Differential proteome analysis of *Mycobacterium avium* subsp. *paratuberculosis* grown *in vitro* and isolated from cases of clinical Johne's disease

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Bovine Johne's disease (paratuberculosis), caused by *Mycobacterium avium* subspecies *paratuberculosis*, poses a significant economic problem to the beef and dairy industry worldwide. Despite its relevance, however, pathogenesis of Johne's disease is still only partially resolved. Since mycobacterial membrane proteins expressed during infection are likely to play an important role in pathogenesis, membrane-enriched fractions, namely mucosa-derived membranes (MDM) and culture-derived membranes (CDM), of *M. avium* subsp. *paratuberculosis* from three cows with clinical paratuberculosis were investigated. An initial analysis by 2D difference gel electrophoresis (2D DIGE) and MALDI-TOF-MS analysis revealed four differentially expressed proteins with only one predicted membrane protein. Due to this limited outcome, membrane preparations were subjected to a tube-gel trypsin digestion and investigated by using nanoflow-liquid-chromatography-coupled tandem MS. Based on this approach a total of 212 proteins were detected in MDM including 32 proteins of bovine origin; 275 proteins were detected in CDM; 59 % of MDM and CDM proteins were predicted to be membrane-associated. A total of 130 of the proteins were detected in both MDM and CDM and 48 predicted membrane proteins were detected in MDM from at least two cows. Four of these proteins were not detected in CDM, implying differential expression in the host. All membrane-associated proteins, especially the four identified as being differentially expressed, might be relevant targets for further analyses into the pathogenesis of bovine paratuberculosis.

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INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* is the causative agent of paratuberculosis (Johne's disease), a chronic and incurable granulomatous enteritis of ruminants (Kreeger, 1991). The disease occurs worldwide (Manning & Collins, 2001) and has considerable economic impact on

the dairy industry (Harris & Barletta, 2001). Involvement of *M. avium* subsp. *paratuberculosis* in some cases of Crohn's disease has been discussed (Mendoza *et al.*, 2009; Pierce, 2009). However, the molecular mechanisms of *M. avium* subsp. *paratuberculosis* pathogenicity are still incompletely understood. Research is hampered by a lack of *M. avium* subsp. *paratuberculosis*-specific antibodies (Santema *et al.*, 2009) and limited knowledge of metabolic pathways and surface-associated proteins functional in the host.

To investigate host-induced protein expression, 2D gel electrophoresis is one possible approach and has been applied to *M. avium* subsp. *paratuberculosis* (Egan *et al.*, 2008; Hughes *et al.*, 2007). Hughes *et al.* (2007) and Egan *et al.* (2008) succeeded in isolating *M. avium* subsp. *paratuberculosis* from gut mucosa of ovine and bovine hosts, respectively. Both groups subsequently analysed bacterial whole-cell lysates by comparative 2D gel electrophoresis and

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Abbreviations: 2D DIGE, 2D difference gel electrophoresis; CDM, culture-derived membranes; MDM, mucosa-derived membranes; MIRU-VNTR, mycobacterial interspersed repetitive unit-variable-number tandem-repeat; MLSSR, multilocus short sequence repeat; nUPLC-ESI Q-TOF-MS/MS, nanoflow-liquid-chromatography-coupled tandem MS.

A supplementary figure and four supplementary tables are available with the online version of this paper.

subsequent silver staining. The work resulted in the identification of a number of proteins possibly important for energy utilization of *M. avium* subsp. *paratuberculosis* in the host.

2D Gel electrophoresis has certain disadvantages as it is time consuming, unsuitable for low-molecular-mass proteins, requires a large amount of sample and often lacks reproducibility (Seibert *et al.*, 2004; Talapatra *et al.*, 2002). Furthermore, membrane proteins are difficult to characterize using a 2D gel electrophoresis approach due to their tendency to aggregate and precipitate during iso-electric focussing and due to their typically lower abundance in the cell compared with soluble proteins (Henningsen *et al.*, 2002; Santoni *et al.*, 2000). In contrast, protein mixtures of considerable complexity can be analysed with high sensitivity, resolution and mass accuracy by direct coupling of liquid chromatography (LC) and electrospray ionization (ESI) MS-MS; (Aebersold & Mann, 2003). Thus, investigating the proteomic profile of two strains of cultured *M. avium* subsp. *paratuberculosis* by fractionation of bacterial cells followed by cation-exchange chromatography and LC MS/MS resulted in detection of 20 % of all ORFs annotated (Radosevich *et al.*, 2007).

In order to identify proteins that may play a role in the direct interaction with host factors we improved the efficacy of *M. avium* subsp. *paratuberculosis* isolation from mucosa, prepared membrane-enriched fractions, and analysed them by using 2D difference gel electrophoresis (2D DIGE) and MALDI-TOF MS as well as by using a nanoflow-liquid-chromatography-coupled tandem mass spectrometry approach (nUPLC-ESI Q-TOF-MS-MS).

METHODS

Origin of animals and culture of *M. avium* subsp. *paratuberculosis*. Three cows of different ages (2.9, 3.6 and 7.9 years) with clinical signs of Johne's disease originating from two different farms around Giessen, Germany, were investigated. Pieces of ileum and jejunum were obtained and cultured for the presence of *M. avium* subsp. *paratuberculosis* and intestines were stored at -80°C until cultural confirmation of infection.

For culture, approximately 300 mg mucosa were homogenized with glass beads (3 mm in diameter, six beads per tube) in 3 vols 0.89 % saline in a Fast Prep instrument for 30 s at intensity setting 4.0; 3 ml homogenized material was mixed with 7.5 ml 0.75 % hexadecylpyridinium chloride (HPC; Merck) by vortexing and allowed to settle, 5 ml supernatant were added to 12.5 ml 0.75 % HPC and incubated at room temperature for 12 h. Decontaminated samples were centrifuged at 3000 g for 15 min, and the sediment resuspended in 150 μl residual fluid was used for inoculation of BBL Herrold's egg yolk agar slants containing 2 μg mycobactin J ml^{-1} (Becton Dickinson) and were incubated at 37°C for 20 days. Subcultures were performed on Middlebrook (MB) agar 7H10 (BD Difco) supplemented with 0.5 % glycerol (v/v), 2 μg mycobactin J ml^{-1} and 10 % oleic acid dextrose catalase (OADC) enrichment [1 l OADC enrichment contains 8.5 g NaCl, 50 g BSA (BSA, fraction V), 20 g glucose, 0.03 g catalase, 0.6 ml oleic acid] and were incubated at 37°C for 40 days. For comparison with mucosa-derived *M. avium*

subsp. *paratuberculosis*, large-scale cultures of the *M. avium* subsp. *paratuberculosis* strains isolated from mucosa were grown on 500 ml MB 7H9 broth (BD Difco) supplemented with 2.5 % glycerol (v/v), 2 μg mycobactin J ml^{-1} and 10 % OADC as surface layer in penicillin flasks at 37°C for 70 days.

Strain typing of bovine isolates of *M. avium* subsp. *paratuberculosis*. High resolution strain typing of mucosa-derived *M. avium* subsp. *paratuberculosis* cultures was done by RFLP analysis using two restriction enzymes (*Bst*EII and *Pst*I), multiple-locus mycobacterial interspersed repetitive unit-variable-number tandem-repeat (MIRU-VNTR) analysis and multilocus short sequence repeat (MLSSR) sequencing as described previously (Möbius *et al.*, 2009).

Isolation of *M. avium* subsp. *paratuberculosis* from surface-layer cultures or directly from mucosa. *M. avium* subsp. *paratuberculosis* surface-layer cultures were grown to confluency and harvested by filtration [250 ml filtration flasks (Millipore)]. Bacteria were collected with a spatula and washed three times in PBS [150 mM NaCl, 9 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , 2.5 mM KCl, (pH 7.4)]. Isolation of *M. avium* subsp. *paratuberculosis* from mucosa was performed as described by Choy *et al.* (1998) with some modifications. Briefly, sections of frozen gut were thawed overnight at 4°C , turned inside-out and washed under flushing water. The mucosal layer was removed on ice using a glass slide, and 5 g of mucosal scrapings were homogenized in 0.2 M sucrose containing 0.1 % Sarkosyl and 1 mM PMSF (solution 1) for 1 min using an Ultra-Turrax. The homogenate was centrifuged (200 g, 20 min, 4°C) and supernatants were collected on ice. The pellets were resuspended in 20 ml solution 1 and recentrifuged (200 g, 20 min, 4°C). All supernatants were combined and centrifuged at 8000 g. Pellets were resuspended in solution 1, purified using a step gradient of 0.3 M sucrose (6000 g, 10 min, 4°C) and twice using a step gradient of 1.5 M KCl (6000 g, 10 min, 4°C); the *M. avium* subsp. *paratuberculosis* pellets obtained were washed three times in PBS and the purity of the preparation was assessed by Ziehl-Neelsen staining and light microscopy.

Preparation of membrane-enriched fractions from *M. avium* subsp. *paratuberculosis*. Membrane-enriched fractions were prepared from mucosa-derived (MDM; mucosa-derived membranes) and culture-derived (CDM; culture-derived membranes) *M. avium* subsp. *paratuberculosis* obtained from the three cows. Two independent repeats of this enrichment were performed using bacteria from independent mucosal scrapings and cultures, respectively. Briefly, 1.2 g wet weight of the *M. avium* subsp. *paratuberculosis* pellets were resuspended in 25 % sucrose, 50 mM Tris/HCl (pH 8.0) containing protease inhibitor cocktail complete (Roche) and mechanically disrupted with 0.1 mm zirconium beads (Biospec Products) in a Fastprep 120 (Thermo Savant) by six cycles of 20 s at 6.5 beats per second with intermittent cooling on ice. Bacterial lysates containing zirconium beads were transferred to a new tube diluted with three vols distilled water and treated with ultrasound (three times for 1 min at setting 4, 40 % duty cycle). The cell debris was removed by centrifugation (30 000 g, 10 min at 4°C). Supernatants were centrifuged at 100 000 g, and the pellets (MDM and CDM) were resuspended in 50 μl standard cell lysis buffer [SCLB; 8 M urea, 2 M thiourea, 30 mM Tris/HCl, 4 % CHAPS (pH 8.5)].

The concentration of solubilized membrane-enriched protein fractions was determined using the 2D Quant kit (GE Healthcare). For a visual assessment of the preparations, 30 μg protein extracts were separated by using SDS-PAGE and stained with Coomassie brilliant blue.

2D DIGE. Independent 2D DIGE experiments were performed for MDM and CDM preparations obtained from cows 2 and 3. Protein

preparations were labelled with cyanine dye Cy3 or Cy5 (GE Healthcare). For each 2D DIGE experiment, equal amounts of protein from each culture-derived membrane-enriched fraction and its mucosa-derived counterpart were pooled as the internal standard and labelled with Cy2 (GE Healthcare); labelling was performed according to the manufacturer's instructions. The internal standard (labelled with Cy2) and two samples (each labelled with either Cy3 or Cy5) were pooled and focussed on the same Immobiline DryStrip (24 cm, nonlinear, pH 3–11; GE Healthcare). Immobiline DryStrips were rehydrated for 14–16 h using 450 µl rehydration buffer [7 M urea (Roth), 2 M thiourea (Sigma), and 4% w/v CHAPS (Roth)] supplemented with 2% of the respective immobilized pH gradient (IPG) buffer and 1.2% (v/v) DeStreak reagent (GE Healthcare). The pooled protein preparations were supplemented with 2% (v/v) of the respective IPG buffer and 2% (v/v) of DeStreak. Samples were loaded into anodal sample cups and subsequently focused using an Ettan IPGphor (GE Healthcare) for 21 h in the following series of time blocks with increasing voltage: 3 h at 150 V, 3 h at 300 V, 6 h at a 1000 V gradient, 3 h at an 8000 V gradient, and 6 h at 8000 V. After isoelectric focussing, the strips were stored at -70°C or directly used for the second dimension as described previously (Buettner *et al.*, 2009).

Scanning, data analysis and protein identification by MALDI-TOF-MS. 2D Gels were scanned on a Typhoon Trio Scanner (GE Healthcare) at a resolution of 100 dots cm^{-1} using filters with specific excitation and emission wavelengths for Cy2 (filter BP 40; 488–520 nm), for Cy3 (filter BP 30; 532–580 nm) and for Cy5 (filter BP 30; 633–670 nm). Fluorescence-labelled spots were analysed by DeCyder (Differential Analysis Software, GE Healthcare), and only proteins with a minimum twofold expression difference were evaluated. The same gels were stained with colloidal Coomassie G-250 and prominent protein spots were excised, trypsinized according to the method of Shevchenko *et al.* (1996) and identified by mass spectrometric methods as described previously (Buettner *et al.*, 2009).

Tube-gel trypsin digestion and nanoflow-liquid-chromatography-coupled tandem mass spectrometry (nUPLC-ESI Q-TOF-MS/MS). For tube-gel trypsin digestion (Lu & Zhu, 2005), 10 µl aliquots containing 100 µg protein from MDM or CDM preparations were mixed with 30 µl acrylamide/bisacrylamide solution [37.5:1; 30% (w/v)] and 30 µl distilled water containing 1% tetramethylethylenediamine (TEMED) and 1% ammonium peroxodisulfate. Subsequently, proteins were fixed with 40% methanol/10% acetic acid, and the gel was cut into small pieces. Proteins were trypsinized as described previously (Shevchenko *et al.*, 1996) supplementing with 0.025% of trypsin enhancer ProteasMax Surfactant (Promega). Extraction of peptides from the gel matrix was performed in 20%, 50% and 80% acetonitrile/water with 0.5% formic acid for 30 min in an ultrasonification bath. Tube-gel trypsin

digestion and nUPLC-ESI Q-TOF-MS/MS were performed twice for each of the independently obtained bacteria preparations per cow and culture.

A 100 µm × 100 mm nUPLC column (ACQUITY UPLC 1.7 µm BEH130 C_{18} ; Waters) was used for peptide separation. Each sample was run over 45 min with a flow rate of 300 nl min^{-1} . Mobile phase A consisted of 0.1% formic acid in 1% acetonitrile. Mobile phase B included 0.1% formic acid in 99% acetonitrile. The gradient was 99% mobile phase A for 0.33 min and then ramped linearly to 65% mobile phase A over 30 min. Over the next minute, it was ramped to 15% phase A and held for 1 min before equilibrating the column with 99% phase A for 13 min. Before every other run, calibration of the Q-TOF MS was performed using [Glu₁]fibrinopeptide B as standard. The capillary voltage was 1800 V and was tuned for signal intensity. The two most intense ions with charge states between 2 and 4 and mass ranges between 450 and 1600 were selected in each survey scan if they met the switching criteria. Different collision energies were used to fragment each peptide ion on the basis of its mass-to-charge (m/z) values. Each sample was run up to eight times. For each new measurement, previously detected peptides in a mass range within ± 100 mDa were excluded from analysis in order to perform fragmentation on peptides represented in weaker signals. By using this approach, each sample was measured until no further increase in the number of identified peptides was observed.

Data processing and bioinformatics. The nUPLC-ESI Q-TOF-MS/MS raw data were processed using ProteinLynx Global Server (Version 2.1, Waters) by searching against the *M. species* Swiss-Prot database downloaded on 25 September 2008 (99 308 entries). The identification of bovine proteins was performed by searching against the NCBI nonredundant database as downloaded on 16 August 2006. All proteins identified in MDM or CDM as being specific for *M. avium* subsp. *paratuberculosis* were copied into separate Microsoft Excel data sheets. The mycobacterial origin of all proteins detected in MDM by a one peptide-hit only was confirmed by searching against the entire NCBI database. Prediction for membrane association or cytoplasmic localization of these proteins was performed with PSORT (<http://psort.ims.u-tokyo.ac.jp/form.html>).

RESULTS

Membrane-enriched fractions of *Mycobacterium avium* subsp. *paratuberculosis* and DNA-based typing of isolates

1D SDS-PAGE and subsequent Coomassie blue staining showed a reproducible pattern for the independent preparations of MDM and CDM, respectively (Fig. 1).

Fig. 1. SDS-PAGE of *M. avium* subsp. *paratuberculosis* membrane-enriched fractions. Coomassie-blue-stained proteins of two preparations of membrane-enriched fraction from mucosa-derived *M. avium* subsp. *paratuberculosis* (MDM 1, MDM 2) from cows 1–3 (lanes 1–3), and the corresponding culture-derived preparations (CDM 1, CDM 2) (lanes 4–6). HupB is the most abundant protein in the prominent band at approximately 30 kDa, and is absent in MDM from cow 1.

The protein pattern was different in the MDM preparations from different cows with the most prominent difference being a protein band at 30 kDa that was almost absent in the MDM of cow 1. The most abundant protein in this band was identified as the *M. avium* subsp. *paratuberculosis*-derived HupB protein (data not shown). However, the protein patterns obtained from two independent MDM preparations from individual cows as well as all CDM preparations were highly similar thereby supporting the reproducibility of the preparation method (Fig. 1).

Characterization of the isolates by high-resolution typing including RFLP, MIRU-VNTR and MLSSR analysis revealed that the *M. avium* subsp. *paratuberculosis* isolate from cow 2 was genetically distinct from the *M. avium* subsp. *paratuberculosis* isolates from cows 1 and 3 which displayed the same typing characteristics (see Supplementary Table S1, available with the online version of this paper).

Identification of proteins by MALDI-TOF-MS after electrophoretic separation using 2D DIGE

Applying 2D DIGE and MALDI-TOF-MS to the membrane-enriched fractions identified four proteins upregulated more than twofold in MDM (Fig. 2). These four proteins were the heparin-binding haemagglutinin (HBHA; 2.3-fold), MAP1205 (2.7-fold), MAP2057 (3.9-fold) and MAP3933c (4-fold). Among these proteins, HBHA was the only protein predicted to be membrane-associated. Therefore, in order to overcome the limitations of gel-based separation techniques in the identification of membrane proteins, nUPLC-ESI Q-TOF-MS/MS was applied as an alternative approach.

Identification of proteins by nUPLC-ESI Q-TOF-MS/MS

nUPLC-ESI Q-TOF-MS/MS was applied to in-tube-digested MDM and CDM of *M. avium* subsp. *paratuberculosis*. Each membrane-enriched fraction was treated with trypsin in two independent reactions resulting in 12 MDM and CDM

protein digests that were subsequently analysed by nUPLC-ESI Q-TOF-MS/MS. In total, 180 different mycobacterial proteins from MDM (see Supplementary Table S2, available with the online version of this paper) and 275 proteins from CDM (see Supplementary Table S3, available with the online version of this paper) were identified. Among the proteins from MDM, an additional 32 proteins were of bovine origin (Fig. 3; see Supplementary Table S4, available with the online version of this paper), and 84 proteins were detected in MDM from at least two cows. The proportion of *M. avium* subsp. *paratuberculosis*-derived peptides among all peptides varied between 18% (cow 1) and 70% (cow 3; see Supplementary Fig. S1, available with the online version of this paper) with the remainder being peptides of bovine origin. Among the proteins from CDM, 155 were identified in at least two distinct isolates. The 84 proteins detected in MDM of at least two different cows were investigated further.

In silico characterization of identified proteins

Among the 84 proteins detected in MDM, 48 were predicted to be membrane-associated (Table 1); out of these, 44 proteins were also identified in culture-derived *M. avium* subsp. *paratuberculosis*, and four proteins (MAP0010c, MAP1775, MAP2643, MAP3968) were not detected in these preparations. Among these four proteins was HBHA (MAP3968) which has also been identified as differentially expressed by 2D DIGE. Further analysis of these 48 proteins by BLAST analysis revealed that 13 of them have significant sequence identities with proteins predicted to be virulence-associated in mycobacteria (AtpF, AtpH, LprG, HBHA, SenX3, Mce, CipA, MAP2121c, MA3305c) or other intracellular pathogens (PepA, HtrA, SecE, MviN), and 13 proteins are annotated as hypothetical with no association with orthologous groups (Table 1).

DISCUSSION

The analysis of membrane-associated proteins of *M. avium* subsp. *paratuberculosis* directly isolated from intestinal

Fig. 2. 2D DIGE gel of membrane-enriched fractions of mucosa-derived (red) and culture-derived (green) *M. avium* subsp. *paratuberculosis* proteins prepared from cow 3. The four spots present only in the mucosa-derived preparation were analysed by MALDI-TOF-MS and identified as MAP3933c (putative short chain dehydrogenase; spot 1), MAP2057 (haloalkane dehalogenase DhmA; spot 2), MAP1205 (methanol dehydrogenase regulator homologue MoxR; spot 3) and MAP3968 (heparin-binding haemagglutinin adhesin-like protein; spot 4).

Fig. 3. Numbers of culture- and mucosa-derived *M. avium* subsp. *paratuberculosis* proteins and predicted cellular localization. Prediction of membrane association or cytoplasmic localization was done by using PSORT (<http://psort.ims.u-tokyo.ac.jp/form.html>).

mucosa of infected cows can give new insights into the expression of potentially virulence-associated factors of *M. avium* subsp. *paratuberculosis*. Therefore, their characterization might improve our understanding of the pathogenesis of Johne's disease. In addition, it might provide targets for novel diagnostic approaches to Johne's disease.

Initial work analysing mucosa-derived *M. avium* subsp. *paratuberculosis* was done by Hughes *et al.* (2007) from ovine hosts and by Egan *et al.* (2008) from bovine hosts. Both groups comparatively analysed mucosa- and culture-derived bacterial whole-cell lysates by 2D gel electrophoresis, with a pH range of 4–7, and subsequent silver staining. The work resulted in the identification of a number of metabolic proteins possibly important for energy utilization of *M. avium* subsp. *paratuberculosis* in the host. In contrast, our approach focussed on membrane-enriched protein preparations of *M. avium* subsp. *paratuberculosis* from mucosa- and culture-derived material.

Comparing *in vivo* with *in vitro* growth has the drawbacks that growth of pathogens in their host is rather uncharacterized and that growth-phase-dependent metabolic changes occur during growth in culture. We chose to use a surface culture of *M. avium* subsp. *paratuberculosis* to prepare CDM since these cultures contain bacteria in different stages of growth. Therefore, proteins identified in MDM only were considered to be more likely to be expressed in a host-dependent rather than a growth-phase-dependent manner.

The method applied for membrane preparations was highly reproducible as indicated by the very similar band pattern after SDS-PAGE of preparations from two independent experiments (Fig. 1). These analyses additionally revealed reproducible qualitative differences in the MDM of the bacteria from the different cows. Differences were articulated particularly by a prominent protein band at 30 kDa

containing the HupB protein as the most abundant component; this band was strongly present in MDM of cows 2 and 3 as well as in all CDM but almost absent in MDM of cow 1. This lower abundance of HupB in MDM of cow 1 was associated with a higher abundance of bovine proteins in both MDM preparations from this cow (Supplementary Fig. S1). Since membrane preparations were reproducible and *M. avium* subsp. *paratuberculosis* isolates were indistinguishable using a highly discriminatory typing scheme (Supplementary Table S1) as well as being derived from animals from the same herd, the qualitative differences observed might reflect differences in pathogen–host-interaction.

Since membrane proteins frequently have an isoelectric point (pI) higher than 7 (Xiong *et al.*, 2005) and since the quantification of silver-stained spots is limited (Patton, 2000; Quadroni & James, 1999), we initially used 2D DIGE with a pH range of 3–11 and analysed spots with an at least twofold difference in expression by MALDI-TOF-MS. Using this approach we were able to identify four differentially expressed proteins only one of which was predicted to be membrane-associated. This limited number of proteins identified was, in part, due to limited availability of membrane-enriched material from mucosa-derived *M. avium* subsp. *paratuberculosis*. In addition, the technical reproducibility of high quality 2D gels is problematic for membrane proteins (Henningsen *et al.*, 2002; Santoni *et al.*, 2000) and, therefore, hampers the identification of low-abundance proteins of interest.

Therefore, we proceeded by performing a qualitative comparison using nUPLC-ESI Q-TOF-MS/MS, an approach previously described as being highly sensitive (Chen & Pramanik, 2009; Old *et al.*, 2005). Since it is also known that mass spectral peak intensities of peptide ions correlate well with protein abundances in complex samples (Chen & Pramanik, 2009) and since the MS search algorithm automatically analyses the most abundant proteins first, this approach appeared suitable for the analysis of differential protein expression. It avoids the limitations of 2D gel electrophoresis in separating membrane proteins and the identification of proteins with a pI higher than 11 and a molecular mass below 15 or above 150 kDa. The principal validity of our approach was supported by the large number of proteins identified and the results of the PSORT algorithm whereby 59 % of mucosa- and culture-derived *M. avium* subsp. *paratuberculosis* proteins were predicted to be membrane-associated (Fig. 3). Among the 41 % of proteins predicted to be cytoplasmic, some may also be membrane-associated or surface-exposed as 12 of them have also been identified after trypsin shaving of *M. avium* subsp. *paratuberculosis* (He & De Buck, 2010).

Among the 48 proteins predicted to be membrane-associated 13 have been associated with virulence of mycobacteria (AtpF, AtpH, LprG, HBHA, SenX3, Mce, CipA, MAP2121c, MAP3305c) or other intracellular pathogens (PepA, HtrA, SecE, MviN) (Table 1); 44 of

Table 1. Membrane proteins of mucosa-derived *M. avium* subsp. *paratuberculosis*, as detected by nUPLC-ESI Q-TOF-MS/MS from at least two cows

No.	Annotation	COG code*	Description†	Homologue (species)	Identity (%)	Reference
1	MAP0007	(–)	Uncharacterized protein	MAV_0007 (<i>M. avium</i> hominissuis)	96	
2	MAP0010c	(–)	Uncharacterized protein	MAV_0012 (<i>M. avium</i> hominissuis)	98	
3	MAP0143	(–)	Uncharacterized protein	MAV_0139 (<i>M. avium</i> hominissuis)	100	
4	MAP0150c	I	Putative acyl-CoA dehydrogenase (FadE25_2)	MAV_0145	100	
5	MAP0184c	(–)	Uncharacterized protein	MaviaA2_00941 (<i>M. avium</i> subsp. <i>avium</i>)	99	
6	MAP0228c	(–)	Cell wall arabinan synthesis protein (EmbB)	MaviaA2_01171 (<i>M. avium</i> subsp. <i>avium</i>)	99	
7	MAP0872	P	Phosphate ABC transporter (PhoS2_2)	MAV_1047 (<i>M. avium</i> hominissuis)	100	
8	MAP0904	M	Uncharacterized protein potentially involved in peptidoglycan biosynthesis	MaviaA2_04709 (<i>M. avium</i> subsp. <i>avium</i>)	100	
9	MAP0920c	M	Large-conductance mechanosensitive channel (MscL)	MAV_1098 (<i>M. avium</i> hominissuis)	100	Talaat <i>et al.</i> (2004)
10	MAP1043	U	Preprotein translocase subunit (SecD)	MUL_1725 (<i>M. ulcerans</i>)	83	
11	MAP1050c	O	Peptidyl prolyl <i>cis trans</i> isomerase (PpiB)	MAV_3462 (<i>M. avium</i> hominissuis)	98	
12	MAP1138c	(–)	22 kDa lipoprotein (LprG)	MAV_3367 (<i>M. avium</i> hominissuis)	100	Gehring <i>et al.</i> (2004)
13	MAP1315c	C	Cytochrome D ubiquinol oxidase, subunit II (CydB)	MAV_3165 (<i>M. avium</i> hominissuis)	99	
14	MAP1316c	C	Cytochrome D ubiquinol oxidase subunit I (AppC)	MAV_3164 (<i>M. avium</i> hominissuis)	99	
15	MAP1775	S	Uncharacterized protein	MAV_2471 (<i>M. avium</i> hominissuis)	99	
16	MAP1826c	(–)	Uncharacterized protein	MAV_2412 (<i>M. avium</i> hominissuis)	99	
17	MAP1830c	U	Twin arginine translocase protein A (TatA)	MAV_2409 (<i>M. avium</i> hominissuis)	100	
18	MAP1925	I	Putative acyl-CoA dehydrogenase (FadD15)	MAV_2307 (<i>M. avium</i> hominissuis)	98	
19	MAP1943	(–)	Uncharacterized protein	MAV_2288 (<i>M. avium</i> hominissuis)	100	
20	MAP1960	(–)	Uncharacterized protein (integral membrane protein)	MAV_2269 (<i>M. avium</i> hominissuis)	99	
21	MAP2083c	R	Uncharacterized protein	MAV_2102 (<i>M. avium</i> hominissuis)	96	
22	MAP2100	V	Putative ABC-transporter	MaviaA2_09030 (<i>M. avium</i> subsp. <i>avium</i>)	98	
23	MAP2121c	(–)	Major membrane protein 1, 35 kDa protein	MAV_2054 (<i>M. avium</i> hominissuis)	100	Bannantine <i>et al.</i> (2003)
24	MAP2454c	C	F ₀ F ₁ ATP synthase subunit delta (AtpH)	MaviaA2_010100006715 (<i>M. avium</i> subsp. <i>avium</i>)	99	Talaat <i>et al.</i> (2004)
25	MAP2455c	C	F ₀ F ₁ ATP synthase subunit B (AtpF)	MAV_1523 (<i>M. avium</i> hominissuis)	100	Talaat <i>et al.</i> (2004)
26	MAP2549c	(–)	Uncharacterized protein	HMPREF0591_5174 (<i>M. parascrofulaceum</i>)	93	
27	MAP2555c	O	Trypsin-like serine protease (HtrA)	MAV_1366 (<i>M. avium</i> hominissuis)	99	Stack <i>et al.</i> (2005)
28	MAP2643	S	Putative isoprenylcysteine carboxyl methyltransferase (Icmt)	MAV_1279 (<i>M. avium</i> hominissuis)	98	
29	MAP3005c	O	Putative thioredoxin-like protein	MAV_3813 (<i>M. avium</i> hominissuis)	100	
30	MAP3210	C	NADH dehydrogenase subunit J (NuoJ)	MAV_4042 (<i>M. avium</i> hominissuis)	99	
31	MAP3260	C	Putative aldo/keto reductase	MAV_4098 (<i>M. avium</i> hominissuis)	99	
32	MAP3305c	(–)	Uncharacterized protein	MAV_4151 (<i>M. avium</i> hominissuis)	99	Patel <i>et al.</i> (2006)
33	MAP3527	O	Trypsin-like serine protease (PepA)	MAV_5187 (<i>M. avium</i> hominissuis)	99	Hauser <i>et al.</i> (1998)
34	MAP3566	C	Putative fatty aldehyde dehydrogenase	MAV_5147 (<i>M. avium</i> hominissuis)	100	
35	MAP3567	IQR	Putative peroxisomal multifunctional enzyme type 2	MAV_5146 (<i>M. avium</i> hominissuis)	98	
36	MAP3610	(–)	Putative Mce associated membrane protein	HMPREF0591_3231 (<i>M. parascrofulaceum</i>)	73	Gioffré <i>et al.</i> (2005)

Table 1. cont.

No.	Annotation	COG code*	Description†	Homologue (species)	Identity (%)	Reference
37	MAP3666c	(–)	Uncharacterized protein	MAV_4942 (<i>M. avium hominissuis</i>)	100	
38	MAP3698c	C	Putative succinate dehydrogenase flavoprotein subunit	MAV_4909 (<i>M. avium hominissuis</i>)	95	
39	MAP3831c	C	Putative ferredoxin, 4Fe–4S	MAV_4817 (<i>M. avium hominissuis</i>)	94	
40	MAP3879c	IQR	Putative short-chain dehydrogenase/reductase SDR	MAV_4766 (<i>M. avium hominissuis</i>)	99	
41	MAP3904	(–)	Putative interferon-induced transmembrane protein	MAV_4740 (<i>M. avium hominissuis</i>)	99	
42	MAP3936	O	60 kDa chaperonin 2 (GroEL2)	MAV_4707 (<i>M. avium hominissuis</i>)	99	
43	MAP3968	(–)	Heparin binding haemagglutinin adhesin-like protein (HBHA)	MAV_4675 (<i>M. avium hominissuis</i>)	99	Pethe <i>et al.</i> (2001)
44	MAP3972c	(–)	Putative CipA homologue	MAV_4671 (<i>M. avium hominissuis</i>)	99	Harrieff <i>et al.</i> (2009)
45	MAP3982	T	Histidine protein kinase, sensor protein (SenX3)	MAV_4661 (<i>M. avium hominissuis</i>)	99	Agarwal <i>et al.</i> (2007)
46	MAP4033c	(–)	Uncharacterized protein	MAV_4608 (<i>M. avium hominissuis</i>)	98	
47	MAP4110	U	Preprotein translocase subunit (SecE)	MAV_4608 (<i>M. avium hominissuis</i>)	99	Camejo <i>et al.</i> (2009)
48	MAP4336	R	Putative virulence factor mvin family protein	MAV_5298 (<i>M. avium hominissuis</i>)	99	Carsiotis <i>et al.</i> (1989)

*The functional classification of proteins identified (COG code) has been adapted from the genome database of *M. avium* subsp. *paratuberculosis* K-10 (<http://www.ncbi.nlm.nih.gov/sutils/coxik.cgi?gi=380>) according to the database Cluster of Orthologous Groups of Proteins (COGs). Energy production and conversion (C); lipid transport and metabolism (I); cell wall/membrane biogenesis (M); post-translational modification, protein turnover, chaperones (O); inorganic ion transport and metabolism (P); secondary metabolites biosynthesis, transport and catabolism (Q); general function prediction only (R); function unknown (S); signal transduction mechanisms (T); intracellular trafficking and secretion (U); defence mechanisms (V); not in COGs (–).

†Descriptions were adopted from the *M. avium* subsp. *paratuberculosis* K-10 gene database, PFAM, CDD or from homologue in *Mycobacterium* species.

these proteins are shown for the first time, to our knowledge, to be expressed by *M. avium* subsp. *paratuberculosis* during natural infection; for four proteins (MAP0150c, MAP2121c, MAP3567, MAP3936) expression during infection had been shown previously (Egan *et al.*, 2008). Among the 48 proteins 13 are still uncharacterized with no orthologues found in the database, making them worthwhile targets for future functional characterization with respect to pathogen–host interaction. Four of the 48 proteins (MAP0010c, MAP1775, MAP2643 and MAP3968) are of particular interest, as they were not found in culture-derived membrane preparations, thereby implying a differential expression during infection in the host.

Of particular interest among these proteins was the HBHA protein (MAP3968) found to be differentially expressed also by the 2D DIGE approach. HBHA has been reported to be expressed in the host by *Mycobacterium tuberculosis* and *Mycobacterium leprae* (de Lima *et al.*, 2009; Pethe *et al.*, 2001; Reddy & Kumar, 2000) and it has been shown to be highly antigenic in *M. tuberculosis* (Masungi *et al.*, 2002; Menozzi *et al.*, 2006; Zanetti *et al.*, 2005). Also, recombinant HBHA of *M. avium* subsp. *paratuberculosis* has been found to be immunogenic and induced antibody responses in cattle with a possible cross-reactivity to *Mycobacterium*

bovis (Sechi *et al.*, 2006). Furthermore, HBHA has been implicated as being involved in systemic dissemination of *M. tuberculosis* (Pethe *et al.*, 2001) and in adherence to epithelial cells by interacting with proteoglycans such as decorin (Delogu & Brennan, 1999).

Among the other differentially expressed proteins are the hypothetical proteins MAP0010c and MAP1775; both are conserved among mycobacteria and completely uncharacterized, as well as protein MAP2643 sharing a 98 % identity to an isoprenylcysteine carboxyl methyltransferase (Icmt) family protein of *M. avium* subsp. *hominissuis*. Icmt is a methyltransferase and the last of three sequentially acting enzymes responsible for the modification of proteins containing a CaaX motif. Protein prenylation, a post-translational modification process initiated by the addition of an isoprenoid lipid, is an important component in the localization and activity of many proteins involved in signal transduction pathways (Peterson *et al.*, 2005).

Taken together, our work has identified a number of previously hypothetical membrane-associated proteins of *M. avium* subsp. *paratuberculosis* that are expressed during natural infection, which may be interesting targets for future work on mycobacterial pathogenesis.

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