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Analysis of the *Anaplasma marginale* Major Surface Protein 1 Complex Protein Composition by Tandem Mass Spectrometry

Henriette Macmillan,[1] Kelly A. Brayton,[1] Guy H. Palmer,[1] Travis C. McGuire,[1] Gerhard Munske,[2] William F. Siems,[2] and Wendy C. Brown[1]*

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

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The protective major surface protein 1 (MSP1) complex of *Anaplasma marginale* is a heteromer of MSP1a and MSP1b, encoded by a multigene family. The *msp1*β sequences were highly conserved throughout infection. However, liquid chromatography-tandem mass spectrometry analysis identified only a single MSP1b protein, MSP1b1, within the MSP1 complex.

The outer membrane proteins (OMPs) of bacterial pathogens mediate interaction with the host, including the immune system. Importantly, the function of OMPs is dependent upon both the conformation of the individual proteins, including oligomerization, and supramolecular interaction with other OMPs through covalent and noncovalent interactions. Among certain intracellular pathogens, most notably those of the genus *Chlamydia*, there is a high degree of intermolecular cross-linking in the outer membrane (7, 19, 24, 37). A similar pattern occurs in the rickettsial pathogen *Anaplasma marginale*, in which the outer membrane proteins associate through extensive disulfide cross-links, forming homomeric and heteromeric complexes (35). The immunologic importance of these complexes is illustrated by the solid protection against *A. marginale* challenge induced by immunization using isolated outer membranes (13, 34), protection which is not fully achieved by immunization with individual proteins alone or in a mixture (1, 27, 29).

In contrast to the failure of individual *A. marginale* OMPs to consistently induce protection, immunization with the major surface protein 1 (MSP1) complex provides significant protection against high-level rickettsemia and disease (26, 27). This MSP1 complex is composed of proteins encoded by genes of two distinct chromosomal loci, *msp1*α and *msp1*β (9). The precise composition of the immunoprotective MSP1 complex has remained undefined, largely due to the presence of multiple *msp1*β genes and partial genes (6, 8, 9, 14, 36), an arrangement that could lend itself to segmental gene conversion, as has been reported for MSP2 and MSP3 (10–12, 23), other immunodominant proteins of *A. marginale*. The recent report of the complete genome sequence of the St. Maries strain of *A. marginale* has provided the unambiguous full complement of *msp1*α and *msp1*β genes and facilitates the mapping of the individual components of the MSP1 complex to the genome (9). The goal of the present study was to characterize the complexity of the MSP1b component in the immunoprotective MSP1 complex.

The genomic organization of the *msp1*α and *msp1*β genes is schematically represented in Fig. 1. Although MSP1a and MSP1b are covalently linked through disulfide bonds (35), the encoding loci are separated by 320 kb in the St. Maries strain genome (9). MSP1a is encoded by a single-copy gene, *msp1*α, and is conserved among strains, with the exception of a set of tandem repeats of 84 or 87 bp at the 5′ end, which vary in number and sequence (4, 8, 21, 25). In contrast, MSP1b is encoded by the polymorphic *msp1*β family, consisting of five paralogous genes (6, 8, 9, 14, 36). Vector NTI (Invitrogen, Carlsbad, CA) was used to align the predicted amino acid sequences of the *msp1*β genes from the St. Maries strain to determine the identities among the genes. The deduced amino acid sequences of the two full-length genes, *msp1*β*1* and *msp1*β*2*, are 92% identical, while the predicted amino acid

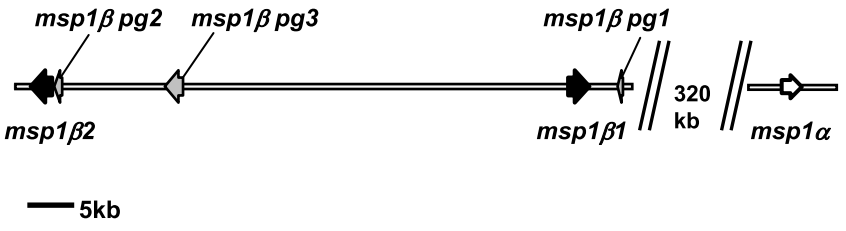

FIG. 1. Genomic organization of the *A. marginale msp1*α and *msp1*β loci in the St. Maries strain genome (9). The white arrow indicates *msp1*α; black arrows indicate full-length *msp1*β genes; gray arrows indicate *msp1*β partial genes pg1, pg2, and pg3.

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

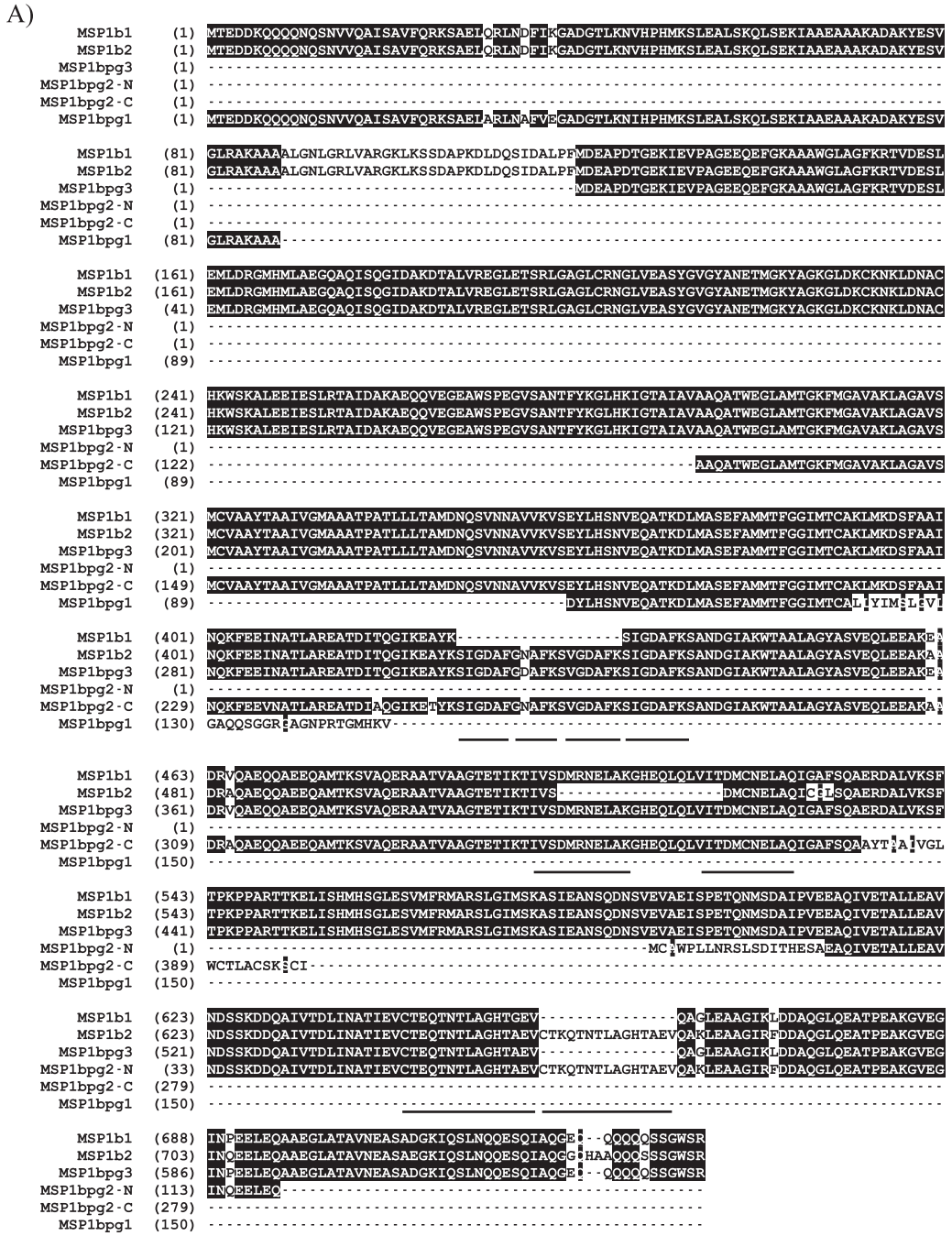

FIG. 2. Predicted amino acid sequences of the MSP1b proteins from *A. marginale* strain St. Maries. For the aligned sequences of MSP1b1, MSP1b2, MSP1bpg3, MSP1bpg2, and MSP1bpg1, identical amino acids are indicated by white letters on a black background, and repeat regions are underlined (A). Nucleotide sequences and corresponding predicted amino acid sequences of the repeat regions (underlined) were identified for the *msp1*β genes (B). Numbers in parentheses indicate the relative positions in the protein or gene sequence.

sequence of the longest partial gene (pg), *msp1*β_{pg3}, shares 97% and 75% identity with MSP1b1 and MSP1b2, respectively. For optimal alignment (Fig. 2A), the predicted amino acid sequence of *msp1*β_{pg2} is divided into two regions (MSP1bpg2-N and MSP1bpg2-C). The N-terminal region of MSP1bpg2 is 66% and 81% identical to the C-terminal region of MSP1b1 and MSP1b2, respectively, while the C-terminal region of MSP1bpg2 is 85% and 82% identical to the N-terminal region of MSP1b1 and MSP1b2, respectively. When the predicted amino acid sequence of the shortest partial gene, *msp1*β_{pg1}, is compared to those of MSP1b1 and MSP1b2, the N-terminal region of MSP1bpg1 is 94% identical to the N-terminal regions of both MSP1b1 and MSP1b2, and the C-terminal region of MSP1bpg1 is 53% identical to those of both MSP1b1 and MSP1b2. Three types of repeats have been identified in MSP1b (5), as in the amino acid alignment of the five MSP1b sequences (Fig. 2A). The repeat

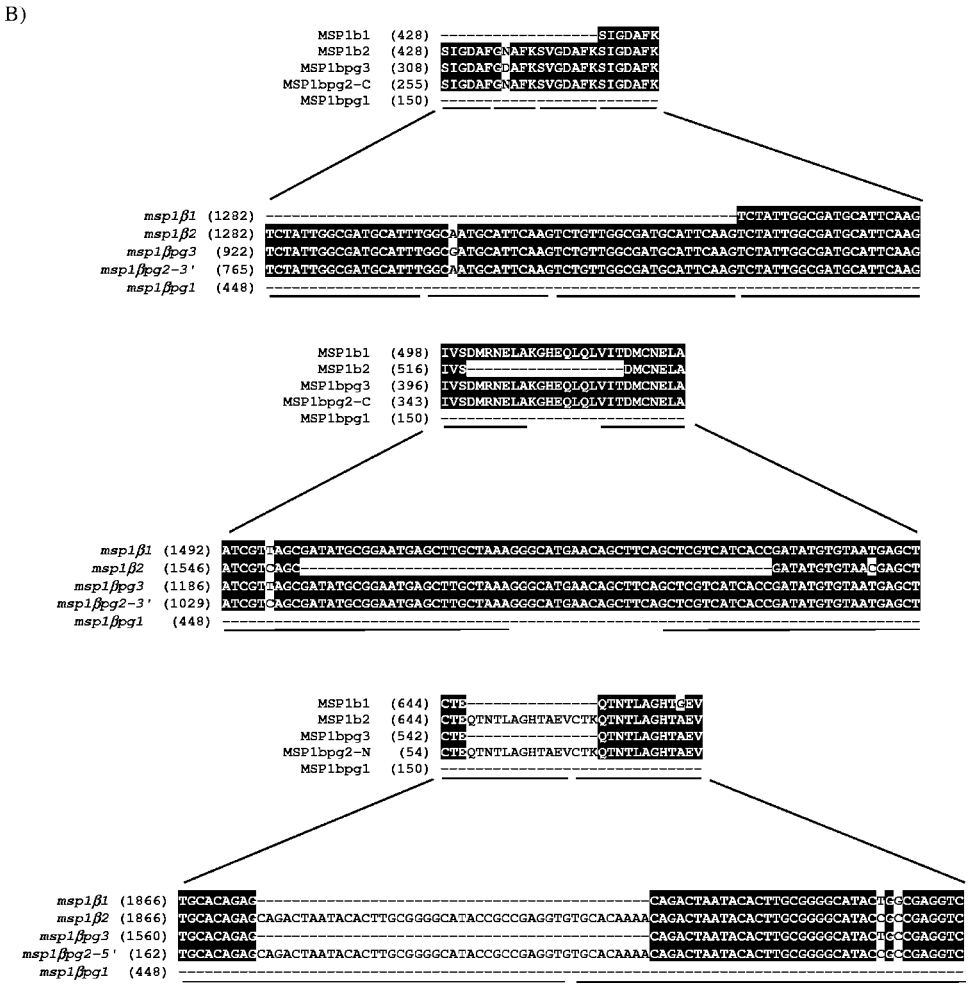

FIG. 2—*Continued.*

regions (Fig. 2B) create an opportunity for recombination events to occur and may lead to duplication or deletion of repeats during replication.

In *A. marginale*, recombination between full-length functional genes and partial genes occurs frequently, as has been described for *msp2* and *msp3* (10–12, 23), generating structural and antigenic variants (1–3, 16–18). To determine if deletion or duplication events occur at the repeat regions in the *msp1*β genes or if recombination occurs among the *msp1*β genes and is selected for during the transmission cycle, we sequenced all five *msp1*β genes at different time points during acute and persistent infection and following tick transmission. Calf C942bl was infected intravenously with an *A. marginale* strain St. Maries stabilate, and ticks (*Dermacentor andersoni*) were allowed to feed on C942b1 and subsequently transmit the infection to calf C956bl. Both calves had peak infections of $\geq 10^9$ *A. marginale* organisms per ml of blood. Calf C956bl was splenectomized 11 months after tick transmission and experienced a second peak of rickettsemia of $\geq 10^9$ *A. marginale* organisms per ml of blood. DNA was isolated from the calves during all three peaks of rickettsemia ($\geq 10^9$ organisms/ml of blood) and at one time point during persistent infection, in which the rickettsemia was $< 10^7$ organisms/ml of blood, and then sequenced. Primers specific for each *msp1*β gene were used (Table 1). Table 2 shows the number of clones sequenced from each calf at each time point. The sequences from these clones were aligned with the sequences derived from the St. Maries strain genome (9) using vector NTI. No clones were sequenced for *msp1*β_{pg1} and *msp1*β_{pg2} during persistent infection due to insufficient amounts of DNA. Although the repeat regions in the *msp1*β genes provide preferential sites for recombination, the clones had no duplications or deletions in the repeat regions. Polymorphisms were limited to nonclustered single-nucleotide substitutions, which occurred at an average of 3×10^{-4} errors per base pair, a rate that is within the range expected for *Taq* polymerase-based amplification. In contrast to the frequent recombinations observed in the *msp2* and *msp3* expression site copies (10–12, 17, 18, 23), our data indicate that either recombination within and among the *msp1*β genes is rare or that there is no strong selective pressure that allows organisms expressing recombined sequences to predominate in the population.

To determine which of the five *msp1*β genes were transcribed, RNA from all three peaks of rickettsemia was isolated and reverse transcribed using random hexamers (Invitrogen). The lack of recombination at the gene level allowed the use of

TABLE 1. Primers used for PCR amplification of *msp1*β genes

Primer[a]	Primer sequence (5′–3′)	Product amplified	Product size (bp)
Msp1b1 TF	CTTGACCAGAGCATTGACGCAC	*msp1*β*1* transcript	1,875
Msp1b1/pg3 TR	AGAAGACTGCTGTTGTTGCTGC	Probe	
Msp1b2 TF	GTAACGAGCTTGCACAAATATGTGGG	*msp1*β*2* transcript	695
Msp1b2 TR	GGAAGAAGACTGCTGTTGTGCAG		
Msp1bpg3 TF	GTCTATTGGCGATGCATTTGGCG	*msp1*β_{pg3} transcript	979
Msp1b1/pg3 TR	AGAAGACTGCTGTTGTTGCTGC		
Msp1bpg2 TF	CCCACCTTGTGTGCATGGC	*msp1*β_{pg2} transcript	1,182
Msp1bpg2 TR	CGCCAAAGTACACCAAAGGCC		
Msp1bpg1 TF	CAGAATTAGAGTATATCGCCCTGTATGCATG	*msp1*β_{pg1} transcript	536
Msp1bpg1 TR	CCCAGTACGGGGGTTTCCC		
Msp1b1/b2 OF	CGGTTATCAAGACATTGTTAAGTAGGTAGGTG	*msp1*β*1* ORF	2,530
Msp1b1 OR	GCCGAATATGCGCAGATGGC		
Msp1b1/b2 OF	CGGTTATCAAGACATTGTTAAGTAGGTAGGTG	*msp1*β*2* ORF	2,338
Msp1b2 OR	CTACCACGTTCTTGTATCCACACAAGG		
Msp1bpg3 OF	GCATAGGGAAGATTGAAGTACCAGC	*msp1*β_{pg3} ORF	2,096
Msp1bpg3 OR	CTACACCGTGTCAGTTAAAGGTAGGG		
Msp1bpg2 OF	CACAGCAGCATATTCAGGAATGTTGAAG	*msp1*β_{pg2} ORF	1,466
Msp1bpg2 OR	GCGATTTTCGCCTCACAGAGC		
Msp1bpg1 OF	CATCATCTCGCAGATGAGCACTCG	*msp1*β_{pg1} ORF	827
Msp1bpg1 OR	GCGATACAACAGCCACAAAGCG		
Msp1b1/b2 EF	ATGACAGAAGACGACAAGCAACAAC	Probe	

[a] TF and TR indicate forward and reverse primers designed to amplify specific *msp1*β transcripts. OF and OR indicate forward and reverse primers designed for the amplification of each *msp1*β open reading frame (ORF). EF indicates a forward primer designed for expression of MSP161 and MSP162.

genome data for designing specific primers for individual *msp1*β transcripts to avoid cross-reactive priming. The optimal annealing temperature for each primer set was determined by performing gradient PCR with the cloned genes as templates (results not shown). PCRs for *msp1*β*1*, *msp1*β*2*, and *msp1*β_{pg2} were performed at an annealing temperature of 60°C, while reactions for *msp1*β_{pg1} and *msp1*β_{pg3} were performed at 70°C and 71.8°C, respectively. The PCR products were visualized on ethidium bromide-stained gels, transferred to nylon membranes, and hybridized with an *msp1*β digoxigenin (DIG)-labeled probe. Primers used to amplify the 2.2-kb probe were designed to anneal to the conserved ends of the full-length genes to ensure that the probe would bind to all five *msp1*β genes. Detection was performed with a DIG luminescent detection kit and CDP-Star (Roche Applied Science, Indianapolis, IN) and visualized by autoradiography. Transcripts for both *msp1*β*1* and *msp1*β*2* were detected during peak infections in calf C942bl and calf C956bl (Fig. 3). A faint band for *msp1*β_{pg3} was observed in the Southern blot for calf C942bl (Fig. 3A), and in two additional experiments, a similar band for *msp1*β_{pg3} was detected for calf C956bl during the postsplenectomy peak of rickettsemia (data not shown). To rule out cross-reactivity and to confirm the specificity of the transcripts, the bands for *msp1*β*1*, *msp1*β*2*, and *msp1*β_{pg3} were cloned and sequenced. The sequences were identical to the genome sequences and confirmed the specificity of amplification.

TABLE 2. Numbers of clones sequenced for each *msp1*β gene during infection

Clone	No. of clones for indicated calf and time of sampling[a]			
	C942bl, acute	C956bl		
		Acute	Persistent	Postsplenectomy (acute rickettsemia)
*msp1*β*1*	13	32	13	25
*msp1*β*2*	29	22	12	28
msp1β_{pg1}	26	10	0	10
msp1β_{pg2}	13	31	0	29
msp1β_{pg3}	13	19	6	5

[a] Blood was obtained from calf C942bl during acute infection (acute) and from C956bl during acute and persistent (persistent) stages of infection and following splenectomy (postsplenectomy), when the rickettsemia again peaked.

To determine if MSP1b1, MSP1b2, and/or MSP1bpg3 associate with MSP1a in the MSP1 complex, our strategy was to immunoprecipitate the MSP1 complex with a monoclonal antibody (MAb), Ana22B1, specific for MSP1a (21), followed by analysis using liquid chromatography-tandem mass spectrometry (LC–MS-MS). To confirm first that LC–MS-MS could distinguish between MSP1b1 and MSP1b2, full-length *msp1*β*1* and *msp1*β*2* were amplified by PCR and cloned into pTrcHis (Invitrogen). The proteins were expressed in *Escherichia coli* and purified with Probond resin (Invitrogen). A FLAG-tag (DYKDDDDK) (22) incorporated at the C-terminal end allowed for additional purification by affinity chromatography using anti-FLAG M2-agarose (Sigma, St. Louis, MO). Ten

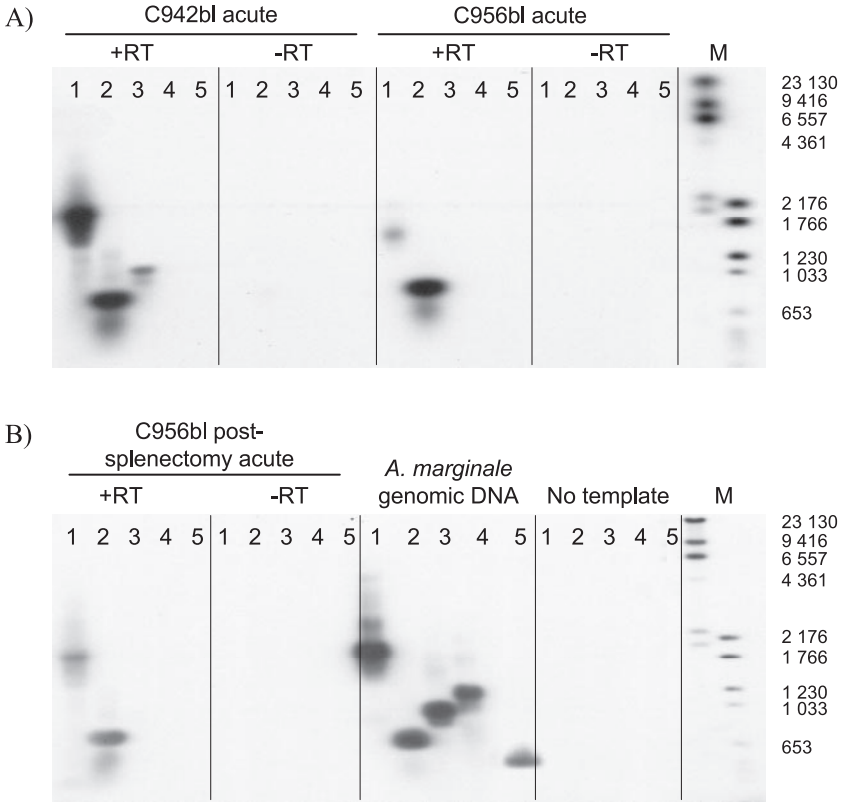

FIG. 3. Analysis of *msp1*β transcripts by Southern blotting with (+RT) and without (−RT) reverse transcription. *A. marginale* strain St. Maries RT-PCR products were hybridized with a DIG-labeled *msp1*β probe. cDNA samples collected from calf C942b1 during acute rickettsemia (A) and from calf C956bl presplenectomy (A) and postsplenectomy (B) were amplified with primers specific for each *msp1*β transcript (Table 1). *A. marginale* genomic DNA was used as a positive control (B). Lanes 1 to 5 represent the primer sets used: 1, *msp1*β*1*; 2, *msp1*β*2*; 3, *msp1*β_{pg3}; 4, *msp1*β_{pg2}; 5, *msp1*β_{pg1}. For the *A. marginale* genomic DNA (B), the image in lane 5 is the result of a longer exposure of the same gel compared to the images of lanes 1 to 4. The sizes of digoxigenin-labeled markers (lane M) are indicated in base pairs on the right of the same gel as shown.

micrograms of each purified protein was separated by sodium dodecyl sulfide-polyacrylamide gel electrophoresis, and the Coomassie blue-stained bands (Fig. 4) were excised for in-gel trypsin digestion (32). The trypsin-digested recombinant proteins were used for LC–MS-MS analysis, which was performed using an LC Packings 180-μm C_{18} 100-Å PepMap column (Dionex, Sunnyvale, CA), an Esquire HCT electrospray ion trap (Bruker Daltonics, Billerica, MA), and an LC Packings Ultimate Nano high-performance liquid chromatography system. A flow rate of 800 nl/min with a 0% to 60% gradient of 0.1% trifluoroacetic acid in 95% acetonitrile was used for 95 min followed by an additional 15 min with a 100% solution of 0.1% trifluoroacetic acid in 95% acetonitrile. The Mascot search engine (Matrix Science) was used to search the peptide fingerprints against the *A. marginale* strain St. Maries genome (9, 20). The Mascot search was performed with carbamidomethyl as the fixed modification of cysteine and variable oxidation of methionine. One missed trypsin cleavage was allowed during the search. Twenty-seven peptides identified for MSP1b1 covered approximately 49% of the MSP1b1 amino acid sequence, with a Mascot score of 2,324. Thirty-one peptides identified for MSP1b2 covered approximately 56% of the MSP1b2 amino acid sequence, with a Mascot score of 4,433. In Mascot, the score for an MS-MS match is based on the abso-

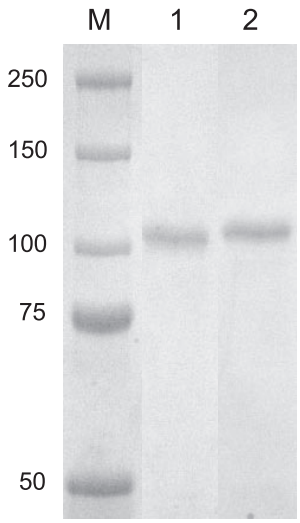

FIG. 4. Expression of recombinant MSP1b1 and MSP1b2. Proteins were purified with an anti-FLAG affinity column. Lane M, dual-color molecular size markers (in kDa); lane 1, recombinant MSP1b1; lane 2, recombinant MSP1b2.

TABLE 3. Unique peptides identified by LC–MS-MS analysis of recombinant MSP1b1 and MSP1b2

Peptide (aa positions)	Sequence of peptide	Ions score of peptide detected at protein amt of[b]:		
		32 pmol	6 pmol	1.2 pmol
MSP1b1				
461–478	EADRVQAEQQAEEQAMTK[a]	16		
465–478	VQAEQQAEEQAMTK	77		
497–508	TIVSDMRNELAK	4		
669–683	LDDAQGLQEATPEAK	85	27	
684–712	GVEGINPEELEQAAEGLATA VNEASADGK	57	19	
713–739	IQSLNQQESQIAQGEQQQQQ QSSGWSR	114	45	58
Mascot score[c]		2,324	250	60
MSP1b2				
428–438	SIGDAFGNAFK	35		
483–496	AQAEQQAEEQAMTK	112	74	
515–535	TIVSDMCNELAQICGLSQAER	82		5
662–676	QTNTLAGHTAEVQAK	53	6	
684–698	FDDAQGLQEATPEAK	93	16	
699–727	GVEGINQEELEQAAEGLATA VNEASAEGK	102	57	6
728–756	IQSLNQQESQIAQGGQHAAQ QQSSSGWSR	116	67	
Mascot score		4,433	837	68

[a] This peptide sequence is the same as the peptide sequence at positions 465 to 478 but has four additional N-terminal amino acids because of a missed trypsin cleavage site.

[b] Ions score is $-10\log(P)$, where P is the probability that the observed match is a random event. Individual ions scores of >25 (32 pmol) and >18 (6 and 1.2 pmol) indicate identity or extensive homology ($P < 0.05$).

[c] Mascot scores reported are the sums of nonredundant individual ions scores of identified peptides.

lute probability (P) that the observed match between the experimental data and the database sequence is a random event. The individual ions score is the absolute probability that the observed match is a random event and is reported as $-10\log_{10}(P)$, where P is the absolute probability. A probability of 10^{-20} thus becomes a score of 200. Individual ions scores of >19 indicate significant identity or extensive homology ($P < 0.05$). The Mascot scores reported are the sums of nonredundant individual ions scores of identified peptides. Mascot scores are derived from ions scores as a nonprobabilistic basis for ranking proteins. Six peptides unique for MSP1b1 and seven peptides unique for MSP1b2 were identified (Table 3). Therefore we could confirm that, although MSP1b1 and MSP1b2 are 92% identical, unique peptides for each full-length MSP1b could be identified by LC–MS-MS from in-gel trypsin-digested proteins.

To immunoprecipitate the MSP1 complex, *A. marginale* organisms were isolated (28) during the postsplenectomy peak of infection from calf C956bl and incubated with MSP1a-specific MAb Ana22B1-coupled Sepharose beads (21, 27, 35). Affinity chromatography using Sepharose-coupled MAb Ana22B1 was previously used to isolate the native MSP1 complex for vaccine trials (26, 27); thus, this immunoprecipitated material mimics the protective immunogen. The immunoprecipitated complex was separated on a 7.5% Criterion gel (Bio-Rad, Hercules, CA) and stained with SYPRO ruby (Bio-Rad) for LC–MS-MS. Stained bands were excised from the gel and pooled from three identical lanes for in-gel trypsin digestion (20, 32). The trypsin-digested samples were analyzed by LC–MS-MS using a flow rate of 1.2 μl/min and a gradient of 10% to 100% of 0.1% trifluoroacetic acid in 95% acetonitrile for 50 min. Mascot was used to search the peptide mass fingerprints against the St. Maries strain genome, as done previously. A band at 75 kDa was confirmed to be MSP1a by a Mascot score of 299, with seven peptides matched and 20% amino acid coverage. The area between 90 and 150 kDa was excised as a block and found to be positive for MSP1b1 with a Mascot score of 58, with two peptides matched and 6% amino acid coverage. Two unique MSP1b1 peptides, LDDAQGLQEATPEAK (ions score, 43) and GVEGINPEELEQAAEGLATAVNE ASADGK (ions score, 29), were identified, and no unique MSP1b2 peptides were identified. Immunoprecipitation with MAb AmR38A6, which recognizes MSP1b (21, 35) and both recombinant MSP1b1 and MSP1b2 proteins (data not shown), was also performed, and the area between 100 and 150 kDa again scored positive for MSP1b1, with a Mascot score of 35, with two peptides matched and 3% amino acid coverage. Only one unique MSP1b1 peptide, LDDAQGLQ EATPEAK (ions score, 35), was identified.

To enrich for MSP1 in the sample used for immunoprecipitation (20), outer membranes were isolated from *A. marginale* organisms (28) obtained from calf C956bl during the postsplenectomy peak of infection and used for immunoprecipitation with MSP1a-specific MAb Ana22B1-coupled Sepharose beads (21, 27, 35). The immunoprecipitated complex was then separated on a 7.5% Criterion gel and either transferred to a nylon membrane for immunoblotting or stained with SYPRO ruby for LC–MS-MS. MSP1a-specific MAb Ana22B1 recognized the predicted 75-kDa protein on the immunoblot (Fig. 5, lane 3), while MSP1b-specific MAb AMR38A6 recognized a predominant band at approximately 100 kDa (Fig. 5, lane 9), indicating that the MSP1 complex could be immunoprecipi-

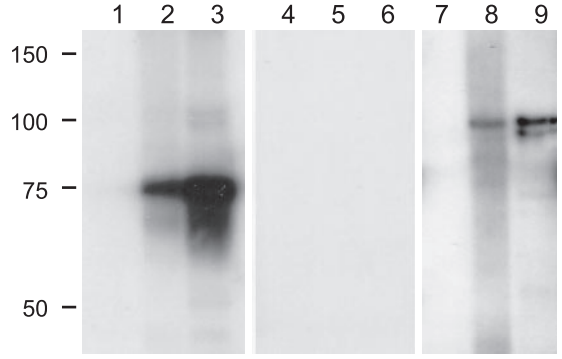

FIG. 5. Detection of MSP1a and MSP1b in *A. marginale* outer membranes immunoprecipitated with MSP1a-specific antibody. Lanes 1, 4, and 7, 1 μg of uninfected red blood cells; lanes 2, 5, and 8, 5 μg of *A. marginale* strain St. Maries organisms; lanes 3, 6, and 9, 30 μg of *A. marginale* strain St. Maries outer membranes immunoprecipitated with MAb Ana22B1 specific for MSP1a. Molecular size markers are indicated in kDa. The Western blot was developed with MAb Ana22B1 specific for MSP1a (lanes 1 to 3), MAb Tryp1E1 specific for *T. brucei* (lanes 4 to 6), and MAb AmR38A6 specific for MSP1b (lanes 7 to 9).

tated as shown by the presence of bands of the expected size and antigenic specificity. No reaction was observed with MAb Tryp1E1 (specific for *Trypanosoma brucei*), which was used as a negative control (Fig. 5, lanes 4 through 6). *A. marginale* strain St. Maries organisms were used as a positive control (Fig. 5, lanes 2, 5, and 8), and uninfected erythrocytes were used as a negative control on the immunoblot (Fig. 5, lanes 1, 4, and 7). All but one of the MSP1b1- and MSP1b2-specific peptides identified by LC–MS-MS are present in MSP1bpg3, so it would be difficult to distinguish MSP1bpg3 from MSP1b1 and MSP1b2 by peptide fingerprinting. However, MSP1bpg3, if translated, is predicted to be 68 kDa, which would allow differentiation between MSP1bpg3, MSP1b1, and MSP1b2 by size. A band of this size was not observed in either the immunoblots or SYPRO ruby-stained gels, suggesting that MSP1bpg3 is either not translated or not part of the MSP1 complex.

Two sequential gel slices representing the region between 95 and 100 kDa were excised from the SYPRO ruby-stained gel, digested with trypsin, and analyzed using LC–MS-MS. Analysis of the upper band revealed peptides from MSP1b1 (Mascot score, 184; five peptides matched and 13% amino acid coverage), with LDDAQGLQEATPEAK (ions score, 21) and GV EGINPEELEQAAEGLATAVNEASADGK (ions score, 56) as the discriminatory MSP1b1-specific peptides. Peptides from an unrelated protein, MSP3 (Mascot score, 292; eight peptides matched and 15% amino acid coverage), were also identified in the upper band. The lower band was positive for MSP3 (Mascot score, 502; 12 peptides matched and 25% amino acid coverage) and also had a lower-scoring hit to MSP1b1 (Mascot score, 36). This could explain the presence of the second lower-intensity band in the Western blot (Fig. 5, lane 9). In a repeated immunoprecipitation with MAb Ana22B1, the LDDA QGLQEATPEAK peptide was again identified for MSP1b1, but MSP1b2-specific peptides were not detected in any of the immunoprecipitations. In a further attempt to identify MSP1b2 in outer membranes not subjected to immunoprecipitation, outer membranes were separated on a 7.5% Criterion gel, and proteins were electroeluted from a block corresponding to 75 to 100 kDa. The electroeluted sample was further separated on a 7.5% Criterion gel, and three bands (approximately 80, 90, and 100 kDa) were excised (data not shown). All three bands were positive for MSP3. The 90-kDa band was also positive for MSP1b1 (Mascot score, 28), with LDDAQGLQE ATPEAK (ions score, 20) as the distinguishing peptide. No MSP1b2-specific peptides were identified.

In general, the limit of protein identification by mass spectrometry is in the low-femtomolar range when proteolytic fragments are introduced into the instrument. The practical sensitivity for identification of proteins in gels is much less because a smaller portion of the protease digestion is analyzed. Radiolabeled recombinant proteins used to systematically evaluate peptide recoveries from in-gel trypsin digestion indicated that at least 80% of the labeled tryptic peptides could be extracted from gel bands containing 1 to 10 pmol of protein (33). To determine the sensitivity of detection in our system, serial dilutions of purified recombinant MSP1b1 and MSP1b2 proteins were separated on a 7.5% Criterion gel. Samples of 10, 2, 0.4, 0.08, and 0.016 μg of protein were loaded per well. The bands were excised and processed for LC–MS-MS. Based on 100% sample recovery and the volume injected for LC–MS-MS, the maximum protein amounts analyzed for MSP1b1 and MSP1b2 were 32 pmol and 31.2 pmol, respectively. At the highest protein amount injected for MSP1b1, six specific peptides were detected (Table 3); at 6.4 pmol, only three peptides specific for MSP1b1 were detected; and at 1.2 pmol, only one specific peptide was detected (Table 3). For MSP1b2, seven specific peptides were detected at 31.2 pmol, the highest amount injected (Table 3); five specific peptides were detected at 6.2 pmol; and two specific peptides were detected at 1.25 pmol (Table 3). At the two lowest amounts of input protein (0.25 and 0.05 pmol), no specific peptides could be detected for either MSP1b1 or MSP1b2. The ions scores of the two MSP1b2-specific peptides detected using 1.25 pmol of input protein are not greater than the statistically significant limit of 18 ($P < 0.05$). However, a significant protein match, with a Mascot score of 68 ($P < 0.05$), could still be established for MSP1b2. Thus, if MSP1b1 and MSP1b2 are present in the MSP1 complex, they should be detected at an amount of 0.96 to 1.25 pmol, based on 80% and 100% recovery, respectively. To determine if any of the unique peptides identified for MSP1b2 were present at low intensities in the MSP1 complex, the *m/z* values of the seven distinguishing peptides of MSP1b2 (listed in Table 3) were extracted from the raw LC–MS-MS data for our samples. However, no peaks for the MSP1b2 peptides were identified. Since unique peptides with similar ions scores were detectable for both MSP1b1 and MSP1b2 using the recombinant proteins, both MSP1b1 and MSP1b2 should be detectable in the MSP1 complex if they were present at the same level. We conclude that if MSP1b2 is linked to MSP1a in the complex, it is below the level of detection.

Additional bands that were observed at 55, 75, and 90 kDa on the SYPRO ruby-stained gel of the immunoprecipitated *A. marginale* outer membranes (data not shown) were also analyzed using LC–MS-MS. The 75-kDa band was confirmed to be MSP1a (Mascot score, 327; 12 peptides matched and 22% amino acid coverage), and the 90-kDa band was positive for MSP3 (Mascot score, 380; 14 peptides matched and 17% amino acid coverage). MSP3 is a highly abundant membrane protein, and its molecular size varies, depending on the length of the central hypervariable domain of a specific variant (23). Thus, detection of MSP3 at molecular sizes of 90, 95, and 100 kDa is consistent with the sizes of the variant domains previously reported and reflects the presence of multiple variants within the *A. marginale* population (3, 23). The detection of MSP3 in the MSP1 complex likely reflects both its abundance in the membrane and its nearest-neighbor relationship with MSP1 (35). Proteins in the MSP1 complex contain glutamine-rich segments, which are thought to form a structural framework for the formation of multimeric complexes (15, 31). The 29- or 30-amino-acid repeat regions of MSP1a contain three consecutive glutamine residues plus an additional one or two glutamine residues (4, 30). MSP1b1 contains four glutamine residues in the first 10 amino acids and an additional four glutamine residues in the next 30 amino acids; at the C-terminal end, 9 of the last 30 amino acids are glutamine (Fig. 2A). These glutamine-rich regions may be responsible for the previously reported noncovalent interaction between the MSP1 complex and MSP3 (35).

The band at 55 kDa was excised to determine if it could be

a product of *msp1*β_{pg3} but was identified by LC–MS-MS as VirB10 (Mascot score, 107; six peptides matched and 20% amino acid coverage). VirB10 is a protein that forms part of the type IV secretion system and was recently identified by LC–MS-MS analysis of antigenic *A. marginale* outer membranes separated by two-dimensional electrophoresis (20). The detection of VirB10 in the complex is novel, as prior studies (35) were limited by the lack of knowledge regarding the full complement of membrane proteins and were restricted to identifying interactions among six known outer membrane proteins using MAbs. How VirB10 is linked to the MSP1 complex and what its immunological significance is are unknown.

It has been proposed that several cysteine-rich proteins in *Chlamydia* spp., including the major outer membrane proteins which contain 7 to 10 cysteine residues (37), form disulfide bonds to provide rigidity to the chlamydial cell wall (7, 19, 37). The *A. marginale* MSP1 complex proteins also contain a notable number of cysteine residues; MSP1a contains nine cysteine residues (4, 30), while MSP1b1 has seven (Fig. 1A). The MSP1 complex has been shown to be disulfide linked, and MSP2 and MSP5 can each occur as monomers or disulfide-linked multimers, consistent with a highly cross-linked outer membrane. MSP1, MSP2, MSP3, and MSP4 are nearest neighbors, and MSP1, MSP2, and MSP5 are also associated through noncovalent interactions (35). Our current findings support the presence of additional outer membrane proteins within these complexes that can now be identified using the complete genome sequence. Analyzing the complexity of and the interactions among membrane proteins is necessary for future immunological studies designed to fully understand the protective nature of *A. marginale* outer membranes.

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