance genes. It was only when a *C. muridarum* strain artificially cured of plasmid was compared with a plasmid-competent *C. muridarum*, was the role of the plasmid noticed. A plasmid-free *C. muridarum* strain was unable to accumulate glycogen within the inclusions and formed smaller plaques compared to its wild-type parent [33]. It is now known that the small plaque size may be due to the inefficiency of the plasmid-free organisms to exit the infected cells rather than defects in entry or intracellular growth [34] while the lack of glycogen is due to the fact that the chromosomal genes required for glycogen synthesis are regulated by the plasmid [18, 19, 24]. More importantly, plasmid-free *C. muridarum* was no longer able to induce upper genital tract pathology hydrosalpinx (oviduct full of liquids due to lumenal occlusion) in C3H/HeouJ mice following an intravaginal inoculation [35]. The plasmid deficiency-mediated attenuation of pathogenicity was further reproduced in the genital tracts of multiple strains of female mice with both *C. muridarum* [36] and *C. trachomatis* [37, 38] and also in the ocular tissues of nonhuman primates with a *C. trachomatis* trachoma serovar [39]. These observations together have firmly established the 7.5kb cryptic plasmid as a chlamydial pathogenic determinant at least for the *C. muridarum* and *C. trachomatis* species.

On the other hand, despite the significant contributions of the plasmid to the chlamydial pathogenicity as discussed above, there are strong evidences that the chlamydial pathogenicity may also depend on the factors that are unrelated to the plasmid.

Not all pathogenic chlamydial species and strains carry the plasmid. The human chlamydial species *C. pneumonia* does not contain the plasmid but can still cause airway infection [40]. Various plasmid-deficient *C. trachomatis* strains have been isolated from humans [28, 29], suggesting that these *C. trachomatis* strains possess the ability to colonize human tissues independently of the plasmid. The role of plasmid in chlamydial pathogenesis is not always obvious. For example, plasmid-competent *C. caviae* GPIC was just as virulent as the plasmid-free GPIC [41]. The plasmid-free *C. trachomatis* organisms were found to be as infectious as the wild organisms in the genital tract of nonhuman primates [42]. Various chlamydial strains/isolates that are either deficient in the plasmid or carry a mutated plasmid have been identified [29], suggesting that the plasmid-encoded functions can be compensated by genes/proteins encoded elsewhere. Experimentally, the plasmid-free *C. muridarum* organisms induced robust pathology when inoculated into the uterine of CBA/1J mice [43], suggesting that the *C. muridarum* ability to induce tubal inflammatory pathologies in this mouse strain was encoded in the chromosome.

Various chromosomal gene mutations have resulted in significant alterations in chlamydial infectivity or pathogenicity in animals, which is independent of the plasmid. For example, gain of function mutations in the chromosomal genes CT135 (*C. trachomatis*) and TC0236 (*C. muridarum*) respectively resulted in enhanced chlamydial infectivity [44, 45]. Similarly, a loss of function mutation in the gene *ctl0233* (*C. trachomatis* L2) that codes for CPAF [46] reduced the L2 organism survival in the mouse lower genital tract [47] while mutations in *tc0668* and/or *tc0237* significantly attenuated the pathogenicity of the *C. muridarum* organisms [48, 49]. Studies based on the cell culture system have revealed many chromosomal genes that may play significant roles in chlamydial pathogenesis [50–56]. Further animal model evaluations of these gene products in chlamydial pathogenicity may dramatically expand the list of chlamydial virulence factors.

The Plasmid Promotes Chlamydial Ascension to and Induction of Inflammation in the Oviduct

Screening 11 strains of mice for susceptibility to *C. muridarum* induction of hydrosalpinx has revealed that both ascending infection and tubal inflammation are required for chlamydial induction of hydrosalpinx in mice [57]. Based on their hydrosalpinx incidence and severity following an intravaginal inoculation with a wild type *C. muridarum*, the 11 mouse strains were categorized into 3 groups: highly susceptible, susceptible or resistant. After an intrauterine inoculation that bypassed cervical barrier, most mice developed significantly more severe hydrosalpinx, indicating a critical role of the cervical barrier in protecting the oviduct from *C. muridarum* induction of pathology. Thus, the chlamydial ability to cross the cervical barrier and to ascend to the oviduct is a key pathogenic determinant. However, regardless of the *C. muridarum* inoculation locations, the A/J mice remained resistant to the hydrosalpinx induction, which correlated with a shortened tubal

inflammatory response [58], suggesting that tubal inflammation may be another determinant that can affect the disease outcome. The plasmid may contribute to both determinants. First, the plasmid-deficiency-mediated attenuation of pathogenicity correlated with reduced activation of Toll-like receptor 2 (TLR2) innate immunity signaling [35] and decreased ascending infection [35, 36]. Second, many mouse strains including CBA/1J were susceptible or highly susceptible to the *C. muridarum* induction of hydrosalpinx following an intravaginal inoculation [57] but the plasmid-free *C. muridarum* failed to induce any significant hydrosalpinx under the same infection condition, which correlated with significantly reduced ascension by the plasmid-free *C. muridarum* [36]. More importantly, by bypassing the cervical barrier, an intrauterine inoculation with the plasmid-free *C. muridarum* induced robust hydrosalpinx in the CBA/1J [48], indicating that the failure of the plasmid-free *C. muridarum* to induce hydrosalpinx after intravaginal inoculation was due to lack of ascending but not lack of the ability to cause tubal damage in the CBA/1J mice. These observations together have demonstrated a critical role of the plasmid in promoting *C. muridarum* to cross the CBA/1J mouse cervical barrier for inducing hydrosalpinx. Third, a significantly reduced inflammatory infiltration was noted in the oviduct tissues of mice infected with the plasmid-free *C. muridarum* [35, 36]. Finally, the plasmid-free *C. trachomatis* L2 was found to be less stimulatory in inducing inflammatory cytokines in the cultured cells [59]. The above observations and analyses suggest that the plasmid-dependent virulence factors may promote chlamydial pathogenicity by aiding in both chlamydial ascension and tubal inflammation. However, the molecular mechanisms remain unknown.

pGP3 is a Key Virulence Factor Encoded by the Plasmid

The chlamydial pGP3 was initially expressed as a 28kDa protein in a bacterial expression system and a rabbit antibody to the recombinant 28kDa localized pGP3 in the outer membrane complex [60]. The recombinant pGP3 was recognized in an ELISA but not Western blot by sera from 81% patients urogenitally infected with *C. trachomatis* [61]. The human serum recognition of pGP3 was later demonstrated to be dependent on the trimeric structure of pGP3 [62, 63]. The trimeric structure of pGP3 was recently determined at a high resolution [64], which revealed a C-terminal trimerization domain (Pgp3c) similar to the receptor binding domain of tumor necrosis factor α (TNFα), a triple helices middle domain (Pgp3m) and a N-terminal domain with a novel structural feature. Careful immunofluorescence assay with monoclonal antibodies raised against the trimeric pGP3 revealed pGP3 in the cytosol of *Chlamydia*-infected cells in addition to its outer membrane localization [27]. Due to the immunodominance in individuals infected with *C. trachomatis* [65, 66], pGP3 has been evaluated as a biomarker for monitoring the *C. trachomatis* exposure in humans [67, 68]. Furthermore, immunization of mice with pgp3 gene or pGP3 protein has been shown to induce protective immunity [69–71]. However, assessing the role of the plasmid-encoded pGP3 in chlamydial pathogenesis was made possible only after the first report on the successful transformation of *C. trachomatis* L2 [72], which has permitted the detailed characterization of the plasmid-encoded ORFs in cell culture [17, 73, 74] and animal models [21] and encouraged the development of various genetic engineering tools for investigating chlamydial biology and pathogenesis [51, 75–78]. The success in transforming *C. muridarum* [19] further enabled the evaluation of the plasmid-encoded pGPs in a female

mouse hydrosalpinx/infertility induction model [79–81]. *C. muridarum* deficient in pGP3 either by deletion of the *pgp3* gene or installation of a premature stop codon in the start region of the gene largely phenocopied the plasmid-free *C. muridarum* [20] although deficiency in pGP5 also reduced *C. muridarum* pathogenicity [82]. The failure of the pGP3-deficient *C. muridarum* to induce hydrosalpinx correlates with its reduced ascension to, decreased survival and attenuated induction of inflammatory infiltration in the oviduct [20].

The Molecular Mechanisms of pGP3-Mediated Pathogenicity

pGP3 is one of the few chlamydial virulence factors that are both well characterized biochemically [27, 62–64, 83] and clearly demonstrated in animal models [20]. However, the precise molecular mechanisms remain unknown. The immundominance of pGP3 in humans infected with *C. trachomatis* [65, 66] may suggest a functional need of the pGP3 protein for *C. trachomatis* colonization in human tissues, the mechanisms of which may be dissected using *C. muridarum* since pGP3 is a highly conserved protein among chlamydial species [27]. The successful transformation of *C. muridarum* [19] coupled with the optimized *C. muridarum* genital tract infection mouse model [20, 36, 57] may have provided the tools necessary for investigating whether and how pGP3 promotes chlamydial ascension and tubal inflammation.

Can pGP3 Promote Chlamydial Ascension?

C. muridarum can spread from the lower genital tract through the cervical barrier, into the endometrial cavity, through the uterotubal junction and into the oviduct, to induce hydrosalpinx. Inoculating *C. muridarum* directly into uterine by bypassing the cervical barrier resulted in more severe hydrosalpinx in mice [57, 84, 85], indicating an important role of cervical barrier in protecting the upper genital tract. However, it is unclear whether the uterotubal junction can also restrict chlamydial ascension although the junction is known to prevent other bacteria from entering the oviduct [86]. Successful ascension requires chlamydial spreading from cell to cell through cell lysis [87], which inevitably exposes the progeny chlamydial elementary bodies (EBs) to the extracellular environment where mucosal effectors can inactivate EBs. During genital tract infection, *Chlamydia*-infected epithelial cells are inflammatory [88, 89] and surrounded by neutrophils [90]. Soluble mucosal effectors such as α-defensins secreted by neutrophils [91, 92] and β-defensins by epithelial cells [92, 93] as well as LL-37/CRAMP from both [92] are detected during chlamydial infection [94–96]. These and other soluble effectors and cellular mechanisms such as phagocytosis [53] and apoptosis [97, 98] may all contribute to the prevention of chlamydial ascension. LL-37 is the C-terminal 37-residue peptide cleaved from hCAP18 (a 18kD human cationic antimicrobial protein), the only identified member of the cathelicidin family [92]. The mouse homologue of LL-37 is CRAMP (cathelin-related antimicrobial peptide [99]). These antimicrobial peptides (AMPs) regulated by the MyD88 pathway can kill microbial pathogens [96, 100]. Indeed, MyD88 deficiency significantly increased mouse susceptibility to *C. muridarum* ascending infection [101, 102]. It is reasonable to hypothesize that the MyD88-dependent mucosal effectors at either the cervical or uterotubal junction barriers may contribute to the prevention of chlamydial ascension, which may select

for chlamydial organisms to evade the MyD88-regulated effectors since AMPs have been shown to select other microbes to evade AMPs [103–105].

The plasmid-encoded pGP3 is a secreted antigen [27] that is both associated with tubal infertility in humans [106] and a known virulence factor for promoting *C. muridarum* ascension in mice [20]. *C. muridarum* with a premature stop codon installed in the *pgp3* gene (designated as CMUT3-pgp3S) is significantly attenuated in ascending. However, it is still unknown whether CMUT3-pgp3S is defective in crossing the cervical or uterotubal junction barriers or both. More importantly, since pGP3 or the Pgp3 middle helix region (pGP3m) have been shown to neutralize LL-37 *in vitro* [83, 107], it will be worth testing whether mice deficient in CRAMP, MyD88 or other innate immunity pathways can rescue the ascending infection of plasmid-free or pGP3-deficient *C. muridarum*.

Can pGP3 Promote Chlamydial Induction of Tubal Inflammation?

Tubal inflammation is another requirement for *C. muridarum* induction of hydrosalpinx [57]. Mice deficient in MyD88 [101] or TLR2 [108] developed severe hydrosalpinx 8 weeks or longer after infection, indicating that MyD88-mediated innate immunity signaling may not be required for the development of the long-lasting hydrosalpinx (but required for preventing ascending infection, see above). Instead, chlamydial organisms are known to activate many inflammatory pathways to selectively promote tubal inflammation since deficiency in some of these pathways resulted in reduced hydrosalpinx but without obvious effects on chlamydial infection course. These pathways include the cell surface receptor-mediated cytokine/chemokine pathways TNF [108, 109], interleukin-1 receptor (IL-1R) [110], IL-17 [111], CXCR2 [112], the intracellular signaling pathways involving caspase-1 [113, 114], STING [115, 116], Nod1 [114] and the NLRP3 inflammasome [117], and the others such as nitric oxidase synthase (iNOS) [118], matrix metalloproteases (MMPs) [119] and complement factor C5 [120]. The role of the tumor necrosis factor receptor 1 (TNFR1) in chlamydial pathogenesis [108, 109] is supported by the observations that the pGP3 C-terminal trimerization domain resembles the receptor binding domain of TNFα [64] and pGP3 is known to activate macrophages and dendritic cells to produce cytokines [27] and the pGP3-deficient *C. muridarum* was less efficient than the pGP3-competent *C. muridarum* in stimulating oviduct inflammation [20]. However, it is still unclear whether pGP3 promotion of tubal inflammation requires the TNFR1 pathway. The role of caspase-1 in chlamydial pathogenicity [113, 121] is consistent with the ability of caspase-1 to promote fibrosis [122]. Chlamydia may activate caspase-1 via both inflammasome-dependent [123, 124] and -independent [110, 114] pathways. However, it is unclear whether pGP3 can activate caspase-1 although bacterial lipopolysacchride (LPS) in the host cell cytosol is known to directly activate other inflammatory caspases [125, 126]. pGP3 [27] and various other chlamydial proteins [127–130] are both associated with pathologies in *Chlamydia*-infected humans [66, 106] and secreted into the cytosol of the infected cells. These proteins may have the opportunity to access the cytosolic inflammatory pathways. Thus, it is reasonable to hypothesize that these secreted chlamydial proteins including the plasmid-encoded pGP3 may be able to promote tubal pathology by activating both extracellular and intracellular inflammatory pathways.

The Role of pGP4 in Chlamydial Pathogenicity

pGP4 was initially described as a master transcriptional regulator of both plasmid and chromosomal genes using a pBRCT vector system by Song *et al.* from the Caldwell group [17] and subsequently confirmed in the pSW2:GFP vector system by Gong *et al.* [18]. The finding provided the molecular basis for an early observation from the Caldwell group that the *C. trachomatis* plasmid is a transcriptional regulator [24]. By comparing the gene expression profiles between the plasmid-free L2R organisms complemented with the complete plasmid versus a plasmid depleted of *pgp4*, *pgp3* expression decreased ~4 folds while 9 chromosomal genes including *glgA* decreased between 2 to 23 folds and 5 other chromosomal genes increased ~2 folds in the absence of *pgp4*. Furthermore, the trend of change in chromosomal gene expression in the plasmid-deficient organisms paralleled with that of the *pgp4*-deleted organisms [17], suggesting that pGP4 is largely responsible for the plasmid-mediated regulation of chromosomal genes.

The above findings raised three immediate questions: First, besides gene regulation, what other functions pGP4 may have? Second, how does pGP4 exert its gene regulation function? Third, what are the roles of the pGP4-reguated genes in chlamydial pathogenesis? Since *pgp4* depletion significantly reduced the expression of *pgp3* [17–20] and lack of pGP3 largely reproduced the phenotype of plasmid-deficiency in mice, it has been difficult to determine whether pGP4 has additional functions beyond its regulation of chlamydial gene expression in animal models. Nevertheless, in cell culture systems, it was found that the plasmid-deficient L2 organisms were less capable of stimulating host cell inflammatory cytokine production [59]. However, it has not been evaluated whether the reduced stimulation was due to lack of pGP3 or pGP4. Furthermore, in contrast to the plasmid-competent L2 that can effectively exit the cytoplasmic inclusion and infected cell for spreading to the neighboring cells, the plasmid-free L2 was found to be incapable of disrupting their inclusions and to remain intracellular as stable mature inclusions [34]. This phenotype was mapped to Pgp4. The chlamydial exit was dependent on protein synthesis and inhibited by the compound C1, an inhibitor of the type III secretion system (T3S), which is consistent with the function of pGP4 as a transcriptional regulator of multiple chromosomal genes, including a few coding for T3S effectors. Furthermore, the failure to lyse the infected cells was rescued by latrunculin B, an inhibitor of actin polymerization, suggesting that a pgp4-regulated chromosomal T3S effector(s) may promote chlamydial exit by depolymerizing F-actin. It will be important to identify the putative T3S effector(s) and to generate the corresponding mutants for evaluation in animal models.

It is worth noting that chromosome-encoded *glgA* expression is dependent on pGP4. The *glgA* expression reduced >8 folds in the absence of pGP4, which is consistent with the observation that there is a lack of glycogen accumulation in inclusions containing chlamydial organisms deficient in plasmid or depleted of *pgp4* [17–20]. Interestingly, the chlamydial *glgA* protein is secreted into both the chlamydial inclusion lumen and host cell cytosol [128], which may partially explain the glycogen accumulation in the lumen of chlamydial inclusions [24, 131]. However, the role of glgA in chlamydial pathogenesis remains unknown. It is possible that the pGP4-dependent chlamydial glycogen synthesis and lytic exit both are for sharing chlamydial glycogen with its host microbiota to promote

chlamydial co-existence within the same host mucosal tissue. Many mucosal microbiota species both synthesize and consume glycogen and glycogen is known to promote microbiota fitness [132]. Fortunately, *glgA* mutants have been produced by the Nelson group [133], which can be used to dissect the roles of the pGP4-dependent chlamydial glycogen synthesis versus lytic exit in chlamydial pathogenicity in mice. Collaborative efforts are underway to address this.

The Roles of pGP5 and pGP8 and their Antisense RNAs in Chlamydial Pathogenicity

Although deletion of the gene coding for pGP5 from *C. trachomatis* serovar L2 did not significantly affect the expression of the plasmid-regulated chromosomal genes [17, 18], deletion of *pgp5* or replacement of *pgp5* with a mCherry gene from *C. muridarum* all led to a significant increase in expression of the plasmid-dependent genes, including *tc0319* (homologue of *ct049*), *tc0181* (homologue of *ct798* or *glgA*), *tc0419* (homologue of *ct142*), *tc0420* (homologue of *ct143*), *tc0421* (homologue of *ct144*) & *tc0357* (homologue of *ct084*). These observations suggest pGP5 may be a negative transcriptional regulator for *C. muridarum* [19]**.** Indeed, these are the same genes that are upregulated by pGP4 [17, 18]. The antisense sRNA-7 encoded in the *pgp5* gene may not be important for the negative transcriptional regulation function since a premature stop codon installation in the *pgp5* gene, which presumably only blocked pGP5 expression without interfering with sRNA-7 transcription, also resulted in an increased expression of these genes [19]. To further test whether the negative regulation of gene expression is unique to *C. muridarum* Pgp5, *C. trachomatis pgp5* was used to replace *C. muridarum pgp5* and the plasmid-dependent expression was monitored in cell culture. The *C. trachomatis pgp5* was as competent as *C. muridarum pgp5* in suppressing the expression of the plasmid-dependent genes during *C. muridarum* infection, demonstrating that inhibition of the plasmid-dependent chromosomal genes is a common property of Pgp5 protein from different chlamydial species. More importantly, when pGP5-deficient *C. muridarum* was evaluated in mice, it displayed a partial but significant attenuation in inducing hydrosalpinx [82], suggesting that pGP5 also contributes to chlamydial pathogenicity in the mouse upper genital tract although not as robust as Pgp3 [20]. The challenge questions are how pGP5 regulates its target gene expression in chlamydial chromosome and whether the attenuated pathogenicity is caused by the increased expression of the chromosomal genes.

pGP5 is a MinD protein with 239 amino acids. MinD is known to bind to ATP and participate in plasmid/chromosome segregation/partitioning. Clearly, the chlamydial plasmid-encoded *minD* is not essential for segregation of either the plasmid or chromosome since deletion of *pgp5* from the plasmid permitted replication of both chlamydial organism and plasmid [17–19]. It remains unknown how Pgp5 selectively suppresses the expression of the plasmid-dependent genes. It is likely that additional factors may be involved in Pgp5-mediated gene regulation. Regardless of how pGP5 works, the discovery of pGP5 as a negative regulator of the same set of genes that are positively regulated by pGP4 suggests that the chlamydial plasmid may help chlamydial organisms to modulate chlamydial gene expression in response to environmental cues. This hypothesis is consistent with the fact that

plasmid is dispensable for chlamydial replication and both pGP4 and pGP5 are dispensable for plasmid maintenance and replication. Thus, *Chlamydia* may be able to afford to use pGP4 and pGP5 for responding to environmental factors from the host, microbiota and co-infection.

pGP8 was initially found to be required for plasmid maintenance since depletion of pgp8 from the pBRCT vector made the plasmid non-viable [17]. However, in a subsequent study [18], when a premature stop codon was introduced into the *pgp8* gene using the pGFP::SW2 vector system, the plasmid was stably maintained in the *C. trachomatis* L2 organisms. This new finding led to the conclusion that the *pgp8* gene sequence but not the pGP8 protein is required for the maintenance of the plasmid. Indeed, the *pgp8* gene also encodes an anti-sense small RNA designated sRNA-2 [22, 23]. sRNA-2 was found to be most abundantly expressed among all the *C. trachomatis* plasmid pORFs [22], indicating the importance of sRNA-2 in regulating *pgp8*. Since pGP8 is not required for plasmid maintenance while plasmids lacking of both the pGP8 protein and sRNA-2 were no longer recoverable [18], sRNA-2 must regulate other elements besides *pgp8* for plasmid maintenance. It will be interesting to directly evaluate whether a plasmid with a loss of function mutation in sRNA-2 that does not affect the expression of a functional pGP8 is viable. The viable mutant could then be compared with the pGP8-deficient mutant for their ability to aid chlamydial colonization in animal tissues.

Finally, deletion of *pgp7* did not cause any significant *in vitro* phenotypes in either *C. trachomatis* L2 [17, 18] or *C. muridarum* [19]. More importantly, pGP7-deficient *C. muridarum* induced as robust hydrosalpinx as the plasmid-competent *C. muridarum* [20, 21]. Thus, pGP7-deficient chlamydial organisms can be used as an internal control for pathogenesis studies. Since either deletion or premature stop engineering of *pgp1*, *pgp2* or *pgp6* genes resulted in loss of plasmid recoverability [17, 18], it has been difficult to evaluate their roles in chlamydial pathogenesis.

Concluding Remarks and Future Directions

Among the chlamydial plasmid-encoded 8 pGPs [24, 27], only pGP3 is both localized in the outer membrane complex [63] and secreted into the host cell cytosol [27]. Since *Chlamydia* can complete their biosynthesis inside an inclusion, the chlamydial proteins secreted out of the inclusion may interact with the host cells. The pGP3 protein is immunodominant [62, 65, 67, 134] and associated with pathologies [66, 106] in the infected individuals. Structurally, it is a stable trimer [63] via its C-terminal trimerization domain (pGP3c) similar to the receptor binding domain of TNFα [64]. pGP3-deficient *C. muridarum* and *C. trachomatis* phenocopied their plasmid-free counterparts [20, 21]. The failure of pGP3-deficient *C. muridarum* to induce hydrosalpinx correlated with a significant reduction in ascension [20] and inflammation, suggesting that pGP3 plays critical roles in both. A pGP3 triple helices middle domain (pGP3m) binds to and neutralizes the antichlamydial activity of host antimicrobial peptide LL-37 [83]. TNFR1 is critical for chlamydial induction of hydrosalpinx [108] and pGP3c possesses a trimeric structure [64] for potentially binding to TNFR1. These analyses suggest that pGP3 can promote both chlamydial ascension via pGP3m-mediated neutralization of mucosal effectors and activation of tubal inflammation

via Pgp3c interacting with host TNFR1. Testing of this hypothesis may significantly advance our understanding of chlamydial pathogenic mechanisms and develop mechanisms-based strategies for attenuating chlamydial pathogenicity. It is worth noting that besides pGP3, further characterization of the remaining plasmid-encoded ORFs will not only promote our understanding of chlamydial pathogenic mechanisms but also facilitate the development of *Chlamydia* transformed with an engineered plasmid as a platform for delivering beneficial factors for human health.

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Outstanding questions

1. Despite the importance of the plasmid-encoded pGP3 in both chlamydial ascension and induction of tubal inflammation, the host targets of pGP3 have not been demonstrated in animal models.
2. pGP3 is both associated with the chlamydial outer membrane and secreted out of chlamydial inclusion into the host cytosol. However, pGP3 lacks clear secretion signal sequence. What secretion pathway is used by pGP3?
3. pGP4 and pGP5 are regulators for multiple chromosomal genes. What are the roles of these gene-encoded proteins in chlamydial pathogenesis?
4. Why is the plasmid not critical for chlamydial colonization in the genital tract despite its significant contribution to chlamydial pathogenicity in the upper genital tract? Although *C. muridarum* deficient in either plasmid or pGP3 is highly attenuated in the mouse upper genital tract pathogenicity, the live organism shedding from the lower genital tract is not significantly different from that of the plasmid-competent *C. muridarum*; Although an ocular serovar of *C. trachomatis* deficient in plasmid is significantly attenuated in infecting monkey eyes, the plasmid-deficient genital serovars of *C. trachomatis* displayed similar infectivity as their plasmid-competent counterparts in the genital tracts of nonhuman primates. These observations seem to suggest that the plasmid is acquired by *Chlamydia* for adaption to tissues other than the genital tract. Addressing this question may fundamentally advance our understanding of chlamydial biology and pathogenesis.

Trends

1. The plasmid is less important for chlamydial growth *in vitro* than during infection *in vivo*, suggesting that *Chlamydia* may have acquired the plasmid for promoting its survival in animals, implicating a plasmid-dependent pathogenicity.
2. pGP3-deficiency reproduced the *in vivo* but not *in vitro* phenotypes of plasmid-deficiency, indicating pGP3 is a key virulence factor for the plasmid-dependent pathogenicity. pGP3 may exert its pathogenicity by promoting both chlamydial ascending infection and induction of tubal inflammation.
3. pGP4 is a master transcriptional regulator for both plasmid genes including *pgp3* and chromosomal genes such as *glgA* and type III secretion effector genes. pGP4 promotes chlamydial exit from infected cells in cell culture.
4. pGP5 appears to be a negative regulator of the chlamydial chromosomal genes that are upregulated by pGP4.