Chlamydia trachomatis Polymorphic Membrane Protein D is a Virulence Factor Involved in Early Host Cell Interactions

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ABSTRACT

*Chlamydia trachomatis* is an obligate intracellular mucosotropic pathogen of significant medical importance. It is the etiological agent of blinding trachoma and bacterial sexually transmitted diseases, infections that afflict hundreds of millions of people globally. The *C. trachomatis* polymorphic membrane protein D (PmpD) is a highly conserved autotransporter and the target of broadly cross-reactive neutralizing antibodies; however, its role in host pathogen interactions is unknown. Here we employed a targeted reverse genetics approach to generate a *pmpD* null mutant that was used to define the role of PmpD in the pathogenesis of chlamydial infection. We show that *pmpD* is not an essential chlamydial gene and the *pmpD* null mutant has no detectable deficiency in cultured murine cells or in a murine mucosal infection model. Notably, however, the *pmpD* null mutant was significantly attenuated for macaque eyes and cultured human cells. Reduction in *pmpD* null infection of human endocervical cells was associated with a deficiency in chlamydial attachment to cells. Collectively, our results show that PmpD is a chlamydial virulence factor that functions in early host-cell interactions. This study is the first of its kind using reverse genetics to evaluate the contribution of a *C. trachomatis* gene to disease pathogenesis.
INTRODUCTION

*Chlamydia trachomatis* is an obligate intracellular mucosotropic bacterium that is the most common cause of preventable blindness (1, 2) and bacterial sexually transmitted infections worldwide (3-5). The study of these medically important pathogens has been severely limited in the past by the lack of genetic tools. However, newly developed genetic approaches enable us to ask definitive questions about the contribution of specific chlamydial genes to pathogenesis (6-8). Polymorphic membrane protein D (PmpD) is one of nine putative autotransporters (AT) encoded in the *C. trachomatis* genome (9). ATs are members of the Gram negative type V secretion system and are important virulence factors that function in host cell interactions and immune evasion (10). PmpD exhibits classical AT processing resulting in a membrane translocator domain that facilitates the presentation of a passenger domain on the chlamydial surface (11). PmpD is highly conserved among all *C. trachomatis* strains and is the target of broadly-neutralizing antibodies (12). Despite its conserved nature, surface localization, and immunological importance, little is known about the function of PmpD in the pathogenesis of *C. trachomatis* infection.

Here, we made a *C. trachomatis pmpD* null mutant using a targeted reverse genetic approach (6). *pmpD* was not essential for *C. trachomatis* growth and the *pmpD* null mutant showed no deficiency in cultured murine cell lines *in vitro* or in a mouse urogenital infection model. However, the *pmpD* null mutant was attenuated in cultured human endocervical and conjunctival cells and in a nonhuman primate model of *C. trachomatis* ocular infection. Our findings show that *C. trachomatis* PmpD is a virulence factor that functions in early host-cell interactions.
MATERIALS AND METHODS

Chemical mutagenesis, library construction and mutation screen. The generation of the low mutagenized *C. trachomatis* serovar D library has been previously described (6). Briefly, McCoy cells in 96-well tissue-culture plates were infected with 10 IFU per well. Chlamydiae were harvested 48 hours post-infection and were used to re-infect McCoy cells in 96-well tissue-culture plates. Infected cells were harvested and passaged for a third time and DNA extracted from the third passage was used to PCR-amplify *pmpD*. Amplicons were heat-denatured, slowly re-annealed, and digested by CEL I. Digestion products were visualized by DNA agarose gel electrophoresis. Mutations were sequenced and the two mutants harboring nonsense mutations in *pmpD* were plaque cloned three times and their genomes sequenced (6).

*Chlamydia trachomatis* strains and cell culture. Plaque cloned *pmpD* mutants and the parental serovar D strain derived from D/UW-3/Cx (6) were propagated in McCoy cells and purified as previously described (13). The immortalized human endocervical cells (A2EN), human conjunctival (HCjE), and primary mouse oviduct cells (Bm12.4) were all cultured as previously described (14-16).

Antibodies and western blot analysis. A rabbit peptide antibody to PmpD (anti-nPmpD antibody) (11) and mouse monoclonal antibodies to B-complex MOMP (L2-I5) and HSP60 (A57-B9) were used in this study. Western blot analysis of PmpD was performed as previously described (11).

Phase microscopy. McCoy cells seeded to 12 mm glass coverslips were infected at an MOI of 0.1 with the WT strain or the *pmpD* null mutant. At 36 hours post-infection coverslips were mounted in PBS and inclusions were evaluated at 40X magnification.
using a Nikon Eclipse TS100 microscope and images were acquired with a Nikon DS-Fi1 camera.

**Immunofluorescence microscopy.** McCoy cells seeded to 12 mm coverslips were infected at an MOI of 0.1 with WT or the *pmpD* null mutant. At 36 hours post-infection cells were washed with PBS and fixed in methanol. Coverslips were blocked with 2% normal goat serum in PBS and inclusions were immunostained with the rabbit anti-PmpD peptide antibody followed by Alexa Fluor 488 goat anti-rabbit IgG (Life Technologies, A11034). Inclusions were also immunostained with the anti-MOMP mouse monoclonal antibody followed by Alexa Fluor 568 goat anti-mouse IgG (Life Technologies, A11004). Nuclei were stained with DAPI (Life Technologies, D3571). Coverslips were mounted in MOWIOL (Sigma-Aldrich, 81381) and evaluated on a Carl Zeiss LSM 710 confocal laser scanning microscope.

**Transmission electron microscopy.** McCoy cells seeded to 12 mm Aclar coverslips were infected at an MOI of 0.5 with the WT strain or the *pmpD* null mutant. At 24 hours post-infection coverslips were washed in PBS and fixed with 2.5% glutaraldehyde in cacodylate buffer (100 mM sodium cacodylate, 50 mM KCl, 2.5 mM CaCl₂) for 30 min at room temperature followed by overnight incubation at 4°C. Samples were processed and imaged as previously described (17).

**Infection and growth analysis in vitro.** McCoy (murine fibroblast), Bm12.4 (primary murine oviduct epithelial), A2EN (human endocervical epithelial), and HCjE (human conjunctival epithelial) cells were each seeded to 24-well plates at a cell density of 1.5x10⁵ cells per well. Twenty-four hours after seeding, cells were inoculated with the *pmpD* null or the WT strain in SPG buffer. All cells were inoculated with 1.5x10⁴ IFU.
per well and plates were centrifuged at 545xg for 1 hour at room temperature. Following infection, inoculum was replaced with supplemented media containing cycloheximide. Cells were either methanol fixed at 36 hours post-infection for inclusion counting or harvested in SPG buffer for enumeration of recoverable IFUs in McCoy cells.

**Murine model of urogenital tract infection.** Eight-week-old female C3H/HeJ mice (Jackson Laboratories) were treated with 2.5 mg medroxyprogesterone acetate at 10 and 3 days prior to urogenital infection with *C. trachomatis*. Groups of ten mice were inoculated intravaginally with doses ranging from $1 \times 10^2$ to $1 \times 10^7$ IFU per mouse in 5 µl SPG. The vaginal vault was swabbed just prior to inoculation. Chlamydial shedding was monitored weekly by swabbing the vaginal vault and culturing the recovered chlamydiae on McCoy monolayers in 48-well plates as previously described (18). The ID$_{50}$ of each strain was calculated using the Reed and Muench method (19, 20). Mice were swabbed until the clearance of infection, indicated by three successive culture negative weeks.

**Murine histopathology.** Female C3H/HeJ mice were infected with 100-times ID$_{50}$ or were mock infected with SPG buffer as described above. Five mice from each infection and mock group were euthanized at 1, 2, 4, 5 and 6 weeks post-infection. Genital tracts were excised, fixed in 10% formalin and embedded into paraffin blocks. Upper genital tract tissue sections including ovaries, oviducts and uterine horns were stained with hematoxylin-eosin (H&E) to visualize inflammatory cell infiltration. Histological scoring of chronic inflammatory cellular infiltrates including lymphocytes, plasma cells and macrophages was performed blinded by a veterinary pathologist using the previously described four-tiered semi-quantitative criteria (18, 21). The range of
cellular infiltration scoring of 0 to 4 was defined as follows: 0 is none, 1 is minimal, 2 is mild, 3 is moderate, and 4 is severe.

**Macaque model of ocular infection.** Adult male and female cynomolgus macaques (*Macaca fascicularis*) were maintained at the Rocky Mountain Laboratories (RML) and cared for under standard practices implemented by the Rocky Mountain Veterinary Branch. Monkeys were housed separately when being used for experimental studies. All handling procedures were approved by the RML Animal Care and Use Committee and the research was conducted in full compliance with the Guide for Care and Use of Laboratory Animals. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care. Ocular infections were conducted by direct inoculation of chlamydiae onto the conjunctival surfaces (20 μl per eye) of both eyes (13). Six macaques were infected with 2x10⁴ IFU/eye of the *pmpD* null mutant and six macaques received 2x10⁴ IFU/eye of the WT strain. Blinded clinical evaluation of ocular disease was performed weekly by RML veterinary staff. Disease was scored based on hyperemia and follicle formation on the upper conjunctival surface of both eyes (13, 22). Hyperemia was scored in the following manner: 0, no hyperemia; 1, mild hyperemia; and 2, severe hyperemia. Sub-epithelial conjunctival follicles were scored as follows: 0, no follicles; 1, 1–3 follicles; 2, 4–10 follicles; 3, >10 follicles; and 4, follicles too numerous to count. A composite disease score for each animal was calculated by adding left eye and right eye pathology scores with a maximum score of 12 (13). Ocular infection was monitored by swabbing the conjunctiva and culturing recoverable IFUs on HeLa cell monolayers as previously described (13). Macaques were monitored for shedding and disease for 100 days post-infection.
Host cell attachment assay in human endocervical cells. Human endocervical cells (A2EN) were infected with $10^7$ IFU of the $pmpD$ null or the WT strain in SPG buffer in a six well plate for one hour at 4°C by centrifugation at 545xg. After infection each inoculum was removed and re-titered in McCoy cells to quantitate unattached infectious organisms. The experiments were repeated using the same inoculum to serially infect A2EN monolayers 2-3 times consecutively before re-titering in McCoy cells.
RESULTS

*pmpD* is a non-essential chlamydial gene. The recently developed targeted reverse genetic approach for *C. trachomatis* (6) was used to generate *pmpD* mutants. We used the *C. trachomatis* serovar D library generated by low level of EMS mutagenesis from our previous work which was known to consist of isogenic or near-isogenic mutants (6). Screening the library revealed 28 *pmpD* mutants, 22 of which had non-synonymous and six of which had synonymous mutations. Two of 28 *pmpD* mutations were premature stop codons. Both of these mutants were plaque cloned and their genomes sequenced (Table 1). The mutant with a C→T transition mutation at nucleotide position 1618 in *pmpD* (*pmpD*<sup>C1618T</sup>) was selected for further analysis as it was near-isogenic with only a single background mutation (Fig. 1A), while the other mutant (*pmpD*<sup>C3280T</sup>) was used to confirm all *in vitro* observations (data not shown). The *pmpD*<sup>C1618T</sup> and the wild type (WT) serovar D parental clones were evaluated by Western blot analysis for *pmpD* expression (Fig. 1B). PmpD-specific antibodies failed to detect any forms of PmpD in the *pmpD*<sup>C1618T</sup> clone including the predicted truncated form produced by the premature stop codon, indicating *pmpD* is not an essential gene for *in vitro* infectivity and growth in McCoy cells. Therefore, the *pmpD*<sup>C1618T</sup> mutant was designated *pmpD* null.

*pmpD*<sup>C1618T</sup> is a null mutant with atypical morphological and ultrastructural phenotypes. Microscopy was performed to determine if the absence of PmpD protein resulted in morphological changes. Phase microscopy of live McCoy cells infected with the *pmpD* null revealed large atypical forms throughout developing inclusions (Fig. 2A). Confocal microscopy also revealed atypical organism distribution including areas that appeared to lack any chlamydial organisms (Fig. 2B). The WT clone exhibited typical


inclusions and organism distribution. Transmission electron microscopy was performed

to evaluate ultrastructural differences between the \textit{pmpD} null and WT clones (Fig. 2C).

Interestingly, \textit{pmpD} null reticulate bodies (RBs) showed reduced association with the

inclusion membrane. At 24 hours post-infection only 6.3\% of the \textit{pmpD} null RBs were

associated with the inclusion membrane compared to 37.6\% of WT RBs with intact

\textit{PmpD}. Other time points were also significantly different (Table S1). Transmission

electron microscopy performed on infected HeLa cells showed similarly reduced RB-

inclusion membrane association. This observation suggests \textit{PmpD} may influence the

RB-inclusion membrane interactions and the absence of \textit{PmpD} destabilizes that close

physical association.

\textit{In vitro} and \textit{in vivo} murine models reveal no detectable role for \textit{C. trachomatis}

\textbf{PmpD}. The ability of the \textit{pmpD} null to infect and propagate in two murine cell lines was

evaluated \textit{in vitro}. McCoy cells (mouse fibroblasts) and Bm12.4 cells (primary mouse

oviduct epithelial cells) (14) were infected with the \textit{pmpD} null or WT strains (Fig. 3A).

Immunostaining of inclusions at 36 hours post-infection revealed no (McCoy cells) or

very little (Bm12.4 cells, 1.22 fold) difference in the ability of the strains to infect the

murine cell lines. Infectious progeny were also enumerated at 36 hours post-infection but

no difference in the number of recoverable IFUs was detected between the strains (Fig.

3B). The ID_{50}, infectious burden and duration of shedding were evaluated for the \textit{pmpD}

null and the WT strains in the C3H/HeJ murine model of urogenital tract infection (18).

Surprisingly, the ID_{50} for the \textit{pmpD} null and the WT strains was almost identical, \textit{10}^{3.025}

and \textit{10}^{3.1742}, respectively (Table S2). The lowest infectious dose that resulted in 100\%

infection was \textit{10}^5 IFU for both strains. The \textit{10}^5 groups were evaluated weekly for
infectious burden and duration of shedding (Fig. 3C). There was no difference in chlamydial burden or duration of shedding between the strains. Histopathological evaluation was conducted at five time points post-infection known to show upper genital tract pathology for *C. trachomatis* (18). No statistical differences in clinical disease between the *pmpD* null and the WT were detected in upper genital tract tissues (Fig. 3DE).

*pmpD null is significantly attenuated in nonhuman primates.* Despite the lack of detectable infection and growth differences in murine model experiments, the conserved and immunogenic nature of *C. trachomatis* PmpD argues that PmpD has an important role in human chlamydial infections. Since *C. trachomatis* PmpD has only a 72% sequence similarity to its ortholog in *C. muridarum*, the natural chlamydial pathogen of mice, we decided to test the *C. trachomatis* *pmpD* null in the nonhuman primate model; a model that mimics human infections much more closely than any murine system. The cynomolgus macaque model of ocular infection was used to evaluate infection and clinical disease *in vivo*. Six animals were infected with the *pmpD* null and six animals with the WT and evaluated at weekly intervals post-infection. Surprisingly, and contrary to the results of the mouse experiment, the chlamydial burden was significantly reduced in monkeys infected with the *pmpD* null compared to monkeys infected with the WT strain during the first two weeks of infection (Fig. 4A). The average total chlamydial burden during the first two weeks of infection for the *pmpD* null infected animals was 558 IFU compared to 7,282 IFU for WT infected animals resulting in a 13-fold reduction in burden. The peak shedding difference was 25-fold at one week post-infection (Table S3). Interestingly, there were no significant differences at any time point after week two.
post-infection. The duration of infections was also similar for \textit{pmpD} null and WT clones. Nevertheless, average total infectious burden during the entire study for the \textit{pmpD} null infected animals was 795 IFU (32 – 1,477 IFU) compared to 7,782 IFU (2,686 – 18,263 IFU) for the WT group ($p=0.002$) resulting in an approximately 10-fold reduction (Table S3). A similar trend in pathological inflammation was observed. Clinical pathology based on hyperemia and follicle formation was significantly reduced in \textit{pmpD} null infected animals at 2 and 3 weeks post-infection compared to WT-infected animals (Fig. 4B). Similar to the infectious burden, pathology at later time points was not significantly different. We re-sequenced the \textit{pmpD} gene of organisms isolated from the mutant infected animals at two, four and six weeks post-infection to test for revertants; none were detected.

\textbf{\textit{pmpD} null is deficient in host cell attachment during early host cell interactions.}

To investigate if the reduced infectivity and virulence in nonhuman primates correlates with a detectable \textit{in vitro} phenotype in human cells we tested a human endocervical cell line, A2EN (15), and a human conjunctival cell line, HCjE (16); both epithelial cells. A2EN and HCjE cells were infected with the \textit{pmpD} null or the WT strains using McCoy cells as infection controls. Contrary to the mouse cell line infections, the \textit{pmpD} null showed an approximately 70% reduction in the ability to infect the human cell lines (Fig. 5A). A similar 70% decrease in recoverable IFUs (Fig. 5B) was observed, indicating that the two chlamydial strains have a similar burst size. These data suggested that the absence of PmpD significantly altered early host cell interactions in human cells while it had no influence on growth and replication \textit{in vitro}. 


To further characterize the role of PmpD during early host cell interactions we investigated the reason the *pmpD* null infections produced reduced IFUs in the human endocervical cells, the primary target cell type during natural *C. trachomatis* serovar D urogenital infections. A2EN cells were infected with the *pmpD* null or the WT strains. After infection the inocula containing the unattached EBs were re-titered in McCoy cells to determine the number of infectious EBs still present. Similar experiments were conducted by serially infecting A2EN monolayers before re-titering in McCoy cells. Interestingly, *pmpD* null inocula contained a high number of viable EBs even after several infections of A2EN monolayers while WT EBs were more than 90% reduced by just one A2EN infection (Fig. 5C). The high number of EBs detected in the *pmpD* null inocula strongly suggests that the absence of PmpD in *C. trachomatis* EBs significantly inhibits chlamydial attachment to human endocervical cells and that this deficiency is likely the cause for the reduced *pmpD* null infectivity in human cells (Fig. 5A).
DISCUSSION

C. trachomatis was historically considered a genetically intractable organism. However, recent advancements in chlamydial genetics now provide new approaches to study gene function (6-8). C. trachomatis PmpD has been implicated as an important chlamydial virulence factor as it is a conserved surface antigen and target of neutralizing antibodies (12). However, direct evidence supporting this hypothesis is lacking. Here, we used a targeted reverse genetics approach to generate a pmpD null mutant and have studied the infection phenotype of this mutant in vitro and in vivo. We showed that pmpD is not an essential chlamydial gene but the absence of PmpD interferes with the RB-inclusion membrane association in both human and murine cells. The pmpD null had no detectable infection or growth deficiencies in cultured mouse cell lines or in a murine chlamydial infection model. However, in human endocervical and conjunctival cells and in a nonhuman primate infection model we observed a significant reduction in infectivity and virulence for the C. trachomatis pmpD null mutant. We also showed that the infection deficiency in human cells and nonhuman primates is likely the result of the null mutant’s significantly impaired chlamydial attachment to host cells. Taken together the findings implicate C. trachomatis PmpD as an important virulence factor that is involved in early host-cell interactions.

The most surprising result of our study was the complete absence of any detectable deficiency in murine infection models. Although PmpD exhibits 72% sequence similarity between human and mouse chlamydial strains, subtle differences may exist in domains that are critical in the molecular interactions at the host-pathogen interface.

Conversely, the cognate PmpD host receptor(s) might exhibit similar diversity that
determines chlamydial interactions. One intriguing hypothesis is that PmpD’s interactions with the host cells are species specific and that *C. trachomatis* PmpD has co-evolved with its natural host to optimize its role in chlamydial pathogenesis. Co-evolution of human and mouse chlamydial strains and their respective hosts has also been described for genes in the organism’s plasticity zone that are thought to function in evasion of host immunity (23). The species specificity of PmpD could be confirmed by generating a *C. muridarum pmpD* null mutant and evaluating its virulence in the mouse model.

Another unexpected finding was that even in nonhuman primates, a model that usually mimics human infection very well, the PmpD Null showed only partial attenuation that was limited to early post-infection while bacterial burden and ocular disease were not significantly altered in later time points. *pmpD* is a member of an expanded and divergent autotransporter family that consists of nine genes (9). This suggests that other Pmps might compensate for loss of PmpD. *C. trachomatis pmps* exhibit phase variable expression patterns during the chlamydial growth cycle (24). Although the significance of the phase variation is not known it could represent a potential compensatory mechanism important to chlamydial pathogenesis. This hypothesis is supported by studies on *Rickettsia*, an intracellular bacterial pathogen with an expanded autotransporter gene family (25). The deletion of the *R. rickettsii* autotransporter outer membrane protein A has been associated with an attenuated phenotype (26). Moreover, other members of this autotransporter gene family are thought to play a role in host tropism that varies between arthropod and mammalian hosts (25). It is possible that the *C. trachomatis pmp* gene family functions similarly by
determining tissue tropism within the human host rather than among disparate mammalian hosts.

The obvious question emerging from our studies is how might PmpD function during chlamydial host interactions? Our current findings support two potential answers to this question. First, our experiments with human endocervical cells suggest that PmpD functions in either attachment or entry. The fact that anti-PmpD antibodies neutralize chlamydial infection \textit{in vitro} (12, 27) is consistent with this possibility. Furthermore, the surface exposed PmpD passenger domain contains an integrin binding RGD motif (11) that might function in either attachment or entry. Interestingly, the PmpD passenger domain of the mouse pathogen \textit{C. muridarum} has this motif substituted with KGD (11), a modification that is known to alter integrin binding specificity (28).

Another possible clue resulting from our work that suggests a specific interaction for PmpD and host cell cytoplasmic membrane receptors was the failure of \textit{pmpD} null RBs to intimately interact with the inner surface of the chlamydial inclusion membrane (Fig. 2C, Table S1). The chlamydial inclusion membrane, although modified by chlamydiae (29, 30), is initially derived from host endocytic vesicles following chlamydial entry (31). Thus, the inability of \textit{pmpD} null RBs to bind the inclusion membrane may be a secondary, infection-independent phenotype that is an indication of an earlier more important PmpD ligand-receptor interaction between EBs and the cytoplasmic membrane that functions in EB attachment or entry. The fact that this intracellular developmental phenotype had no measurable effect on the \textit{in vitro} growth of the organism in cultured cells argues against an alternative function important to nutrient acquisition or chlamydial protein secretion; but one cannot exclude that such a host-pathogen relationship might be
more important to the pathogenesis of chronic or persistent chlamydial infections. The lack of in vitro growth defect is also inconsistent with the contact-dependent hypothesis that suggests the detachment of the RB from the inclusion membrane provides the signal for late differentiation of RB into EB (32, 33).

Further experimentation is needed to fully elucidate the molecular interactions between PmpD and the host cells. Unfortunately our efforts to complement the null mutant and express PmpD on a shuttle vector in the pmpD null, or in serovar L2, were unsuccessful; no viable transformants were found after numerous attempts, while identical shuttle vectors carrying similar-sized inserts were successful. Our current efforts are focused on generating null mutants for other members of the pmp family in combination with the pmpD null mutation to investigate their potential redundant functions, generating a C. muridarum pmpD null mutant to confirm the species specificity of PmpD, and introducing nonsynonymous SNPs into potentially key parts of the protein, like the RGD motif. In summary, our study suggests that the C. trachomatis PmpD is an important chlamydial virulence factor that primarily functions in host cell attachment and entry.
ACKNOWLEDGEMENTS

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FIGURE LEGENDS

FIG 1  *pmpD*<sup>C1618T</sup> is a null mutant. (A) Stick drawing summarizing the principle forms of WT PmpD and sequencing information showing the location of the *pmpD*<sup>C1618T</sup> stop codon. (B) Western blot analysis of whole EB proteins with a PmpD-specific antibody showed strong reactivity with WT and no reactivity with *pmpD*<sup>C1618T</sup>. Anti-HSP60 antibody was used as a loading control.

FIG 2  The *pmpD* null mutant exhibits unique morphological and ultrastructural phenotypes *in vitro*. (A) Phase microscopy of McCoy cells at 36 hours post-infection revealed atypical organism distribution associated with the *pmpD* null but not the WT strain. (B) Confocal immunofluorescence microscopy at 36 hours post-infection showed no immunostaining of *pmpD* null with a PmpD-specific antibody. Immunostaining of MOMP and DAPI stained DNA confirmed the atypical organism distribution in *pmpD* null inclusions. (C) Transmission electron microscopy at 24 hours post-infection showed reduced association of *pmpD* null RBs with the inclusion membrane compared to the WT strain (Table S1).

FIG 3  No detectable role for *C. trachomatis* PmpD in murine infection models. (A) Enumeration of inclusions in murine fibroblasts (McCoy) and primary murine oviduct (Bm12.4) cells revealed no (McCoy cells) or very little difference (Bm12.4 cells, 1.22 fold) in the ability of the *pmpD* null to infect *in vitro* compared to WT. (B) Recoverable IFUs harvested from McCoy and Bm12.4 cells at 36 hours post-infection also showed no differences in the ability of the *pmpD* null mutant to grow in these cell lines compared to
WT. (C) Recoverable IFUs and duration of chlamydial shedding following urogenital infection of C3H/HeJ mice were similar for \textit{pmpD} null (n=10) and the WT strain (n=10). No statistical difference was found at any time point (two tailed t-test). (D) H&E staining of C3H/HeJ upper genital tract tissues (ovaries, oviducts and uterine horns) infected with \textit{pmpD} null or the WT strain showed no histopathological differences up to 6 weeks post-infection. Average clinical disease scores with standard deviations are shown (n=5). (E) H&E staining of oviducts (OD) are shown for \textit{pmpD} null, WT and mock infected animals.

FIG 4 \textit{pmpD} null is attenuated in a nonhuman primate infection model. Six cynomolgus macaques were infected ocularly with \textit{pmpD} null and six with the WT strain to evaluate infection, duration of shedding, and clinical pathology. (A) Ocular infection was monitored by swabbing the conjunctiva and culturing recoverable IFUs on HeLa cell monolayers. Averages and standard deviations are shown on a log\(_{10}\) scale. Detailed chlamydial shedding data are shown in Table S3. (B) Disease was scored based on hyperemia and follicle formation on the upper conjunctival surfaces (score 0 = no disease, score 12 = maximum disease). Averages and standard deviations are shown. Macaques were monitored for shedding and disease for 100 days post-infection. * indicates statistically significant difference (p<0.05, two tailed Mann-Whitney U Test, n=6).

FIG 5 \textit{pmpD} null is deficient in attachment to human cells. (A) Infection of human endocervical (A2EN) and conjunctival (HCjE) cells with \textit{pmpD} null yielded significantly
fewer inclusions than WT (p<0.05, two tailed Mann-Whitney U Test, n=3). *pmpD* null inclusions are shown as a percentage of the WT control for each cell line. (B) A decrease in recoverable IFUs concomitant with decreased inclusions was detected in both human cell lines following infection by *pmpD* null and WT strain (p<0.05, two tailed Mann-Whitney U Test, n=3). *pmpD* null recoverable IFUs are shown as a percentage of the WT control for each cell line. (C) A2EN cells were infected zero, one, two or three times consecutively with the same inocula before re-titering the non-attached EBs in the inocula in McCoy cells. The number of viable EBs remaining in the inocula after the A2EN infections as determined by re-titering in McCoy cells are shown (average and standard deviation). Unattached *pmpD* null IFUs remaining in the inocula after 1, 2 and 3 infections of A2EN cells were statistically significantly higher (p<0.05, two tailed Mann-Whitney U Test, n=6).
Table 1. De novo genome sequencing of *pmpD* mutants.

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<th>Mutant</th>
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</tbody>
</table>

*SNP location refers to genomic location in the *C. trachomatis* D-LC annotated reference sequence (NC_017436.1). † IG: intergenic region.