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Proteomic analyses of *Ehrlichia ruminantium* highlight differential expression of MAP1-family proteins

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ABSTRACT

The *Rickettsiales Ehrlichia ruminantium* (ER) is the causative agent of heartwater, a fatal tick-borne disease of livestock in sub-Saharan Africa and in the Caribbean, posing strong economical constraints to livestock production. In an attempt to identify the most prominent proteins expressed by this bacterium, especially those encoded by the major antigenic protein 1 (*map1*) multigene family, a proteome map of ER cultivated in endothelial cells was constructed by using two dimensional gel electrophoresis combined with mass spectrometry. Among the sixty-four spots detected, we could identify only four proteins from the MAP1-family; the other proteins detected were mainly related to energy, amino acid and general metabolism (26%), to protein turnover, chaperones and survival (21%) and to information processes (14%) or classified as hypothetical proteins (23%).

Additional studies on MAP1-family protein using immunochemical labeling also revealed that these proteins are differentially expressed along the bacterium life cycle, presenting different structural organization. Interestingly, when infectious elementary bodies (EBs) are released from host cells, MAP1 appears to be organized in SDS and heat-resistant dimers and trimers stabilized by disulfide bridges.

Overall, the results presented herein not only reveal the first partial proteome map of ER but provide new insights on the expression ER MAP1-family proteins in host endothelial cells.

1. Introduction

The *Rickettsiales Ehrlichia ruminantium* (ER) is a small (0.2–2.5 μm), Gram-negative obligatory intracellular bacterium transmitted by *Amblyomma* ticks and the causative agent of heartwater, a fatal tick-borne disease of ruminants in sub-Saharan Africa and in the Caribbean (Allsopp, 2010). Within the mammalian host cells, ER presents a complex life cycle with two distinct developmental forms: an intracellular replicative reticulate body (RB) and an extracellular infectious elementary body (EB) responsible for infection (Jongejan et al., 1991). To successfully survive and multiply in these different environments,

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ER requires life cycle stage-specific adaptation, which may result in differential protein expression.

Advances in ER genome annotation offer now the opportunity to increase knowledge on ER biology (Collins et al., 2005; Frutos et al., 2006). Still, due to the lack of genetic tools for ER manipulation, little is currently known on whether these genes are actually expressed in living organisms. Until now, the *map1* (major antigenic protein 1) cluster is the most studied family of ER genes. The protein MAP1 is a well-studied immunodominant outer membrane protein expressed in the mammalian host and it is encoded by a member of a multigene family comprising 16 paralogs (Sulsona et al., 1999). MAP1-family proteins are considered as priority targets for candidates vaccines (Frutos et al., 2006) being potentially involved in the adaptation of ER to the mammalian host and vector tick (Postigo et al., 2008). However, few data are currently available on the expression and characterization of the MAP1-family proteins along this bacterium life cycle.

Herein, we used a proteomic approach to analyze the expression of MAP1-family proteins in ER. First, a two-dimensional (2D) electrophoresis analysis coupled to mass spectrometry (MALDI-TOF–TOF) was performed to identify the most abundantly expressed proteins by ER in bovine host endothelial cells. Afterwards, the expression of the MAP1-family proteins, detected first in the 2D analysis, was analyzed during the bacterium life cycle using Western blot and immunofluorescence microscopy methodologies using newly raised polyclonal antibodies and a monoclonal antibody against MAP1.

2. Materials and methods

2.1. Ethics statement

All animal experiments (polyclonal antibody production) were conducted according to internationally approved OIE standards, under authorizations set forth by the director of the veterinary services of Guadeloupe on behalf of the Prefect of Guadeloupe on August 2006 (authorization number: A-971-18-01). Certificates of authorization are available from the authors upon request.

2.2. ER cultivation and purification

ER Gardel strain (isolated at CIRAD, Guadeloupe, French West Indies) was routinely propagated in bovine aortic endothelial (BAE) cells as previously described (Marcelino et al., 2005). To obtain the maximum amount of "pure" populations, ER culture was synchronized (Marcelino et al., 2005) and representative samples of infectious EB harvested at 120 h post infection were partly purified with several centrifugation/washing steps at 20,000 × *g* (Marcelino et al., 2007). For protein expression analysis along the bacteria life cycle, ER samples were taken at several time-points post infection as described in Marcelino et al. (2005). The protein content of all samples was measured using the BCA kit 23235 (Pierce, USA), according to the manufacturer's instructions for 96-well protocol. Samples were stored in aliquots at −80 °C with an anti-protease cocktail (Roche, Germany) until further analysis.

2.3. 2D gel electrophoresis

Isoelectric focusing (IEF) followed by SDS–PAGE was used to separate proteins, as previously performed in our laboratories (Almeida et al., 2010). Briefly, EB protein extract (125 μg) was diluted in 200 μl of 3-[(3-cholamidopropyl)dimethylammonium]-1-propane sulfonate (CHAPS) (4%, w/v), urea (8 M), thiourea (2 M), IPG buffer pH 3–10 (0.8%, v/v) and dithioerythritol (DTE) (60 mM) and separated in the first dimension using a linear pH 3–10 IPG strip (11 cm, GE, Uppsala, Sweden). IEF was performed in the IPGphor system (Amersham Biosciences, Uppsala, Sweden) and the program was used according to the manufacturer's instruction: rehydration of the strips was carried out for 12 h at 30 V, followed by a step-and-hold running condition from 100 V until 150 Vh, 250 V until 250 Vh, 1000 Vh until 1500 Vh, 2500 V until 2500 Vh with a gradient step at 800 Vh for 30 min and a final step at 8000 V until 24,000 Vh is reached. After IEF, strips were stored at −20 °C until further use. Before SDS–PAGE, IEF strips were equilibrated in two steps using 10 ml of Tris–HCl (pH 8.8; 50 mM) with urea (6 M), glycerol (30%, v/v) and SDS (2%, w/v) for 15 min under slow agitation either with dithiothreitol (10 mg/ml, Step 1) or iodoacetamide (25 mg/ml, Step 2). SDS–PAGE was performed in 12.5% (w/v) acrylamide large slab gels (two strips per gel) in an Ettan six DALT system (GE, Uppsala, Sweden) according to the manufacturer's instructions. Gels were stained for 48 h using Colloidal Coomasie Blue and subsequently washed 3 times in double distilled water. Gels were stored at 4 °C in a 20% (w/v) ammonium sulfate solution until image acquiring and spot excision. In this experiment, one gel was run from four different EB culture batches. Digital images of the gels were acquired in an ImageMaster digital scanner (Amersham Biosciences, Uppsala, Sweden).

2.4. In gel digestion and MALDI-TOF–TOF MS

Spots present in at least 3 of the replicates and absent from the control gels (uninfected BAE cells) were processed as previously described (Almeida et al., 2010). Briefly, protein spots were excised from gel, distained, reduced with dithiothreitol, alkylated with iodoacetamide, and dried in a speedvac. Gel pieces were rehydrated with digestion buffer (50 mM NH_4HCO_3) containing trypsin (6.7 ng/μl) (Promega, Madison, WI, USA) and incubated overnight at 37 °C. The buffered peptides were acidified with formic acid, desalted and concentrated using homemade reversed phase microcolumns (POROS R2, Applied Biosystems, Foster City, CA, USA). The peptides were eluted onto a MALDI plate using a matrix solution that contained 5 mg/ml α-cyano-4-hydroxycinnamic acid dissolved in 70% (v/v) acetonitrile/0.1% (v/v) trifluoroacetic acid. The mixture was allowed to air-dry. Protein identification was done by MALDI-TOF–TOF analysis performed with an Applied Biosystem 4800 Proteomics Analyser (Applied Biosystems, Foster City, CA, USA) in both MS and MS/MS mode. Positively charged ions were analyzed in the reflectron mode over the *m*/*z* range of 800–3500 Da. Each MS spectrum was obtained in a result independent acquisition mode with a total of 800 laser shots per spectra and a fixed laser intensity of 3500 V, being externally

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calibrated using des-Arg-Bradykinin (904.468 Da), angiotensin 1 (1296.685 Da), Glu-Fibrinopeptide B (1570.677 Da), ACTH (1–17) (2093.087 Da), and ACTH (18–39) (2465.199) (Calibration Mix from Applied Biosystems). Ten s/n best precursors from each MS spectrum were selected for MS/MS analysis. MS/MS analyses were performed using CID (Collision Induced Dissociation) assisted with air, using a collision energy of 1 kV and a gas pressure of 1×10^6 Torr. Two thousand laser shots were collected for each MS/MS spectrum using a fixed laser intensity of 4500 V. Raw data were generated by the 4000 Series Explorer Software v3.0 RC1 (Applied Biosystems, Foster City, CA, USA) and all contaminant *m*/*z* peaks originating from human keratin, trypsin autodigestion, or matrix were included in the exclusion list used to generate the peptide mass list used in database search.

2.5. Data searching and data interpretation

The generated mass spectra were used to search the NCBI predicted protein database. Searches were conducted using two algorithms: Paragon from Protein Pilot software v 2.0 (Applied Biosystems, MDS Sciex) and Mowse from MASCOT-demon 2.1.0 Software (Matrix-Science). Protein identifications were accepted if protein score was above 2.0 ($p < 0.01$) for Paragon and a threshold of 95% ($p < 0.05$) for Mowse. For Protein Pilot, search parameters were set as follows: enzyme, trypsin; Cys alkylation, iodoacetamide; special factor, urea denaturation; species, none; and ID focus, biological modification. As for Mascot, the interpretation of the combined MS + MS/MS data was carried out using the GPS Explorer Software (Version 3.5, Applied Biosystems, Foster City, CA, USA), using the following parameters: missed-cleavage, one; peptide tolerance, 50 ppm; fragment mass tolerance, 0.25 Da; fixed modification, carbamidomethylation of cysteine; and variable modification, methionine oxidation. From the predicted protein database, the theoretical molecular mass and p*I* of the identified proteins was obtained using the Expasy Mw/pI Tool (http://www.expasy.org/tools/pi_tool.html). The identified proteins were only considered if a MASCOT protein score above 68 ($p < 0.05$) was obtained. For searches done with Protein Pilot proteins were only considered if having at least one peptide with 95% confidence. Protein identifications with only one peptide with 95% confidence were further validated with manual inspection of the assigned sequence. Quality criteria for manual confirmation of MS/MS spectra were the assignment of major peaks, occurrence of uninterrupted y- or b-ion series at least with 3 consecutive amino acids and the presence of a2/b2 ion pairs.

2.6. Antibodies

The primary antibodies used were: mouse monoclonal antibody (mAb) anti-MAP1 (4F10B4) kindly provided by Prof. F. Jongejan (Utrecht University, Netherlands) and rabbit polyclonal antibodies anti-MAP1-6, MAP1+1 and MAP1-14. The polyclonal antibodies were obtained as followed: ER genomic DNA used to amplify *map1+1*, *map1-14* and *map1-6* genes was isolated from EB using the QIAamp DNA mini kit (QUIAGEN GmbH, Hilden Germany) according to the manufacturer's instruction. Primer sequences, melting and annealing temperatures were used as previously described (Raliniaina et al., 2010). Amplicons were cloned into the pGEM-T vector as specified by the manufacturer (Promega, Madison, WI) and sequenced using the Big Dye kit and an ABI PRISM automated sequencer (PE-Applied Biosystems, Foster City, CA). MAP1+1, MAP1-6 and MAP1-14 were cloned in the pRSET expression vector and expressed as His-tagged fusion proteins according to the manufacturer's specifications (Invitrogen Carlsbad CA, USA) in *Escherichia coli* were grown at 37 °C at 180 rpm, induced with 1 mM IPTG at OD600 = 0.8 and harvested by centrifugation after 4 h induction. Recombinant proteins were purified from bacterial lysates with the ProBond purification system (Invitrogen). Expression, molecular mass (MM) and identity of the target proteins were confirmed by SDS–PAGE and mass spectrometry. The purified proteins were used separately to produce polyclonal antiserum by immunization of rabbits. Previous to the immunization, a blood sample was obtained from the ear marginal vein (negative control). Afterwards, each purified protein was emulsified in Montanide ISA 50 adjuvant (kindly provided by Seppic SA, France), a mineral oil-based adjuvant which has been considered as safe by the Committee for Veterinary Medical Products (CVMP) for use in immunological products, and then injected intramuscularly in rabbit to produce immune sera. Sixty days after the injection of each emulsified recombinant protein in different Creole rabbits, blood samples were collected by puncture of the ear marginal vein and centrifuged at 3000 rpm for 10 min at room temperature and polyclonal serum (supernatant) was collected. The pre-immune serum and immune antiserum were then adsorbed against *E. coli* prior to use in Western blot; only immune serum was able to recognize the recombinant proteins (data not shown). The secondary antibodies used were: goat anti-mouse-HRP and anti-rabbit-HRP for Western blot (both from Sigma–Aldrich) and anti-rabbit AlexaFluor 594, anti-rabbit FITC and anti-mouse AlexaFluor 488 for confocal microscopy. As negative controls for immunofluorescence labeling, ER-infected BAE cells were incubated with the secondary conjugated antibodies; there was no detectable labeling, indicating that labeling with anti-MAP antibodies was specific.

2.7. SDS–PAGE and Western blot

To follow the production kinetics and characterize the structure of the MAP1-family proteins, SDS–PAGE analysis was performed followed by Western blot. Protein separation of ER samples (10^7 ER per lane) and uninfected BAE (used as negative control, 5 μg) was performed using precast NuPage 4–12% Novex® Bis-Tris gels. Briefly, samples were solubilized in NuPAGE® LDS Sample Buffer, treated under reducing or non-reducing conditions (to detect folded monomers, dimers and trimers (Surrey et al., 1996)) and subjected to electrophoresis under denaturing conditions for 35 min in NuPage® MES Buffer (all from Invitrogen, UK). Each gel included pre-stained molecular mass markers (SeeBlue 2, Invitrogen, UK). After separation by SDS–PAGE, proteins were transferred to PVDF (Millipore, USA) membranes in a Semiphor Transpher blotter

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unit (Amersham Pharmacia Biotech, USA). The membranes were blocked for 1 h in PBS (pH 7.4) with 0.05% (v/v) Tween 20 (PBS-T) and 5% (w/v) skimmed milk (Merck, Germany) (PBS-TM), incubated overnight with the specific primary antibodies and then for 1 h with the appropriate phosphatase alkaline-conjugated secondary antibodies (Santa Cruz Biotechnology, Germany). Antibodies were diluted in PBS-TM and washings after each incubation step were done in PBS-T. Finally, the membranes were developed using BCIP/NBT substrate (Pierce, USA). Each experiment was performed 2 times using biological samples from two independent cultures, with identical results.

2.7.1. Thermal stability of MAP1 protein in EBs

Thermal stability was investigated by incubating purified EBs (5 μg) at different temperatures (37, 56, and 70 °C) from 10 to 60 min. EBs samples were afterwards analyzed under denaturing conditions in a pre-cast NuPage 4–12% Novex® Bis-Tris gels followed by immunoblotting using mAb anti-MAP1, as described above.

2.7.2. Trypsin digestion of MAP1 in EBs

To test MAP1 susceptibility to protease digestion, purified EBs (5 μg) were incubated at room temperature for 10 min or at 56 °C for 30 min and was subsequently treated with trypsin (2 μg/ml, Sigma, St. Louis, MO). The reaction mixtures were incubated in 100 mM Tris–HCl (pH 8.5) at 37 °C during 2–30 min. The products of the digestion were loaded in pre-cast NuPage 4–12% Novex® Bis-Tris gels and analyzed by Western blotting using mAb anti-MAP1, as described above.

2.8. Immunofluorescence microscopy

To analyze ER protein expression along the bacterial life cycle, we applied an immunofluorescence protocol optimized by our laboratories (Marcelino et al., 2007) and used mAb anti-MAP1 (4F10B4) and rabbit polyclonal antibodies MAP1-6 and MAP1-14 (produced in house, as described above). Slides were mounted in Vectashield H-1000 (Vector Lab, USA) and examined using a Leica SP5 confocal microscope. Merge between channels and maximum *z*-projection were created using the open source ImageJ software version 1.38 (http://www.rsb.info.nih.gov/ij/).

3. Results

3.1. Identification of E. ruminantium proteins by 2DE gels and MALDI-TOF–TOF

The gels for BAE cells and EB samples are presented in Fig. 1A and B, respectively. Proteins presented isoelectric

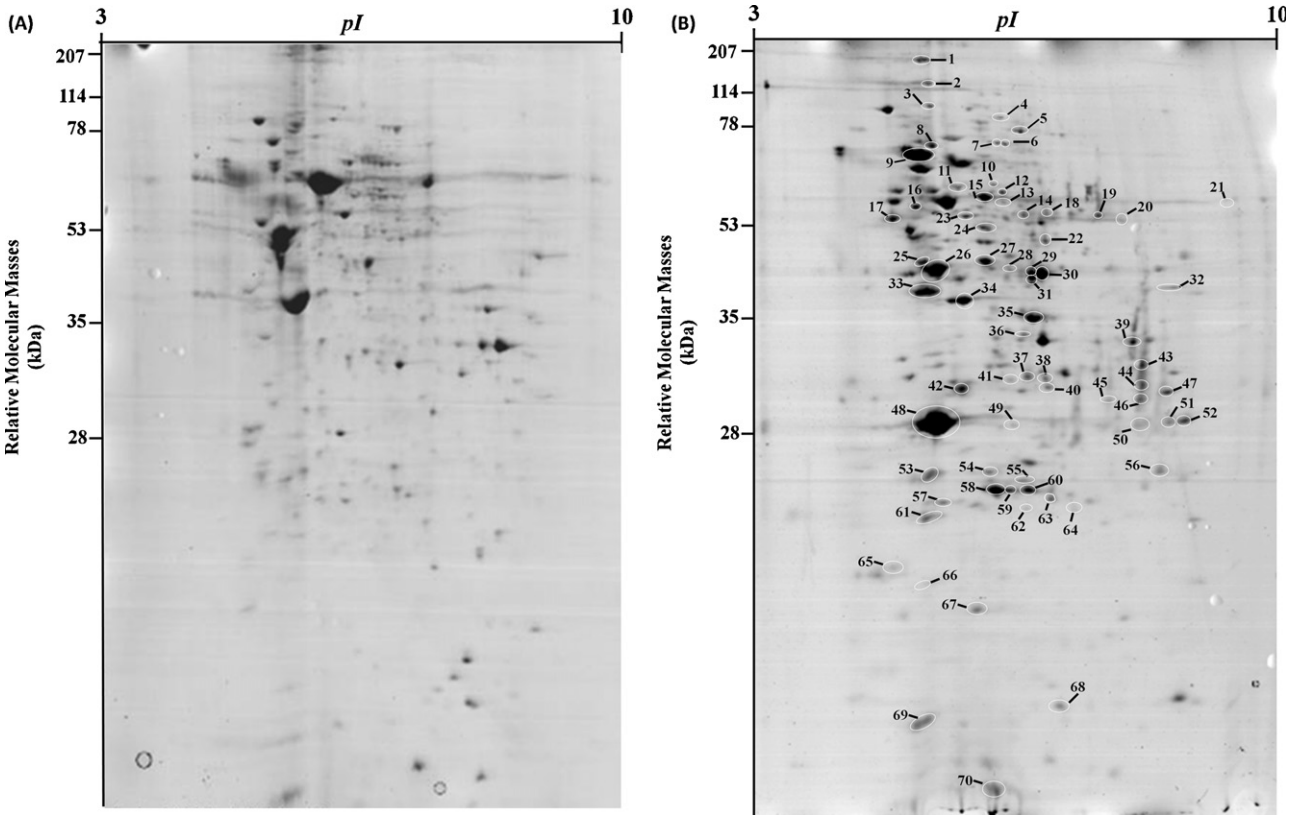

Fig. 1. Two-dimensional electrophoretic map of uninfected bovine aortic endothelial (BAE) cells (A) and purified infectious *E. ruminantium* Gardel elementary bodies (EBs) proteins expressed at 120 hpi (B). The crude extract was separated using a pH 3–10 IPG strip in the first dimension, followed by a 12.5% SDS–PAGE in the second dimension. The 2D gels were stained using Colloidal Coomasie Blue. A representative gel of four different BAE and purified EB batches is shown. Position of molecular masses markers (in kDa) is indicated on the left side.

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points (p*I*) ranging from 4.5 to 9.5 (most of them having an acidic p*I*) and apparent molecular mass (MM) ranging from 10 to 176 kDa. After image analysis between BAE and EB gels, only the spots differentially detected in EB were considered for mass spectrometry analysis. After spot digestion, the resulting mass spectra were searched against the NCBI general protein database and the identified proteins were then classified according to Collins et al. (Collins et al., 2005), the COG functional categories (http://www.ncbi.nlm.nih.gov/COG/old/index.html) and the online pathway tools on the Kyoto Encyclopedia of Genes and Genomes web site (KEGG) (www.genome.jp/kegg/) (Table S1) and compiled into a schematic drawing (Fig. 2).

Among the sixty-four spots detected exclusively in ER (Fig. 1B), we could identify only four members from the MAP1-family: MAP1-6 (spot 21), MAP1+1 (spots 43–46, and 62), MAP1-14 (spot 37) and MAP1 (spots 48 and 53), the most abundant spots in the 2D gel. Interestingly, MAP1-6 (with a theoretical molecular mass of 30.4 kDa) was detected at ~60 kDa, MAP1+1 (32 kDa) was detected as 5 isoforms, one of them at a lower molecular mass (~25 kDa); MAP1 and MAP1-14 presented apparent molecular mass corresponding to the theoretical value (Table S1). Eight other ER proteins were also found in multiple spots (from 2 to 5 spots *per* protein), such as the NADP-dependent malic enzyme (Tme, spots 6 and 7), the integrase/recombinase (XerC, spots 13 and 14), serine hydroxymethyltransferase (GlyA, spots 28–31), putative peptide deformylase (Def2, spots 50 and 60), 10 kDa chaperonin (GroES, spots 27 and 70), putative peroxiredoxin (Tsa, spots 58 and 59), protein of the Type IV Secretion System (T4SS) (VirB9, spots 51 and 52) and hypothetical outer membrane protein (spots 18–20) (Fig. 1B and Table S1).

The other identified proteins belonged to several COG functional categories (Table S1). Within the "energy, amino acid production and general metabolism" category, we detected enzyme complexes typical of aerobic respiration, including AtpA (spot 23), two enzymes involved in the tricarboxylic acid cycle (TCA) (SucA, spot 4 and E3, spot 22) and the SdhA protein (spot 10) that allows linkage between the TCA and the aerobic electron transport chain (Fig. 2). From the central intermediary metabolism, only NADP-dependent malic enzyme (Tme, spots 6 and 7) was detected. Five proteins involved in amino acid metabolism were identified: PutA (spot 3), DapE (spot 33), ArgJ (spot 61), GlyA (spots 28–31) and GlnA (spot 24). Two proteins involved in co-factors production were identified: FolD (spot 40) and FolP (spot 47). Ten proteins related to protein turnover processes were also identified: PepA (spot 17) and ClpP (spot 57) are involved in the processing and regular turnover of proteins; ClpB (spot 5), DnaK (spot 9) and DnaJ (spot 32) are crucial for cell physiology protection. DksA (spot 65) and SurE (spot 49) are involved in protein stabilization. TsaA (spots 58 and 59) and ElbB (spot 54) are involved in cell redox homeostasis. Only five proteins related to DNA metabolism were detected (XerC-spots 13/14, SsB-spot 66, RpoB-spot 2, RpoZ spot-68 and PyrE-Spot 63). Four proteins were found to be involved in translation and modification of nascent peptides: RpsA (spot 11), FusA (spot 8), Def2 (spots 50 and 60) and TrpS

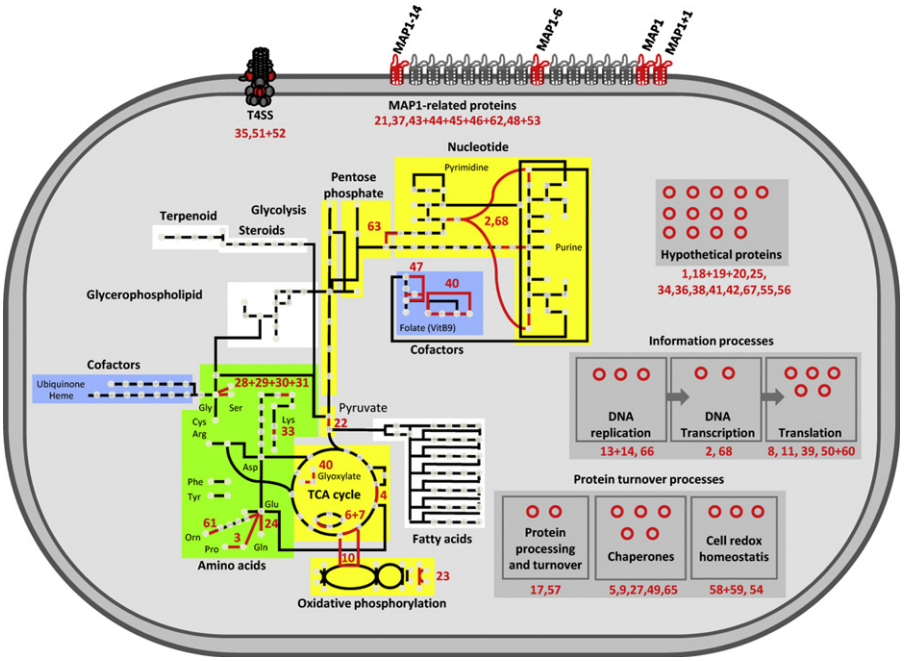

Fig. 2. Metabolic pathways of *E.ruminantium* Gardel elementary body (EBs) proteins, based on genomic and proteomic information. Each node represents a metabolite, each link represents a protein and each box represents a pathway. This overview map is color-coded to indicate proteins that were identified based on the proteome analysis of *Ehrlichia ruminantium* elementary bodies and on the data from KEGG database (http://www.genome.jp/kegg/pathway.html). The networks of some of the more important pathways are shown in colored boxes: yellow, central and intermediary metabolites; white, lipid metabolism; blue, cofactors; green, amino acids; gray, hypothetical proteins and proteins involved in information and protein turnover processes. Membrane proteins are shown in the membrane. Gray nodes and links are present in *E. ruminantium* genome whereas red nodes and links indicate proteins that have been identified in our proteomic study.

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(spot 39). We also identified two pathogenesis-associated proteins: VirB11 (spot 35) and VirB9 (spots 51 and 52), that are components of a Type IV Secretion System (T4SS), an important virulence factor used by Gram-negative bacteria to inject effectors into host cells.

To improve the functional annotations of hypothetical proteins, we performed a manual re-annotation of proteins with unknown functions. This additional analysis revealed proteins potentially important for bacterial infection and survival such as zinc protease (spot 25), a peptidase (spot 56), diguanylate cyclase (spot 41), histidine kinase (spot 42) and ankyrin A (spot 1), a periplasmic receptor associated to TRAP (Tripartite ATP-independent Periplasmic Transporter) family (spot 38), and the gene Q5HAN9 was identified as coding for an oxidoreductase (spot 55). Genes Q5FH07 (spots 18–20), Q5FGU8 (spot 34) and Q5HA54 (spot 67) present a high sequence homology with other outer membrane proteins from other *Anaplasmataceae* family members. No function was assigned to the gene Q5FFH4 (spot 36).

3.2. MAP1-family proteins are differentially expressed and present distinct structural organization along the ER life cycle

To provide additional information on protein structural organization along the ER developmental cycle in BAE cells, samples were first analyzed under non-reducing conditions. The Western blot analyses revealed, as shown in Fig. 3, that none of the studied OMPs were detected from 24 hpi up to 48 hpi which corresponds to the lag and early exponential phase of ER growth (Marcelino et al., 2005). MAP1, MAP1-6 and MAP1-14 were detected from 72 hpi onwards (mid-exponential phase in ER growth development) until extracellular ER elementary bodies release from the host cells at around 120 hpi (Marcelino et al., 2005). More specifically, from 72 to 120 hpi, MAP1 was detected as a diffuse band ranging from 25 to 31 kDa, corresponding to the monomeric form of the protein. From 120 hpi, two additional bands with higher apparent molecular mass (MM, ~50 kDa and 90 kDa) were also detected, probably the dimeric and trimeric forms of MAP1. Contrary to MAP1, the proteins MAP1-6 and MAP1-14 were detected only as monomers, with apparent MM of approximately 31 kDa and 28 kDa, respectively. For MAP1-6, an additional band at approximately 21 kDa was also detected, presumably due to the natively folded monomer previously shown for other OMPs to present an increased mobility due to its more compact structure (Kleinschmidt, 2006) as only the band at 31 kDa was detected in reducing conditions (data not shown).

In order to confirm the expression pattern of MAP1 related proteins along the ER life cycle, confocal immunofluorescence microscopy was performed (Fig. 4). The detection of MAP1 protein (Fig. 4A, green), bacterial and host-cell DNA (stained with DAPI, blue) and host-cell membrane (labeled with anti-caveolin-1, red), allowed us to devise the distribution map of MAP1, distinguishing between localization in extracellular EB and intracellular RB. In Fig. 4A, it is possible to observe that at 24 hpi, MAP1 is faintly detected; this could be to adherent but non-internalized ER on host cells. From 48 to 96 hpi, during the late-exponential phase of ER growth kinetics, there was an increased in MAP1 detection, concomitantly with amplified detection of bacterial DNA. At 120 hpi, when host cells are lysed and EBs released, there was still co-localization of bacterial DNA with MAP1 protein. Using immunofluorescence microscopy, MAP1-6 and MAP1-14 proteins presented a detection pattern similar to MAP1 (Fig. 4A), as exemplified in Fig. 4B for MAP-1-14.

3.3. MAP1 oligomers in EBs are linked by disulfide bonds

To provide more insights on the native conformation of MAP1, we ran SDS–PAGE gels of purified EBs under different conditions (Fig. 5A). When EBs were heated without DTT (Fig. 5A, lane 2), a migration pattern similar to control sample (lane 1) was detected although the band corresponding to MAP1 monomer was detected at 31 kDa. When the sample was incubated at room temperature with DTT (Fig. 5A, lane 3), a strong band is observed at between 25 and 31 kDa, with a weaker band at 50 kDa, no band at 90 kDa being detected, indicating that MAP1 dimer and

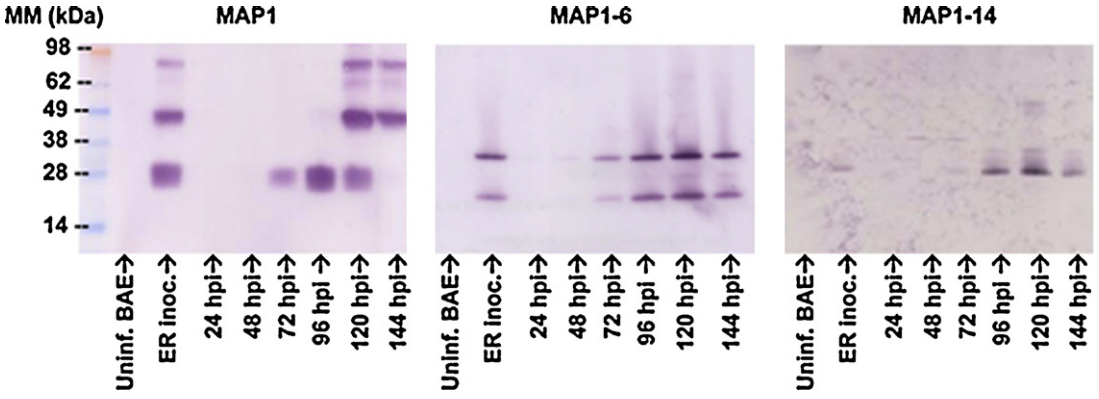

Fig. 3. Differential expression of MAP1, MAP1-6 and MAP1-14 proteins in *E. ruminantium* Gardel grown in host cells evaluated by Western blotting analysis. ER extracts prepared from infected monolayers harvested at different hours post infection (hpi) were processed in non-reducing conditions and proteins separated by SDS–PAGE. ER protein levels were normalized by the number of bacteria as determined by real time PCR (10^7 ER per lane). BAE host cells extracts were used as negative control and contained the maximum amount of total protein applied a gel, corresponding to 5 μg per lane. Experiments were performed 2 times with different ER extracts, with identical results. "MM" stands for molecular mass.

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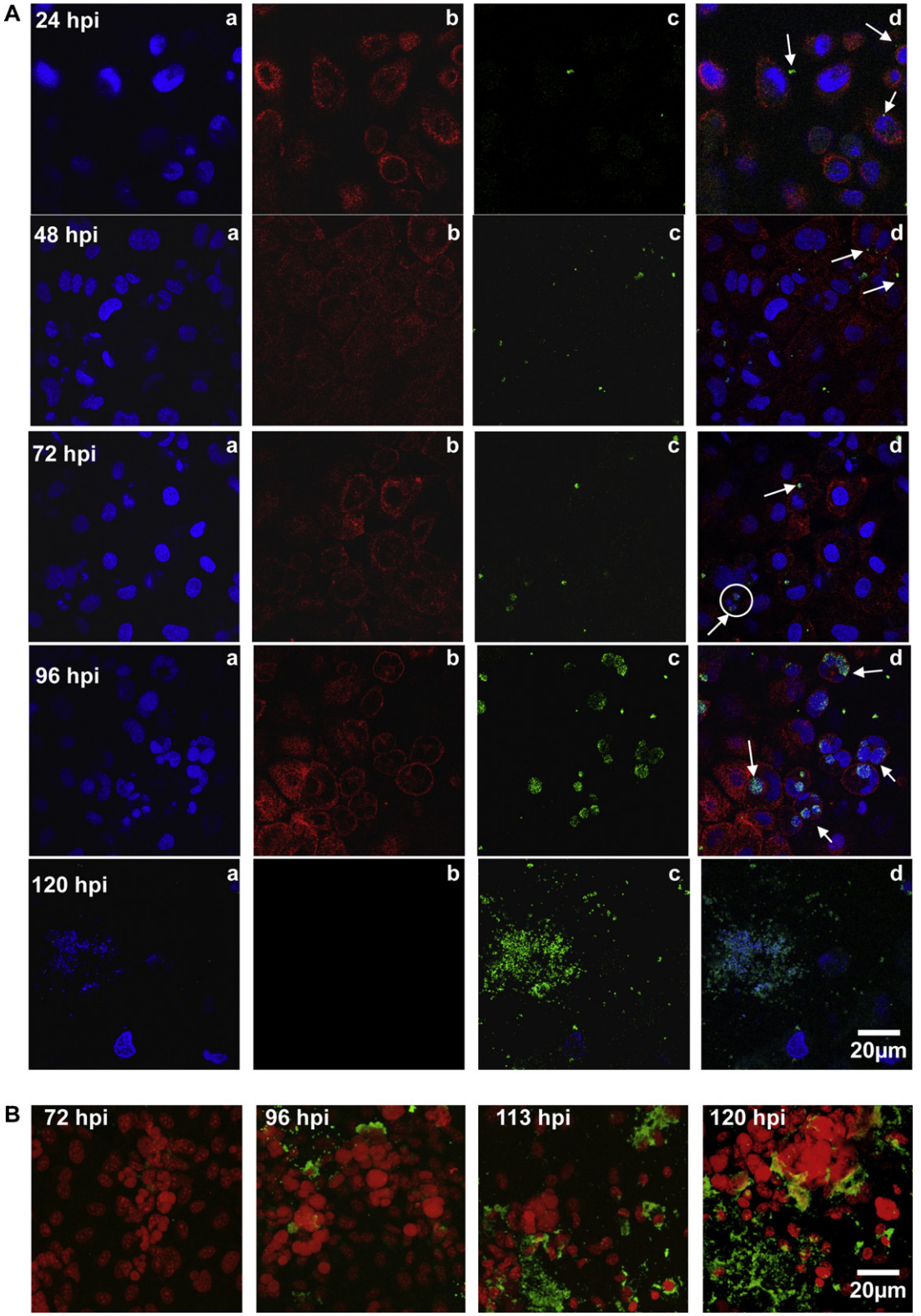

Fig. 4. Expression of *E. ruminantium* MAP1-family proteins evaluated by confocal immunofluorescence microscopy at different time post infections. (A) MAP1 detection: a – DNA staining with DAPI (blue); b – BAE cells labeled with a monoclonal anti-caveolin antibody (cav-1) (red); c – MAP-1 labeled with a monoclonal anti-MAP1B antibody (green); d – merge; (B) MAP1-14 detection: DNA stained with PI (red); MAP1-14 labeled with a polyclonal anti-MAP1-14 antibody (green).

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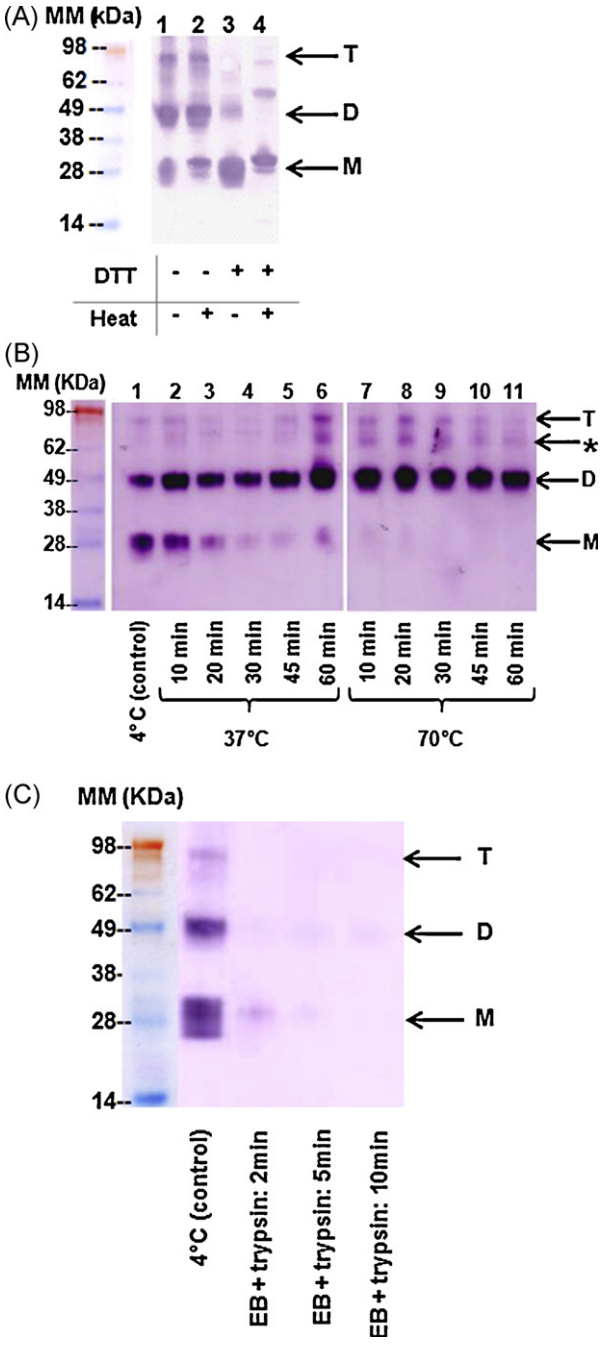

Fig. 5. *E. ruminantium* MAP1 stability in purified EBs after DTT incubation (A), heating (B) and trypsin digestion (C). (A) DTT incubation: purified EBs (5 μg) were incubated at room temperature or heated at 70 °C for 10 min in the presence or absence of 50 mM DTT as indicated at the bottom of each lane. (B) Heating: aliquots of 5 μg of purified EBs were heated at different temperatures (37, 56 and 70 °C) from 10 to 60 min. (C) Trypsin digestion: 5 μg of purified EBs was incubated with trypsin (2 μg/ml) with (lanes 2 and 3) or without (lanes 5–7) a pre-treatment at 56 °C during 30 min. Experiments were performed 2 times with different ER extracts, with identical results. "MM" stands for molecular mass while "M", "D" and "T" stand for monomer, dimer and trimer, respectively. "*" indicates the presence of an additional band at 62 kDa.

trimer are DTT sensitive. When the sample was incubated at room temperature with DTT (Fig. 5A, lane 3), a strong band is observed at between 25 and 31 kDa, with a weaker band at 50 kDa, no band at 90 kDa being detected, indicating that MAP1 dimer and trimer are DTT sensitive. Interestingly, when RB samples were submitted to DTT and heat, no changes in the detection profile was observed (data not shown). These results indicate that MAP1 dimers and trimers in ER elementary bodies are linked by disulfide bonds.

3.4. MAP1 oligomers in EBs are heat resistant but trypsin sensitive

When EB samples were incubated at least 30 min incubation at 37 °C (Fig. 5B) a substantial decrease in MAP1 monomer detection is observed, while no changes were observed for the dimers and trimers migration patterns. Interestingly, for all temperatures tested, an additional band at approximately 62 kDa is observed with the decrease on MAP1 monomer detection along time. From Fig. 5C lane 3, when EB samples were first incubated at 56 °C prior to trypsin treatment, no signal is observed for MAP1 detection. When EBs were not heated prior to trypsin digestion, faint bands at 30 kDa were still visible at 2 min post incubation, dimers being still visible up to 30 min incubation (data not shown). These results suggest that the decrease in detection of MAP1 monomeric form at later post-infection time (144 hpi) (Fig. 3) could be related to heat sensitive properties of MAP1 monomers, and not related to degradation phenomena associated to protease exposure.

4. Discussion

E. ruminantium was first identified more than 80 years ago, but few data on its biology is currently available. Herein, by using a proteomic approach, we identified some of the most prominent proteins expressed in infectious ER elementary bodies (Gardel strain) cultivated in infected bovine endothelial cells.

Among the forty-eight different EB proteins identified, only 4 proteins belonged to the MAP1-family (MAP1, MAP1-6, MAP-14, MAP1+1), MAP1 being the most abundant spots in the 2D gel which could explain its high immunodominance characteristic, as already observed *in vivo* (Jongejan and Thielemans, 1989) and *in vitro* (Postigo et al., 2008). The other proteins detected in the 2D gel are mainly related to energy, amino acid and general metabolism (26%), to post-translational modification, protein turnover, chaperones and survival (21%), lesser proteins being related to DNA replication, transcription and translation (14%) (Table S1). The remaining spots correspond to proteins involved in cell mobility, secretion and pathogenesis (4%) and to hypothetical proteins (23%). We could also identify two components of a T4SS, namely VirB9 (basal body) and VirB11 (energy source), providing the first evidence in ER of a macromolecular multicomponent system for translocation of virulence factors as previously observed in other bacterial pathogens such as *A. marginale* (Lopez et al., 2007) and *E.*

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chaffeensis (Lin et al., 2011). Among the several proteins detected, ER was found to share several proteins with other obligate intracellular bacteria (Chao et al. (2004), Hajem et al. (2009), Marques et al. (2008), Ramabu et al. (2010), Renesto et al. (2005), Samoilis et al. (2007), Seo et al. (2008) and Vandahl et al. (2001), Table S2) such as DnaK, PepA and Ssb (detected on 11 bacteria of 12 total analyzed), AtpA/FusA/ClpB/GroS (8/12), Lpd/ClpP (7/12) and SdhA/RpoB/TrpSRpsA/ClpB (6/10). Tme and TsaA were exclusively found in *R. parkeri* and ER and other were exclusively found in *Anaplasmataceae*: ArgJ was only found in *E. chaffeensis* (using the nano-LC–MS/MS method) and ER, XerC in *A. phagocytophilum* and ER, SurE in the *E. chaffeensis*, *A. phagocytophilum* and ER. ElbB was exclusively detected in ER, deserving thus additional functional studies to further characterize their role in ER biology and more globally in *Anaplasmataceae*. Overall, we only detected 5.1% of the 950 predicted CDS for ER (Gardel strain). While 1D-SDS–PAGE with nano-LC–MS/MS and multidimensional LC–MS/MS could allow a highest number of identified proteins (Lin et al., 2011), 2D-PAGE has advantages in the possible separation of protein isoforms and quantitative differential profiling (Reinders et al., 2006). Indeed, we observed that 25% of the analyzed spots are isoforms, including MAP1-family members such as MAP1+1 and MAP1, suggesting that post-translational modifications (PTMs) might be important in EB for the regulation of cellular processes such as host cell recognition, signaling, metabolism and in determining antigenicity as previously observed in other *Rickettsiales* (Ogawa et al., 2007; Pornwiroon et al., 2009).

As above mentioned, only 4 out of 16 *map1* paralogs were found to be expressed in EBs cultivated in host endothelial cells. Previous studies using ER Gardel CTVM (a subset of strain Gardel with mutations in the *map1* operon) showed that all *map1* paralogs are transcribed *in vitro* in endothelial cells (Bekker et al., 2005) while only 3 immunodominant proteins from the *map1* cluster was found to be expressed *in vitro* in endothelial cells (Postigo et al., 2008). Our results reinforce the idea that ER *map1* gene cluster can be differentially expressed according to the microenvironment (host-tick-extracellular milieu). In other *Ehrlichia* species such as *E. chaffeensis* and *E. canis*, the causative agents of human and canine monocytic ehrlichiosis, respectively, and the pathogens most closely related to ER, it was found that p28/omp1 (*E. chaffeensis*) and p30 (*E. canis*) gene clusters (orthologous to the MAP1 multigene family (van Heerden et al., 2004)) were also differentially expressed. For instance, it was demonstrated that the P28 and P30 proteins expressed *in vitro* in infected canine macrophage cell cultures included the orthologs of the *map1* (p28-Omp19 and p30-1) and *map1+1* (p28-Omp20 and p30-20) genes (Singu et al., 2005). More recently, for *E. chaffeensis* cultured in HL60, it was reported that all the 22 paralogous tandemly arranged p28/omp1 genes family proteins were expressed at the protein level (Lin et al., 2011), contrary to only 4 paralogs detected in this work for *E. ruminantium*. Although the reasons for this are not yet clear, this could be due to an increased sensibility of 1D-SDS–PAGE with nano-LC–MS/MS to detect proteins with lower expression levels compared to 2D electrophoresis map or to a factual differential protein expression profile between these two closely related bacteria.

When we further analyzed the expression of MAP1, MAP1-6 and MAP1-14 along the ER developmental cycle in host mammalian cells, we also observed that these proteins are differentially expressed. Contrary to MAP1-6 and MAP1-14, MAP1 presented a differential structural organization between 96 (intracellular RBs) to 120 hpi (when EB are released from host cells) from a monomer to a multimeric structure (dimers and trimers), suggesting a possible role for MAP1 multimeric structures in the infection mechanism, namely as a porin. Additional studies on MAP1 susceptibility to reducing conditions, heating and trypsin digestion reinforce this hypothesis. Indeed, MAP1 complexes were shown (i) to be DTT sensitive (Fig. 5A) suggesting that sulfhydryl groups are an important element in the conformation of this hypothetical porin as observed for other Gram-negative bacteria (Kleinschmidt, 2006), (ii) to exhibit a heat-modifiable gel migration behavior (Fig. 5B) similar to that of the *E. coli* OmpA protein (Wexler, 1997) and (iii) to be susceptive to the proteolytic cleavage, suggesting that the mAb used along this work may recognize a surface exposed trypsin-sensitive conformational epitope of the MOMP trimer involved in the binding between monomers to form dimers and trimers. Still, the ultimate role of MAP1 as a porin still needs to be confirmed by additional biochemical and biophysical studies.

Overall, the work presented herein reports for the first time the partial proteome of ER infectious elementary bodies and provides new information on ER MAP1-proteins family expression. Considering to the lack of genetic tools to manipulate ER, this proteome map may offer a useful knowledge to support further infection-studies on ER (for comparative scrutiny between ER strains, non-infectious replicative RB and infectious EBs forms, and the virulent versus the attenuated forms of the bacterium) to be further complemented by transcriptomic analyses using SCOTS methodology (Emboule et al., 2009; Pruneau et al., 2011).

Authors' contributions

IM, AMA, CB, PMA, MJTC, AVC, DM, NV and TL conceived and designed the experiments. IM, AMA and MB performed the experiments. Production of ER was done by IM and MB. Production of antibodies was done by CS, NV, TL and DM. Immunological studies were performed by IM, MB and CB. AMA, IM and CFF have done Proteome analyses. IM, AMA, CB, MB, CFF and DFM have analyzed the data. IM, AMA, CB, DFM and NV wrote the paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetmic.2011.11.022.

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