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The expression, processing and localization of polymorphic membrane proteins in *Chlamydia pneumoniae* strain CWL029

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Abstract

Background: Chlamydiae are obligate intracellular bacteria, which are important human pathogens. Genome sequences of *C. trachomatis* and *C. pneumoniae* have revealed the presence of a *Chlamydia* specific gene family encoding polymorphic outer membrane proteins, Pmps. In *C. pneumoniae* the family comprises twenty-one members, which are all transcribed. In the present study, the expression, processing and localisation of the sixteen full-length Pmps in *C. pneumoniae* strain CWL029 have been further investigated by two-dimensional gel electrophoresis and immunofluorescence microscopy.

Results: Ten Pmps were identified in elementary bodies (EBs). Eight of these were investigated with respect to time dependent expression and all were found to be up-regulated between 36 and 48 hours post infection. Antibodies against Pmp6, 8, 10, 11 and 21 reacted with chlamydiae when infected cells were formalin fixed. Pmp6, Pmp20 and Pmp21 were found in cleaved forms, and the cleavage sites of Pmp6 and Pmp21 were identified.

Conclusions: The Pmps are heavily up-regulated at the time of conversion of RB to EB, and at least ten Pmps are present in EBs. Due to their reaction in formalin fixation it is likely that Pmp6, 8, 10, 11 and 21 are surface exposed. The identified cleavage sites of Pmp6 and Pmp21 are in agreement with the theory that the Pmps are autotransporters.

Background

Chlamydiae are pathogenic gram-negative bacteria of which *C. pneumoniae* causes upper and lower respiratory tract infections in humans [1]. Going through a developmental cycle the chlamydiae alternate between infective elementary bodies (EBs) and replicative reticulate bodies (RBs) [2]. The bacteria are obligate and intracellular, residing inside a specialized phagosome, named the chlamydial inclusion. The duration of the developmental

cycle for *C. pneumoniae* cultivated in cell culture is about 72 hours [3].

The *C. pneumoniae* CWL029 genome sequence revealed the presence of a gene family, the *pmp* family, consisting of 21 members [4] that were paralogous to the *pmps* found in *C. trachomatis* [5] and *C. psittaci* [6–9]. The *C. psittaci* Pmps have been analysed by two-dimensional electrophoresis in an earlier study [37]. The Pmps are two-domain proteins with similarity to autotransporter proteins [4,10,11]. They are characterized by a high frequency of the two sequences FxxN and GGAI in the N-terminal part (twelve and seven repeats on average, respectively) [4], and their C-terminal part shows the characteristics of a β-barrel [10]. The GGAI motif and the prediction of a C-terminal β-barrel suggest that the Pmps are autotransporters, transporting an N-terminal passenger domain through a pore formed by their C-terminal part [10,12]. Seventeen *C. pneumoniae* Pmps contain a signal peptidase I cleavage site directing transport over the inner membrane, and two Pmps contain a signal peptidase II cleavage site suggesting lipid modification [4].

The Mw of most Pmps in *C. pneumoniae* strain CWL029 is predicted to be just below 100 kDa, but three are larger: Pmp6 is 142 kDa, Pmp20 is 178 kDa and Pmp21 is 167 kDa and Pmp12 is only 56 kDa. Four genes (*pmp3*, *4*, *5* and *17*) contain a mutation resulting in premature stop. In *C. pneumoniae* strain AR39 [13] and J138 [14]*pmp6* contains 393 less base pairs, and in strain J138 Pmp2 and Pmp4 contain frame shift mutations [14].

Variation of membrane protein expression is thought to provide protection against the immune system of the host [15] and it has been suggested that the Pmps may provide such a protection of chlamydiae. This was indicated by the findings of Birkelund *et al.* [16] and Pedersen *et al.* [17] who observed differential expression of Pmp10.

Transcripts have been detected from all Pmp genes in *C. trachomatis* [18] as well as *C. pneumoniae* [19]. As part of a large-scale proteome analysis [20], ten Pmps (Pmp2, 6, 7, 8, 10, 11, 13, 14, 20 and 21) or fragments of these were identified by mass spectrometry (MS) in *C. pneumoniae* CWL029 EBs. The positions of these in the 2-D protein profile can be viewed at [http://www.gram.au.dk]. In addition to these, Montigiani *et al.* [21] identified Pmp16 by MS. Grimwood *et al.* [19] found Pmp2, 6, 7, 8, 9, 11, 13, 14, 15, 16, and 18 to be present in *C. pneumoniae* CWL029 EBs by immunoblotting (IMB) using antibodies raised against synthetic 20-mer peptides. Pmp1, 19, 20 and 21 were not detected although full-length genes encode these. Pmp6 migrated around 100 kDa although it theoretically should be 144 kDa.

The detection of Pmp6 forty kDa below the predicted value has been interpreted by Henderson and Lam [10] as an indication that an N-terminal passenger domain is cleaved off, which is frequently seen for autotransporters. In strains AR39 and TW183, sharing the 393 base pair deletion, Pmp6 was detected at the resulting theoretical full-length position of 130 kDa by Grimwood *et al.* [19].

In the study described in this paper, 2-D PAGE was used to elucidate the expression pattern of the *C. pneumoniae* Pmps in CWL029 at different times during the developmental cycle. Pmp6, Pmp20 and Pmp21 were found to be cleaved and the cleavage sites of Pmp6 and Pmp21 were identified. IMF was used to determine whether Pmps could be detected at the surface of the bacteria.

Results

The proteome study by Vandahl *et al.* [20] was not conclusive on the absence of any Pmps. Hence, we performed 2-D immnoblotting (IMB) to identify the 2-D PAGE position of expressed Pmps not earlier mapped. Pmp3, 4, 5, 12 and 17 were omitted from the study because these are truncated. Polyclonal antibodies (pAbs) were obtained by immunization of rabbits with recombinant proteins produced in *E. coli* (table 1). Antigens were raised against the N-terminal part of Pmp6, 7, 8, 10, 13, 14, 15, 16, 18, 19, 20 and 21 because antibodies raised against full length Pmps showed cross-reactivity. The N-terminal part is the most variable and has been detected at the surface for *C. psittaci* Pmps [9]. All the obtained pAbs reacted in 1-D IMB with the recombinant Pmps to which they were raised (data not shown). The antigen for IMB was EB lysate. Reacting spots (figure 1A) were identified by comparison to a reference gel upon autoradiography of the blot (figure 1B) [22] or by MS analysis of spots excised from corresponding gels.

The proteins detected by each antibody are listed in table 1 using bold for strong reaction and plain for weaker reaction. PAb203-rPmp10 was mono-specific for Pmp10. The antibodies against rPmp2, rPmp7, rPmp8 and rPmp11 recognized these Pmps (respectively), and additional Pmps as listed in table 1. PAb221-rPmp6 recognized the 91 kDa spot that was earlier identified as originating from Pmp6 [20] and in addition another Pmp6 spot at 60 kDa marked X in figure 1A. PAb236-rPmp20 recognized a spot located just above the 90 kDa Pmps, which was earlier identified as originating from Pmp20 [20]. PAb237-rPmp21 recognized the 91 kDa Pmp6 spot, Pmp13 and a 48 kDa Pmp21 fragment marked Y in figure 1C. The results with antibodies against rPmp1 and rPmp9 were inconclusive and MS identification of these Pmps could not be obtained. Antibodies against rPmp16 and rPmp18 both reacted with Pmp8 and Pmp10 but no other proteins. Antibodies against rPmp15 and rPmp19 did not re-

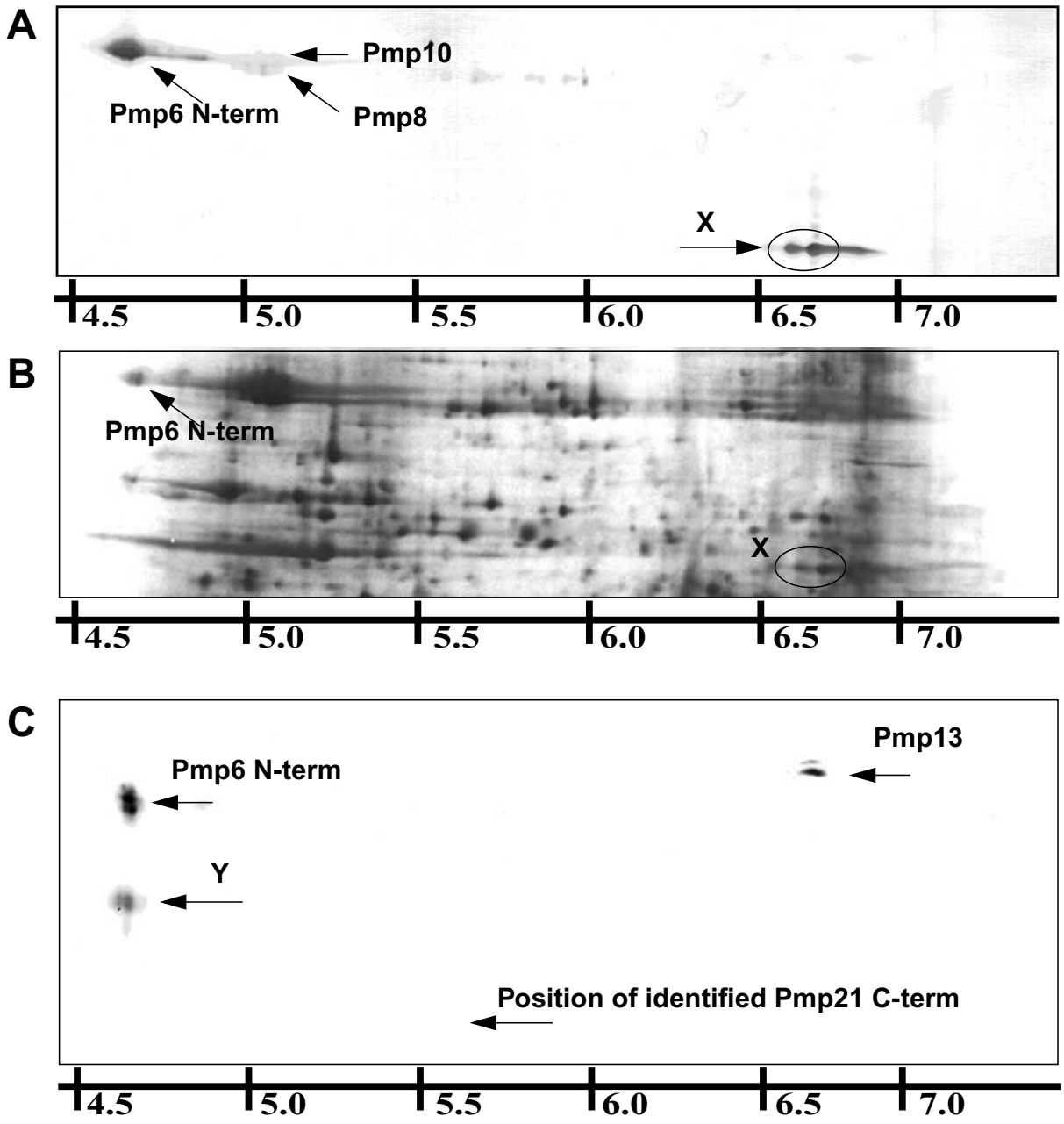

Figure 1
2-D immunoblotting of EB proteins Radiolabeled proteins from EBs purified 72 hpi were separated by 2-D PAGE and electroblotted onto PVDF membranes. The membranes were reacted with polyclonal rabbit antibodies raised against recombinant Pmp. Bound antibodies were visualized by color reagents and membranes were afterwards exposed to X-ray films so that reacting spots could be identified by comparison to an annotated gel. **A:** PVDF blot of EB proteins reacted with Pmp6-pAb221, X shows a C-terminal fragment of Pmp6 recognized by Pmp6-pAb221; **B:** Autoradiography of A; **C:** PVDF blot of EB proteins reacted with pAb237-rPmp21, Y shows an N-terminal fragment of Pmp21 recognized by pAb237-rPmp21.

Table 1: Overview of Pmps, antibodies and immunoblot reactions

pAb	Pmp	Cl.	Fam.	L	aa	cloned	MS	G	M	IMB	MeOH	Form.
204	**1**	I	G	I	923	**26–923**	-	-	I	I?**2** 6 7**8** **10** 13 14 20 21	X	-
207	**2**	I	G	I	842	**20–842**	X	S	M	**2** 6 **8** **10**	X	-
-	**3***	I	G	I								
-	**4***	I	G	I								
-	**5***	I	G	I								
221	**6**	II	G	I	1408	18–905	N+C	S	M	**6** 8 10**X**	X	X
220	**7**	II	G	I	937	25–545	X	S		**2** **6** **7** 8 10 11 13	X	-
201	**8**	II	G	I	931	21–931	X	S	M	**8** **10**	X	X
208	**9**	II	G	I	929	**27–929**	-	S	I	2 **6** **7** **8** 9? 10 11 13 14 20	X	u
203	**10**	II	G	II	929	**20–929**	X	-	M	**10**	X	X
195	**11**	II	G	II	929	**1–929**	X	W	M	**6** **11**	X	X
-	**12#**	II	-	I								
222	**13**	II	G	I	974	20–542	X	S	M	*not performed*	X	-
228	**14**	II	H	I	979	25–492	X	S	M	*not performed*	X	-
229	**15**	III	EF	I	939	18–478	-	W	I	-	-	-
230	**16**	III	EF	N	935	1–472	-	S	M	8 10	-	-
-	**17***	III	EF	I								
231	**18**	III	EF	N	893	1–566	-	P		8 10	-	-
234	**19**	IV	A	I	948	22–501	-	-		-	-	-
236	**20**	IV	BC	I	1724	22–1272	X	-	M	20 13	-	-
237	**21**		D	I	1610	52–1129	N+C	-	M	**6** 13**Y**	X	X

pAb: Antibody number; **Pmp:** Pmp number: *, genes containing a premature stop; #, naturally short gene; shading indicates either * or #. **Cl.**: Cluster in the *C. pneumoniae* genome.**L**: Predicted leader sequences: I, signal peptidase I cleavage site; II, signal peptidase II cleavage site; -, no predicted cleavage site. **Fam.**: Closest related *C. trachomatis* Pmp. **aa:** Amino acids in full-length Pmp. **cloned:** First and last amino acid in the recombinant protein. **MS:** Mass spectrometry identification by: X, peptides dispersed along the entire sequence; N, peptides localized to the N-terminal part; C, peptides localized to the C-terminal part; -, not identified by MS. **G:** Detection in 1D IMB by Grimwood *et al.*: S, strong reaction; W, weak reaction; P, possible reaction; -, no reaction. **M:** Detection by Montigiani *et al.* [24]: M, MS and immunoblot identification; I, immunoblot detection alone. **IMB:** Immunoblotting results: bold numbers indicate strong reaction; X is a C-terminal fragment of Pmp6; Y is an N-terminal fragment of Pmp21; results marked ? were inconclusive. **MeOH:** MeOH fixation IMF at 72 hpi: X, reaction; -, no reaction. **Form.**: Formalin fixation IMF at 72 hpi: X specific reaction; u, unspecific reaction; -, no reaction.

act with any EB proteins. We were thus not able to detect Pmp 15, 16, 18 and 19 by IMB using EB lysates as antigen.

Identification of cleaved Pmps

The antibodies against the high molecular weight Pmps (Pmp6, Pmp20 and Pmp21) recognized protein spots at lower molecular weight than expected. PAb221-rPmp6 recognized a set of spots, X, at 60 kDa (figure 1A) in addition to the earlier identified 91 kDa fragment of Pmp6. MS analysis of a tryptic digest of X revealed peptides from the C-terminal part of Pmp6 (singly underlined peptides, figure 2). In contrast, the mass spectra from the Pmp6 spot at 91 kDa was found to contain peptides solely from the N-terminal part of Pmp6 (doubly underlined peptides, figure 2). To verify the identifications, gels were electroblotted onto PVDF membranes and N-terminal amino acid sequences of the proteins were assessed by Edman degradation. The sequence obtained from X (xVPVVPVAP) confirmed that it was a C terminal fragment of Pmp6 as it matched an internal sequence of Pmp6 (^{872}SVPVVPVAP) (figure 2). The sequence obtained from the N-terminal fragment (xNTDLxSSD) matched a sequence of Pmp6 (^{24}ANTDLSSSD) that confirmed cleavage at the signal peptidase I cleavage site predicted by Signal-P [23,24] (figure 2). As seen in figure 2, these cleavage sites explain the observed pI and Mw coordinates. Also in figure 2, a model of Pmp6 is shown where the internal cleavage site is mapped relative to the predicted domains.

PAb237-rPmp21 reacted with a novel spot pattern, Y, at 66 kDa (figure 1C). A tryptic digest of Y was found to contain solely N-terminal peptides of Pmp21 by MS analysis (amino acids 122–130, 181–202, 203–213, 251–268, 493–510, and 530–548). The earlier identified Pmp21 spot at 48 kDa [20] was found to contain solely C-terminal peptides (amino acids 1205–1223, 1251–1273, 1279–1289, 1295–1311, 1312–1326, 1315–1326, 1400–1417, 1447–1457, 1464–1483, 1465–1483, 1484–1494, 1502–1521, 1504–1521, 1525–1540, 1594–1606). No reaction was observed with the latter spot (figure 1C) explained by the fact that pAb237-rPmp21 was raised against a recombinant protein covering only the N-terminal part of Pmp21, up to amino acid 1129 (table 1). Edman degradation of the 48 kDa spot revealed a sequence matching an internal sequence of Pmp21 (^{1185}SSPTPNKDKA) thereby identifying the cleavage site.

```
0001 MKYSLPWLLT SSALVFSLHP LMAANTDLSS SDNYENGSSG SAAFTAKETS DASGTTYTLT
0061 SDVSITNVSA ITPADKSCFT NTGGALSFVG ADHSLVLQTI ALTHDGAAIN NTNTALSFSG
0121 FSSLLIDSAP ATGTSGGKGA ICVTNTEGGT ATFTDNASVT LQKNTSEKDG AAVSAYSIDL
0181 AKTTTAALLD QNTSTKNGGA LCSTANTTVQ GNSGTVTFSS NTATDKGGGI YSKEKDSTLD
0241 ANTGVVTFKS NTAKTGGAWS SDDNLALTGN TQVLFQENKT TGSAAQANNP EGCGGAICCY
0301 LATATDKTGL AISQNQEMSF TSNTTTANGG AIYATKCTLD GNTTLTFDQN TATAGCGGAI
0361 YTETEDFSLK GSTGTVTFST NTAKTGGALY SKGNSSLTGN TNLLFSGNKA TGPSNSSANQ
0421 EGCGGAILAF IDSGSVSDKT GLSIANNQEV SLTSNAATVS GGAIYATKCT LTGNGSLTFD
0481 GNTAGTSGGA IYTETEDFTL TGSTGTVTFS TNTAKTGGAL YSKGNNSLSG NTNLLFSGNK
0541 ATGPSNSSAN QEGCGGAILS FLESASVSTK KGLWIEDNEN VSLSGNTATV SGGAIYATKC
0601 ALHGNTTLTF DGNTAETAGG AIYTETEDFT LTGSTGTVTF STNTAKTAGA LHTKGNTSFT
0661 KNKALVFSGN SATATATTTT DQEGCGGAIL CNISESDIAT KSLTLTENES LSFINNTAKR
0721 SGGGIYAPKC VISGSESINF DGNTAETSGG AIYSKNLSIT ANGPVSFTNN SGGKGGAIYI
0781 ADSGELSLEA IDGDITFSGN RATEGTSTPN SIHLGAGAKI TKLAAAPGHT IYFYDPITME
0841 APASGGTIEE LVINPVVKAI VPPPQPKNGP IASVPVVPVA PANPNTGTIV FSSGKLPSQD
0901 ASIPANTTTI LNQKINLAGG NVVLKEGATL QVYSFTQQPD STVFMDAGTT LETTTTNNTD
0961 GSIDLKNLSV NLDALDGKRM ITIAVNSTSG GLKISGDLKF HNNEGSFYDN PGLKANLNLP
1021 FLDLSSTSGT VNLDDFNPIP SSMAAPDYGY QGSWTLVPKV GAGGKVTLVA EWQALGYTPK
1081 PELRATLVPN SLWNAYVNIH SIQQEIATAM SDAPSHPGIW IGGIGNAFHQ DKQKENAGFR
1141 LISRGYIVGG SMTTPQEYTF AVAFSQLFGK SKDYVVSDIK SQVYAGSLCA QSSYVIPLHS
1201 SLRRHVLSKV LPELPGETPL VLHGQVSYGR NHHNMTTKLA NNTQGKSDWD SHSFAVEVGG
1261 SLPVDLNYRY LTSYSPYVKL QVVSVNQKGF QEVAADPRIF DASHLVNVSI PMGLTFKHES
1321 AKPPSALLLT LGYAVDAYRD HPHCLTSLTN GTSWSTFATN LSRQAFFAEA SGHLKLLHGL
1381 DCFASGSCEL RSSSRSYNAN CGTRYSF
```

Theoretical values			Observed values		
Fragment	**kDa**	**pI**	**Fragment**	**kDa**	**pI**
Full length	**142.3**	**5.28**	**Full length**	***not observed***	
N-terminal	**84.8**	**4.64**	**Acidic spot**	**91.3**	**4.63**
C-terminal	**57.5**	**6.75**	**Basic spot**	**60.0**	**6.67**

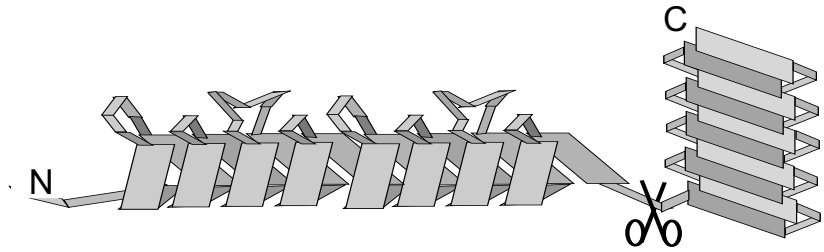

Figure 2
Pmp6 fragments Peptides identified from the acidic protein spot by MS are doubly underlined. Peptides identified from the basic spot are singly underlined. Amino acids from N-terminal sequences obtained by Edman degradation of the fragments are shown in bold. GGAI/L repeats are boxed. Notice that all repeats are located N-terminally of the cleavage site. Theoretical pI and Mw values of fragments were calculated by the Compute pI/Mw tool [http://www.expasy.ch/tools/pi_tool.html]. A schematic drawing of the predicted domains is shown at the bottom, the pair of scissors representing the internal cleavage site.

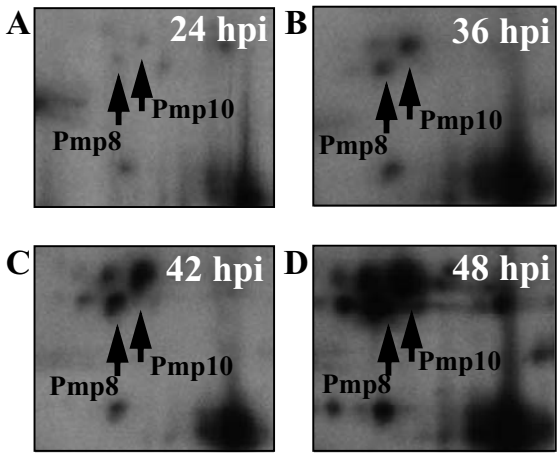

Figure 3
Visualization of Pmp8 and Pmp10 by two-hour labeling and 2-D PAGE Sections from autoradiographs of 2-D PAGE separated proteins from *C. pneumoniae* infected cells labeled with [^{35}S]methionine by two-hour cultivation in a [^{35}S]methionine medium added cycloheximide.

Cleavage at this site results in a fragment of a theoretical Mw of 51 kDa, which is in good agreement with the observed Mw of 48 kDa. For the protein spot at 66 kDa containing the N-terminal peptides, cleavage at the signal peptidase cleavage site predicted by Signal-P was confirmed at the amino acid 30 (^{30}AHSLHSSELD). The theoretical molecular weight of a fragment starting at this position and ending at the cleavage site at amino acid 1185 is 116 kDa. As no peptides were identified at the C-terminal side of amino acid 548 in the N-terminal fragment, further cleavage or degradation from the C-terminal end will be a likely explanation of the discrepancy between expected and observed values.

Pmp20 was identified just above the 90 kDa Pmps, but as no molecular weight markers were present above 90 kDa, the exact molecular weight of the protein cannot be deduced. However, it is found much lower than the expected 178 kDa and the earlier obtained MS identification of this protein spot was based on peptides located in the C-terminal part of Pmp20 (residues 1057–1069, 1387–1400, 1421–1432, 1506–1527, 1557–1571, 1587–1597, and 1644–1654) indicating that also Pmp20 is cleaved.

2-D PAGE analysis of time dependent expression

The time of expression of the Pmps identified in the 2-D protein profile was investigated by pulse labeling with [^{35}S]methionine for two-hour-periods followed by 2-D PAGE and autoradiography. Samples were labeled at 12, 24, 36, 42 and 48 hours post infection (hpi). The earliest time for detection of Pmps was 24 hpi, at which time the highest abundant Pmps in the gels (Pmp8 and Pmp10) were only barely visible (figure 3A). The amount of Pmps expressed in a two-hour labeling period increased during the developmental cycle as illustrated for Pmp8 and Pmp10 in figure 3A,3B,3C,3D. In figure 4 the volumes of Pmp spots (Pmp2, 7, 8, 10, 11, 13, 14 and the C-terminal part of Pmp6) are plotted as percentages of the total spot volumes of the gels at 24, 36 and 48 hpi. The remaining identified Pmps were omitted from this analysis since they could not be confidently quantified due to overlapping spots. Spot volumes were calculated as density integrated over Gaussian area of spots visualized by autoradiography and detected by use of the Melanie II software. The volume of each spot was divided by the number of methionines of the protein. The corrected volume percentages are arbitrary, as the total spot volumes of the gels could not be corrected for methionine content of every protein present in the gel. However, the numbers do reflect the mutual proportion of the measured proteins and the time dependent change in their contribution to the total protein synthesis. Over time, the expression of all the Pmps in figure 4 increased from below 0.05 units at 24 hpi to between 0.15 and 1.35 units at 48 hpi. The most pronounced increase was from 36 to 48 hpi. Pmp10 was found to be expressed at the highest rate (1.5 units at 48 hpi) followed by Pmp8 (0.6 units at 48 hpi). It must be noted that the apparent level of expression is influenced by the properties of the proteins with respect to separation by 2-D PAGE. However, the Pmps must be expected to exhibit comparable behaviour.

A parallel analysis of the expression of MOMP and DnaK was performed for comparison to the Pmps (figure 4). MOMP is expressed during the growth fase of *Chlamydiae* [25]. DnaK is an early protein [26] and has been suggested to be RB specific and hence to show decreased expression late in the developmental cycle [27]. The expression of MOMP was found to increase at the same rate from 24 to 36 hpi as from 36 to 48 hpi (figure 4). The expression of DnaK was similar to that of MOMP at 24 hpi, but showed no increase from 24 to 36 hpi and decreased from 36 to 48 hpi (figure 4).

It can be concluded that all Pmps investigated with respect to time dependent regulation (Pmp2, 6, 7, 8, 10, 11, 13, 14) were found to be strongly up-regulated between 36 and 48 hpi, coinciding with the conversion of RB to EB. This is in agreement with what has been reported for *C. trachomatis* and *C. psittaci* POMPS [28,29]. At 48 hpi Pmp8 and Pmp10 were several fold higher expressed than any other Pmp.

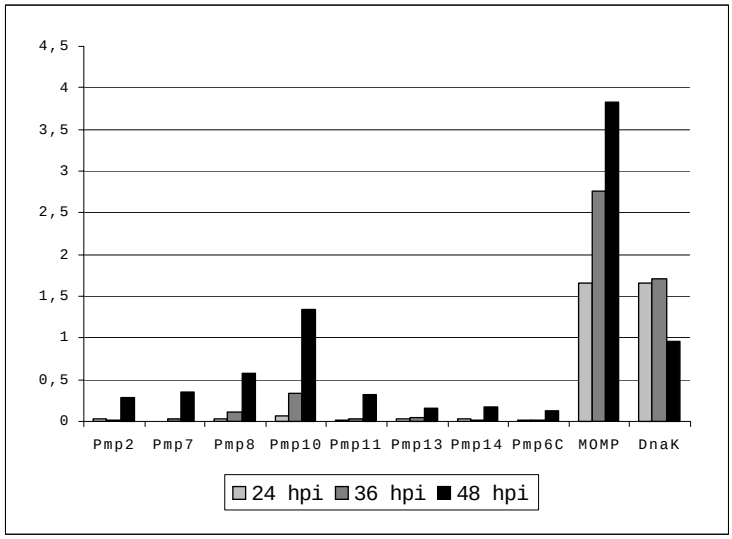

Figure 4
Time dependent expression of Pmps Histogram showing the contribution of selected Pmps to the total amount of protein synthesized in a two-hour labeling period. *Chlamydia* proteins were selectively labeled with [^{35}S]methionine under the addition of cycloheximide to stop host cell protein synthesis, separated by 2-D PAGE and visualized by autoradiography. Infected cells were labeled at 24, 36 and 48 hours post infection. Spots were detected by using the MelanieII software. Spot volumes were calculated as density integrated over Gaussian area, and the percentage of total spot volume in each gel was calculated. The percentages were corrected for the methionine content of each protein. The corrected spot volume percentages are given in arbitrary units at the Y-axis. Only Pmps located in the gel at positions where no overlapping protein spots hindered quantification are depicted. MOMP and DnaK are included as controls.

Immunofluorescence microscopy

In order to investigate whether the Pmps absent from EBs could be detected during growth in cell culture, the antibodies against these Pmps were used in IMF on *C. pneumoniae* infected HEp-2 cells. Cells cultivated on coverslips were infected and fixed in methanol at 72 hpi. The investigated antibodies were visualized with a FITCH conjugated secondary antibody. The antibodies against rPmp15, 16, 18 and 19, which did not react in IMB, did not react in IMF either (table 1). Furthermore, Pab236-rPmp20 was found not to react in IMF (table 1) although Pmp20 was identified in EB gels by IMB and MS. Examples are shown in figure 5.

All Pmp antibodies were used in IMF of infected cells that were formalin fixed at 72 hpi. Formalin cross-linking of outer membrane proteins prevents antibodies from penetrating the bacteria so that only proteins in the outer membrane are accessible. MAb18.1-DnaK was included as a control of the impermeability as described earlier [30]. As the DnaK epitope recognized by MAb18.1 is formalin insensitive [30], the lack of reaction of MAb18.1 (figure 5, row 1) must be due to the prevention of reaction of interior proteins by the fixation.

The pAbs raised against recombinant Pmp1, 2, 7, 13, 14, 15, 16, 19 and 20 did not react after formalin fixation. Due to the described cross-reaction of the Pmp antibodies, the reacting antibodies were absorbed with recombinant proteins. The Pmp antibodies were absorbed with rPmps corresponding to the Pmps to which they cross-reacted in IMB and with the rPmps to which they were raised, respectively. The reaction of pAb208-rPmp9 could be prevented by addition of any of the Pmps 6, 8, 10, 11 or 21 (table 2), and hence it must be considered non-specific. The pAbs raised against rPmp6, 8, 11 and 21 were found to react specifically in formalin fixation (table 2), as their reaction was prevented by the recombinant Pmp that they were raised agains, but not by the ones to which they cross reacted in IMB. Pmp8 is shown as an example in figure 5 (6). Pab203-rPmp10 was not investigated by competition IMF as it was shown to be mono-specific for Pmp10 by IMB.

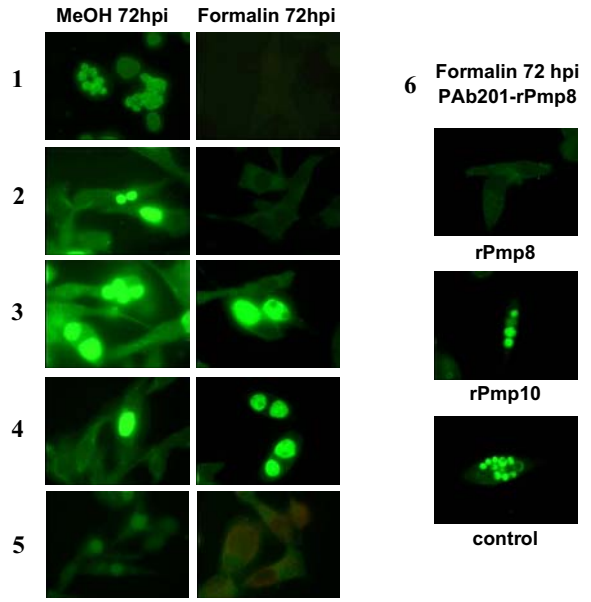

Figure 5
Immunofluorescence microscopy of *C. pneumoniae* infected HEp-2 cells Immunofluorescence microscopy pictures of infected HEp-2 cells fixed in methanol or 3.7% formaldehyde at 72 hours post infection (hpi). Bound antibody was detected with a FITCH conjugated secondary antibody. Fixed cells reacted with **1:** Monoclonal antibody recognizing DnaK (MAb18.1), **2:** pAb116-rMOMP, **3:** pAb110-rOmp2, **4:** pAb237-rPmp21, **5:** pAb234-rPmp19. **6:** Competition IMF. Reaction with pAb201-rPmp8 in the presence of: **rPmp8**: rPmp8, to which the antibody was raised, **rPmp10**: rPmp10, to which the antibody cross reacted in IMB, **control**: no recombinant proteins. As rPmp8 prevents reaction of pAb201-rPmp8 whereas rPmp10 does not affect the reaction, we conclude that the observed reaction is specific for Pmp8.

Pmp10 [30,17] and Pmp11[30] have earlier been shown to be surface exposed and the results indicate that this is also the case for Pmp6, 8 and 21. Other Pmps may also be at the surface, but in that case the epitopes recognized by our antibodies have been destroyed by the fixation or in some way masked.

PAb116-rMOMP did not react after formalin fixation (figure 5, row 2), which is in agreement with earlier reports on the lack of surface exposed linear epitopes of *C. pneumoniae* MOMP [31]. Pab110-rOmp2 did react (figure 5, row 3) indicating the presence of surface exposed epitopes of Omp2 in *C. pneumoniae* in opposition to *C. trachomatis* where Omp2 is probably located at the inner surface of the outer membrane complex [32,33].

Discussion

In the present study, cleavage sites of Pmp6 and Pmp21 have been identified, and it is suggested that also Pmp20 is cleaved. Cleavage of the these Pmps explain why they are found at different molecular weight in different studies. Grimwood *et al.* [19] detected the N-terminal fragment of Pmp6 at 90 kDa that was also identified by Vandahl *et al.* [20], whereas Montigiani *et al.* [21] found the lower molecular weight C-terminal fragment of Pmp6. Only a C-terminal fragment of Pmp21 has been described previously [20,21].

There is no similarity in amino acid sequence between the cleavage sites in Pmp6 and Pmp21, but both sites are located between the C-terminal predicted β-barrel [10] and the N-terminal predicted parallel β-helix fold [34]. This position is in agreement with the theory that the Pmps may be autotransporters; as such often cleave off their N-terminal part [10]. However, if the N-terminal part is liberated it would not be detected in IMB using proteins from purified EBs as antigens, and as the N-terminal fragment is detected in EB gels for Pmp6 and Pmp21 it must be concluded that it remains bound to the EB.

Pab236-rPmp20 was raised against a fragment of Pmp20 covering the N-terminal part and some of the C-terminal fragment, but only the C-terminal part could be detected in IMB (data not shown), suggesting that the N-terminal part may be liberated. Pab236-rPmp20 did not react in IMF. It was raised against unfolded recombinant protein and may not recognize the correctly folded protein, especially not if the N-terminal part is liberated or degraded.

The fragments of Pmp6, Pmp20 and Pmp21 were detected after a labeling period of two hours, and no increase in the amount of cleavage products was observed after a chase period of six hours (data not shown). This suggests that the cleavage occurs rapidly after synthesis. We consider it unlikely that the cleavage should be an artificial phenomenon as the infected cells were harvested in a lysis solution containing 7 M urea, 2 M thiourea and reducing agents, and as the results were highly reproducible. The lack of detection of full-length products of the high molecular weight Pmps may be caused by low resolution in 2-D PAGE of such proteins, but full-length products of these were also absent in 1-D IMB by Grimwood *et al.* [19]. Interestingly, Grimwood *et al.* [24] detected Pmp6 from strains TW183 and AR39 at a Mw of 130 kDa, which is the theoretical Mw of Pmp6 in these strains, due to a deletion of the bases encoding amino acids 429 to 559, which does not include the cleavage site.

In the present study we did not detect any of the Pmps15-18, originating from cluster III. Pmp16 and Pmp18 are the only Pmps lacking a leader sequence. Pmp17 is truncated

Table 2: Competition immunofluorescence

	none	rPmp6	rPmp11	rPmp8	rPmp9	rPmp21	rPmp7	rPmp10
pAb221-rPmp6	**+++**	**0**	**+++**					
pAb195-rPmp11	**+++**	**+**	**0**					
pAb201-rPmp8	**+++**			**0**				**+++**
pAb208-rPmp9	**++**	**0**	**0**	**0**	**0**		**0**	
pAb237-rPmp21	**+++**	**+++**				**0**		

The results of competition IMF in which the recombinant Pmp listed in the top row was added to an IMF reaction using the polyclonal antibodies listed in the first column. The reactions were carried out only for the shaded table cells: **0**, no reaction; **+**, weak reaction; **++**, moderate reaction; **+++**, strong reaction.

and was thus not investigated. Montigiani *et al.* [21] have reported identification of Pmp16 in *C. pneumoniae* from a clinical isolate, but Grimwood *et al.* [19] found that both Pmp16 and Pmp18 were unstable in CWL029, which may explain that these Pmps were not observed in our study. However, it could also be speculated whether differences have been introduced through laboratory passages. We observe expression of Pmp10 in strain CWL029 whereas it was not detected in strain CWL029 by Grimwood *et al.* [19] due to a frame shift mutation. Grimwood *et al.* [19] have described differences in the expression of Pmp1 and Pmp3 between strains CWL029 and TW183 as determined by immunoblotting, meaning that at least some Pmps are differentially expressed between strains.

Pmp6, Pmp8, Pmp10, Pmp11 and Pmp21 were detected at the surface of the bacteria by formalin fixation IMF. Apart from Pmp21, all the Pmps detected at the surface show greatest similarity to PmpG [4], a constituent of the outer membrane of *C. trachomatis* L2 [35,28]. An explanation of the expansion of the number of *C. pneumoniae* Pmps similar to PmpG could be that *C. pneumoniae* varies its surface to escape the immune defence of the host by changing the expression of Pmps. However, if Pmp expression is changed in order to vary the surface, this is most likely a consequence of the surface localization of Pmps rather than the very reason for the existence of Pmps.

The most highly expressed among all the investigated Pmps at all points in time is Pmp10, which is known to contain surface exposed epitopes [30]. Pmp10 is differentially expressed in infected cell culture [17] and mice [16]. Pmp10 still being the most highly expressed Pmp suggests that it carries out an important function. This function would presumably have to be supplemented in bacteria not expressing Pmp10. Pmp10 contains a predicted signal peptidase II cleavage site directing lipid modification and *pmp11*, which is located next to *pmp10* (but in the opposite direction), encodes the only other predicted lipid modified Pmp. If the function of Pmp10 depends on lipid modification, and this function is needed in bacteria lacking Pmp10, Pmp11 would be the obvious alternative.

The finding that all Pmps are heavily upregulated at the time of conversion of RBs into EBs indicates that the function of these is structural but they may also be needed with respect to attachment or entry of EBs. An N-terminal triangular beta-layer motif could provide the bacteria with a shielding lattice and ensure proper spacing to a host cell or an epitope exposed to the complement system. If the lipid modifications of Pmp10 and Pmp11 are used as anchors inserted into the host cell membrane, subsequent action of other entry molecules would probably depend on proper spacing. However, all theories on functions of the Pmps remain very speculative.

Conclusions

The Pmps investigated with respect to time dependent regulation (Pmp2, Pmp6, Pmp7, Pmp8, Pmp10, Pmp11, Pmp13, and Pmp14) were found to be up-regulated late in the developmental cycle. Due to their reaction in formalin fixation we propose that Pmp6, Pmp8, Pmp10, Pmp11, and Pmp21 are surface exposed. Pmp6, Pmp20 and Pmp21 were found in cleaved forms and the identified cleavage sites of Pmp6 and Pmp21 are in agreement with the theory that the Pmps are autotransporters.

Methods

Organisms and cultivation

C. pneumoniae CWL029 (also known as VR1310) (ATTC) was cultivated in semi confluent monolayers of Hep-2 cells (ATCC) as described [20]. Elementary bodies were purified 72 hpi essentially as described [30] with the exception that Visipaque replaced Urografin for gradient making.

PCR and cloning

PCR enzymes were Expand™ High Fidelity (Roche) and reaction conditions were as recommended by the manufac-

turer. As the Ligation-Independent Cloning (LIC) kit (Novagen) was used for cloning of PCR products, all primers (DNA Technology) were designed with LIC specific 5'-sequences: Forward primer: 5' GAC GAC GAC AAG AT *pmp*-sequence 3'. Reverse primer: 5' GAG GAG AAG CCC GGT *pmp*-sequence 3'. Primers for amplification of *pmp* genes were placed as listed in table 1 and the sequences were obtained from GeneBank (AE001363). In those cases where only the N-terminal of the Pmp gene was cloned a stop codon was introduced. The pET 30Ek LIC vector (Novagen) was used for cloning and expression of recombinant protein according to the protocol provided by the manufacturer. Cloning was performed in *E. coli* NovaBlue.

Protein expression and purification

Plasmid DNA was prepared from NovaBlue cells by the alkaline lysis method and used for transformation of *E. coli* BL21(DE3) by eletroporation. Clones were tested for correct insert sequence by PCR with vector specific primers. The PCR product was used for sequence reactions using the Terminator Ready Reaction Mix (Perkin Elmer), and sequencing performed on an ABI PRISMTM 377 DNA Sequencer (Perkin Elmer). IPTG (1 mM) induction of expression of His-tagged fusion proteins was performed for two hours at 37°C when A_{600} reached 0.4. Recombinant protein was purified from pelleted cells using HiTrap Ni^{2+} columns (Amersham Pharmacia). The purity of the recombinant proteins was tested by SDS PAGE and the protein concentration measured by use of the Bradford Protein Assay (BioRad).

Production of antibodies

New Zealand White rabbits were immunized: i) intramuscularly on days 1, 8 and 15 using 10 μg of protein in PBS and 50% Freunds incomplete adjuvant; ii) intravenously on days 29, 36 and 43 using 10 μg of protein in PBS. The rabbits were bled on day 60.

Labeling

Pulse labeling of infected cell cultures was performed by using 100 μCi/mL radioactive methionine (Amersham Pharmacia) in a methionine-free RPMI 1640 medium as described previously [20]. 40 μg/mL cycloheximide was added to inhibit host cell protein synthesis during labeling. Cell cultures were labeled for two-hour-periods at 6, 12, 24, 26, 42, 48 and 54 hpi and harvested immediately after labeling by scraping off in lysis solution (7 M urea, 2 M thiourea, 4% w/v CHAPS, 40 mM Tris base, 65 mM DTE and 2% v/v Pharmalyte 3–10 (Amersham Pharmacia)). Before purification of EB the same labeling periods were used to ensure incorporation of radioactivity into proteins synthesized at all times during the developmental cycle.

Electrophoresis

One-dimensional SDS gels were run as described [30]. The protocol for two-dimensional gel electrophoresis was as described [20]. First dimension was carried out using non-linear Immobiline Drystrips pH 3–10 (Amersham Pharmacia). Proteins were focused in the strips using an IPGphor (Amersham Pharmacia) applying a voltage of 20 V for rehydration of strips and 120 kVh for focusing. For second dimension 9–16% T gradient polyacrylamide SDS gels were used. Comparative gels were loaded with 300.000 cpm as determined by TCA precipitation and scintillation counting. Gels for N-terminal sequencing were loaded with 600 μg protein from purified unlabeled EB. Gels for MS identification were loaded with 3.000.000 cpm and a total of 600 μg protein – all from purified EB. Gels for IMB were loaded with 75 μg protein from purified unlabeled EB and 300.000 cpm labeled EB protein. Protein spots in gels were visualized by autoradiography as described [20]. Comparative gels were treated with Amplify (Amersham Pharmacia) before exposure to BioMax MR X-ray films (Kodak).

Immunoblotting

Two-dimensional gels were washed for five min. in double-distilled water and soaked for 30 min. in transfer buffer. PVDF membranes (Immobilon-P, pore size 0.45 μm, Millipore) were soaked for one min. in methanol and then for 30 min. in transfer buffer. The transfer buffer contained: 50 mM Tris, 50 mM boric acid, 0.02% SDS and 10% methanol. The transfer was performed at 90 V and 10°C for four hours. Membranes were blocked in a buffer containing 20 mM Tris, 150 mM NaCl, and 3% gelatine, pH 7.5, washed in washing buffer (20 mM Tris, 500 mM NaCl, 0.05% tween-20) and incubated with polyclonal antibodies (pAbs) diluted 1/300 in antibody buffer (washing buffer added 0.2% gelatine) for one hour at 37°. After three washing steps membranes were incubated with secondary antibody (goat-anti-rabbit-IgG-AP-conjugate, BioRad) diluted 1/2000 for another hour at 37°C. After washing the blots, bound antibody was visualized with 270 μL NBT (50 mg/mL in 70% DMF) and 270 μL BCIP (25 mg/mL in 100% DMF) in 45 mL buffer containing 100 mM NaCl, 5 mM MgCl and 100 mM Tris-HCl at pH 9.5. As a fraction of the EB proteins was radiolabeled, reacting spots could be identified by comparison to a reference gel [20] upon autoradiography of the PVDF membrane [22].

Mass spectrometry

Protein spots excised from preparative gels were identified by MALDI TOF mass spectrometry as described [20].

N-terminal sequencing

Protein spots excised and pooled from eight Coomassie Brilliant Blue stained PVDF membranes each loaded with

600 μg of protein from purified EB were analyzed using an Applied Biosystems 404 protein sequencer (Perkin Elmer).

Immunofluorescence microscopy

IMF was performed as described [36] analyzing infected cells fixed in methanol or formalin at 72 hpi. Formalin fixed cells were permeabilized with 0.2% Triton X-100 for 10 minutes at room temperature. Bound primary antibody was visualized with a secondary FITCH conjugated antibody (DAKO), which was absorbed to prevent cross-reaction.

Authors' contributions

B.B.V. carried out the 2-D PAGE, IMB and IMF experiments, analyzed the Edman degradation results and drafted the manuscript. A.S.P. made the recombinant Pmps. K.G. and J.V. carried out the MS analysis. A.H. carried out the Edman degradation. G.C and S.B. participated in design and coordination of the study. All authors read and approved the final manuscript

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