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Proteomic Characterisation of Two Strains of *Mycoplasma mycoides* subsp. *mycoides* of Differing Pathogenicity

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Abstract

Mycoplasma mycoides subsp mycoides (*Mmm*) is a major pathogen of cattle, causing contagious bovine pleuropneumonia (CBPP), a severe–frequently fatal-disease that is widespread in sub-tropical countries. Despite recognition as a pathogen in the 19th century and advances made through genome sequencing and synthetic biology, many of the molecular characteristics of *Mmm* remain poorly characterized. Using a proteomics approach, this study aimed to define *Mmm* major protein determinants, including surface membrane proteins and those involved in host colonization and pathogenicity. For this purpose, both whole cells and a Triton X-114 enriched membrane fraction of a highly pathogenic African field strain (N6) and a vaccine strain (KH3J) were analysed and compared using gel-based methodologies i.e. 2-DGE and SDS-PAGE in combination with MALDI-MS/MS and LC-ESI-MS/MS.

A total of 318 proteins, (around 31% of the predicted proteome) were identified, comprising lipoproteins, prolipoproteins and hypothetical proteins with predicted membrane locations and possible roles in pathogenicity. One hundred and forty-five of these proteins, with different predicted sub-cellular locations and functions but also including uncharacterized proteins, have not previously been reported as expressed by *Mmm*. Many more proteins were detected by LC-ESI-MS/MS (n=315) than by 2-DGE and MALDI-MS/MS (n=52), however 3 proteins were detected only by the latter approach.

Most proteins were observed to be common to both strains although a substantial number of proteins were detected exclusively in only one strain with possible implications for fitness and pathogenicity. To our knowledge, this study represents the most extensive proteomics characterization of *Mmm* to date and has identified characteristics on which to base future studies of the physiology and pathogenicity of this prokaryote.

Keywords: Contagious bovine pleuropneumonia; Mycoplasma mycoides subsp. Mycoides; 2-DGE; LC-ESI-MS/MS; Protein identification

Abbreviations: Mmm: Mycoplasma mycoides subsp. mycoides; CBPP: Contagious Bovine Pleuropneumonia; OIE: Organisation Internationale de la Sante Animal (World Organisation for Animal Health)

Introduction

Members of the class Mollicutes are recognised as the simplest prokaryotic organisms capable of culture outside their host species. Although capable of growth on laboratory media, these organisms are highly fastidious and dependent on their hosts to supply many essential nutrients as a result of loss of many metabolic pathways during adaptation to their hosts; correspondingly, these organisms have reduced genome–and hence, proteome–content. Major genera of Mollicutes include *Acholeplasma*, *Spiroplasma*, *Ureaplasma* and *Mycoplasma*, which all show dependence upon their hosts to provide an environment suitable for their survival. Among these diverse groups, *Mycoplasma* species are common causes of infection of animals where they can colonise mucous membranes of respiratory or urogenital tracts. Typically infection is persistent although some *Mycoplasma*s are associated with severe, acute diseases often resulting in a high rate of mortality. Mycoplasmas belonging to the "*Mycoplasma mycoides* cluster" represent a group of pathogens affecting ruminants that share phenotypic and genetic characteristics. Within these is *Mycoplasma mycoides* subsp. *mycoides* small-colony type (*Mmm*) which is the aetiological agent of contagious bovine pleuropneumonia (CBPP), a highly contagious respiratory disease affecting cattle and buffaloes [1].

CBPP is currently absent from Europe and the USA but outbreaks have occurred in southern Italy (1990-1993), Spain (1994) and Portugal (1999). CBPP is now considered the most significant disease of cattle in Africa–particularly sub-Saharan regions where it can cause high morbidity and mortality and great economic losses [2,3]. CBPP is included in the list of animal diseases that must be reported to the World Organization for Animal Health (OIE) and international regulations impose restrictions on cattle trade from infected territories.

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CBPP is therefore a disease of considerable socioeconomic importance in developing countries.

Despite the significance of CBPP, much is unknown about the molecular determinants of colonisation, pathogenesis and virulence of *Mmm*. Approaches for the control of CBPP remain dependent upon serodiagnostic tools and vaccines which are considered unreliable. Development of better control tools can be gained through increasing the understanding of genome and proteome content of these pathogens and related investigations of immune responses [4,5]. The sequencing of the *Mmm* type strain PG1 genome [6] opened the way to post-genomic research and a better understanding of *Mmm* function and pathogenic mechanisms. The *Mmm* PG1 genome is characterized by a single circular chromosome of 1,211,703 bp with a very low G + C content (24 mole %) and encodes 1017 putative proteins (NC_005364.2; http://www.ncbi.nlm.nih.gov/bioproject/10616). Recently, a second *Mmm* genome of the Australian strain Gladysdale has been annotated (Accession No. CP002107; http://www.ncbi.nlm.nih.gov/bioproject/27713). This genome is 1,193,808 bp, encoding for 1068 putative proteins and it is highly similar to *Mmm* PG1 strain, differing mainly in single nucleotide polymorphisms [7]. The *Mmm* genome contains a very high density of insertion sequences (13% of the genome size) and this has given rise to some genetic variation between strains and duplication of regions. Of note, the *Mmm* genomes possess several large repeated regions which confer some inter-strain variability [8] resulting in duplication and attrition of some genes leading to some genomic divergence particularly in regions adjacent to insertion sequences.

A variety of potential fitness, immune evasion and virulence factors have been identified through genomic and functional analyses. Defining the proteome of an organism is pivotal to defining its biological activities and interactions with its host species.

Previous studies applied proteomics approaches to characterize differences in protein content during adherent compared to planktonic growth of *Mmm* [9]. In a more recent study Krasteva et al. [10] used polyacrylamide gel electrophoresis and mass spectrometry to characterize the Triton X-114 soluble proteome of nine *Mmm* strains including T1/44 vaccine, PG1 reference and field strains from Europe and Africa: a total of 250 proteins were identified. Further, immunoproteomics approaches looking at seroreactive proteins, identified a total of thirty seven proteins [11]. *Mmm* recombinant protein arrays have also been used to systematically survey immunogenicity of selected proteins [12]. Even if providing relevant information, none of these studies incorporated a highly detailed survey of *Mmm* proteomes and hence this work aimed to provide a more extensive characterization of the protein profiles of *Mmm* strains.

The study focused on two *Mmm* strains KH3J and N6 originating from African countries (Sudan and Botswana respectively) and isolated five decades apart (1940 and 1996 respectively). KH3J has been used as a vaccine strain and is of low pathogenicity whilst N6 is a field isolate of high pathogenicity [13-17]. Complementary gel-based and liquid chromatography-based approaches were utilized for characterisation of the entire proteomes of two *Mmm* strains differing in virulence.

Materials and Methods

Strains and growth conditions

The two *Mmm* African strains N6 and KH3J were obtained from laboratory stock cultures stored at -70°C in Eaton broth and grown in Eaton broth at 37°C in an atmosphere containing 5% CO_2 as described previously [9]. Cells from 1,000 ml cultures were harvested by centrifugation at 8,000×*g* for 40 min at 4°C. Cell pellets were subsequently washed 3 times with a semi-defined medium [18] without serum to remove any remaining medium components-mainly serum proteins. *Mycoplasma* cells were harvested by centrifugation and the cell pellet was re-suspended in 200 ml of fresh semi-defined medium then incubated at 37°C overnight into log phase. The cells were separated from this medium by centrifugation at 8,000×*g* for 40 min at 4°C. The supernatant was carefully removed and placed in a sterile bottle; the pellets were washed 3 times with PBS then whole cell pellets and supernatants were stored at -70°C until required.

Two-dimensional gel electrophoresis and Image analysis

Whole cell lysates of N6 and KH3J were prepared by mechanical disruption of *Mycoplasma* pellets resuspended in PBS using a Ribolyser™ (Hybaid) and 2-DGE was carried out using Ettan IPGphor and Multiphor II systems (GE Healthcare). Samples were subjected to 2-D Clean-Up Kit (GE Healthcare) according to the manufacturer's instructions. Protein samples were thoroughly solubilised in isoelectric focusing buffer consisting of 8 M urea, 4% v/v CHAPS, 40 mM Tris Base, Protease inhibitor cocktail (Roche), 1% DTT w/v, 1% v/v IPG buffer pH 3-10 non linear (GE Healthcare) and 0.025% w/v bromophenol blue. Insoluble material was removed by centrifugation at 15,000×*g* for 15 min.

Protein concentration was determined using a Bicinchoninic acid (BCA) [19] assay, reading absorbance at 562 nm using a Dynatech MR 5,000 plate reader. BSA was used to prepare a concentration standard curve.

Proteins (500 µg) were separated by IEF on pH 3-10 non linear IPG strips (18 cm) (GE Healthcare), which were rehydrated for 12 h and focused for 30,000 Vh. Prior to electrophoresis in the second dimension, strips were equilibrated for 15 min in equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS, bromphenol blue w/v 0.002%) containing 1% w/v DTT. This step was repeated using equilibration buffer supplemented with 4% v/v iodoacetamide. Strips were applied to 12-14% pre-cast (245 mm×180 mm×0.5 mm) gels (GE Healthcare). Electrophoresis was performed at 600V/20 mA/40W for 15-20 min then at 40 mA per gel at 12°C until the dye front reached the bottom of the gel. Gels were fixed in 40% v/v methanol, 10% v/v acetic acid for 1 h and then stained overnight in Coomassie Brilliant Blue R (Sigma). Gels were destained in 25% v/v methanol for 1 h. Triplicate biological samples (i.e. different batches of cultures) for each strain were run and analysed using Phoretix 2D software version 2004 (Non-linear Dynamics).

MALDI-MS and MS/MS

For identification of proteins by MALDI-MS/MS spots were excised from the gels and washed three times for 15 min at room temperature in 50 mM ammonium bicarbonate in 50% acetonitrile (ACN) using a vortex mixer. The solution was removed and the gel pieces covered with 100% ACN to dehydrate for 10 min. Supernatant was removed and the gel pieces vacuum-dried for 20 min. The gel spots were then rehydrated in trypsin digest solution (10 ng/µL sequencing grade modified trypsin) in 25 mM ammonium bicarbonate and incubated at 37°C for at least 16 h. Tryptic peptides were applied to a polished stainless steel target plate in a solution of 10 mg/ml α-Cyano-4-hydroxycinnamic acid (CHCA) in 0.1% trifluoroacetate (TFA) and 50% ACN. MS spectra were obtained using an Ultraflex-II TOF/TOF instrument (Bruker Daltonics) operated in the reflectron mode for both MS and MS/MS analyses. Each spectrum was produced by accumulating data from

10×100 consecutive laser shots. Peptides were identified by matching the measured monoisotopic masses to theoretical monoisotopic masses generated using the MASCOT search engine (see below). From protein samples that remained unidentified, selected peptides were fragmented in MS/MS mode. The search parameters were: maximum of one missed cleavage by trypsin, variable modification of: oxidation of methionine, modification of cysteine by propionamidation and carbamidomethylation and a peptide tolerance of ± 50 ppm. Using these parameters and searching the *Mmm* PG1 database, probability scores greater than 43 were considered significant ($p<0.05$).

Phase separation of integral membrane proteins in Triton X-114 solution

Triton X-114 detergent phase fractionation of broth-grown *Mmm* N6 and KH3J was performed [20]. Protein concentrations of *Mycoplasma* cell pellets resuspended in PBS were determined using a Bicinchoninic acid (BCA) as described above. An aliquot of *Mycoplasma* suspension containing 1 mg of protein, was collected, centrifuged at 8,000×*g* for 40 min at 4°C and the cell pellets suspended in 900 µl of cold TS buffer (154 mM NaCl, 10 mM Tris, pH 7.4) containing protease inhibitor cocktail (Roche) and 100 µl of cold 10% (v/v) Triton X-114. The solution was incubated at 37°C for 5 min during which time the solution became cloudy, indicating the condensation of detergent. The samples were then centrifuged for 3 min at 8,000×*g* at room temperature and the aqueous phase removed and discarded. Cold TS buffer was added to the detergent phase and incubated for 3 to 5 min on ice and then incubated at 37°C for 5 min to condense detergent. Samples were centrifuged at 8,000×*g* at room temperature for 3 min and the upper aqueous phase was discarded and detergent phase retained and washes were repeated 3 times. Following the final wash the upper aqueous phase was removed and 900 µl cold methanol was added to the condensed detergent phase. Samples were placed at -70°C overnight, then centrifuged at 12,000×*g* for 10 min in the cold to precipitate proteins; the methanol phase was removed completely and protein pellets dried prior to storing at -70°C.

SDS-PAGE and LC-MS/MS

These procedures were carried out on both *Mycoplasma* whole cells and on Triton-X 114 membrane enriched fraction as described previously in similar surveys of bacterial proteomes [21-25] summarized as follows:

Sample preparation: Approximately 15 µg of each *Mmm* sample material (whole cells and Triton-X 114 membrane enriched fraction) were loaded into single sample wells of a discontinuous Tris/glycine SDS-PAGE mini-gel (4% stacking gel; 12% resolving gel) and separated at 130V (constant voltage) over ~ 90 min using a Mini-Protean™ II Dual Slab Cell (BioRad). Resolved proteins were visualized using SimplyBlue Safe Stain™ (Invitrogen). The stained gel lane was excised and then sliced horizontally from top to bottom to yield a series of ~25 equal gel slices of 2.5 mm deep. Each of the resulting 25 gel slices was then subjected to standard in-gel destaining, reduction, alkylation and trypsin digestion procedures [26].

LC-ESI-MS/MS: Liquid chromatography was performed using an Ultimate 3000 nano-HPLC system (Dionex) comprising a WPS-3000 well-plate micro auto sampler, a FLM-3000 flow manager and column compartment, a UVD-3000 UV detector, an LPG-3600 dual-gradient micropump and an SRD-3600 solvent rack controlled by Chromeleon chromatography software (Dionex). A micro-pump flow rate of 246 µl/min^{-1} was used in combination with a cap-flow splitter cartridge, affording a $^{1}/_{82}$ flow split and a final flow rate of 3 µl/min^{-1} through a 5 cm×200 m ID monolithic reversed phase column (Dionex) maintained at 50°C. Samples of 4 µl were applied to the column by direct injection. Peptides were eluted by the application of a 15 min linear gradient from 8-45% solvent B (80% v/v acetonitrile, 0.1% (v/v) formic acid) and directed through a 3 nl UV detector flow cell. LC was interfaced directly with a 3-D high capacity ion trap mass spectrometer (Esquire HCTplus™, Bruker Daltonics) via a low-volume (50 µl/min^{-1} maximum) stainless steel nebuliser (Agilent, cat. no.G1946-20260) and ESI. Parameters for tandem MS analysis were set as previously described [21-25].

Database mining: Deconvoluted MS/MS data was submitted to an in-house MASCOT server and searched against a fully annotated *Mmm* PG1 genomic database (http://www.ncbi.nlm.nih.gov) using the MASCOT search algorithm http://www.matrixscience.com/. The high extent of genomic similarities among *Mmm* strains makes the PG1 genome a relevant resource for assigning protein identities. The presentation and interpretation of MS/MS data was performed in accordance with published guidelines [27]. To this end, fixed and variable modifications selected were carbamidomethyl (C) and oxidation (M) respectively and mass tolerance values for MS and MS/MS were set at 1.5 Da and 0.5 Da respectively. Molecular weight search (MOWSE) scores attained for individual protein identifications were inspected manually and considered significant only if: a) two peptides were matched for each protein, and b) each peptide contained an unbroken "*b*" or "*y*" ion series of a minimum of four amino acid residues.

Results and Discussion

Contagious bovine pleuropneumonia (CBPP) is recognized as one of the major constraints on cattle welfare and productivity in many sub-tropical countries, especially in Africa. Despite reportedly being first isolated as the etiological agent of CBPP as early as 1896 [28], much about the physiology and pathogenicity of *Mmm* remains poorly defined. There is, therefore, continuing need for improved phenotypical characterization and understanding of these organisms in order to advance novel control measures. In the present investigation we have taken dual approaches of 2-DGE combined with MALDI-MS/MS and SDS-PAGE combined with LC-ESI-MS/MS to characterize the entire proteomes of two *Mmm* strains, this representing the most extensive proteome survey to date of these organisms.

For this purpose we have selected one strain of low pathogenicity (KH3J) and another of high pathogenicity (N6), both originally isolated in African countries. Strain KH3J was originally isolated in Sudan in 1940 and has been used as a vaccine strain on account of its low pathogenicity whereas strain N6 is a more recent field isolate (1996) from Botswana and retains high pathogenicity [17]. Although *Mmm* strains show some genomic differences-especially between strains of African, Australian and European origins [29,30]-strains from diverse origins remain antigenically similar [31]. Of note, genotypic characteristics of these strains are similar to that of the reference strain PG1 [32] which provides the original genome sequence [6]; furthermore, the recent formal release of a second *Mmm* genome for strain Gladysdale [7] defines only SNPs as genomic differences between strains. This was further confirmed by recent phylogenetic analyses conducted using next-generation sequencing technologies that compared *Mmm* strains of different origins [30]. Hence the selected strains are broadly representative of those available in the *Mmm* research community.

Compilation of *Mmm* N6 and KH3J proteins identified

In total, 318 *Mmm* proteins were identified in the two strains through the combined methodologies employed in this study (Supplementary Table 1). One hundred and forty-five out of the 318 proteins identified, with different predicted sub-cellular location and function, have not ever before been reported as being expressed by *Mmm*. Since the *Mmm* genome contains several large duplicated regions, several proteins are represented by more than one open reading frame encoding identical or indistinguishable proteins–accordingly the numbers of open reading frames from which proteins were detected ranges from 310 to 318. These proteins represent approximately 31% of the deduced proteome of this organism and this survey greatly extends the proteome coverage compared to earlier investigations. Multiple functional classes were represented among the proteins detected and, as is common among global proteome analyses of prokaryotes, proteins of the class "translation, ribosomal structure and biogenesis" were most common (representing approx 21% of proteins detected), followed by "not classified" (approx 14%). A further 4 classes of proteins involved in "transport" and "metabolism" (COG categories G and F, 8% and 7% respectively), "energy production and conversion" (C, 7%), "replication, recombination and repair" (L, 7%) were next most frequently represented as were proteins of "general function prediction only" (approx. 9%). Using PSORTb v2.0 bacterial localization prediction tools to predict sub-cellular location, the majority of detected proteins were predicted to be cytoplasmic (n=114; 36%) or membrane–associated proteins (n=52; 16%). No location was predicted for the remaining proteins (n=152). As anticipated, many more proteins were detected by LC-ESI-MS/MS (n=315) than by 2-DGE (n=52), nonetheless 3 proteins were detected only by the latter and not the former methodology thus re-emphasizing the benefits of complementary methods in characterizing microbial proteomes.

2-DGE electrophoresis and MALDI-MS/MS analyses of *Mycoplasma* whole cells: Representative 2-DGE gels for N6 and KH3J whole cell lysates are shown in Figure 1. A comparison of 2-DGE gel spot volumes using the Kruskal-Wallis Test showed that all p-values were greater than p-calculated (Supplementary table 2), indicating that there is no statistical difference between the spots of the six 2-DGE gels (corresponding to three gels from each of two strains); i.e. spots were consistently observed between gels and between strains.

A total of 113 protein spots were detected on the reference gel by 2D Phoretix software and 81 of these spots were identified by MALDI-MS/MS corresponding to 52 different proteins (Table 1) which were mostly soluble cytoplasmic proteins. In 2-DGE, some proteins (namely putative phosphonate ABC transporter (MSC_0079), NADH oxidase (MSC_0263), pyruvate dehydrogenase lipoamide alpha chain (MSC_0265), pyruvate dehydrogenase (lipoamide) beta chain (MSC_0266), PTS system glucose-specific IIA component (MSC_0274), L-lactate dehydrogenase (MSC_0527), hypothetical protein MSC_0587, glyceraldehyde 3-phosphate dehydrogenase (MSC_0679), glycerone kinase (MSC_0747), triosephosphate isomerase (MSC_0823), thymidine phosphorylase (MSC_0830), purine nucleoside phophorylase (MSC_0835), uracil phosphoribosyltransferase (MSC_0893), ribose-phosphate pyrophosphokinase (MSC_0952) and glucose-inhibited division protein A (MSC_1017)) presented isoforms with charge differences, while others, (namely 30S ribosomal protein S6 (MSC_0027), AhpC/TSA family protein (MSC_0053) and translation elongation factor Tu (MSC_0160)) presented charge and mass variations (Figure 1 and Table 1). In most cases, two or three forms were identified for a given

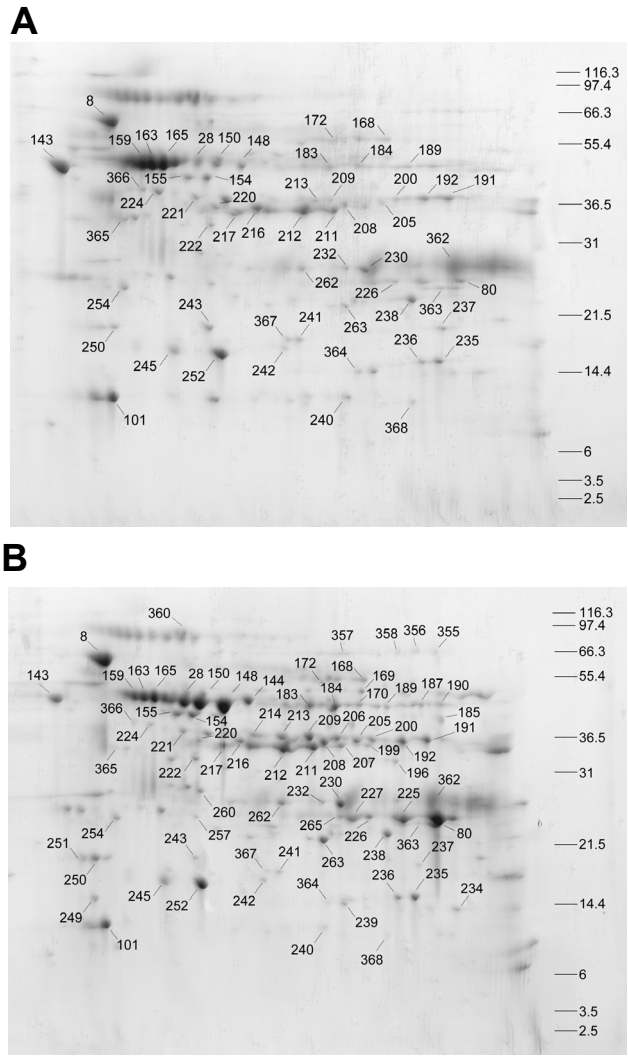

Figure 1: Representative 2-DGE gels for *Mycoplasma mycoides* subsp. *mycoides* strains KH3J-low pathogenicity-(A) and N6-high pathogenicity-(B).

protein, but, in the cases of 30S ribosomal protein S6 (spots 235, 236, 239 and 364), uracil phosphoribosyltransferase (spots 80, 225, 227 and 265), ribose-phosphate pyrophosphokinase (spots 206, 209, 213 and 214), translation elongation factor Tu (spots 159, 163, 165, 365 and 366) and thymidine phosphorylase (spots 28, 144, 148 and 150), a greater number of corresponding spots were found. Migration patterns of isoforms suggest that post-translational modifications could account for observed changes–among these, acylation is widely described in mycoplasmas including *Mmm* [33], protein phosphorylation and acetylation have been noted as common post translational modifications (PTMs) for *M. pneumoniae* [34,35] and can be presumed to be PTMs in *Mmm*. Furthermore, carbamylation of proteins, producing altered isoelectric points ("carbamylation trains") should also be considered [36]. Three proteins, not previously described, were identified through 2-DGE only: MSC_0051 (hypothetical protein) is an uncharacterized conserved protein of unknown function; MSC_0335 (ribosome binding factor) is a cytoplasmic protein essential for efficient processing of 16S rRNA; MSC_0894 (glycine hydroxymethyl transferase) is also a cytoplasmic protein playing an important role in the biosynthesis of

Spot[a]	Locus tag[b]	Protein[c]	MW[d]	pI[e]	Score[f]
235	MSC_0027	30S ribosomal protein S6	16094	6.77	109
236	MSC_0027	30S ribosomal protein S6	16094	6.77	107
239	MSC_0027	30S ribosomal protein S6	16094	6.77	66
364	MSC_0027	30S ribosomal protein S6	16094	6.77	124
154	***MSC_0051***	***Hypothetical protein MSC_0051***	***21553***	***5.59***	***47***
249	MSC_0053	AhpC/TSA family protein	17397	4.82	46
250	MSC_0053	AhpC/TSA family protein	17397	4.82	84
251	MSC_0053	AhpC/TSA family protein	17397	4.82	114
187	MSC_0079	Prolipoprotein, putative phosphonate ABC transport	50087	9.24	63
189	MSC_0079	Prolipoprotein, putative phosphonate ABC transport	50087	9.24	193
365	*MSC_0110*	*Utp-glucose-1 phosphate uridylyltransferase*	*32745*	*5.02*	*129*
196	*MSC_0139*	*Fructose-bisphosphate aldolase class II*	*32766*	*6.32*	*152*
262	*MSC_0149*	*Thymidine kinase*	*23927*	*5.74*	*48*
360	*MSC_0159*	*Elongation factor G*	*76137*	*5.29*	*227*
159	MSC_0160	Translation elongation factor Tu	43277	5.11	190
163	MSC_0160	Translation elongation factor Tu	43277	5.11	138
165	MSC_0160	Translation elongation factor Tu	43277	5.11	209
365	MSC_0160	Translation elongation factor	43277	5.11	92
366	MSC_0160	Translation elongation factor Tu	43277	5.11	72
199	*MSC_0218*	*Guanylate kinase*	*34253*	*6.43*	*143*
170	MSC_0263	NADH oxidase	50133	6.12	102
183	MSC_0263	NADH oxidase	50133	6.12	69
184	MSC_0263	NADH oxidase	50133	6.12	150
206	*MSC_0264*	*Lipoate protein ligase*	*38463*	*6.17*	*121*
154	MSC_0265	Pyruvate dehydrogenase lipoamide, alfa chain;	41849	5.31	186
155	MSC_0265	Pyruvate dehydrogenase lipoamide alfa chain	41849	5.31	199
205	MSC_0266	Pyruvate dehydrogenase(lipoamide), beta chain	36186	5.93	83
208	MSC_0266	Pyruvate dehydrogenase (lipoamide), beta chain	36186	5.93	117
168	*MSC_0267*	*Dihydrolipoamide S-acetyltransferase*	*45798*	*6.22*	*95*
216	*MSC_0269*	*Phosphate acetyltransferase*	*35673*	*5.62*	*69*
242	MSC_0274	PTS system ,glucose-specific IIA component	16849	5.35	70
245	MSC_0274	PTS system, glucose-specific IIA component	16849	5.35	84
252	MSC_0274	PTS system,glucose-specific IIA component	16849	5.35	104
238	*MSC_0276*	*Glycerone kinase*	*22750*	*6.33*	*105*
237	*MSC_0296*	*Transcription elongation factor*	*17439*	*6.77*	*146*
362	*MSC_0301*	*Oxidoreductase*	*26507*	*7.74*	*67*
368	***MSC_0335***	***Ribosome-binding factor A***	***13803***	***6.62***	***98***
357	*MSC_0349*	*Translation initiation factor IF-2*	*68760*	*6.09*	*106*
243	*MSC_0353*	*Hypotetical protein MSC_0353*	*19505*	*5.40*	*87*
254	*MSC_0451*	*Elongation factor P*	*20461*	*4.96*	*45*
263	*MSC_0474*	*Adenine phosphoribosyltransferase*	*19281*	*5.91*	*126*
257	*MSC_0493*	*Copper homeostasis protein*	*25578*	*5.44*	*131*
240	*MSC_0498*	*Histidine triad protein*	*15482*	*6.06*	*53*
169	*MSC_0509*	*Glyceraldehyde - 3-phosphate dehydrogenase (NADP)*	*51944*	*8.35*	*182*
220	MSC_0527	Lipoate-protein ligase A	39777	5.45	122
221	MSC_0527	Lipoate-protein ligase A	39777	5.45	51
207	MSC_0532	L-lactate dehydrogenase	34626	5.86	58
211	MSC_0532	L-lactate dehydrogenase	34626	5.86	119
212	MSC_0532	L-lactate dehydrogenase	34626	5.86	144
241	MSC_0587	Hypothetical protein MSC_0587	16394	5.69	81
367	MSC_0587	Hypothetical protein MSC-0587	16394	5.69	114
143	*MSC_0588*	*Cell division protein FtsZ*	*41467*	*4.45*	*46*
363	*MSC_0600*	*Ribosome recycling factor*	*20799*	*6.85*	*80*
222	*MSC_0607*	*Elongation factor Ts*	*32525*	*5.46*	*149*
185	*MSC_0609*	*Heat shock protein DnaJ(chaperone)*	*41934*	*8.40*	*98*
8	*MSC_0610*	*Heat shock protein 70*	*63915*	*4.9*	*268*
187	*MSC_0678*	*Phosphoglycerate kinase*	*44387*	*6.93*	*137*
191	MSC_0679	Glyceraldehyde 3-phosphate dehydrogenase	36990	7.01	171
192	MSC_0679	Glyceraldehyde-3- phosphate dehydrogenase	36990	7.01	152
200	MSC_0679	Glyceraldehyde-3-phosphate dehydrogenase	36990	7.01	130

217	*MSC_0721*	*DNA-directed RNA polymerase alpha subunit*	*34989*	*5.52*	*229*
224	MSC_0747	Glycerone kinase	35842	5.11	61
366	MSC_0747	Glycerone kinase	35842	5.11	48
172	*MSC_0761*	*Aspartyl/glutamyl-tRNA amidotransferase subunit B*	*56178*	*6.46*	*212*
230	MSC_0823	Triosephosphate isomerase	27417	6.22	155
232	MSC_0823	Triosephosphate isomerase	27417	6.22	60
262	MSC_0823	Triosephosphate isomerase	27417	6.22	81
28	MSC_0830	Thymidine phosphorylase	48896	5.64	224
144	MSC_0830	Thymidine phosphorylase	48896	5.64	220
148	MSC_0830	Thymidine phosphorylase	48896	5.64	237
150	MSC_0830	Thymidine phosphorylase	48896	5.64	228
226	MSC_0835	Purine nucleoside phophorylase	24496	6.23	103
265	MSC_0835	Purine nucleoside phosphorylase	24496	6.23	120
80	MSC_0893	Uracil phosphoribosyltransferase	25531	8.62	117
225	MSC_0893	Uracil phosphoribosyltransferase	25531	8.62	134
227	MSC_0893	Uracil phosphoribosyltransferase	25531	8.62	119
265	MSC_0893	Uracil phosphoribosyltransferase	25531	8.62	60
190	***MSC_0894***	***Glycine hydroxymethyltransferase***	***45660***	***7.17***	***104***
234	MSC_0895	Ribose 5-phosphate isomerase, RpiB	18293	9.20	115
206	MSC_0952	Ribose-phosphate pyrophosphokinase	40104	5.71	81
209	MSC_0952	Ribose-phosphate pyrophosphokinase	40104	5.71	193
213	MSC_0952	Ribose -phosphate pyrophosphokinase	40104	5.71	143
214	MSC_0952	Ribose-phosphate pyrophosphokinase	40104	5.71	186
260	*MSC_0962*	*Transcription antitermination protein nusg*	*24080*	*5.22*	*178*
101	*MSC_1007*	*50S ribosomal protein L7/L12*	*12868*	*4.95*	*132*
205	*MSC_1015*	*Asparagine synthetase Asn A*	*38004*	*6.13*	*120*
355	MSC_1017	Glucose-inhibited division protein A	70697	7.96	196
356	MSC_1017	Glucose-inhibited division protein A	70697	7.96	101
358	MSC_1017	Glucose -inhibited division protein A	70697	7.96	95

Footnotes:
[a]spot number as defined by 2-DE;
[b]locus tag as defined for *M. mycoides* subsp. *mycoides* strain PG1 (accession number NC_005364.2)
[c]protein annotation as defined for *M. mycoides* subsp. *mycoides* strain PG1 (accession number NC_005364.2)
[d]calculated molecular mass (Da) based on annotation for *M. mycoides* subsp. *mycoides* strain PG1 (accession number NC_005364.2)
[e]calculated isoelectric point based on annotation for *M. mycoides* subsp. *mycoides* strain PG1 (accession number NC_005364.2)
[f]MOWSE score obtained through searching MASCOT database for *M. mycoides* subsp *mycoides* strain PG1.

Table 1: Proteins from *Mycoplasma mycoides* subsp. *mycoides* identified by MALDI-MS/MS following separation by 2-DGE. Proteins are ordered by Locus tab (MSC_nnn) and spot numbers are indicated in the leftmost column. Identified proteins represented by a single spot are indicated in *italics*; proteins identified through 2-DGE only are indicated in ***bold italics***.

purines, thymidylate, methionine, and other important biomolecules.

SDS-PAGE and LC-ESI-MS/MS analyses of *Mycoplasma* whole cells and TritonX-114 enriched membrane protein fraction: Whilst the 2-DGE approach is very useful for profiling and comparison of protein content, several classes of proteins - including proteins with extremes of pI and molecular weight, low abundance proteins and integral membrane proteins - tend to be under-represented in 2-DGE analysis [37,38]. The proteins identified in the two *Mmm* strains reflect this anticipated under-representation hence a complementary "shotgun" approach was taken to identify proteins in whole cell and membrane-associated compartments. Representative SDS-PAGE gels for N6 and KH3J whole cells and Triton X-114 membrane fraction are shown in Supplementary Figure 1**.** The application of SDS-PAGE combined with LC-ESI-MS/MS greatly extended the proteome coverage and 315 of the proteins detected were identified by this methodology, including many membrane-associated proteins which tend to be under-represented in 2-DGE. Two hundred and eighty proteins were identified in whole cell lysates using SDS-PAGE and LC-ESI-MS/MS analysis, 49 of which were also identified by 2-DGE and MALDI-MS/MS analysis.

The membrane is the primary point of contact between *Mycoplasma* and their host cells. *Mmm* membrane proteins are recognized by host immune responses and variation in membrane protein content offers a means for evading host immunity [39,40] therefore it is important to determine in detail the protein composition of this bacterial compartment. Membrane-associated proteins were enriched by Triton X-114 fractionation and subsequent SDS-PAGE – LC-ESI-MS/MS analysis identified 81 proteins, 46 of which were also identified in whole cell lysates using the same approach. Among these 46 proteins, common to whole cell lysates and TritonX-114 enriched fraction, 36 were identified only by SDS-PAGE – LC-ESI-MS/MS while 10 proteins were also detected using 2-DGE – MALDI-MS/MS (Supplementary Table 1). Of the 81 proteins identified, twenty were annotated as lipoproteins or prolipoproteins, a number almost equal to that indicated by a recent study [41] in an LC-MS/MS approach however in that study no protein identities were specified. Our results reflect recent findings in Triton X-114 enriched membrane protein fractions of 9 *Mmm* strains where up to 23 lipoproteins were identified [10], including all of the lipoproteins found in the present study. In the current survey, a further 22 proteins within the membrane-enriched fraction were annotated as "hypothetical", 10 of which (MSC_0065, MSC_0107, MSC_0374, MSC_0389, MSC_0457, MSC_0606, MSC_0671, MSC_0708, MSC_0757, MSC_0916; have putative membrane locations as predicted by PsortB (Table 2). These proteins

GI number[a]	Locus tag[b]	Protein[c]	Score[d]	MW[e]	pI[f]	SC[g]
gi|42560565	MSC_0005	Hypothetical purine NTPase	651	42545	9.50	42
gi|42560568	MSC_0008	Ribose/Galactose ABC transporter, permease component II	127	34301	9.95	9
gi|42560571	MSC_0011	Ribose/galactose ABC transporter, substrate-binding component	548	60956	8.99	32
gi|42560573	MSC_0013	Prolipoprotein	165	62638	9.21	7
gi|42560589	MSC_0029	Acyl carrier protein phosphodiesterase	234	22304	5.47	32
gi|42560592	MSC_0032	Hypothetical protein MSC_0032	965	120763	9.29	29
gi|42560595	MSC_0035	Malate permease	79	41593	9.83	7
gi|42560599	MSC_0039	ATP-dependent zinc metallopeptidase FtsH	66	71813	6.24	13
gi|42560622	MSC_0062	Hypothetical protein MSC_0062	112	11218	10.41	25
gi|42560625	MSC_0065	Hypothetical protein MSC_0065	222	31193	9.64	14
gi|42560638	MSC_0079	Prolipoprotein, putative phosphonate ABC transporter	2451	50144	9.24	62
gi|42560662	MSC_0103	Hypothetical protein MSC_0103	600	33237	8.96	47
gi|42560666	MSC_0107	Hypothetical protein MSC_0107	148	82675	9.19	5
gi|42560670	MSC_0111	Hypothetical protein MSC_0111	172	17423	9.28	54
gi|42560697	MSC_0145	Glycerophosphodiester phosphodiesterase	175	28264	8.21	23
gi|42560710	MSC_0158	30S ribosomal protein S7	205	17888	9.99	34
gi|42560712	MSC_0160	Translation elongation factor Tu	564	43391	5.11	29
gi|42560715	MSC_0163	Leucyl aminopeptidase	506	50202	6.02	28
gi|42560736	MSC_0184	Oligopeptide ABC transporter, substrate-binding component	825	119593	8.82	19
gi|42560775	MSC_0225	Spermidine/putrescine ABC transporter permease component	228	37270	9.46	9
gi|42560776	MSC_0226	Spermidine/putrescine ABC transporter, permease and substrate-binding component	87	120224	9.28	3
gi|42560801	MSC_0253	Phosphopyruvate hydratase	265	49620	5.40	22
gi|42560804	MSC_0256	Hypoxanthine phosphoribosyltransferase	114	22184	5.74	26
gi|42560805	MSC_0257	Glycerol uptake facilitator	147	27130	9.56	12
gi|42560806	MSC_0258	Glycerol kinase	177	57524	6.02	21
gi|42560811	MSC_0263	NADH oxidase	301	50418	6.12	24
gi|42560813	MSC_0265	Pyruvate dehydrogenase (lipoamide), alpha chain	235	42191	5.31	25
gi|42560814	MSC_0266	Pyruvate dehydrogenase (lipoamide), beta chain	322	36528	5.93	29
gi|42560815	MSC_0267	Dihydrolipoamide S-acetyltransferase	258	45855	6.22	20
gi|42560819	MSC_0271	Prolipoprotein	95	75375	8.43	9
gi|42560905	MSC_0361	Hypothetical protein MSC_0361	401	41424	8.43	26
gi|42560916	MSC_0374	Hypothetical protein MSC_0374	82	27437	9.87	9
gi|42560929	MSC_0389	Hypothetical protein MSC_0389	199	32752	9.49	28
gi|42560937	MSC_0397	Prolipoprotein	1174	25600	9.58	42
gi|42560944	MSC_0405	Hypothetical protein MSC_0405	141	34320	6.85	11
gi|42560966	MSC_0431	Prolipoprotein	673	40825	9.00	40
gi|42560969	MSC_0434	ABC transporter, permease and ATP-binding componen	127	71336	9.37	5
gi|42560992	MSC_0457	Hypothetical protein MSC_0457	32	201027	9.37	1
gi|42561015	MSC_0480	Fatty acid/phospholipid synthesis protein	504	36936	8.67	46
gi|42561034	MSC_0499	Prolipoprotein	113	80958	6.12	3
gi|42561035	MSC_0500	Hypothetical prolipoprotein	1521	108023	9.14	46
gi|42561054	MSC_0519	Prolipoprotein B	2446	69865	8.73	68
gi|42561065	MSC_0532	L-lactate dehydrogenase	313	34911	5.86	35
gi|42561091	MSC_0558	Sodium:solute symporter family	91	63071	9.63	4
gi|42561100	MSC_0570	Prolipoprotein	135	25758	5.92	23
gi|42561105	MSC_0575	Hypothetical prolipoprotein	444	39186	9.40	29
gi|42561128	MSC_0598	Hypothetical protein MSC_0598	192	172496	9.38	5
gi|42561135	MSC_0606	Hypothetical protein MSC_0606	262	23657	9.60	33
gi|42561146	MSC_0617	Prolipoprotein	611	94536	8.91	20
gi|42561153	MSC_0624	Hypothetical protein MSC_0624	206	57665	9.57	9
gi|42561154	MSC_0625	Prolipoprotein	735	98369	8.72	29
gi|42561156	MSC_0627	Prolipoprotein	1556	96773	8.40	41
gi|42561163	MSC_0635	Prolipoprotein	199	99687	8.70	10
gi|42561181	MSC_0653	Prolipoprotein	339	44220	9.11	30
gi|42561198	MSC_0671	Hypothetical protein MSC_0671	245	36135	9.99	15
gi|42561205	MSC_0679	Glyceraldehyde-3-phosphate dehydrogenase	225	37218	7.01	25
gi|42561216	MSC_0690	Hypothetical protein MSC_0690	133	32756	8.98	21
gi|42561233	MSC_0707	Hypothetical protein MSC_0707	105	17564	9.91	19
gi|42561234	MSC_0708	Hypothetical protein MSC_0708	225	59074	9.61	10

gi|42561236	MSC_0710	Hypothetical protein MSC_0710	793	75095	9.27	40
gi|42561237	MSC_0711	Prolipoprotein	320	29981	7.63	22
gi|42561242	MSC_0716	ABC transporter, permease component (vitamin B12?)	318	38050	9.89	16
gi|42561282	MSC_0757	Hypothetical protein MSC_0757	117	29415	9.22	10
gi|42561300	MSC_0775	Prolipoprotein	1362	83310	9.13	37
gi|42561301	MSC_0776	Conserved hypothetical prolipoprotein	674	90741	9.21	29
gi|42561313	MSC_0790	Alkylphosphonate ABC transporter, substrate-bindin	639	55681	8.88	33
gi|42561327	MSC_0804	ABC transporter, substrate-binding component	975	54136	8.89	35
gi|42561358	MSC_0837	Lysophospholipase	285	35035	9.53	19
gi|42561379	MSC_0860	PTS system, glucose-specific IIBC component	2005	73945	8.10	29
gi|42561392	MSC_0873	PTS system, glucose-specific, IIBC component	1274	73973	8.10	28
gi|42561406	MSC_0888	ATP synthase delta chain	161	20608	9.52	20
gi|42561407	MSC_0889	ATP synthase b chain	340	20456	9.12	33
gi|42561411	MSC_0893	Uracil phosphoribosyltransferase	343	25702	8.62	24
gi|42561429	MSC_0911	CDP diacylglycerol-glycerol-3-phosphate 3-phosphatidyltransferase	309	23516	9.82	23
gi|42561434	MSC_0916	Hypothetical protein MSC_0916	185	64579	9.28	11
gi|42561473	MSC_0957	Hypothetical prolipoprotein	1049	49226	9.11	53
gi|42561520	MSC_1006	50S ribosomal protein L10	360	18124	9.37	33
gi|42561521	MSC_1007	50S ribosomal protein L7/L12	109	12868	4.95	26
gi|42561534	MSC_1021	Prolipoprotein Q	267	52108	9.52	23
gi|42561557	MSC_1046	Prolipoprotein Q	267	52108	9.52	23
gi|42561574	MSC_1066	Putative inner membrane protein translocase component YidC	331	45566	9.90	20

Footnotes:

[a]gene identifier number as defined by NCBI

[b]locus tag as defined for *M. mycoides* subsp. *mycoides* strain PG1 (accession number NC_005364.2)

[c]protein annotation as defined for *M. mycoides* subsp. *mycoides* strain PG1 (accession number NC_005364.2)

[d]MOWSE score obtained through searching MASCOT database for *M. mycoides* subsp. *mycoides* strain PG1.

[e]calculated molecular mass based on annotation for *M. mycoides* subsp. *mycoides* strain PG1 (accession number NC_005364.2)

[f]calculated molecular mass based on annotation for *M. mycoides* subsp. *mycoides* strain PG1 (accession number NC_005364.2)

[g]% sequence coverage

Table 2:. Proteins identified from *Mycoplasma mycoides* subsp. *mycoides* membrane protein-enriched fraction (Triton X-114) analyzed by LC-ESI-MS/MS.

may have roles in pathogenicity and represent important targets for future investigations.

As indicated above, a feature of mycoplasmas, including *Mmm*, is the variable expression of surface proteins. These proteins have been described as important for colonization and adaptation to the host environment at different phases of infection. Phase variation, antigenic variation or epitope masking have been previously described as possible mechanisms used by mycoplasmas to evade the host immune response [42-44].

Among the recognized variable surface proteins of *Mmm*, the proteomics approaches used in this study identified LppA (MSC_0013), LppB (MSC_0519) and LppQ (MSC_1021). It should be noted that while several proteins are annotated as LppA, these actually differ in sequence; *lppB* is present in a single copy; and *lppQ* is present in duplicate in the genome – MSC_1021 and MSC_1046 (both 445 amino acids). Because lipoproteins are known to stimulate the release of proinflamatory cytokines and are strongly immunodominant, they are considered to be prime target antigens for the development of specific and sensitive serological diagnostic tests. LppA is a highly conserved antigen of *Mmm* capable of stimulating both humoral and cell mediated response and as such is a potential candidate for a future CBPP vaccine [45-47]. LppB is expressed in African and Australian but not in European clusters [10,29]. This protein is able to elicit a strong humoral response while its involvement in disease pathogenesis remains to be elucidated. LppQ is a lipoprotein specific to *M. mycoides* subsp. *mycoides*. A combination of specificity and powerful antigenicity saw LppQ exploited in the development of an indirect ELISA [48]. Conversely, immunization of animals with LppQ resulted in an increased susceptibility to CBPP lesion development, suggesting a role for this protein in immunopathology [49].

Noteworthy is the absence of many of the other reported variably expressed membrane proteins including LppC (MSC_0122/MSC_0177), MSC_0117, MSC_0364, MSC_0390, MSC_0809, MSC_0810, MSC_0812, MSC_0813, MSC_0815, MSC_0816, MSC_0817, MSC_0818, MSC_0847, MSC_1005, MSC_1033, MSC_1058. Similarly, results are described in the recent investigation of 9 *Mmm* strains by LC-MS/MS, where these proteins were also absent with the exception of MSC_0364 and MSC_1005 which were identified only in 3 strains, while MSC_0847 was found only in T144 strain [10].

Assuming that the absence of these proteins is not attributable to the limitations of the technique used, these results may reflect the lack of immune or other selective pressures on *Mmm* during growth *in vitro*. A comparative study of the *Mmm* proteome following multiple passages in serial culture would be of value for the purposes of demonstrating qualitative shifts in mycoplasma protein expression during *in vitro* propagation.

Another characteristic feature of the *Mmm* genome is the presence of insertion sequences (ISs) and duplications. Prominent in the genome are three large repetitive regions flanked by ISs [8] and proteins from each of these large repeat regions were detected: PhnD–MSC_0790/MSC_0804; PtsG–MSC-0860/MSC_0873; GalE–MSC_0971/MSC_0978; GlfG–MSC_0977/MSC_0984; AsnA–MSC_1015/MSC_1040; GidA–MSC_1017/1042; LppQ–MSC_1021/MSC_1046. As a result of the very high sequence conservation in the proteins

encoded by these duplicated regions, it is not possible to selectively define from which of the repeated genomic regions the detected proteins are derived. Despite detecting peptides representing these aforementioned repeat region proteins, not all proteins in these regions were detected (Glk (MSC_0863 & MSC_0875), Arc (MSC_0700, MSC_0864 & MSC_0877), MgtA (MSC_0868, MSC_0881, MSC_0907), PncA (MSC_1020 & MAC_1045), OppF (MSC_0183, MSC_0968, MSC_0975, MSC_0983) and PhnB (MSC_0788) and it is therefore unclear whether this represents differential expression of these proteins or a reflection of abundance of these proteins in the proteome milieu.

Among the *Mmm* proteins detected was PtsG (MSC_0860/MSC_0873), encoding a glucose phosphotransferase system permease. A previous study [50] did not detect this protein in strain KH3J using a specific antibody and also could not select KH3J variants that did express PtsG at appreciable levels. Therefore the present study is the first to report that this low pathogenicity strain can express this protein. Whether this represents heterogeneity in this strain through storage and passage in different laboratories, or is another example of a protein variably expressed under adaptive selection remains to be defined. Both *Mmm* strains N6 and KH3J originate from Africa and therefore possess an intact glycerol uptake system encoded by GtsABC (MSC_0516-MSC_0518). However, this component was not detected in this study even though glycerol was a component of the growth medium used in preparing *Mmm* cultures.

A recent comparative analysis of the genomes of *Mmm* and related *Mycoplasma* species within the "*mycoides* cluster" [41] highlighted the presence of frameshifts, substitutions or insertion sequences resulting in pseudogenes in *Mmm*. Despite these alterations, peptides representing five of the pseudogene products were detected in that study and in the present investigation peptides of MSC_0103 (47% sequence coverage) and MSC_0710-0711 (40% and 22% respectively) have been detected indicating that these, too, are expressed by both strains N6 and KH3J, at least during culture of *Mmm in vitro*. In addition, proteins representing additional pseudogene products which were not detected in the study of Thiaucourt et al. [41] were detected in the current study: MSC_0235 (leucyl aminopeptidase; 19% sequence coverage; detected in strain KH3J) and MSC_0242 (Hypothetical NTPase; 55% sequence coverage; detected in strain N6). This raises several possibilities including (i) possible genome sequencing errors leading to artefactual nucleotide substitutions or frameshifts or (ii) potential variation in expression of these genes among strains. Such polymorphisms could lead to functionally relevant inter-strain differences in, for instance, surface lipoprotein (MSC_0103; MSC_0710-0711) expression with consequent effects on pathogenicity and/or immunogenicity. Short sequence polymorphisms are not mutually exclusive with altered protein expression since even single nucleotide alterations can affect protein expression via both mis-sense and non-sense - which of these possibilities operates in *Mmm* requires further systematic investigation of protein expression under selective pressure.

Comparison of the proteomes of *Mmm* N6 and KH3J strains

As indicated above, the total number of proteins identified in *Mmm* strains N6 and KH3J was 318 (including proteins duplicated in the genome). For any given protein, a frequency of occurrence of at least 2 out of 3 biological replicates in any one strain was set as a strict criterion for inclusion. Applying this rule, a total of 239 proteins was shared by both strains of which 53 were detected only in strain N6 and a further 26 only in strain KH3J. However, it should be born in mind that both the *Mycoplasma* culture and the LC-ESI-MS/MS methods used are subject to variation between replicates. Accordingly, these numbers may over-represent proteins that are specific to, or preferentially expressed, in any particular strain. Thus to reduce the likelihood of falsely assigning "strain-specificity" *per se* to any given protein, the inclusion criteria for a strain-specific proteins was adjusted such that any protein occurring at a frequency of only 1 out of 3 biological replicates in either strain was excluded. This increased stringency reduced the number of putative strain-specific proteins to 25 and 15 for strains N6 and KH3J respectively.

Proteins unique to each strain represent multiple functional classes. Different metabolic enzymes were particular to each strain although within an individual strain no clear common metabolic role was apparent. It is noteworthy that 12 and 5 hypothetical proteins

Strain[a]	Locus tag[b]	Protein[c]
N6	**MSC_0004**	**Dimethyladenosine transferase KsgA**
KH3J	***MSC_0008***	***Ribose/Galactose ABC transporter, permease component II***
N6	MSC_0009	Ribose/Galactose ABC transporter, permease component I
N6	**MSC_0013**	**Prolipoprotein**
N6	**MSC_0018**	**Transcriptional regulator, RpiR family**
KH3J	*MSC_0031*	*Heat shock protein (33 kDa chaperonin)*
KH3J	***MSC_0032***	***Hypothetical protein MSC_0032***
KH3J	***MSC_0034***	***D-lactate dehydrogenase***
N6	MSC_0050	Guanosine 5'-monophosphate oxidoreductase
N6	**MSC_0065**	**Hypothetical protein MSC_0065**
N6	MSC_0066	Seryl-tRNA synthetase
KH3J	***MSC_0085***	***tRNA modification GTPase***
N6	**MSC_0096**	**Dephospho-CoA kinase**
N6	**MSC_0105**	**Endodeoxyribonuclease IV**
N6	MSC_0133	Hypothetical protein MSC_0133
N6	**MSC_0153**	**Hypothetical protein MSC_0153**
N6	MSC_0218	Guanylate kinase
KH3J	*MSC_0219*	*DNA methylase*
KH3J	*MSC_0221*	*Translation initiation factor IF-3*
N6	**MSC_0224**	**Spermidine/putrescine ABC transporter ATP-binding component**
N6	MSC_0226	Spermidine/putrescine ABC transporter, permease and substrate-binding component
N6	MSC_0230	Transposase ISMmy1B
KH3J	*MSC_0235*	*Leucyl aminopeptidase*
N6	**MSC_0242**	**Hypothetical protein MSC_0242**
N6	**MSC_0255**	**Hypothetical protein MSC_0255**
N6	**MSC_0256**	**Hypoxanthine phosphoribosyltransferase**
KH3J	***MSC_0257***	***Glycerol uptake facilitator***
KH3J	***MSC_0259***	***Glycerol-3-phospate oxidase***
N6	MSC_0272	Pantetheine-phosphate adenylyltransferase
N6	**MSC_0295**	**Oxidoreductase**
N6	**MSC_0337**	**Riboflavin kinase**
N6	**MSC_0347**	**30S ribosomal protein S15**
KH3J	*MSC_0354*	*Nitroreductase family protein*
N6	**MSC_0387**	**Ribosomal large subunit pseudouridine synthase B**
N6	**MSC_0389**	**Hypothetical protein MSC_0389**
KH3J	***MSC_0405***	***Hypothetical protein MSC_0405***
N6	MSC_0410	DNA-binding protein HU (HB)
KH3J	***MSC_0434***	***ABC transporter, permease and ATP-binding componen***
N6	**MSC_0448**	**Hypothetical protein MSC_0448**
N6	MSC_0458	ThiJ/PfpI family protein
N6	MSC_0461	Hypothetical protein MSC_0461
KH3J	*MSC_0479*	*Ribonuclease III*
KH3J	*MSC_0500*	*Hypothetical prolipoprotein*
N6	MSC_0507	Hypothetical protein MSC_0507

KH3J	***MSC_0526***	***NADH dependent flavin oxidoreductase***
KH3J	***MSC_0533***	***N-acetylglucosamine-6-phosphate deacetylase***
N6	**MSC_0544**	**Dihydrolipoamide dehydrogenase**
KH3J	***MSC_0558***	***Sodium:solute symporter family***
N6	MSC_0560	50S ribosomal protein L27
N6	MSC_0570	Prolipoprotein
N6	**MSC_0571**	**Transposase ISMmy1F**
KH3J	***MSC_0577***	***1-acyl-sn-glycerol-3-phosphate acyltransferase***
N6	**MSC_0581**	**dCMP deaminase**
KH3J	***MSC_0596***	***Phenylalanine-tRNA ligase alpha subunit***
KH3J	***MSC_0598***	***Hypothetical protein MSC_0598***
N6	**MSC_0612**	**Heat-inducible transcription repressor HrcA**
N6	MSC_0614	Putative hydrolase of the HAD family
KH3J	*MSC_0616*	*Ham1 family protein*
N6	**MSC_0621**	**Hypothetical protein MSC_0621**
KH3J	*MSC_0624*	*Hypothetical protein MSC_0624*
N6	**MSC_0635**	**Prolipoprotein**
N6	**MSC_0653**	**Prolipoprotein**
N6	MSC_0709	Leucine-tRNA ligase
KH3J	*MSC_0718*	*ABC transporter, ATP-binding component (vitamin B12)*
N6	MSC_0723	30S ribosomal protein S13
N6	MSC_0725	Adenylate kinase
N6	MSC_0727	50S ribosomal protein L15
N6	MSC_0762	Glutamyl-tRNA amidotransferase subunit A
N6	MSC_0764	DNA ligase
N6	MSC_0808	Transposase ISMmy1C
N6	MSC_0894	Glycine hydroxymethyltransferase
N6	**MSC_0896**	**Hypothetical protein MSC_0896**
N6	MSC_0916	Hypothetical protein MSC_0916
N6	MSC_0943	Excinuclease ABC subunit A
KH3J	*MSC_0945*	*Hypothetical protein MSC_0945*
N6	MSC_0954	Peptidyl-tRNA hydrolase
N6	MSC_0955	50S ribosomal protein L9
N6	MSC_1017	Glucose-inhibited division protein A
KH3J	***MSC_1064***	***LICA protein***

Footnotes:
[a]strain in which protein was identified
[b]locus tag as defined for *M. mycoides* subsp. *mycoides* strain PG1 (accession number NC_005364.2)
[c]protein annotation as defined for *M. mycoides* subsp. *mycoides* strain PG1 (accession number NC_005364.2)

Table 3: Proteins detected in only *Mycoplasma mycoides* subsp. *mycoides* strain N6 (high pathogenicity) or KH3J (low pathogenicity). Proteins are presented in locus tag (MSC_nnn) order. Proteins detected in 3 of 3 or 2 of 3 biological replicates of strain N6 and in either 1 of 3 (standard text) or 0 of 3 (bold text) biological replicates of strain KH3J are unitalicized. Proteins detected in 3 of 3 or 2 of 3 biological replicates of strain KH3J and in either 1 of 3 (standard text) or 0 of 3 (bold text) biological replicates of strain N6 are presented in *italics*.

were differentially detected in strains N6 and KH3J respectively and 3 lipoproteins were detected in N6 only (Table 3). For instance, LppA (MSC_0013), and prolipoproteins MSC_0635 and MSC_0653 were detected only in strain N6-(highly pathogenic) as was an additional hypothetical predicted membrane protein (MSC_0065). Of these (pro)lipoproteins, only LppA has been investigated to any extent and reported as an immunogen as mentioned above [46,47]. One could assume that these differences may correlate with differences in pathogenicity between the two strains, although no role in virulence has been defined for LppA and no information is available on the other three proteins. Furthermore these proteins were recently found not only in African and European pathogenic field strains, but also in the T1/44 vaccine and the PG1 reference strains [10]. Thus, despite the differences observed between the two strains in the current study, it is not possible to outline any correlation between the presence/absence of these proteins and differences in pathogenicity of N6 and KH3J. However, given the significant role of lipoproteins in *Mmm* virulence, the differential detection of proteins of this class could be a relevant feature with important roles in *Mycoplasma*-host interactions.

Transport proteins were also among those differentially detected in the two *Mmm* strains. Spermidine/putrescine transport ATP-binding component MSC_0224 was detected only in strain N6 although the permease and substrate-binding components of this system (respectively MSC_0225 and MSC_0226) were detected in both N6 and KH3J strains. Conversely, several transport-associated proteins were detected only in strain KH3J: MSC_0008 (galactose/ribose transporter permease component), MSC_0405 (predicted to be a substrate-specific energy-coupling factor (ECF) transporter component-IPR024529), MSC_0434 (ABC transporter ATP-binding permease protein), MSC_0558 (sodium-solute symporter) and MSC_0598 (FtsX-like lipid transport permease protein). Although not defined herein, it is possible that the attenuation of KH3J involves a higher requirement for several substrates hence the over-abundance of transport-associated proteins relative to the more pathogenic N6. The extent to which this is the case will require further systematic evaluation including assessment of additional strains of differing virulence.

Glycerol metabolism has been reported as an important virulence-associated metabolic activity of *Mmm* [51] and several enzymes involved in this pathway were detected in only KH3J: notably, MSC_0259 (GlpO) – which metabolises glycerol to release H_2O_2 and mediate cytoxicity [52-54] in addition, MSC_0577 (PlsC - 1-acyl-sn-glycerol-3-phosphate acyltransferase) and MSC_0259 (GlpF - glycerol-3-phosphate oxidase) were also detected in KH3J but not N6. Since the glycerol uptake system GtsABC (MSC_0516-0518) was detected in neither KH3J nor N6, the presence of these glycerol metabolising proteins in KH3J may represent a futile function. This, together with the presence of multiple substrate uptake mechanisms, could be interpreted as a metabolic dysregulation in strain KH3J which could contribute to its attenuation.

Transcriptional regulators were also differentially detected in the 2 strains examined and, given their role in controlling multiple functions through modification of gene expression, this has potential implications for pathogenicity. Specifically, the regulatory proteins RpiR (MSC_0018) and HrcA (MSC_0612), both of which are negative regulators of gene transcription, were observed in *Mmm* strain N6 only. A function for RpiR has not been described in *Mycoplasma* spp. although roles have been demonstrated in regulation of metabolic and virulence activities in diverse bacterial genera [55-57]. HrcA has been shown to contribute to "heat shock" responses in *Mycoplasma hyopneumoniae* [58] and *M. genitalium* [59] and has been correlated with resistance to antimicrobial peptides in *M. pulmonis* [60]. Despite detection of HrcA in only N6, several proteins up-regulated during heat shock and involving HrcA-dependent regulation (i.e. DnaJ (MSC_0609); DnaK (MSC_0610); ClpB (MSC_0613) and Lon (MSC_0454) were detected in both strains of *Mmm* investigated in this study.

Other proteins detected differentially in the two strains were GidA (MSC_1017) in strain N6 - the sole differential protein encoded on the large repetitive elements and GnsAB (MSC_0032), a deduced membrane protein involved in phospholipid synthesis (PFAM08178, IPR012563) in only strain KH3J. This re-emphasises that a range of protein functional classes may be identified through comparison of prokaryote strains of differing phenotype (such as virulence) and any functional relevance to these differences can only be presumed

until proved. Nevertheless, the differences observed in proteomes between strains support the multifactorial nature of *Mycoplasma*-host interactions and is broadly in keeping with the covert nature of pathogenesis of these prokaryotes.

Concluding Remarks

Herein, the first extensive proteome analysis of *Mmm* strains has been presented. Two strains of differing pathogenicity have been examined: strains N6 and KH3J of high and low pathogenicity respectively. Our goal was not to provide a comprehensive, in-depth comparative analysis of *Mmm* proteomes but was, rather, to initiate an extensive survey to identify proteins as a basis for further targeted analyses of *Mmm* physiology, pathogenicity and immunity with this intractable pathogen. Using two simple complementary methodologies, more than 300 proteins, representing approximately 31% of the proteome, were detected, extending current knowledge of *Mmm* protein expression.

Proteins representing multiple functional categories were identified comprising many metabolic, immunogenic and virulence-associated factors including several of the membrane lipoproteins which have key roles in *Mycoplasma*-host interactions. Also present were 59 hypothetical proteins including proteins represented by pseudogenes. Overall, therefore, this proteome analysis re-emphasises the complexity of *Mycoplasma* expression and adaptation even in the absence of host-imposed selective pressures, and supports the need for further systematic investigations of *Mmm* expression and function including inter-strain comparisons.

This study also initiated direct comparison between two strains of differing pathogenicity. Although differences in protein content were observed, the biological significance of these observations requires further study. Even though not a definitive appraisal of proteins contributing to virulence/attenuation, the identification of differences in proteome content between these two strains highlights a substantial number of *Mmm* proteins, including regulators of gene expression and a number of possible proteins that are subject to variable expression. Further investigation of inter-strain heterogeneity and of selected proteins will be necessary to define which or whether any of these correspond to fitness and pathogenicity. Our own studies are now extending the range of *Mmm* strains to incorporate field strains in order to define conserved proteins and further advance understanding of *Mycoplasma mycoides* subsp. *mycoides* interaction with the bovine host.

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