INTRODUCTION

Chlamydia trachomatis is an obligate intracellular pathogen that is responsible for significant worldwide morbidity associated with ocular and sexually transmitted diseases (STD). The developmental cycle of the organism is biphasic beginning with the adhesion of the elementary body (EB), an infectious and metabolically inert form, to the host cell. After endocytosis, the EB differentiates ~2 to 12 h post infection (p.i.) into a larger, non-infectious and metabolically active reticulate body (RB), which initiates intracellular replication by binary fission within a vacuole called an inclusion body. At ~30 to 36 h p.i., RBs reorganize into new EBs, which are released by host cell lysis or exocytosis at 48 to 72 h p.i. that initiates another infectious cycle [1,2].

The disease spectrum of C. trachomatis ranges from conjunctivitis and ocular trachoma to tubal factor infertility, ectopic pregnancy and infant pneumonia [3,4]. C. trachomatis serological variants (serovars) are grouped within two human biological variants (biovars) according to characteristics of disease presentation: the trachoma biovar, including serovars A to C and Ba, which cause conjunctivitis and trachoma, and serovars D to K and Ba, Da, Ia and Ja, which cause conjunctivitis, urogenital infections and infant pneumonitis, and the lymphogranuloma venereum (LGV) biovar (serovars L1 to L3 and L2a). The latter serovars are more invasive, causing genital ulceration, lymphadenitis and proctitis [3,5]. However, serotyping of the major outer membrane protein (MOMP), and phylogenetic reconstructions of this protein and the corresponding gene (ompA) [6,7] do not group serovars by trachoma, non-invasive urogenital or invasive LGV disease groups.

The molecular mechanisms behind these biological differences among serovars (or strains) are not well understood. Genome sequences of reference strains D/UW-3 [8] and A/Har-13 [9], as well as ongoing C. trachomatis genomic sequencing are providing information on specific genes and proteins that may explain tissue tropism and virulence differences for the three disease groups. C. trachomatis contains a partial tryptophan operon (trpRBA) where urogenital strains, but not trachoma strains, can synthesize tryptophan from mucosal substrates [10]. The toxin gene possesses...
an intact N-terminal region that encodes an active enzymatic domain for the urogenital strains but not for trachoma or LGV strains [11].

Research on the nine member polymorphic membrane protein (Pmp) gene (pmp) family has revealed phylogenetic reconstructions where six pmps (pmpB, pmpC, pmpE, pmpF, pmpH and pmpK) form clades that correspond to the three disease groups [12–14]. At the gene expression level, previous RT-PCR analyses of reference strains D/UW-3 and L2/434 [15], and microarray analysis of D/UW-3 [16] found that all nine pmps were transcribed starting at 8 h.p.i. Yet, based on real-time quantitative (k)RT-PCR, we found expression as early as 2 h.p.i. for pmpC for reference strains Ba/ Apache-2, G/UVW-57 and L2/434, and a differential expression profile with earlier up-regulation of pmpC for L2/434 [17]. Also, another study based on kRT-PCR, Kiselev et al. [18] detected pmpD expression as early as 2h.p.i. for L2/434. Proteomics analyses have also shown that all Pmps of L2/434 are detected as outer membrane constituents [19–22]. There is also evidence that some Pmps are antigens for human sera [15,23]. We observed a heterogeneous immunoreactivity to recombinant (r)PmpC using sera from patients infected with different C. trachomatis strains, suggesting a role for PmpC in antigenic variation [17]. More recently, Pmps have been considered autotransporters based on bioinformatics analyses [24,25]. Wehr et al. [26] has experimentally demonstrated the autotransporter model for the C. pneumoniae ortholog of C. trachomatis PmpD, Pmp21. Further, using immunofluorescence microscopy, Western blotting and penicillin treatment, the results of Kiselev et al. [18] for L2/434 PmpD are in general agreement with the autotransporter model for this protein. PmpD has also been shown to be a species-common neutralizing antigen [27], while PmpF has been implicated as a potential target of the host immune response as it contains several predicted major histocompatibility (MHC) epitopes within the N-terminal domain [9].

Despite the potential importance of Pmps in chlamydial biology, there is a lack of expression data for the pmp genes as well as an insufficient understanding of the host immune response to their proteins. Here, we profile the expression of all pmps throughout development for reference strains E/Bour and L2/434, representing the two C. trachomatis biovars. We chose E/Bour because it is the most prevalent strain worldwide, although the mechanisms of its ecological success are not yet understood. L2/434 was selected as it has been widely studied with a plethora of biological information for comparative analyses. The biological uniqueness of these two strains in vivo is reflected in their differential tissue tropism, virulence and disease presentation. In light of our recent findings that reference strains do not represent the same genetic composition of clinical strains that are circulating among human populations today [28], we also compared the nine pmp expression levels for four C. trachomatis clinical strains, representing ompA genotypes of E and L2. Further, we examined the immunoreactivity of sera from adolescents with and without C. trachomatis urogenital infections against rpmps to further define their potential importance in human disease.

MATERIALS AND METHODS

C. trachomatis cell culture of reference strains and clinical strains

C. trachomatis reference strains E/Bour and L2/434, three clinical strains belonging to ompA genotype E (designated as E/537C-05, E/S-141 and E/CS-500-96) and one clinical strain belonging to ompA genotype L2 were evaluated in this study. E/537C-05 and E/S-141 were collected from patients with vaginal discharge, E/CS-500-96 from a patient with cervicitis, and L2 from a patient with proctitis. Each was propagated in HeLa 229 cell monolayers using standard techniques as previously described [4,29]. EBs were harvested at 48–72 h.p.i. and purified by discontinuous density centrifugation in Renografin [30].

Confluent HeLa cells were either mock-infected or infected with a multiplicity of infection of one for each reference strain or clinical strain in SPG prior to incubation with culture medium [4,29]. Eight T25 flasks (one for each time point of 2, 6, 12, 18, 24, 36, and 48 h and mock-infected) per strain were inoculated and placed at 37 °C in 5% CO2 [17]. Cultured cells were harvested at each time point, and total RNA was extracted as previously described [17].

Reverse Transcription and Quantitative Real-Time PCR (kPCR)

RNA was quantified by O.D. measured at 260. cDNA was generated from 500 ng of each RNA sample using TaqMan RT Reagents (Applied Biosystems, Foster City, CA) and random hexamers, and was quantified by O.D. measured at 260.

Quantitation of pmp expression was achieved using the ABI 7000 SDS (Applied Biosystems), SYBR Green chemistry, and the standard curve method for relative quantitation, using reagents and thermocycling as previously described [17]. 16SrRNA was used as the endogenous control since normalizing the data against 16SrRNA provides a control for the number of organisms (EBs and RBs) and, therefore, for the differential growth rate of each strain. ompA was included as a quality control for kRT-PCR results since it has been widely used for gene expression studies [16,17].

Primers for each of the nine pmps (Table 1) were designed using Primer Express (Applied Biosystems). Primers for ompA, 16SrRNA, and pmpC were used as previously designed (Table 1) [17].

Each plate contained two replicates of each sample cDNA, three different negative controls and standard curves for each gene as previously described [17]. For all experiments, the amount of target and control gene was determined from the respective standard curve by conversion of the mean threshold cycle values. Normalization was obtained by dividing the quantity of the target gene by the quantity of the control gene. The specificity of the amplified products was verified by analysis of the dissociation curves generated by the ABI7000 software based on the specific melting temperature for each amplicon. The results were based on three independent experiments for reference strains E/Bour and L2/434, and for the four clinical strains.

Genetic analysis of the pmpFE operon for C. trachomatis reference and clinical strains

Based on the considerable expression disparities between pmpF and pmpE (which belong to the same operon) for reference strain L2/434 and mostly for the clinical strains (see results below), we sequenced the pmpFE operon as well as the upstream 164 base pair (bp) pmpG/pmpF intergenic region (IGR) that likely contains the operon regulatory region. In the C. trachomatis chromosome, pmpF and pmpE are located on the minus strand; pmpE is located upstream of pmpE, with a 2 bp IGR. pmpF was sequenced for the six strains (Genbank Accession Numbers EF490370 for E/537C-05, EF490371 for E/CS-500-96, EF490372 for E/S-141, EF490373 for L2, EF490374 for E/Bour, and EF490375 for L2/434), while pmpG/pmpF IGR and pmpF were sequenced only for the clinical strains (Genbank Accession Numbers EF490366 for L2, EF490367 for E/CS-500-96, EF490368 for E/S-141, and EF490369 for E/537C-05), as the sequences for the reference strains were available from our previous study (GenBank...
Given the expression dissimilarities obtained in this study for er.html and http://www.prodoric.de/vfp/vfp_promoter.php.

prediction programs: http://www.fruitfly.org/seq_tools/promot-

described in the literature and also by using two promoter

using EditSeq software (DNASTAR, Madison, WI) for sequences

IGR, where we used primer 5

performed as previously described [12], except for the

434) [12]. The amplification and sequencing strategies were

a putative promoter search for the

structure and sequence variability of promoter regions [31–34],

AG-3

Accession Number AY887650 for E/Bour and L2/434 (GenBank Accession No. X52557 and M14738, respectively).

ompA

ompA-9

OmpA-10

16SrRNA

16SRNA-9

16SRNA-10

Table 1. Oligonucleotide primers used for kRT-PCR

| Gene     | Primers          | Primer sequence (5’ to 3’) | Gene Location | Base pair size |
|----------|------------------|----------------------------|---------------|---------------|
| pmpA     | pmpA-3           | TGCTAGGGGAAAGATGGTGCAGAATAG | 1434–1457     | 51            |
|          | pmpA-4           | TGAACGGGTGGTTAAAAAATCCG    | 1484–1463     |               |
| pmpB     | pmpB-5           | CGACTACATAGCAAATAACACTGCTAA | 2120–2144     | 102           |
|          | pmpB-6           | TACGGAGGCTCTCAGGATATTCGTT  | 2221–2195     |               |
| pmpC     | pmpC-11          | TTATGTGCTCCCTACAGACCTACATCA | 4150–4174     | 56            |
|          | pmpC-12          | CCCGTGACTATTATTTGCCTGGT    | 4205–4181     |               |
| pmpD     | pmpD-3           | GCCTGTGCTGCTGCCGAAATAAT    | 4455–4476     | 51            |
|          | pmpD-4           | ACTGTCGTGAGTGAAGACCTCCATT  | 4505–4480     |               |
| pmpE     | pmpE-1           | CATAAGCGCTCTCCGGGATA      | 2140–2160     | 51            |
|          | pmpE-2           | GTGTTTCTGGCTCGTATCATC      | 2190–2169     |               |
| pmpF     | pmpF-5           | TCTTATGTTGTGATCCGATTGCT    | 2520–2541     | 69            |
|          | pmpF-6           | CTCGGCATGTATGTTGGTCCA      | 2588–2566     |               |
| pmpG     | pmpG-1           | TGGGTATTTCGGAAGTCTGGAAT    | 2221–2242     | 51            |
|          | pmpG-2           | ACCTAAGAATCGGCGGTCAT       | 2271–2252     |               |
| pmpH     | pmpH-15          | TGATACCGCAGTATAATGACAA      | 2486–2511     | 61            |
|          | pmpH-16          | TGCTAAGACTATCCGCAATGAT     | 2546–2525     |               |
| pmpi     | pmpi-1          | GGAAAGTGTGCGGATCGAT        | 2176–2195     | 51            |
|          | pmpi-2          | GAAAGTGGTGGCGCGATCGAT      | 2226–2207     |               |
| ompA     | OmpA-9           | TGGCGCTTGGATGGTCTGTT       | 33–52         | 75            |
|          | OmpA-10         | GTGCAATGCTATGGCGTGGATCAG    | 108–86        |               |
| 16SrRNA  | 16SRNA-9        | GCCAAGGCGCTTACCATAATTTAT   | 734–756       | 76            |
|          | 16SRNA-10       | CCAGGGCTATCTAATCTGTGTGCT   | 809–786       |               |

*Primers designed based on each pmp sequence of reference strains E/Bour and L2/434 [12].
*Primers designed only for strain L2.
*Primers designed based on the ompA sequence of reference strains E/Bour and L2/434 (GenBank Accession No. X52557 and M14738, respectively).
*Primers designed based on the 16SrRNA sequence of reference strains E/Bour and L2/434 (GenBank Accession No. D85722 and U68443, respectively).

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Immunoreactivity of patient sera against Pmp fusion proteins

We generated fusion proteins for PmpD and PmpF because the latter displayed such high mRNA expression for L2/434 and the clinical strains, and the former was expressed late in development for all strains under study, being the last up-regulated protein for four of the six strains analyzed. Also, PmpD has been associated with neutralizing epitopes [27]. The rMOMP fusion protein was available from a previous study [50]. The PET30 expression system (EMB Biosciences, San Diego, CA) was used for cloning PCR products containing pmpD or pmpF generated from strain E/Bour genomic DNA as we have described [17]. The forward and reverse primers were 5’-GACGCCGCGAACAGATAGTGGTCCCGA-

GAAAGATATA-3’ and 5’-AATGCTGGATTGCGATTGACGCTC-CTTCTCC-3’ for pmpD, respectively, and 5’-GACGCCGCGAACAGATAGTGGTCCCGA-

GAAAGATATA-3’ and 5’-AATGCTGGATTGCGATTGACGCTC-CTTCTCC-3’ for pmpF, respectively. Sequencing confirmed that the insert was in frame with the S-tag and His-tag as we have described version 4.4 (http://rna.urmc.rochester.edu/rnastructure.html) due to the regulatory or processing role of stem-loop structures in premature transcription termination as well as in mRNA degradation and maturation mechanisms [45–49], respectively.
previously for rPmpC [17]. The clones were transformed into *E. coli* BL21 and induced using 0.1 µM IPTG during the exponential growth phase. Ni-agarose (Sigma, St. Louis, MO) was used for fusion protein purification according to the package insert. Recombinant proteins were determined to be the correct molecular weight (calculated at ~160.6 kDa for rPmpD and ~112.3 kDa for rPmpF) by immunoblot using AP-conjugated S-protein, which binds to the S-tag peptide with a distinct band at the correct molecular weight for each. Optimal protein concentrations were determined and standardized using BCA (Pierce, Rockford, IL) before analyzing the clinical sera. The optimal protein concentration for rPmpD was 50 ng and 100 ng for rPmpF. Sera from 39 consented female adolescents 14 to 19 years of age attending clinics in Oakland, CA, were used at a 1:50 dilution for immunoblotting as described previously [17]. The Institutional Review Board of Children’s Hospital Oakland approved the study, and all patients provided written consent for all clinical samples that were obtained and used in this study. The blots were blocked with Blotto prior to reacting with patient sera and alkaline phosphatase-conjugated anti-human IgG (R&D Systems, Minneapolis, MN). The chemiluminescent substrate ECF (Amersham, Piscataway, NJ) was used to visualize reactive bands. Twenty six (67%) of the 39 adolescents were infected with a single *ompA* genotype: 3 Ba, 3 D, 8 E, 5 F, 1 G, 1 Ia, 2 J, and 3 K. There was no evidence for mixed infections. The original cervical samples were used for sequencing (see ref 17) to best determine the presence of a mixed infection, since propagation may result in one strain overgrowing another. On inspection of the electropherograms, none of the samples had ambiguous results. All nucleotides were represented by single, clear peaks with extremely low background and without evidence for double peaks in a nucleotide position where different *ompA* genotypes differ, which might suggest a mixed infection.

**RESULTS**

Real-time quantification for *pmp* expression

The results of specificity assays revealed no non-specific products, and indicated the presence of the expected amplicon for each gene. Standard curves for all 11 genes had slope values between -3.1 and -3.3, which represents efficiencies between 93 and 100%. There were only minor variations in the slope for each standard curve among independent experiments, indicating a highly reproducible qPCR as we have also shown in previous experiments [17]. We defined the gene expression profile as the qualitative gene expression pattern throughout development where quantitative values are not considered. For example, one expression profile would show increasing expression up to a peak with tapering down of the expression after the peak. An expression peak was defined as the time point at which a noticeable expression increase occurred.

For both reference strains, *ompA* had remarkably higher mRNA values at all time points than for the *pmp* (Fig. 1). In contrast to most *pmps*, *ompA* revealed a similar gene expression profile for both reference strains.

Expression profile of the nine *pmp* genes throughout development for *C. trachomatis* clinical strains

The four clinical strains had a similar *pmp* expression profile (Fig. 2), which showed decreasing mRNA levels after the expression peak to 48 h. mRNA levels were detected at 2 h p.i. for all *pmps*, although the scale limits visualization. Overall, *pmpF* peaked at 18 h for L2 (Fig. 2A) and E/C500-96 (Fig. 2D), and at 36 h for clinical strains E/C573C-05 (Fig. 2B) and E/S-141 (Fig. 2C). Similar to reference strains E and L2, *ompA* was expressed late in development (together with other *pmps*) for the other two clinical E strains under study. *pmpF* had the highest expression among all of the *pmps* for the clinical strains (Fig. 2). In fact, there was a 27-fold higher expression of *pmpF* compared with the least expressed gene (*ompA*) for L2. For clinical E strains, there was a 19.2- and 22.6-fold higher expression of *pmpF* compared with the least expressed gene (*pmpI*) for E/C573C-05 and E/C500-96 respectively, and a 54.2-fold higher expression than *ompA* for E/S-141. Although no relevant dissimilarities were observed for *pmpF* between clinical L2 and L2/434, there were considerable expression differences among the clinical strains and E/Bour with up to 11.7-fold higher mRNA values for E/C500-96 than for E/Bour. For *ompA*, mRNA levels peaked at 36 h for E/C573C-05 and E/S-141, and at 18 h for E/C500-96 and L2, declining thereafter (Fig. 2). The most striking example of differential mRNA levels between *ompA* and *pmps* occurred for E/S-141, where *ompA* had a 232.0-fold higher value compared with the least expressed gene, *ompA*. However, all clinical strains except E/C500-96 had lower *ompA* expression levels for all time points compared with the corresponding reference strains.

Genetic analysis of *pmpFE* operon for *C. trachomatis* reference strains and clinical strains

The *pmpF*, *pmpE* and *pmpG/pmpF* IGR sequences for the three clinical E strains were 100% similar to the corresponding E/Bour sequences, while L2 showed 4 nucleotide (nt) differences to L2/434 but only for *pmpE*. Compared to both L2 strains, the four E strains showed 317 (10.2%) nt and 106 (10.3%) amino acid (aa) differences for *pmpF* as well as 36 (1.9%) nt and 21 (20 to L2) (2.1%) aa differences for *pmpF*. For the *pmpG/pmpF* IGR, which comprises the ~164 bp upstream region of *pmpF*, there were 5 nt differences between the L2 and the E strains, although none of them fell within the putative promoter region for the *pmpFE*
operon (Fig. 3). The putative promoter is located within a 100% conserved stretch of the pmpG/pmpF IGR for both reference and all clinical strains (Fig. 3). The -10 promoter element (TAAAAT) identified in this study was identical to the one that was previously characterized for the L2/434 and D/UW-3 ltuB promoter, while the -35 region (TTGCAT) was 100% similar to the hctA promoter of the same chlamydial reference strains [32].

Analysis of the pmpFE operon sequence revealed several putative hairpin loop structures although the actual RNA folding in those regions functioning as a classic rho-independent type transcriptional terminator [49] cannot be assumed. At least 41 putative RNase E cleavage sites were identified throughout the pmpFE operon, 13 of which were not conserved between L2/434 (and L2) and the four E strains (Fig. 4). One of these non-conserved sites involved the pmpF/pmpE IGR, and is specific for the E strains. The search for an RBS revealed a perfect prokaryotic Shine-Dalgarno sequence (AGGAGG) located 17 nts upstream of the start codon of pmpE, which is approximately 3000 bp below the last bp in Figure 3 and, therefore, is not shown. This RBS is in close proximity to the above-described putative RNase cleavage site shared only by the four E strains. However, the best approach for a putative RBS sequence for pmpF has two mismatches when compared with the ones described in the literature, and is unusually distant from the start codon (Fig. 3). Two additional putative RNase E cleavage sites, one of which was in close proximity to this RBS, were identified within the pmpF regulatory region (Fig. 3).

Immunoreactivity of patient sera with Pmp fusion proteins

Table 2 shows the clinical and microbiologic characteristics of the 39 adolescents enrolled in the study and the results of their serum immunoreactivity to rPmpD and rPmpF. All sera from patients infected with chlamydial clinical strains Ba, E, F and K (n = 15; 57.7%), but none with D, Ia, J or G (n = 11; 42.3%), were reactive to rPmpD while sera from uninfected patients were nonreactive with rPmpD (p = 0.001). Figure 5 shows the immunoblot results of representative sera from patients infected with Ba, D, E, F, G, Ia, J and K to rPmpD. Because pmpD is highly conserved among all
reference strains [12], constructing rPmpD using the *pmpD* sequence of reference strain E/Bour should not have contributed to the observed differences in immunoreactivity. Further, cross-reactivity between strains was unlikely since the patients were infected with only a single strain, and sera that were reactive to rPmpD were not reactive to rPmpF. In our previous study, sera from the same individuals infected with clinical strains D, E and G reacted with rPmpC [17]. Surprisingly, none of the sera reacted to

**Figure 2. Expression profile of *pmp* and *ompA* genes throughout the development of *C. trachomatis* clinical strains.** (A) Strain L2 shares the same *ompA* genotype as L2/434; and strains E/537C-05 (B), E/S-141 (C) and E/CS-500-96 (D) share the same *ompA* genotype as E/Bour. Values represent the mean±SEM based on three independent experiments for time points of 2, 6, 12, 18, 24, 36, and 48 h post infection. See methods for details.

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rPmpF (Figure 5), not even sera from the eight patients infected with strain E, although all sera from infected patients and one uninfected patient reacted with rMOMP as previously shown [17].

**DISCUSSION**

In this study, we determined the gene expression profile of the nine *pmp* genes throughout development for reference strains L2/434 and E/Bour, and four clinical strains belonging to *ompA* genotypes E and L2. The reference strains had significant gene expression differences where E/Bour had relatively lower mRNA levels and generally sustained expression from 24 to 48 h compared with L2/434 (Fig. 1). Surprisingly, in contrast to clinical L2, the three clinical E strains showed a dissimilar *pmp* expression profile to E/Bour (Fig. 2). These remarkable expression dissimilarities are generally supported by our recent comparative genomics findings where the laboratory adapted reference strains did not reflect the same genetic makeup of strains that are circulating among human populations today and currently exposed to immune selection [28].

It is well known that the developmental stages for reference E strains occur at later time points than for reference L2 strains [51]. This is supported by the *ompA* expression for E/Bour, which is shifted ~6 hours later than for L2/434. However, the differential growth rate between these two reference strains does not explain the dissimilar *pmp* expression, as most *pmps* were up-regulated at the same time point for both (Fig. 1). For E/Bour, almost all of the *pmps* had increased expression during the second half of development with comparable mRNA levels at these stages, suggesting a similar involvement in RB division and RB to EB transformation. For L2/434, although most *pmps* showed a general up-regulation of transcription at the exponential growth phase of RB division when new membranes are being formed, *pmpC*, *pmpE* and *pmpF* appeared to play a more important role during this phase. Thus, the gene expression results of both E/Bour and L2/434 suggest their potential importance in membrane integrity. However, some Pmps may have more specific functions than others, depending on the chlamydial strain. In support of this, a proteomics study by Shaw et al. [21] detected five Pmps among reference strains A/HAR-13, D/UW-3, and L2/434, where PmpF was only detected for L2/434. In another proteomics study, only *pmpE*, *pmpG*, and *pmpH* were detected for L2/434 [22]. However, it is possible that these studies reflect a lack of sensitivity in detecting Pmps since a recent study was able to detect all Pmps for L2/434 [19].

Interestingly, *pmpA* had, in general, the lowest expression levels of all *pmps* at each time point except that it had one of the highest levels at 12 h p.i. (Fig. 1 and 2), suggesting a greater importance of PmpA during early stages of development. This is supported by shotgun proteomics where Skipp et al. [19] identified PmpA exclusively in RBs, whereas all other Pmps were detected in both RBs and EBs for L2/434. Additionally, for PmpD, the late up-regulation at 36 h corresponds to RB transformation into EBs, suggesting a role in EB outer membrane structure. In support of this, PmpD has a cysteine content considerably higher than any other Pmp [12]. There are 26 conserved cysteine residues in PmpD for all 19 *C. trachomatis* reference strains, while the mean for all other Pmps is only 13.9 [SE 2.3]. Cysteine residues are responsible for the highly disulfide cross-linked proteins of the outer membrane complex of EBs. Previous studies found that PmpD is surface located and cross-linked in the chlamydial outer membrane complex of EBs. Previous studies found that PmpD is surface located and cross-linked in the chlamydial outer membrane complex of EBs.
Table 2. Clinical and microbiologic characteristics of female adolescents from whom sera was used for determining the immunoreactivity against rPmpD and rPmpF

| ompA genotype* (n) | Clinical diagnosisb (n) | Immunoreactivity of sera against recombinant fusion proteins |
|-------------------|-----------------------|-------------------------------------------------------------|
|                   |                       | rPmpD (%)         | rPmpF (%)         |
| Ba (3)            | Cervicitis            | 3/3 (100)        | 0/3 (0)          |
| D (3)             | Cervicitis Dischargec (1/3) | 0/3 (0)        | 0/3 (0)        |
| E (8)             | Cervicitis            | 8/8 (100)        | 0/8 (0)         |
| F (5)             | Cervicitis Dischargec (4/5) | 1/5 (20)        | 0/5 (0)        |
| G (1)             | Cervicitis Dischargec (1/1) | 0/1 (0)        | 0/1 (0)        |
| Ia (1)            | Cervicitis            | 0/1 (0)          | 0/1 (0)        |
| J (2)             | Cervicitis            | 0/1 (0)          | 0/1 (0)        |
| K (3)             | Cervicitis            | 3/3 (100)        | 0/3 (0)        |
| Uninfected (13)   | No clinical signs or symptoms | 0/13 (0)       | 0/13 (0)       |

*Patients were adolescents 14–19 years of age who had a C. trachomatis infection with only one ompA genotype as described in methods or were uninfected;

The diagnosis of cervicitis was based on physical findings consistent with cervicitis as determined by the examining physician; all adolescents infected with C. trachomatis had cervicitis, and none of these patients complained of any symptoms;

A cervical discharge was noted by the examining physician; none of these patients had clinical signs or symptoms consistent with upper genital tract disease.

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membrane complex through disulide bonds [52]. Furthermore, the N-terminal domain of C. pneumoniae Pmp21, the C. trachomatis PmpD ortholog, was shown to be non-covalently bound to other components of the EB surface [26]. Additionally, PmpD has shown species-specific neutralizing activity [27]. These collective data are supported by our findings that sera from C. trachomatis infected patients were reactive to rPmpD (Fig. 5). Our results were remarkably consistent for sera from patients infected with the same strain. For example, sera from all eight patients infected with strain E were reactive as were sera from three patients infected with strain Ba and three infected with strain K, although only one of the five patients with strain F were reactive; none of patients with strains D, Ia, J or G were reactive. Additional research is required to determine epitopes on PmpD that may correlate with the differential immune responses we observed.

Overall, considering both reference strains and clinical strains, pmpD and pmpF were the least expressed genes, while pmpF was the most highly expressed, although pmpE and pmpG also had similar expression levels for E/Bour. We previously found that PmpF is the most polymorphic protein among the C. trachomatis Pmps for both reference and clinical strains [28,12]. Consistent with the observed protein diversity, phylogenetic analyses of PmpF grouped C. trachomatis strains by tissue tropism properties [12]. Further, comparative analyses of PmpF reveal distinct domains that may be associated with a specific disease group.

The outer membrane exposure of the N-terminus has been experimentally demonstrated for some C. pneumoniae Pmps [26,53], suggesting that these proteins may be subjected to host immune pressure. The N-terminal half for C. trachomatis PmpF also contains numerous non-synonymous amino acid changes at locations of predicted MHC epitopes [9], indicating that it may be involved in eliciting a cellular immune response. Our findings that none of the sera from infected patients reacted with rPmpF suggest that this protein is not associated with the humeral immune response. Strain origin (E/Bour) of rPmpF did not seem to be an issue as sera from the eight patients infected with strain E were non-reactive. Furthermore, sensitivity was unlikely to be an issue given the immunoreactivity of the same sera with rPmpC and rMOMP, as we have previously described [17], and with rPmpD in this study. The occurrence of highly repeated GGAI motifs in the N-terminus suggests that Pmps may be associated with cell adhesion [54], which has been reported for Pmp21 of C. pneumoniae [26]. These cumulative findings suggest that Pmps are expressed with a differential immune response for patients infected with a specific strain. These findings and the remarkable pmpF expression dissimilarities among L2/434, E/Bour and the clinical strains suggest that there may be differential biological functions across strains and within the same strain for PmpF, either as a structural component to maintain membrane integrity, as part of a large pool of polymorphic antigens to elicit cellular immunity, or as an adhesin.

In our study, the pmpF sequences for the three clinical E strains were found to be 100% similar to the E/Bour sequence as was L2 to L2/434. Since it is highly unlikely that identical proteins have diverse functions, we hypothesized that there may be differential regulation at the promoter level or regulation involving variations in mRNA processing and/or degradation, which would yield distinct mRNA amounts according to strain-specific needs. It is well known that point mutations in regulatory regions, such as promoter regions and RBS, can affect transcription and translation levels. However, analysis of the putative promoter region and RBS for pmpF showed that they are 100% conserved for both reference and the clinical strains (Fig. 5), suggesting that the observed pmpF expression heterogeneity may result from variations.

![Figure 5. Dot-Blot of serum immunoreactivity against recombinant (r)PmpD and rPmpF](image)

Sera was obtained from adolescents singly infected and uninfected with a different C. trachomatis clinical strain as described previously [17] (see also methods). rPmpD and rPmpF concentrations were standardized for use on the blots. Immunoreactivity to each fusion protein for sera from patients infected with strain Ba (n = 3), D (n = 3), E (n = 8), F (n = 5), G (n = 1), Ia (n = 1), J (n = 2) or K (n = 3) are shown. Of note is that immunoreactivity was consistent for sera from patients infected with the same clinical strain except for strain F (Table 2); all eight patients infected with strain E were reactive to rPmpD.

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in mRNA processing and/or degradation. In fact, regulatory systems of gene expression acting at both the transcriptional and translational levels are well represented in the chlamydial genome, including homologues of endoribonucleases E, III, G, and P, exonucleases II and PNPase, and oligoribonuclease [8,9]. These are known to control mRNA stability and processing as well as translational efficiency in other bacteria, such as Escherichia coli and Staphylococcus aureus [44,48,55–57]. The susceptibility of mRNA to ribonuclease attack may be influenced by events occurring not only at any stage during ribosome binding, but also during translation elongation or termination [40].

We identified two conserved putative RNase E cleavage sites in the pmpG/pmpF IGR, one of which is in close proximity to the putative RBS (Fig. 3). It is known that RBS sequence variability and sequestering by competitive regulatory proteins or conformational impediments can affect ribosome binding/loading and, thus, mRNA lifetime [40]. Considering this, a hypothetical initial cleavage by RNase E could reduce the affinity of the pmpF translation initiation region for ribosomes, thereby allowing subsequent mRNA degradation/processing by endo- and exonucleases, preferentially for E/Bour when compared to the other strains. A similar regulation has already been reported for sodB mRNA of E. coli at low iron concentrations [58]. However, this hypothetical mechanism, although possible, is speculative and lacks experimental evidence. pmpF and pmpE belong to the same operon, yet had remarkably dissimilar mRNA levels for L2/434, and more so for all clinical strains with up to 8.4-fold higher expression for pmpE than for pmpF (Fig. 1A and 2). This did not occur for the pmpGH operon. We speculated that the expression heterogeneity within the pmpFE operon may be generated by premature termination of transcription, rapid mRNA processing, or mRNA degradation primarily of the downstream gene (pmpE) of this large operon transcript (>6 Kb). Similar regulatory mechanisms have already been suggested to explain the existence of multiple transcripts within other bacterial polycistronic operons [55], such as those of Bacillus subtilis ara [59], Nitrosomonas europaea cbb [60], and Borrelia burgdorferi ospA/B and bmpA/B [61,62].

Although we cannot assume that the putative stem-loop structures found within the pmpFE operon sequence may act as classic rho-independent type transcriptional terminators [49], the possibility of hairpin formation (a common phenomenon in mRNA, mainly on large transcripts) cannot be ignored nor can its hypothetical processing role in mRNA degradation and maturation be discounted. Furthermore, several putative RNase E cleavage sites were identified throughout the pmpFE operon (Fig. 4), which is expected for policistronic operons, although it is well known that RNase E cleaves mRNA only at a limited number of sites [55]. Interestingly, some of the RNase E sites were not conserved between L2 and E strains, suggesting that targeted mRNA degradation or rapid processing events may occur in this large transcript. Curiously, one of these non-conserved recognition sites involved solely the pmpFE/pmpE IGR of the four E strains. Thus, if RNase E uses this cleavage site, subsequent degradation or processing events from this point would only occur for E strains and could hypothetically yield an mRNA decay of pmpE. Yet, as above, this mechanism is speculative and lacks experimental evidence. However, in a previous study, differential transcript quantities were reported for the MMSO genes of E. coli that contained a consensus RNase E cleavage site in the intergenic regions of the operon, suggesting complex mRNA processing [63].

Overall, the heterogeneous expression levels among pmps and among strains highlight the importance of this gene family in chlamydial biology. In particular, the unique expression disparity for the pmpFE operon with relatively high pmpF mRNA levels for 5 of the 6 strains under study, as well as the differential immunoreactivity of patient sera to rPmpD, suggest that some Pmps may explain phenotypic differences among strains for antigenicity, virulence and tissue tropism. Furthermore, our findings that clinical E strains do not correlate with reference strain E/Bour at the gene expression level are supported by our previously reported genomic data [28], reinforcing the need to examine clinical along with reference strains to advance our understanding of the role of pmps in chlamydial biology and disease pathogenesis.

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Author Contributions

Conceived and designed the experiments: DD AN JG MB. Performed the experiments: AN CF JG SM HC MB AN. Analyzed the data: DD AN JG MB AN. Contributed reagents/materials/analysis tools: DD MB. Wrote the paper: DD JG AN.

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