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Identification of *Chlamydia trachomatis* Outer Membrane Complex Proteins by Differential Proteomics▽†

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The extracellular chlamydial infectious particle, or elementary body (EB), is enveloped by an intra- and intermolecular cysteine cross-linked protein shell called the chlamydial outer membrane complex (COMC). A few abundant proteins, including the major outer membrane protein and cysteine-rich proteins (OmcA and OmcB), constitute the overwhelming majority of COMC proteins. The identification of less-abundant COMC proteins has been complicated by limitations of proteomic methodologies and the contamination of COMC fractions with abundant EB proteins. Here, we used parallel liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) analyses of *Chlamydia trachomatis* serovar L2 434/Bu EB, COMC, and Sarkosyl-soluble EB fractions to identify proteins enriched or depleted from COMC. All well-described COMC proteins were specifically enriched in the COMC fraction. In contrast, multiple COMC-associated proteins found in previous studies were strongly enriched in the Sarkosyl-soluble fraction, suggesting that these proteins are not COMC components or are not stably associated with COMC. Importantly, we also identified novel proteins enriched in COMC. The list of COMC proteins identified in this study has provided reliable information for further understanding chlamydial protein secretion systems and modeling COMC and EB structures.

Bacteria in the phylum *Chlamydiae* are characterized by their complex intracellular developmental cycles. *Chlamydiae* must assume at least two functionally distinct morphotypes, the intracellular, replicative reticulate body (RB) and the extracellular, infectious elementary body (EB), to replicate and be transmitted to new hosts (50). The divergence of distinct RB and EB forms may have been driven by the different pressures these pathogens face inside host cells during replication and outside host cells during transmission. For example, the outer membrane of EB contains a poorly immunogenic truncated lipopolysaccharide (LPS) (14, 30) and immunodominant epitopes of the major outer membrane protein (MOMP) vary substantially among closely related chlamydial strains (13). EB also lack detectable peptidoglycan (2, 20, 60), although functional murein biosynthetic enzymes (2, 5, 16, 21, 32, 43, 45, 46) are expressed in RB during productive and persistent infection (44). To compensate for the loss of murein, EB are enveloped by a protein P-layer, which lends osmotic stability to the infectious particle (29).

Attempts to identify components of the P-layer and outer membrane proteins of *Chlamydia* were advanced by the observation that these layers can be separated from many soluble EB proteins using the detergent *N*-lauroyl sarcosine (Sarkosyl). Caldwell et al. dubbed the Sarkosyl-insoluble fraction the chlamydial outer membrane complex (COMC) and noted that purified COMC maintained the shape of intact EB and contained a complete outer membrane, and they reported that a single outer membrane protein, MOMP, accounted for more than 60% of total COMC protein content (15). Other studies revealed that the COMC is stabilized by extensive disulfide bonds between MOMP monomers (26, 27, 53) and between MOMP and two abundant cysteine-rich COMC components (26, 28). Other studies revealed that the COMC is stabilized by extensive disulfide bonds between MOMP monomers (18, 29, 52) and the EB surface (3, 19, 47, 67). More recent data suggest that not all EB outer membrane (OM) proteins are disulfide cross-linked to the COMC. For example, polymorphic membrane protein D localizes to the surface of EB but can be extracted from intact EB with gentle detergents in the absence of reducing agents (17, 61). Thus, not all COMC proteins are exposed on the EB surface, nor are all EB OM proteins components of the COMC.

Beyond these well-described and abundant COMC components, other studies have indicated that additional proteins localize to the EB surface and/or COMC of *Chlamydia trachomatis* (7, 28, 36, 51, 57, 64, 67, 70). However, confirming that specific proteins localize to the COMC or OM of EB can be challenging due to factors such as the contamination of EB preparations with RB proteins and technical limitations of proteomic and surface-labeling protein identification methods (29, 56).

Here, we used differential proteomics to identify proteins specifically enriched in the COMC. Isolated COMC were dissolved in 8 M urea, and the extracted proteins were digested with trypsin. The resulting peptides were analyzed by high-sensitivity liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) to identify low-abundance proteins. Sarkosyl-soluble fractions and whole EB were analyzed in parallel with COMC, and protein assignments were com-

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pared among three replicate runs of each fraction. In total, peptides from 329 L2 proteins were identified. The differential analysis of protein abundance indicated the enrichment of 17 proteins in the COMC. Our results define the cadre of low-abundance COMC proteins, provide a starting point for the identification of surface-exposed EB proteins, and identify EB proteins that are likely to be recognized by innate immunity receptors and/or capable of eliciting neutralizing antibodies in vivo. Finally, our findings and data from other recent studies permit the refinement of existing models of EB and COMC structure.

MATERIALS AND METHODS

***Chlamydia* cultivation.** *C. trachomatis* serovar L2 (strain 434/Bu) was cultivated in HeLa-229 cells, and EB were purified by Renografin density gradient (DG) centrifugation as previously described (15). EB were enumerated by dilution titration on HeLa 229 cells and were visualized by immunofluorescent microscopy using the monoclonal antibody EVI-H1, which targets chlamydial LPS (8).

Sarkosyl fractionation of EB. COMC were prepared essentially as described by Caldwell et al., with minor modifications (15). Approximately 5 mg of DG-purified *C. trachomatis* L2 EB were suspended in phosphate-buffered saline (PBS) (pH 7.4) containing 2% (wt/vol) sodium *N*-laurosyl sarcosine (Sarkosyl; Sigma) and 1.5 mM EDTA. The suspensions were sonicated for 2 min and incubated at 37°C for 1 h. The Sarkosyl-insoluble fraction was pelleted by centrifugation at 12,000 × *g* for 10 min, and the Sarkosyl-soluble fraction was carefully separated from the compacted pellet. Following a second identical extraction, the combined supernatants from the first two centrifugation steps constituted the Sarkosyl-soluble fraction later analyzed by LC-MS/MS. The Sarkosyl-insoluble pellets were treated with DNase I and RNase A at 37°C for 2 h. Pellets from these digestions were suspended in PBS, extensively sonicated, and pelleted twice to remove proteins weakly associated with the COMC. Protein constituents of residual COMC pellets were extracted and analyzed by LC-MS/MS.

Electron microscopy. The purity of COMC preparations was confirmed by electron microscopy as described previously, with minor modifications (15). Samples were fixed in 1.5% glutaraldehyde in PBS (pH 7.4) for 1 h at 4°C and then were postfixed in 1% osmium tetroxide for 1 h at 4°C. The samples were dehydrated in a gradient of ethanol dilutions (30, 50, 70, 90, 95, and 100%) and propylene oxide and were embedded in Epon 812. Silver-to-gold sections were cut and stained with uranyl acetate and lead citrate and were examined using a JEOL model JEM 1010 electron microscope.

SDS-PAGE and Western blotting. Samples were suspended in Laemmli sample buffer supplemented with 5% (wt/vol) β-mercaptoethanol and were denatured by boiling for 10 min. Proteins were separated by 10% reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were visualized by Coomassie brilliant blue staining using standard procedures. COMC purity was evaluated by the Western blotting of EB, COMC, and soluble fractions using monoclonal antibodies targeting chlamydial MOMP (L2-I45) (1) and HSP60 (49) or rabbit polyclonal immune sera prepared against a *C. trachomatis* PmpD peptide (61). Mouse polyclonal sera against recombinant glutathione *S*-transferase (GST) fusion proteins of *C. trachomatis* PulD/YscC and PmpH were prepared by immunizing mice with 50 μg of each recombinant protein essentially as we have described previously (37).

Proteomic sample preparation. An overview of our proteomic strategy is depicted in Fig. 1. Proteomic sample preparation differed slightly among EB, soluble fractions, and COMC fractions. Both EB (dissolved in 2% SDS) and soluble fractions were exchanged into a digestion buffer containing 50 mM ammonium bicarbonate and 8 M urea. COMC were reconstituted directly in the digestion buffer. Protein concentrations were determined by the Bradford assay. Dithiothreitol (DTT) was added at a molar ratio of 40:1 (DTT:protein), and samples were incubated for 2 h at 37°C to reduce protein disulfide bonds. Samples then were chilled on ice and reacted with iodoacetamide (IAM) at a molar ratio of 80:1 (IAM:protein) in complete darkness for 2 h. Subsequently, a 40-fold excess cysteine was added (at 25°C for 30 min) to inactivate residual DTT and IAM. Samples were diluted 4-fold with 50 mM NH_4HCO_3, pH 8.0, followed by the addition of 2% (wt/wt) L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin. Samples then were incubated overnight at 37°C. After enzymatic digestion, peptides were desalted by solid-phase extraction using Oasis hydrophilic-lipophilic balance cartridges (Waters Inc., Milford, MA) and vacuum dried until further analysis.

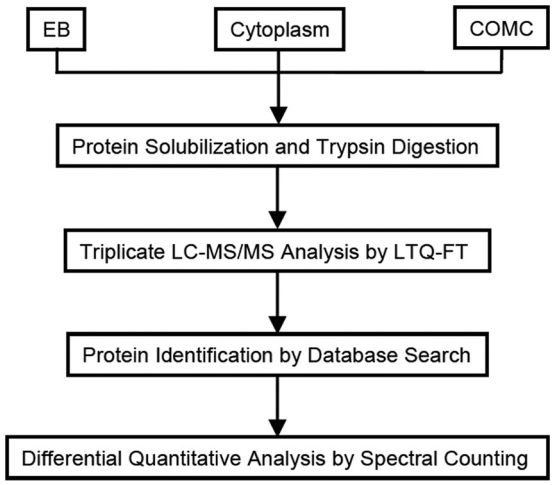

FIG. 1. Schematic of the proteomic approach used for profiling COMC proteins.

Nanoflow LC-MS/MS. Nanoflow reverse-phase LC separation was carried out on an UltiMate 3000 LC system (Dionex, Sunnyvale, CA), as previously described (40). The capillary column (75 μm by 130 mm; Picofrit; New Objective, Woburn, MA) was packed in house. A methanol slurry containing 5 μm 100-Å Magic C18AQ silica-based particles (Microm BioResources Inc., Auburn, CA) was forced through an empty capillary (with a frit in the end) using a bomb. Tryptic peptides were dissolved in high-performance liquid chromatography (HPLC)-grade water, and 300 ng of sample was loaded onto the analytical column in a single LC-MS/MS experiment. The mobile phase was comprised of solvent A (97% water, 3% acetonitrile [ACN], and 0.1% formic acid [FA]) and solvent B (100% ACN and 0.1% FA). LC separation was carried out with the following gradient: solvent B was increased from 6 to 20% in 60 min and then was raised to 28% in 20 min; solvent B was rapidly ramped to 90% and was maintained for 10 min, and then 100% solvent A was used for column equilibration. Peptides eluted from the capillary column were electrosprayed directly onto a hybrid mass spectrometer (LTQ-FT Ultra; ThermoElectron, San Jose, CA) for MS/MS analysis. A data-dependent mode was enabled for peptide fragmentation. One full MS scan with the FT analyzer was acquired in parallel with the fragmentation of the three most intense peptide ions by collision-induced dissociation (CID). MS/MS scans were acquired with a linear ion trap mass spectrometer. Dynamic exclusion was enabled to preclude repeated analyses of the same precursor ion. COMC, EB, and soluble fractions were analyzed in triplicate, and statistical analyses were performed later with respect to peptide and protein identifications. Trapping and analytical columns were washed extensively between samples to prevent carryover from previous experiments.

Protein identification and differential abundance analysis. MS/MS scans were processed with commercial software (Bioworks Browser, ThermoElectron, San Jose, CA) to generate DTA (data) files. DTA (data) files were searched against the chlamydial (L2) protein database using MASCOT (Matrix Science Ltd., London, United Kingdom) (66). Peptide and protein assignments were filtered to retain identifications with scores above 32. Protein relative abundance in EB, COMC, and soluble fractions was assessed by spectral counting. MS/MS spectral counts associated with protein assignments in individual fractions were divided by the sum of spectral counts for all assigned proteins in that fraction. Percentages of spectral counts thereby represented the relative abundance of a given protein in the fraction. An enrichment factor was assigned for each protein by dividing the percentage values in different fractions; standard deviations were calculated based on variability in triplicate runs.

RESULTS

Isolation of COMC from purified EB. COMC were extracted from EB with Sarkosyl and were imaged by transmission electron microscopy (TEM). No intact EB or intermediate forms were present, the outer membrane complex shells appeared to be devoid of cytosolic contents, and COMC were

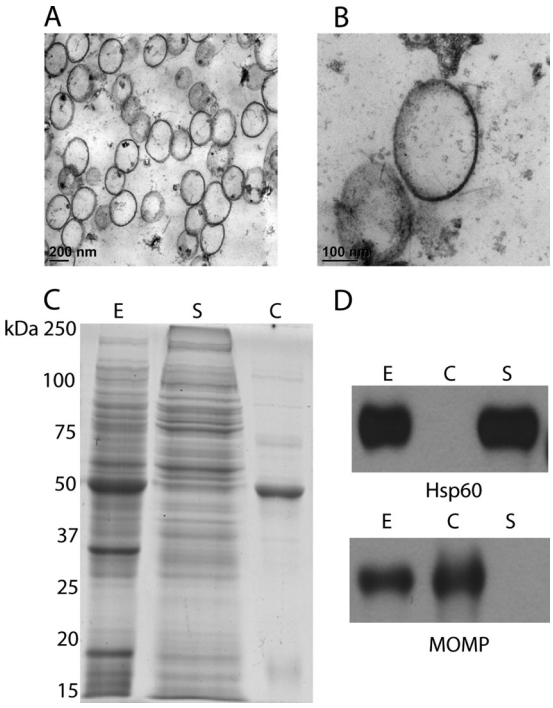

FIG. 2. Evaluation of enrichment strategy by TEM, SDS-PAGE, and Western blotting. TEM of COMC thin sections is shown. (A and B) Micrographs of COMC following 2% Sarkosyl extraction at ×20,000 magnification (A) and ×75,000 magnification (B). (C) SDS-PAGE analysis of EB (E), COMC (C), and Sarkosyl-soluble fractions (S). Note the depletion of the dominant ~40-kDa band corresponding to MOMP in the Sarkosyl-soluble fraction compared to that of whole EB and COMC. (D) Western blot analysis of EB (E), COMC (C), and Sarkosyl-soluble fractions (S) with monoclonal antibodies against MOMP and HSP60. Note the depletion of MOMP and HSP60 from the soluble and COMC fractions, respectively.

morphologically indistinguishable from images previously published by Caldwell et al. (15) (Fig. 2A and B). The EB, Sarkosyl-soluble (soluble), and COMC (insoluble) fractions were further analyzed by SDS-PAGE (Fig. 2C). The complexity of the COMC was substantially reduced compared to that of the soluble fraction or intact EB. MOMP was almost undetectable in the soluble fraction, whereas it was clearly enriched in the COMC. COMC contained additional less intense but clear groups of protein bands in the ranges of approximately 30, 45, 60, and 70 kDa and a large number of minor bands in the range of 90 to 150 kDa, which is consistent with previous reports (7, 51, 64). We blotted the fractions with monoclonal antibodies targeting MOMP and the abundant soluble protein HSP60 (4) to evaluate the purity of COMC. Western blot analysis confirmed the segregation of HSP60 and MOMP into the Sarkosyl-soluble and COMC fractions, respectively (Fig. 2D). These results indicated that fractionation was successful and that the COMC was depleted of Sarkosyl-soluble proteins.

Parallel proteomic analysis of COMC, soluble fraction, and EB. LC-MS/MS was used to comprehensively identify proteins in whole EB and soluble and COMC fractions. To assess reproducibility, triplicate LC-MS/MS experiments were conducted on each fraction. Confident peptide (MASCOT score cutoff of 32) and protein assignments are summarized in Table 1. A complete list of identified peptides and proteins is provided in Table S1 in the supplemental material.

On average, 221, 276, and 25 proteins were identified per the LC-MS/MS experiment of whole EB and soluble and COMC fractions, respectively, corresponding to 740, 990, and 216 peptide identifications, respectively. In total, we identified peptides corresponding to 329 proteins predicted from the L2 genome sequence (data from all LC-MS/MS runs), which compares favorably with other published *C. trachomatis* proteomes (6, 57, 58) (see Table S1 in the supplemental material). Most

TABLE 1. Proteins enriched in the COMC fraction

L2[a]	D[b]	Protein	L2 accession no.	Mass (Da)	Pep[c]	Spec[d]	C/S[e]	*t* test[f]
ctl0050	*CT681*	Momp	YP_001654141	43,036	36	200	17.7	0.002
ctl0702	*CT443*	OmcB	YP_001654774	60,783	51	134	36.6	0.001
ctl0250	*CT871*	PmpG	YP_001654335	108,032	30	46	16.7	0.016
ctl0251	*CT872*	PmpH	YP_001654336	108,119	27	35	14.2	0.002
ctl0248	*CT869*	PmpE	YP_001654333	105,500	19	18	11.5	0.001
ctl0043	*CT674*	PulD/YscC	YP_001654134	100,574	14	15	6.5	0.002
ctl0082	*CT713*	PorB	YP_001654173	37,839	7	9	10.2	0.010
ctl0671	*CT414*	PmpC	YP_001654743	187,729	10	8	5.3	0.005
ctl0887	*CT623*	CTL0887	YP_001654178	48,560	9	11	2.3	0.018
ctl0703	*CT444*	OmcA	YP_001654775	10,013	3	9	9.5	0.021
ctl0249	*CT870*	PmpF	YP_001654334	113,209	8	8	3.7	0.002
ctl0071	*CT702*	PmpB	YP_001654742	184,085	10	9	6.0	0
ctl0541	*CT289*	CTL0541	YP_001654618	42,241	6	8	12.7	0.004
ctl0626	*CT372*	OprB	YP_001654701	49,929	10	12	12.7	0.028
ctl0493	*CT241*	OMP85	YP_001654570	88,943	8	4	28.0	0.063
ctl0645	*CT389*	CTL0645	YP_001654718	47,018	3	2	NC[g]	ND
ctl0863	*CT600*	Pal	YP_001654932	21,532	1	1	NC[g]	ND

[a] Serovar L2 434/Bu gene designation.
[b] Serovar D UW-3/CX gene designation.
[c] Number of unique peptides observed for a given protein in the COMC fraction during triplicate LC-MS/MS analysis.
[d] Average spectral counts observed in the COMC fraction in a single LC-MS/MS experiment.
[e] Enrichment factor calculated by dividing the percent spectral count in the COMC by that in the soluble fraction.
[f] Values obtained from the Student *t* test with the null hypothesis that the mean percent spectral counts from the soluble and COMC fractions are the same. ND, not done.
[g] NC, not calculated. No peptides corresponding to the protein were identified in the soluble fraction.

TABLE 2. Proteins enriched in the Sarkosyl-soluble fraction

L2[a]	D[b]	Protein	Accession no.	Mass (Da)	Pep[c]	Spec[d]	S/C[e]	*t* test[f]
ctl0574	*CT322*	Ef-Tu/TufA	YP_001654650	43,309	26	50	19.7	0.005
ctl0365	*CT110*	GroEL	YP_001654449	58,090	31	40	NC[g]	0.008
ctl0842	*CT579*	CopD	YP_001654912	43,935	13	23	NC	0.009
ctl0652	*CT396*	DnaK/HSP70	YP_001654725	70,843	26	40	NC	0.001
ctl0255	*CT875*	CTL0255	YP_001654339	65,689	20	28	33.4	0.000
ctl0112	*CT743*	Hc1	YP_001654203	13,567	6	11	2.2	0.131
ctl0850	*CT587*	CTL0850	YP_001654920	45,303	12	15	NC	0.005
ctl0566	*CT314*	RpoB′	YP_001654642	154,813	31	36	10.5	0.010
ctl0567	*CT315*	RpoB	YP_001654643	140,057	27	26	10.1	0.003
ctl0254	*CT874*	PmpI	YP_001654338	92,935	5	3	1.6	0.019
ctl0183	*CT812*	PmpD	YP_001654273	156,783	5	4	NC	0.651

[a] Serovar L2 434/Bu gene designation.
[b] Serovar D UW-3/CX gene designation.
[c] Number of unique peptides observed for a given protein in the COMC fraction during triplicate LC-MS/MS analysis.
[d] Average spectral counts observed in the COMC fraction in a single LC-MS/MS experiment.
[e] Enrichment factor calculated by dividing the percent spectral count in the soluble by that in the COMC fraction.
[f] Values obtained from the Student *t* test with the null hypothesis that the mean percent spectral counts from the soluble and COMC fractions are the same.
[g] NC, not calculated. No peptides corresponding to the protein were identified in COMC.

proteins were assigned based on multiple peptide identifications. The average number of peptide assignments per protein was 3 in whole EB and was increased to 8 for COMC proteins (per individual LC-MS/MS experiment), reflecting a high level of confidence owing to the decreased complexity of the later fraction. The top two assignments in COMC were MOMP and the 60-kDa cysteine-rich protein OmcB, which were recognized by 34 and 47 unique peptides, corresponding to 84 and 77% sequence coverage, respectively. In contrast to the most frequently observed COMC proteins, elongation factor Tu (EF-Tu), GroEL (HSP60), and DnaK (HSP70) were the top assignments in the soluble fraction. Peptides corresponding to these five proteins also were the most abundant components of whole EB.

Identification of proteins enriched in the COMC by differential analysis. Quantitative information about protein assignments was obtained through a spectral counting method. Spectral counts represent the total number of repeated identification of peptides for a given protein during the entire analysis and provide a semiquantitative measurement of protein abundance (39). An enrichment factor was calculated by dividing the percentage of individual proteins in COMC by that in the soluble fraction. Table 2 lists proteins selectively enriched in the COMC fraction and corresponding enrichment factors.

In total, 17 proteins were reproducibly enriched more in the COMC than in the soluble fraction. The enrichment of known COMC proteins, including MOMP (15), OmcA, OmcB (28), PorB (36), OprB (7), some polymorphic membrane proteins (Pmp) (discussed below) (51, 57, 64), and the type II/III secretion outer membrane ring protein PulD/YscC (CTL043) (70), ranged from approximately 10- to 37-fold. A variety of abundant proteins that have identified as possible COMC components (7) or in COMC in other studies reviewed by Raulston (56) were absent or infrequent in our COMC preparation fractions. For example, nine proteins identified in COMC in a recent study (7) (Table 2) were detected exclusively in (GroEL, CopD, HSP70, CTL0850, and PmpD) or were highly enriched in (TufA, RpoB, RpoC, and CTL0255) Sarkosyl-soluble fractions in this study (see Table S1 in the supplemental material). Many of these proteins were among the most abundant components in the soluble fraction. Considering that the enrichment strategy robustly identified all well-established COMC proteins, these data suggest that these proteins are not likely to be covalently associated with the COMC. The data also strengthen the interpretation that the novel COMC components we identified are unlikely to represent contaminants from other EB fractions.

Polymorphic membrane proteins. Chlamydial Pmp proteins are members of the type V-secreted autotransporter (AT) super family (72). *C. trachomatis* encodes and expresses nine Pmp proteins (5, 60), whereas *C. pneumoniae* may encode as many as 21, although some of these may be pseudogenes (23, 24, 31, 71). Pmp subdomains include an N-terminal passenger domain, a central conserved domain of unknown function, and a C-terminal β-barrel domain (31, 71). The β-barrel domain of ATs (31, 71) mediate the translocation of the central and N-terminal domains of these proteins from the periplasm to the bacterial surface; presumably, Pmp proteins are secreted by the same mechanism (72). Eight of nine Pmp proteins predicted from the *C. trachomatis* serovar L2 genome sequence (60) have been observed in whole EB or COMC and soluble fraction proteomes, and all nine Pmp proteins have been detected in late inclusions by Western blotting (63). However, our results indicated that Pmp proteins differed in their abundance and distribution among EB fractions.

PmpB, PmpC, PmpE, PmpF, PmpG, and PmpH all were significantly enriched in COMC, which is in agreement with previous reports (7, 51, 63, 64, 69). Peptides corresponding to these proteins were enriched from approximately 4-fold (PmpC) to 17-fold (PmpG) in the COMC compared to that in the soluble fraction (Table 1). Peptide abundance and the corresponding locations of these peptides within different Pmp proteins further sorted the Pmp into groups. PmpE, PmpG, and PmpH were most frequently detected and highly enriched in COMC, which is consistent with previous reports (51, 64). Interestingly, peptides corresponding to the β-barrel domains of these proteins were detected only in whole EB and COMC (Fig. 3 and see Table S1 in the supplemental material). Multiple peptides and spectra corresponding to the N-terminal domains of each protein were detected in replicate analyses of

MQTSFHKFFL SMILAYSCCS LNGGGYAAEI MVPQGIYDGE TLTVSFPYTV
IGDPSGTTVF SAGELTLKNL DNSIAALPLS CFGNLLGSFT VLGRGHSLTF
ENIRTSTNGA ALSNSAADGL FTIEGFKELS FSNCNSLLAV LPAATTNKGS
QTPTTTSTPS NGTIYSKTDL LLLNNEKFSF YSNLVSGDGG AIDAKSLTVQ
GISKLCVFQE NTAQADGGAC QVVTSFSAMA NEAPIAFVAN VAGVRGGGIA
AVQDGQQGVS SSTSTEDPVV SFSRNTAVEF DGNVARVGGG IYSYGNVAFL
NNGKTLFLNN VASPVYIAAK QPTSGQASNT SNNYGDGGAI FCKNGAQAGS
NNSGSVSFDG EGVVFFSSNV AAGKGGAIYA KKLSVANCGP VQFLRNIAND
GGAIYLGESG ELSLSADYGD IIFDGNLKRT AKENAADVNG VTVSSQAISM
GSGGKITTLR AKAGHQILFN DPIEMANGNN QPAQSSKLLK INDGEGYTGD
IVFANGSSTL YQNVTIEQGR IVLREKAKLS VNSLSQTGGS LYMEAGSTLD
FVTPQPPQQP PAANQLITLS NLHLSLSSLL ANNAVTNPPT NPPAQDSHPA
VIGSTTAGSV TISGPIFFED LDDTAYDRYD WLGSNQKINV LKLQLGTKPP
ANAPSDLTLG NEMPKYGYQG SWKLAWDPNT ANNGPYTLKA TWTKTGYNPG
PERVASLVPN SLWGSILDIR SAHSAIQASV DGRSYCRGLW VSGVSNFFYH
DRDALGQGYR YISGGYSLGA NSYFGSSMFG LAFTEVFGRS KDYVVCRSNH
HACIGSVYLS TQQALCGSYL FGDAFIRASY GFGNQHMKTS YTFAEESDVR
WDNNCLAGEI GAGLPIVITP SKLYLNELRP FVQAEFSYAD HESFTEEGDQ
ARAFKSGHLL NLSVPVGVKF DRCSSTHPNK YSFMAAYICD AYRTISGTET
TLLSHQETWT TDAFHLARHG VVVRGSMYAS LTSNIEVYGH GRYEYRDASR
GYGLSAGSRV RF

FIG. 3. Peptides corresponding to the β-barrel domains of PmpE, PmpG, and PmpH are absent from the soluble fraction. PmpG peptides detected in COMC and soluble fractions were mapped to the predicted protein sequence. Double-underlined amino acid residues (amino acid residues 737 to 999) correspond to the β-barrel domain of PmpG. Boldfaced amino acid residues were present in peptides identified in COMC fractions. Single-underlined amino acid residues were present in peptides detected in soluble fractions. Amino acid residues not boldfaced or underlined were not detected in COMC or soluble fractions. The similar segregation of β-barrel peptides between COMC and soluble fractions was observed for PmpE and PmpH but not other Pmp proteins (see Table S1 in the supplemental material).

the soluble fraction (4, 7, and 6 spectra average per LC-MS/MS run, respectively) (Fig. 3). Peptides corresponding to the β-barrel domains of PmpB, PmpC, and PmpF were detected in the soluble fraction, although fewer peptides corresponding to these proteins were detected overall (Table 1; also see Table S1 in the supplemental material). This suggests that although most Pmp can locate to the EB OM, not all of these proteins are necessarily strongly attached to the COMC, as has been suggested previously for some *C. psittaci* Pmp proteins (11).

Peptides corresponding to PmpI and PmpD were detected in relatively low quantities compared to that of the Pmp mentioned above, and the pattern of peptide identifications differed between Pmp and the COMC-enriched Pmp. PmpD was not detected in the COMC, whereas multiple PmpD peptides were identified in replicate LC-MS/MS runs of whole EB and soluble fractions (Table 2). These results are consistent with reports that PmpD is located on the surface of EB (17, 61, 63). Two PmpI peptides were detected in COMC in one of three replicate LC-MS/MS runs. In contrast, multiple PmpI peptides were detected in replicate analyses of EB and soluble fractions, although the enrichment of PmpI in the soluble fraction was not statistically significant (Table 2).

The differential distribution of Pmp in soluble and COMC fractions was confirmed in a Western blotting assay using antibodies against Pmp proteins (PmpD and PmpG) and the COMC-enriched type II/III secretion family protein PulD/YscC. PmpD was detected only in EB and soluble fractions, whereas PmpH and PulD/YscC were detected only in EB and COMC (Fig. 4). These observations are consistent with the hypothesis that PmpD is associated with the EB surface without being covalently linked to COMC proteins. This hypothesis is supported by the fact that PmpD lacks cysteine residues in its

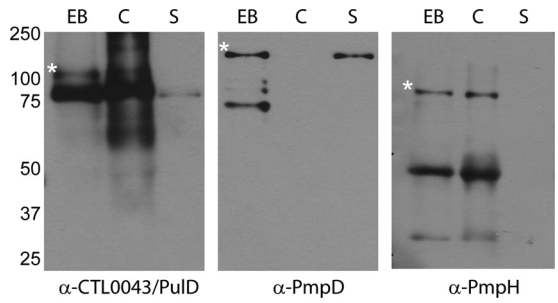

FIG. 4. Western blot of EB, COMC, and Sarkosyl fractions. Equal amounts of protein from L2 EB, COMC, and Sarkosyl fractions were separated by SDS-PAGE, transferred to nitrocellulose, and blotted with specific antibodies against CTL0043 (YscC/PulD), PmpD, and PmpH. EB, COMC (C), and Sarkosyl-soluble (S) fractions are indicated above lanes in individual blots. Approximate molecular masses in kDa are indicated at the left. Primary antibodies (designated α-CTL0043/PulD, α-PmpD, and α-PmpH) are indicated below. Asterisks indicate the approximate predicted sizes of the mature proteins.

β-barrel domain (amino acids 1245 to 1507), whereas the other Pmp contain two to six cysteine residues. This observation may be pertinent to the association of Pmp with the COMC in general, because these proteins are cysteine rich compared to the AT of other bacteria (72), and this is consistent with the interpretation that some Pmp are covalently attached to COMC proteins by disulfide linkage.

CTL0493, CTL0645, and Pal. CTL0493 (Omp85) was one of the most strongly enriched (28-fold) proteins in COMC. Consistently with COMC localization, CTL0493 contains a high-confidence signal peptide (0.997 by SignalP 3.0) with a most-probable cleavage site predicted between amino acids 23 and 24 (0.409). This interpretation is also supported by a report that Omp85 is surface exposed on EB and that chlamydial infectivity can be blocked with anti-Omp85 antibodies (59). CTL0493 shares strong homology with two-partner secretion (TPS)/outer membrane protein 85 (Omp85) superfamily proteins (NCBI Blast result) (34). These proteins are ubiquitous Gram-negative bacterial outer membrane proteins that insert proteins into the outer membrane, translocate folded proteins from the periplasm to the bacterial surface (TPS subfamily specifically), and act as porins (33, 34, 72).

CTL0645 and Pal were indentified exclusively in COMC fractions, although these proteins were in low abundance relative to other COMC components based upon total numbers of unique peptide identifications and spectral counts, relative to total numbers of theoretical trypsin fragments in each protein. Two unique peptides (5 spectrum counts) corresponding to CTL0645 were detected. CTL0645 was recently identified as an EB-specific protein in a proteomic survey of RB and EB (58). CTL0645 has a high-confidence signal peptide (0.976 by SignalP 3.0) and a predicted cleavage site between amino acids 24 and 25 (0.951). The 44-kDa mature protein is predicted to contain 13 cysteine residues (3.2% of amino acids), which is comparable to other COMC proteins, including MOMP (2.3%), OprB (2.0%), PorB (2.4%), and OmcB (4.3%). CTL0645 is highly conserved in all *C. trachomatis* genomes sequenced to date (>99% amino acid amino acid identity, NCBI Blast result) but does not share strong homology with characterized proteins outside *Chlamydia*.

A single peptide corresponding to Pal (peptidoglycan-associated lipoprotein) was identified in each of the three COMC fractions. This is interesting because Pal orthologs maintain the outer membrane (22) and are potent agonists of eukaryotic Toll-like receptors (38, 68). Chlamydial Pal is also cysteine rich (2.1%) and was also reported to be an EB-specific protein by Skipp et al. (68). SignalP 3.0 predicted a confident N-terminal secretion sequence in Pal (0.997), although the predicted cleavage site between residues 22 and 23 of the protein was of lower confidence (0.549). Multiple alignment of *C. trachomatis* Pal with orthologs indicated absolute conservation of the cysteine and serine residues in positions 22 and 23 (numbering based upon *C. trachomatis* Pal) (Fig. 5). These residues correspond to the first two amino acids in Pal orthologs which are lipidated (cysteine, +1), and which direct Pal to the outer membrane (serine, +2) (22, 54). Thus, the alignment suggested that the signal sequence cleavage site in *C. trachomatis* Pal lies between amino acids 20 and 21. Interestingly, alignment also revealed absolute conservation of 8 of 10 residues in *C. trachomatis* Pal which were recently shown to mediate interactions of *H. influenzae* Pal with a synthetic peptidoglycan precursor (UDP-*N*-acetylmuramyl-L-Ala-*R*-D-Glu-*m*-Dap-D-Ala-D-Ala) (Fig. 5) (54).

Although CTL0493, CTL0645, and Pal have not previously been associated with COMC, their designation as probable COMC components is based upon the following criteria: (i) the presence of predicted signal sequences (all three proteins), (ii) high concentrations of cysteine residues (CTL0645 and Pal), (iii) their similarity to known outer membrane proteins (CTL0493 and Pal), and (iv) previous reports identifying these proteins as EB surface antigens (CTL0493) or EB-specific proteins (CTL0645 and Pal).

CTL0887. CTL0887 shares extensive amino acid identity with a 76-kDa *C. pneumoniae* protein reported to be an EB surface antigen based upon the observation that monoclonal antibodies targeting this protein partially neutralized *C. pneumoniae* EB (55). Tanzer and Hatch previously identified CTL0887 (designated Orf 623 according to the *C. trachomatis* serovar D genome annotation) in L2 COMC and reported that this protein was labeled by a lipophilic OM-specific reagent but was unaffected by the trypsin treatment of whole EB (64). Birkelund et al. also identified four peptides corresponding to CTL0887 during the proteomic analysis of the L2 COMC (7). We detected 10 unique peptides and 95 spectra corresponding to CTL0887. CTL0887 was enriched 2.3-fold in COMC compared to level of the soluble fraction, but this protein clearly was an abundant component of whole EB, COMC, and soluble fractions. Peptides identified in each fraction were similar and corresponded to regions throughout CTL0887, suggesting that full-length or nearly full-length protein was present in each fraction (data not shown). SignalP 3.0 predicted a high confidence signal sequence (1.000) and cleavage site between amino acids 25 and 26 (0.997), indicating that CTL0887 is secreted. Unlike other COMC proteins, CTL0887 is relatively cysteine poor (0.2%) and lacks obvious transmembrane helices, although the observation that this protein is labeled with a lipophilic reagent indicates it is an OM protein (64). Collectively, these observations suggest that CTL0887 locates to the EB OM but may not be covalently attached to other COMC proteins.

DISCUSSION

COMC proteins maintain the integrity of the chlamydial infectious particle and are among the first chlamydial proteins that interact with host cells during infection. Understanding the composition of the COMC is relevant to both chlamydial biology and vaccine design, because COMC proteins are prominent targets of neutralizing antibodies elicited during chlamydial infection in vivo (9, 48).

The composition of the COMC has been investigated using a variety of methods, but many of the proteins identified as components of this fraction have not been verified by other means aside from simple detection in Sarkosyl-insoluble EB fractions. We chose to use comparative proteomics based on reports that COMC can be contaminated with soluble proteins during purification (7, 29, 56). Our COMC purification was preoptimized by standardizing times and intensities of sonication and DNase I treatment steps while monitoring COMC, soluble, and whole EB fractions in parallel with Western blotting and TEM. Three observations are pertinent to replicating our results: (i) extensive sonication was critical to reduce levels of known cytoplasmic proteins in COMC, (ii) mild detergent pretreatments altered the COMC proteome, and (iii) the trypsin pretreatment of EB altered the COMC proteome (data not shown). Our observations agree with reports that PmpD can be extracted from EB with mild detergents (61), and that detergent (29) and trypsin pretreatment (25) alter the COMC proteome. Caveats of our relatively stringent extraction approach include small amounts of EDTA in our Sarkosyl extraction buffer and intensive sonication. Previous reports suggest that such steps could impact outer membrane stability and solubilize outer membrane proteins not covalently attached to the COMC (3). Thus, the identification of a protein as soluble in our data set does not rule out outer membrane or EB surface localization but likely does indicate a lack of direct covalent association with cysteine cross-linked proteins that form the backbone of COMC.

As suggested by others (29, 56), we suspect that discrepancies in COMC association among different studies relate to differences in protocols. Anticipated limitations of the exclusion of NP-40 and trypsin pretreatments from our protocol include the nonspecific RB protein contamination of COMC and nonspecific precipitation of membrane proteins, which could incorrectly be interpreted as COMC components. We believe our protocol was effective based upon various observations, including (i) inclusion membrane proteins, such as Inc proteins, were absent in our data set, (ii) most L2 proteins with predicted outer membrane secretion signal sequences and/or transmembrane helices were identified only in EB and the soluble fraction, (iii) our method robustly enriched all known COMC components, and (iv) a parallel analysis of *C. muridarum* and *C. trachomatis* serovar D COMC yielded essentially identical results (data not shown). Nonetheless, our results are a guide to likely COMC localization; it is essential that novel COMC proteins be confirmed by independent methods such as EB neutralization assays (12) and EB surface labeling (64, 65).

Birkelund and coworkers recently reported a proteomic analysis of the *C. trachomatis* L2 COMC while our study was under way. Rather than a comprehensive proteome profiling, their studies were targeted to determine in vivo proteolytic

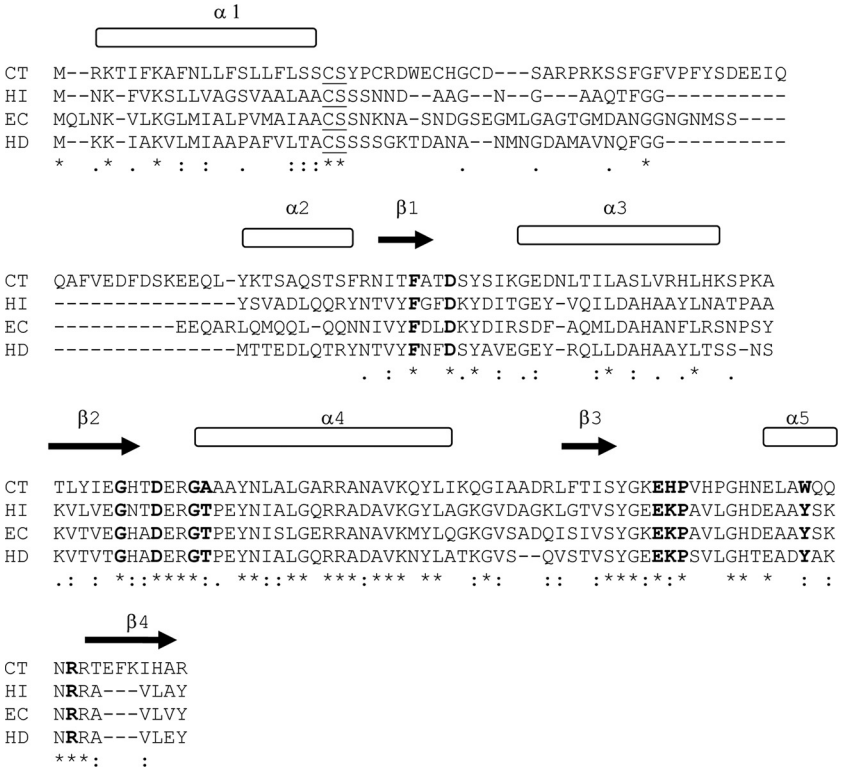

FIG. 5. PSI-PRED structural prediction and alignment of Pal homologs. Predicted α-helical regions are indicated by white cylinders, and β-strands are indicated by black arrows above the sequences (10, 35). The abbreviations of the aligned sequences (accession numbers are in parentheses) are the following: CT, *Chlamydia trachomatis* serovar L2 434/Bu (YP_001654932); HI, *Haemophilus influenzae* (NP_438542); EC, *Escherichia coli* K-12 (POA914); and HD, *Haemophilus ducreyii* (NP_874137). Identical residues are indicated by an asterisk, similar residues by a colon, and low-similarity residues by a dot. Underlined residues correspond to the +1 cysteine and +2 serine residues in mature *E. coli* Pal. Boldfaced residues indicate broadly conserved residues in diverse Pal orthologs, and many of these residues recently were shown to mediate *H. influenzae* Pal interaction with a synthetic peptidoglycan precursor (54).

cleavage sites of COMC proteins, and thus N-terminal peptides were enriched prior to mass-spectrometric analysis (7). This method yields a single N-terminal peptide per protein unless the protein is processed at multiple cleavage sites. In either case, the selective analysis of N-terminal peptides to some extent compromises the confidence of protein identification, because most peptides are not N terminal and are filtered prior to analysis. Nonetheless, our results are largely complementary with those of the Birkelund study. Notable differences include (i) few proteins detected in our COMC fractions corresponded to known or likely cytoplasmic proteins (<3% of total sequences in our study versus ~36% in the Birkelund study, as assessed by spectral counts); (ii) multiple proteins designated cytoplasmic contaminants of the COMC in the Birkelund study were confirmed here to be enriched in the soluble fraction; (iii) COMC proteins were identified with greater confidence here based upon peptide scores, total numbers of unique peptides, and spectral counts; (iv) increased numbers of unique peptides and corresponding spectral counts in our study permitted the analysis of the differential localization of subdomains of some proteins (such as β-barrels and effector domains of Pmp); (v) the overall greater depth of LC-MS/MS analysis permitted confident detection of additional COMC enriched proteins; and finally, (vi) our proteomic data expand the known proteome of *C. trachomatis* L2 (58) (see Table S1 in the supplemental material).

In addition to strengthening the case for a number of putative COMC proteins observed in only one or a few studies, we identified three additional COMC proteins (CTL0493, CTL0645, and Pal). All three proteins either have been identified in EB by proteomic analysis (58), have been predicted as outer membrane components in silico, or have been shown to locate to the EB surface by other approaches, such as neutralization assays (59). The known functions of orthologs of two of these proteins, CTL0493 (Omp85) and Pal, seem especially consistent with their localization to COMC. Omp85 family proteins facilitate the insertion of proteins into the outer membrane (34). We speculate that Omp85 mediates the translocation of abundant COMC porins such as MOMP, OprB, and PorB to the COMC during the RB-to-EB transition. The identification of Pal in COMC is interesting, because orthologs anchor the outer membrane to peptidoglycan (54) and are involved with the assembly of the Tol complex, a supermolecular structure that connects the inner and outer membranes in other gram-negative bacteria (22). Multiple-sequence alignment indicated that residues in *H. influenzae* Pal that interact with peptidoglycan were almost absolutely conserved in chlamydial Pal (Fig. 5) (54). This suggests that Pal is a suitable

ligand to isolate chlamydial peptidoglycan. Further, this observation suggests that peptidoglycan components are present in COMC. We currently are exploring this possibility.

Our proteomic comparisons indicated that Pmp proteins differ in their distributions in COMC and soluble fractions. PmpD and PmpI were enriched in EB and soluble fractions (Table 2, Fig. 4). This indicates that other proteins on the EB surface likely were identified in the soluble fraction in our data set and could explain the observation of the low enrichment of some Pmp and CTL0887 in COMC. PmpA was not detected in any experiment, which is consistent with reports that this protein is RB specific (58) and is not an outer membrane protein (60, 66). However, recent data indicate that PmpA is present on the RB outer membrane of RB late during infection (62). In contrast to the soluble Pmp discussed above, peptides corresponding to the β-barrels of PmpE, PmpG, or PmpH were absent from the soluble fraction. Taken together, the observations described above indicate that Pmp proteins are differentially associated with or are differentially oriented within the COMC. Observations from other studies support this hypothesis. For example, *C. psittaci* Pmp differ in their trypsin sensitivity in intact EB and possibly in their requirements for intermolecular disulfide bonding with purified COMC (65). Another study indicated that the amino-terminal effector domains of some Pmp locate to the cytoplasmic face of COMC in EB (11), although this is controversial (41, 42).

Whether groups of Pmp are oriented or attached differently with COMC proteins or the outer membrane should be investigated further, because this scenario suggests a mechanism by which effector domains of these proteins could be sequentially displayed to host cells. We propose a model detailing this hypothesis, based upon results of this study and the previous literature. Pmp not covalently attached to the COMC but located in the EB outer membrane would be available for immediate delivery to host cells and would not need to be restricted to the locality of the entering EB, as has been suggested for PmpD (61). Pmp whose effector domains locate to the EB surface and that are disulfide-linked to COMC by β-barrel cysteine residues would be immediately available for host-cell interactions but presumably would remain EB associated until the intracellular reduction of the COMC. Finally, observations of the differential sensitivity of Pmp to trypsin treatment (65) and a report that effector domains of Pmp orient toward the interior face of COMC (11) suggest that the effector domains of some Pmp are not displayed on the EB surface. Thus, we speculate that effector domains of such Pmp translocate to the EB surface after the reduction of the COMC is completed later in the developmental cycle. In summary, this model details a route by which Pmp effector domains could be delivered sequentially to the EB surface and temporally regulate host-pathogen interactions during the earliest phases of chlamydial attachment and invasion.

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