Identification of antigenic proteins from *Mycobacterium avium* subspecies *paratuberculosis* cell envelope by comparative proteomic analysis

Shanmugasundaram Karuppusamy,¹ Lucy Mutharia,² David Kelton,³ Niel Karrow⁴ and Gordon Kirby¹,*

**Abstract**

Johne’s disease (JD) is a contagious, chronic granulomatous enteritis of ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). The aim of this study was to identify antigenic proteins from the MAP cell envelope (i.e. cell wall and cytoplasmic membranes) by comparing MAP, *M. avium* subsp. *hominissuis* (MAH) and *M. smegmatis* (MS) cell envelope protein profiles using a proteomic approach. Composite two-dimensional (2D) difference gel electrophoresis images revealed 13 spots present only in the image of the MAP cell envelope proteins. Using serum from MAP-infected cattle, immunoblot analysis of 2D gels revealed that proteins in the 13 spots were antigenic. These proteins were identified by liquid chromatography tandem mass spectrometry as products of the following genes: *sdhA*, *fadE25_2*, *mkl*, *citA*, *gapdh*, *fadE3_2*, *moxR1*, *mpp*, *purC*, *mdh*, *atpG*, *fbpB* and *desA2* as well as two proteins without gene names identified as transcriptional regulator (MAP0035) protein and hypothetical protein (MAP1233). Protein functions ranged from energy generation, cell wall biosynthesis, protein maturation, bacterial replication and invasion of epithelial cells, functions considered essential to MAP virulence and intracellular survival. Five MAP cell envelope proteins, i.e. *SdhA*, *FadE25_2*, *FadE3_2*, MAP0035 and DesA2 were recombinantly expressed, three of which, i.e. *SdhA*, *FadE25_2* and *DesA2*, were of sufficient purity and yield to generate polyclonal antibodies. Immunoblot analysis revealed antibodies reacted specifically to the respective MAP cell envelope proteins with minimal cross-reactivity with MAH and MS cell envelope proteins. Identification and characterization of MAP-specific proteins and antibodies to those proteins may be useful in developing new diagnostic tests for JD diagnosis.

**INTRODUCTION**

Johne’s disease (JD)/paratuberculosis is an incurable chronic granulomatous enteritis of ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) grouped under the *M. avium* complex (MAC) [1]. JD is global in distribution and incurs huge economic losses to the cattle industry [2]. Currently, JD is diagnosed by detection of MAP bacteria in faeces or milk via culture, detection of MAP bacterial DNA by PCR and immunological responses to MAP by serological assays. Faecal culture is time-consuming, costly and lacks sensitivity particularly in low shedders or in the pre-clinical stage of infection [3]. PCR analyses that amplify MAP-specific sequences such as IS900 and ISMAP02 are used for detection of MAP DNA in faeces, milk and tissues [3]. However, test sensitivity and specificity is diminished due to difficulties in recovering high-quality MAP DNA as well as PCR interference due to inhibitors in the faeces. ELISA is commonly used for JD diagnosis by measuring milk or serum antibodies to complex mixtures of MAP proteins such as whole-cell sonicated protein preparations, cell wall preparations and/or purified protein derivatives. However, these preparations contain cross-reacting proteins to other genetically related environmental mycobacteria. In addition, seroconversion typically occurs at later stages of JD infection such that current commercially available ELISA diagnostic tests are not useful in detecting sub-clinically infected animals [4]. Therefore, rapid, sensitive and specific diagnostic tests are needed to identify animals infected with MAP to prevent and control MAP transmission and JD. In view of this, there is a need to identify MAP species-specific antigenic proteins that could be used as diagnostic markers.

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**Abbreviations:** 2-DE, two-dimensional electrophoresis; DIGE, difference gel electrophoresis; JD, Johne’s Disease; LC-MS/MS, liquid chromatography tandem mass spectrometry; MAC, *M. avium* complex; MAH, *Mycobacterium avium* subsp. *hominissuis*; MAP, *Mycobacterium avium* subsp. *paratuberculosis*.

Two supplementary figures are available with the online version of this article.
antigens that can be incorporated into JD diagnostic tests with improved sensitivity and specificity [5].

In spite of genotypic similarities, there are major phenotypic differences that exist between bacterial strains within the MAC. While MAP and M. avium subsp. hominisuis (MAH) share genetic similarity of more than 95% at their nucleotide level, MAP is very slow growing, mycobactin J-dependent and an obligate intracellular pathogen of intestinal tissues. In contrast, MAH is a fast growing and opportunistic pathogen of the respiratory tract [6]. In view of this, we speculate that there may be unique antigenic-proteins or epitopes among these species, particularly in the cell envelope (i.e. cell wall and cytoplasmic membrane), that could be used in diagnostic test development. The cell envelope plays a vital role in host cell entry, modulation of the phagosome membrane, defence against the host immune system, and environmental survival [7].

Proteomic comparisons using gel-based two-dimensional electrophoresis (2-DE) and gel-free approaches have revolutionized protein identification and provide a useful complement to gene expression studies [8]. In particular, 2-DE is a valuable tool for differential comparisons of cell membrane proteins from closely related bacterial strains [9]. In addition to characterization of isoelectric points, molecular weights, isoforms and post-translational modifications, the identity and antigenicity of proteins can be achieved by combining 2-DE with liquid chromatography tandem mass spectrometry (LC-MS/MS) and immunoblotting [10, 11]. In addition, difference gel electrophoresis (DIGE) is a useful technological refinement involving labelling of proteins with fluorescent Cy dyes and subsequent 2-DE that allows for the assessment of quantitative differences in proteins between samples. Indeed, a comparison of membrane proteins of three strains of Mycoplasma agalactiae by 2D-DIGE showed considerable protein differences between strains [9].

The aim of this study was to identify antigenic MAP species-specific proteins from the cell envelope of MAP organisms by comparing MAP, MAH and M. smegmatis (MS) cell envelope protein profiles using 2D-DIGE, 2-DE immunoblot and LC-MS/MS proteomic approaches. To begin biomarker validation, selected MAP-specific proteins were recombinantly expressed and antibodies generated to these proteins were used to determine MAP-specificity by immunoblot analysis.

METHODS

Source bacterial strains, media and growth conditions

Three bacterial species, M. smegmatis strain MC²155, M. avium subsp. hominisuis (MAH) strain 104 and M. avium subsp. paratuberculosis (MAP) (Madonna, gc86 and gd30 strains), graciously provided by Dr L. Mutharia, University of Guelph, were used in the experiments. The environmental mycobacteria M. smegmatis was examined as a possible antigen for the production of antibodies that are cross-reactive to MAP as most cattle are exposed to this bacterium in their environment. M. smegmatis was grown in Luria–Bertani (LB) broth medium (BD Difco, NJ, USA) as described by [12]. MAH is another common opportunistic pathogen in the environment and has genetic similarity with other members of MAC such as M. avium subsp. avium and MAP. Exposure of cattle to this bacterium also generates cross-reacting antibodies and affects MAP diagnosis. MAH was cultured as previously described [13]. Three MAP strains (Madonna, gc86 and gd30 strains) isolated from clinical cases from southern Ontario (Canada) were used instead of the MAP-K10 reference strain. The advantages of clinical strains for proteomic comparison have been well documented [14]. MAP strains were grown in Middlebrook 7H9 broth medium supplemented with glycerol (0.5% v/v), 10% (v/v) oleic acid-albumin-dextrose-catalase and mycobactin-J (2 µg ml⁻¹) (Allied Monitor Inc, Fayette, MO, USA) at 37°C for 6 to 8 weeks. All bacterial cultures were harvested by centrifugation at 3500 r.p.m. for 30 min at 4°C and washed three times with ice-cold PBS (pH 7.4). Bacterial pellets were then washed with 0.16 M sodium chloride solution and the weight of wet cells was determined [7]. The bacterial pellets were preserved at −80°C until further analysis.

Subcellular fractionation and protein extraction

Subfractionation of M. smegmatis, MAH and MAP was done to obtain cell wall core and cytoplasmic membranes as described [7, 15] with a few modifications. Briefly, 2 ml of lysis buffer (0.05 M potassium phosphate, 0.022% (v/v) β-mercaptoethanol, pH 6.5) was added to each gram of frozen bacterial cells (wet cell weight). Lysozyme (final concentration 4.8 mg ml⁻¹ w/v) (Sigma-Aldrich, Oakville, CA) and a complete protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) were also added to the cells and incubated for 4 h at 37°C with continuous stirring. Bacterial lysates were transferred into 2 ml screw-capped centrifuge tubes containing sterile zirconium-silica beads (0.1 mm) (Biospec Products) and disrupted by 12 cycles of high velocity bead beating with intermittent cooling on ice after each bead beating. Lysates were centrifuged at 1000 g for 15 min at 4°C to remove unbroken cells and debris. Supernatants were centrifuged at 20000 g for 30 min at 4°C to obtain the pelleted cell walls and the supernatants containing cytoplasmic membrane and cytosolic fractions. Supernatants were centrifuged at 150000 g for 90 min at 4°C to obtain cytoplasmic membranes and cytosolic fractions. Pellets containing cell walls and membrane fractions (cell envelope) were then washed twice with PBS (pH 7.4) containing a protease inhibitor cocktail to remove cytoplasmic contamination.

Cell envelope pellets were re-suspended in standard cell lysis buffer containing 7 M urea (Sigma-Aldrich, St. Louis, MO, USA), 2 M thiourea (GE Healthcare, Montreal, QC, Canada), 30 mM Tris/HCl, 4% CHAPS (pH 8.5) (Affymetrix, Maumee, OH, USA), incubated on ice for 30 min with
intermittent mixing and centrifuged at 10 000 g for 20 min at 4 °C to remove insoluble materials. Supernatants containing soluble proteins were stored at −20 °C until further 2D-DIGE analysis.

2D-difference gel electrophoresis (2D-DIGE)

Protein labelling
Prior to 2D-DIGE analysis, proteins samples were processed with a 2-DE clean-up kit (GE Healthcare, Montreal QC, Canada) as per manufacturer protocols and protein concentrations were estimated using the Bio-Rad protein assay (Bio-Rad Laboratories, USA). For analytical 2D-DIGE gel analysis, 50 µg of protein samples from MAP (pooled proteins from three strains), MAH and M. smegmatis cell envelopes were labelled with 100 pM of Cy2, Cy3 and Cy5 CyDye DIGE fluors (GE Healthcare, Montreal QC, Canada) respectively according to the minimal dye labelling procedure described by the manufacturer. Protein samples were incubated with CyDyes on ice for 1 h in the dark followed by 10 min on ice with 1 µl of 10 mM lysine in the dark to stop the labelling reaction.

First and second dimensional isoelectric focussing
Labelled protein samples were pooled and mixed with 450 µl of rehydration buffer ([7 M urea, 2 M thiourea and 2 % w/v CHAPS supplemented with 1 % w/v DTT] (Fisher Scientific, Pittsburgh, USA) and 0.5 % v/v Pharmalytes (GE Healthcare, Montreal QC, Canada) supplemented with 1 % w/v DTT for 15 min under shaking conditions, and again in 10 ml of equilibration buffer supplemented with 2.5 % w/v iodoacetamide for another 15 min under shaking conditions. For second dimensional separation, pH gradient strips were rinsed with SDS running buffer and placed onto 12.5 % SDS-PAGE gels with a lane allocated for pre-stained molecular markers (ECL Plex Rainbow; GE Healthcare). Electrophoresis was carried out at 6 W/gel for 17 h at 20 °C in a DALT six gel electrophoresis unit (GE Healthcare). Gel images were obtained by a Typhoon 9410 scanner (GE Healthcare). Cy2, Cy3 and Cy5 images were scanned with laser excitation at 488, 532 and 633 nm respectively; and with emission filters at 520, 580 and 670 nm respectively. Gel images were transferred to DeCyder software 6.5 (GE Healthcare), and data analysis was performed for the proteomes of all three mycobacterial species as previously described [9].

Animal sera
A total of 15 serum samples were used in this experiment. Overall, 12 serum samples were obtained from cows on dairy farms participating in the Ontario Johne’s Education and Management Assistance Program that tested positive using Paracheck milk, IDEXX milk and IDEXX serum and IDEXX hyper ELISAs. Dr Niel Karrow (University of Guelph) generously provided three serum samples from calves experimentally infected with MAP and control serum from 2-month-old Jersey and Holstein calves from herds with no reported cases of JD.

Immunoblot analysis of 2-DE gels of MAP cell wall and membrane proteins
For immunoblot analysis, 150 µg of MAP cell envelope proteins in standard cell lysis buffer underwent isoelectric focussing (IEF) and equilibration as per the 2D-DIGE protocol mentioned earlier. Equilibrated 24 cm strips were cut into two 12.5 cm pieces in order to perform the second-dimension separation, i.e. 12.5 % SDS-PAGE gel electrophoresis at 10 W/gel for 3 h using a Hoefer SE600 Ruby electrophoresis unit (GE Healthcare). Proteins were then electrophoretically transferred onto nitrocellulose membranes and incubated in blocking buffer [2 % BSA in Tris-buffered saline pH 7.6 containing 0.1 % Tween 20 (TBST)] at room temperature (RT) for 1 h and then incubated with JD ELISA-positive, pooled cattle serum (1 : 100 in 2 % BSA in TBST buffer) for 3 h at 4 °C on a shaker. Membranes were then incubated with horseradish peroxidase-conjugated affinity-purified rabbit anti-bovine IgG (Jackson Immunoresearch Laboratories, West Gove, PA) antibodies (1 : 10 000 in 0.5 % BSA-TBST buffer) for 1 h at RT. After washing with TBST and TBS buffers, membranes were visualized by enhanced chemiluminescence.

Protein identification
2-DE: preparatory gel
In order to identify the antigenic proteins that were observed on 2-DE immunoblots, a total of 200 µg of unlabelled MAP proteins was loaded into a sample cup on Immobiline DryStrips (GE Healthcare), placed on a IPG-phor isoelectric focussing unit (GE Healthcare) and rehydrated at 30 V for 14–16 h. First dimensional focussing occurred over 21 h under the following conditions: 150 V for 3 h, 300 V for 3 h, 1000 V for a 6 h gradient, 8000 V for a 3 h gradient, 8000 V for 6 h and 500 V for 5 h [16]. After first dimensional isoelectric focussing, strips were equilibrated in 10 ml of equilibration buffer containing 70 mM Tris-HCl (pH 8.8), 2 % SDS, 6 M urea, 30 % glycerol, supplemented with 1 % w/v DTT for 15 min under shaking conditions, and again in 10 ml of equilibration buffer supplemented with 2.5 % w/v iodoacetamide for another 15 min under shaking conditions. For second dimensional separation, pH gradient strips were rinsed with SDS running buffer and placed onto 12.5 % SDS-PAGE gels with a lane allocated for pre-stained molecular markers (ECL Plex Rainbow; GE Healthcare). Electrophoresis was carried out at 6 W/gel for 17 h at 20 °C in a DALT six gel electrophoresis unit (GE Healthcare). Gel images were obtained by a Typhoon 9410 scanner (GE Healthcare). Cy2, Cy3 and Cy5 images were scanned with laser excitation at 488, 532 and 633 nm respectively; and with emission filters at 520, 580 and 670 nm respectively. Gel images were transferred to DeCyder software 6.5 (GE Healthcare), and data analysis was performed for the proteomes of all three mycobacterial species as previously described [9].
LC-MS/MS analysis
SYPRO Ruby-stained gel plugs were processed to extract peptides for LC-MS/MS analysis as previously described [17]. Samples containing the peptides were desalted and concentrated using C18 Zip Tips (EMD, Millipore). The samples were run on a linear ion trap-Orbitrap hybrid analyser (LTQ-Orbitrap) outfitted with a nano-spray source and an EASY-nLC split-free nano-LC system (Thermo-Fisher). Peptide mixtures were dissolved in 0.1% formic acid and loaded at constant 800 Bar onto a column for injection for mass spectrometry analysis. Thermo Easy Spray 75 μM × 15 cm PepMax RSLC Easy-Spray columns filled with 3 μM C18 beads were used. Peptides were eluted over 60 min at a rate of 250 nL min⁻¹ using a 0–35% ACN gradient in 0.1% formic acid. One MS full scan (400–1500 m/z) in the Orbitrap mass analyser was performed. Automatic gain control target of 500 000, max injection time of 100 ms, one micro scan, resolution of 60 000 were performed. Six data-dependent scans, automatic gain control target of 30 000, max injection time of 50 ms were applied. Minimum ion intensity of 1000 was required to trigger a MS/MS event. Dynamic exclusion was applied using a maximum exclusion list of 500 with one repeat count; a repeat duration of 30 s and exclusion duration of 10 s were applied.

Database searching
Tandem mass spectra were extracted using Scaffold Software version 4.3.0. Charge state deconvolution and deisotoping were not performed. All MS/MS samples were analysed using Sequest (Thermo Fisher Scientific, San Jose, CA, USA; version 1.4.0.288) and X! Tandem (The GPM, thegpm.org; version CYCLONE 2010.12.01.1). Sequest was set up to search Uniprot-Mycobacterium paratuberculosis. fasta (unknown version, 9222 entries) assuming the digestion enzyme trypsin. X! Tandem was set up to search the Uniprot-Mycobacterium avium subsp. paratuberculosis database (unknown version, 17 721 entries) also assuming trypsin. Sequest and X! Tandem were searched with a fragment ion mass tolerance of 0.60 Da and a parent ion tolerance of 2.0 Da. Deamidation of asparagine and glutamine, oxidation of methionine and carbamidomethylation of cysteine were specified in Sequest as variable modifications. Glu->pyro Glu of the n-terminus, ammonia-loss of the n-terminus, gln->pyro Glu of the n-terminus, deamidated of asparagine and glutamine, oxidation of methionine and carbamidomethyl of cysteine were specified in X! Tandem as variable modifications.

Criteria for protein identification
Scaffold (version Scaffold_4.3.0, Proteome Software, Portland, OR) was used to validate MS/MS based peptide and protein identifications. The false discovery rate (FDR) for peptide-spectrum match and protein identification was set to 1% and was determined by using the reversed peptide sequences (target-decoy-search strategy) to account for any false matches. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the PeptideProphet algorithm [18]. Protein identifications were accepted if they could be established at greater than 99.9% probability and contained at least two identified peptides. Protein probabilities were assigned by the ProteinProphet algorithm [19]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Codon optimization and PCR amplification of synthetic DNA fragments
DNA sequences of six MAP genes, i.e. MAP3698c (succinate dehydrogenase-iron-sulfur subunit-sdhA), MAP0150c (acyl-CoA dehydrogenase-fadE25_2), MAP129C (ribonucleotide-transport ATP-binding protein ABC transporter-mkl), MAP3651c (acyl-CoA dehydrogenase-fadE3_2), MAP1233 (hypothetical protein) and MAP2698c (acyl-ACP desaturase-desA2) were retrieved from the GenBank database. The selected genes were designed for recombinant expression with Nde I (CATATG) and Xho I (CTCGAG) restriction enzyme sites at 5’ and 3’ prime ends of the selected genes respectively and a polyhistidine (6× His) tag incorporated in the C-terminal region of the ORF. Rare codons of the selected genes were then optimized with E. coli to maximize their expression. Genes were synthesized as strings of DNA fragments by Invitrogen, Life Technologies (Burlington, ON, Canada). Synthetic DNA fragments were dissolved in 10 mM Tris buffer (pH 8.5) to a final concentration of 20 ng μl⁻¹. Primers and cycling conditions are indicated in Table 1. Synthetic string DNA fragments were PCR-amplified by Q5High-Fidelity DNA Polymerase (New England BioLabs, Whitby, ON, Canada) and PCR-amplified products were gel-purified as per standard protocols.

Cloning of MAP cell envelope protein coding sequences
Purified PCR-amplified products and circular pET 30a (+) vector (Novagen, Darmstadt, Germany) were double digested with NdeI and Xhol restriction enzymes (New England BioLabs, Whitby, ON Canada). RE-digested pET 30a (+) vector and PCR products were gel-purified and ligated by T4 DNA ligase (Invitrogen, 5791 Van Allen Way Carlsbad, CA, USA) overnight at 26°C. Escherichia coli BL21 (DE3) strain (New England BioLabs, Whitby, ON Canada) were prepared by the Inoue method as described by [20]. Ligated products were transformed into E. coli BL21 (DE3) competent cells [20]. Transformed cells were plated on LB agar containing kanamycin 30 μg ml⁻¹ and incubated overnight at 37°C. To confirm the transformation and ligation, four colonies from each plate was screened by colony PCR using gene-specific and universal T7 primers. Insert-confirmed colonies were grown in LB broth supplemented with kanamycin 30 μg ml⁻¹ overnight at 37°C and plasmids were purified by alkaline lysis. Plasmids were sequenced to confirm in-frame insertion of the coding sequence into the pET 30a (+) expression vector.

Protein expression and purification
Protein expression and solubility were tested as previously described [21]. To obtain large quantities of pure
recombinant proteins, *E. coli* BL21 (DE3) carrying recombinant clones were grown in LB broth supplemented with kanamycin (30 µg ml⁻¹) overnight at 27 °C in an orbital shaker (200 r.p.m.). Cells were induced (OD₆₀₀ of 0.5 to 0.7) with 0.1 M IPTG and incubated at 37 °C with vigorous shaking at 300 r.p.m. for 4 h. Induced cells were centrifuged and bacterial pellets were frozen on dry ice and stored at −80 °C until further use. Bacterial pellets were subsequently resuspended in lysis buffer (50 mM NaH₂PO₄, 500 mM NaCl, 5 to 10 mM imidazole pH 7.4), supplemented with lysozyme (2 mg ml⁻¹) (Sigma-Aldrich, 3050 Spruce street, St. Louis, MO, USA) and a complete protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) and incubated at 37 °C for 30 min. Following three freeze-thaw cycles, cells were pulse sonicated on ice with 90 s rests after each sonication, centrifuged at 24 000 r.p.m. for 25 min at 4 °C, transferred to new tubes and stored on ice awaiting further processing. Up to this stage, the procedure was common for all the soluble recombinant proteins. FadE25_2 and DesA2 recombinant proteins were purified by PureProteome Nickel magnetic beads (EMD Millipore, Temecula, CA, USA) as per the manufacturer’s instructions. Because SdhA and FadE3_2 proteins failed to bind to PureProteome Nickel magnetic beads, these two recombinant proteins were purified by the gravitational flow method using HiTrap TALON crude resins (GE Healthcare, Bio-Sciences AB, Uppsala, Sweden) as per the manufacturer’s instructions. Due to low solubility, hypothetical protein was purified under denaturing conditions. Recombinant Mkl protein did not respond well to induction with IPTG showing minimal expression and reduced growth and enhanced toxicity in *E. coli* cells. As a result, Mkl protein was not pursued further. Purified proteins were analysed by SDS-PAGE gels stained by rapid Coomassie staining procedures [22] to examine for protein purity. Purified fractions were dialysed using a Slide-A-Lyzer mini dialysis device (10K MWCO, 0.1 ml) (Pierce Biotechnology, Thermoscientific) in 10 mM PBS (pH 7.2) with five exchanges at RT and desalted recombinant proteins were stored at −80 °C.

### Immunization of rats with recombinant protein antigens

Six female Sprague-Dawley rats (two rats/protein) were used to generate polyclonal antibodies against recombinant SdhA, FadE25_2 and DesA2 because these three proteins had enhanced yield and purity following purification by affinity chromatography. In brief, 100 µg (50 µg/rat) of purified and desalted proteins were mixed with an equal volume of TiterMax gold adjuvant (Sigma-Aldrich, St. Louis, MO, USA) and vortexed 20 min at 4 °C to prepare an emulsion for the primary immunization. Following collection of pre-immune serum samples, rats were injected intramuscularly with emulsions. For subsequent booster immunization, 50 µg (25 µg/rat) of protein antigens were mixed with Freund’s incomplete adjuvant (Sigma-Aldrich, St. Louis, MO, USA) and vortexed to prepare 1:1 emulsions. Three booster immunizations were given on days 14, 28 and 40 respectively. On the third day following the last booster immunization, serum was prepared from whole blood collected from rats and stored at −80 °C. Immunization protocols, the use and care of animals in this experiment were approved by the Animal Care and Use Committee of the University of Guelph.

### Immunoblot analysis of rat anti-SdhA, FadE25_2 and DesA2 polyclonal antibodies against recombinant proteins

For immunoblot analysis, three purified proteins (SdhA, FadE25_2 and DesA2) were electrophoretically separated on duplicate gels, transferred onto nitrocellulose membranes and blocked against non-specific protein with 5% skim milk in TBST for 1 h at RT. One set of membranes was incubated overnight at 4 °C with pre-immune serum samples diluted 1:500 with 5% skim milk in TBST). The second set of membranes were incubated overnight at 4 °C with immune serum (1:5000 in 5% skim milk in TBST) from rats immunized with SdhA, FadE25_2 and DesA2. Membranes were incubated with anti-rat IgG horse radish peroxidase (HRP)-linked secondary antibody (Cell Signaling Technology, MA01923, USA) (1:5000 in 5% skim milk in TBST) for 1 h at RT. After each step, membranes were

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primers (underline indicates RE sites)</th>
<th>Cycling conditions</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>sdhA</em></td>
<td>Fp: 5′-gaaggagatacatagaggggag-3′</td>
<td>Denaturation at 95°C – 4 min, 25 cycles of 95°C – 1 min, 63°C – 30 sec, 72°C – 1 min, final ext. at 72°C – 5 min</td>
<td>1998</td>
</tr>
<tr>
<td><em>mkl</em></td>
<td>Rp: 5′-gggggggggctccgtttct-3′</td>
<td></td>
<td>1050</td>
</tr>
<tr>
<td><em>desA2</em></td>
<td>Fp: 5′-gaaggagatacatagaggggag-3′</td>
<td>Denaturation at 95°C – 4 min, 25 cycles of 95°C – 1 min, 60°C – 30 s, 72°C – 1 min, final ext. 72°C – 5 min</td>
<td>865</td>
</tr>
<tr>
<td><em>fadE25_2</em></td>
<td>Rp: 5′-gtggtggtggtgctcaca-3′</td>
<td>Denaturation at 95°C – 4 min followed by 25 cycles of 95°C – 1 min, 65°C – 30 s, 72°C – 1 min. Final ext. 72°C – 5 min</td>
<td>1246</td>
</tr>
<tr>
<td><em>fadE3_2</em></td>
<td>Fp: 5′-gaaggagatacatagaggggag-3′</td>
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<td>1248</td>
</tr>
<tr>
<td>Hypothetical (MAP1253)</td>
<td>Rp: 3′-gtggtggtggtgctcaca-3′</td>
<td>Denaturation at 95°C – 4 min, 25 cycles of 95°C – 1 min, 61°C – 30 s, 72°C for 1 min. Final ext. 72°C – 5 min</td>
<td>733</td>
</tr>
</tbody>
</table>

Table 1. List of primers and PCR cycling conditions
washed with TBST and incubated with Clarity Western ECL substrate (Bio-Rad Laboratories, USA) as per the manufacturer's instructions. Signals were detected using a ChemiDoc imaging system (Bio-Rad Laboratories, USA).

**Immunoblot analysis of polyclonal antibodies against MAP, MAH and *M. smegmatis* cell envelope proteins**

To test the specificity of the rat anti-SdhA, FadE25_2 and DesA2 polyclonal antibodies to the MAP cell envelope proteins antigens, one-dimensional electrophoresis (1-DE) immunoblot analysis was performed. In brief, 15 µg of MAP, MAH and *M. smegmatis* cell envelope protein extracts were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Non-specific sites were blocked with 5% skim milk in TBST and incubated overnight at 4°C with rat anti-SdhA, FadE25_2 and DesA2 antibodies (1:5000 in 2% BSA in TBST). Membranes were washed with TBST and incubated with anti-rat HRP-linked conjugate (1:2000 dilution in 5% skim milk in TBST) for 1 h at RT and membranes were washed with TBST and then probed with Clarity Western ECL substrate (Bio-Rad Laboratories, USA).

**2-DE immunoblot analysis of polyclonal antibodies against MAP cell envelope proteins**

For 2-DE immunoblot analysis, 125 µg of MAP cell envelope proteins in standard cell lysis buffer underwent IEF on 24cm, non-linear, pH 3–7 Immobiline DryStrips (GE Healthcare) and equilibration as per the 2D-DIGE protocol mentioned earlier. The second-dimension separation was performed on 12.5% SDS-PAGE gels at 10W/gel for 3 h. Proteins were then electrophoretically transferred onto nitrocellulose membranes. Membranes were blocked with 5% skim milk in TBST for 1 h at RT and incubated with rat anti-SdhA, FadE25_2 and DesA2 antibodies (1:5000 in 2% BSA in TBST) overnight at 4°C. Membranes were washed with TBST and incubated with anti-rat HRP-linked conjugate (1:2000 dilution in 5% skim milk in TBST) for 1 h at RT and then probed with Clarity Western ECL substrate (Bio-Rad Laboratories, USA).

**RESULTS**

**2-DE proteome map of MAP, MAH and *M. smegmatis***

Proteins were separated on Immobilon strips pH 3 to 7 NL, 24 cm long to cover a wide range of cell envelope proteins from MAP, MAH and *M. smegmatis*. A 2-DE proteomic analysis map clearly resolved numerous cell envelope proteins for all three mycobacterial species (Fig. 1). The coloured spots shown in Fig. 1 indicate differences in the proteome profiles for each *Mycobacterium* species with only a few protein spots (white) that are shared by all three bacterial species. More importantly, we observed that many protein spots (blue) had pI and MW characteristics that were specific to the MAP cell envelope. By acquiring the images under a single channel for each Cy dye the labelled protein profiles were visualized separately also indicating differences in protein profile patterns (Fig. S1a–c, available in the online version of this article). For all three mycobacterial species, molecular masses of the proteins ranged from 12 to 150 kDa and were located between ~pH 3.5 and pH 7 ranges with most located in the acidic region (~pH 4–6). Several strings of protein spots with the same mass but with different pI values were observed in the 2-DE map of all three mycobacterial species suggesting possible post-translational modifications.

**Assessing antigenicity of mycobacterial cell envelope proteins**

Routine exposure of animals to *M. smegmatis* and MAH in the environment can produce cross-reacting antibodies which may recognize MAP epitopes and interfere with the sensitivity and specificity of serological assays used for JD diagnosis. We validated this concept via immunoblot analysis by probing MAP, *M. smegmatis* and MAH cell envelope proteins, separated by 1D-PAGE, with JD-positive cattle serum. Our results revealed that serum reactivity to many MAP proteins (Fig. 2) was also considerable reactivity to *M. smegmatis* and MAH cell wall and membrane proteins (Fig. 2).

The antigenicity of MAP-specific proteins identified by 2D-DIGE was determined by immunoblot analysis with JD-positive cattle serum in order to identify antigen candidates for JD diagnostic purposes. Results revealed serum reactivity to many MAP proteins (Fig. 3a), many of which aligned with the MAP-specific protein spots identified by 2D-DIGE (Fig. 3b). In total, 13 MAP protein spots were subsequently selected for further analysis based on: (i) DIGE analysis indicating their specificity to MAP; (ii) immunoblot analysis indicating their antigenicity; and (iii) 2D-PAGE analysis...
The following six MAP cell envelope proteins were selected for recombinant expression: \textit{sdhA}, \textit{fdeE25}_2, \textit{fdeE3}_2, \textit{mkl}, \textit{desA2} and hypothetical protein (MAP1233). The rationale for selecting these six genes is that they have not been extensively studied to date and knowledge of their MAP-specificity and utility in JD diagnostic test development is limited. High level of expression was induced in four of the five genes with 0.1 M IPTG in LB media, however, this was not possible with Mkl protein due to toxicity to \textit{E. coli} cells upon induction with IPTG. Further attempts to express this protein under different conditions such as growing cells at lower temperatures (23 to 30 °C), addition of glucose into the media, altering plating methods, conducting plasmid stability tests, and induction with low concentrations of IPTG (2.5 to 10 µM) were unsuccessful. Testing of the solubility of the expressed proteins revealed that SdhA, FadE25_2, FadE3_2 and DesA2 were soluble and hypothetical protein was insoluble likely due to an inclusion body. Therefore, SdhA, FadE25_2, FadE3_2 and DesA2 proteins were purified under native conditions by using immobilized metal affinity chromatography techniques (IMAC) (Fig. S2). Hypothetical protein was purified with Ni-NTA resins, isolated on sucrose gradients and eluted in low pH (4.5) urea denaturation buffer (Fig. S2).

**Generation of antibodies against MAP recombinant proteins and immunoblot analysis**

Three recombinant proteins (SdhA, FadE25_2 and DesA2) were selected for polyclonal antibody generation. Antibodies were not generated for three recombinant proteins including FadE3_2, Mkl and hypothetical protein (MAP1233) for the following reasons. Recombinant Mkl was minimally expressed and was toxic to \textit{E. coli} cells upon induction with IPTG. As a result insufficient amounts of high-quality Mkl protein were available for immunization. During the purification of FadE3_2 recombinant protein, host cell proteins (Fig. S2) were present which may have eventually affected the downstream application of antibodies. Hypothetical protein was denatured (8 M urea, pH 4.5) during elution in the purification process and as a result did not refold into the native form and was unstable. Immunoreactivity to SdhA, FadE25_2 and DesA2 was tested periodically after each immunization as described in Methods. While pre-immune serum samples showed no antigen antibody reactions to the target proteins (data not shown), post-immune serum samples reacted strongly with SdhA, FadE25_2 and DesA2 recombinant proteins (Fig. 4a–c).

**1-DE and 2-DE immunoblot analysis of polyclonal antibodies against MAP cell envelope proteins**

To further determine the antigenicity of the three recombinant MAP cell envelope proteins and the specificity of the polyclonal antibodies to those proteins, 1-DE and 2-DE immunoblotting was performed using total MAP cell envelope extracts. Results of 1D immunoblot analysis revealed single bands with molecular weights corresponding to SdhA, FadE25_2 and DesA2 with negligible cross-reactivity with MAH and \textit{M. smegmatis} cell envelope proteins (Fig. 5a–c). In 2-DE immunoblot analysis, polyclonal antibodies reacted specifically with SdhA, FadE25_2 and DesA2 proteins as indicated by a correlation with pI and molecular weight coordinates in the 2-DE proteome map of MAP (Fig. 6a–d). Polyclonal antibodies against SdhA detected several MAP protein spots (Fig. 6b) that were subsequently confirmed as SdhA by LC-MS/MS analysis. Similarly, polyclonal antibodies against FadE25_2 protein detected one...
additional MAP protein spot (Fig. 6c) which was confirmed as FadE25_2 by LC-MS/MS.

**DISCUSSION**

The objective of this study was to identify MAP cell envelope proteins that are uniquely antigenic to MAP and not other mycobacterial species. To the best of our knowledge, this is the first study to use 2D-DIGE to compare the cell envelope proteomes of three mycobacterial species, i.e. MAP, MAH and *M. smegmatis*.

Our proteomic analysis showed that several strings of distribution patterns were similar in the three mycobacterial species. While many of the MAP proteins identified in this study have homologous gene sequences with other species of the MAC, the majority of cell envelope proteins were found to have distinct coordinates in the three mycobacterial species. This finding may be due to subtle differences in amino acid sequences, splice variants or post-translational modifications of homologous proteins across species that cause shifts in DIGE coordinates. A previous study which compared the proteomes of MAP and MAA whole cell
lysates by 2-DE analysis revealed that expression levels of 32 proteins were increased in MAP in comparison to MAA [23]. Out of these 32 proteins, the gene sequences of 19 proteins had little or no differences with MAA gene sequences. Another comparative qualitative proteomic analysis of purified protein derivatives from MAP (PPDj), *M. avium* subsp. *avium* (PPDa) and *M. bovis* (PPDb) showed that 12 proteins were specific to PPDj, one of which (MAP1718c) had no homologue in the MAA genome, whereas the remaining 11 proteins had homologous gene sequences with more than 98 % protein similarity with MAA.

Sequence homology alone may not be a good predictor of protein function as single amino acid changes can lead to differences in protein function [24, 25]. For example, bioinformatic analysis of two forms of Cpn60 protein (cpn60.1 and 2) in *Mycobacterium tuberculosis* (MTB), with >60 % nucleotide sequence similarity have markedly different functions in MTB pathogenesis [24]. Structural and functional relationships of proteins, protein–protein interactions, catalytic functions of enzymes, formation of protein complexes and antigenic epitopes are often modified by post-translational modifications (PTM) [26]. This suggests that differential expression patterns or isoforms may represent specific epitopes or protein regions that may elicit pathogen-specific immune responses in their hosts. Accordingly, authors of a recent study suggest that diversity in PTM amongst closely related mycobacterial species affords uniqueness to the proteins and/or species-specific epitopes [27]. As such, we speculated that these unique epitopes can elicit MAP-specific immune responses in their hosts. This rationale led us to hypothesize that some of the distinct spots on the MAP 2D gels may be useful to assess humoral responses to MAP infection and to generate polyclonal antibodies for detection of MAP micro-organisms in infected cattle.

LC-MS/MS results were also suggestive of different isoforms or PTM for three proteins that were represented by multiple spots on 2D-DIGE. These include iron-sulfur subunit of succinate dehydrogenase (identified in spots 1, 2 and 3), acyl-CoA dehydrogenase (identified in spots 4 and 5) and fibronectin-binding antigen 85 complex B (spots 11, 12 and 13). We identified more than one protein for some of the spots with a high percentage of protein coverage for each individual protein. For example,

<table>
<thead>
<tr>
<th>Spot no.</th>
<th>Protein name</th>
<th>Protein molecular weight (kDa)</th>
<th>Exclusive unique peptide count</th>
<th>Exclusive unique spectrum count</th>
<th>Total spectrum count</th>
<th>Percentage of sequence coverage</th>
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<tr>
<td>1</td>
<td>Succinate dehydrogenase iron-sulfur subunit</td>
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<td>71</td>
<td>56</td>
<td>91</td>
<td>92</td>
<td>74</td>
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<tr>
<td>3</td>
<td>Succinate dehydrogenase iron-sulfur subunit</td>
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<td>67</td>
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<td>4</td>
<td>Acyl-CoA dehydrogenase</td>
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<td>7</td>
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<td>21</td>
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<td>19</td>
<td>23</td>
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Table 3. Characteristics of identified MAP cell envelope proteins

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<tr>
<th>Protein accession nos.</th>
<th>Protein name</th>
<th>Gene name</th>
<th>Locus name MAP</th>
<th>MAH orthologue</th>
<th>MS orthologue</th>
<th>MW (kDa)</th>
<th>Theoretical PI</th>
<th>Sub-cellular location</th>
<th>Function</th>
<th>JD antigen</th>
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</thead>
<tbody>
<tr>
<td>R4MT83_MYCPC</td>
<td>Succinate dehydrogenase iron-sulfur subunit</td>
<td>sdhA</td>
<td>MAP3698c</td>
<td>MAV_4909/MAH_4321</td>
<td>MSMEG_0418</td>
<td>71</td>
<td>5.7</td>
<td>Cell surface</td>
<td>Energy generation - involvement in TCA cycle and electron transport chain</td>
<td>ND</td>
</tr>
<tr>
<td>R4NF35_MYCPC</td>
<td>Acyl-CoA dehydrogenase</td>
<td>fadE25_2</td>
<td>MAP0150c</td>
<td>MAH_0161</td>
<td>MSMEG_0531</td>
<td>44</td>
<td>5.58</td>
<td>Cell wall</td>
<td>Energy generation - β-oxidation of cholesterol</td>
<td>Yes</td>
</tr>
<tr>
<td>R4NP6_MYCPC</td>
<td>Acyl-CoA dehydrogenase</td>
<td>fadE3_2</td>
<td>MAP3651c</td>
<td>MAH_4370</td>
<td>MSMEG_1991</td>
<td>44</td>
<td>6.15</td>
<td>Cell wall</td>
<td>Energy generation - involvement in fatty acid metabolism</td>
<td>Yes</td>
</tr>
<tr>
<td>MDH_MYCPA</td>
<td>Malate dehydrogenase</td>
<td>mdh</td>
<td>MAP2541c</td>
<td>MAH_1241</td>
<td>Msmyc_04842</td>
<td>34.6</td>
<td>4.87</td>
<td>Cell surface</td>
<td></td>
<td>ND</td>
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<tr>
<td>Q74K8_MYCPA</td>
<td>Citrate synthase II</td>
<td>citA</td>
<td>MAP0827c</td>
<td>MAH_0868</td>
<td>MSMEG_5676</td>
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<td></td>
<td></td>
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<tr>
<td>ATPG_MYCPA</td>
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<td>atpG</td>
<td>MAP2452c</td>
<td>MAH_1331</td>
<td>MSMEG_4937</td>
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<td>Cell wall</td>
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<tr>
<td>R4NC8_MYCPA</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
<td>gapdh</td>
<td>MAP1164</td>
<td>MAV_3341/MAH_1965</td>
<td>MSMEG_3084</td>
<td>36</td>
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<td>Cell surface</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>R4NC48_MYCPA</td>
<td>Ribonucleotide-transport ATP-binding protein ABC transporter</td>
<td>mkl</td>
<td>MAP4129</td>
<td>MAH_3912</td>
<td>MSMEG_1366</td>
<td>37</td>
<td>5.27</td>
<td>Cell wall, hypothetical protein</td>
<td>Provision of energy for cell membrane transport of ribonucleotides</td>
<td>ND</td>
</tr>
<tr>
<td>Q9RA4_MYCPA</td>
<td>35kDa protein</td>
<td>mnp</td>
<td>MAP2121c</td>
<td>MAH_1673</td>
<td>MSMEG_4537</td>
<td>34</td>
<td>4.96</td>
<td>Cell surface</td>
<td>Involvement in bacterial invasion of epithelial cells</td>
<td>Yes</td>
</tr>
<tr>
<td>R4N1Q_MYCPA</td>
<td>Phosphoribosylaminomimidazole-succinocarboxamide synthase</td>
<td>purC</td>
<td>MAP0614</td>
<td>MAH_0633</td>
<td>MSMEG_5841</td>
<td>33</td>
<td>4.92</td>
<td>Cell wall</td>
<td>Bacterial replication through biosynthesis of purine</td>
<td>ND</td>
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<tr>
<td>R4NAF7_MYCPA</td>
<td>Transcriptional regulatory protein</td>
<td>marR</td>
<td>MAP1205</td>
<td>MAH_0065</td>
<td>MSMEG_3147</td>
<td>41</td>
<td>6.0</td>
<td>Cell surface</td>
<td>Involvement in protein maturation through chaperone-like function</td>
<td>ND</td>
</tr>
<tr>
<td>Q8VU82_MYCPA</td>
<td>Fibrinectin-binding antigen 85 complex B</td>
<td>fbpB</td>
<td>MAP1609c</td>
<td>MAH_2470</td>
<td>MSMEG_2078</td>
<td>35</td>
<td>5.33</td>
<td>Cell wall and surface</td>
<td>Biosynthesis of cell wall lipids</td>
<td>Yes</td>
</tr>
<tr>
<td>R4N4U1_MYCPA</td>
<td>Acyl-ACP desaturase</td>
<td>desA2</td>
<td>MAP2598c</td>
<td>MAH_1084</td>
<td>MSMEG_5248</td>
<td>31</td>
<td>4.86</td>
<td>Cell wall</td>
<td>Biosynthesis of cell wall mycolic acids</td>
<td>Yes</td>
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<tr>
<td>R4N381_MYCPA</td>
<td>Transcriptional regulatory protein</td>
<td></td>
<td>MAP0035</td>
<td>MAH_0065</td>
<td>MSMEG_0052</td>
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<td>5.40</td>
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<td>R4MZS5_MYCPA</td>
<td>Hypothetical protein</td>
<td></td>
<td>MAP1233</td>
<td>–</td>
<td>–</td>
<td>26</td>
<td>5.38</td>
<td></td>
<td></td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not described and tested as candidate antigen.
three proteins, ribonucleotide-transport ATP-binding protein ABC transporter, citrate synthase II and glyceraldehyde 3-phosphate dehydrogenase were found in spot 6 and 35 kDa protein, phosphoribosylaminoimidazole-succinocarboxamide synthase and malate dehydrogenase were both present in spot number 8. Identification of more than one protein in single spots excised from 2D gels was in agreement with other studies [23, 28, 29].

Fig. 4. Assessment of immunogenicity of recombinant MAP proteins against polyclonal antibodies. Immunoblot analysis of MAP SdhA, FadE25_2 and DesA2 recombinant proteins (indicated by arrows) with rat anti-SdhA (a), anti-FadE25_2 (b) and anti-DesA2 (c) polyclonal antibodies respectively.

Fig. 5. Assessment of specificity of polyclonal antibodies to MAP compared to other mycobacterial species. Immunoblot analysis with rat anti-SdhA (a), rat anti-FadE25_2 (b) and rat anti-DesA2 (c) antibodies against MAP, MAH and MS cell envelope proteins (indicated by arrows). Note: antibodies reacted with MAP-specific proteins with no or minimal cross-reactivity with MAH and MS.
While the gene loci of the proteins in the MAP cell envelope identified in this study are known, often little is known about their functional roles. For this reason, protein function is often inferred by knowledge of similar proteins in other mycobacterial species including MTB. In many instances, identified proteins play essential roles in maintaining viability of mycobacterial species and/or show evidence of immunogenicity. The following are brief descriptions of the functional roles of the MAP cell envelope proteins identified in this study.

Succinate dehydrogenase iron-sulfur subunit (SdhA; spot numbers 1 to 3): In MTB, succinate dehydrogenase (along with fumarate reductase) is essential in maintaining the proper growth rate by fine-tuning the respiratory rate, in allowing MTB to adapt to hypoxic environments, in maintaining the membrane potential, in synthesizing ATP and for bacterial persistence [30].

Acyl-CoA dehydrogenase (FadE25_2; spot numbers 4 and 5 and FadE3_2; spot number 7) is an enzyme that participates in the first dehydrogenase step of the \( \beta \)-oxidation pathway in cholesterol metabolism to produce carbon as an energy source [31, 32]. This may be essential for MAP survival and persistence in the host, particularly dependence on cholesterol for growth within macrophages, under nutrient-deprived conditions [16, 31, 32]. FadE3_2 significantly induced INF-\( \gamma \) immune responses in sheep subclinically infected with MAP in comparison with healthy animals [33].

Tryptophan synthase beta chain (TrpB spot 5) is an integral component of the tryptophan synthase complex with involvement in the last step of \( L \)-tryptophan biosynthesis by catalysing the condensation of indole and serine to form tryptophan. This biosynthetic pathway is present in prokaryotes and absent in mammals and infectivity is attenuated in \( M. tuberculosis \) organisms lacking tryptophan biosynthesis [34].

Ribonucleotide-transport ATP-binding protein ABC transporter (Mkl; spot number 6) is encoded by the MAP4129 gene locus however, little is known about its functional role. In MTB, Rv0655ORF encodes the mkl protein which is necessary for the survival of MTB within macrophages in infected mice [35].

Citrate synthase II (CitA; spot number 6): Overexpression of CitA increases the growth rate of MTB under hypoxic and iron-limited stress conditions by diverting acetyl CoA into the TCA cycle and reducing triacylglycerol (TAG) synthesis [36]. Thus, CitA may play a vital role in mycobacterial replication under favourable conditions and help bacterial spread within the host. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; spot number 6) is a glycolytic enzyme that, through an alternative pathway, helps cells to acquire iron.

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**Fig. 6.** Assessment of specificity of polyclonal antibodies to \( M. avium \) subsp. \( paratuberculosis \). (a) 2-DE proteome map of \( M. avium \) subsp. \( paratuberculosis \). (b–d) Immunoblot (2D-PAGE) of cell envelope proteins of MAP probed with rat anti-SdhA (b), rat anti-DesA2 (c) and rat anti-FadE25,2 (d) polyclonal antibodies.
by acting as a transferrin receptor [37, 38]. Transferrin receptors are essential in importing iron into M. avium vacuoles from macrophages [39].

Transcriptional regulatory protein (MAP0035 loci; spot number 10) MAP0035 shares 85% amino acid identity with Rv0023 (transcriptional regulatory protein) of MTB. Overexpression of MTB Rv0023 results in up-regulation of ndh [40], a gene that is essential in replenishing the NAD+ pool necessary for cellular oxidation [41] in hypoxic, non-replicating MTB. 35 kDa protein, major membrane protein (MMP, spot number 8) is located on the MAP cell surface and is involved in bovine epithelial cell invasion [42]. MMP expression is increased under stress conditions, such as low oxygen levels and high osmolality, encountered by MAP in the intestinal environment [42]. In calves experimentally infected with MAP, serum antibodies against this protein have been detected as early as 1 month post-infection [43].

Phosphoribosylaminomimidazole-succinocarboxamide syn- thase (SAICAR; spot 8): SAICAR, encoded by the purC gene, is an intermediate in purine nucleotide biosynthesis [44] and has been previously reported in mycobacterial cell envelopes [45]. Inactivation of purC by mutation in M. bovis BCG and M. tuberculosis 103 affects growth of these mutant strains in inactivated murine macrophage [46]. Malate dehydrogenase (Mdh; spot number 8) is a MAP species-specific enzyme that catalyses the conversion of malate to oxaloacetate in the citric acid cycle [47]. Mdh is antigenic and serum antibodies to mdh from cows clinically infected with MAP has been documented [47] suggesting its potential utility for new JD diagnostics. ATP synthase gamma chain (atpG; spot number 9) is an essential component of the F0F1 ATP synthase complex that is crucial in generating ATP and plays a key role in activity, assembly or stability of mycobacteria [27]. Hypothetical protein (MAP1233 loci; spot number 10): BLAST analysis revealed protein sequence similarity with the methyltransferase FkBM family that are associated with cell wall biogenesis and remodelling, however, further functional studies are required. Fibronectin-binding antigen 85 complex B (FbpB; spots 11, 12 and 13) has 80–83% homology with M. bovis, M. leprae and M. tuberculosis [48]. Immunization of goats and calves with a cocktail of recombinant proteins containing fbpB85B induced Th1 immune responses and conferred protection against MAP infection [49, 50]. Acyl-ACP desaturase (DesA2; spot number 13); functional loss ofDesA2 protein by conditional mutations and gene disruptions affects the viability of pathogenic mycobacteria and is necessary for their survival [51, 52]. In silico analysis indicates that DesA2 carries several T and B cell epitopes and is an immunogenic protein [53]. Indeed, sheep with MAP infection develop lymphoproliferative and IFN-γ responses and serum antibodies to DesA2 [53].

To assess the relevance of these results, we cross-referenced our findings with three other studies of mycobacterial species, the intent of which was to identify total MAP cell surface, cell wall and envelope and MAH cell surface proteins [7, 13, 54]. Comparison showed that the proteins that we identified are located on the cell surface, cell wall and cell envelope complexes suggesting that the protein extraction method used in this study was effective in isolating and enriching MAP cell envelope proteins for proteomic analysis. However, some of the proteins that we identified (i.e. SdhA, Gapdh, FbpB, FadE25_2, FadE3_2 and Mdh) have been reported to exist in multiple cellular locations (i.e. in the secretome and cytoplasm) [55, 56]. This may be due to undefined translocation mechanisms as explained in earlier studies [55] and suggests that these proteins may have more than one function by acting as so-called ‘moonlighting proteins’ [57]. Moonlighting properties of highly conserved glycolytic, metabolic enzymes and bacterial adhesion proteins are well documented and are essential for bacterial survival [58]. For example, antigen 85B (FbpB) is involved in multiple functions such as mycobacterial cell wall synthesis, bacterial adhesion and pathogenesis. The translocation mechanism of moonlighting proteins is currently not fully understood as these proteins often have diverse and unrelated conformations. However, several hypotheses have been proposed [57]. One hypothesis is that moonlighting proteins are secreted by as yet uncharacterized mechanisms involving post-translational modifications with subsequent binding to the cell surface as anchorless proteins. Other evidence suggests moonlighting proteins detach or leach from dead or stressed cells and subsequently re-associate on the cell surface. It is possible that some MAP proteins may have been released during cell lysis and/or cell death with subsequent attachment to the envelope complexes. There are limited reports available on moonlighting properties of mycobacterial proteins and further studies are warranted.

Because one antigen may not be immunogenic throughout the course of MAP infection [23] serum samples are often pooled for immunoproteomic analysis [55, 59]. In view of the fact that the JD-positive cattle and experimentally infected calves from which serum was obtained for this study were at different stages of development of JD, the serum was pooled in order to improve the efficiency of detection of antigenic MAP cell envelope proteins.

Mkl, Gapdh, FadE3_2 and MoxR1 reported in the present study have been shown to be immunoreactive by previous proteomic studies of MAP and M. tuberculosis [47, 59, 60]. Moreover, some of the proteins we identified including Mdh, FadE3_2 and DesA2 have been reported as MAP-specific based on comparative proteomic analysis [23, 61] and in silico epitope prediction [53]. While differences in spot coordinates on 2D gels do not confirm species specificity of MAP cell envelope proteins, the structural changes of some of the proteins may be profound enough to prevent cross-reactivity of antibodies. Thus, our proteomic data provide a list of candidate MAP cell envelope proteins that we hypothesized could potentially stimulate the production of antibodies that would be able to distinguish MAP from other environmental mycobacteria.
To test this hypothesis, six proteins, i.e. SdhA, FadE25_2, FadE3_2, Mkl, hypothetical protein (MAP1233) and DesA2, were recombinantly expressed, three of which (SdhA, FadE25_2 and DesA2) were of sufficient quality and quantity for polyclonal antibody generation in rats. Our 2-DE immunoblot analysis of MAP cell envelope protein extracts confirmed that antibodies against SdhA, FadE25_2 and DesA2 detected their MAP target antigens at the expected pI and molecular weights. More importantly, 1-DE results indicated that these polyclonal antibodies detected each of the recombinant proteins with very minimal or no cross-reactivity with MAH and M. smegmatis cell envelope proteins. This reflects the antigenic distinctiveness of SdhA, FadE25_2 and DesA2 within MAC cell envelope proteins. Anti-SdhA polyclonal antibodies showed slight cross-reactivity with MAH cell envelope proteins suggesting that MAP SdhA protein may contain some conserved regions and/or epitopes that are shared with MAH SdhA proteins or other proteins of similar molecular weight.

There are some limitations in the experimental approaches used in this study. We recognize that 2D gel electrophoretic separation of proteins with LC tandem MS may reveal only a fraction of the proteins that are unique to the MAP cell envelope proteins. However, in view of our study objective, identification of antigenic MAP cell envelope proteins by immunoblot analysis of gels was more advantageous and appropriate than using a gel-free proteomics approach. In addition, in vitro culture of MAC bacteria examined in this study may have resulted in protein expression that is different from that which occurs in the host. Moreover, pooling of serum samples from various individual cattle eliminated the opportunity to examine the immunoreactivity of MAP cell envelope proteins in individual cattle. Because our comparison was limited to three MAC bacterial species, it is possible that some of the proteins identified as MAP-specific may share epitopes with other mycobacterial species not examined in this study. In view of this, a shot gun proteomic analysis of several mycobacterial bacterial species would complement the gel-based proteomics approach used in this study, however, antigenic determination would still be problematic.

In conclusion, MAP-specific proteins and candidate antigens were identified using a proteomic approach involving 2D-DIGE and immunoblotting with sera from JD-positive and experimentally infected animals. Polyclonal antibodies were generated to three recombinantly expressed antigenic MAP cell envelope proteins (SdhA, FadE25_2 and DesA2). Subsequent immunoblotting confirmed that these antibodies reacted specifically to MAP proteins SdhA, FadE25_2 and DesA2 with very minimal cross-reactivity with MAH and M. smegmatis cell envelope proteins. The utility of recombinantly expressed SdhA, FadE25_2 and DesA2 and associated polyclonal antibodies in identifying MAP infection is currently being investigated in dairy cows.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

References
17. Gundry RL, White MY, Murray CI, Kane LA, Fu Q et al. Preparation of proteins and peptides for mass spectrometry analysis in a...
bottom-up proteomics workflow. Curr Protoc Mol Biol 2009;Chap-
ter 10:Unit10.25.

18. Keller A, Nesvizhskii AI, Kolker E, Aebersold R. Empirical statisti-
cal model to estimate the accuracy of peptide identifications
made by MS/MS and database search. Anal Chem 2002;74:5383–
5392.

19. Nesvizhskii AI, Keller A, Kolker E, Aebersold R. A statistical
model for identifying proteins by tandem mass spectrometry. Anal

20. Sambrook J, Russell DW. The Inoue method for preparation and
transformation of competent E. coli: “Ultra Competent” cells. CSH

21. Walls D, Loughran GT. Purification of poly-histidine-tagged pro-

22. Simpson RJ. Rapid coomassie blue staining of protein gels. Cold

23. Hughes V, Bannantine JP, Denham S, Smith S, Garcia-Sanchez A
et al. Immunogenicity of proteome-determined Mycobacterium
avium subsp. paratuberculosis-specific proteins in sheep with par-

24. Henderson B, Martin A. Bacterial virulence in the moonlight: mul-
titasking bacterial moonlighting proteins are virulence determi-


of protein post-translational modifications in bacteria. J Proteomics

27. Facciulo A, Mutharia LM. Mycobacterial glycoproteins: a novel
subset of vaccine candidates. Front Cell Infect Microbiol 2014;4:
133.

High resolution quantitative proteomics of HeLa cells protein spe-
cies using stable isotope labeling with amino acids in cell culture
(SILAC), two-dimensional gel electrophoresis(2DE) and nano-liquid
chromatography coupled to an LTQ-OrbitrapMass spectrometer.

Descriptive proteomic analysis shows protein variability between
closely related clinical isolates of Mycobacterium tuberculosis.

Role of intragenic binding of cAMP responsive protein (CRP) in regu-
lation of the succinate dehydrogenase genes Rv0249c-Rv0247c in

The reactivity of Mycobacterium avium subspecies paratuberculosis
from the intestine of infected ruminants for proteomic evaluation.

32. Griffin JE, Gawronski JD, DeJesus MA, Loerger TR, Akerley BJ
et al. High-resolution phenotypic profiling defines genes essential
for mycobacterial growth and cholesterol catabolism. PLoS Pathog
2011;7:e1002251.

33. Hughes V, Denham S, Bannantine J, Chianini F, Kerr K et al.
Interferon gamma responses to proteome-determined specific
recombinant proteins: potential as diagnostic markers for ovine

34. Shen H, Yang Y, Wang F, Zhang Y, Ye N et al. Characterization of
the putative tryptophan synthase B-subunit from Mycobacterium

35. Content J, Peirs P. The ABC transporter systems. The Mycobacterial
199.

36. Sassetti CM, Boyd DH, Rubin EJ. Genes required for mycobacte-
rial growth defined by high density mutagenesis. Mol Microbiol

37. Raje CL, Kumar S, Harle A, Nanda JS, Raje M. The macrophage
cell surface glyceraldehyde-3-phosphate dehydrogenase is a

38. Kumar S, Sheokand N, Mhadeshwar MA, Raje CL, Raje M. Charac-
terization of glyceraldehyde-3-phosphate dehydrogenase as a
199.

39. Lombard JE, Garry FB, McCluskey BJ, Wagner BA. Risk of
removal and effects on milk production associated with paratu-

and manipulating the Mycobacterium tuberculosis transcriptome
using a transcription factor overexpression-derived regulatory

41. Rao SP, Alonso S, Rand L, Dick T, Pethe K. The protonotive
force is required for maintaining ATP homeostasis and viability
of hypoxic, nonreplicating Mycobacterium tuberculosis. Proc Natl Acad

42. Bannantine JP, Huntley JF, Milten E, Stabel JR, Bermudez LE.
The Mycobacterium avium subsp. paratuberculosis 35 kDa protein
plays a role in invasion of bovine epithelial cells. Microbiology

43. Määttänen P, Trost B, Scuten E, Potter A, Kuslik A et al. Diver-
gent immune responses to Mycobacterium avium subsp. para-
tuberculosis infection correlate with korne responses at the site of

44. Brown AM, Hoopes SL, White RH, Sarisky CA. Purine biosynthesis

45. Marques MAM, Neves-Ferreira AGC, da Silveira EKX, Valente RH,
Chapeaurouge A et al. Deciphering the proteomic profile of Myco-

46. Jackson M, Phalen SW, Lagranderie M, Ensergueix D, Chavarot P
et al. Persistence and protective efficacy of a Mycobacterium

47. Piras C, Soggiu A, Bonizz L, Greco V, Ricchi M et al. Identification
of immunoreactive proteins of Mycobacterium avium subsp. para-

Evaluation of immune responses and protective efficacy in a
mouse model following immunization with a cocktail of recombinant
antigens and a polypeptide of Mycobacterium avium subsp. paratu-

49. Kathaperumal K, Kumanan V, McDonough S, Shen LH, Park SU
et al. Vaccine immunization with recombinant Mycobacterium avium
subsp. paratuberculosis proteins induces differential immune responses
and protects calves against infection by oral challenge. Vaccine

50. Wolfe LM, Veeraraghavan U, Idicula-Thomas S, Schüer S,
Wennerberg K et al. A chemical proteomics approach to profiling
the ATP-binding proteome of Mycobacterium tuberculosis. Mol Cell

51. Dyer DH, Lyle KS, Raymond I, Fox BG. X-ray structure of putative
acyl-ACP desaturase DesA2 from Mycobacterium tuberculosis

52. Gurung RB, Purdie AC, Begg DJ, Whittington RJ. In silico identifi-
cation of epitopes in Mycobacterium avium subsp. paratuberculosis
proteins that were upregulated under stress conditions. Clin

53. Leite FL, Reinhardt TA, Bannantine JP, Stabel JR. Envelope pro-
tein complexes of Mycobacterium avium subsp. paratuberculosis


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