

Transcriptional analysis of in vitro expression patterns of *Chlamydomphila abortus* polymorphic outer membrane proteins during the chlamydial developmental cycle

Nicholas WHEELHOUSE*, Kevin AITCHISON, Lucy SPALDING, Morag LIVINGSTONE, David LONGBOTTOM

Moredun Research Institute, Pentlands Science Park, Bush Loan, Edinburgh, Midlothian, EH26 0PZ, United Kingdom

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Abstract – *Chlamydomphila abortus* is the aetiological agent of ovine enzootic abortion. Sequencing, annotation and comparative analysis of the genome of *C. abortus* strain S26/3 has revealed variation in the loci encoding the polymorphic membrane proteins (Pmps). These Pmps resemble autotransporter proteins of the type V secretion system, suggesting an important role in chlamydial pathogenesis. The purpose of this study was to characterise the transcriptional expression patterns of this family during the developmental cycle of *C. abortus*. McCoy cells were infected with *C. abortus* and analysed for *pmp* mRNA expression over a 72 h period. Few *pmp* transcripts were detected in the early stages of the developmental cycle. Peak expression occurred at 48 h post-infection (p.i.) other than for *pmp5E*, where it was observed at 24 h p.i. Overall, expression of *pmps 5E*, *18D* and *10G* were found to be 40 to 100-fold higher than the lowest expressing *pmps (6H, 13G and 15G)* at 24 h p.i., while *pmps 18D* and *17G* were 14 to 16-fold higher than the lowest (*11G, 14G and 15G*) at 48 h. Levels of expression for all the other *pmp* genes were below one copy per genome at any time point. The expression of all the *pmps* reduced to near base-line levels by 60 h p.i. These results demonstrate that *pmp* expression in *C. abortus* is mid to late cycle, consistent with conversion of the reticulate body to the elementary body. The low level of *pmp* transcription may be indicative of heterogeneity in expression, suggesting a possible role for some of the Pmps in antigenic variation and chlamydial pathogenesis.

***Chlamydomphila abortus* / *pmp* / gene expression / antigenic variation**

1. INTRODUCTION

Chlamydomphila abortus is the aetiological agent of ovine enzootic abortion (OEA), the single most common infectious cause of ovine abortion in the United Kingdom [1]. It is also a major cause of lamb mortality throughout Europe and is endemic in most sheep-rearing areas of the world. In addition *C. abortus* is

zoonotic and poses a potential risk to the health of pregnant women [16]. In common with other members of the Chlamydiaeae, *C. abortus* is a Gram-negative obligate intracellular pathogen that undergoes a biphasic life-cycle. The infectious form of the organism, the elementary body (EB), enters the host cell where it resides within a vacuole known as an inclusion. Within this inclusion the EB undergoes conversion to the metabolically active reticulate body (RB), which replicates through binary fission. Between 48 and 72 h following infection the

* Corresponding author: nick.wheelhouse@moredun.ac.uk

RB re-condense to EB and at the end of the cycle the inclusion and host cell are lysed releasing infectious organisms that infect neighbouring cells [16].

Sequencing, annotation and comparative analysis of the genome of *C. abortus* strain S26/3 has revealed variation in the loci encoding a family of outer membrane proteins known as polymorphic membrane proteins or Pmps [31], which were originally identified through their immuno-reactivity with convalescent sheep sera obtained from aborted ewes [14, 15]. The expression of Pmps is not restricted to *C. abortus* and *pmp* genes have been identified in all pathogenic chlamydial species sequenced to date [2, 23, 24, 27, 31]. Whilst all sequenced Chlamydiaceae species possess *pmp* genes, there is widespread heterogeneity in the array of the genes carried by each species. While *C. trachomatis* and the closely related *C. muridarum* genomes encode 9 *pmp* genes (termed A-I), *C. pneumoniae*, *C. abortus*, *C. caviae* and *C. felis* genomes encode 21, 18, 17 and 20 *pmps*, respectively. These Pmps resemble autotransporter proteins (AT) of the type V secretion system in which the gene encodes a single precursor containing three functional domains, an N-terminal signal sequence, a passenger (effector) and a carboxy-terminal β -barrel translocator domain [11]. In silico analyses of the Pmps have identified homology with several proteobacterial virulence factors secreted via the TypeV secretion apparatus, including the *Escherichia coli* adhesin AIDA-I [10]. Indeed a role in bacterial adhesion has been ascribed to PmpD [33] and yet whilst little else is understood about the functions of the Pmps it has also been suggested through genetic comparisons of *C. trachomatis* strains that they may play important roles in chlamydial pathogenesis [28] and niche specificity [7, 21]. Immunologically phase-variation in the expression of the Pmps through slip-strand slippage may play a role in the evasion of host immune responses [22, 31]. In terms of their importance to the host immune response to chlamydial infection, Pmps induce both antigen-specific T cell responses [8, 26], essential in the clearance of primary infection [5] and specific humoral responses [12, 15] making them potential vaccine [17] and diagnostic candidates [25].

Little is known about the regulation or function of the Pmps in *C. abortus* and in *Chlamydia* generally. Given the potential importance of the Pmps in the virulence and pathogenesis of *C. abortus* and their potential in the development of vaccine and diagnostic reagents, the purpose of this study was to further characterise this important protein family by studying their expression during the developmental cycle through transcriptomic analysis.

2. MATERIALS AND METHODS

2.1. Cell culture and *C. abortus* propagation

McCoy cells were grown in RPMI1640 medium supplemented with 5% heat inactivated fetal calf serum. HEp2 cells used for the propagation and titration of *C. abortus* stocks were routinely grown in Iscove's Modified Dulbecco's Medium (Invitrogen, Paisley, UK) supplemented with 10% heat inactivated fetal calf serum (PAA Laboratories Ltd, Yeovil, Somerset, UK).

2.2. Infection of cells and nucleic acid extraction

The *C. abortus* strain S26/3 was grown in HEp2 cells and titrated according to a previously published protocol [9]. To investigate the in vitro expression of *pmps* in *C. abortus*, McCoy cells were grown to subconfluence in T25 tissue culture flasks (Corning Costar, High Wycombe, UK). Duplicate flasks were infected with *C. abortus* at an estimated multiplicity of infection (MOI) of two in infection medium consisting of RPMI 1640 containing 2% FCS and 1 μ g/mL cycloheximide (Sigma Aldrich, Dorset, UK). After 2 h, the medium was removed and replaced with fresh infection medium. At 6, 12, 24, 36, 48, 60 and 72 h post-infection (p.i.) total DNA and RNA were isolated from paired flasks using DNeasy[®] and RNeasy⁺[®] mini kits (Qiagen, Crawley, West Sussex, UK). Experiments were conducted on three separate occasions.

2.3. Nucleic acid quantitation and reverse-transcription (RT)

DNA and RNA concentrations were quantified at 260 nm using a nanodrop ND-1-1000 spectrophotometer (ThermoFisher Scientific, Loughborough, Leicestershire, UK) and the quality of total RNA

was assessed on an Agilent 2100 Bioanalyzer (Agilent, West Lothian, UK) prior to reverse transcription using an Omniscript[®] RT Kit (Qiagen) and random primers (Invitrogen).

2.4. Generation of plasmids for standard curves

Unique regions of each of the *pmp* genes were amplified by PCR using specific primers (Tab. I) and an Expand Long-Template PCR kit (Roche Diagnostics, Burgess Hill, UK). PCR products were purified using the QIAquick[®] gel extraction kit (Qiagen) prior to cloning into the appropriate vector. Purified *16S*, *5E* and *omp1* PCR products were cloned into pGEM[®]-T Easy (Promega, Southampton, Hampshire, UK) the remaining *pmp* products had been previously cloned into the pET30-Ek/Lic vector (Merck chemicals Ltd. Nottingham, UK). Plasmids were either transformed into NovaBlue (Merck chemicals Ltd; pET30-Ek/Lic constructs) or JM109 (Promega; pGEM[®]-T Easy constructs) competent *E. coli* cells according to standard procedures, and transformants were selected on the basis of appropriate antibiotic resistance. Colonies were selected for overnight culture and plasmids were purified using the QIAprep[®] spin miniprep kit (Qiagen). The presence of correct sequences was verified by sequencing on a MegaBACE[™]-500 capillary DNA sequencer using DYEnamic[™] ET Dye Terminator technology (GE Healthcare, Little Chalfont, Bucks, UK).

2.5. Real-time polymerase chain reaction (qPCR)

Unique primers and probes were designed for each of 15 *pmps* and *16S* rRNA genes (Tab. II) using Primer Express v2.0 (Applied Biosystems, Warrington, Lancashire, UK). Real-time PCR protocols for quantitation of both *omp1* [13] and *C. abortus* genomes [4] have been previously described. Assays were set up using TaqMan[®] Universal PCR Master Mix and run on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) according to standard procedures. In all RNA determinations, both forward and reverse primers were used at 900 nM and probe at 250 nM final concentration. Samples were analyzed in triplicate either with 1 μ L eluted DNA (for genome determination) or 5 ng RNA from each time point. For quantitation, relevant standard curves were also included on each plate. The number of mean copies per well were calculated against standard curves (10^8 – 10^2) derived either from purified genomic DNA (to determine number of *C. abortus* genome copies) as previously

described [13] or from plasmid (to determine transcript levels) for each relevant assay. Total numbers of recoverable genomes and transcripts were calculated per flask and transcripts were normalised against genomes for each time point from the duplicate flasks.

2.6. Statistics

Data were analysed by ANOVA (Genstat version 7 statistical package) using Fisher's least significant difference test to separate the means at both the 5% and 1% probability levels.

3. RESULTS

3.1. RNA profiles

Total RNA was prepared from *C. abortus* infected McCoy cells at 6, 12, 24, 36, 48, 60 and 72 h p.i. Figure 1 shows typical RNA profiles for two of the time points, 24 and 48 h p.i. Between 0 and 36 h there was little observed prokaryotic ribosomal RNA visible, however the eukaryotic RNA was intact. At 48 h there was a substantial increase in the levels of prokaryotic RNA observed and this coincided with a reduction in the levels of host RNA which was visible as a decrease in the overall intensity of the signals produced on the electropherogram. Post 48 h (60 and 72 h) during which time there was significant levels of cell lysis there was an abrupt decrease in the levels of both eukaryotic and prokaryotic RNA.

3.2. Expression of 16S rRNA and *omp1* transcripts

As an initial step in the analysis of the *pmps*, the expression of 16S rRNA and *omp1*, both of which are considered to be constitutively expressed throughout the chlamydial developmental cycle, was determined and normalized relative to that of chlamydial genome number (Fig. 2). At the earliest time-points of 6 and 12 h p.i. there was negligible expression of the 16S rRNA gene (Fig. 2A). There was a significant rise above the base-line levels of 16S between 12 and 24 h p.i. ($p < 0.05$), which continued to increase at 36 h ($p < 0.01$ above

Table 1. Primers used to clone *pmp* plasmid standards used in RT-PCR.

| Gene | Forward primer (5'-3')* | Reverse primer (5'-3')* | Amplicon size (bp) |
|---------------|---|--|--------------------|
| <i>pmp1B</i> | GACGACGACAAGATCTCGATTACAGTATTGGAGAAC | GAGGAGAAAGCCGGTTTAGAATATACATACGTGCGCCGAGTTAG | 5351 |
| <i>pmp2A</i> | GACGACGACAAGATTTCACTCGCACCACTGACTC | GAGGAGAAAGCCGGTTTAGAAACTTAGAGATATACCAAC | 2792 |
| <i>pmp3E</i> | GACGACGACAAGATTTTACCTCTATTCTCGGAAGCC | GAGGAGAAAGCCGGTCTAAAAAATTAACGAGCTCCCTGC | 2937 |
| <i>pmp4E</i> | GACGACGACAAGATCGAACTCAATTTGCTTGTAAAG | GAGGAGAAAGCCGGTCTAAAAATGTAATTCACCTTG | 2807 |
| <i>pmp5E</i> | ATAAGACTGA GAAAACGATATGAAATCCAC | AGTTGCTAGAGAGTTTAGAAGGAAAAGTTTGCCCCACG | 1114 |
| <i>pmp6H</i> | GACGACGACAAGATTTGCTTATGCACTCGGGGTGG | GAGGAGAAAGCCGGTTTAGAATGTGCCTTGCAAGCCTGTGATG | 2930 |
| <i>pmp7G</i> | GACGACGACAAGATTAACGGGACCTTAGAGAGATGTTTC | GAGGAGAAAGCCGGTTTAGAATCGAAATTACTTCTAC | 3002 |
| <i>pmp10G</i> | GACGACGACAAGATGACGGTCTCACAGGGACC | GAGGAGAAAGCCGGTTTAAAGAAATTCGCCATTGCCCTGA | 2408 |
| <i>pmp11G</i> | GACGACGACAAGATTTCTGGCACTGGATTCAACGGC | GAGGAGAAAGCCGGTTTAAATGCACCCCTGCTG | 2495 |
| <i>pmp13G</i> | GACGACGACAAGATCACGAACAGTGAGCCATTCAATC | GAGGAGAAAGCCGGTTTAGAAGCCAAATGACTGAAAGAGC | 2399 |
| <i>pmp14G</i> | GACGACGACAAGATCGACGCAACGGGAGCAITTCAG | GAGGAGAAAGCCGGTTTAGAATGCGACCTTAGCG | 2699 |
| <i>pmp15G</i> | GACGACGACAAGATAAGCTTAGCGATAATTACGATGG | GAGGAGAAAGCCGGTTTAGAATTTGTATCTACCC | 4110 |
| <i>pmp16G</i> | GACGACGACAAGATCTCCTATGCAAGCAAAAGATTCTCC | GAGGAGAAAGCCGGTTTAAAGCCAAATGACTGAAGAGC | 2321 |
| <i>pmp17G</i> | GACGACGACAAGATCTCCTTTGCAAGGAAAGATTTCAGG | GAGGAGAAAGCCGGTTTAGGTTCTGTGAAGATCCCCTGAGC | 2324 |
| <i>pmp18D</i> | GACGACGACAAGATGCTACATTTTCTCACTCCG | GAGGAGAAAGCCGGTTTAGAAGATCAAGCGCATACCAATTC | 4598 |
| <i>omp1</i> | CATATGCTGTAGGGAACCCAGCTGAACC | GGCGGAATTCITATGCGAATGGAT | 1245 |
| <i>16S</i> | GGCGGGCTGGATGAGGC | GAGTGACTCTCTAGCGGGCA | 868 |

* For constructs cloned into pET30-Ek/Lic vector: forward primers contain 5'-GACGACGACAAGAT-3'; and reverse primers contain 5'-GAGGAGAAAGCCGGT-3'.

Table II. Real-time PCR primers and probes.

| Gene | Forward primer (5'-3') | Reverse primer (5'-3') | Taqman probe (5'-3') | Amplicon size (bp) |
|---------------|-------------------------------|-------------------------------|-------------------------------------|--------------------|
| <i>Pmp1B</i> | GCTACAGAGGATCGGGATTAAGTG | TCCGCTTGCATCTTGTGTTTG | TTCAGATTCTGCATCAAGAATTTTCACGCA | 77 |
| <i>pmp2A</i> | TTTAGCGAAAACTCCGCAGAA | AATGGGCTGGAAAGCTATAAGG | CGTGGAGCTATAACCGGAGCAG | 74 |
| <i>pmp3E</i> | GGTGGCAAGGCTTGGT | CGGAATCTGGCACACCTA | ACGGCTATCCACCAAGCGAATCGC | 61 |
| <i>pmp4E</i> | AGGATACATTCCTCCACCCCAT | TAGGGGGCTTGGCCAAAGA | ATCGTGGAGATCTTGTGGCTAATG | 65 |
| <i>pmp5E</i> | TCCGTCACTTTGGGAATTAGAAG | TCCGTCACTTTGGGAATTAGAAG | TGCGTATAAACCTAACGCCCGTCCGAA | 74 |
| <i>pmp6H</i> | TGCCTCATGCAATATCGAAAAT | GCCGCAAGCAGCAAAGTT | ACTCTGGGGCTATCTCGTTTTCT | 66 |
| <i>pmp7G</i> | TGCTGCTGAGGTAAGTAGCGTTA | GCTATTCGCTTGATTGGTTACAGA | CGTCGACTGCAGCTACTGCGGGC | 72 |
| <i>pmp10G</i> | GCCAAACCCTCCTTAATATTGAAGA | TGGTTTTGCCATCCGTATTTG | ATCGTCTACAGTCAAGGAGCTGCTATCAG | 81 |
| <i>pmp11G</i> | TGCCTTATCCACTGCTACCCAAA | GGAGCCGTTGAATCCAGTTG | TTCTTGCAGATGCCGACTCTGTCAACC | 72 |
| <i>pmp13G</i> | TCTTTAATCCTTAAAGATGGTGTCACTCT | CGTAGTCCCTAGGTCATGACAA | AAATCCTTACAGCAAACAGAGGGCGC | 89 |
| <i>pmp14G</i> | GGAGCATTCAGTCCGCAATC | TCTACAATAGAAATATCACTCTCGACGTT | ACAAGCACTGCGGGAGGAACGATTT | 76 |
| <i>pmp15G</i> | CCCTTATCTTTCACCCGCATCA | TCCGTTGGTGTTTTTGGTTTCA | CGGATGTTCGCAATGAGCATGGC | 75 |
| <i>pmp16G</i> | CTCAATGCTAAAGAAGTTTTGGTATT | GAGAAAGCATCTTCTGTATAAGTAGTG | TCTTTTATGACCCCTATCGTAAACACAGGAGGATC | 124 |
| <i>pmp17G</i> | GGCACGGGAATTTACAAAAC | CTCCCCCAAGTAATAGGGTCAATAGA | ACGTGCTAAAGACGGCTTCGGAATTTTC | 75 |
| <i>pmp18D</i> | TCCACTGGGATGATCACCAATA | GCATAGAAAGCGTATCGAATATCAC | TGATAAATTATATGGTTATGTACCAGCTTC | 81 |
| <i>omp1</i> | GCGGCATTCAAAGCTCGTT | CCTTGAGTGTATGCCTACATTTGG | TGTTAAAGGATCCCTCATAGCAGCTGATCAG | 85 |
| <i>16S</i> | AATCTTTCCGAAATGGAGAAA | ACAACCCTAGAGCCTTCAATCACA | TCTGACGAAAGCAAGCGCCGGC | 66 |

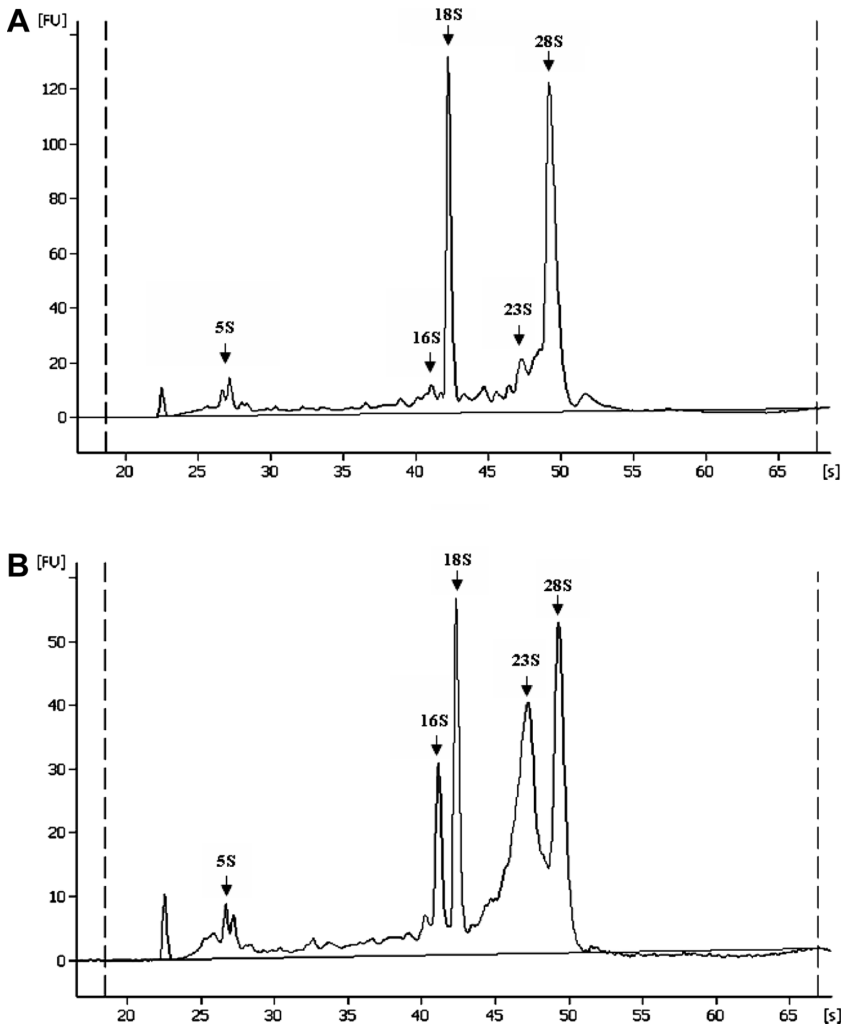


Figure 1. Electropherograms of total RNA isolated from *C. abortus*-infected McCoy cells at two time points in the chlamydial developmental cycle. The presence of both eukaryotic (28S, 18S, 5S) and prokaryotic (chlamydial) (23S, 16S, 5S) RNA is indicated at 24 h p.i. (A) and 48 h p.i. (B).

baseline) and peaking at 48 h p.i. ($p < 0.01$). After 48 h there was a dramatic fall in 16S rRNA transcript levels, which were not significantly different from baseline levels.

The pattern of *omp1* mRNA expression was similar to that of 16S (Fig. 2B). Levels of transcripts were negligible at both 6 and 12 h p.i. Between 12 and 36 h p.i. there was a significant increase in the transcript levels ($p < 0.05$). Lev-

els again increased to peak at 48 h ($p < 0.01$) before decreasing to near base-line levels by 60 h.

3.3. Expression of *pmp* transcripts

Prior to determining relative *pmp* mRNA expression levels, each of the designed *pmp* primer/probe sets (Tab. I) was tested for

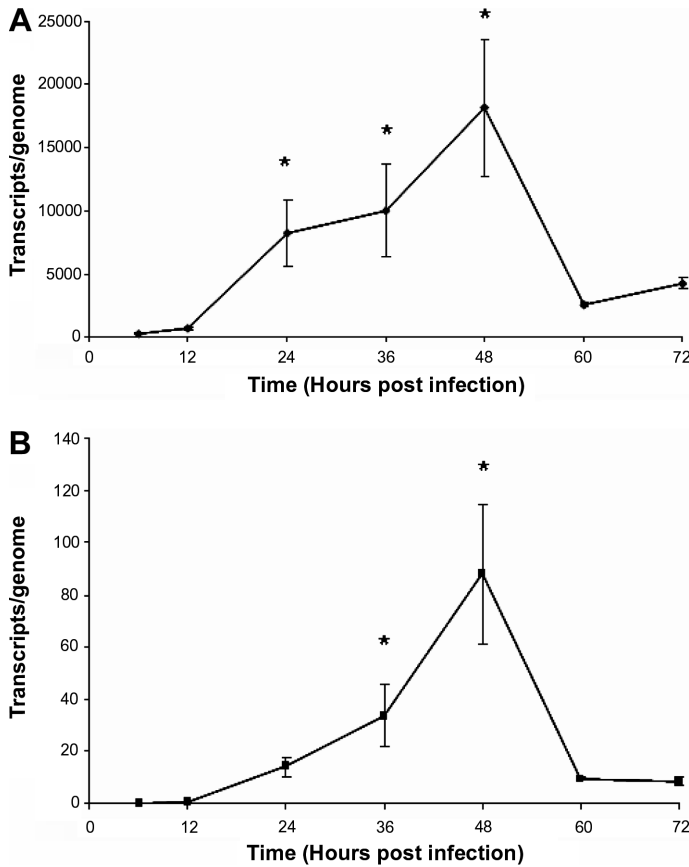


Figure 2. Transcriptional expression of *C. abortus* 16S rRNA (A) and *omp1* (B) RNA. Cells were infected with *C. abortus* (MOI 2). RNA and DNA were harvested between 6 and 72 h p.i. RNA transcript levels were analyzed by reverse transcription quantitative PCR and normalized against numbers of chlamydial genomes (see Materials and Methods). \pm SEM of three independent experiments (see Materials and Methods). * $p < 0.05$ above baseline levels.

cross-reactivity against all 15 cloned plasmid DNA. No cross reactivity was observed with any of the primer/probe sets, each being specific for the relevant plasmid DNA. The results of the real-time PCR assays on the RT reaction mixes for each of the 15 *pmp* genes, relative to total genome copies at each of the time points in the chlamydial developmental cycle, are shown in Figure 3. No significant *pmp* gene expression was observed at either the 6 or 12 h time points. Several *pmp* genes had a statistically significant increase in expression at 24 h, specifically 2A ($p < 0.05$), 10G

($p < 0.05$), 11G ($p < 0.05$) and 5E ($p < 0.01$). However, only *pmp5E* exhibited peak expression at this time point, with its expression decreasing throughout the remaining period of the cycle. In contrast, expression of 2A, 10G and 11G continued to stay elevated albeit at a relatively low level until 48 h, after which expression decreased. For *pmp18D* expression at 24 h was much greater than for all other *pmps*, other than *pmp5E*, and continued to increase until 48 h p.i. Maximum expression was observed for all *pmps*, other than *pmp5E*, at 48 h p.i. ($p < 0.05$). As observed for both

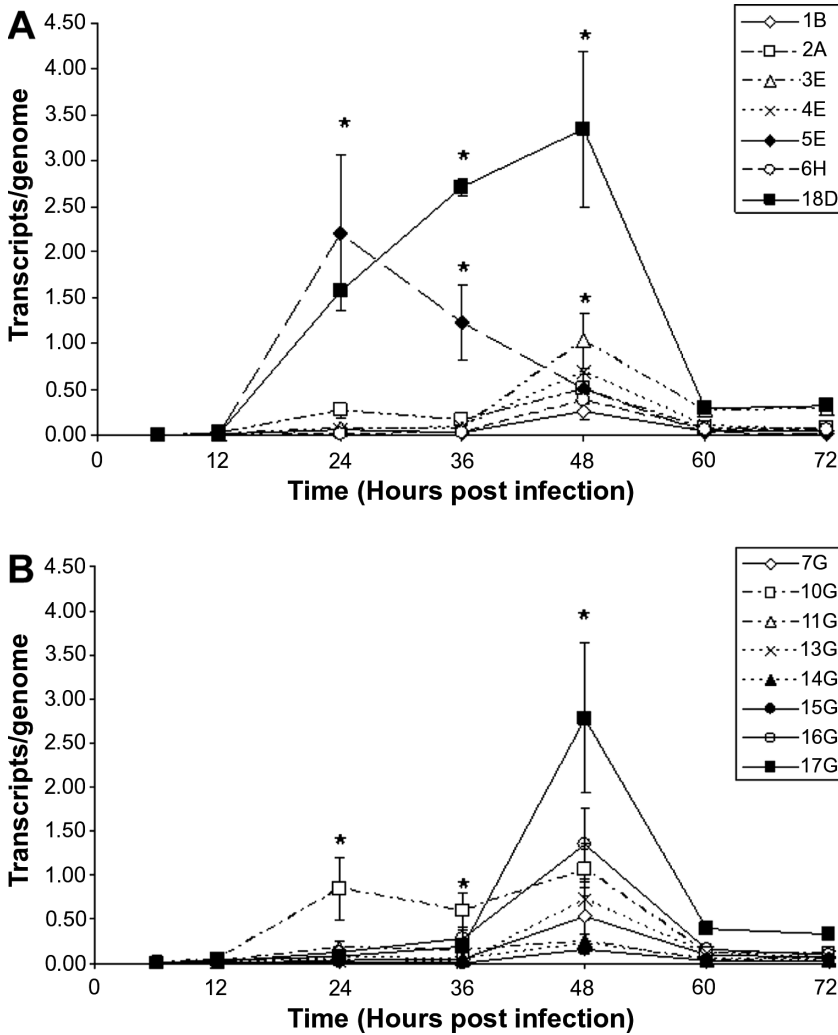


Figure 3. Transcriptional expression of *C. abortus pmp* mRNA. Cells were infected with *C. abortus* (MOI 2). RNA and DNA were harvested between 6 and 72 h p.i. RNA levels were analyzed by reverse transcription quantitative PCR and normalized against numbers of chlamydial genomes (see Materials and Methods). For clarity *pmp* results have been divided into panels A and B. Panel A depicts non-*pmpG* family members, while panel B depicts *pmpG* family members. Results are presented as the mean transcript levels normalized against genome copy number \pm SEM of three independent experiments (see Materials and Methods). * $p < 0.05$ above baseline levels.

16S and *omp1*, the expression of all the *pmps* reduced to near base-line levels at the 60 h time-point and remained low at 72 h. Interestingly, throughout the developmental cycle, transcript levels for all *pmps* other than *pmp5E*,

pmp16G, *pmp17G* and *pmp18D*, were determined to be below one transcript per genome. Overall, expression of *pmps 5E*, *18D* and *10G* were found to be 40 to 100-fold higher than the lowest expressing *pmps (6H, 13G* and

15G) at 24 h p.i., while *pmps* 18D and 17G were 14 to 16-fold higher than the lowest (11G, 14G and 15G) at 48 h.

4. DISCUSSION

This study has demonstrated the transcriptional expression of 15 *pmp* genes. Although 18 genes were identified from the sequencing of the *C. abortus* genome [31], one was a duplicate (*pmp12G*) of *pmp17G*, while another two (*pmp8G* and *pmp9G*) had unrecoverable frame-shifts, and so were not included in this study. Transcripts for all 15 *pmp* genes were analysed at different stages in the chlamydial developmental cycle: defined as early (0–12 h p.i.); mid to late cycle (during the end phase of the growth and multiplication of RB) (12–24 h p.i.); late (terminal differentiation of RB to EB) (24–48 h p.i.); and end of cycle (48–72 h p.i.).

Few *pmp* transcripts were detected in the early stages of the developmental cycle. Indeed the level of transcripts for the majority of the *pmp* genes was very low (less than 1 copy per genome). This could be due to the asynchronicity of the developmental cycle, although this was kept to a minimum by ensuring a low passage level. It is also highly probable that across the population of *C. abortus* there will be considerable variation in the levels of Pmp expression in individual cells and across the population of cells, which could play an important role in contributing towards antigenic variation. In addition, although McCoy cells are widely used for the growth of *C. abortus*, this organism can infect a wide variety of other cell types in which a different expression profile may have been observed. This will require further investigations, as well as to determine comparative protein expression patterns.

Based upon the premise that 16S rRNA transcripts are constitutively expressed throughout the cycle, other studies have used this against which to normalise their gene of interest [18, 20]. In those studies fixed concentrations of chlamydial RNA were used in the final analyses and it is highly probable that ribosomal RNA does make up a stable component of the chlamydial RNA pool. Whilst this approach illus-

trates comparative differences in transcript levels of particular genes of interest within a fixed pool of RNA at a particular time point, it is more difficult to compare transcript levels across time points due to fluctuations in the levels of total RNA and 16S rRNA. The methodology adopted in this study, where both DNA and RNA were isolated from duplicate cultures, allowed the direct determination of transcript numbers relative to the number of organisms present (as determined by genome copy number). The results clearly demonstrate that 16S rRNA transcript levels vary throughout the cycle, increasing to around 48 h and thereafter falling back to low levels reflecting conversion of the RB to EB and consequential reduction in metabolic activity.

All *pmps* demonstrated a peak in transcript levels during the mid to late cycle (24–48 h p.i.). These results are in agreement with previous studies carried out at both the proteomic [29, 30, 32] and transcriptomic levels [19] for other chlamydial species (*C. trachomatis*, *C. psittaci* and *C. pneumoniae*). The function(s) of the Pmps remains to be elucidated however it is attractive to speculate that the relatively late timing of their mRNA expression is consistent with their presence in either the infectious EB or their requirement in part of the differentiation process, and so potential roles in adhesion, antigenic variation and immune evasion.

Whilst transcript levels of several *pmps* were significantly up-regulated by 24 h the level of expression of *pmp5E* at this point was most dramatic. The structure of this particular Pmp is divergent from the other family members in that the protein consists of only a beta-barrel and autochaperone domain and no apparent passenger domain (effector protein). Given the lack of a passenger domain it has been hypothesised that *pmp5E* represents an orphan gene [31]. However, in the closely related *C. caviae* sequence there is also a *pmpE/F* gene encoding a truncated Pmp [24]. It is plausible that the truncated Pmp5E may fulfil an as yet undefined role in chlamydial development and could hypothetically function as part of a two-partner secretion system with a yet to be identified partner or could also act as a porin rather than as a classical autotransporter.

The original characterised Pmp proteins identified in *C. abortus* were those now termed Pmp12/17G (Pomp90A/B), Pmp13G (Pomp91A) and Pmp16G (Pomp91B) [15]. Pmps 12/17G and 16G were identified as being highly expressed through their immunogenicity with convalescent sheep serum [14] and shown to be highly immunodominant components of a protective chlamydial outer membrane protein preparation [15]. This is consistent with the elevated levels of transcriptional expression observed for *pmps* 12/17G and 16G, relative to the other *pmps* (with the exception of *pmp18D*). Interestingly, Pmp16G was identified in the recently published *C. abortus* genome as a pseudogene by virtue of a frame-shift in a centrally located polyguanine tract [31], while no such frame-shift was observed when originally identified through expression analysis and sequencing [15]. Indeed, Pmp16G has also been reported to be expressed as a full length protein following 2-dimensional gel electrophoresis of whole *C. abortus* EB preparations [6]. In this current study, while we have demonstrated the expression of *pmp16G* transcripts we have not examined whether any of them contain a frame-shift mutation that would result in a truncated protein. Phase-variation of protein expression has been observed in the *pmp* genes of other chlamydial species containing homopolymeric tracts [22] and so could also be a mechanism by which they contribute to antigenic variation.

In comparison with *omp1*, the expression of each of the *pmps* was very low (up to 450-fold at 48 h). However in comparison to the other *pmps* the expression of *pmp18D* was relatively high from mid to late in the cycle. PmpD is the most highly conserved of all the Pmps at both the DNA and amino acid level across the *Chlamydiaceae* and perhaps this level of conservation combined with the relatively high abundance of transcripts may point to an essential role in the chlamydial developmental cycle. A putative role for PmpD in the adhesion of *Chlamydia* to host cells has been hypothesised, as antibodies directed against the N-terminal domain have been at least partially successful in inhibiting the in vitro infection of both *C. pneumoniae* [33] and *C. trachomatis* [3]. However, the expression of this gene earlier

in the cycle would perhaps suggest an additional role for this protein during the late replicative/early differentiation phase.

The results presented in this study represent the most extensive transcriptional characterisation of the *C. abortus pmps* during the developmental cycle. The data indicates that most of the Pmps are expressed during the later stages of the cycle and thus are likely to play important roles in either the RB or the conversion of RB to EB and that the low level of expression may be indicative of a mechanism of antigenic variation. Whilst this is an important step forward in our understanding of the regulation of *pmp* gene expression in *C. abortus* it will be important to follow up these analyses with further studies aimed at investigating protein expression.

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