FULL PAPER *Bacteriology*

Identification of Novel Immunogenic Proteins in *Mycoplasma capricolum* subsp. *Capripneumoniae* Strain M1601

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ABSTRACT. The purpose of this study was to obtain immunogenic proteins and potential proteins of interest that were isolated from *Mycoplasma capricolum* subsp. *capripneumoniae* (Mccp) by MALDI-TOF mass spectrometry. One-dimensional SDS-PAGE and two-dimensional gel electrophoresis of whole cell preparation were conducted, and membrane proteome maps were prepared by immunoblotting. One-dimensional SDS-PAGE identified three immunogenic proteins with molecular masses in the range 29–97.2 kDa, two of which were in the membrane protein fraction. After two-dimensional gel electrophoresis, 20 highly immunogenic proteins were identified in the whole cell protein preparation while 9 immunogenic proteins were identified in the membrane protein fraction. This indicated that membrane proteins were the principle immunogenic proteins in Mccp. These proteins may have potential for the development of improved diagnostic tests and possible vaccines.

KEY WORDS: homology, identification, immunoblotting, immunogenic proteins, *Mycoplasma capricolum* subsp. *Capripneumonia.*

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Mycoplasma capricolum subsp. *capripneumoniae* (Mccp) is the causative agent of contagious caprileuropneumonia in goats [13], which is a disease characterized by coughing, dyspnea, lagging behind the herd, lying down, fever (40.5–41.5°C) and mouth breathing in the terminal stages [21]. In chronic cases, animals present sporadic coughing, emaciation, and diarrhea. Animals that recover from this form of the disease do not show any signs or lesions after recovery. Although there are no sequelae in recovered animals, the survivor goats may remain carriers of the infection.

The genome sequence of Mccp has been completed but not annotated by Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences. There have been few efforts to identify and characterize Mccp antigens, which might potentially be useful for immunodiagnosis and/or vaccination. These antigens could also be virulence factors and their characterization may help to elucidate the mechanisms of pathogenicity. There is an obvious need for better antigens to improve the serological methods that are currently used for goat herd surveillance, which is an important step before the implementation of preventive or therapeutic measures.

Proteome analysis is a useful complementary method for studying bacteria, which supports genome annotation and protein identification [9, 22], and it could be used to characterize differentially expressed or post-translationally modified antigenic proteins [11]. This approach is particularly attractive in mycoplasmae, because the high A + T content and the use of TGA as a tryptophan codon rather than as a stop codon prevent a genetic approach using *Escherichia coli* expression libraries. Two-dimensional gel electrophoresis (2DE) mapping of Mccp proteins will improve genome annotation, and it will help to characterize the extent of post-translational modifications, as well as identifying potential virulence factors.

In this study, we isolated three dominant immunogenic proteins in Mccp with SDS-PAGE and immunoblotting, 2 of which were membrane proteins. To further elucidate the identity of these proteins, 2DE maps of the whole cell protein preparation and the membrane protein fraction were produced, followed by immunoblotting. We identified 20 novel immunogenic proteins in the whole cell protein preparation and 9 immunogenic proteins in the membrane protein fraction. These important results have established the immunogenic protein profile of Mccp for the 1st time, which will be valuable for studying its pathogenicity and developing vaccines and serodiagnostic markers in the future.

MATERIALS AND METHODS

Bacterial strains and cultivation: The Mccp strain was isolated from an infected goat in GanSu, China. The strain

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was cultivated at 37°C in KM_2 broth containing 0.5% w/v Minimum Essential Medium, 0.5% w/v lactalbumin hydrolysate, 0.5% w/v yeast extract, 15% v/v horse serum, 0.08% w/v phenol sulfonphthalein, 0.01% w/v thallium acetate, and 2,000 IU/l^{-1} penicillin. The pH was maintained at 7.4–7.6.

Preparation of the whole cell protein samples: Cells were harvested at a density of 10^8 CCU ml^{-1} by centrifugation at 12,000 × *g* for 20 min and resuspended in 1 m*l* 25 mM Tris–HCl, pH 7.2. Cell suspensions were then lysed by sonication (25 Hz in a VC601 Sonics and Materials Inc. sonicator, Sydney, Australia) in an ice bath using five 30 sec cycles with a 1 min interval between pulses. Proteins were quantified using the Bradford method (Bio-Rad Protein Assay, Bio-Rad, Hercules, CA, U.S.A.).

Preparation of the membrane protein fraction: Membrane proteins were prepared essentially as previously described, with slight modifications [16, 25]. Briefly, the harvested cells were washed twice with pre-cooled 50 mM Tris-HCl buffer, pH 7.2, and resuspended in the same buffer. Cell lysis was performed by sonication, and undisrupted cells were removed by centrifugation at 12,000 × *g* for 20 min. Membrane proteins were then collected by centrifugation at 100,000 × *g* for 30 min and washed 3 times with distilled water. The pellet was resuspended in distilled water.

Convalescent sera: Five Mccp-free goats were inoculated tracheally with Mccp at a dose of 2.5×10^{10} CCU goat^{-1}. Seven weeks after the 1st injection, blood was collected, and serum samples were evaluated by ELISA [26]. Specific titers were obtained from 1:5,000 to 1:10,000, and the serum with the highest titer was used in the immunoblotting experiments. The pre-infection serum was used as a negative control. The Institutional Animal Care and Use Committee at Lanzhou Veterinary Research Institute approved the study.

One-dimensional SDS-PAGE and immunoblotting: The whole cell protein preparation and membrane protein fraction of Mccp were separated by 1-dimensional SDS-PAGE using a PROTEAN1 II xi 2D Cell (Bio-Rad). Proteins were electroblotted onto PVDF membranes (Amersham Biosciences, Waukesha, WI, U.S.A.). Blotted membranes were blocked with 3% w/v albumin bovine V (Roche) in phosphate buffered saline (PBS) (10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, 137 mM NaCl and 2.7 mM KCl) and then incubated with goat anti-Mccp serum diluted 1:50 in blocking solution. Membranes were washed 3 times in PBS for 5 min, incubated with a secondary antibody (anti-goat IgG HRP, Sigma; 1:3,000), washed and developed with 4-chloro-1-naphthol (Sigma) according to the manufacturer's instructions. Controls were carried out using the corresponding pre-infection serum.

Two-dimensional gel electrophoresis and immunoblotting: Protein samples were solubilized in an isoelectric focusing buffer (IEF buffer) containing 7 M urea, 2 M thiourea, 4% w/v CHAPS, 1% w/v dithiothreitol (DTT), and 0.2% v/v ampholytes, pH 3–10 (Bio-Rad). We passively rehydrated 17 cm immobilized pH gradient (IPG) strips (pH 3–10, Bio-Rad) for 16 hr with 300 μl cell extract samples containing 1–4 mg of protein. Isoelectric focusing was performed in a Protean IEF cell system (Bio-Rad) with up to 50,000 VH at a maximum voltage of 10,000 V. Strips were equilibrated for 15 min in equilibration buffer I (30% v/v glycerol, 6 M urea, 1% DTT, and a trace of bromophenol blue) and for 15 min in equilibration buffer II (equilibration solution I where DTT was replaced with 4% iodoacetamide). In the 2nd dimension, IPG strips were run vertically onto 12% SDS-PAGE gels using a PROTEAN II xi 2D Cell (Bio-Rad). Gels were subsequently stained with Coomassie Brilliant Blue R250 (Bio-Rad) or with silver. The stained gels were scanned with an Image Scanner (Amersham Biosciences) and analyzed using the PDQuest Basic 8.0 program (Bio-Rad), followed by additional visual analysis. Immunoblotting was carried out as described above.

Protein identification and homology analysis: Protein spots were manually excised from Coomassie-stained gels and digested in-gel with trypsin. Gel plugs received three washing steps with 180 μl of 50% acetonitrile and 50 mM ammonium bicarbonate for 15 min, followed by one washing step with 180 μl of acetonitrile. After the washing procedures, gel plugs were dried by vacuum centrifugation and digested for 18–24 hr at 37°C using 12 μl of 10 μg ml^{-1} modified porcine trypsin (sequencing grade modified trypsin, Promega Corporation, Madison, WI, U.S.A.) diluted to 25 mM in NH_4HCO_3. After tryptic digestion, peptides were extracted in two washing steps with 50 m*l* 50% acetonitrile and trifluoroacetic acid (TFA) for 1 hr. Extracted peptides were dried and resuspended in 10 μl of Milli-Q water. A sample of 1 μl of crude digest was mixed with 1.0 μl of α-cyano-4-hydroxycinnamic acid (10 mg ml^{-1} in 0.1% TFA in 1:1 acetonitrile/methanol), and an aliquot of 0.5 μl was delivered to the target plate, and then dried at room temperature. MS was conducted using an AB 4700 TOF/TOF Proteomics Analyzer (Applied Biosystems), operated in the reflector mode for MALDITOF peptide mass fingerprinting (PMF) or collision-induced dissociation-MALDI-MS/MS (MS/MS). Each spectrum was based on the accumulating data from 2,000 consecutive laser shots. All samples that were analyzed by PMF were further analyzed by MS/MS, where up to 5 PMF precursor ions per sample were selected and submitted to MS/MS analysis. Peptides were identified using the MASCOT search engine (http://www.matrixscience.com) by searching against the NCBI database (release 7442,25/3/2010, Mycoplasma). Maximum mass errors of 100 and 200 ppm were allowed for valid PMF and MS/MS protein identifications, respectively. No functions of Mccp proteins have been previously described, so a BLAST search was performed for the identified proteins using the sequences obtained.

RESULTS

One-dimensional SDS-PAGE and immunoblotting: A western blot was generated using convalescent serum from goats. We repeated this assay 3 times, with a very high degree of reproducibility (data not shown). Three dominant immunogenic proteins were detected with molecular masses in the range 29–97.2 kDa, two of which were in the membrane protein fraction (Fig. 1). Controls were carried out using the

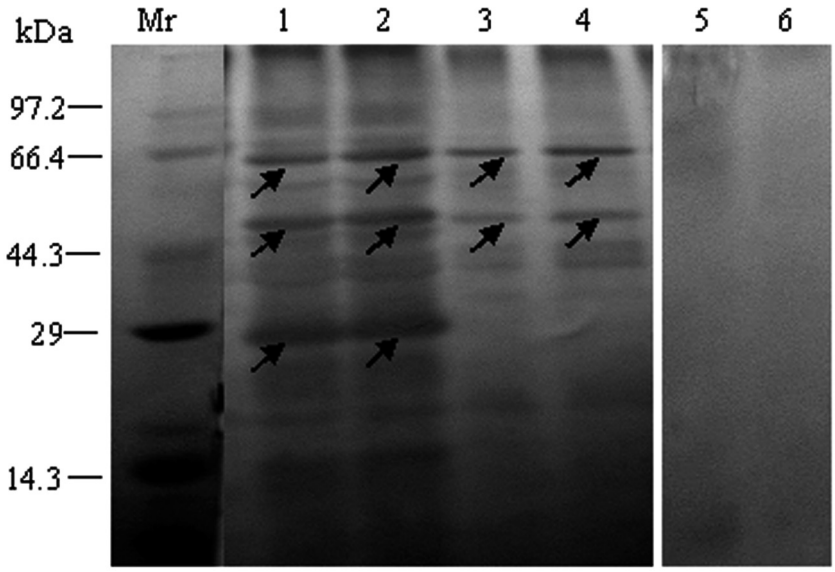

Fig. 1. Western-blot of antiserum of *Mycoplasma capricolum* subsp. *Capripneumoniae*(the detected immunogenic proteins were marked with arrows) Mr: Protein molecular weight marker;1, 2:M1601 whole cell Protein sample;3, 4:M1601 membrane protein fraction; 5, M1601 whole cell Protein sample against pre-infection serum; 6, M1601 membrane protein fraction against pre-infection serum.

corresponding pre-infection serum, and no immunoreactive protein was detected .This showed that membrane proteins were the principle immunogenic proteins.

Two-dimensional gel electrophoresis: To identify these immunogenic proteins, we performed 2DE of the whole cell protein preparation and the membrane protein fraction. As technical controls, we repeated all the protein preparation and subsequent 2DE experiments 3 times, with a very high degree of reproducibility (data not shown). About 300 prominent protein spots were resolved in Coomassie-stained 2DE gels with pH 3–10 IPG strips (Fig. 2a), while 150 protein spots were detected in the membrane protein fraction (Fig. 3a).

Identification of immunogenic proteins by mass spectrometry and homology analysis: Immunoblot assays of the 2DE gels were performed using the serum of an infected goat to identify novel immunogenic proteins (Fig. 2b and Fig. 3b). Controls were carried out using the corresponding pre-infection serum, and no immunoreactive protein spots were detected (data not shown). We repeated this assay 3 times, with a very high degree of reproducibility . The convalescent serum produced IgG recognition patterns containing 40 spots in the whole cell protein preparation with 15 spots in the membrane protein fraction. These spots were identified and they corresponded to 20 different proteins and 9 different proteins with post-translational modifications. Nine immunogenic proteins (heat shock protein 70; cytosol aminopeptidase family, catalytic domain protein; L-lactate dehydrogenase; four members of the pyruvate dehydrogenase complex; NADP-dependent glyceraldehyde-3-phosphate dehydrogenase; elongation factor Tu) that were identified in the membrane protein fraction were also identified in the whole cell protein preparation. No Mccp proteins have been previously annotated, so we performed a BLAST search of the identified proteins using the sequences obtained. The results showed that the homology to proteins from related species was all>92.5%. In particular, 9 proteins had homologies>99%, as follows: 4 members of the pyruvate dehydrogenase complex, heat shock protein 70, transketolase, elongation factor G, phosphoenolpyruvate-protein phosphotransferase, and glutamyl-tRNA (Gln) amidotransferase subunit A. Others had homologies>98%, such as L-lactate dehydrogenase, cytosol aminopeptidase family catalytic domain protein, aldehyde dehydrogenase (NAD) family protein, thioredoxin reductase (NADPH), elongation factor Tu, and peptidase M24 family. The homologies of the remaining proteins were between 92.5% and 96.5% (Table 1 and Table 2).

DISCUSSION

We have described the results of an immunoproteome-based analysis of Mccp pathogenic strain M1601. One-dimensional SDS-PAGE and immunoblot analysis identified 3 principal immunogenic proteins, with molecular masses in the range 29–97.2 kDa, 2 of which were also present in the membrane protein fraction. The whole cell protein 2DE facilitated the resolution of over 300 protein spots in the pH range 3–10, with 150 protein spots in the membrane protein fraction. However, multiple spots may correspond to a single gene, if post-translational modification occurs, e.g., chemical modification or proteolytic cleavage [4, 15, 25]. This

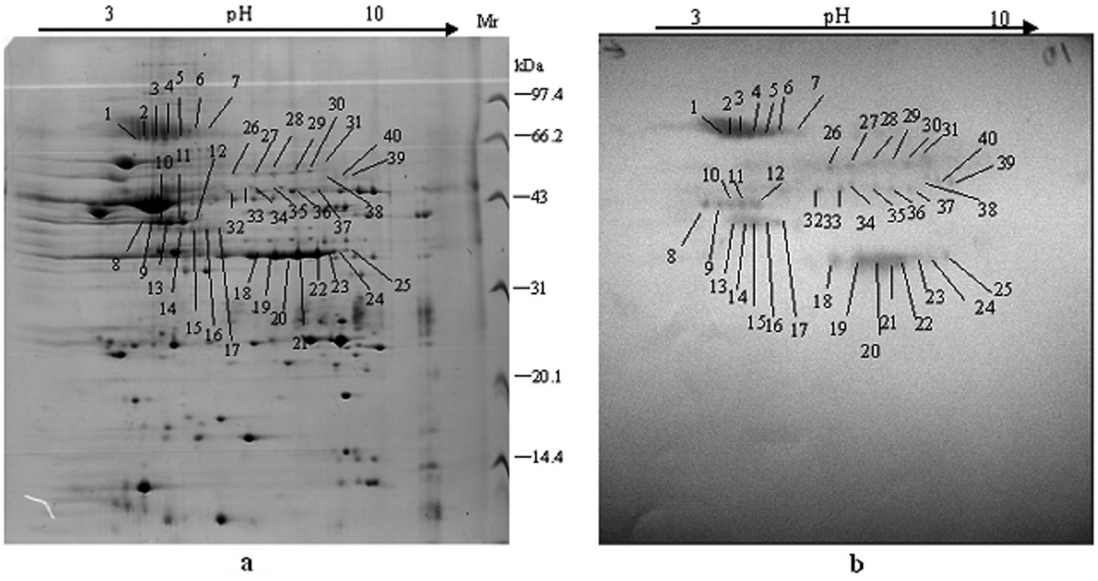

Fig. 2. a. 2DE gels of whole cell protein sample from *Mycoplasma capricolum* subsp. *capripneumoniae* strain M1601. Proteins were separated by IEF using 17 cm IPG strips, pH 3–10, followed by SDS-PAGE on 12% gels, and stained with 0.1% Coomassie Brilliant Blue R250. The approximate molecular weights are shown on the right of the gel and the acid to alkaline gradient is from left to right. The spot numbers correspond to those identified by MS and are listed in Table1. b. Identification of immunogenic proteins from the whole cell protein sample from *Mycoplasma capricolum* subsp. *capripneumoniae* strain M1601. Proteins resolved by 2DE as in Fig. 2.a were electroblotted onto PVDF membranes, and probed with an anti-Mccp goat antiserum (1:50 dilution). Anti-goat IgG HRP (1:3,000 dilution) was used to develop antigen-antibody reactions. The acid to alkaline gradient is from left to right. Groups of immunogenic spots are indicated by numbers.

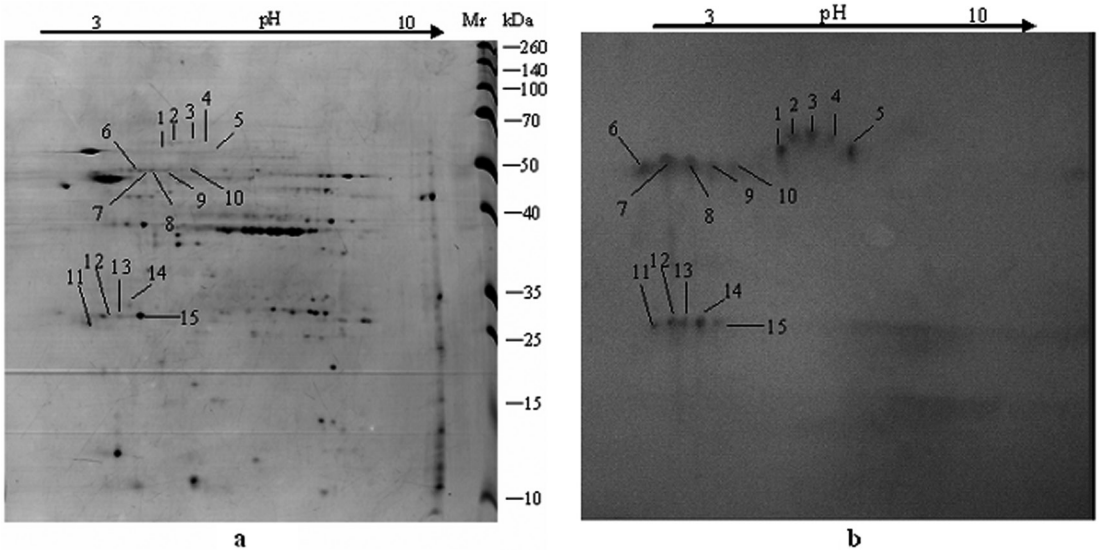

Fig. 3. a. 2DE gels of the membrane protein fraction from *Mycoplasma capricolum* subsp. *capripneumoniae* strain M1601. Proteins were separated by IEF using 17 cm IPG strips, pH 3–10, followed by SDS-PAGE on 12% gels, and stained with 0.1% Coomassie Brilliant Blue R250. The approximate molecular weights are shown on the right of the gel and the acid to alkaline gradient is from left to right. The spot numbers correspond to those identified by MS and are listed in Table 2. b. Identification of immunogenic proteins from the membrane protein fraction of *Mycoplasma capricolum* subsp. *capripneumoniae* strain M1601. Proteins resolved by 2DE as in Fig. 3.a were electroblotted onto PVDF membranes, and probed with an anti-Mccp goat antiserum (1:50 dilution). Anti-goat IgG HRP (1:3,000 dilution) was used to develop antigen-antibody reactions. The acid to alkaline gradient is from left to right. Groups of immunogenic spots are indicated by numbers.

Table 1. Proteins identified in the whole cell protein sample by MS that reacted with convalescent sera in the immunoblotting experiments

Locus tag in corresponding genome/ Gene name	Protein name/origin	Spot no.	*Mycoplasma capricolum* subsp. *capripneumoniae* strain M1601 identity (%)
MSC_0532/ldh	L-lactate dehydrogenase/ *Mycoplasma mycoides* subsp. *mycoides* SC str. PG1	20/21	98
MSC_0265/pdhA	Pyruvate dehydrogenase E1 component alpha subunit/ *Mycoplasma mycoides* subsp. *mycoides* SC str. PG1	15/16/17	99.1
MCAP_0226/pdhB	Pyruvate dehydrogenase complex E1component beta subunit/ *Mycoplasma capricolum* subsp. *capricolum* ATCC 27343	22	99.7
MCAP_0227/pdhC	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex/*Mycoplasma capricolum* subsp. *capricolum* ATCC 27343	8/9	99.2
MCAP_0228/pdhD	Dihydrolipoamide dehydrogenase/ *Mycoplasma capricolum* subsp. *Capricolum* ATCC 27343	29/30/31	99.4
MCAP_0369/dnaK	Heat shock protein 70/ *Mycoplasma capricolum* subsp. *capricolum* ATCC 27343	13/14	99.4
MSB_A0207	Cytosol aminopeptidase family, catalytic domain protein/ *Mycoplasma leachii* PG50	11	98.2
MCAP_0461	Aldehyde dehydrogenase (NAD) family protein/ *Mycoplasma capricolum* subsp. *capricolum* ATCC 27343	12	98.3
MCAP_0610/tkt	Transketolase/ *Mycoplasma capricolum* subsp. *capricolum* ATCC 27343	25	99.1
MSC_0938/trxB	Thioredoxin reductase (NADPH)/ *Mycoplasma mycoides* subsp. *mycoides* SC. PG1	23	98.3
MSC_0263/nox	NADH oxidase/ *Mycoplasma mycoides* subsp. *mycoides* SC str. PG1	10	93.8
MSC_0214/tufA	Elongation factor Tu / *Mycoplasma mycoides* subsp. *mycoides* SC str. PG1	18/19	98
MCAP_0153/fusA	Elongation factor G / *Mycoplasma capricolum* subsp. *capricolum* ATCC 27343	1/2/3	99.7
MSB_A0355	Peptidase, M24 family/ *Mycoplasma leachii* PG50	24	98.9
MSB_A0517	Putative RNA polymerase sigma factor RpoD/ *Mycoplasma leachii* PG50	32	94.5
MCAP_0233/ptsI	Phosphoenolpyruvate-protein phosphotransferase/ *Mycoplasma capricolum* subsp. *capricolum* ATCC27343	26/27/28	99.0
MMCAP1_0508	Glutamyl-tRNA (Gln) amidotransferase subunit A/ *Mycoplasma mycoides* subsp. *capri* str. GM12	33	100
MMCAP1_0451	NADP-dependent glyceraldehyde-3-phosphate dehydrogenase/ *Mycoplasma mycoides* subsp. *capri* str. GM12	38/39/40	96.5
MMS_A1021	DNA topoisomerase/ *Mycoplasma mycoides* subsp. *mycoides* SC str. Gladysdale	4/5/6/7	96.1
MMCAP1_0064 /lysS	Lysine-tRNA ligase/ *Mycoplasma mycoides* subsp. *capri* str. GM12	34/35/36/37	92.5

result was similar to that reported from proteomic studies of other mycoplasmae [7, 9, 18, 20, 22, 24].

We identified 20 novel Mccp antigens in the whole cell protein preparation while 9 novel antigens were identified in the membrane protein fraction. The analysis showed that these proteins had>92.5% homology to proteins from related species. In particular, 9 proteins all had>99% homology to proteins in the database, as follows: 4 members of the pyruvate dehydrogenase complex, heat shock protein 70, transketolase, elongation factor G, phosphoenolpyruvate-protein phosphotransferase, and glutamyl-tRNA (Gln) amidotransferase subunit A. Others had homology>98%, such as L-lactate dehydrogenase, cytosol aminopeptidase family catalytic domain protein, aldehyde dehydrogenase (NAD) family protein, thioredoxin reductase (NADPH), elongation factor Tu, and peptidase M24 family. The homologies of the remaining proteins were between 92.5% and 96.5%. Previous studies have demonstrated the immunogenicity of the pyruvate dehydrogenase complex. Pyruvate dehydrogenase appears to be located on the cell surface where it mediates adhesion in some mycoplasmae and other bacteria [3, 5, 6, 10, 12, 14, 17, 19, 23]. Elongation factor Tu has recently been described as immunogenic, and it is also surface-localized, mediating *M. pneumoniae* binding to fibronectin [5]. Heat shock protein 70 has been reported to be surface-localized in other mycoplasmae where it mediates host receptor binding [2]. Another vaccine candidate is glyceraldehyde-3-phosphate dehydrogenase in *M. pulmonis*, which is surface-localized and capable of binding to mucin [1]. The conserved sequence of this protein makes it a possible vaccine candidate. Interestingly, 4 members of the pyruvate dehydrogenase complex, elongation factor Tu, heat shock

Table 2. Proteins identified in the membrane protein fraction by MS that reacted with convalescent sera in the immunoblotting experiments

Locus tag in corresponding genome/ Gene name	Protein name/origin	Spot no.	*Mycoplasma capricolum* subsp. *capripneumoniae* strain M1601 identity (%)
MCAP_0369/dnaK	Heat shock protein 70/ *Mycoplasma capricolum* subsp. *capricolum* ATCC 27343	8	99.4
MSB_A0207	Cytosol aminopeptidase family, catalytic domain protein/ *Mycoplasma leachii* PG50	9	98.2
MSC_0532/ldh	L-lactate dehydrogenase/ *Mycoplasma mycoides* subsp. *mycoides* SC str. PG1	11/13	98
MSC_0265/pdhA	Pyruvate dehydrogenase E1 component alpha subunit/ *Mycoplasma mycoides* subsp. *mycoides* SC str. PG1	7	99.1
MCAP_0226/pdhB	Pyruvate dehydrogenase complex E1component beta subunit/ *Mycoplasma capricolum* subsp. *capricolum* ATCC 27343	12	99.7
MCAP_0227/pdhC	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex/*Mycoplasma capricolum* subsp. *capricolum* ATCC 27343	6	99.2
MCAP_0228/pdhD	Dihydrolipoamide dehydrogenase/ *Mycoplasma capricolum* subsp. *Capricolum* ATCC 27343	1/2/3/4/5	99.4
MMCAP1_0451	NADP-dependent glyceraldehyde-3-phosphate dehydrogenase/ *Mycoplasma mycoides* subsp. *capri* str. GM12	10	96.5
MSC_0214/tufA	Elongation factor Tu / *Mycoplasma mycoides* subsp. *mycoides* SC str. PG1	14/15	98

protein 70, and glyceraldehyde-3-phosphate dehydrogenase were all identified in the whole cell protein preparation and membrane protein fractions in Mccp. This confirms that membrane proteins are the primary immunogenic proteins and these proteins are abundant in the cell.

We identified only 9 immunogenic proteins in the membrane protein fraction, perhaps because of limiting factors including: (i) the dynamic range of 2DE prevents the detection of low abundance proteins; (ii) difficulties with the solubilization of membranes; and (iii) the poor resolution of basic proteins and proteins with a molecular mass<10 kDa or>150 kDa [20]. Thus, different approaches are required to investigate Mccp further, such as enrichment using Triton X-114 fractionation [15], synthesis of potential immunogenic peptide epitopes [8], or the heterologous expression of potential antigens.

In order to elucidate the exact role of these potential colonization factors and their use as vaccine antigens, future experiments should focus on the dynamics of humoral immune responses during the disease course and the differences between infected animals with acute disease and those that recover.

The functions of only a few Mccp proteins have been reported. Thus, the immunogenic proteins described here will facilitate comparative studies to identify their localization and their role in colonization by this important disease.

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