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Altered developmental expression of polymorphic membrane proteins in penicillin-stressed *Chlamydia trachomatis*

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Summary

Late *Chlamydia trachomatis* inclusions express each member of the surface-exposed polymorphic membrane protein family (Pmp subtypes A through I) with a reproducible distribution of fully-on, fully-off and intermediate phenotypes. This observation is consistent with observed variable Pmp antibody profiles in *C. trachomatis*-infected patients and has led to the hypothesis that the *pmp* gene family forms the basis of a phase variation-like mechanism of antigenic variation. Here we investigate and compare the developmental expression of each of the nine *pmp* genes under conditions of optimal *in vitro* growth with that under conditions that promote prolonged survival of chlamydiae when exposed to penicillin-induced stress. We demonstrate that the *pmp* gene family includes distinct transcriptional units that are differentially expressed along development and differentially responsive to stress. In particular, our results indicate that expression of *pmpA*, *pmpD* and *pmpI* is uniquely unaffected by stress, suggesting that the PmpA, PmpD and PmpI proteins play a critical role in the pathogenesis of *C. trachomatis*.

Introduction

Members of the *Chlamydiaceae* cause widespread infections in humans and animals. *Chlamydia trachomatis*, the world's most common sexually transmitted bacterial pathogen (WHO, 2001), is also the agent of trachoma, the world's leading cause of preventable blindness (Whitcher *et al*., 2001). *Chlamydia pneumoniae* is ubiquitous in animals and humans where it causes frequent respiratory infection (Ekman *et al*., 1993; Grayston *et al*., 1993; Heiskanen-Kosma *et al*., 1999) and is associated with coronary disease (Campbell *et al*., 1995; Kuo *et al*., 1995; Kontula *et al*., 1999; Wyplosz *et al*., 2006), the world's number one killer disease of humans. Contrasting with the diversity of hosts and disease sites is the highly conserved biphasic developmental cycle that constitutes the backbone of the unique obligate intracellular biology of *Chlamydia* (Rockey and Matsumoto, 1999). A chlamydial infection begins when an infectious but metabolically dormant elementary body (EB) attaches to and enters into a mucosal epithelial cell of a eukaryotic host. A critical early event is *Chlamydia*'s unique ability to escape phagolysosome fusion, enabling survival of the internalized EB and its subsequent differentiation into the 'first' reticulate body (RB), the metabolically active, replicating form of the organism. RBs divide by binary fission strictly within the confines of a phagosome-derived vacuole termed the inclusion. After several rounds of exponential growth, RBs that may now number in the hundreds in the replete inclusion start to differentiate to EBs asynchronously; such that the mid cycle inclusion contains many replicating RBs and few EBs, while the late inclusion contains few remaining RBs and a majority of fully differentiated, fully infectious EBs. The infectious progeny may then be released and disseminated to new mucosal sites upon lysis of the infected host cell or exocytosis (Todd and Caldwell, 1985; Hybiske and Stephens, 2008) thereby closing the developmental cycle.

Chlamydial development may be altered *in vitro* upon exposure to antibiotics (Tamura and Manire, 1968; Matsumoto and Manire, 1970; Dreses-Werringloer *et al*., 2000; 2001), depletion of essential nutrients such as amino acids (Allan and Pearce, 1983; Allan *et al*., 1985; Beatty *et al*., 1994a) or iron (Raulston, 1997; Al-Younes *et al*., 2001), exposure to IFN-γ (Beatty *et al*., 1993; Pantoja *et al*., 2001), heat shock (Engel *et al*., 1990a; Kahane and Friedman, 1992) or phage infection (Hsia *et al*., 2000). Under these conditions, RBs undergo a classical stress response characterized by upregulated expression of heat shock chaperonins (Engel *et al*.,

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1990b; Molestina *et al*., 2002; Belland *et al*., 2003) and coincidental inhibition of cell division. This results in the generation of aberrantly enlarged, multinucleated RB forms termed aberrant RBs (Beatty *et al*., 1993). Because aberrant RBs may survive *in vitro* for extended periods of time, yet are able to revert to normal RBs (and later differentiate to EBs) upon removal of the stressor (except for phage-induced stress), the chlamydial stress response has been suggested to play a role in persistent or chronic infection *in vivo* (Beatty *et al*., 1994c), a hallmark of chlamydial disease. Among these, persistence induced *in vitro* by penicillin has provided a convenient, albeit controversial, model for persistent chlamydial infections in humans. An uncontested benefit of *in vitro* persistence models, however, is that comparative studies of developmental growth under stressed and normal conditions have provided unique insight into pathways that determine the fate of a chlamydial infection and its impact on the host response (Beatty *et al*., 1994c; Belland *et al*., 2003).

We have recently reported unique regulatory properties of a polymorphic membrane protein (Pmp) gene family (Grimwood and Stephens, 1999) present in all species of the *Chlamydiaceae* (Stephens *et al*., 1998; Kalman *et al*., 1999; Read *et al*., 2000; 2003; Thomson *et al*., 2005; Azuma *et al*., 2006). Under normal culture conditions, each of the nine Pmp proteins of *C. trachomatis* (Pmp subtypes A through I) was variably expressed at the surface of chlamydiae in individual inclusions (Tan *et al*., 2010). Inclusions observed at 48 h post infection (hpi) displayed a reproducible distribution of fully-on, fully-off and intermediate Pmp phenotypes. These observations together with observed variable Pmp subtype-specific antibody profiles in *C. trachomatis*-infected patients (Tan *et al*., 2009) are consistent with our hypothesis that the *pmp* gene family of *C. trachomatis* forms the basis of a phase variation-like mechanism of antigenic variation, presumably for the dual purpose of immune evasion and adaptation to different niches within the infected host(s) (Tan *et al*., 2010).

These findings are significant in view of the reported or predicted functions of the *pmp* gene family. Recent reports have identified PmpD of *C. trachomatis* (Crane *et al*., 2006; Swanson *et al*., 2009), and Pmp6, Pmp20 and the PmpD orthologue, Pmp21, of *C. pneumoniae* (Wehrl *et al*., 2004; Molleken *et al*., 2010) as possible adhesins for both these organisms. Indeed motifs similar to the GGA(I,L,V) and FXXN repeat motifs present in the Pmps have also been found in adhesins of *Anaplasma phagocytophilum* (Girard and Mourez, 2006). These properties and the independent immunoproteomic identification of Pmp peptides as protective antigens (Karunakaran *et al*., 2008), has highlighted the potential of the surface-exposed Pmp family in the development of a multi-component *Chlamydia* vaccine.

Here, we examine the expression of each member of the *pmp* gene family of *C. trachomatis* at the transcriptional and phenotypic levels along normal development and under conditions of penicillin-induced stress. Results suggest that multiple levels of regulation exist including transcriptional and post-transcriptional mechanisms with several *pmp* genes differentially expressed along development and six of nine *pmp* genes strongly downregulated in stressed cultures. Moreover, the identification of several Pmps commonly expressed under normal and stressed conditions provides new incentive for further investigation into the potential of Pmp proteins in the development of a multi-component vaccine against chlamydial genital infections.

Results

pmpABC*,* pmpFE *and* pmpGH *are cotranscribed in* in vitro*-grown* C. trachomatis

In view of the colinearity of *pmpA*, *B* and *C*, *pmpF* and *E*, and *pmpG* and *H* (Fig. 1A), we first investigated the presumed cotranscription of these genes by RT-PCR. Because our previous immunofluorescence (IF) studies of *in vitro*-grown *C. trachomatis* serovar E have shown that each Pmp protein subtype is strongly expressed at late developmental times (Tan *et al*., 2010), we evaluated transcription in similar cultures at 24, 32 and 48 hpi. Total RNA was used to amplify the intergenic regions between members of each gene pair by RT-PCR. Figure 1B shows that the *pmpA-B, pmpB-C*, *pmpF-E* and *pmpG-H* intergenic regions are amplified at 24, 32 and 48 hpi, confirming that these loci are transcribed at late developmental times and indicating that *pmpABC*, *pmpFE* and *pmpGH* are organized in operons. Transcript levels were markedly lower for *pmpA-B* and *pmpB-C* than for *pmpF-E* and *pmpG-H*.

pmp *transcription is developmentally regulated*

Transcription of each *pmp* gene was first examined at 2 hpi, then at 6 h intervals during the active growth phase of *C. trachomatis* (2–24 hpi) and at longer intervals thereafter using RT-qPCR (Fig. 2). The essential gene *tufA* encoding elongation factor EF-Tu involved in protein synthesis was used for comparison as it is not only well-expressed throughout development but is also a reliable measure of exponential growth. Conveniently, EF-Tu is also used as a target antigen for IFA staining of inclusions in subsequent experiments in this study (see below) (Zhang *et al*., 1994). After a lag corresponding to early EB-to-RB differentiation, *tufA* transcription expectedly rose swiftly until 18 hpi, levelled off between 18 and 24 hpi and sharply decreased beyond 24 hpi as RBs differentiate

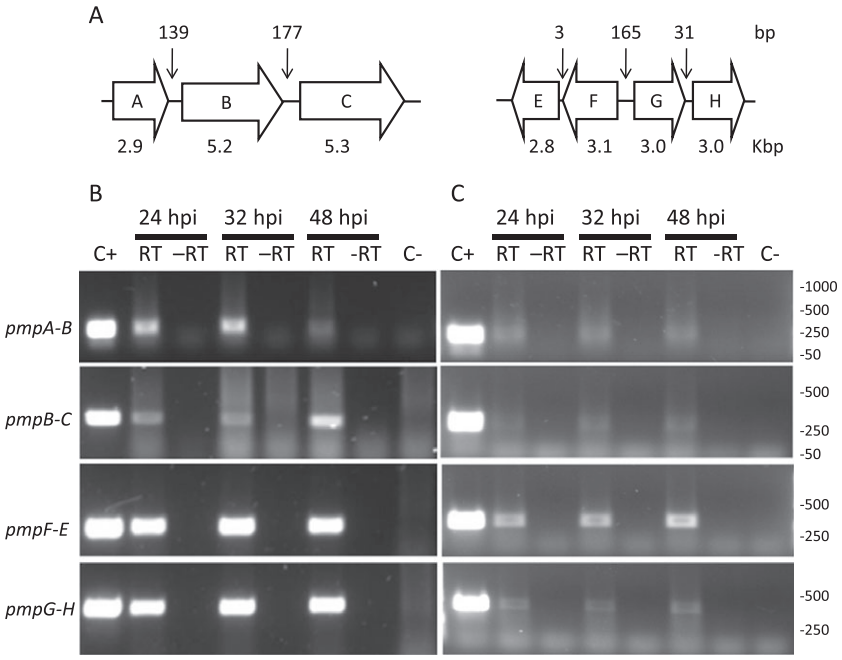

Fig. 1. *pmpABC*, *pmpEF* and *pmpGH* are single transcriptional units. (A) Organization of the *pmpABC*, *pmpFE* and *pmpGH* putative operons with gene sizes and intergenic distances (not drawn to scale). Total RNAs from *C. trachomatis* cultures grown under normal conditions (B) or with 200U penicillin (C) were obtained at different times post infection as indicated and amplified by RT-PCR with specific primers flanking the intergenic region of *pmpA-pmpB*, *pmpB-pmpC*, *pmpF-pmpE* and *pmpG-pmpH* respectively. C+, positive control *C. trachomatis* genomic DNA. C–, negative control cDNA from uninfected HeLa cells; RT/-RT, with/without reverse transcriptase. Molecular sizes were obtained by alignment with a 50 bp ladder (Fermentas).

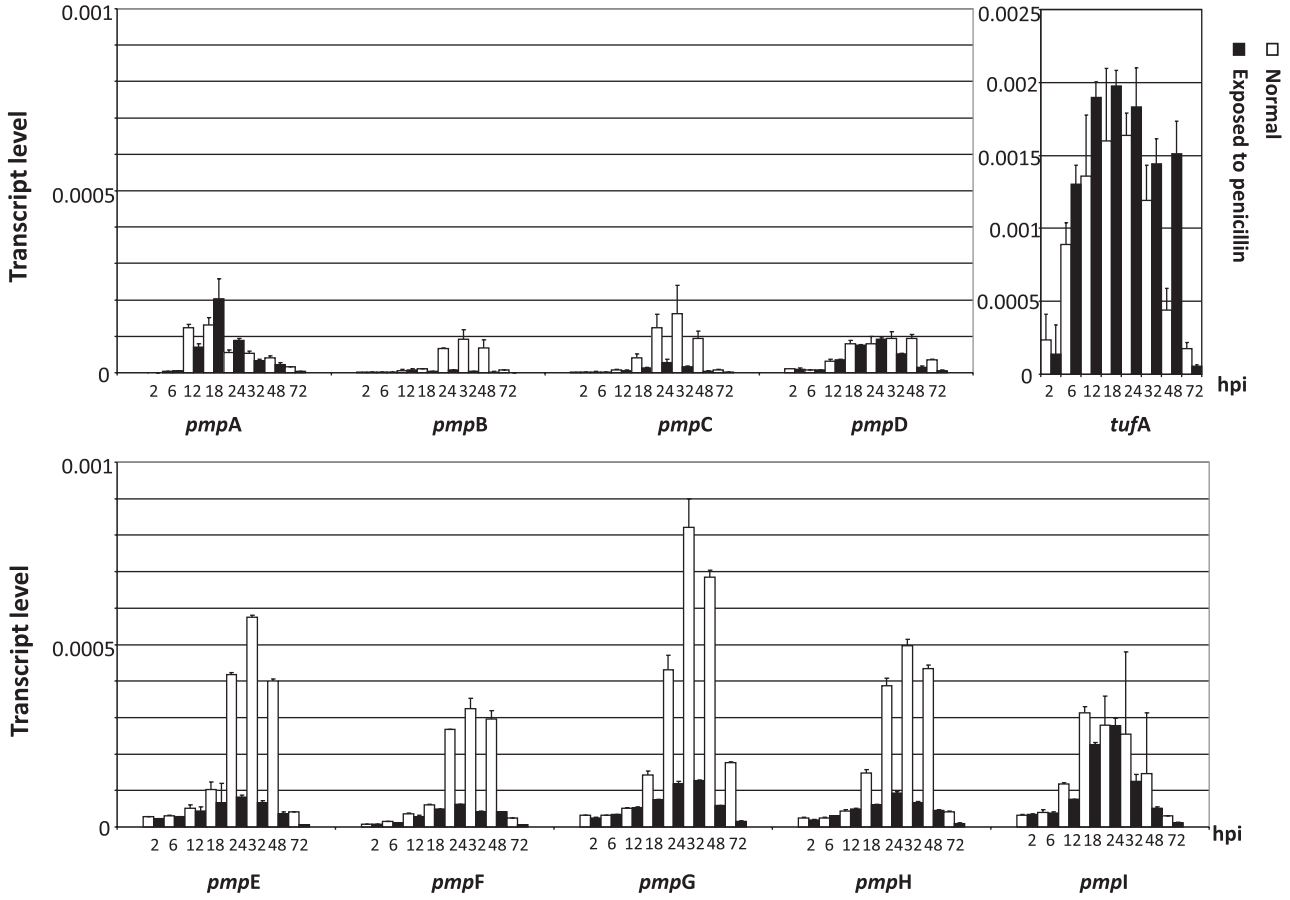

Fig. 2. Transcription of specific *pmp* genes of *C. trachomatis* is differentially regulated along development and upon exposure to penicillin. Transcript levels were measured by real-time RT-qPCR in *C. trachomatis* grown under normal conditions (white bars) and with penicillin (black bars) at 2, 6, 12, 18, 24, 32, 48 and 72 hpi. Transcript levels of the *tufA* gene encoding EF-Tu are used for comparison.

into EBs and protein synthesis gradually shuts down in a growing number of differentiating RBs within the inclusion (Fig. 2). Although detectable for all *pmp* genes, 2 hpi transcript levels were expectedly very low, particularly for *pmpA-C.* Consistent with the results of RT-PCR amplification of intergenic segments (Fig. 1B), expression was highest for the linked *pmpEFGHI* genes with a ninefold difference between the most and least expressed *pmp* genes (*pmpG* and *pmpB*, respectively) and a fourfold average difference between genes of the *pmpEFGHI* and *pmpABC* loci. In sharp contrast with *tufA* expression, transcription of all *pmp* genes except *pmpA*, *pmpD* and *pmpI* was highest at late (24–48 hpi) developmental times with a characteristic peak at 32 hpi followed by a decline at 48 hpi and beyond (Fig. 2). Expression of the unlinked *pmpA* and *pmpI* rose significantly at 12 hpi, peaked at mid development (18 hpi) and declined thereafter. Transcript levels of *pmpD* uniquely continued to rise until 48 hpi and declined thereafter.

pmp *transcription is altered in penicillin-induced persistent* C. trachomatis

In experiments using *C. trachomatis* grown under normal conditions, we infrequently observed inclusions containing aberrantly enlarged chlamydiae at late developmental times with altered or shut off *pmp* expression (Fig. 3). Because of the physical likeness of these rare inclusions to stress-induced *in vitro*-persistent inclusions, we investigated the expression of each *pmp* gene comparatively in *C. trachomatis* grown under normal conditions and under penicillin-induced stress by RT-qPCR. *tufA* again provided an appropriate control for these experiments as a chlamydial gene unlikely to be influenced by penicillin and whose expression is maintained past 24 hpi in stressed chlamydiae (Belland *et al*., 2003). As expected, expression of *tufA* was not significantly affected by exposure to penicillin until the late developmental time of 48 hpi when expression was upregulated under penicillin stress. Expression

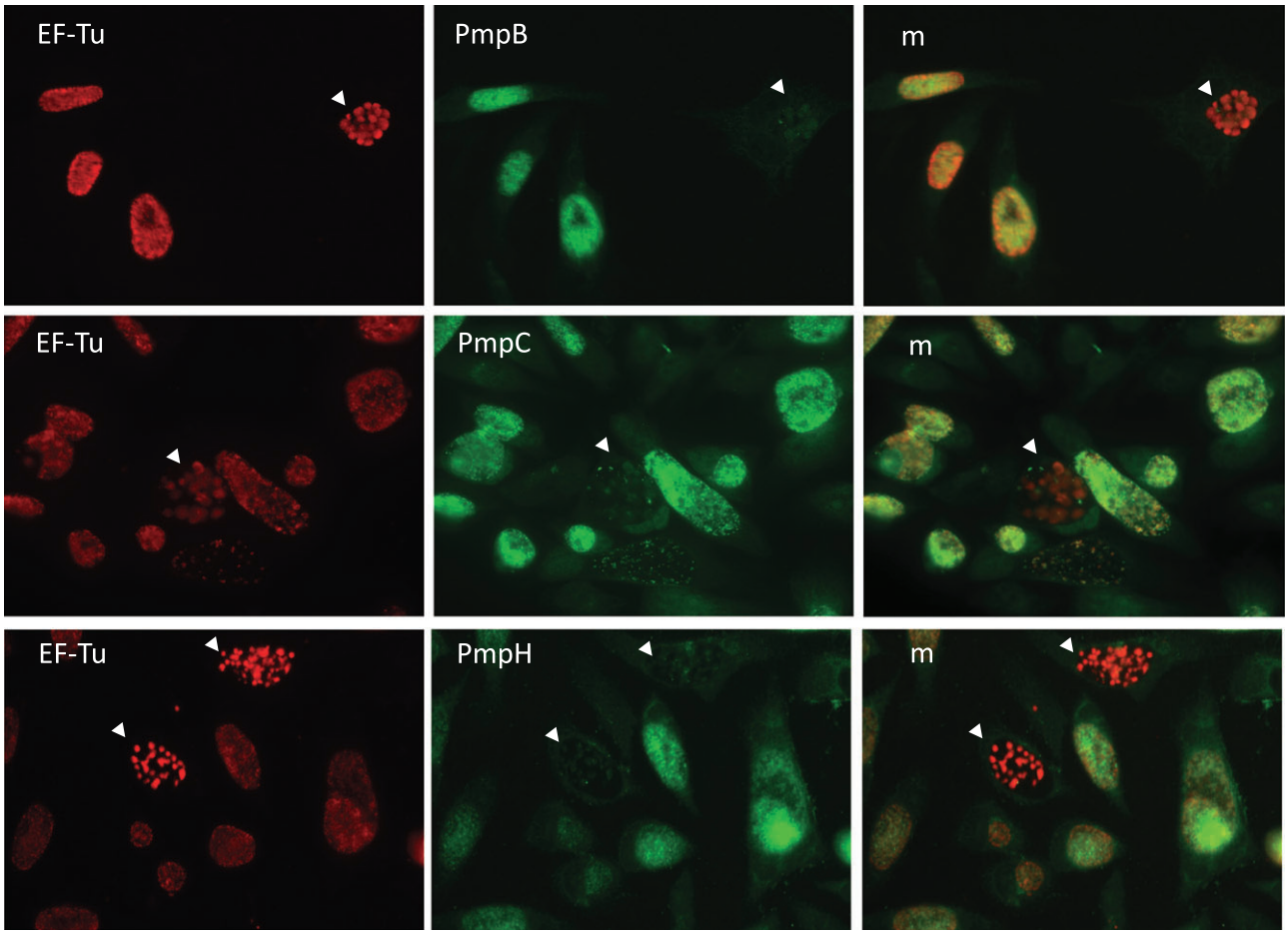

Fig. 3. Aberrant inclusions are present in normal cultures. Infected HeLa cells were fixed at 42 hpi and double-stained with anti-EF-Tu antibody and antibodies specific for PmpB, PmpC or PmpH as indicated, and visualized at 400 × magnification. Single channel and merged images (m) are shown. White arrowheads indicate aberrant inclusions.

of *tufA* appeared to fall sharply at the latest developmental time of 72 hpi under both growth conditions; however, this was due to an unexpected loss of monolayer integrity rather than to specific downregulation (not shown).

Exposure of infected monolayers to penicillin led to the downregulated expression of all *pmp* genes except *pmpA*, *pmpD* and *pmpI*. The largest drop in expression was seen for *pmpB* and *pmpC* at 32 hpi (27- and 21-fold, respectively). Expression of *pmpA, pmpD* and *pmpI* was relatively unchanged upon exposure to penicillin except for a partial decrease at early times for *pmpA* and *pmpI* and at late times for *pmpD* in penicillin-induced stress vs. normal conditions. Both RT-PCR (Fig. 1C) and RT-qPCR (Fig. 2) experiments revealed similarly reduced transcript levels for *pmpE, F, G* and *H* under penicillin exposure that were approximately twofold lower than that for *pmpI* by RT-qPCR.

Pmp production is altered in penicillin-stressed C. trachomatis *inclusions*

Although RT-qPCR is suitable to observe changes in gene expression at the population level, this method is inappropriate to evaluate production from the expressed gene in individual inclusions. To address this question, we investigated the production of each Pmp subtype in *C. trachomatis*-infected HeLa 229 monolayers with or without exposure to penicillin by IF using a panel of monospecific polyclonal antibodies (pAbs) and monoclonal antibodies (mAbs) and methodology that has been previously described (Tan *et al*., 2010). Measurements of overall fluorescence intensity were also obtained to further quantify visual evaluations. Although penicillin-induced stress is not encountered by chlamydiae during a natural untreated infection, a significant benefit of this model is that only growing chlamydiae, not host cells, are affected by the drug. Although early inclusions were too small to evaluate Pmp production, the off frequency of inclusions stained for each Pmp subtype was similar to our earlier results (Tan *et al*., 2010) at each developmental time at which whole inclusions could be observed (approximately 12 hpi and beyond) (not shown). Inclusions at the two developmental times of 24 and 48 hpi are shown in Fig. 4. These, respectively, represent inclusions near the onset of late differentiation and mature inclusions in unstressed cultures, and inclusions at two stages of persistent growth in cultures exposed to penicillin. The 24 and 48 hpi times allowed for direct comparison of inclusions containing non-dividing aberrant RBs in the two stressed cultures with inclusions, respectively, containing a majority of undifferentiated RBs or mostly differentiated EBs in the unstressed culture (Fig. 4). Double staining with EF-Tu-specific mAb and Pmp subtype-specific pAb or mAb yielded distinct patterns of Pmp production in individual stressed inclusions at the two developmental times. In agreement with the qRT-PCR results, neither PmpB (Fig. 4B) nor PmpC (not shown) were produced to any significant levels by IF at any time in stressed cultures (Fig. 4E). Consistent with qRT-PCR results, PmpA staining intensity was similar in stressed and unstressed cultures (Fig. 4E). In unstressed inclusions, PmpA-specific and EF-Tu-specific staining were mostly colocalized in the inclusion lumen, suggesting that a major portion of the PmpA protein remains associated or in close proximity to the chlamydiae (Fig. 4A). However, in penicillin-stressed inclusions, while EF-Tu staining was concentrated on aberrant RBs, PmpA staining was distributed over the whole inclusion suggesting that PmpA antigen is diffusible under these conditions (Fig. 4A). In contrast, the production of PmpD and PmpI, although predicted to be mostly unchanged in penicillin-stressed cultures by RT-qPCR, was altered with respect to both the distribution and level of production of the proteins (Fig. 4C and D). PmpD and I antigen appeared to concentrate in the luminal space between mostly unstained aberrant RBs with higher amounts of the proteins concentrating at the periphery of the aberrant RBs. Overall production of PmpD and PmpI was also decreased by 43% and 47% at 24 hpi and 50% and 40% at 48 hpi, respectively, under exposure to penicillin (Fig. 4E), perhaps reflecting the observed late downregulation of *pmpD* transcription (Fig. 2) and decreased stability of both proteins in stressed cultures. Production of the genetically linked PmpE, G and H proteins, predicted to be dramatically reduced by RT-qPCR, was expectedly reduced by IF at the two developmental times (not shown and Fig. 4E). However, while PmpE antigen was rarely detected, PmpG and H proteins were detected concentrated in the luminal space surrounding aberrant RBs similar to the pattern observed for PmpD and I (e.g. PmpI in Fig. 4). PmpF, although predicted to be reduced by RT-qPCR, was produced at similar levels in both stressed and unstressed cultures (Fig. 4E), perhaps reflecting elevated stability of the protein under stress conditions, and was also distributed similar to PmpD and PmpI (Fig. 4C and D). It was also noted that while the overall level of production of PmpD, G, H and I was generally lower at 48 than 24 hpi, there was significant inclusion-to-inclusion variation at either time.

Discussion

The observed, uncoordinated on/off switching of each member the *C. trachomatis pmp* gene family (Tan *et al*., 2010) suggests complex, multilayered mechanisms involving transcriptional and post-transcriptional regulation. In this study, we investigate the regulation of *pmp* expression as a consequence of two essential physiological functions: the specific requirement of chlamydiae to

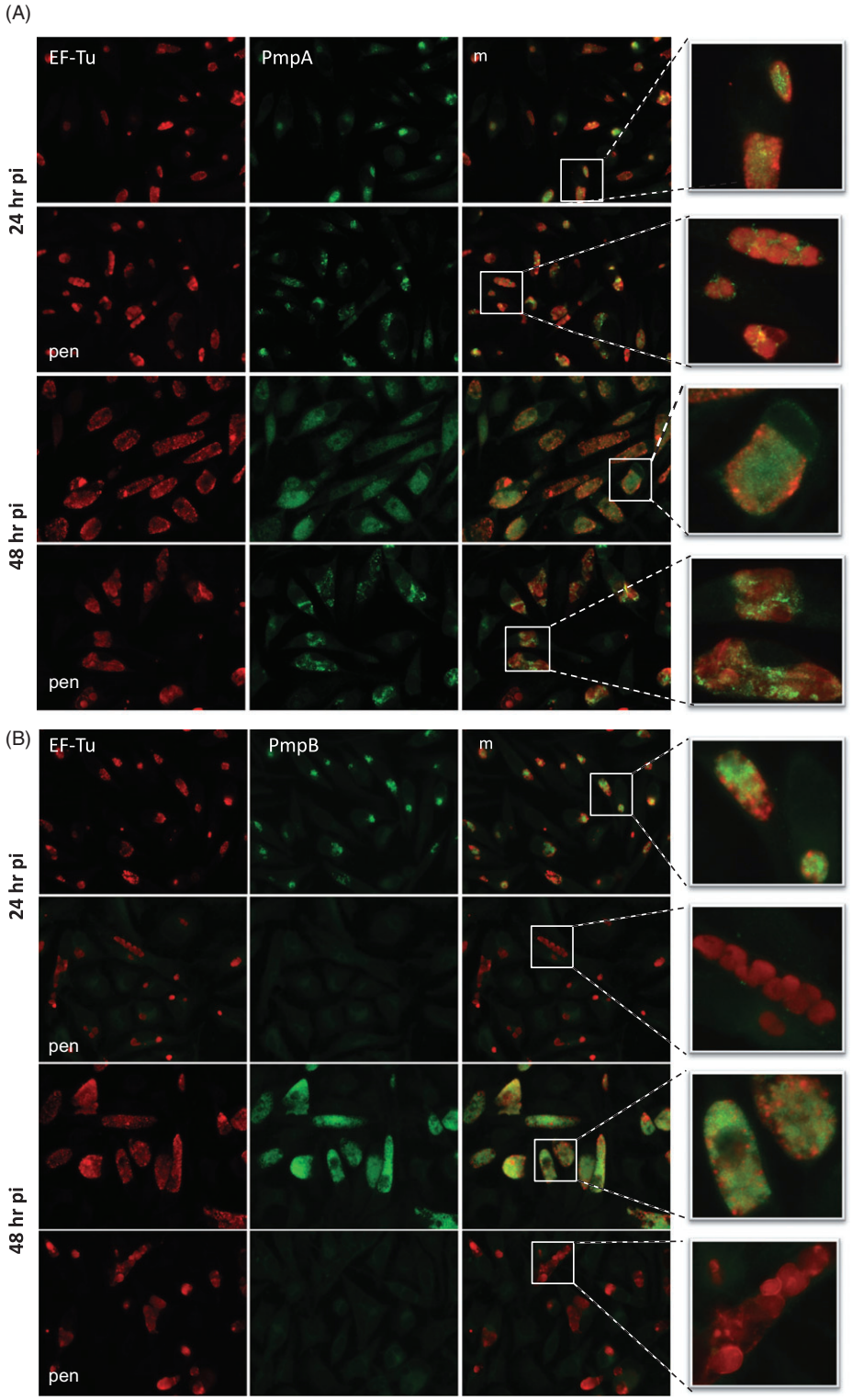

Fig. 4. Production of Pmps is altered in stressed inclusions. *C. trachomatis*-infected HeLa cells were fixed at 24 and 48 hpi, and double-stained with EF-Tu-specific antibody and Pmp-specific antibody as indicated. Merged images (m) and magnified insets thereof are shown in the two right-most columns. For each Pmp, inclusions grown under normal culture conditions or penicillin-induced stress conditions (pen) are shown. Only results obtained for PmpA (A), PmpB (B), PmpD (C) and PmpI (D) are shown as they are representative of all other Pmps. Staining patterns at 24 and 48 hpi obtained for PmpC, PmpE, PmpF, PmpG and PmpH (not shown) were, respectively, similar to those obtained for PmpB, PmpD, PmpA, PmpD and PmpD. (E) IF staining quantified at 24 and 48 hpi under normal (white) and penicillin-stressed (black) conditions. Statistically significant differences ($P < 0.01$) are indicated with *.

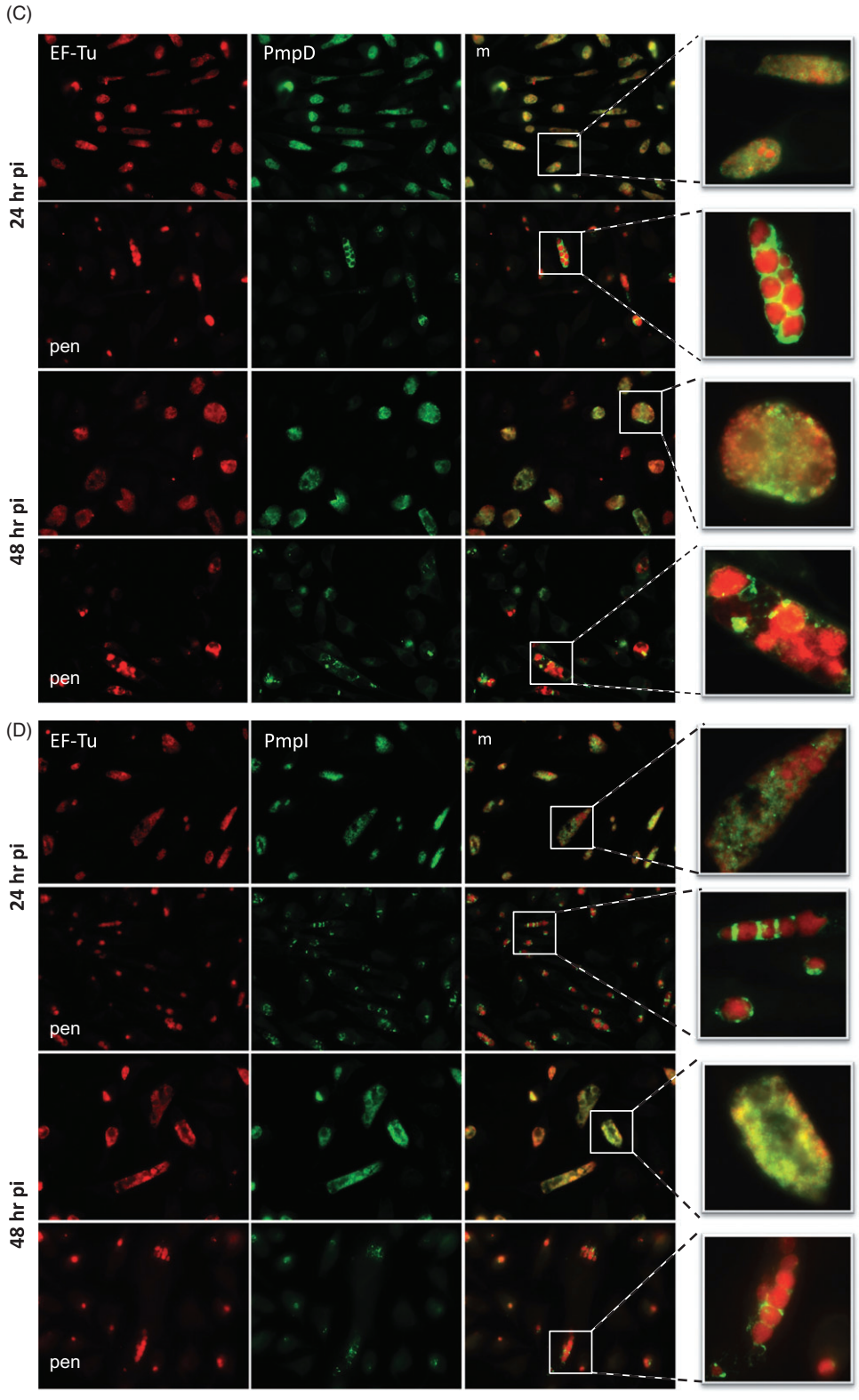

Fig. 4. *Continued.*

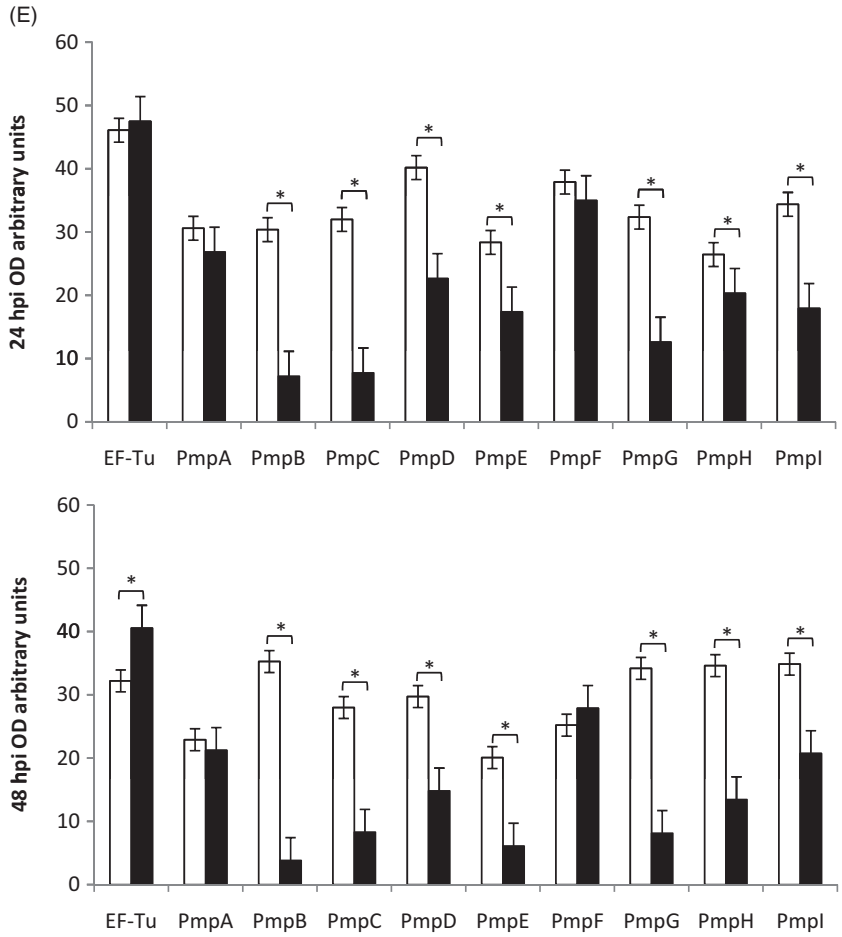

Fig. 4. *Continued.*

differentiate from replicating RBs to infectious EBs, and the chlamydial survival response to stress.

In order to assess transcriptional regulation, we first undertook to identify transcriptional units. The presumed operon structures of the *pmpABC*, *pmpFE* and *pmpGH* were confirmed by detection of intergenic cDNAs. Although cotranscription of *pmpBC*, *pmpFE* and *pmpGH* is consistent with transcriptional regulation as the basis of the putative on/off switching mechanism as the observed Pmp/off frequencies within each gene pair are similar (respectively 5–10/5–10, 1–2/5–10 and 1–10/1–2%), dissimilar off frequencies for PmpA (0.1–1%) and PmpB/C (5–10%) and the observation that PmpB/on-PmpC/off and PmpB/off-PmpC/on inclusions coexist in the same culture (Tan *et al*., 2010) suggest that at least for the *pmpABC* operon, distinct regulatory mechanisms are operating for each gene.

The uniqueness of the *pmpABC* operon was further highlighted by the distinct developmental profile of *pmpA* whose transcript peaked at the mid developmental times of 12 and 18 hpi, sharply contrasting with most other *pmp* transcripts, including those of the putatively cotranscribed *pmpB* and *C* that peaked late (Fig. 2). A possible explanation for this result is that *pmpA* may be under the control of a second *pmpA*-specific promoter in addition to the *pmpABC* operon promoter. Alternatively, post-transcriptional mechanisms affecting mRNA stability or epigenetic modification may be responsible for the observed differential developmental transcription within the *pmpABC* operon. Similar to *pmpA*, *pmpI* transcription also peaked at the early-late developmental time of 18 hpi, suggesting that multiple, distinct regulatory mechanisms govern expression at the *pmpEFGHI* locus. The *pmpBC* and the highly expressed *pmpFE* and *pmpGH* cotranscribed pairs as well as the unlinked *pmpD* gene displayed similar transcription profiles along development with a typical peak at 32–48 hpi, suggesting a role of the corresponding gene products either at late stages of development or during the early steps of infection. Notwithstanding differences in the developmental transcription profiles of *pmp* genes and operons, transcripts were detected for all *pmp* genes at late developmental

times, consistent with the detection of *pmp* gene products at the EB surface in late inclusions (Tan *et al*., 2010) and with the presence of antibodies to one or more Pmps in *C. trachomatis*-infected patients (Tan *et al*., 2009).

The expression profile of the *pmp* gene family was noticeably altered in response to stress. Whereas transcription of *pmpBC, pmpFE* and *pmpGH* was markedly downregulated when infected cells were exposed to penicillin, transcription of *pmpA*, *pmpD* and *pmpI* was mostly unaffected with only partial, statistically significant reduction of *pmpD* transcription at 48 hpi (Fig. 2). Penicillin-stressed transcript levels were generally concordant with expressed levels of the gene products detected by IF in penicillin-stressed infected cells (Fig. 4). A similar expression profile was independently observed for *pmp*D of *C. trachomatis* serovar L2 (Kiselev *et al*., 2007), suggesting that these findings hold true across biovars, serovars and strains. It is interesting that *pmpA*, *pmpD* and *pmpI*, which are the most conserved *pmp* genes in *C. trachomatis* (respectively 99.6%, 99.1% and 99.2% similarity at the amino acid sequence level), are also uniquely expressed under stress conditions. It suggests that these three proteins may play a critical role above and beyond that of the other Pmps under conditions that are least amenable to chlamydial growth, i.e. as chlamydiae are faced with the need to survive rather than grow. It is also noteworthy that these three Pmps were subject to the lowest Pmp/off frequencies in inclusions by as much as a log when compared with the other Pmps (Tan *et al*., 2010). One may speculate that under conditions of stress encountered in the infected host, chlamydiae expressing these three Pmps may be at an advantage and that their maintained production under stress conditions may reflect the continuous selection for survival of the fittest chlamydiae in the infected host. Moreover, the observation that the Pmp subtype off frequency did not change over developmental times is consistent with our previous observation of PmpB/on and PmpB/off inclusions in the same cell (Tan *et al*., 2010), suggesting that the Pmp phenotype of a given inclusion is a property inherent to the EB that led to its formation.

The results presented herein have shed light on possible regulatory mechanisms governing the expression of the *pmp* gene family. The significance of our findings however rests on the role of the chlamydial stress response in the context of infection and disease of humans. The initial observation that chlamydiae persist as non-dividing RBs for extended periods of time when exposed to stress and that removal of the stressor allows resumption of normal development to fully infectious EBs, was tentatively interpreted as the *in vitro* reflection of persistent, subclinical infection *in vivo* and ensuing chronic disease (Beatty *et al*., 1994c). This was strengthened further by the observations that IFN-gamma, high levels of which are found at the site of chlamydial infections, induce a potent stress response in chlamydiae through depletion of the essential amino acid tryptophan (Beatty *et al*., 1994a,b). Although a direct role of the stress response in the establishment of persistent infection is debated, non-dividing aberrant RBs can be occasionally observed in unstressed cultures both *in vitro* (Fig. 3) and *in vivo*, in the context of disease (Whittum-Hudson *et al*., 2006; Pospischil *et al*., 2009). It is therefore likely that stress-induced aRBs are an integral part of the biology of chlamydial infections regardless of their hypothetical role in persistence in the infected host. The observed maintained expression and production of PmpA, PmpD and PmpI under conditions of stress (Figs 2 and 4) may thus be highly significant for survival of the organism in mucosal or cellular sites that are least hospitable for chlamydial growth. The combined identification of PmpI as an antigen more frequently recognized by antibodies of women with pelvic inflammatory disease than of adolescent and young adult women with primary genital *C. trachomatis* infection ($P < 0.0001$) (Tan *et al*., 2009), and whose expression is unchanged under conditions of penicillin-induced stress, provides incentive for further investigation into the potential of high titre PmpI-specific antibodies as serologic predictors of chronic or more severe chlamydial disease. Moreover the identification of three Pmps (PmpA, D and I) that are commonly expressed under normal and stress-induced persistent conditions highlights the potential of these Pmps in the development of a component vaccine against *C. trachomatis* infection.

Experimental procedures

Culture of C. trachomatis

Chlamydia trachomatis serovar E strain UW5-CX was propagated in HeLa monolayers without cycloheximide as previously described (Tan *et al*., 2009). In some experiments, penicillin (200U) was added immediately after infection.

RNA methods

RNA extraction. Confluent HeLa 229 monolayers with and without added penicillin were mock-infected or infected with *C. trachomatis* at a multiplicity of infection of 1, placed at 37°C in 5% CO2 and harvested at 2, 6, 12, 18, 24, 32, 48, 72 hpi. Where appropriate, penicillin (200U ml^{-1}) was added immediately after infection. Monolayers were lysed by addition of 2 ml TRIzol reagent and incubation at room temperature for 5 min. After addition of chloroform and vigorous shaking for 30 s, lysates were further incubated at room temperature for 5 min and centrifuged at 12 000 *g* for 15 min at 4°C. Total RNA was then successively precipitated from the aqueous phase with isopropyl alcohol, washed with ice-cold 75% ETOH, air-dried at room temperature, resuspended in 200 μl RNase-free water and stored at

–70°C. RNA was visually examined by electrophoresis in agarose gels (1.5%) equilibrated with DEPC water, 5X RNA running buffer and formaldehyde (10%).

RT-PCR. Total RNA extracted at 24, 32 and 48 hpi was used to generate cDNA using SuperScript II RT (Invitrogen) following the manufacturer's instructions. The cDNA was then used to amplify the intergenic region between adjacent *pmp* genes by RT-PCR using specific primers designed using the FastPCR Professional 5.1.83 and OligoAnalizer 3.1 programs (Table S1). *C. trachomatis* genomic DNA was used as a positive control while HeLa 229 total RNA and non-reverse-transcribed *C. trachomatis* total RNA were used as negative controls.

RT-qPCR. After DNAse treatment, cDNA was generated from 1 μg RNA using SuperScript II RT using random hexamers. Expression of each *pmp* gene was measured using the iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories), iQ SYBR Green Supermix (Bio-Rad Laboratories), specific primers for each *pmp* gene (Table S1) and the standard curve method for relative quantification. 16S rRNA cDNA was used for normalizing the data as it provides a control for the number of organisms (EBs and RBs) (Nunes *et al*., 2007). Alternate normalization with 16S rRNA genomic DNA gave similar results although peak transcription of several *pmp* genes was shifted from 32 hpi to the earlier 18 or 32 hpi times (i.e. still considered late developmental times). Gene *tufA* encoding elongation factor Tu (EF-Tu) was included as an additional reference gene for RT-qPCR experiments. EF-Tu was also used as a positive control in correlated IF experiments (see below). Primers for each of the nine *pmp* genes and 16S rRNA were designed as previously described (Nunes *et al*., 2007). Primers for *tufA* (Table S1) were designed using the FastPCR Professional 5.1.83 and OligoAnalizer 3.1 programs. Each plate contained two replicates of each cDNA sample and three different negative controls. Standard curves were generated for each gene as previously described (Gomes *et al*., 2005). For each experiment, the amount of target and control gene was determined from each respective standard curve by conversion of the mean threshold cycle values. Normalization was obtained by dividing the amount of the target gene transcript by the amount of 16S rRNA. Specificity of the amplified products was verified by analysis of the dissociation curves generated by the iQ5 Optical System Software (Version 2.0) based on the specific melting temperature for each amplicon. The results were based on two independent experiments (i.e. four independent RT-qPCR reactions).

IF microscopy

HeLa monolayers grown to confluence on coverslips were infected with *C. trachomatis* serovar E at a multiplicity of infection of 0.5 in 24-well plates under normal culture conditions or in medium containing 200 U penicillin (added immediately after infection), incubated at 37°C in 5% CO2 and fixed with paraformaldehyde at 24, 48 or 72 hpi. Fixed monolayers were then permeabilized with PBS containing 0.1% Triton X-100, 0.05% SDS and 0.2% BSA and double-labelled with anti-EF-Tu monoclonal antibody (to stain inclusions) and Pmp-specific antibodies (Table S2). Guinea pig PmpA/B/C/E/F/G/H-specific antisera were pre-adsorbed with partially purified insoluble His-tagged recombinant beta-galactosidase to remove non-specific reactivity as before (Tan *et al*., 2010). For PmpA, B, C, E, F, G and H, double-stained IF micrographs were obtained by staining first for inclusions using mouse α-EF-Tu mAb as the primary antibody and Alexa 594-conjugated goat anti-mouse IgG as the secondary antibody, and second for a specific Pmp subtype using pre-adsorbed guinea pig Pmp-subtype-specific antiserum as the primary antibody and Alexa 488-conjugated goat anti-guinea pig IgG as the secondary antibody. For PmpD and I, double-stained IF micrographs were obtained by staining first for the specific Pmp subtype using mouse PmpD/I-specific mAb as the primary antibody and Alexa 488-conjugated goat anti-mouse IgG as the secondary antibody, and second for inclusions using mouse α-EF-Tu mAb conjugated to Alexa Fluor 594. The occurrence of PmpD and PmpI-negative inclusions (Tan *et al*., 2010) provided evidence of no cross reactivity between Alexa 488-conjugated goat anti-mouse IgG and mouse α-EF-Tu mAb. Stained inclusions were observed on a Zeiss Axio Imager Z1 fluorescence microscope (Carl Zeiss MicroImaging)

Measurements of average fluorescence intensity were obtained using ImageJ software 1.43u (Rasband W. NIH, USA, http://rsb.info.nih.gov/ij) for 30 to 50 inclusions in three independent experiments. Statistically significant differences were calculated for three independent experiments using *t*-test.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Primers designed for this study.
Table S2. Antibodies used in this study.

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