Molecular characterization and subcellular localization of macrophage infectivity potentiator, a *Chlamydia trachomatis* lipoprotein

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Running title: Molecular characterization of a *Chlamydia* lipoprotein
Abstract

Macrophage infectivity potentiator (MIP) was originally reported to be a chlamydial lipoprotein from experiments showing incorporation of radiolabeled palmitic acid into the native and recombinant MIP, inhibition of post-translational processing of recombinant MIP by globomycin, known to inhibit signal peptidase II, and solubility of native MIP in Triton X-114. However, the detailed structural characterization of the lipidic moiety on MIP has never been fully elucidated. In this study, bioinformatics and mass spectrometry analysis, as well as radiolabeling and immunochemical experiments were conducted to further characterize MIP structure and subcellular localization. In silico analysis showed that the amino acid sequence of MIP is conserved across Chlamydiae species. A potential signal sequence with a contained lipobox was identified and a recombinant variant C20A was prepared by substituting the probable lipobox cysteine for an alanine. Both incorporation of [U-$^{14}$C]-esterified glycerol and [U-$^{14}$C]-palmitic acid as well as post-translational processing that was inhibitable by globomycin, were observed in wild type recombinant MIP, but not in the C20A recombinant MIP variant. Fatty acid content of native and recombinant MIP were analyzed by gas chromatography-mass spectrometry and the presence of amide-linked fatty acids was investigated by alkaline methanolysis in recombinant MIP. These results demonstrated a lipid modification in MIP similar to that of other procaryotic lipoproteins. In addition, MIP was detected in outer membrane preparation of Chlamydia trachomatis elementary bodies, and was shown to be present at the surface of elementary bodies by surface biotinylation and surface immunoprecipitation experiments.

Introduction
Chlamydiae are obligate intracellular Gram negative bacteria that are major human pathogens capable of causing a wide range of diseases (1, 12, 24, 34, 43). All Chlamydiae share a characteristic, biphasic cycle of development with infectious, spore-like elementary bodies (EB) and intracellular dividing reticulate bodies (RB) that are metabolically active and inhabit a non-fusogenic inclusion (46). The mechanisms by which Chlamydiae induce diseases are poorly understood but may include a proinflammatory immune response to chlamydial antigen (22) even if this antigen has yet to be unequivocally revealed (62). In many bacterial diseases, lipoproteins play a prominent role in the pathogenesis with a ubiquitous presence as major constituents of the bacterial cell wall. However in Chlamydiae, little is known about the actual existence and role of lipoproteins. One of the few lipoproteins characterized so far in Chlamydia trachomatis, is macrophage infectivity potentiator (MIP) which was identified as a lipoprotein by incorporation of radiolabeled palmitic acid into the native and recombinant proteins (41), inhibition of post-translational processing of recombinant (r)MIP by globomycin (a specific inhibitor of signal peptidase II (31)), and solubility of native MIP in Triton X-114 (42). Other studies have shown that C. trachomatis MIP is a 27 kDa membrane protein located in both EB and RB (38), with a COOH-terminal region showing a high homology with eukaryotic and prokaryotic FK506 binding proteins (FKBP) (41) and exhibiting peptidyl-prolyl cis-trans isomerase (PPIase) activity (40). However, evidence supporting the presence of a classical lipoprotein feature in MIP, similar to murein lipoprotein from Escherichia coli (25), the best characterized bacterial lipoprotein, has not been shown. As Chlamydiae are notably very atypical bacteria, phylogenetically separated from other eubacteria (48), we have conducted a detailed structural characterization of MIP. The probable signal sequence was determined by in silico analysis and the cysteine in position 20 (cysteine²⁰) was predicted to be the
lipobox cysteine (33, 44). To assess the involvement of cysteine\textsuperscript{20} in lipid modification, a
rMIP variant was prepared, by substituting the cysteine\textsuperscript{20} with alanine (C20A rMIP) using
site-directed mutagenesis (17). To characterize the structure of the lipidic moiety, [U-
\textsuperscript{14}C]-esterified glycerol and [U-\textsuperscript{14}C]-palmitic acid attachments to the lipobox cysteine, as
well as peptide signal release, were examined in both wild type (WT) rMIP and the C20A
rMIP variant. Fatty acid contents of native and WT rMIP were analyzed by gas
chromatography-mass spectrometry and amide linkage of any fatty acid present was
investigated by alkaline methanolysis in WT rMIP. To define its subcellular localization
and possible surface exposure, the presence of native MIP was examined in a
chlamydial outer membrane complex (COMC) preparation, and its presence at the
surface of elementary bodies was investigated by surface biotinylation and surface
immunoprecipitation experiments.
MATERIALS AND METHODS

Sequence alignments. The sequences of MIP or putative MIP were found in Swiss-Prot/TrEMBL database. To study homologies among the five MIP sequences, the multiple sequence alignment program CLUSTAL W (69) (available at www.ebi.ac.uk/clustalw/) was used with Gonnet 250 as a matrix. To study homologies between two sequences, binary sequence alignment program SIM (29) (available at www.expasy.org/tools/sim-prot.html) was used with Blosum62 as a comparison matrix.

In silico analysis. Three different computer prediction programs were used to detect the pattern characterizing bacterial lipoprotein precursors in MIP sequences. The pattern is a well conserved cysteine-containing 'lipobox' of four residues within the lipoprotein signal peptide sequence. The bioinformatics tools were: PROSITE (accession number: PS000013, www.expasy.org/prosite/)(30), DOLOP (Database Of bacterial LipOProteins with [LVI][ASTVI][GAS][C] as consensus pattern (available at www.mrc-lmb.cam.ac.uk/genomes/dolop/)(44), and LipoP (accessible at www.cbs.dtu.dk/services/LipoP/)(33).

Protein homologues of those required for precursor export and lipoprotein biosynthesis in E. coli were searched into the complete proteomes of five different species of Chlamydiae. Complete proteomes deduced from genomic sequences (28, 35, 52, 53, 59, 63) were available at HAMAP (high-quality automated and manual annotation of microbial proteomes) site (http://www.expasy.org/sprot/hamap/).

Bacteria. Elementary bodies of C. trachomatis LGV2 strain 434 (inactivated by a photochemical treatment affecting genome of bacteria) was either purchased from Biodesign International (Milan Analytica AG, Le Milan, Switzerland) or prepared according to Boleti et al. (6).
Preparation of WT rMIP and C20A rMIP variant. Preparation of WT rMIP from *C. trachomatis* has been previously described (2). C20A rMIP variant was prepared by in vitro site-directed mutagenesis as follows. Template DNA for the PCR was obtained from purified WT rMIP plasmid clone (2). Replacement of bases coding for cysteine by those coding for alanine was performed with the QuickChange site-directed mutagenesis kit from Stratagene (Amsterdam Zuidoost, The Netherlands). For the amplification reaction, oligonucleotide primers containing the desired mutation (in bold) were: 5'-TGCCTATCGTAGGAGCTGATAACGGAGGCGGTTTCG-3' for the 5' end and 5'-GCGAACCGCCTCCGTTATCAGCTCCTACGATAGGCA-3' for the 3' end. Other conditions were previously described (2).

Immunization of rabbits with WT rMIP and development of polyclonal antibodies. Two female New Zealand White rabbits, (weight 2.0-2.5 kg), were used by Covalab (Lyon, France) to produce antibodies to WT rMIP. Before immunization, the preimmune sera were collected for use as control. For the first immunization, each rabbit was injected intradermally with purified endotoxin-free WT rMIP (50 µg). The injected proteins (0.5 ml) were emulsified with an equal volume of Freund’s complete adjuvant. Animals were subsequently injected three times at 3-week intervals with the same immunogen emulsified with incomplete Freund’s adjuvant. Injections were performed intradermally, except the last which was done subcutaneously. Antisera of immunized animals were collected 11 days after the third and the fourth booster immunization and examined for the presence of MIP-specific antibodies by ELISA. Rabbits were sacrificed 82 days following the first injection. The IgG fraction of the antiserum was isolated using an immobilized Protein A-agarose column.

Immunoblot analysis. Proteins from EB, *E. coli* clones expressing either WT rMIP or C20A rMIP were separated on 12% Tris-glycine SDS polyacrylamide gels under
reducing conditions, electrophoretically transferred onto a nitrocellulose membrane, and
probed with rabbit polyclonal anti-MIP IgG diluted 1/5000 and HRP goat anti-rabbit IgG
(Santa Cruz Biotechnology, Inc., LabForce AG, Nunningen, Switzerland) diluted
1/10000. The membrane was developed with the enhanced chemiluminescence (ECL)
immunoblot detection system before visualization with Hyperfilm (Amersham
Biosciences, Buckinghamshire, UK).

**In vivo radiolabeling of WT rMIP and C20A rMIP variant with phosphatidic acid, L-
α-dipalmitoyl, [U-\(^{14}\)C]-glycerol, and [U-\(^{14}\)C]-palmitic acid.** Experimental conditions
were described by Lundemose et al. (42). Briefly, cultures in Luria-Bertani broth (LB)
medium of recombinant *E. coli* M15[pREP4] expressing WT rMIP and C20A rMIP variant
were induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG)(Eurogentec,
Geneva, Switzerland). One µCi of phosphatidic acid, L-α-dipalmitoyl, \([\text{U-}^{14}\text{C}]\)-glycerol
(specific activity, 140 mCi/mmol; Perkin-Elmer, Schwerzenbach, Switzerland), or 1 µCi
of \([\text{U-}^{14}\text{C}]\)-palmitic acid (specific activity, 780 mCi/mmol; Amersham Biosciences),
dissolved in toluene, dried with N\(_2\) gas and suspended in phosphate-buffered saline
(PBS) with 1% Triton X-100 were added to 100 µl of culture, and cultivation continued
for 1 h. To some samples, globomycin (generous gift of Dr. Shunichi Miyakoshi, Sankyo,
Tokyo, Japan) dissolved in 95% ethanol at 20 mg ml\(^{-1}\) (final concentration, 100 µg ml\(^{-1}\))
was added 10 min before induction. The control cultures received an equivalent volume
of 95% ethanol. Cells were centrifuged, resuspended in SDS sample buffer, heated for 5
min at 95°C, subjected to 12% SDS-PAGE with
low range rainbow molecular weight (MW) markers \([^{14}\text{C}])\)-labeled (2 500-45 000 Da)
(Amersham Biosciences). The same amounts of induced WT rMIP and C20A rMIP
variant upper bands were loaded on the gel (in preliminary experiments, the
 corresponding volumes of cells were determined after band quantification using a
densitograph and OptiQuant software). Gels were transferred onto nitrocellulose membranes and visualized by autoradiography before being probed with anti-penta his mAb (QIAGEN AG, Basel, Switzerland).

**N-terminal amino acid sequencing.** Purified WT rMIP was separated on 18% SDS-PAGE gels (Novex, Invitrogen, Basel, Switzerland) under reducing conditions and transferred to polyvinylidene difluoride membrane (Immobilon-PSQ, Millipore, Volketswil, Switzerland) using semi-dry transfer cell (Novex, Invitrogen) in 10 mM 3-(cyclohexylamino)-1-propane sulphonic acid (CAPS) buffer, pH 11 containing 10% (v/v) methanol. Blot was stained with Ponceau Red S, dried, and bands of interest were excised. Samples were then subjected to four cycles of Edman degradation (Atheris Laboratories, Geneva, Switzerland).

**Purification of native MIP by immunoprecipitation.** A total of $2 \times 10^{10}$ EB were harvested by centrifugation, resuspended in 1 ml of a solution containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, protease inhibitor cocktail (Complete® EDTA-free, Roche Diagnostics AG, Rotkreuz, Switzerland), and disrupted by sonication before addition of an equal volume of buffer containing 1% SDS, 2% Triton X-100, 20 mM β-mercaptoethanol. After 1 h extraction at 4°C, insoluble material was removed by centrifugation at 20,000 x $g$ for 5 min at 4°C. Seize X Protein A immunoprecipitation kit (Pierce, Perbio Science, Lausanne, Switzerland) was then used according to the manufacturer's instructions to purify native MIP. Briefly, purified IgG antibodies raised in rabbit against WT rMIP were used to probe native MIP from the EB lysate. Antibodies were chemically cross-linked to a Protein A gel with the bifunctional reagent disuccinimidyl suberate. The EB lysate was then incubated with the immobilized antibody to form the immune complex. After washing, native MIP was eluted with 0.1 M glycine (pH 2.5) and immediately neutralized with Tris-HCl.
Identification of fatty acids associated with native and WT rMIP after acid- and base-hydrolysis by gas chromatography-mass spectrometry analysis. To evaluate the proportion of ester-linked and amide-linked fatty acids, two types of hydrolysis were performed. Fatty acids bonded through ester or amide linkages were liberated by strong acid hydrolysis whereas only esterified fatty acids were released by a base treatment. Strong acid hydrolysis was performed in 6 M HCl at 100°C overnight under nitrogen and was followed by a fatty acid saponification in KOH. Alkaline hydrolysis was performed in 5% KOH in aqueous methanol at 100°C overnight under nitrogen. After acidification, fatty acids were subsequently recovered by repeated extraction with methylene chloride and purified using anion exchange solid-phase extraction cartridges (Separtis AG, Grellingen, Switzerland), highly selective for acidic compounds. Then, fatty acids were methyl-esterified with 14%, v/v, boron trifluoride in methanol (Fluka AG, Buchs, Switzerland) and recovered by repeated extraction with methylene chloride. At last, hydroxyl groups were further derivatized to trimethylsilyl ethers by the addition of N,O-bis(trimethylsilyl)trifluoroacetamide, to identify possible hydroxylated fatty acids. All the analytes were then analyzed with a DB-5 fused silica capillary column (30 m long by 0.25 mm inner diameter by 0.25 µm film thickness) (Agilent J & W Scientific, Basel, Switzerland). Analytical gas–liquid chromatography was carried out on a Hewlett-Packard 5890 gas–liquid chromatograph interfaced with a quadrupole mass spectrometer (Trio-2 VG, Masslab, Manchester, UK). For sample analysis, the column temperature was held at 80°C for 1 min before it was raised to 300°C at a rate of 3°C min⁻¹. Electron impact mass spectra were obtained at 70 eV and individual fatty acid methyl esters were identified by their fragmentation pattern and ion masses. Fatty acid methyl esters are given as a percentage of the total peak area recovered on the chromatogram.
Mild alkaline hydrolysis of [U-\(^{14}\)C]-palmitic acid-labeled WT rMIP. E. coli cells expressing WT rMIP were grown in LB medium in the presence of 0.4 mM IPTG and 100 µg ml\(^{-1}\) globomycin, and labeled with [U-\(^{14}\)C]-palmitic acid. Bacteria were pelleted, washed, treated at 37 °C for 2 h with 0.1 N NaOH in 90% methanol and neutralized. Proteins were next separated by 12% SDS-PAGE and silver stained with Silver Stain Plus Kit (Bio-Rad, Reinach, Switzerland). Gels were dried and submitted to autoradiography. Digital images were processed using a densitograph and OptiQuant software.

Preparation of Chlamydial outer membrane complex (COMC). Preparation of COMC was performed as described (49). Briefly, C. trachomatis L2 EB were suspended (5 mg ml\(^{-1}\) of EB protein) in PBS, pH 7.4, containing 2% (w/v) Sarkosyl (Sigma, Fluka AG) and 1 mM EDTA. This suspension was incubated at 37°C for 1 h with mixing every 5 min, and then centrifuged at 100,000 x g for 1 h. The insoluble pellet was resuspended in the same buffer with 10 mM dithiothreitol (DTT), incubated for 1 h at 37°C, with mixing every 5 min, and centrifuged as described above. The pellet was washed twice in PBS to remove excess detergent. This Sarkosyl insoluble material consisted of COMC (11). Proteins from Sarkosyl-soluble and –insoluble fractions were subjected to 12% SDS-PAGE analysis, and transferred onto a nitrocellulose membrane. MIP was detected with polyclonal anti-MIP IgG diluted 1/5000, major outer membrane protein (MOMP) with anti-MOMP mAb (clone 165) (Biodesign International) diluted 1/5000 and soluble S1 with anti-S1 ribosomal protein mAb 124.4 (39) diluted 1/1000. Membranes were next probed with HRP goat anti-rabbit or anti-mouse IgG (Santa Cruz Biotechnology) diluted 1/10000, developed with the ECL immunoblot detection system before visualization with Hyperfilm (Amersham Biosciences). Digital images were processed using a densitograph and OptiQuant software.
Elementary body surface biotinylation. For total protein biotinylation, $10^9$ EB were solubilized in lysis buffer containing 1% Triton X-100 in PBS and EDTA-free protease inhibitor cocktail (Complete®, Roche). Solubilization was carried out by rotation overnight at 4°C. For surface biotinylation, $10^9$ EB were washed three times with ice-cold PBS (pH 8.0), and resuspended in PBS. The biotinylation reaction was performed with 2 mM sulfosuccinimidyl-6-(biotinamido)hexanoate (Sulfo-NHS-LC-Biotin) (Pierce) for 1 min on ice. In EB lysate, the residual Sulfo-NHS-LC-Biotin was quenched with 10 mM Tris and inactivated biotin was eliminated by buffer exchange using desalting column. In surface biotinylated EB, quenching was performed by three washings with 10 mM Tris and inactivated biotin was removed by two washings with PBS (pH 8.0). Surface biotinylated EB were subsequently lysed with 1% Triton X-100. Biotin-labeled proteins were separated from unlabeled proteins by affinity purification using streptavidin-agarose beads (Amersham Biosciences). The beads were first pre-incubated in 10 mM Tris and 3% bovine serum albumin in order to avoid non specific protein binding. After capture of biotinylated proteins, elution by boiling in Laemmli sample buffer, SDS-PAGE analysis and membrane transfer, MIP was probed with rabbit polyclonal anti-MIP IgG and HRP goat anti-rabbit IgG (Santa Cruz Biotechnology) and hsp60 with mAb to hsp60 kindly provided in ascitic fluid by Ian Maclean (University of Manitoba, Canada) and HRP goat anti-mouse IgG (Santa Cruz Biotechnology). Labeled proteins were detected with the ECL immunoblot detection system before visualization with Hyperfilm (Amersham Biosciences).

Surface immunoprecipitation of native MIP. To investigate possible surface exposition of C. trachomatis MIP, a surface immunoprecipitation of MIP was performed according to Shang et al. (58). Briefly, $0.5 \times 10^{10}$ intact EB were mixed with rabbit polyclonal anti-MIP IgG or biotinylated mAb 124.4 to soluble S1 ribosomal protein (39).
and gently shaken for 2 h at 30°C. Agglutinated EB were pelleted at 2,000 x g for 15 min, resuspended in 5 mM MgCl₂ in PBS, pelleted again at 2,000 x g for 15 min, and then resuspended in 450 µl of solution containing 10 mM Tris HCl (pH 8.0), 1 mM EDTA and a protease inhibitor cocktail (Complete® EDTA-free, Roche). To this suspension 50 µl of 10% protein grade Triton X-100 (Calbiochem, Juro Supply, GmbH, Lucerne, Switzerland) was added, and the mixture was agitated overnight at 4°C. The insoluble material was removed by centrifugation at 16,000 x g for 20 min. Sodium deoxycholate (0.2% final concentration), SDS (0.1 %) and 20 µl of a slurry of Protein G Sepharose or streptavidin-agarose beads (Amersham Biosciences) were added to the supernatant. These mixtures were gently agitated for 1 h at 4°C. The Protein G Sepharose or streptavidin-agarose–antibody–antigen complexes were washed three times in 10 mM Tris HCl (pH 7.5), 500 mM NaCl, 1% Triton X-100, 0.2% deoxycholate, 1 mM EDTA, once in 10 mM Tris-HCl, and finally resuspended in sample buffer. Samples were then processed by SDS-PAGE and immunoblotting with rabbit polyclonal anti-MIP IgG or anti-S1 ribosomal protein mAb 124.4 and HRP IgG conjugates (Amersham Biosciences). Labeled proteins were visualized by chemiluminescence. Immunoprecipitation control experiments were performed in parallel under the same conditions as those described above except that either no antibody or pre-immune serum were mixed with intact EB. As an additional control S1 ribosomal protein was also immunoprecipitated from EB lysates.
RESULTS

**MIP is conserved among five different species of Chlamydiae.** To determine the degree of homology of MIP within five different species of Chlamydiae, alignment of amino acid sequences was performed. The five MIP-like protein sequences, deduced from the complete genomic sequences of five different strains: *Chlamydia trachomatis* (strain D/UW-3/Cx) (63), *Chlamydophila pneumoniae* (strains AR39, CWL029, J138 and TW-183) (35, 52, 59), *Chlamydia muridarum* (strain MoPn/Nigg) (52), *Chlamydophila caviae* (strain GPIC) (53), and *Parachlamydia sp. subsp. Acanthamoeba sp.* (strain UWE25) (28) were available in Swiss-Prot/TrEMBL database. In 243-290 residue overlap, 78 were identical in all five MIP sequences, showing a high degree of sequence conservation (Fig. 1). Pairwise alignments of MIP sequences from pathogenic Chlamydiae (*C. trachomatis, muridarum, caviae, and pneumoniae*) resulted in 58 to 88% identity with 88% for the two *Chlamydia*, and 71% for the two *Chlamydophila* MIP sequences. Lowest scores (36 to 40%) were obtained when pairwise alignments were performed between MIP from pathogenic Chlamydiae and MIP from the environmental Chlamydiae: *Parachlamydia sp. subsp. Acanthamoeba sp.* (28)(data not shown). Even though lower levels of homology were observed between these strains, it shows that the mip gene was already present in the ancient core gene set of Chlamydiae because *Parachlamydia* is a *Chlamydia*-related symbiont of free-living amoebae, representing an evolutionary early-diverging sister group (about 700 million years ago) of present-day pathogenic Chlamydiae (28).

**Prediction of MIP sequences as putative lipoprotein precursors according to different bioinformatics analyses.** To examine whether NH$_2$-terminal regions of the selected MIP sequences comprised the typical prokaryotic lipoprotein precursor...
patterns, three different computer prediction programs were used. All these MIP proteins were predicted to be possible lipoproteins by one or several prediction methods, with clear identification of their lipobox sequences. The cysteine in position 20 was predicted to be the lipobox cysteine of *C. trachomatis* MIP (Table 1).

**Presence of protein homologues of those required for precursor export and lipoprotein biosynthesis in *E. coli*.** Based on previous analysis originally performed in *E. coli* and now commonly used a model (26), homologues of proteins required for precursor export and lipoprotein biosynthesis were found in the complete proteomes of five different species of Chlamydiae. As an example, *C. trachomatis* protein homologues are presented in Table 2. Even when proteins were identified as putative or probable, they appeared as highly conserved sequences within the five different species of Chlamydiae. As an example, the *C. trachomatis* protein homologues appeared to be 45 to 65% similar and 28 to 45% identical to *E. coli* proteins (data not shown).

**MIP has classical lipoprotein feature.** By analogy to other bacterial lipoproteins and according to established prediction methods, fatty acids were thought to be attached to a glycerol molecule linked by a thioether bond to cysteine$^{20}$. To determine if cysteine$^{20}$ is the lipobox cysteine of MIP, a C20A rMIP variant was prepared after site-directed mutagenesis where the cysteine$^{20}$ was changed to alanine (17). The two different recombinant proteins were prepared with a 6xHistidine (His(6)) tag in COOH-terminal position. To assess whether WT and C20A rMIP His(6) were comparable in MW with native MIP, an immunoblot analysis was performed using MIP-specific polyclonal antibodies. One band of about 26.6 kDa was detected in *C. trachomatis* EB whereas two bands of about 27.6 and 32 kDa were detected in WT rMIP His(6), and only one band of about 32 kDa in C20A rMIP His(6) (Fig. 2). The band of about 27.6 kDa was consistent in MW to the mature-like form, obtained after signal peptide release, as the MW was
similar to the 26.6 kDa of the only band observed in *C. trachomatis* EB. The 1 kDa difference observed corresponded to the weight of the His(6) tag present in COOH-terminal position of WT rMIP. The band of 32 kDa, absent in *C. trachomatis* EB, was consistent in MW to precursor-like forms of MIP, although this requires further confirmation.

To confirm the presence of the predicted lipid modification, in vivo labeling experiments of *E. coli* clones expressing WT and C20A rMIP were initially performed in the presence of phosphatidic acid, L-α-dipalmitoyl, [[U-\(^{14}\)C]-glycerol] which is known as a substrate for the enzyme diacylglycerol transferase (56). This precursor was used instead of [U-\(^{14}\)C]-glycerol to avoid unspecific labeling by glycerol catabolite products. When this esterified glycercyldonor was used, a main radioactive band of 32 kDa and a faint band of 27.6 kDa were detected in the total cell extract of the WT rMIP clone whereas no radioactive band was detected in the C20A rMIP clone (Fig. 3). These results indicated that a substantial amount of [U-\(^{14}\)C]-esterified glycerol was used in WT rMIP lipidation and allowed identification of the radioactive 32 kDa band as the diacylglycerolphosphoprotein, and the radioactive 27.6 kDa band as the diacylglycerol-modified lipoprotein obtained after peptide signal release.

To further assess the presence of fatty acids in WT rMIP and its dependency on the lipobox cysteine\(^{20}\), *E. coli* clones expressing WT and C20A rMIP were radioactively labeled in vivo using [U-\(^{14}\)C]-palmitic acid as an acyl donor. Only one radioactive band of 27.6 kDa was detected in the total cell extract of the WT rMIP clone whereas no radioactive band was detected in the C20A rMIP clone (Fig. 4). These results showed that the radioactive 27.6 kDa band represents an acyl-modified lipoprotein which may be either diacylated or triacylated according to analogies made with other bacterial lipoproteins.
To confirm that the precursor form of WT rMIP is processed by signal peptidase II, [U-\(^{14}\)C]-palmitic acid radiolabeling was performed in the presence of globomycin, a signal peptidase II inhibitor (31)). In these conditions, [U-\(^{14}\)C]-palmitic acid incorporation was observed in both the upper and lower bands (Fig. 4), as previously reported for \(C.\) \textit{trachomatis} MIP (42). The radiolabeled upper band corresponds to an accumulation of the diacylglycerol-modified prolipoprotein due to the inhibition of the peptide signal cleavage by globomycin. These results demonstrate that in \(E.\) \textit{coli}, WT rMIP is processed by signal peptidase II. As expected, globomycin had no detectable effect in immunoblot on the size of C20A rMIP, showing that replacement of cysteine\(^{20}\) eliminates processing by lipoprotein signal peptidase II. To confirm that the upper band was the precursor form with the signal peptide still present, micro-sequencing was performed on the 32 kDa band of WT rMIP that demonstrated an intact NH\(_2\)-terminal sequence corresponding to the uncleaved MIP precursor (data not shown). All these results confirm that \(E.\) \textit{coli} enzymes recognize the MIP lipoprotein motif and add a lipid tail to the recombinant protein.

To further analyze the lipid component of MIP, fatty acids were identified in purified native as well as WT rMIP by gas chromatography-mass spectrometry analysis. Native MIP was purified by immunoprecipitation and WT rMIP by nickel chelate affinity chromatography (2). The types and distributions of fatty acids present in the native and WT rMIP were very similar, comprising C12:0-C22:0 fatty acids with three major fatty acids (accounting for over 92% of total fatty acids) corresponding to nC18:0, nC16:0, and nC14:0. Branched i.e. iso- and anteiso-saturated fatty acids were found with odd carbon numbers (C15 and C17) in very small amounts (Table 3). The presence of characteristic fatty acids iC15:0 and aiC15:0 in \(C.\) \textit{trachomatis} has been previously described (4, 19). No unsaturated or hydroxylated fatty acids were identified. The same
fatty acids, in the same proportion, were predominant in acid and base hydrolysates of native and WT rMIP, indicating that the *E. coli* vector system is capable of providing a reliable source of rMIP that has very similar fatty acid composition to the native MIP.

To determine whether amide-linked fatty acids were present, the mass ratios of total fatty acids/ester-linked fatty acids were estimated. They were found to be equal to 1 for WT rMIP and to 1.12 for native MIP. These results are consistent with no amide-linked fatty acids being present in WT rMIP, and a small proportion present in native MIP. To further investigate this possibility, the presence of amide-linked fatty acids in WT rMIP was examined by mild alkaline methanolation, a treatment that releases ester-linked but not amide-linked fatty acids (47). Treatment was performed in WT rMIP labeled with [U-\(^{14}\)C]-palmitic acid in the presence of globomycin that blocked lipoprotein maturation in order to obtain both precursor and mature forms of MIP. The precursor form was used as a negative control for amide-linked fatty acid, because the presence of signal peptide prevented the addition of such amide-linked fatty acid. After SDS-PAGE separation, gel silver staining showed that no protein was lost during treatment whereas autoradiography demonstrated a notable decrease in radioactivity (Fig. 5). Analysis of results obtained after image digitalization revealed that alkaline methanolation released a significantly higher amount of [U-\(^{14}\)C]-palmitic acid radioactivity from the upper than from the lower band (92 ± 2% vs 82 ± 5%; mean of four experiments, p = 0.01). These results indicate that if there are some amide-linked fatty acids present, the NH\(_2\)-acylation is not completed.

**MIP is present in *C. trachomatis* outer membrane complex (COMC).** As *C. trachomatis* MIP was reported to be a membrane protein located in both EB and RB (38), the presence of MIP was investigated in the outer membrane of intact EB by treatment with the weak anionic detergent sodium lauryl sarcosinate (Sarkosyl). After
immunoblot analysis using polyclonal anti-MIP IgG, anti-MOMP and anti-S1 mAb, image
digitalization showed that 60% of native MIP was present in the Sarcosyl-soluble and
40% in the Sarcosyl-insoluble fraction. In contrast, 85% of MOMP was found in the
Sarcosyl-insoluble fraction whereas 100% of the soluble S1 ribosomal protein was found
in the Sarcosyl-soluble fraction (Fig. 6). Two bands were obtained for MOMP as already
reported in similar experiments (11, 37, 66, 67). These results suggest that native MIP
might have a dual localization, both in the inner (Sarcosyl-soluble) and outer (Sarcosyl-
insoluble fraction) membranes (20), as shown for other bacterial lipoproteins (5, 10, 23,
27, 58, 61, 64). To eliminate the possibility that native MIP could be part of a Sarcosyl-
insoluble supramolecular complex by disulfide cross-linking (18), treatment was carried
out in the presence of DTT as a reducing agent.

**MIP is exposed at the surface of C. trachomatis elementary bodies.** The presence
of native MIP in COMC did not indicate if MIP is in the inner or the outer leaflet of the
outer membrane. Its possible exposure at the surface was determined by two
independent approaches involving exposure of intact EB to biotinylation reagent and to
a MIP-specific polyclonal antibody. Elementary body surface proteins were biotinylated
during 1 min using Sulfo-NHS-LC-Biotin (Pierce), a water-soluble, membrane
impermeable reagent. An EB lysate was prepared and surface biotin-labeled proteins
were separated from unlabeled proteins by affinity purification using streptavidin-
agarose beads, and subjected to SDS-PAGE analysis and membrane transfer. MIP-
specific polyclonal antibody detected native MIP in this fraction whereas mAb anti-hsp60
did not reveal the cytosolic protein hsp60 (3) (Fig. 7A and B). This demonstrated that
under these experimental conditions, Sulfo-NHS-LC-Biotin did not label the intracellular
protein hsp60 in intact EB. As a control, the experiment was performed in an EB lysate
where hsp60 could be visualized as biotin-labeled protein (Fig. 7B). In addition, it should
be noted that the MIP band obtained from total EB lysate displayed a slightly upper MW than the band obtained from surface-biotinylated EB, indicating that probably more biotinylated sites (i.e. lysine residues) were accessible to biotinylation reagent in total EB lysate than in intact EB. These results support the idea that MIP is partly exposed at the surface and argues against the possibility that Sulfo-NHS-LC-Biotin gained access to intracellular proteins under our experimental conditions.

To further assess MIP surface exposure, a surface immunoprecipitation technique was used (58). Intact EBs were incubated either with polyclonal IgG specific for MIP or biotinylated mAb to soluble S1 ribosomal protein followed by gentle washes to remove unbound antibodies. The MIP antigen-antibody complex was isolated using protein G-Sepharose beads and S1 antigen-biotinylated mAb complex using streptavidin-agarose beads. After solubilization, complexes were analyzed by electrophoresis and immunoblotting. As shown in figure 8, a large amount of MIP was found in the surface-immunoprecipitated material showing that MIP is accessible to antibody on the surface of intact EB (Fig. 8A) whereas soluble S1 ribosomal protein was only detected in EB lysate (Fig. 8B). These results indicate that native MIP is surface exposed and complement those obtained after EB surface biotinylation.
DISCUSSION

The present work describes, for the first time, the lipid component of a Chlamydia lipoprotein and demonstrates that the structure is similar to E. coli murein lipoprotein, the best characterized bacterial lipoprotein (25). This conclusion is supported by several lines of evidence coming from bioinformatics analysis and radiolabeling experiments. In silico analysis of MIP amino acid sequence revealed the presence of a probable signal peptide according to DOLOP and LipoP prediction programs, but not according to the strictful predictive rule of the Prosite pattern PS00013 (30). The predicted lipobox was IVGC with cysteine\textsuperscript{20} being the lipobox cysteine. First experimental evidence came from Lundemose et al. who have previously reported the incorporation of radiolabeled palmitic acid into native MIP (41). Further experimental evidence has been provided in this study, and was mainly performed with an E. coli clone expressing WT rMIP, because of technical difficulties in working with Chlamydia. The first step of the MIP synthesis, i.e. the addition of a diacylglycerol moiety to lipobox cysteine\textsuperscript{20} was demonstrated by the incorporation of radiolabeled esterified glycerol and radiolabeled palmitic acid in WT rMIP whereas no labeling was obtained into C20A rMIP variant. However, a difference of WT rMIP labeling was observed between the two precursors: when phosphatidic acid, L-\(\alpha\)-dipalmitoyl, [[U-\textsuperscript{14}C]-glycerol] was used as esterified glycerol donor, both the mature and precursor forms of WT rMIP were radioactive whereas when [U-\textsuperscript{14}C]-palmitic acid was used as fatty acid precursor, only the mature form was radioactive. The accumulation of the precursor form, (i.e. of diacylglyceryl-modified prolipoprotein) observed when phosphatidic acid, L-\(\alpha\)-dipalmitoyl, [[U-\textsuperscript{14}C]-glycerol] was used as precursor implies that signal peptidase II was a rate-limiting step. One explanation could be the wrong configuration of the dipalmitoyl glycerol moiety transferred from phosphatidic acid to the prolipoprotein. Such a scenario is possible as
an asymmetric C atom is present at the 2 position of the esterified glycerol. The commercial preparation has the *R* configuration whereas the natural substrate has the *S* configuration (65). The next step involving the cleavage of the signal peptide was demonstrated by the inhibition of WT rMIP processing in presence of globomycin, a specific inhibitor of signal peptidase II. The subsequent step involving the aminoacylation of the cysteine residue by an amide-linked fatty acid was investigated by both gas chromatography-mass spectrometry analysis of native and WT rMIP and by mild alkaline methanolysis of WT rMIP. Both procedures demonstrated that aminoacylation was not completed. Gas chromatography-mass spectrometry analysis of WT rMIP found no amide-linked fatty acid whereas a little amount was found by alkaline methanolysis, a treatment that releases ester-linked but not amide-linked fatty acids (47). Indeed, about 10% of [U-14C]-palmitic acid radioactivity remained linked to WT rMIP after alkaline methanolysis whereas 33% should remain in case of total acylation with one amide-linked and two ester-linked acyl chains. Even in native MIP, the NH₂-terminal cysteine residue did not appear to be completely modified with amide-linked fatty acids because a mass ratio of total fatty acids/ester-linked fatty acids of 1.12 was obtained whereas it should be equal to 1.5 in the case of total acylation. The present ratio equal to 1.12 indicated that only 24% of cysteine²₀ in native MIP had amide-linked acyl chains. Taken together, this would imply that, even if native MIP appears to have a slightly higher proportion of amide-linked acyl chains, both forms of MIP are similar and are products of incomplete post-translational modifications as was previously reported for other bacterial lipoproteins (7, 8, 14, 15).

Overall, these results confirm that *E. coli* processes WT rMIP using an identical pathway to its own lipoproteins with the exception that posttranslational modifications can become rate-limiting steps as *E. coli* expressed WT rMIP both as a precursor and
mature forms, as previously reported (7, 13, 15, 36, 70). In C. trachomatis, the lipoprotein biosynthetic pathway is not known. The fact that protein homologues of all essential components of the Sec general secretion pathway in E. Coli were found in Chlamydiae, supports the notion that pro-lipoprotein translocation across the inner membrane involves Sec translocase (16). As homologues of enzymes required for lipoprotein biosynthesis in E. Coli were also found in Chlamydiae, subsequent lipid modification most likely involves the same pathway to that identified in E. coli.

The final point of investigation in the present study was the subcellular localization of native MIP. This was found to be present both in inner and outer membrane of EB and was shown to be present at the surface of EB by biotinylation and immunoprecipitation experiments. These results are consistent with previous reports demonstrating that at least a portion of MIP is exposed on the chlamydial surface (38, 41, 45, 55), and suggest that the rules and mechanisms regulating MIP sorting and transport to EB outer surface have yet to be fully characterized. The « +2 rule » for lipoprotein sorting in the E. coli cell envelope (72) cannot be applied to MIP. Indeed, the second amino acid of mature MIP, immediately after cysteine, is aspartic acid, residue considered to be specific for inner membrane retention (71). However, other lipoproteins have also been reported to have different sorting signals from the ones dictating inner or outer membrane localization in E. coli lipoproteins (50, 54, 57, 73).

One important question relating specifically to MIP cellular location, is the exact function in vivo of this protein in C. trachomatis MIP. If a subset of MIP remains attached to the inner membrane, it could act as a PPIase and be involved in the folding of periplasmic or outer membrane proteins after their translocation across the cytoplasmic membrane. If MIP is located at the surface of EB, it might play a key role in pathogenesis and host immunity as was shown for other lipoproteins implicated in several important bacterial
infections, including *Mycobacterium tuberculosis* (9), *Treponema pallidum* (68), *Listeria monocytogenes* (21), *Hemophilus influenzae* (60), *Campylobacter jejuni* (32), and *Borrelia burgdorferi* (51). As a surface PPIase, MIP might also be involved in initiating chlamydial productive infection (40). However, the exact contribution of the PPIase activity to the functional properties of MIP is still unclear since no bacterial or host substrates for MIP have been identified so far.

In conclusion, the present work shows that MIP is a classical lipoprotein, partly exposed at the surface of EB. However, similarly to other lipoproteins (73), the sequence of molecular events leading from inner membrane expression to exposition at the surface remains unknown as well as the nature of MIP function. These are important issues that deserve further efforts of investigation.
ACKNOWLEDGMENTS

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REFERENCES


lipoproteins and synthetic lipopeptides activate monocytes/macrophages. J Immunol 734


Figure legends

**FIG. 1.** Sequence alignment of *Chlamydia trachomatis* (accession number: P26623), *Chlamydia muridarum* (Q9PJK1), *Chlamydophila pneumoniae* (Q9ZP3), *Chlamydophila caviae* (Q824R2), and *Parachlamydia sp. subsp. Acanthamoeba sp.* (Q6ME92) MIP as performed by the CLUSTAL multiple alignment program. Identities are indicated as asterisks and similarities as a period (: means conserved substitutions and . semi-conserved substitutions) in the lines below the sequences.

**FIG. 2.** Immunodetection of MIP in *C. trachomatis* EB, and in cultures of *E. coli* M15[pREP4] overexpressing WT rMIP His(6) and C20A rMIP His(6) variant. Proteins were separated by 12% Tris-glycine SDS-PAGE, blotted on a membrane and detected with rabbit polyclonal anti-MIP IgG and HRP goat anti-rabbit IgG. Molecular-mass markers (kDa) are indicated on the left. In cultures of rMIP, two forms of the protein were detected. The black arrow indicates the position of a protein of about 32 kDa, consistent in MW to the precursor-like forms and present in both WT rMIP His(6) and C20A rMIP His(6) variant. The white arrow indicates the position of a protein of about 27.6 kDa, was consistent in MW to the mature like-form and present only in WT rMIP His(6). In *C. trachomatis* EB, MW of mature form is about 1 kDa lower than in WT rMIP due to the absence of the 6xHis tag in COOH-terminal position.

**FIG. 3.** Immunodetection and diacylglycerol radiolabeling of rMIP in cultures of *E. coli* M15[pREP4] overexpressing WT rMIP His(6) and C20A rMIP His(6) variant. Cells were grown in LB medium. Expression of WT rMIP and C20A rMIP were induced by the addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and further labeled with phosphatidic acid, L-α-dipalmitoyl, [[U-^{14}C]-glycerol]. Proteins were separated by SDS-
PAGE, 6xHis tag proteins were detected with anti-penta his mAb and labeled proteins were visualized by autoradiography. The black arrow indicates the position of a protein of about 32 kDa, corresponding to the precursor forms and present in both WT rMIP His(6) and C20A rMIP His(6) variant. The white arrow indicates the position of a protein of about 27.6 kDa, corresponding to the mature form and present only in WT rMIP His(6).

**FIG. 4.** Immunodetection and palmitic acid radiolabeling of rMIP in cultures of *E. coli* M15[pREP4] expressing WT rMIP His(6) and C20A rMIP His(6) variant. Cells were grown in LB medium, either in the presence (+) or absence (-) of 0.4 mM IPTG, vehicle (ethanol), or 100 µg ml\(^{-1}\) globomycin. Proteins were subjected to SDS-PAGE, 6xHis tag proteins were detected with anti-penta his mAb and labeled proteins were visualized by autoradiography. Black arrows indicate the pro-lipoprotein forms and white arrows the mature form of WT rMIP.

**FIG. 5.** Mild alkaline hydrolysis of [U-\(^{14}\)C]-palmitic acid labeled WT rMIP. *E. coli* M15[pREP4] cells expressing WT rMIP were grown in LB medium in the presence of 0.4 mM IPTG, and 100 µg ml\(^{-1}\) globomycin and labeled with [U-\(^{14}\)C]-palmitic acid. Bacteria were pelleted, washed, treated by either 0.1 N NaOH in methanol for 2 h, and neutralized. Proteins were separated by 12% SDS-PAGE and silver stained and the gel dried and submitted to autoradiography. Results obtained from one representative experiment are shown.

**FIG. 6.** Native MIP subcellular localization. Chlamydial outer membrane complex (COMC) was prepared after EB treatment in the weak anionic detergent sodium lauryl
sarcosinate (Sarkosyl). Proteins from Sarkosyl-soluble and -insoluble fractions (COMC) of intact EBs were separated on 12% SDS–PAGE and transferred onto a nitrocellulose membrane. An equivalent amount of starting EBs was present in both Sarkosyl-soluble and -insoluble fractions. MIP was detected with rabbit polyclonal anti-MIP IgG, MOMP with anti-MOMP mAb (clone 165), and soluble S1 ribosomal protein with anti-S1 mAb 124.4 (39).

**FIG. 7.** Immunoblot analysis of streptavidin-bound fraction from non biotinylated, surface-biotinylated and lysate-biotinylated EB. Intact EB were either untreated or biotinylated with Sulfo-NHS-LC-Biotin (Pierce) for 1 min on ice and lyzed. Intact EB were also lyzed before been biotinylated. All biotinylated molecules were captured with streptavidin-agarose. Streptavidin-bound fraction was analyzed by SDS-PAGE and immunoblotting. MIP was probed with polyclonal IgG anti-MIP (A) and hsp60 with mAb anti-hsp60 (B).

**FIG. 8.** Surface immunoprecipitation of native MIP. Intact EB were incubated with either no antibody, pre-immune serum, polyclonal IgG anti-MIP or biotinylated mAb 124.4 to soluble S1 ribosomal protein (39) and lyzed. Immunoprecipitation of MIP was performed in the presence of Protein G Sepharose and the capture of S1 antigen-biotinylated antibody complex performed with streptavidin-agarose. Samples were then processed by SDS-PAGE and immunoblotting with rabbit polyclonal anti-MIP IgG or streptavidin-HRP conjugate. Labeled proteins were visualized by chemiluminescence (A and B). A lysate of EB was also prepared and mixed with biotinylated mAb 124.4 to soluble S1 ribosomal protein and then processed as described above (B).
### Tables

**Table 1.** Prediction of Chlamydiae Macrophage Infectivity Potentiators (fkbp-type peptidyl-prolyl cis-trans isomerases) as putative lipoproteins according to different bioinformatics tools

<table>
<thead>
<tr>
<th>Computer prediction programs</th>
<th></th>
</tr>
</thead>
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<tr>
<td>Chlamydiae species</td>
<td>Gene ordered locus names</td>
</tr>
<tr>
<td>-----------------------------------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td><em>Chlamydia trachomatis</em> (strain D/UW-3/Cx)</td>
<td>CT541</td>
</tr>
<tr>
<td><em>Chlamydia muridarum</em> (strain MoPn/Nigg)</td>
<td>TC0828</td>
</tr>
<tr>
<td><em>Chlamydophila caviae</em> (strain GPIC)</td>
<td>CCA00078</td>
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<tr>
<td><em>Chlamydophila pneumoniae</em> (strains AR39, CWL029, J138 &amp; TW-183)</td>
<td>CPn0661, CP0086, CpB0687</td>
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<tr>
<td><em>Parachlamydia sp.</em> subsp. Acanthamoeba sp. (strain UWE25)</td>
<td>pc0383</td>
</tr>
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</table>

**Table 2.** Gene name, accession number and definition in Swissprot/TrEMBL of *Chlamydia trachomatis* proteins homologues of those required for secretory machinery and lipoprotein biosynthesis in *Escherichia coli*. 

---

**Gene name** | **Gene ordered locus names** | **Primary accession number (Swissprot/TrEMBL)** | **Definition (Swissprot/TrEMBL)**

*Chlamydia trachomatis* (strain D/UW-3/Cx)
<table>
<thead>
<tr>
<th>Gene</th>
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<th>Description</th>
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<tr>
<td>fh</td>
<td>CT025</td>
<td>Signal recognition particle GTPase</td>
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<tr>
<td>secA</td>
<td>CT701</td>
<td>Preprotein translocase secA subunit</td>
</tr>
<tr>
<td>secD/secF</td>
<td>CT448</td>
<td>Protein export</td>
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<tr>
<td>secE</td>
<td>CT321</td>
<td>Preprotein translocase subunit</td>
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<tr>
<td>secY</td>
<td>CT510</td>
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<tr>
<td>Int</td>
<td>CT534</td>
<td>Apolipoprotein N-acyltransferase (EC 2.3.1.-)</td>
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<tr>
<td>lspA</td>
<td>CT408</td>
<td>Lipoprotein signal peptidase (EC 3.4.23.36) (Signal peptidase II)</td>
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<tr>
<td>lgt</td>
<td>CT252</td>
<td>Prolipoprotein diacylglyceryl transferase (EC 2.4.99.-)</td>
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Table 3. Types and distributions of fatty acids identified from purified native and WT rMIP

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<thead>
<tr>
<th>Fatty acids released</th>
<th>Native MIP</th>
<th>after base hydrolysis</th>
<th>after acid hydrolysis</th>
<th>WT rMIP</th>
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<th>after acid hydrolysis</th>
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<td>nC12:0</td>
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<td>1.84</td>
<td>0.97</td>
<td>1.27</td>
<td>1.27</td>
<td>0.92</td>
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<td>0.47</td>
<td>0.13</td>
<td>0.92</td>
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<td>6.91</td>
<td>6.91</td>
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<tr>
<td>iC15:0</td>
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<td>0.44</td>
<td>0.44</td>
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<td>0.37</td>
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<td>iC17:0</td>
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<td>0.11</td>
<td>0.2</td>
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<tr>
<td>nC20:0</td>
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<td>0.67</td>
<td>0.71</td>
<td>0.6</td>
<td>0.6</td>
<td>0.6</td>
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<tr>
<td>nC22:0</td>
<td>0.15</td>
<td>0.11</td>
<td>0.1</td>
<td>0.12</td>
<td>0.12</td>
<td>0.12</td>
</tr>
</tbody>
</table>

*a* expressed in % of total fatty acids released

**n:** linear (straight chain)

*i:* methyl branched-chain with iso-structure: methyl group at the penultimate carbon atom

*ai:* methyl branched-chain with anteiso-structure: methyl group on the third carbon from the end

number of carbon:number of double bounds
FIG. 2

Immunodetection with MIP-specific pAb
Radiolabeling with phosphatidic acid, L-\(\alpha\)-dipalmitoyl, [[U-\(^{14}\)C]-glycerol]

Immunodetection with anti-penta his mAb

FIG. 3
Radiolabeling with \( [U^{14}C] \)-palmitic acid

Immunodetection with anti-penta his mAb

WT rMIP | C20A rMIP
---|---
IPTG | - + + + | - + + +
vehicle (ethanol) | - - + - | - - + -
globomycin | - - - + | - - - +

FIG. 4
FIG. 5

Silver staining

Autoradiography

kDa  0  0.1 N NaOH

30

30
 FIG. 6

- **S1 ribosomal protein**
- **MOMP**
- **MIP**

**kDa**
- 45
- 30
- 20

**Sarkosyl-soluble fraction**
**Sarkosyl-insoluble fraction**
A
Intact elementary bodies incubated with:
- No antibody
- Pre-immune serum
- Polyclonal IgG anti-MIP
- Monoclonal anti-S1 ribosomal protein

B
Intact and lysed elementary bodies incubated with anti-S1 ribosomal protein antibodies: