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Identification of *Anaplasma marginale* Outer Membrane Protein Antigens Conserved between *A. marginale* Sensu Stricto Strains and the Live *A. marginale* subsp. *centrale* Vaccine▽

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Live vaccination with *Anaplasma marginale* subsp. *centrale* (synonym for *Anaplasma centrale*) induces protection against severe disease upon challenge with *A. marginale* sensu stricto strains. Despite over a century of field use, the targets of protective immunity remained unknown. Using a broad proteomic approach, we identified the proteins in a challenge sensu stricto strain that were bound by the relevant antibody isotype induced by live vaccination with *Anaplasma marginale* subsp. *centrale*. A core of 15 proteins was identified in vaccinated animals across multiple major histocompatibility complex (MHC) haplotypes. This core separated into two structural/functional classes: "housekeeping" proteins involved in replication and metabolism and outer membrane proteins (OMPs). Orthologous proteins of both classes were identified within the vaccine strain and among sensu stricto strains. In contrast to the broad conservation among strains in the sequences of the housekeeping proteins, there was significantly greater divergence in the OMPs and greater divergence in both OMP sequences and the encoding locus structure between the vaccine strain and the sensu stricto strains than among the sensu stricto strains. The OMPs bound by live vaccine-induced antibody overlapped with OMPs that were immunogenic in animals vaccinated with inactivated vaccines and subsequently protected against bacteremia and disease. The identification of this core set of OMPs is consistent with the hypothesis that "subdominant" immunogens are required for vaccine-induced protection against *A. marginale* and provides clear direction for development of a safer, more effective vaccine.

Sir Arnold Theiler described what was later recognized as the first rickettsial pathogen, *Anaplasma marginale*, in 1908 as the cause of a fatal infectious disease of cattle in South Africa (32, 39). Remarkably, within 2 years of this initial discovery, Theiler isolated a naturally occurring less virulent strain, which he designated *A. marginale* subtype *centrale* (the organism has been redesignated *A. marginale* subsp. *centrale*, a synonym for *Anaplasma centrale*, in keeping with current taxonomic convention), and demonstrated that vaccination with this attenuated strain induced protection against virulent *A. marginale* (40). This vaccine has continued in use in multiple countries throughout Africa, Asia, Australia, and the Americas (22). Despite these 100 years of field use, the basis for protective immunity against virulent strains remains unknown. Understanding this basis is relevant for two independent reasons. The first is that the *A. marginale* subsp. *centrale* vaccine is a live, blood-based vaccine and carries with it risk of vaccine-induced disease as well as transmission of known and unknown contaminating pathogens (13, 36). The latter concern has prevented its licensure in both the United States and the European Union. Identification of the antigens responsible for inducing immunity would allow development of safer inactivated recombinant-based vaccines. The second reason is that immunity is not uniform against all strains, and outbreaks have been reported even in vaccinated populations (3, 41, 43). Knowledge of the vaccine antigens responsible for protection would allow detection of emergent strains that differed in these key determinants and reformulation of the vaccine to ensure continued efficacy.

We recently completed the fully annotated genome sequence of the *A. marginale* subsp. *centrale* vaccine strain, which allowed *in silico* identification of genes predicted to encode proteins orthologous to those in virulent *A. marginale* sensu stricto strains (21). However, this bioinformatic approach is limited to recognition of proteins already shown to have a role in protective immunity and does not address whether the proteins are actually expressed by the vaccine strain, whether the degree of conserved sequence identity reflects epitope conservation, or whether the proteins induce relevant immune effectors in the vaccinated animals. Consequently, we opted for a broader approach that queries an unbiased complement of vaccine strain antigens that induce the relevant effectors in vaccinates and then uses a proteomic approach to identify the specific protein in both the vaccine and sensu stricto strains. Herein, we report the identification of these antigens and discuss the results in the context of vaccine efficacy and development of improved vaccines.

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MATERIALS AND METHODS

Immunization and challenge. Seronegative and *msp5*-negative (as determined by PCR) calves were immunized by intravenous inoculation with 10^8 organisms of the Israel vaccine strain of *A. marginale* subsp. *centrale* (no. C6171, C6175,

C6187, C6188) or the St. Maries *A. marginale* sensu stricto strain (no. C6170). The major histocompatibility complex (MHC) haplotypes were determined by PCR-restriction fragment length polymorphism (RFLP) typing (42) and were different among the calves. Bovine leukocyte antigen DRβ3 RFLP patterns for the five calves were as follows: vaccinate no. C6170, 16/16; no. C6171, 22/8; no. C6175, 16/8; no. C6187, 22/24; and no. C6188, 16/22. Sera were collected at 61 days postimmunization, just prior to challenge, by feeding *Dermacentor andersoni* ticks ($n = 50$ per calf) infected with the St. Maries strain of *A. marginale* (C6170 and C6171 were not challenged). The immunization and challenge have previously been reported in detail (20). The postimmunization/prechallenge sera were used to identify antigens in the challenge St. Maries strain by a combination of two-dimensional (2-D) electrophoresis, immunoblotting, and tandem mass spectrometry.

Two-dimensional electrophoresis and immunoblotting. Bacteria isolated directly from blood infected with the St. Maries strain of *A. marginale* (23, 33) were resuspended in a rehydration sample buffer (Bio-Rad, Hercules, CA) containing 7 M urea, 2 M thiourea, 1% (wt/vol) amidosulfobetaine-14, 40 mM Tris, 0.001% bromophenol blue, and 2 mM tributyl phosphine. Complete Mini protease inhibitors (Roche, Indianapolis, IN) were also added to the buffer. Whole *A. marginale* organisms were lysed by sonication (Branson digital sonifier 450) at 100% output for 2 min total in 15-s intervals or until the lysate was clear. To degrade the remaining nucleic acids in the sample, 1 mM $MgCl_2$ and 10 U benzonase (Sigma, St. Louis, MO) were added, and the sample was incubated for 10 min at room temperature. The lysate was centrifuged at 17,000 × *g* at 20°C to pellet insoluble material. Samples were processed with a ReadyPrep reduction and alkylation kit (Bio-Rad), followed by processing with a ReadyPrep 2-D cleanup kit (Bio-Rad). Samples were solubilized in isoelectric focusing buffer containing 5 M urea, 2 M thiourea, 2% 3-[(3-chloramidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 2% SB 3-10 (*N*-decyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate), 2 mM tributyl phosphine (TBP), 0.001% bromophenol blue, and 0.5% Bio-Lyte 3-10 ampholytes (Bio-Rad). Total protein concentration was determined using an RC DC protein assay (Bio-Rad), and 150 μg of bacterial lysate was applied to 11-cm immobilized pH gradient (IPG) ReadyStrips, pH 5 to 8 (Bio-Rad). Overnight rehydrated strips were focused for 40,000 V · h using a Protean isoelectric focusing (IEF) cell system (Bio-Rad). The second dimension was performed on 10.5 to 14% gradient gels using a Criterion gel system (Bio-Rad). After electrophoresis, gels were either stained in Sypro ruby protein gel stain (Bio-Rad) overnight or transferred to polyvinylidene fluoride (PVDF) membranes using a Criterion blotting system. PVDF membranes were allowed to air dry and were stained with Sypro ruby protein blot stain (Bio-Rad). After imaging the stained blots, PVDF membranes were blocked for 1 h in I-Block (Applied Biosystems, Bedford, MA) blocking reagent containing 0.5% Tween 20 and were probed with a 1:100 dilution of immune sera from calves C6171, C6175, C6187, and C6188, immunized by inoculation with the *A. marginale* subsp. *centrale* vaccine strain, or calf C6170, immunized by inoculation with the St. Maries strain. Binding of IgG2 was detected by a 1:20,000 dilution of horseradish peroxidase (HRP)-conjugated sheep anti-bovine IgG2 antibody (Serotec, Raleigh, NC). An Amersham ECL Plus Western blotting system (GE Healthcare, Fairfield, CT) was used according to the manufacturer's directions to detect immunoreactive spots.

Liquid chromatography and tandem mass spectrometry. Sypro ruby-stained blot images were aligned to the corresponding two-dimensional immunoblot using PDQuest software (Bio-Rad). The identified immunoreactive spots were manually excised from the gel, dehydrated in 200 μl acetonitrile for 5 min, and dried completely by vacuum centrifugation. Samples were rehydrated in a 50-ng/μl trypsin (Trypsin Gold, mass spectrometry grade; Promega, Madison, WI) solution suspended in 50 mM ammonium bicarbonate (Sigma). Excess trypsin was removed, and samples were digested overnight in 50 mM ammonium bicarbonate at 37°C on an orbital shaker. After a brief centrifugation, the supernatants were collected individually, and 30 μl of 0.1% trifluoroacetic acid (Pierce, Rockford, IL) was added to the remaining gel piece and incubated at 37°C for 45 min. A second extraction was performed by adding 30 μl of 30% acetonitrile and 0.1% trifluoroacetic acid and incubating as before, followed by a final extraction using 30 μl of 60% acetonitrile and incubating at 20°C for 30 min. The supernatants from each extraction were pooled and then dried by centrifugation under vacuum. The samples were then analyzed by liquid chromatography and tandem mass spectrometry (LC/MS-MS). MASCOT (Matrix Science) was used to match *A. marginale* peptides with LC/MS-MS data using the completely sequenced and annotated genomes of the St. Maries strain of *A. marginale* and the Israel vaccine strain of *A. marginale* subsp. *centrale* (4, 21). Proteins containing one or more peptides with a MASCOT ion score greater than 20 indicated that there is a <5% chance that the peptide match is a random event. The MASCOT search was performed with the following settings: carbamidomethylation of cysteine residues as a fixed modification and oxidation of methionine residues as a variable modification.

Identification of orthologues in *A. marginale* subsp. *centrale* and sensu stricto *A. marginale* strains. The positions of conserved Msp2 N- and C-terminal regions were determined by aligning translated full-length *msp2* variant sequences using AlignX software included with the Vector NTI suite (Invitrogen) to identify a common consensus sequence at the N- and C-terminal regions of the Msp2 variant sequences. The same approach was used to define the N and C termini present within the full-length Msp3 sequence. To identify orthologs of immunoreactive proteins, the translated protein products of the St. Maries strain gene sequences were entered into BLASTP to search against the protein databases of the following organisms: *A. marginale* subsp. *centrale* (NCBI taxonomy identification no. [taxid] 574556), *A. marginale* sensu stricto strain Florida (taxid 320483), *A. marginale* sensu stricto strain Mississippi (taxid 403779), *A. marginale* sensu stricto strain Puerto Rico (taxid 517437), and *A. marginale* sensu stricto strain Virginia (taxid 517436). The Mississippi, Puerto Rico, and Virginia strain sequences were generated by pyrosequencing and covered approximately 96% of each genome (12). Percent sequence identity values were obtained by dividing the total number of amino acid sequence identities by the length of the shortest sequence used in the comparison. For genes with multiple short regions of identity, the sum of nonoverlapping identities, derived from significant BLASTP hits (using an expect [E] value threshold of <1), was divided by the length of the shortest sequence.

Mapping of vaccine-induced antibody binding to conserved versus variant Msp2 domains. Msp2 peptides were expressed as His-tagged recombinant proteins in *Escherichia coli* and purified by immobilized metal affinity chromatography. The Msp2 N-terminal region, C-terminal region, and full-length protein were cloned into pBAD/TOPO ThioFusion (Invitrogen), and Msp2 hypervariable regions were cloned into pDEST17 (Invitrogen) using the Gateway Expression System (Invitrogen). Primers 9H1F-1 (5′ G GGG ACA AGT TTG TAC AAA AAA GCA GGC TTA AAT GCC ATA GAG AGT GCT 3′) and 9H1R-2 (5′ GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA CTA TGT CGT GTT GAT GGT GTC 3′) were used for cloning the Msp2 9H1 variant hypervariable region; primers 9H1F-1 and STMR-3 (5′ GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA CTA CTG CAA GCT GAT GGT GTT A 3′) were used for cloning the Msp2 Ψ1 hypervariable region; 9H1F-1 and STMR-4 (5′ GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA CTA CTG CAA GTT GAT GGT GTC T 3′) were used for cloning the Msp2 Ψ2 hypervariable region; STMF-2 (5′ G GGG ACA AGT TTG TAC AAA AAA GCA GGC TTA ACT ACT GTA GAA GCT GCT 3′) and STMR-5 (5′ GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA CTA CGA CGT ACT GAT GTT GCT AC 3′) were used for cloning the Msp2 E6/F7 hypervariable region; STMF-3 (5′-G GGG ACA AGT TTG TAC AAA AAA GCA GGC TTA AAT GCA GTA GAG AAT GCT −3′) and STMR-6 (5′ GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA CTA TGT CGT GTT GAT GGT GGT A 3′) were used for cloning the Msp2 P1E6 hypervariable region; MSP2 FL (5′ ATG AGT GCT GTA AGT AAT AGG 3′) and Msp2 Rd1 (5′ ACC CCA CTT CTT AGC CTC ACT 3′) were used for cloning the conserved amino-terminal region of Msp2; Msp2 Fd1 (5′ AAA GAT GAG AAG GCC ATA GTT 3′) and MSP2 RL (5′ GAA GGC AAA CCT AAC ACC CAA 3′) were used for cloning the conserved carboxy-terminal region of Msp2; and MSP2 FL and MSP2 RL were used for cloning the full-length Msp2 9H1 sequence. pDEST17 and pBAD/TOPO ThioFusion constructs were transformed into *E. coli* BL21-A1, and transformants were grown in LB broth containing 50 μg/ml carbenicillin. Expression of recombinant protein was induced by adding L-arabinose to a final concentration of 0.2%. His-tagged fusion proteins were purified using the Probond purification system (Invitrogen). Recombinant proteins were separated by SDS-polyacrylamide gel electrophoresis using 4 to 20% precast gels (Bio-Rad). Proteins were transferred to PVDF, probed with 1:100-diluted immune serum as described above, and incubated with 1:20,000-diluted HRP-conjugated sheep anti-bovine IgG2 antibody (Serotec). Amersham ECL Plus (GE Healthcare) was used as described above to detect serum IgG2 bound to recombinant Msp2 domains.

RESULTS

Proteomic identification of antigens targeted by live vaccination using *A. marginale* subsp. *centrale*. To identify *A. marginale* sensu stricto antigens recognized by the relevant antibody isotype induced by live vaccination with *A. marginale* subsp. *centrale*, organisms of the St. Maries strain of *A. marginale* were lysed, separated by two-dimensional electrophoresis,

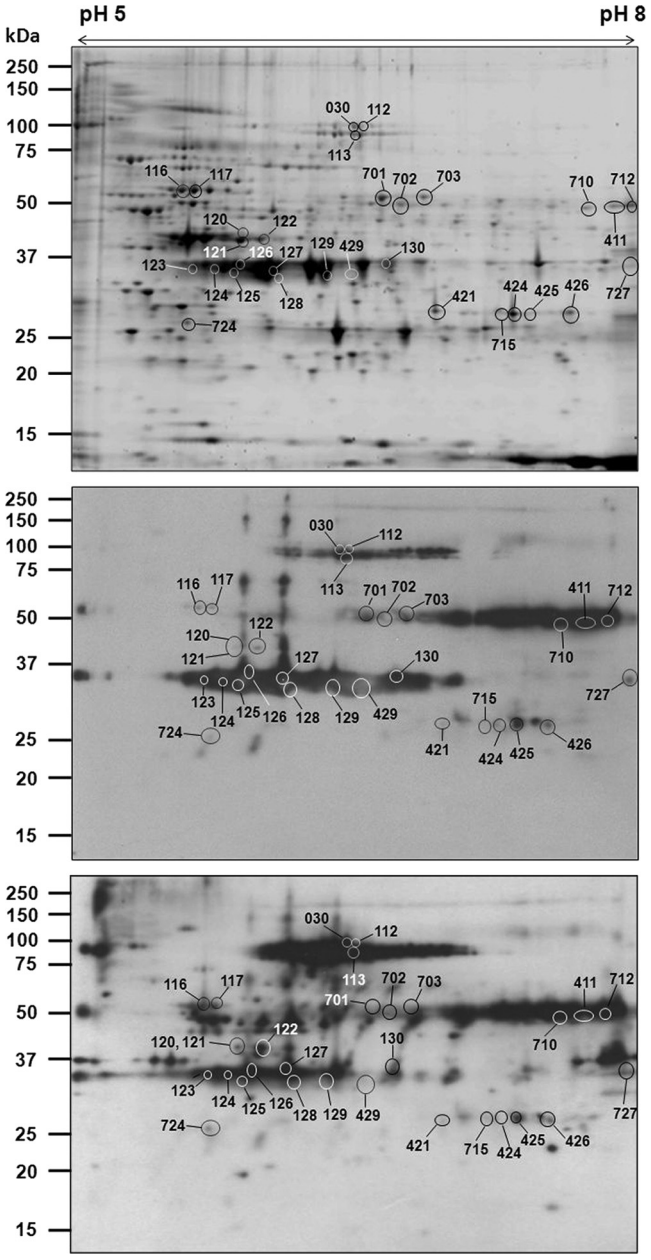

FIG. 1. Identification of *A. marginale* strain St. Maries proteins recognized by IgG2 induced by immunization with the *A. marginale* subsp. *centrale* vaccine strain. Gels were either stained with Sypro ruby for visualization of protein (top) or used for immunoblotting to detect bound IgG2 present in serum from *A. marginale* subsp. *centrale* vaccinate (sera from vaccinate C6175) (middle). As a homologous strain positive control, immunoblotting was performed using serum from an animal infected with the St. Maries strain, vaccinate C6170 (bottom). The pH gradient for all three images is given at the top of the figure. The molecular mass in kilodaltons (kDa) is indicated on the left-hand side of the images. Circles and numbers correspond to protein spots that were identified by LC/MS and are listed in Table 1.

and probed for binding of IgG2 in sera from calves immunized by inoculation with the Israel vaccine strain of *A. marginale* subsp. *centrale* (Fig. 1). Two-dimensional immunoblots were overlaid with an image from the respective Sypro ruby-stained gel, and immunoreactive spots were excised, trypsin digested, and analyzed by LC/MS-MS. Screening vaccinates with different MHC haplotypes identified 15 *A. marginale* St. Maries strain proteins that contained epitopes bound by all sera (Table 1). These 15, which represent a core set of proteins with epitopes shared between the *A. marginale* subsp. *centrale* vaccine strain and the challenge sensu stricto St. Maries strain, separated into the following two groups: (i) "housekeeping" proteins involved in metabolism and replication and (ii) outer membrane proteins (OMPs). In addition to the core protein set, 9 proteins contained epitopes recognized by IgG2 from one or more, but not all, vaccinates, including proteins belonging to the *Anaplasma* appendage-associated protein family (AM878, AM879, and AM880), AM1010 (a conserved lipoprotein family protein), chaperone protein DnaK, malonyl coenzyme A (CoA)-acyl carrier protein transacylase (FabD), transcription elongation factor (GreA), inorganic phosphatase protein (Ppa), and single-strand binding protein (Ssb).

Identification of orthologues in the vaccine and challenge strains. To confirm that there were corresponding orthologous proteins in the vaccine strain, the *A. marginale* subsp. *centrale* genome was searched using BlastP; orthologues were identified for each of the 15 core immunogenic proteins (Table 2). The percentage of amino acid sequence identity was >90% between the vaccine strain and the St. Maries strain for all proteins involved in replication and metabolism, unsurprising given the highly conserved functions of these proteins. In contrast, the percentage of identity among orthologous OMPs was significantly lower ($P < 0.001$; Student's t test). Sequence divergence between the vaccine and challenge strain orthologues is present in both OMPs encoded by single-copy genes (AM779, AM854, Omp11, Omp13, Omp14) and those encoded by more complex gene families (Omp7 to Omp9, Msp2, Msp3) (Table 2). All *sensu stricto* strains as well as *A. marginale* subsp. *centrale* contain single expression sites for, individually, Msp2 and Msp3 and a variable set of 5 to 10 donor alleles distributed throughout the chromosome that generate antigenic variants by a gene conversion process in which a donor allele hypervariable domain (HVR) is recombined into the expression site (2, 4, 5, 6, 12, 17, 21, 26). Thus, the percent identities in the Msp2 and Msp3 donor alleles between the two strains range from 18 to 74% (Tables 3 and 4).

Conservation of vaccine strain OMPs among *A. marginale* sensu stricto strains. To determine if the identified vaccine strain OMPs were broadly conserved, the genomes of the Florida, Puerto Rico, Mississippi, and Virginia strains were searched. The 10 core vaccine strain OMPs (Msp2, Msp3, Omp7/8/9, Omp11, Omp13, Omp14, AM779, AM854) were identified in each strain. The identities between the vaccine strain and the sensu stricto strains ranged from 49 to 93% (Table 2); there was significantly greater divergence between the vaccine strain and the sensu stricto strains than within the sensu stricto strains (Tukey-Kramer multiple comparison test, $P < 0.001$). For the three strains with completely sequenced and annotated genomes, St. Maries, Florida, and the vaccine strain, the locus positions could be compared. The loci for all OMPs were highly conserved for the Florida and St. Maries strains, with greater divergence in the vaccine strain (Fig. 2). As previously noted, the three-gene locus encoding Omp7, Omp8, and Omp9 in the sensu stricto strains is collapsed into a single-gene locus in *A. marginale* subsp. *centrale* (21).

TABLE 1. *Anaplasma marginale* St. Maries strain proteins bound by serum IgG2 induced by live vaccination

Protein type	Spot no.[a]	Protein annotation	Mascot score(s) (standard)[b]	No. of peptides with *P* values of <0.05	% sequence coverage	Mol wt (kDa)	Theoretical pI
Membrane	727	AM1220 Omp7	84	3	9	41.9	8.9
	727[c]	AM1221 Omp8	42	1	2	42.9	9.2
	122, 727[c]	AM1222 Omp9	79, 42	2, 1	6, 2	43.1	8.2
	424, 425, 426	AM1255 Omp11	534, 267, 365	10, 5, 9	35, 23, 33	34.8	6.7
	421, 715	AM1258 Omp13	139, 65	3, 1	6, 12	38.0	7.6
	727	AM075 Omp14	30	1	2	43.1	7.6
	710, 411, 712	AM779 surface-expressed protein	128, 38, 65	3, 1, 1	8, 2, 3	57.4	7.2
	724	AM854 peptidoglycan-associated lipoprotein	138	3	14	25.9	5.3
	123–130	AM1144 Msp2	132–640	2–16	7–16	42.1	6.0
	30, 112, 113	AM1063 Msp3	593, 84, 48	14, 2, 1	14, 2, 2	104.5	6.0
Housekeeping	122	AM231 succinyl-CoA synthetase beta chain (SucC)	92	1	11	41.8	5.5
	121, 122	AM254 elongation factor Tu (TufB)	647, 422	14, 8	36, 30	43.0	5.4
	702	AM1131 isocitrate dehydrogenase (Icd)	40	1	11	53.5	5.8
	116, 117	AM944 Hsp chaperonin (GroEL)	480, 218	9, 5	25, 18	58.4	5.2
	701, 703, 411	AM1174 F1-ATP synthase alpha subunit (AtpA)	290, 188, 29	7, 3, 1	21, 7, 1	54.6	6.0

[a] Spot no. refers to the identification illustrated in Fig. 1.
[b] Standard MASCOT scores are the sum of probability-based MASCOT ion scores for each individual peptide.
[c] OMP8 and OMP9 hits for spot no. 727 are from the same peptide.

Vaccine-induced antibody binding to conserved versus variant Msp2 domains. The binding of antibody induced by *A. marginale* subsp. *centrale* vaccination to epitopes on St. Maries strain Msp2 and Msp3 (Fig. 1) was clearly detectable, consistent with prior analysis using sensu stricto *A. marginale* strains (1, 16, 23, 30). However, it was unknown whether the binding was to the relevant, surface-exposed central HVR domain or the conserved N- and C-terminal but non-surface-exposed membrane domains (15, 44). The binding of vaccine strain-induced IgG2 to full-length Msp2, the full set of donor HVRs, and the N- and C-terminal regions was tested using Msp2, as there was greater conservation in the Msp2 domains (including the HVRs and the N and C termini) between the strains than among the Msp3 domains (Tables 2, 3, and 4). Consequently, domain-specific cross-reactivity was more likely to be detected with Msp2 than with Msp3. Each of the unique HVRs encoded by the *msp2* donor alleles of the St. Maries strain was expressed as a His-tagged recombinant protein, purified, and tested for reactivity with sera from each of the vaccine strain-immunized animals. The conserved N- and C-terminal regions shared by all St. Maries strain Msp2 variants were also expressed, purified, and tested using the same protocol. Serum from an animal inoculated with only the St. Maries strain served as a positive control; IgG2 binding to the complete Msp2 containing the St. Maries strain 9H1 HVR, the 9H1 HVR alone, and each of the conserved terminal domains was detected (Fig. 3E). In contrast, binding of the sera from each of the vaccinates consistently bound only the whole protein and the N-terminal domain, with weak binding to the C-terminal domain by two sera (Fig. 3A to D). As a loading control for the recombinant proteins, an identically processed membrane was probed with anti-His antibody (Fig. 3G). The analysis was repeated for each of the additional four unique St. Maries strain HVRs encoded by donor alleles 1, 3H1, E6/F7, and P1; there was no detectable binding of any of the sera to any of the HVRs (data not shown).

DISCUSSION

Previous studies revealed that protection against challenge with homologous *A. marginale* strains correlates with IgG2 antibody binding to the pathogen surface; bound IgG2, the predominant opsonizing subclass of bovine immunoglobulin, then mediates macrophage uptake and bacterial killing (11, 31). Consistent with this mechanism of homologous strain protection, live immunization with the Israel vaccine strain of *A. marginale* subsp. *centrale* induced IgG2 that bound a core set of OMPs expressed by the heterologous St. Maries sensu stricto strain. Excluding, for the moment, the highly variant Msp2 and Msp3, the amino acid sequence identities among the remaining core OMPs ranged from 60 to 84% between the vaccine and the challenge strains. This range emphasizes the need to actually test and confirm cross-reactivity using relevant immune effectors rather than attempt to predict cross-reactivity based solely on *in silico* analysis. Interestingly, both the amino acid sequence identities of the OMPs (Table 2) and locus positions (Fig. 2) of the encoding genes are clearly more divergent between the vaccine strain and the sensu stricto strains than among the sensu stricto strains. There are three genes, *omp7* to *omp9*, encoding closely related proteins in the sensu stricto strains that are collapsed into a single gene in the *A. marginale* subsp. *centrale* vaccine strain (4, 21). These differences in genomic organization and OMP identity may well reflect the true genetic distance between sensu stricto strains and *A. marginale* subsp. *centrale*. However, there is a caveat in that all of the sensu stricto *A. marginale* strains sequenced to date were isolated in North America. Analysis of strains isolated from other continents with regions where *A. marginale* is endemic

TABLE 2. Comparison of *Anaplasma marginale* subsp. *centrale* vaccine strain antigens with *A. marginale* sensu stricto orthologs

Israel vaccine strain protein annotation	St. Maries strain protein annotation	% identity[a]				
		St. Maries strain	Florida strain	Mississippi strain	Puerto Rico strain	Virginia strain
Membrane proteins						
ACIS_00178 Omp7	AM1220 Omp7	70, 100	68, 86	69, 94	67, 94	68, 99
ACIS_00178 Omp7	AM1221 Omp8	66, 100	64, 90	66, 90	76, 100	64, 88
ACIS_00178 Omp7	AM1222 Omp9	64, 100	64, 99	–	–	64, 98
ACIS_00140 Omp11	AM1255 Omp11	60, 100	60, 97	54, 87	61, 97	–
ACIS_00138 Omp13	AM1258 Omp13	63, 100	57, 98	60, 94	59, 97	–
ACIS_00068 Omp14	AM075 Omp14	71, 100	71, 97	73, 99	–	71, 97
ACIS_00557 undefined product	AM779 surface-expressed protein	84, 100	84, 100	84, 99	84, 100	84, 100
ACIS_00486 peptidoglycan-associated lipoprotein	AM854 peptidoglycan-associated lipoprotein	79, 100	79, 100	79, 100	79, 100	79, 100
ACIS_00229 Msp2 N-terminal conserved region	AM1144 Msp2 N-terminal conserved region	84, 100	84, 99	83, 99	84, 100	82, 98
ACIS_00229 Msp2 C-terminal conserved region	AM1144 Msp2 C-terminal conserved region	90, 100	91, 100	86, 97	93, 97	93, 97
ACIS_00229 Msp2 hypervariable regions	AM1144 Msp2 hypervariable regions	33–74[b], 100	36–67, 35–100[b]	–	–	–
ACIS_00617 Msp3 N-terminal conserved region	AM1063 Msp3 N-terminal conserved region	52, 100	51, 74	55, 77	49, 77	53,77
ACIS_00617 Msp3 C-terminal conserved region	AM1063 Msp3 C-terminal conserved region	84, 100	85, 98	88, 99	78, 96	71, 88
ACIS_00617 Msp3 hypervariable regions	AM1063 Msp3 hypervariable regions	18–48[b], 100	13–44, 14–100[b]	–	–	–
Housekeeping proteins						
ACIS_01060 succinyl-CoA synthetase (SucC)	AM231 succinyl-CoA synthetase (SucC)	98, 100	99, 100	97, 99	98, 99	–
ACIS_01040 elongation factor Tu (TufB)	AM254 elongation factor Tu (TufB)	97, 100	97, 100	97, 100	98, 100	97, 100
ACIS_00241 isocitrate dehydrogenase (Icd)	AM1131 isocitrate dehydrogenase (Icd)	99, 100	100, 99	99, 99	100, 99	100, 99
ACIS_00394 chaperonin GroEL	AM944 60-kD chaperonin (GroEL)	99, 100	99, 100	99, 100	99, 100	99, 100
ACIS_00219 Atp synthase subunit alpha (AtpA)	AM1174 F1-ATP synthase alpha subunit (AtpA)	93, 100	93, 100	93, 100	93, 99	93, 100

[a] The St. Maries, Florida, Mississippi, Puerto Rico, and Virginia strains were compared to the Israel and St. Maries strains. The first number indicates sequence identity to the Israel strain, and the second number indicates sequence identity to the St. Maries strain. The Mississippi, Puerto Rico, and Virginia strain comparisons were performed using pyrosequencing data that comprise approximately 96% of the full sequence. Only partial sequences of ortholog gene products were available (12). A dash (–) indicates a partial sequence that does not allow comparison, not the absence of the gene.

[b] For Msp2 and Msp3 hypervariable regions, a range of sequence identities is given that represents the lowest and highest sequence identities observed when comparing all donor alleles in the genome.

TABLE 3. Amino acid sequence identities between *Anaplasma marginale* subsp. *centrale* (Israel vaccine strain) and *A. marginale* strain St. Maries Msp2 hypervariable region variants

Israel vaccine Msp2 HVR variant	% identity with St. Maries Msp2 HVR variant:				
	Ψ1	Ψ2[a]	9H1	P1[b]	E6/F7
G1[c]	36	48	58	48	63
C	38	54	49	53	54
F	37	48	51	50	42
B1	66	48	33	53	35
A22	43	40	38	38	42
AF	61	54	53	43	67
A1	74	37	43	52	46

[a] St. Maries strain donor allele Ψ2 is duplicated, with the duplicate, 3H1, having an identical sequence (7).

[b] St. Maries donor allele Ψ P1 is duplicated, with the duplicate, G11, having an identical sequence (7).

[c] *A. marginale* subsp. *centrale* (Israel) donor allele Ψ G1 is duplicated, with the duplicate, G2, having an identical sequence (21).

will be required to clearly interpret the full diversity within the species (12).

The relevance of the core OMPs identified using IgG2 induced by live vaccination is supported by their identification in two prior studies using antibody induced by inactivated vaccine preparations. All core OMPs except for Omp13 were shown to

TABLE 4. Amino acid sequence identities between *Anaplasma marginale* subsp. *centrale* (Israel vaccine strain) and *A. marginale* strain St. Maries Msp3 hypervariable region variants

Israel vaccine Msp3 HVR variant	% identity with St. Maries Msp3 HVR variant:						
	1	2	3	4	5	6	7
1	30	36	42	43	33	19	29
2	22	34	53	41	42	24	40
3	26	38	31	45	39	34	41
4	30	31	19	35	34	31	34
5	21	34	48	29	32	23	34
6	24	20	27	23	32	33	23
7	27	26	31	47	44	31	33
8	22	25	37	18	30	18	30

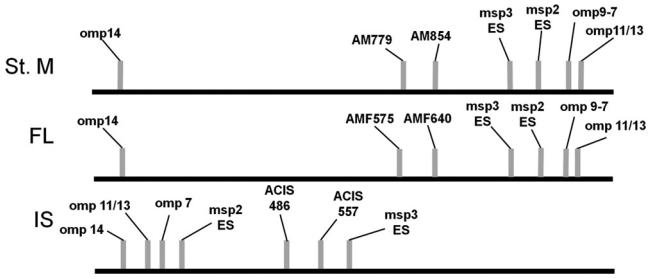

FIG. 2. Locus positions of the immunoreactive *A. marginale* strain St. Maries (St. M) outer membrane proteins compared with orthologues present in the sensu stricto Florida strain (FL) and Israel vaccine strain (IS) of *A. marginale* subsp. *centrale*. The complete circular chromosome is shown as linear for illustration purposes, starting with the origin of replication. AM779, AMF575, and ACIS557 are orthologues; AM854, AMF640, and ACIS486 are orthologues. msp2 ES and msp3 ES represent the positions of the *msp2* and *msp3* expression sites, respectively.

be present in either a purified outer membrane immunogen or cross-linked surface complexes that significantly protected against bacteremia upon homologous strain challenge (23, 28, 29). Of the 10 core OMPs recognized by IgG2 in the present study using sera from animals given the live *A. marginale* subsp. *centrale* vaccine, eight of these were also bound by IgG2 following immunization with a protection-inducing outer membrane protein immunogen (23). Thus, these eight OMPs (Am854, Msp2, Msp3, Omp7 to Omp9, Omp11, Omp14) are bound by IgG2 in immunized and protected animals regardless of whether a live vaccine or a killed immunogen with adjuvant was used.

Notably, the current study indicates that the live vaccine does not induce relevant antibody to Msp1, Msp4, and Msp5 of the challenge St. Maries sensu stricto strain. While the lack of binding to Msp1 is consistent with the structural differences between sensu stricto strains and *A. marginale* subsp. *centrale* (21), Msp4 and Msp5 are broadly conserved among all sensu lato *A. marginale* strains (27), and the lack of antibody binding appears to reflect poor immunogenicity in the context of live vaccination. This appears to be intrinsic to the vaccine rather than the host genetics, as there was no detectable recognition in vaccinates with different MHC haplotypes.

Despite strong IgG2 binding to full-length Msp2, more detailed mapping indicated that reactivity was attributable to binding the conserved membrane domains and not the surface-exposed HVR domains that are relevant for immunity. Very minimal binding to only the 9H1 HVR was detected for two vaccinates, while sera from the remaining vaccinates showed no detectable binding to any of the HVRs. Interestingly, while the amino acid sequence of the C-terminal region is more conserved between the vaccine strain and the challenge St. Maries strain than is the N-terminal region sequence, the primary cross-reactivity was to the N terminus. This preferential reactivity of the N terminus over the C terminus is not specific to live immunization nor to the immunoblot assay, as enzyme-linked immunosorbent assay (ELISA) mapping following outer membrane immunization yielded the same conclusion (1, 44). While Msp3 was not similarly mapped, the shared gene locus structure, HVR recombination mechanism, and pattern of conserved N- and C-terminal regions with a central, surface-exposed HVR domain supports that lessons learned from Msp2 are applicable to Msp3 (7, 9, 18, 26).

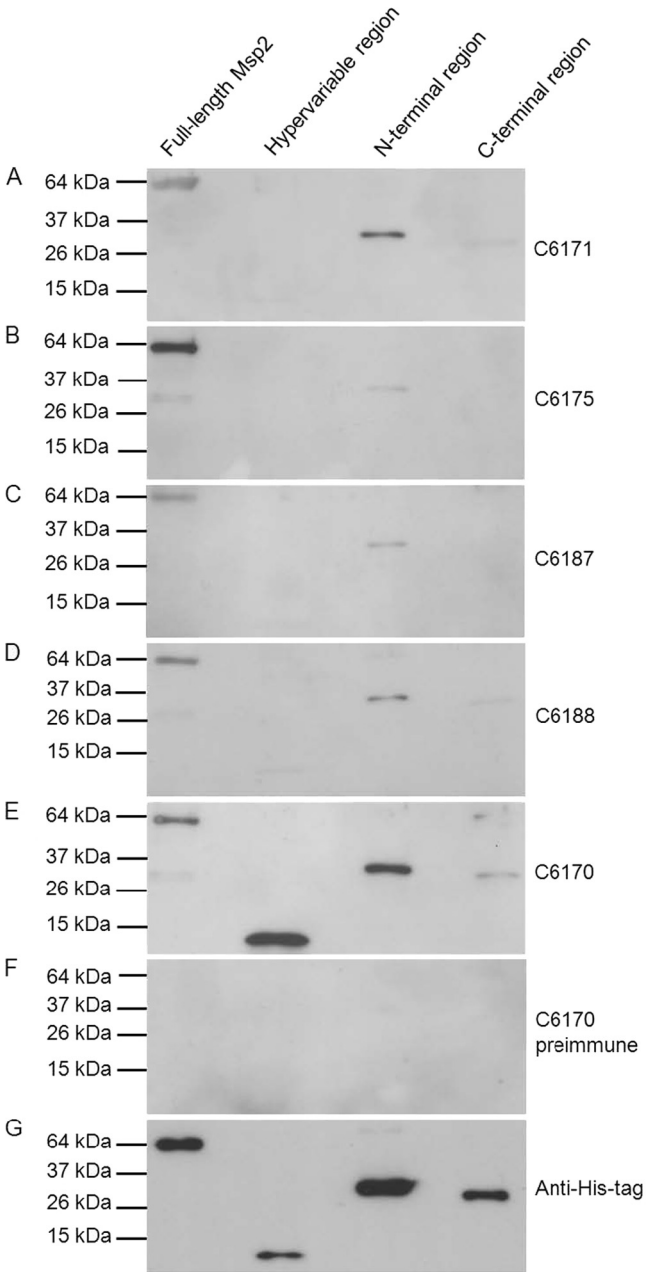

FIG. 3. Mapping of IgG2 binding to conserved membrane regions versus variant surface domains of Msp2. Ten picomoles of recombinant full-length Msp2, 9H1 hypervariable region, N-terminal conserved region, and C-terminal conserved region were immunoblotted and probed for serum IgG2 binding from each of the *A. marginale* subsp. *centrale* vaccinates: C6171 (A), C6175 (B), C6187 (C), and C6188 (D). Serum from a St. Maries strain-infected animal, C6170 (E), was used as a positive control; preinfection serum from the same animal was used as a negative control (F). Probing with anti-His antibody was used as a positive control for the presence of each recombinant protein domain (G). The position and size of molecular mass standards are indicated to the left of the images.

The failure of IgG2 induced by the live *A. marginale* subsp. *centrale* vaccine to bind to the North American St. Maries

strain HVR is consistent with the divergence in the Msp2 HVR repertoire (defined by the genomic complement of Msp2 alleles) among strains (19, 35). While this divergence allows a strain with a unique HVR repertoire to escape existing Msp2-based immunity against the predominant endemic strain and establish strain superinfection (19), it also may limit the capacity of Msp2 to induce protective immunity against a broad range of strains. Finally, although vaccine-induced IgG2 against the challenge strain Msp2 HVRs was not required at the time of challenge, a role for the Msp2 conserved regions in priming the immune response cannot be ruled out. The N- and C-terminal regions, while not surface exposed, contain $CD4^+$ T lymphocyte epitopes conserved between the vaccine strain and sensu stricto strains, including the St. Maries strain (1, 8, 38). These epitopes prime helper T cells which may enhance *de novo* antibody production against the heterologous HVR upon challenge (34).

In addition to the core OMPs bound by each of the individual vaccinated animals, there was also a core group of five proteins generally classified as having housekeeping functions (Table 1). Although bacterial proteins engaged in replication and metabolism, such as elongation factor Tu, have, unexpectedly, been identified to be surface exposed, none of the four other *A. marginale* housekeeping proteins have been identified by radioisotopic surface labeling, surface-specific chemical cross-linking, or proteomic analysis of purified membranes (14, 23, 29, 33, 37). Furthermore, the identities among numerous *Alphaproteobacteria* make it unlikely that these proteins have a specific role in immunity against *A. marginale*.

In summary, six vaccine strain OMPs (Acis557, Acis486, Omp7, Omp11, Omp13, Omp14) induced relevant IgG2 antibody that bound eight orthologues (Am779, Am854, Omp7 to Omp9, Omp11, Omp13, Omp14) in the challenge St. Maries sensu stricto strain. Their identification is consistent with two hypotheses that have been proposed relative to protective immunity against *A. marginale*: (i) immunity is attributable to subdominant antigens rather than the most immunodominant antigens, such as Msp2 and Msp3, and (ii) proteins associated with each other in the membrane are capable of inducing enhanced IgG2 through linked T cell-B cell recognition (10, 23, 24, 25). Testing these hypotheses with these specific OMPs is a clear next step in developing a safe and effective vaccine.

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