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Identification of Novel Antigenic Proteins in a Complex *Anaplasma marginale* Outer Membrane Immunogen by Mass Spectrometry and Genomic Mapping

Job E. Lopez,[1] William F. Siems,[2] Guy H. Palmer,[1] Kelly A. Brayton,[1] Travis C. McGuire,[1] Junzo Norimine,[1] and Wendy C. Brown[1]*

Department of Veterinary Microbiology and Pathology[1] and Department of Chemistry,[2] Washington State University, Pullman, Washington 99164

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Immunization with purified *Anaplasma marginale* outer membranes induces complete protection against infection that is associated with CD4$^+$ T-lymphocyte-mediated gamma interferon secretion and immunoglobulin G2 (IgG2) antibody titers. However, knowledge of the composition of the outer membrane immunogen is limited. Recent sequencing and annotation of the *A. marginale* genome predicts at least 62 outer membrane proteins (OMP), enabling a proteomic and genomic approach for identification of novel OMP by use of IgG serum antibody from outer membrane vaccinates. Outer membrane proteins were separated by two-dimensional electrophoresis, and proteins recognized by total IgG and IgG2 in immune sera of outer membrane-vaccinated cattle were detected by immunoblotting. Immunoreactive protein spots were excised and subjected to liquid chromatography-tandem mass spectrometry. A database search of the *A. marginale* genome identified 24 antigenic proteins that were predicted to be outer membrane, inner membrane, or membrane-associated proteins. These included the previously characterized surface-exposed outer membrane proteins MSP2, operon associated gene 2 (OpAG2), MSP3, and MSP5 as well as recently identified appendage-associated proteins. Among the 21 newly described antigenic proteins, 14 are annotated in the *A. marginale* genome and include type IV secretion system proteins, elongation factor Tu, and members of the MSP2 superfamily. The identification of these novel antigenic proteins markedly expands current understanding of the composition of the protective immunogen and provides new candidates for vaccine development.

Bacteria of the genera *Anaplasma* and *Ehrlichia* cause acute infection in immunologically naïve hosts and are major causes of tick-borne disease in animals and humans. Studies with both bovine and murine models support the induction of a type 1 immune response leading to antigen-specific CD4$^+$ T-lymphocyte proliferation, gamma interferon (IFN-γ) production, and immunoglobulin G2 (IgG2) antibody directed against the pathogen surface as being necessary for protection (10, 16, 25, 26, 34, 38, 61). However, the critical targets of this protective immune response have not been definitively identified for any of the pathogens in these two genera.

In several studies with *Anaplasma marginale*, immunization of cattle with purified outer membranes has been shown to induce protection against high levels of rickettsemia and clinical disease, and in one study, complete protection against infection was attained (16, 58). This protection is associated with high IgG2 titers against the immunodominant major surface protein 2 (MSP2) (16) and CD4$^+$ T-lymphocyte proliferative responses to MSP1, MSP2, and MSP3 (16) as well as MSP4 (W. C. Brown, unpublished observations). However, none of these individual proteins has induced protection equivalent to that of the complex outer membrane immunogen (1, 16, 43, 44, 48, 49, 58). This discrepancy in ability to induce protection suggests at least two possible explanations. The first is that the critical outer membrane protein (OMP) antigens have not yet been identified. The limited repertoire identified to date has been biased by the methods used, primarily surface chemistry labeling and screening with panels of monoclonal antibodies (MAb) (45, 48, 50). However, the isolation of IFN-γ-secreting CD4$^+$ T-lymphocyte clones that responded specifically to outer membranes but did not recognize any of the previously identified MSPs (MSP1 to MSP5) indicates the presence of additional T-cell epitopes on as-yet-unidentified OMPs (17). The second possibility is that a combination of OMPs, alone or in a membrane context, is needed to induce complete protection. Addressing either possibility requires a more comprehensive knowledge of the composition of the protective outer membrane immunogen.

The recent availability of the complete genome sequence of the St. Maries strain of *A. marginale* allows new approaches to identify previously unknown OMPs in the complex outer membrane immunogen. In the present work, we report a combined immunologic, proteomic, and genomic approach for identification of novel antigenic OMP. The separation of the complex immunogen by using two-dimensional (2D) electrophoresis followed by detection of proteins bound by total IgG and IgG2 from outer membrane-immunized cattle restricted the identification to antigens relevant to the induction of an immune response. This approach also permitted identification of subdominant antigens that were weakly reactive with immune sera. Immunoreactive proteins were excised and subjected to liquid chromatography-tandem mass spectrometry (LC–MS-MS) for definitive identification by mapping to the annotated genome.

* Corresponding author. Mailing address: Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, WA 99164-7040. Phone: (509) 335-6067. Fax: (509) 335-8529. E-mail: wbrown@vetmed.wsu.edu.

MATERIALS AND METHODS

Preparation of *A. marginale* outer membranes. Two splenectomized calves, C942b1 and C986b1, were inoculated intravenously with 1.8 ml cryopreserved blood stabilate mixed with 4.0 ml normal bovine serum. The stabilate was prepared from calf C533b1 infected with the St. Maries, Idaho, strain of *A. marginale* (20). On day 21 postinfection, when the levels of rickettsemia reached 23% and 24% for calves C942b1 and C986b1, respectively, fresh blood was obtained from each calf and cryopreserved in liquid nitrogen as previously described (20) for preparation of outer membranes to be used for immunization and fractionation. *A. marginale* organisms were isolated from thawed infected erythrocytes as previously described, with minor modifications (47). Briefly, infected erythrocytes were washed at least five times with phosphate-buffered saline (PBS) and centrifuged at 30,000 × *g* for 30 min after each wash. Infected erythrocytes were resuspended in PBS containing Complete Mini protease inhibitors by following the manufacturer's instructions (Roche, Penzberg, Germany). *A. marginale* organisms released from infected erythrocytes by sonication were centrifuged at 30,000 × *g* for 20 min and resuspended in 20% sucrose in 10 mM HEPES containing 50 μg per ml each of DNase I and RNase A. Outer membranes were prepared by sucrose density gradient centrifugation as previously described (58). Isolated *A. marginale* was disrupted by sonication, centrifuged at 1,500 × *g* for 15 min to pellet residual organisms, and layered onto the top of a sucrose step density gradient containing 2 ml of 32, 38, 44, 48, and 52% sucrose. Following centrifugation at 82,000 × *g* for 20 h at 4°C, three bands were visualized at 1.15 g per cm^3, 1.17 g per cm^3, and 1.22 g per cm^3. The bands were collected and resuspended in ice-cold 10 mM HEPES buffer and centrifuged at 177,000 × *g* at 4°C for 1 h. This was repeated twice, and after a final wash, outer membrane pellets were either resuspended in PBS with 5 μg per ml gentamicin for use in immunization or processed through a ReadyPrep 2D cleanup kit (Bio-Rad, Hercules, Calif.) prior to 2D electrophoresis.

Outer membrane fractionation and immunoblotting. Infected erythrocytes, *A. marginale* organisms, and purified outer membrane fractions were solubilized and denatured by being boiled for 5 min in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (0.025 mM Tris-HCl [pH 6.8], 2% SDS, 15% glycerol, 2.5% β-mercaptoethanol, 0.02% bromophenol blue), and serial dilutions ranging from 0.008 to 1 μg protein were applied to a 4 to 20% gel. Following electrophoresis, proteins were transferred to nitrocellulose membranes by using a Criterion blotter (Bio-Rad). Membranes were then blocked overnight in I-Block blocking reagent (Applied Biosystems, Bedford, Mass.) containing 0.5% Tween 20 and probed for 1 h with either MSP5-specific MAb ANAF16C1 (60) or anti-erythrocyte MAb ANA8A, diluted to 2 μg per ml. Immunoreactivity was detected using goat anti-mouse secondary antibody from Western-Star chemiluminescent immunoblot detection systems (Applied Biosystems, Foster City, Calif.) according to the manufacturer's specifications.

Immunoblotting was performed similarly to detect MSP1a in the outer membrane fraction. Either 5 μg uninfected erythrocyte membranes or 1.25 and 2.5 μg outer membranes were electrophoresed, transferred to nitrocellulose, reacted with 2 μg per ml MAb ANA22B1 specific for MSP1a (4), and developed as described above.

Immunization. Three 7-month-old neutered male Holstein calves, designated 04B90, 04B91, and 04B92, with bovine lymphocyte antigen (BoLA) *DRB3* haplotypes common within Holstein-Friesian breeds (54), were selected. *DRB3* alleles were defined by PCR restriction fragment length polymorphism analysis of exon 2 (59). Sequencing of the BoLA *DQA* genes was performed as previously described (51). The nomenclature of bovine class II genes can be found at http://www.projects.roslin.ac.uk/bola and http://www.ebi.ac.uk/ipd/mhc/bola. BoLA *DRB3* and *DQA* haplotypes for the calves in this study are as follows: for calf 04B90, *DRB3 *1101/*1501*, *DQA *10011/*2206*, and *DQB *10011/*22021*; for calf 04B91, *DRB3 *1201/*2703*, *DQA *12011/*2201*, and *DQB *0101/*22031*; and for calf 04B92, *DRB3 *0201/*1201*, *DQA *0203*, and *DQB *12011/*2201*.

Calves were immunized four times with outer membranes from the 1.22-g-per-cm^3 fraction of the sucrose density gradient described above. Briefly, each calf received a subcutaneous inoculation at weeks 0, 2, 4, and 8 of 60 μg total protein of membranes resuspended in 1.3 ml PBS containing 6 mg saponin (16, 58). The antibody titer following immunization was determined by immunoblotting using pre- and postimmunization sera as previously described, with minor modifications (16). Briefly, 150 μg of whole bacteria or uninfected erythrocytes (negative control) was solubilized in SDS-PAGE sample buffer, electrophoresed in a 10 to 20% Tris-HCl Criterion precast gel (Bio-Rad), and then transferred to a nitrocellulose membrane. After membranes were incubated overnight at 4°C in I-Block reagent, preimmune and immune sera from calves 04B90, 04B91, and 04B92 were diluted to 1:200, 1:1,000, and 1:5,000 in I-Block reagent and incubated with the membranes for 1 h, followed by a 1-h incubation with a 1:3,000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-bovine IgG antibody (Kirkegaard and Perry, Gaithersburg, Md.). Immunoblots were washed with I-Block reagent with 0.5% Tween 20 for 1 h, and antigen-antibody binding was detected by chemiluminescence using an ECL Western blotting detection system (Amersham Biosciences, Piscataway, N.J.) according to the manufacturer's instructions.

2D electrophoresis and immunoblotting. Purified outer membranes in the 1.22-g-per-cm^3 density band from sucrose density centrifugation as described above were processed with a ReadyPrep 2D cleanup kit (Bio-Rad) and solubilized in 5 M urea, 2 M thiourea, 4% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, 0.5% Bio-Lyte 3/10 ampholytes (Bio-Rad), 2 mM tributyl phosphine reducing agent, and 2% 3-(decyldimethyl-ammonio) propanesulfonate (SB3-10). Protein determinations were performed using a Quick Start Bradford (10a) protein assay (Bio-Rad) according to manufacturer specifications. Isoelectric focusing (IEF) was performed using a Protean IEF cell system (Bio-Rad) and 11-cm immobilized pH gradient (IPG) strips, pH 5 to 8, according to manufacturer specifications. Briefly, strips were rehydrated with 150 μg of solubilized outer membranes and focused for 30,000 V · h. The second dimension was performed on 10 to 20% SDS-PAGE gradient gels (Bio-Rad) using a Criterion cell system (Bio-Rad), per the manufacturer's instructions. Protein gels were stained with SYPRO Ruby (Bio-Rad) or transferred to a nitrocellulose membrane by using a Criterion blotter (Bio-Rad), blocked overnight in I-Block reagent with 0.5% Tween 20, and probed with immune serum from calf 04B90, 04B91, or 04B92 diluted at 1:200 in I-Block reagent. Antigen binding was detected with a 1:3,000 dilution of HRP-conjugated goat anti-bovine IgG antibody or HPR-conjugated sheep anti-bovine IgG2 antibody (Serotec, Raleigh, N.C.) followed by chemiluminescence as described above. The 2D gel and 2D immunoblot images were overlaid with aid from the 2D imaging software PDQuest (Bio-Rad), and corresponding spots were manually picked and prepared for LC–MS-MS.

In-gel trypsin digestion, LC–MS-MS, and *A. marginale* database search. For LC–MS-MS analysis, IgG-reactive protein spots were excised from SYPRO Ruby-stained gels and processed as previously described, with minor modifications (55). Individual protein spots were destained overnight in 10% methanol and 7% acetic acid at room temperature and then in 200 μl of high-pressure-liquid-chromatography-grade acetonitrile (ACN) (CH_3CN) for two 5-min washes. ACN was completely evaporated by vacuum centrifugation, and gel pieces were reduced in 10 mM dithiothreitol for 30 min, alkylated in 50 mM iodoacetamide for 30 min, dehydrated with two 5-min incubations in ACN, rehydrated for 10 min in 100 mM ammonium bicarbonate, dehydrated again with two 5-min incubations in ACN, and completely dried by vacuum centrifugation. Gel pieces were rehydrated for 10 min in 20 ng per μl ice-cold sequencing-grade modified porcine trypsin (Promega, Madison, Wisc.) solution (solubilized in 50 mM ammonium bicarbonate) and overnight in 50 mM ammonium bicarbonate at 37°C with continuous shaking. Following centrifugation, the protein was extracted from individual gel spots for 10 min with 60 μl 5% formic acid in 50% ACN and pooled with the corresponding overnight supernatant. The extraction was repeated and pooled with the previous extract, and samples were evaporated by vacuum centrifugation until <25 μl remained. Samples were then subjected to LC–MS-MS analysis by use of an Esquire HCT electrospray ion trap (Bruker Daltonics, Billerica, Mass.) and an LC Packings (Dionex, Sunnyvale, Calif.) Ultimate nano-high-pressure-liquid-chromatography system with a Famos microautosampler and a Swichos microcolumn switching module (Dionex). The trap column was a C_{18} PepMap, 300 μm by 1 mm, 5 μm (LC Packings), and the nanoanalytical column was a C_{18} PepMap, 75 μm by 150 mm, 3 μm, 100 Å (LC Packings). For separation, 0.1% TFA with 90% ACN as solvent A was used. The column was rinsed with 5% solvent A for 3 min, and separation was performed at a flow rate of 250 nl per min by using a binary gradient starting at 20% solvent A and rising to 70% in 42 min. After elution, the column was rinsed with 90% solvent A for 10 min and subsequently equilibrated with 5% solvent A for 10 min. Nanospray tips were made in house by etching fused silica capillaries (20 μm by 360 μm) with hydrofluoric acid. Data were acquired in the Bruker Auto(MS2) mode, using a repeated cycle of a single MS survey scan followed by two MS-MS scans of parent ions determined from a dynamically updated list. Batches of MS-MS fragment ion lists were submitted to our local MASCOT server (www.matrixscience.com) for comparison to the protein sequences of the St. Maries strain of *A. marginale*. Protein identifications were based on one or more tryptic peptides yielding MASCOT ion scores greater than 16, corresponding to a probability of 95% or greater that the peptide match is not a random event.

In silico analysis of identified proteins. To predict the cellular locations of immunostimulatory proteins, posttranslation and topology prediction programs were used, including a signal peptide prediction algorithm, SignalP (http://www.cbs.dtu.dk/services/SignalP/) (9), and a transmembrane prediction algorithm,

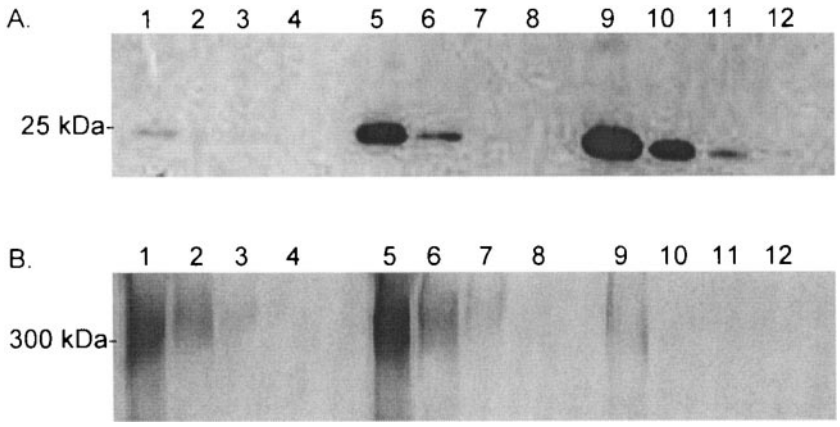

FIG. 1. Enrichment of *A. marginale* outer membranes. Decreasing concentrations of 1.0, 0.2, 0.04, and 0.008 μg per lane *A. marginale* infected erythrocytes (lanes 1 to 4), whole *A. marginale* organisms (lanes 5 to 8), and purified *A. marginale* outer membranes (lanes 9 to 12) were separated using SDS-PAGE and transferred to a nitrocellulose membrane. MAb ANAF16 (A), reactive with MSP5, and MAb ANA8A (B), reactive with bovine erythrocyte membranes, were used to probe the samples for MSP5 enrichment and bovine erythrocyte contamination. Binding was detected using alkaline phosphatase-labeled anti-mouse antibody followed by chemiluminescence.

transmembrane hidden Markov model (TMHMM) (http://www.cbs.dtu.dk/services/TMHMM-2.0/) (56). Furthermore, protein similarity searches were performed for protein products by using Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/) and the public NCBI database. As a guideline, proteins with BLAST values of <1e-20 were considered to be similar to proteins in other organisms. Where applicable, Vector NTI suite 9 (Invitrogen) was used to predict pIs.

RESULTS

Evaluation of isolated *A. marginale* outer membranes. Immunoblot analysis of the different sucrose density gradient bands of sonicated *A. marginale* reacted with MAb specific for outer membrane protein MSP5 (60) revealed that, as described previously (58), the 1.22-g-per-cm^3 band was the most highly enriched for outer membrane proteins (data not shown). Immunoblot analysis of infected erythrocytes, whole bacteria, and the outer membrane-enriched fraction from the 1.22-g-per-cm^3 sucrose density gradient band, using MAb specific for *A. marginale* MSP5, revealed a 125-fold enrichment of MSP5 in outer membranes compared to infected erythrocytes and a 25-fold enrichment of MSP5 in outer membranes compared to *A. marginale* whole bacteria (Fig. 1A). Immunoblot analysis of the same antigens by use of a bovine erythrocyte-specific MAb that detects an undefined high-molecular-weight protein indicated there was a 25-fold decrease in erythrocyte contamination compared to infected erythrocytes and to whole *A. marginale* (Fig. 1B).

To further verify the presence of outer membrane proteins in the fraction to be used to immunize cattle and to perform 2D electrophoresis, immunoblotting was performed to detect MSP1a (Fig. 2). MSP1a is present in purified outer membranes but not in membranes from uninfected erythrocytes.

Seroconversion of outer membrane vaccinates. Three calves with common major histocompatibility complex class II haplotypes were selected for this study based on *DRB3* allelic frequencies that are expressed in a majority of Holstein and Friesian cattle (54). To evaluate antibody responses after immunization with outer membranes, immunoblotting of *A. marginale* with serially diluted pre- and postimmunization sera from the three calves was performed. Preimmune sera were unreactive with *A. marginale* antigens at all dilutions. However, postimmunization sera diluted 1:5,000 from all three animals reacted with *A. marginale* antigens ranging from approximately 15 to 250 kDa (data not shown). As a negative control, immunoblotting was performed similarly, using uninfected bovine erythrocytes, and pre- and postimmunization sera from all three animals were negative at all dilutions.

Identification of novel antigenic OMP. To identify antigenic OMP, 2D electrophoresis and immunoblotting were performed with sera from the outer membrane vaccinates. Immunoblots were developed with anti-IgG (Fig. 3A to C) or anti-IgG2 (data not shown) antibody. Spots of interest were defined by overlay analysis of the stained 2D gels and immunoblots. A

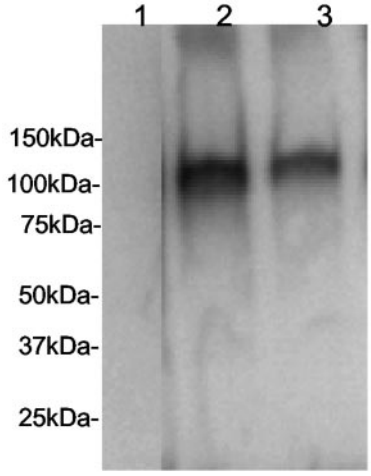

FIG. 2. Identification of MSP1a in purified outer membranes of *A. marginale*. Uninfected erythrocyte membranes (lane 1, 5 μg) or *A. marginale* outer membranes (lane 2, 2.5 μg; lane 3, 1.25 μg) were separated using SDS-PAGE and transferred to a nitrocellulose membrane. MSP1a-specific MAb ANA22B1 was used to probe the blot. Binding was detected using alkaline phosphatase-labeled anti-mouse antibody followed by chemiluminescence.

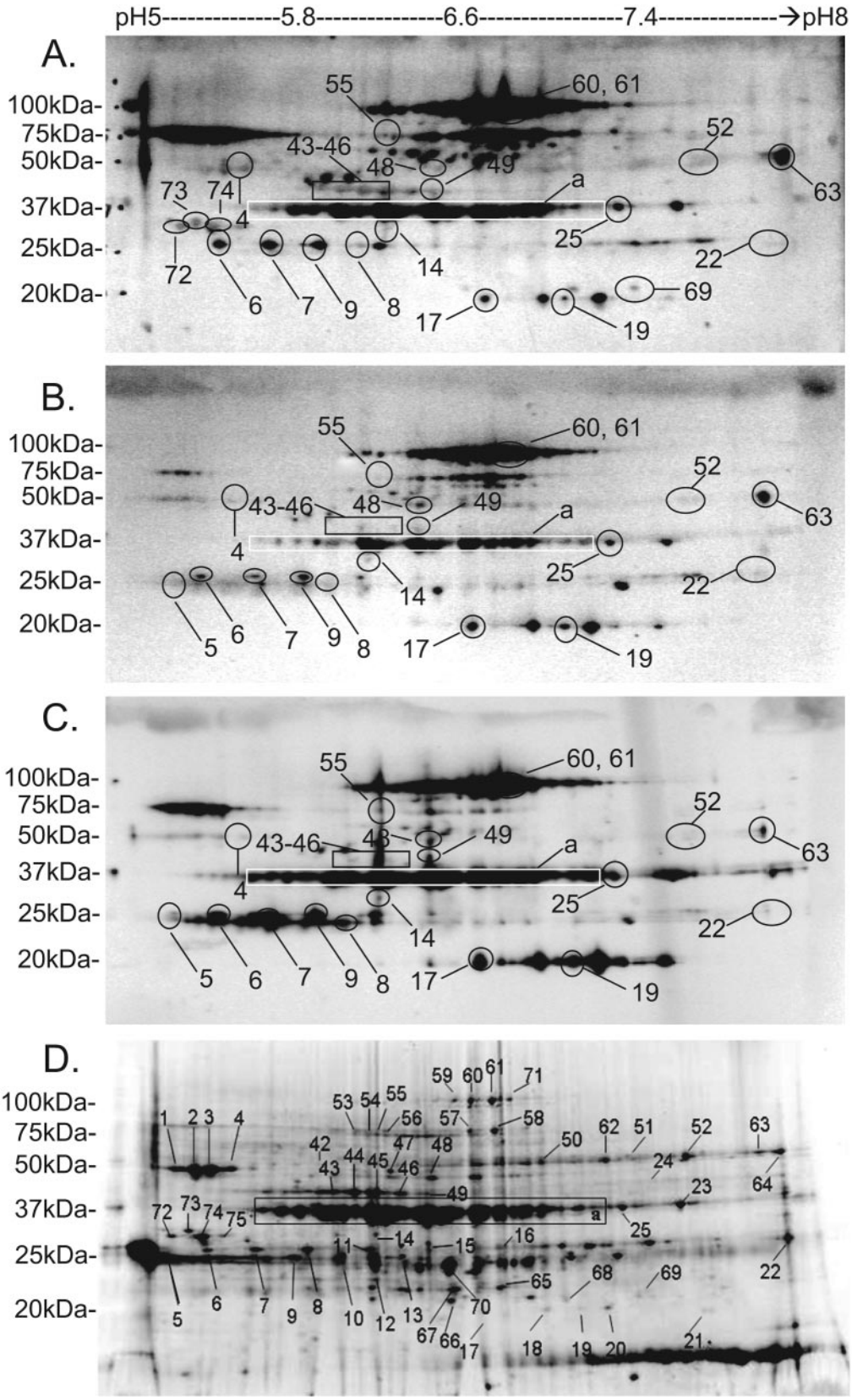

FIG. 3. Identification by immunoblotting of antigenic *A. marginale* outer membrane proteins separated by 2D electrophoresis. Outer membrane proteins were separated by 2D electrophoresis. One gel was stained with SYPRO Ruby for total protein detection (D), while proteins from three other gels were transferred to nitrocellulose membranes. Membranes were probed with immune sera diluted 1:200 from 04B90 (A), 04B91 (B),

TABLE 1. *A. marginale* proteins identified by LC–MS-MS

Spot no.	Genome identity	Protein annotation	No. of distinct peptide matches[a]	% Sequence coverage[b]	MASCOT ion score[c]	Predicted location	Molecular mass (kDa)[d]
4	AM197	Undefined product	1	10	23	Outer membrane	47
5	AM366	Undefined product	5	2	27	Outer membrane	25
7, 8	AM097	Conjugal transfer protein	6, 9	29	387, 534	Membrane associated	30
9	AM854	Undefined product	2	11	137	Outer membrane	25
14	AM1314	VirB10	4	10	194	Inner membrane	31
17	AM387	Undefined product	3	1	31	Membrane associated	20
	AM236	MSP5	1	8	30	Outer membrane	20
19	AM127	Undefined product	5	3	17	Outer membrane	20
22	AM1142	OpAG2	7	19	347	Outer membrane	30
	AM1315	VirB9	3	24	59	Outer membrane	30
	AM1220	OMP7	1	4	44	Outer membrane	30
25	AM1164	OMP4	2	8	213	Outer membrane	37
	AM075	OMP14	1	4	24	Outer membrane	37
26–41	AM1144	MSP2	3–27[e]	10–29[e]	90–821[e]	Outer membrane	37
43, 45, 46	AM254	Elongation factor Tu	5, 6, 2	18	433	Membrane associated	43
48	AM956	PepA cytosol amino peptidase	1	2	71	Outer membrane	49
49	AM254	Elongation factor Tu	3	18	250	Membrane associated	43
	AM1096	OMA87	1	6	18	Outer membrane	43
55	AM1223	OMP10	1	5	18	Outer membrane	75
60, 61	AM1063	MSP3	8, 3	2	73	Outer membrane	87
69	AM529	Undefined product	1	10	22	Outer membrane	24
72	AM072	Undefined product	4	9	21	Membrane associated	31
73, 74	AM878	*Anaplasma* appendage-associated protein family	5	11	202		
	AM879		4	13	165	Membrane associated	34, 31
	AM880		5	17	180		

[a] Number of peptides identified from each protein.
[b] Percentage of coverage by the identified peptides.
[c] MASCOT ion scores greater than 16 are significant (P value of <0.05).
[d] Molecular masses of protein spots picked for LC–MS-MS.
[e] For MSP2 proteins, ranges in MASCOT ion score, percentage of coverage, and number of distinct peptide matches are provided.

total of 75 spots (Fig. 3D), which coincided with the image overlay analyses, were excised from the gels, trypsinized, and processed by LC–MS-MS to obtain molecular masses of peptide fragments. Mass spectral data from the 75 spots were searched against the annotated *A. marginale* St. Maries strain genome database, yielding strong identification from 38 spots which contained a total of 24 proteins (Table 1). Protein scores in Table 1 are the sums of nonredundant individual ion scores of identified peptides, as reported by MASCOT. The table also reports, for each protein, the number of distinct peptides identified by the MS-MS analysis. Multiple proteins were identified in spots 17, 22, 49, 73, and 74. Of the 24 proteins identified by

and 04B92 (C), and binding was detected by HRP-conjugated anti-bovine IgG secondary antibody followed by a chemiluminescent substrate. To identify spots of interest, images from the SYPRO Ruby gel and the immunoblots were overlaid. Immunoreactive spots are labeled with numbers or blocks. Block a represents protein spots 26 to 41, identified as MSP2. Spots 60 and 61 represent MSP3, and spot 17 represents MSP5. The remaining immunogenic protein spots have been labeled on the immunoblots. Spots 12 and 70 were not immunoreactive but were used as two of several reference spots to determine reproducibility of outer membrane separated 2D gels. Spots 6, 63, and 17 were used to align 2D immunoblots. See Table 1 for a complete list of identified proteins.

LC–MS-MS, MSP2, MSP3, and MSP5 have previously been described as immunogenic in cattle (2, 3, 13, 14, 16, 17, 39, 48, 49, 58). Of the 21 remaining proteins, 15 (AM075, AM097, AM127, AM197, AM254, AM387, AM854, AM956, AM1096, AM1142, AM1164, AM1220, AM1223, AM1314, and AM1315) were recognized by IgG antibodies from all three animals, and 5 (AM072, AM529, AM878, AM879, and AM880) of the 21 proteins were recognized by IgG from only calf 04B90. One protein (AM366) was recognized by IgG from calves 04B91 and 04B92 (Fig. 3). Immunoblotting was also performed using immune sera from outer membrane vaccinates to detect IgG2 (data not shown). The same pattern of spots was revealed, but in some cases the reactivity was weaker than with total IgG. In addition, total IgG from calf 04B90 detected four additional spots, but not enough LC–MS-MS data were obtained for a positive score when searching the annotated *A. marginale* genome. Otherwise, there were no differences in the numbers of spots detected when anti-IgG2 or anti-IgG was used (data not shown). Figure 4 summarizes the antibody recognition by all three animals.

To predict the cellular location of the newly identified proteins, topology prediction algorithms, including TMHMM and posttranslational modification prediction algorithm SignalP, were used (Table 1). Of the 21 putative antigenic proteins, 10 were predicted by SignalP (9) to contain a signal peptide. Thirteen of the remaining proteins that were not predicted to have a signal peptide were predicted by TMHMM (56) to contain transmembrane domains (Table 1). A single predicted internal membrane protein, AM1314 (VirB10), was identified. Proteins predicted to be within the outer membrane included the undefined proteins AM197, AM366, AM529, AM854, AM127, and AM956. Previously defined proteins also included AM075 (OMP14), AM1096 (OMA87), AM1142 (OpAG2), AM1223 (OMP10), AM1315 (VirB9), AM1220 (OMP7), and AM1164 (OMP4) (Table 1). Membrane-associated proteins included the undefined products AM072 and AM387, as well as AM097 (conjugal transfer protein), AM254 (elongation factor Tu [EF-Tu]), and the protein family of AM878, AM879, and AM880 (*Anaplasma* appendage-associated proteins) (Table 1).

Protein BLAST database searches (http://www.ncbi.nlm.nih.gov/BLAST/) were performed on undefined proteins to determine identity with protein sequences deposited in the NCBI database since the annotation of the *A. marginale* genome (Table 2). BLAST searches were also performed on annotated proteins other than MSP2, MSP3, and MSP5, in order to determine sequence identity in related organisms (Table 2). Three undefined proteins (AM197, AM854, and AM127) and nine defined proteins (AM075, AM097, AM254, AM956, AM1096, AM1142, AM1164, AM1314, and AM1315) had significant BLAST scores to putative proteins in *A. ovis*, *A. phagocytophilum*, *Ehrlichia canis*, *E. chaffeensis*, or *E. ruminantium* (Table 2). The remaining proteins (AM072, AM366, AM387, AM529, AM878, AM879, AM880, AM1220, and AM1223) did not have a significant BLAST score to any putative protein.

DISCUSSION

This study used a combined immunologic, proteomic, and genomic approach to discover new antigenic proteins in the protection-inducing outer membrane immunogen of *A. marginale*. Evidence for a protective role of humoral immunity against rickettsial pathogens has been established (8, 24, 29, 33), and more specifically there is evidence for a protective role of OMP-specific IgG2 antibodies. Complete protection against homologous strain challenge in *A. marginale* outer membrane-immunized cattle was associated with high IgG2 titers against OMP (16). Furthermore, passive transfer of OMP-specific immune sera, comprised mainly of IgG2a, IgG2b, and IgG3 antibodies, from immunocompetent mice provided SCID mice with significant protection from infection with *E. chaffeensis* (34, 35). Therefore, a type 1 immune response regulated by IFN-γ that influences isotype switching to *A. marginale* OMP-specific IgG2 may be important to prevent bacterial attachment and penetration of host cells and to enhance phagocytosis and complement fixation (21, 40).

There have been several recent attempts to identify novel proteins in *A. marginale* by using various antigen preparations (6, 53). In one study, cattle were immunized with membrane proteins fractionated by isoelectric point. Five proteins were identified in a fraction containing proteins with pIs ranging from pH 7.7 to 9.6, which induced partial protective immunity. These novel proteins were designated Ana43, Ana37, Ana32, Ana29, and Ana17 based on their molecular masses in kDa (53). Of these, sequence analysis of Ana29 predicted this to be an inner membrane protein (30), and partial amino acid sequencing of Ana32 identified this protein as OpAG3 (11, 53). In a separate study, proteins from *A. marginale* were separated by one- or two-dimensional electrophoresis and immunoblots were probed with sera from cattle that had been immunized with *A. marginale* and then challenged, in order to identify IgG2-reactive proteins (6). Using mouse antibodies specific for MSP1a, MSP2/MSP3, or MSP5, the investigators identified these proteins on one-dimensional gel immunoblots. Immune bovine serum detected proteins of similar sizes on both one- and two-dimensional gel immunoblots, including numerous proteins that comigrated with proteins of the same size as MSP2, as well as unknown proteins designated Am56, Am50, Am38, and Am28, based on their molecular masses in kDa (6). However, neither amino acid nor encoding gene sequences were identified for any of these proteins. Thus, in this study, the recognition of MSP1a, MSP2, MSP3, and MSP5 by postimmunization and challenge bovine serum was tentatively based on apparent molecular masses of immunoreactive proteins (6).

In the present study, a strategy was implemented to identify antigenic proteins in outer membrane preparations which have been shown to induce protection against homologous strain challenge in cattle (16, 58). The annotated *A. marginale* genome (11) provided a searchable database that was used to identify the gene sequences of 24 proteins based on LC–MS-MS analysis of immunoreactive spots. Among these were the well-characterized proteins MSP2, MSP3, and MSP5 (2, 3, 13, 14, 17, 46, 48, 60). Of the 75 spots selected, 16 were identified as MSP2, consistent with the high abundance of this protein in the outer membrane (43). The large number of MSP2 proteins reflects the generation during acute and persistent infection of antigenic variants of MSP2 that vary slightly in size (23). In a recent study, we identified nine different *msp2* transcripts in acute-infection blood from several challenged cattle (1), which is consistent with the identification of multiple MSP2 protein spots by 2D electrophoresis and immunoblot-

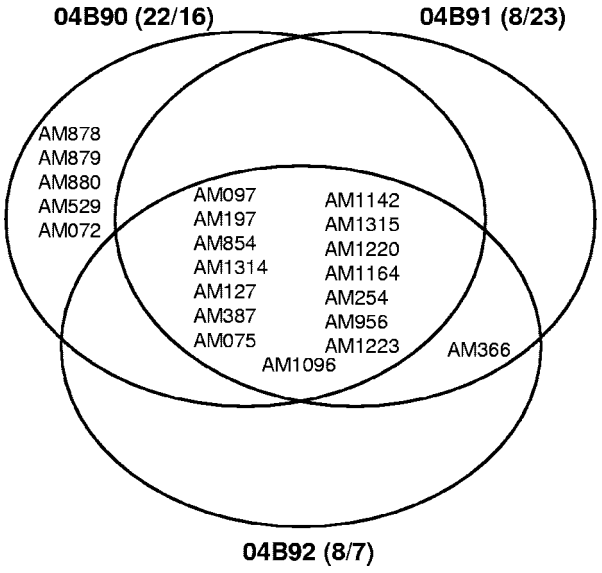

FIG. 4. Comparison of proteins recognized by individual immune sera. A Venn diagram illustrates the individual proteins recognized by sera from the three outer membrane-immunized cattle. The BoLA *DRB3* restriction fragment length polymorphism haplotypes are indicated in parentheses.

ting. Antigenic variation in MSP2 occurs by gene conversion of whole pseudogenes and small segments of pseudogenes into a single expression site (5, 12), resulting in the expression of proteins that have conserved amino- and carboxy-terminal regions and a central hypervariable region. Thus, the different variants would be expected to have similar molecular masses and different pIs, consistent with our data and those of Riding et al. (53). All of the 16 MSP2 spots yielded peptide sequences of VEVEVGYER (amino acids [aa] 130 to 138), AVEGA EVIE (aa 279 to 287), and/or DTGIASFNFAYFGGELGVR (aa 388 to 406), from the highly conserved amino or carboxy regions of MSP2, where relative amino acid sequence position is based on the *msp2* 11.2 genomic clone (46). MSP1a and MSP1b (43, 44) were not identified, perhaps because these proteins were not sufficiently abundant to be detected by LC–MS-MS or because the predicted pI of the *A. marginale* St. Maries strain MSP1a is 4.99 and those of MSP1b1 and MSP1b2 are 4.78 and 4.87, which would fall just outside of the range of the pH 5 to 8 gradient used for IEF. The presence of MSP1a in the outer membrane preparation used for 2D electrophoresis was verified by immunoblotting whole outer membranes (Fig. 2). Furthermore, $CD4^+$ T cells from the vaccinated cattle responded specifically to native MSP1 protein and to selected peptides of MSP1a (unpublished observations). Additionally, while we were able to identify MSP4 by LC–MS-MS analysis of spots selected by molecular mass (data not shown), MSP4 was surprisingly not reactive with immune sera. However, it has been reported that MSP4 may not be very antigenic in cattle immunized with *A. marginale* (6).

Twenty-one of the proteins identified in our study had not previously been characterized immunologically, although two of these were reportedly expressed. The product of the OpAG2 gene of the *msp2* operon was shown to be expressed on the surface of *A. marginale* during infection of bovine erythrocytes as well as within tick tissue (36). Also, an *Anaplasma* appendage-associated protein localizes with actin filaments on the surface of *A. marginale* during intracellular infection (57). However, the present study is the first report that either of these proteins induces an IgG response in outer membrane-immunized cattle. Of the remaining 19 newly identified proteins, 7 were undefined, in that they were annotated in the genome as putative proteins but had no known function. BLAST searches were performed on

TABLE 2. Comparative amino acid sequence analysis of newly identified *A. marginale* proteins and homologs in related organisms

Protein annotation	BLAST identity and score[a]	Organism identity[b]
Undefined product (AM197)	Predicted membrane protein (1e-24)	*E. canis* (YP_303304)
Undefined product (AM366)	No significant BLAST score	
Undefined product (AM854)	OMP and peptidoglycan associated protein (5e-30)	*E. canis* (YP_303196)
Undefined product (AM387)	No significant BLAST score	
Undefined product (AM127)	Hypothetical protein (1e-77)	*E. chaffeensis* (ZP_00544751)
Undefined product (AM529)	No significant BLAST score	
Undefined product (AM072)	No significant BLAST score	
OpAG2 (AM1142)	2e-23	*E. chaffeensis* (AAK28661)
OMP4 (AM1164)	6e-32	*A. ovis* (AAG37873)
OMP7 (AM1220)	No significant BLAST score	
OMP10 (AM1223)	No significant BLAST score	
OMP14 (AM075)	8e-40	*A. phagocytophilum* (AAO30097)
Conjugal transfer protein (AM097)	2e-96	*E. canis* (YP_303416)
VirB9 (AM1315)	2e-80	*A. phagocytophilum* (AAM00421)
VirB10 (AM1314)	1e-118	*A. phagocytophilum* (AAM00418)
Elongation factor Tu (AM254)	0.0	*E. ruminantium* (YP_180474)
OMA87 (AM1096)	0.0	*E. chaffeensis* (ZP_00544838)
PepA cytosol amino peptidase (AM956)	2e-173	*E. ruminantium* (CAI28112)
Anaplasma appendage-associated protein (AM878)	No significant BLAST score	
Anaplasma appendage-associated protein (AM879)	No significant BLAST score	
Anaplasma appendage-associated protein (AM880)	No significant BLAST score	

[a] BLAST searches were performed on undefined and annotated proteins to determine the percent sequence identity with proteins in closely related organisms. BLAST E values of <1e-20 were considered to be significant and are indicated.

[b] GenBank accession numbers of the proteins identified in related organisms are provided in parentheses.

these undefined proteins to identify homologous sequences in other organisms that had been deposited in the GenBank database since the *A. marginale* genome was annotated. Two sequences had identity to hypothetical proteins in *E. canis* (AM197 and AM854) and one sequence had identity to a hypothetical protein in *E. chaffeensis* (AM127), while AM072, AM366, AM387, and AM529 did not have sequence similarity to any published proteins.

Potential functions of the remaining proteins were determined by comparison with known proteins in other bacteria. Proteins with designated functions include VirB9, VirB10, conjugal transfer protein, EF-Tu, and PepA cytosol amino peptidase. VirB9, VirB10, and conjugal transfer protein are considered part of the type IV secretion system (TFSS), which mediates secretion or cell-to-cell transfer of macromolecules, proteins, or DNA-protein complexes in gram-negative bacteria, such as *Bordetella pertussis*, *Legionella pneumophila*, *Rickettsia prowazekii*, *Ehrlichia chaffeensis*, and *Anaplasma phagocytophilum* (7, 18, 42). While the role of the TFSS in *A. marginale* has not been defined, the *virB* operon in *Brucella suis* was shown to be required for intracellular survival after phagocytosis (22, 41) and may therefore be critical to *A. marginale* survival in erythrocytes.

The identification of PepA cytosol amino peptidase and EF-Tu was somewhat unexpected because both are believed to be cytoplasmic proteins. PepA cytosol amino peptidase was so designated by genomic annotation (11) but by TMHMM was predicted to be anchored through the outer membrane and exposed to the outside environment. In gram-positive *Staphylococcus epidermidis*, this protein appears to be cell surface associated (52), but its location in gram-negative bacteria is unclear. EF-Tu was also predicted by TMHMM analysis to be surface associated, and the presence of EF-Tu in various compartments of bacteria has been previously documented. EF-Tu associates with the cell surface of *Escherichia coli* (32) and that of *Lactobacillus johnsonii* (28). Furthermore, in *L. johnsonii*, EF-Tu had properties of an adhesion factor, mediating attachment of bacteria to human intestinal cells (28). In *Mycobacterium leprae* and *Mycobacterium pneumoniae*, EF-Tu was identified as a cell wall-associated protein (19, 37). Thus, in some organisms EF-Tu appears to function in the bacterial cytoplasm for protein synthesis, whereas in other organisms EF-Tu appears to localize to the surface, where it can function in bacterial attachment to host cells.

Proteins with unknown functions include OMP4, OMP7, OMP10, OMP14, and OMA87. With the exception of OMA87, these proteins were designated members of the MSP2 superfamily because of sequence identity to a family of surface antigens known as pfam01617 (11). However, their functions in *A. marginale* are unclear.

The approach of using LC–MS-MS analysis of immunoreactive protein spots excised from 2D gels has several limitations. One unpredicted result from this study was that only 52% of the protein spots submitted for LC–MS-MS were positively identified as *A. marginale* proteins. Those spots that did not yield a positive identification in the *A. marginale* genome were also searched against the NCBI database, and spots 1 to 3, 51, 52, 62 to 65, and 67, comprising 13% of the immunoreactive spots, were determined to be of bovine origin (data not shown). It is probable that contamination with bovine protein in these spots quenched any identification of *Anaplasma* proteins, as it is unlikely that cattle would develop an antibody response to self-antigens following immunization. In fact, sera from calves immunized with outer membranes did not react on immunoblots to uninfected erythrocytes (data not shown). The failure to positively identify the additional protein spots by LC–MS-MS may have resulted from poor solubilization of very hydrophobic outer membrane proteins and therefore incomplete trypsinization.

There were several examples of differences between actual and predicted molecular masses or pIs. These discrepancies could result from posttranslational modification (as is known to occur with MSP1a, which is glycosylated [4, 27]) or partial protein degradation. Furthermore, algorithms to predict pIs are not completely accurate. There were also examples of identifying the same protein in more than one spot. Finding MSP2 and MSP3 in multiple spots was expected, because multiple variants of these proteins, which can differ slightly in molecular masses and pIs, are present during infection (1–3, 13–15, 17, 43, 44, 48). The peptides identified in spots 73 and 74 were conserved peptides derived from the closely related appendage-associated protein family members, so that the analysis was unable to assign an individual member to an individual spot. Additionally, the presence of conjugal transfer protein in spots 7 and 8 and of EF-Tu in spots 43 to 46 and 49 may have resulted from aberrant migration on the gels if these proteins were complexed with their substrates. EF-Tu interacts with tRNA during protein synthesis, and high-affinity binding to tRNA might have impeded the removal of tRNA during outer membrane preparation. Similarly, as a component of the TFSS, conjugal transfer protein may mediate secretion or cell-to-cell transfer of macromolecules and protein-DNA complexes. Thus, residual DNA and/or RNA bound to these two proteins may have affected their mobilities.

The identification of novel antigens in the complex outer membrane immunogen has expanded the number of targets recognized by immune serum IgG and thus provided a number of vaccine candidates for further study. For an effective vaccine, it is important that these proteins can be recognized by serum antibody and $CD4^+$ T lymphocytes from a majority of cattle within a vaccinated population upon infection and that these proteins are conserved among different strains of *A. marginale*. Animals selected for this study expressed common major histocompatibility complex class II *DRB3* alleles that are present in over 50% of Holstein-Friesian cattle (54). Furthermore, the majority of the novel antigenic proteins were recognized by IgG2 from all three animals (Fig. 4), indicating the stimulation of T-helper-lymphocyte responses that promote switching to IgG isotypes. If protective, these vaccine candidates may be useful at the population level.

The availability of the sequences encoding these proteins from other *A. marginale* strains will enable determination of protein sequence conservation among the different strains. Additionally, identification of these antigenic proteins may offer insight to immunoreactive proteins in related pathogens, such as *A. phagocytophilum*, *E. chaffeensis*, *E. canis*, and *E. ruminantium*. *A. phagocytophilum* contains proteins highly similar to *A. marginale* MSP2 (23, 31, 62), and BLAST searches of the *A. marginale* proteins identified in this study indicate that several have sequence identity to proteins in *A. phagocytophilum*

and species within the genus *Ehrlichia*. Thus, the identification of these immunogenic *A. marginale* proteins may also provide direction towards specific antigens for vaccine development against related animal and human pathogens.

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