

Characterization of Outer Membrane Proteins in *Chlamydia trachomatis* LGV Serovar L2

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We used a photoactivatable, lipophilic reagent, 3'-(trifluoromethyl)-3-(*m*-[¹²⁵I]iodophenyl)diazirine, to label proteins in the outer membrane of elementary bodies of *Chlamydia trachomatis* LGV serovar L2 and mass spectrometry to identify the labeled proteins. The identified proteins were polymorphic outer membrane proteins E, G, and H, which were made late in the developmental cycle, the major outer membrane protein, and a mixture of 46-kDa proteins consisting of the open reading frame 623 protein and possibly a modified form of the major outer membrane protein.

The cell envelope of *Chlamydia* species resembles that of other gram-negative bacteria, consisting of an outer membrane (OM), an inner membrane (IM), and a periplasmic space. Two unusual features of the chlamydial envelope are a deficient and perhaps novel peptidoglycan structure (reviewed in references 6, 10, and 21) and the presence of a disulfide-bond-cross-linked major outer membrane protein (MOMP) in the OM and cross-linked cysteine-rich proteins (CRPs) in the periplasm (reviewed in reference 12).

The MOMP is the only chlamydial OM protein that has been well characterized. It is a porin (2, 30), is surface exposed (7, 28), and may play a role in the attachment of *Chlamydia trachomatis* to host cells (27, 28). Genes that potentially encode a family of proteins, referred to as polymorphic outer membrane proteins (Pomps), have been identified in the genomes of *Chlamydia* species: nine Pomp genes (*pmpA* to *pmpH*) are present in the genomes of *C. trachomatis* serovars D and L2, 21 genes (*pmp1* to *pmp21*) are in *C. pneumoniae*, and at least six genes (Pomp90 and Pomp98 families) are encoded by the genomes of ovine strains of *C. psittaci* (15, 20, 26). However, only two Pomps have been shown to be produced in *C. trachomatis* and *C. pneumoniae*, and only four Pomps have been identified in *C. psittaci* (11, 16, 20, 22, 29). The functions of the Pomps are not known, nor is it known when *C. trachomatis* Pomps are made during the developmental cycle. Several other OM proteins can be predicted from chlamydial genomic sequences (15, 26); however, none of these proteins have been experimentally identified in OMs.

The OM of chlamydiae is poorly characterized, in part because of the difficulty of growing large quantities of chlamydiae but mainly because chlamydial OMs cannot be separated from IMs by density gradient centrifugation. Criteria that have been used to identify chlamydial OM proteins include surface exposure, as detected by susceptibility to trypsin or reaction with antibodies following treatment of infectious elementary bodies (EBs) with these reagents, and insolubility in the weak anionic detergent sodium lauryl sarcosinate (Sarkosyl). Although these

methodologies have been useful, they can yield deceptive results. For example, damage to EBs or contamination of EBs with osmotically fragile reticulate bodies during harvesting and purification can expose proteins that are not on the surface of EBs, and the failure to observe positive reactions will result if a surface-exposed protein lacks a trypsin-sensitive site or an immunodominant epitope. Insolubility in Sarkosyl is also subject to misinterpretation. The technique was originally developed by Filip et al. (9) to remove cytoplasmic membrane proteins from well-characterized integral OM proteins of *Escherichia coli* and was adapted to chlamydial studies by Caldwell et al. (4) and Hatch et al. (14). However, the reason for the differential solubility of IM and OM proteins in Sarkosyl is not known, and it is likely that some non-OM proteins fractionate in the Sarkosyl-insoluble fraction and that some OM proteins are released from OMs by Sarkosyl. For these reasons we chose to identify *C. trachomatis* LGV serovar L2 OM proteins on the basis of their reaction with 3'-(trifluoromethyl)-3-(*m*-[¹²⁵I]iodophenyl)diazirine ([¹²⁵I]TID), a photoactivatable lipophilic reagent developed by Brunner and Semenza (3) to label amino acid side chains and other ligands in the lipophilic environment of cytoplasmic membranes of eukaryotic cells. Because TID is lipophilic, it penetrates the OM of gram-negative bacteria, where it becomes trapped and incapable of passing through the hydrophilic periplasm and beyond (8).

Identification of OM proteins in *C. trachomatis* L2. EBs were harvested at 48 h after infection of L929 cells, treated with 1% Nonidet P-40 (Sigma Chemical Co., St. Louis, Mo.) to eliminate osmotically fragile reticulate bodies, and purified by centrifugation for 30 min at 80,000 × *g* on a three-step gradient of 29, 34, and 40% Hypaque-76 (Nycomed Inc., Princeton, N.J.). Purified EBs from 2 × 10⁸ cells were reacted with 25 μCi of [¹²⁵I]TID, as previously described (8), and incubated for 30 min at 37°C in the presence or absence of 12 μg of trypsin (type III from bovine pancreas; Sigma Chemical Co.) in 200 μl of phosphate-buffered saline (pH 7.4), followed by the addition of 24 μg of trypsin inhibitor (type II-O from chicken egg white; Sigma). After the EBs were washed once in trypsin inhibitor, one half of the EB preparation was solubilized by heating to 90°C in Laemmli buffer (18) containing 5% β-mercaptoethanol and 10 mM dithiothreitol, and the other half was extracted with 500 μl of 0.5% Sarkosyl (Sigma) in phosphate-buffered

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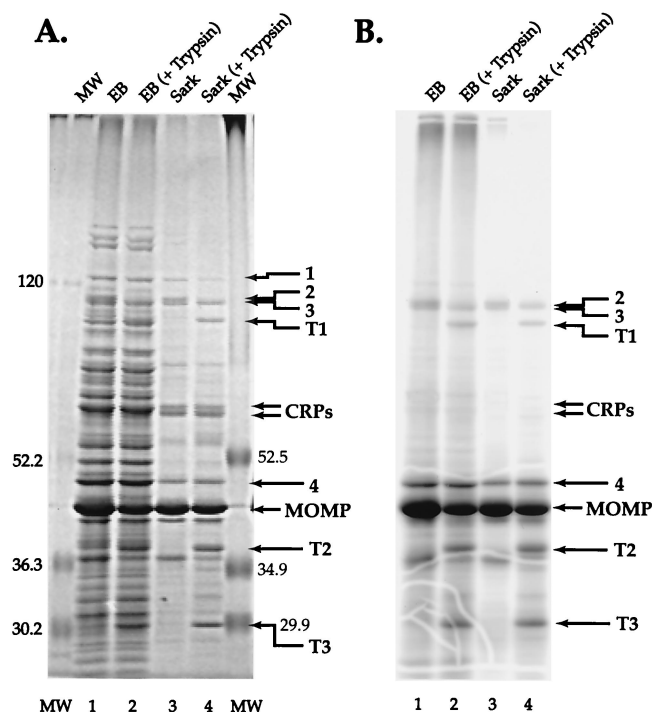


FIG. 1. Identification of proteins labeled with [125 I]TID by SDS-PAGE. (A) Coomassie-stained 7.5 to 15% polyacrylamide gel, with prestained protein standard sizes shown in kilodaltons at each side; (B) phosphorimage of the gel. Lanes: 1, whole EBs; 2, whole EBs following treatment with trypsin; 3, the Sarkosyl-insoluble fraction; 4, the Sarkosyl-insoluble fraction of EBs treated with trypsin. Arrows point to proteins discussed in the text.

saline for 30 min at 37°C. The Sarkosyl-insoluble fraction was collected by centrifugation ($14,500 \times g$ for 20 min) and washed twice in 500 μ l of 0.5% Sarkosyl before it was solubilized in Laemmli buffer with reducing agents. Proteins in whole EBs and the Sarkosyl-insoluble fraction of EBs were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5 to 15% gradient gels, and incorporation of 125 I into proteins was detected by phosphorimaging of the dried gels.

The protein profiles of whole EBs and the Sarkosyl-insoluble fraction of EBs are shown in Fig. 1. A number of proteins were seen in the Coomassie brilliant blue-stained Sarkosyl-insoluble fraction (Fig. 1A, lane 3), including the 40-kDa MOMP and its likely degradation products (28), the 60-kDa CRP doublet, and several unknown proteins. The unknown proteins included one of about 120 kDa (protein 1), a poorly resolved doublet of about 100 kDa (proteins 2 and 3), and a protein of approximately 46 kDa (protein 4). Only the MOMP, likely degradation products of the MOMP, and proteins 2 to 4 were labeled extensively with TID; the periplasmic CRPs, notably, were not labeled (Fig. 1B, lane 3). The TID-labeled, Sarkosyl-insoluble profile was similar to that of the TID-labeled whole EB protein profile (Fig. 1B, lanes 1 and 3). Treatment of EBs with trypsin prior to the preparation of the Sarkosyl-insoluble fraction reduced the intensity of the protein doublet 2-3, with the appearance of new peptides, designated tryptic fragments T1 to T3.

In order to better resolve the high-molecular-weight pro-

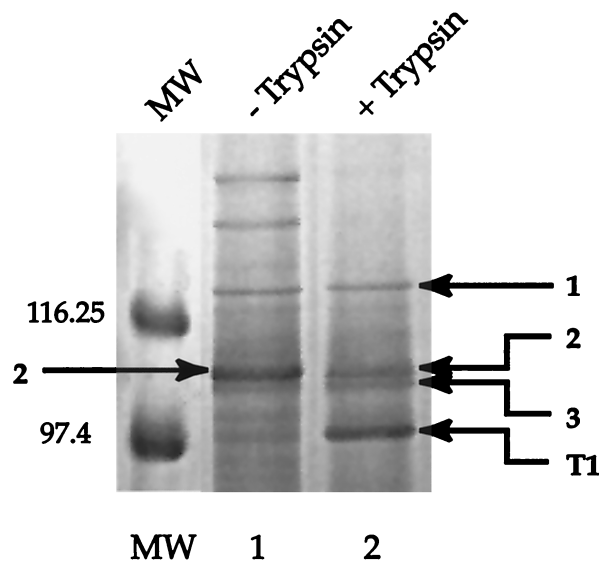


FIG. 2. Zinc-imidazole-stained 5 to 7.5% polyacrylamide gel of high-molecular-weight proteins present in the Sarkosyl-insoluble fraction of EBs. Lanes: 1, Sarkosyl-insoluble fraction of EBs; 2, Sarkosyl-insoluble fraction of EBs treated with trypsin. Sizes of prestained protein standards in kilodaltons are shown on the left. Arrows point to bands (numbered the same as in Fig. 1) that were analyzed by mass spectrometry.

teins, the Sarkosyl-insoluble fraction of EBs was fractionated on a 5.0 to 7.5% gel and proteins were visualized with the reversible, negative zinc-imidazole stain described by Castellanos-Serra et al. (5). Under this condition of analysis, the intensity of protein 2 was reduced when the EBs were treated with trypsin, suggesting that the band may consist of a mixture of two or more proteins (Fig. 2). Gel slices containing proteins 1 to 3 and trypsin fragment T1 were excised from the gel shown in Fig. 2 and prepared for analysis by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry after in-gel digestion with trypsin, as described by Shevchenko et al. (25). Protein 4, the MOMP, and trypsin fragments T2 and T3 were similarly analyzed following electrophoresis of the Sarkosyl-insoluble fraction on 7.5 to 15% gels. Because the *C. trachomatis* L2 genome has not been completely sequenced and annotated, we identified proteins with the *C. trachomatis* D database located on the Protein Prospector website (<http://prospector.ucsf.edu>). Our findings are summarized in Table 1.

When EBs were not first treated with trypsin, protein bands 2 and 3 contained a mixture of Pumps E, G, and H. After treatment of EBs with trypsin, these bands were resolved to a mixture Pumps E and H, with PumpE being the predominant protein in band 2 and PumpH being the predominant protein in band 3; fragment T1 was identified as a degradation product of PumpG. We concluded that the trypsin-sensitive PumpG and the trypsin-insensitive PumpE comigrate as band 2, with contamination with Pump H (protein 3) due to smearing during electrophoresis. Pumps G and H were previously identified by Mygind et al. (22) in the Sarkosyl-insoluble fraction of *C. trachomatis* L2.

The best identification of band 1 was *C. trachomatis* D open reading frame (ORF) 664 (26). The predicted peptide of ORF

TABLE 1. Proteins identified in the Sarkosyl-insoluble fraction by mass spectrometry^a

Band	Identification
1.....	ORF 664
2 (- trypsin).....	Mixture of Poms E, G, and H
2 (+ trypsin).....	Mixture of Poms E and H
3 (- trypsin).....	Mixture of Pomp G and H
3 (+ trypsin).....	PompH
T1.....	PompG
4.....	Mixture of MOMP and ORF 623
T2.....	MOMP
T3.....	MOMP

^a Zinc-imidazole negatively stained bands were excised from gels, and samples were prepared for MALDI-TOF mass spectrometry by in-gel digestion with trypsin as described by Shevchenko et al. (25).

664 possesses a potential FHA (forkhead-associated) domain, thought to be important in protein-protein interactions, and is weakly homologous to several proteins found in eukaryotes and prokaryotes; however, its function in chlamydiae is not clear. PSORT analysis (<http://psort.nibb.ac.jp>) predicted an uncleavable signal sequence and an IM location for the protein encoded by ORF 664, which is consistent with the lack of labeling of protein 1 by [¹²⁵I]TID. The predicted molecular weight of the mature protein encoded by ORF 664 is 89,649, considerably less than the relative molecular weight (120,000) determined by SDS-PAGE. The predicted pI of the mature protein is 4.32, and the acidic nature of the protein, including several stretches of high-density negative charge, may be responsible for its anomalous migration rate. Interestingly, ORF 664 is weakly paralogous to PompC (E value = 5e-06); however MALDI-TOF analysis failed to identify PompC of either *C. trachomatis* D or *C. trachomatis* L2 (only a partial sequence is available) in band 1.

Protein band 4 (relative molecular weight of about 46,000) was found to be a mixture of the MOMP and ORF 623 (26) by MALDI-TOF analysis, with the tryptic peptides with the highest counts being assigned to the MOMP. N-terminal amino acid analysis confirmed the presence of the MOMP and ORF 623 in protein band 4, with the MOMP being present in a ratio of about 2 to 1. ORF 623 encodes a predicted chlamydia-specific protein of unknown function; it possesses a predicted signal sequence, which was confirmed by the N-terminal sequence analysis, and the characteristics of an OM protein as determined by PSORT analysis. The predicted molecular weight of the mature form is 45,669. The presence of the MOMP in protein band 4 may be the result of smearing of the abundant 40-kDa MOMP during electrophoresis or may be due to the presence of a posttranslationally modified form of the MOMP in the band. Kuo and colleagues (17) have presented evidence that the 40-kDa MOMP of *C. trachomatis* L2 is N-linked glycosylated with a high-mannose oligosaccharide. It is possible the carbohydrate identified by Kuo et al. (17) was the result of contamination of unmodified 40-kDa MOMP with modified 46-kDa MOMP. However, in preliminary studies, we failed to detect alteration in the migration rate of any protein in the Sarkosyl-insoluble fraction when EBs were treated with calf intestinal alkaline phosphatase (Boehringer-Mannheim; 5 U in 200 μ l of phosphatase buffer for 1 h at 37°C), N-glycosylase F (Boehringer-Mannheim; 2 U in 200 μ l of phosphate-

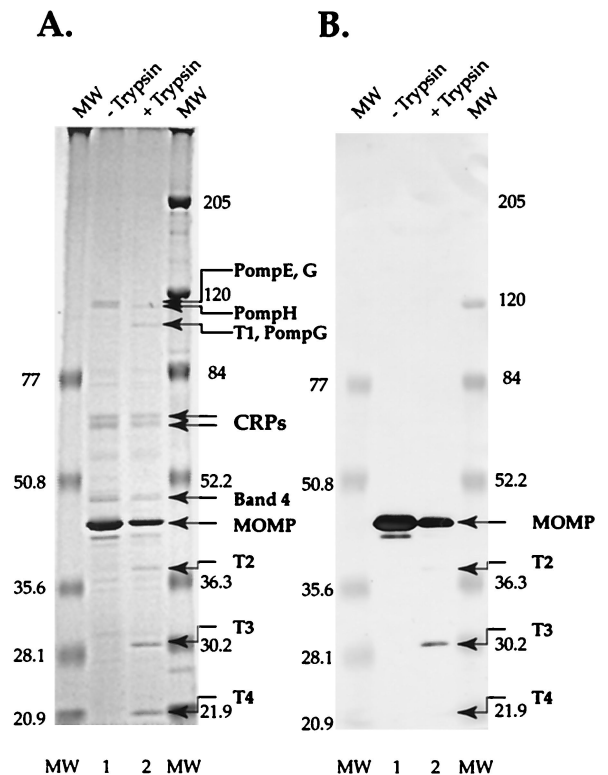


FIG. 3. SDS-PAGE (A) and immunoblot (B) analysis of the Sarkosyl-insoluble fraction of EBs incubated in the presence (lane 2) and absence (lane 1) of trypsin. The blot was treated with monoclonal antibody L2I-45, which reacts with the serovar-specific epitope in the MOMP of *C. trachomatis* L2 (1). Trypsin fragment T4 was not analyzed by mass spectrometry; however, the intensity of the band suggests that it was derived from the MOMP.

buffered saline for 1 h at room temperature, followed by the addition of Triton X-100 to 0.25% and incubation at room temperature for 30 min and then overnight at 4°C), and endoglycosylase H (Boehringer-Mannheim; 2 mU, same incubation conditions as for N-glycosylase F) (data not shown).

In a further attempt to identify the MOMP in band 4, an immunoblot was treated with monoclonal antibody L2I-45, which recognizes the serovar-specific epitope in the MOMP of *C. trachomatis* L2 (1). Only the abundant 40-kDa MOMP and degradation products of the MOMP were recognized by the antibody (Fig. 3).

Stage-specific synthesis of Poms. We recently demonstrated that three Poms of the ovine *C. psittaci* Pomp90 family and three other potential Poms are synthesized cotemporally with the late-stage-specific CRPs in *C. psittaci* 6BC (29). We examined stage-specific synthesis of *C. trachomatis* L2 Poms by adding 275 μ Ci of [³⁵S]Cys-[³⁵S]Met (Protein Labeling Mix; NEN, Boston, Mass.) to 150-cm² monolayers of infected cells (2 \times 10⁷) at 14 h postinfection (p.i.) and preparing a Sarkosyl-insoluble fraction directly from the infected cells at 16, 18.5, and 23 h p.i. and from the harvested medium supernatant fluid and cells remaining attached to the flasks at 48 h p.i. Cycloheximide (0.5 μ g/ml) was present during the entire infection to inhibit incorporation of label into host cells. Incorporation of label into chlamydial proteins in the Sarkosyl-insoluble frac-

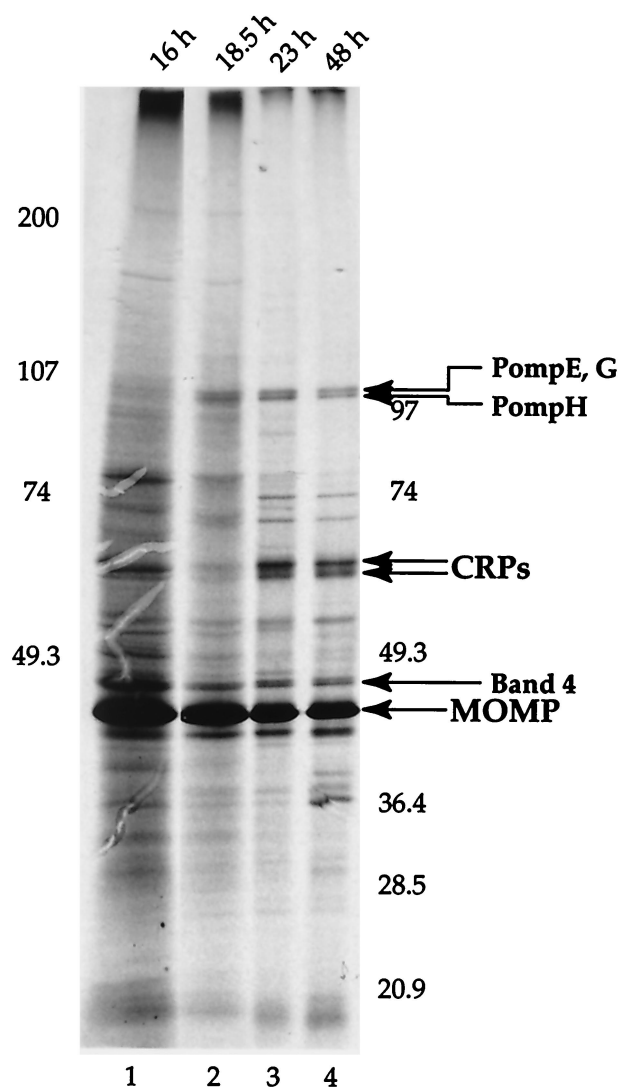


FIG. 4. Autoradiograph of a 7.5 to 15% polyacrylamide gel showing incorporation of [^{35}S]Cys-[^{35}S]Met into the Sarkosyl-insoluble fraction from 14 to 48 h p.i. The Poms appear to be made in greater amounts late in the cycle, relative to the MOMP, but at detectable levels sooner than the late-stage-specific 60-kDa CRPs.

tion was assessed by autoradiography of a dried polyacrylamide gel, with the amount of material loaded onto the gel adjusted so that the amount of MOMP (present in relatively constant amounts at all times p.i.) for each sample was the same (Fig. 4). The results indicated that the Poms are made at 18.5 h and later times, whereas synthesis of the late-stage-specific CRPs (13, 23) is not obvious until 23 h. The 16 h p.i. sample was difficult to interpret; the large amount of material loaded resulted in a high background and included proteins which comigrated with the CRPs that were not present at late times p.i. Nonetheless, it appears that the Poms, at least relative to the MOMP, are not made in significant amounts between 14 and 16 h p.i.

Conclusions. [^{125}I]TID labeling of *C. trachomatis* L2 EBs identified three Poms (E, G, and H), the MOMP, and another protein or proteins that migrate during SDS-PAGE with

a relative molecular mass of about 46 kDa. It is likely that additional proteins are present in the OM but in insufficient amounts to be detected by the amount of TID label that we used. This is almost certainly the case for PompF, which is encoded immediately upstream of PompE on the same operon, with the UAA stop codon of PompF located only 2 bp upstream of the AUG start codon of PompE (26). Also, in a preliminary study Lindquist and Stephens (19) detected transcripts of the genes encoding Poms B, D, I, and G by reverse transcription-PCR in *C. trachomatis* L2. The Poms that we detected are encoded by genes that are clustered in the same region of the genome: the operon encoding Poms F and E (*pmpFE*) and three divergently transcribed genes encoding Poms G, H, and I. It is interesting that the divergently transcribed Pomp genes are cotemporally expressed.

Mass spectrometric and N-terminal amino acid analysis indicated that the MOMP was a major constituent of the TID-labeled, 46-kDa protein band, suggesting that the protein may be a posttranscriptionally modified form of the MOMP, perhaps identical to the modified form described by Kuo et al. (17). However, the presence of the MOMP was not confirmed by immunoblot analysis and we found no evidence of glycosylated or phosphorylated protein in either the 46- or the 40-kDa protein. Alternatively, the TID-labeled protein may be encoded by ORF 623, a predicted OM protein of unknown function. Definitive identification of the proteins in the 46-kDa band will require additional investigation. Whatever the identity of the TID-labeled 46-kDa protein is, there is evidence that it may be antigenic in that Newhall et al. (24) identified a band of similar size that reacted on immunoblots of multiple serovars of *C. trachomatis* with the sera of infected patients. Although the proteins in the 46-kDa band did not possess a surface-exposed trypsin-sensitive site, their presence in the OM suggests they may be useful as diagnostic tools or in the development of a subunit vaccine.

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