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Anaplasma marginale Major Surface Protein 3 Is Encoded by a Polymorphic, Multigene Family

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The immunodominant surface protein, MSP3, is structurally and antigenically polymorphic among strains of *Anaplasma marginale*. In this study we show that a polymorphic multigene family is at least partially responsible for the variation seen in MSP3. The *A. marginale msp3* gene *msp3-12* was cloned and expressed in *Escherichia coli*. With *msp3-12* as a probe, multiple, partially homologous gene copies were identified in the genomes of three *A. marginale* strains. These copies were widely distributed throughout the chromosome. Sequence analysis of three unique *msp3* genes, *msp3-12*, *msp3-11*, and *msp3-19*, revealed both conserved and variant regions within the open reading frames. Importantly, *msp3* contains amino acid blocks related to another polymorphic multigene family product, MSP2. These data, in conjunction with data presented in previous studies, suggest that multigene families are used to vary important antigenic surface proteins of *A. marginale*. These findings may provide a basis for studying antigenic variation of the organism in persistently infected carrier cattle.

Anaplasma marginale is a rickettsial hemoparasite which invades the erythrocytes of cattle, causing severe anemia, weight loss, abortion, and even death. The disease that it causes, anaplasmosis, has a global distribution and results in major worldwide economic losses in cattle (10, 14). In acute stages of the disease, the rickettsial levels exceed 10^9 infected erythrocytes per ml and can easily be detected by microscopic examination of blood smears. However, animals that survive the infection remain carriers and maintain a low level of rickettsemia which cannot be detected microscopically (21, 25). These carrier cattle serve as a perpetual source of infection for susceptible cattle (22, 25). Ticks become infected by feeding on carriers, the organism multiples in the vector, and vector transmission is easily and effectively accomplished (8).

Cyclic rickettsemia has been detected and quantitated in carrier cattle by nucleic acid probe hybridization (7, 12). The level of rickettsemia varied markedly at bimonthly intervals from $<10^3$ to $>10^5$ infected erythrocytes per ml of blood (7). The number of infected erythrocytes gradually increased over a 10- to 14-day period and then precipitously decreased (12). The length and consistency of the cycles suggest that recurrence is due to continual antigenic variation by the organism followed by development of primary immune responses by the host.

Major surface protein 3 (MSP3) is an immunodominant, 86-kDa antigen of *A. marginale* (15, 20). However, multiple, related 86-kDa polypeptides were identified within a given strain of *A. marginale*. Some of these polypeptides share common epitopes as identified by anti-MSP3 monoclonal antibodies (MAbs) and rabbit-anti-MSP3 immune sera (1). However, size and charge polymorphism and antigenic variation of MSP3 among strains were also identified. In this paper, we report the cloning and expression of *msp3* from the Florida (FL) strain of *A. marginale*. Southern blot analysis of genomic DNA identified multiple related copies of *msp3* distributed throughout the genomes of three different strains. Sequence analysis of three unique *msp3* genes revealed defined regions of heterogeneity within related copies for the FL strain. Significant amino acid sequence homology was also identified between MSP3 and a second major surface protein of *A. marginale*, MSP2. Limited amino acid sequence homology was identified between the gene product of *msp3-12* and the M protein of *Streptococcus pyogenes*. These findings support the hypothesis that the organism extensively uses multigene families to antigenically vary important, immunodominant surface antigens.

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MATERIALS AND METHODS

***A. marginale* organisms.** Three strains of *A. marginale* were used in this study and are designated by their original location of isolation (16): FL, Virginia (VA), and south Idaho (SI). Strains were stored in liquid nitrogen as cryopreserved stabilates (13) before being used to infect splenectomized calves. Thawed stabilate (20 ml) from each isolate was injected intramuscularly into 6-month-old, male Holstein calves. The calves were monitored daily for percent rickettsemia by microscopic examination of blood smears and packed cell volume. Infected, whole blood was collected in EDTA from calves during periods of peak rickettsemia (FL, 70%; VA, 36%; and SI, 42%) and centrifuged at 10,000 × *g* for 15 min, and the serum and buffy coat were removed. Packed erythrocytes were washed three times in phosphate-buffered saline (0.14 M NaCl, 2.68 mM KCl, 8.26 mM K_2HPO_4, pH 7.4), resuspended to a packed cell volume of 50%, and stored at −70°C.

Antibodies. A panel of four anti-MSP3 MAbs was used: AMG75C2, AMG76B1, 43/19, and 43/23. The reactivity of one of these (AMG75C2) has been described elsewhere (15). The other MAbs were produced from cloned hybridomas by immunization of mice with affinity-purified MSP3 (FL strain) as previously described (15). The reactivity of these MAbs was identified by binding to 86-kDa antigens in immunoblot assays using proteins from an FL strain separated by single-dimension and two-dimensional gel electrophoresis as previously described (data not shown) (1). A MAb specific for the variable surface glycoprotein of *Trypanosoma brucei* (TRYP1E1) was used as a negative control in immunoblot experiments.

***msp3* cloning.** Genomic DNA was isolated from an FL strain of *A. marginale* by phenol-chloroform extraction and ethanol precipitation as previously described (4, 5). The library was composed of mechanically sheared genomic DNA which was ligated with *Eco*RI adaptors and inserted into lambda ZAP bacteriophages (Stratagene, La Jolla, Calif.). *Escherichia coli* XL-1 Blue cells (Stratagene) were transformed with recombinant lambda ZAP, and ampicillin-resistant colonies were screened for MSP3 expression with a pool of anti-MSP3 MAbs (43/19 and 43/23) as previously described (4). Positive colonies were identified, and inserts were subcloned into pBluescript SK(−) plasmids (Stratagene). *E. coli*

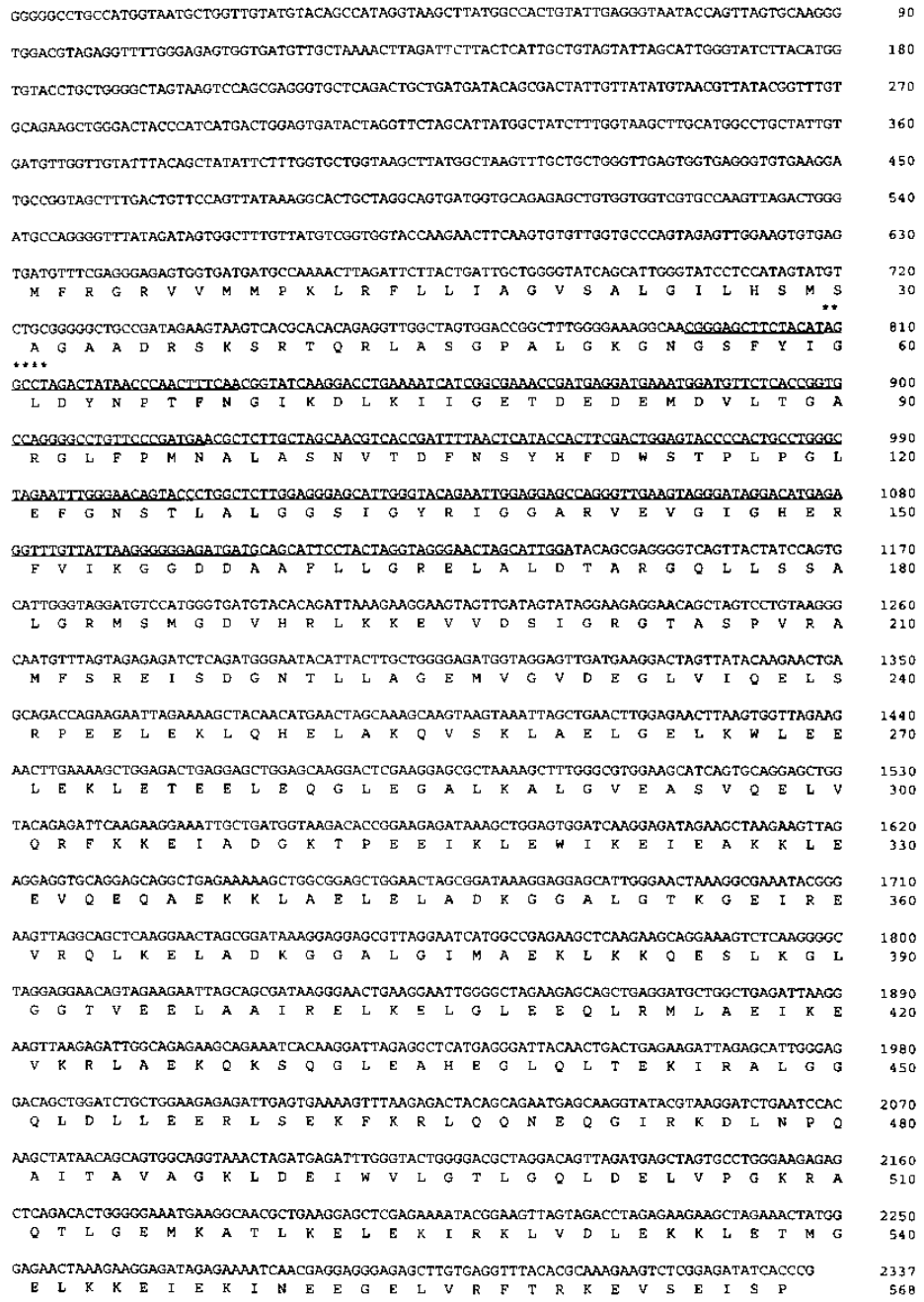


FIG. 1. Nucleotide sequence and translation of *msp3-12* (pBluescript-cloned copy). The entire nucleotide sequence and the translation of the open reading frame of pBluescript *msp3-12* are illustrated. The area homologous to the *msp2* gene is underlined. Asterisks above nucleotides 809 to 814 indicate the *Stu*I cleavage site.

XL-1 Blue cells were transformed with recombinant pBluescript, and ampicillin-resistant colonies were screened with MAbs for MSP3 expression. Three recombinant colonies were identified, and clones were designated *msp3-11*, *msp3-12*, and *msp3-19*.

Immunoblotting of recombinant MSP3. *E. coli* cells (Epicurian Coli XL1-Blue) (Stratagene) were transformed with pBluescript containing each of the three *msp3* genes. Transformed cells were plated on Luria agar containing 50 μg of ampicillin per ml and selected by blue-versus-white screening. *E. coli* cells were transformed with nonrecombinant pBluescript for use as a negative control. Selected colonies were grown overnight in Luria broth containing 50 μg of ampicillin per ml. Transformed *E. coli* cells were suspended in phosphate-buffered saline; lysed in one-half their volume of a 3× sample buffer containing 0.1 M Tris (pH 6.8), 5% (wt/vol) sodium dodecyl sulfate (SDS), 50% glycerol, 7.5% β-mercaptoethanol, and 0.00125% bromophenol blue; and heat denatured at 100°C for 3 min. *E. coli* lysates or similarly prepared FL *A. marginale* lysates were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose as previously described (1). Membranes were reacted with 5 μg of anti-MSP3 MAb AMG75C2 or the negative control MAb TRYP1E1. Antigen-antibody reactions were visualized by enhanced chemiluminescence as previously described (1).

***msp3* sequencing.** Plasmid DNA was extracted from single clones of *msp3-12*, *msp3-11*, and *msp3-19* and used for double-stranded sequencing using sequentially derived primers initiating dideoxynucleotide chain termination reactions. DNA sequence analysis using the Genetics Computer Group package from the University of Wisconsin, version 7.3, was performed on a Vax11/785 computer. Amino acid sequence analysis was performed using the PSIGNAL program of the PC/GENE software package, version 6.6, from the University of Geneva, Geneva, Switzerland. This analysis was based on a method described by Von Heinje (24). A BLAST search for homologous sequences was performed and analyzed by previously reported methods (3).

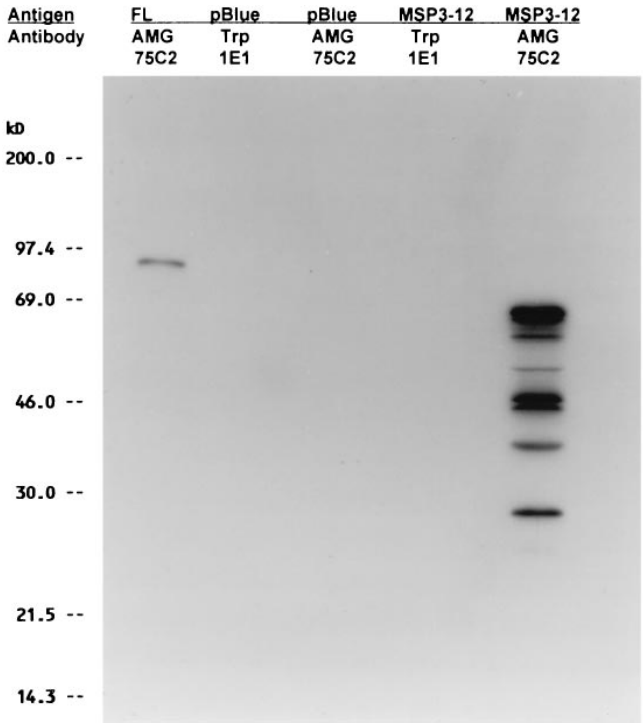


FIG. 2. Expression of MSP3 polypeptides by clone *msp3-12* detected with MAbs. Reactivity of an anti-MSP3 MAb (AMG75C2) and a negative isotype control (Trp1E1) with expressed proteins from clone *msp3-12* is shown. Nonrecombinant pBluescript plasmid (pBlue) is used as a negative control. *A. marginale* FL is used as a positive control.

Digoxigenin labeling of pBluescript *msp3-12*. A digoxigenin-labeled probe of pBluescript *msp3-12* was prepared by digestion of 5 μg of pBluescript *msp3-12* with *Eco*RI and *Stu*I (Boehringer Mannheim Corp., Indianapolis, Ind.). *Eco*RI digestion removed the insert from the plasmid, and digestion with *Stu*I removed 815 bases from the 5′ end of the insert. This eliminated the noncoding region of the clone along with the first 182 bases of the 5′ end of the open reading frame (see Fig. 1). The 1.5-kbp insert of the *msp3-12* gene was gel purified and then extracted from agarose by ion-exchange chromatography using a QIAquick gel extraction kit (Qiagen Inc., Chatsworth, Calif.). A probe was made by random prime labeling 200 ng of *msp3-12* DNA with digoxigenin by using the Genius System Nonradioactive DNA Labeling Kit (Boehringer Mannheim).

Representation of *msp3-12* in the *A. marginale* genome. To verify that the cloned pBluescript *msp3-12* was an accurate representation of genomic *msp3*, multiple restriction sites of pBluescript *msp3-12* and genomic *A. marginale* DNA (FL) were compared by using restriction enzymes which cut within the *msp3* gene. Restriction enzymes *Nco*I (Boehringer Mannheim), *Bsp*M (New England Biolabs, Beverly, Mass.), and *Eae*I (New England BioLabs) were chosen to produce defined fragments from different areas in the *msp3* gene (see Fig. 3, top). Digestions of genomic *A. marginale* DNA (1.0 μg) and pBluescript *msp3-12* (0.1 μg) were performed according to the manufacturer's specifications. Digests were Southern blotted under prehybridization and hybridization conditions of 65°C in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt's solution (1× Denhardt's solution is 0.02% [wt/vol] Ficoll, 0.02% [wt/vol] polyvinylpyrrolidone, and 0.02% [wt/vol] bovine serum albumin), 0.5% (wt/vol) SDS, and 200 μg of sheared herring sperm DNA per ml as previously described (17). Membrane washes (three times for 30 min each) were performed under highly stringent conditions at 65°C in 0.5× SSC–0.1% SDS. Bound probe was detected by enhanced chemiluminescence using alkaline phosphatase-conjugated antidigoxigenin antibody according to the manufacturer's recommendations (Genius System Luminescent Detection Kit; Boehringer Mannheim). The membranes were exposed to Hyperfilm-MP (Amersham International, plc, Buckinghamshire, England) to visualize bound antibodies. The molecular sizes of comigrating cloned and genomic fragments were determined by comparison to lambda DNA-*Hin*dIII fragments (GIBCO BRL, Gaithersburg, Md.) and 1-kbp DNA ladder (GIBCO BRL) molecular size standards.

Identification of multiple *msp3* gene copies. *A. marginale* genomic DNA from either the FL, VA, or SI isolate was extracted as described above, and aliquots of 1 μg of DNA were digested with *Hin*cII (New England Biolabs), *Sac*I (Boehringer Mannheim), *Nde*I (Boehringer Mannheim), or *Sph*I (Boehringer Mannheim). These restriction endonucleases do not cut within the known sequence of the *msp3-12* gene. Calf thymus DNA was digested identically as a control. Nonrecombinant pBluescript and pBluescript *msp3-12* DNAs (0.1 μg) were digested with *Eco*RI and used as negative and positive controls, respectively, for probe hybridization. Digested fragments were separated by gel electrophoresis in a 1% agarose gel containing 0.1 μg of ethidium bromide and photographed. Southern blotting was performed under prehybridization and hybridization conditions as described above, and blots were probed with digoxigenin-labeled pBluescript *msp3-12*. Bound probe was detected by enhanced chemiluminescence.

Distribution of *msp3* gene copies in the *A. marginale* chromosome. The locations of multiple *msp3* copies in the chromosome were determined by Southern blotting large *A. marginale* genomic fragments separated by clamped homogeneous electric field (CHEF) electrophoresis. *A. marginale* strains used in CHEF electrophoresis studies were prepared from infected erythrocytes, embedded in agarose plugs, and digested with *Not*I (Boehringer Mannheim) and *Sfi*I (New England Biolabs) as previously described (2, 6). Uncut *Mycobacterium bovis* chromosomal DNA (Promega Corp., Madison, Wis.) was digested identically as a control for restriction endonuclease activity.

CHEF gel electrophoresis was done on the CHEF DRII system (Bio-Rad Laboratories, Richmond, Calif.). Plugs of digested DNA were electrophoresed in 1% agarose gels in 0.5× Tris-borate-EDTA buffer at 14°C. Electrophoretic conditions were set at 180 V with a 10-s switch rate and a 16-h run time. Delta 39 lambda ladders (Promega Corp.) and lambda DNA-*Hin*dIII fragments (GIBCO BRL) were used as size standards. The gels were stained for 30 min in 0.5 μg of ethidium bromide per ml and photographed. Southern blotting and hybridization of the separated bands were performed as described above except that the gel was depurinated in 0.25 M HCl for 15 min prior to washing and transferred to nylon membranes. Prehybridization, hybridization with digoxigenin-labeled pBluescript *msp3-12*, and detection by chemiluminescence were performed as described above.

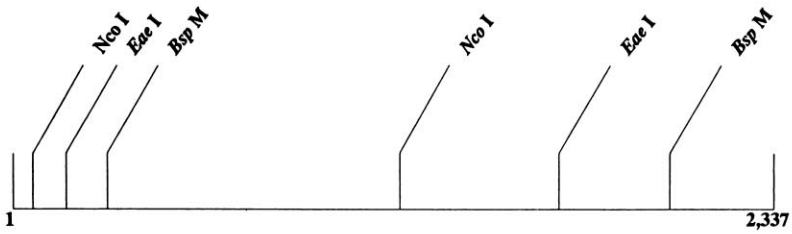
Nucleotide sequence accession numbers. The nucleotide sequences of the *msp3* genes have been assigned the following GenBank accession numbers: *msp3-12*, U60778; *msp3-19*, U60779; and *msp3-11*, U60780.

RESULTS

The *msp3-12* gene was identified by screening a genomic library of an FL strain of *A. marginale* DNA with a pool of anti-MSP3 MAbs. The nucleotide and deduced amino acid sequences of the pBluescript *msp3-12* insert are shown in Fig. 1. This gene contained the coding region for the N terminus of MSP3 with the open reading frame extending for 1,704 bp. In addition, *msp3* contained 633 bp upstream of the open reading frame. Amino acid sequence analysis of the open reading frame predicted a signal peptide at the N terminus with a cleavage site between amino acids 33 and 34. A potential transmembrane helix was identified just before the cleavage site extending from amino acids 12 to 33.

To verify the relatedness of recombinant MSP3 to the native molecules, lysates from *E. coli* cells transformed with pBluescript containing *msp3-12* or with nonrecombinant plasmid were separated by SDS-polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose and reacted with anti-MSP3 MAb (AMG75C2). Initial bodies from an FL strain of *A. marginale* were used as a positive control for MAb AMG75C2. MAb AMG75C2 reacted only with native MSP3 and recombinant polypeptides expressed by clone *msp3-12* (Fig. 2). The largest recombinant MSP3 polypeptide had a molecular size of approximately 65.0 kDa, which corresponds to the calculated size of the protein expressed by the open reading frame of *msp3-12*. Isotype control MAb TRYP1E1 showed no reactivity to *msp3-12*. No reactivity was observed between MAb AMG75C2 or MAb TRYP1E1 and nonrecombinant pBluescript.

To verify that the cloned pBluescript *msp3-12* faithfully represented a genomic copy, genomic DNA from an FL isolate of *A. marginale* and pBluescript *msp3-12* DNA were digested with restriction enzymes to yield predicted fragments of specified lengths. The enzymes used, the restriction sites in pBluescript *msp3-12*, and the predicted sizes of the fragments are illustrated (Fig. 3, top). Digested DNA fragments were Southern blotted to nylon filters and probed with digoxigenin-labeled



Restriction Enzyme	Cleavage site in pBluescript MSP3-12	Size of co-migrating plasmid and genomic fragments
Nco I	12/1,188	1,176
Eae I	52/1,762	1,710
Bsp M	193/2,076	1,886

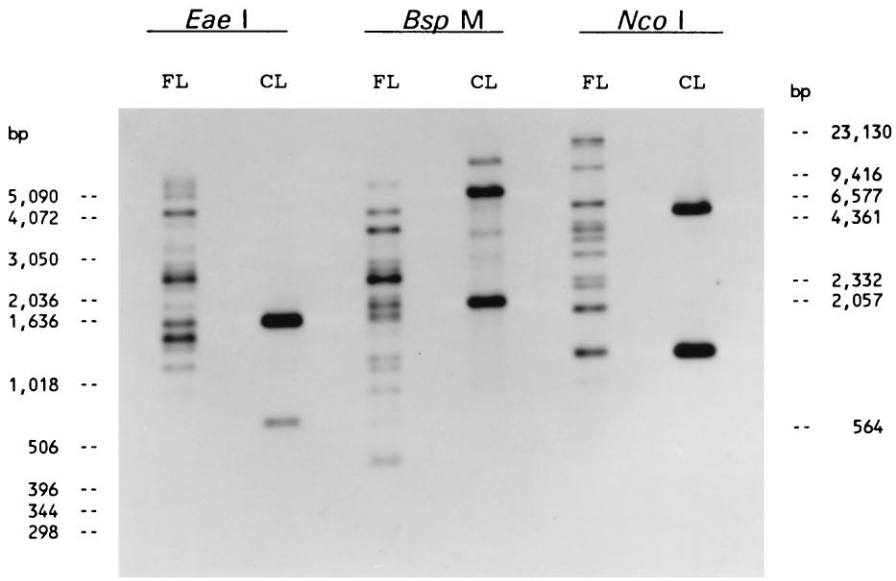


FIG. 3. Restriction map (top) and genomic representation (bottom) of clone *msp3-12*. (Top) Restriction enzyme sites and the resulting fragments used to map *msp3-12* to the genome. The nucleotide cleavage site for each restriction enzyme is illustrated diagrammatically and by nucleotide number. Sizes of resulting fragments in base pairs are also provided. (Bottom) Southern blot of genomic DNA from the FL strain of *A. marginale* or pBluescript *msp3-12* (CL), digested with restriction enzyme *Eae*I, *Bsp*M, or *Nco*I and probed with digoxigenin-labeled *msp3-12*.

msp3-12. All enzymes produced pBluescript *msp3-12* fragments of the predicted size which comigrated with a fragment in the genomic DNA (Fig. 3, bottom). Comigration of large fragments was seen in digests extending from the 5′-terminal region to the 3′-terminal region of the gene. Fragments of clone which do not comigrate with genomic DNA result from clone fragments which also contain variable portions of the vector.

To determine if one or more copies of *msp3* were present, *A. marginale* genomic DNA from either FL, SI, or VA isolates was digested with restriction enzymes, Southern blotted to nylon membranes, and probed with digoxigenin-labeled *msp3-12*. Enzymes *Sph*I, *Nde*I, *Sac*I, and *Hin*cII were chosen because they do not cut within the sequence of the *msp3-12* gene. This should produce a single band if only one genomic copy of *msp3* is present. *Sac*I cuts once within the open reading frame of *msp3-19* (at nucleotide 498), potentially producing two observable bands for a single-copy gene. Multiple bands were observed on Southern blot hybridizations, indicating the presence of multiple, partially homologous *msp3* copies (Fig. 4). The exact number of copies cannot be determined since restriction site polymorphism may exist in other *msp3* copies, resulting in more than one band from a single copy. Similar intensities of many of the bands within a single isolate may indicate that

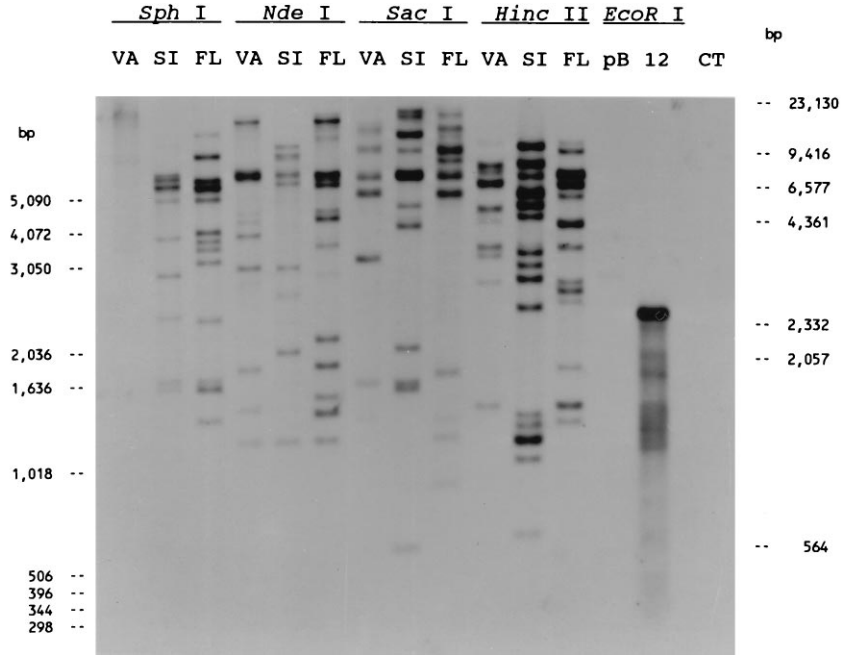


FIG. 4. Presence of multiple copies of *msp3* in genomic *A. marginale* DNA. Genomic DNAs from VA, SI, and FL strains of *A. marginale* were digested with the restriction enzymes indicated above the lanes and Southern blotted. *Eco*RI-digested pBluescript *msp3-12* (12) was used as a positive control for probe hybridization. *Eco*RI-digested, empty pBluescript (pB) and undigested bovine calf thymus DNA (CT) were used as negative controls. The blots were hybridized with digoxigenin-labeled pBluescript *msp3-12*.

extensive homology exists between some of the copies. Restriction fragment length polymorphism (RFLP) was seen when comparing enzyme digests of each of the three isolates of *A. marginale*.

To determine the distribution of *msp3* in the genome, intact, *A. marginale* genomic DNA from three isolates (FL, SI, and VA) was digested into large fragments with restriction enzymes *Sfi*I and *Not*I and separated by CHEF electrophoresis. The gel was stained and photographed (data not shown). These fragments are nonoverlapping and have been previously shown to represent the entire 1,250-kbp *A. marginale* genome (2). The *Sfi*I and *Not*I fragments were identical to those previously reported (2), with *Sfi*I digestion of the FL isolate producing 12 bands ranging in size from 14 to 170 kbp. This same gel was then Southern blotted to nylon filters and probed with digoxigenin-labeled *msp3-12* (Fig. 5). The probe hybridized to multiple fragments of *Not*I and *Sfi*I digests of all three isolates. Most of the gene copies in the FL isolate were contained within two large *Sfi*I fragments with previously reported band sizes of 137.5 and 170 kbp (2). However, the results did indicate that the *msp3* gene was widely distributed throughout the genome in all three isolates tested. Gene copies were present on six of the FL and VA *Sfi*I-digested fragments and five of the fragments from the SI isolate (Fig. 5). *Not*I digestions yielded eight bands in the FL isolate which hybridized to the *msp3-12* probe, seven bands in the VA isolate, and five bands in the SI isolate (Fig. 5). RFLP was also observed between isolates on CHEF gels.

Two additional, unique *msp3* clones were identified by screening the FL strain genomic DNA library with the pool of anti-MSP3 MAbs. Clones *msp3-11* and *msp3-19* lacked the 5′ end of the open reading frame, but each contains the termination codon for the C terminus and 1,473 and 2,480 bp, respectively, downstream of the 3′ end of the open reading frame. The schematic representation of the three genes in Fig. 6 shows three unique genes with extensive areas of homology. This confirms that multiple copies of partially homologous *msp3* genes are present in the genome.

Homologous areas were identified throughout the three *msp3* genes (Fig. 6). Areas common to all three genes were at bases 1191 to 1472 and bases 1818 to 2008 of the *msp3-12* clone. An area at the 3′ end of *msp3-12* between bases 2090 and 2280 was also found in *msp3-11* but was absent in *msp3-19*. The last 200 bp at the 3′ end of the open reading frame of genes *msp3-11* and *msp3-19* were homologous.

The *msp2* gene encodes a 36-kDa surface protein and is known to be expressed by a polymorphic, multigene family (19). Areas of homology between the 5′ end of the *msp2* gene and *msp3-12* are indicated in Fig. 6. The areas of amino acid sequence homology between *msp3-12* and *msp2* (amino acids 55 through 176) showed 65.6% similarity and 54.9% identity (Fig. 7). There was a homologous area of more than 500 bp between *msp3-11* and *msp2* (Fig. 6). However, this area was outside of the open reading frame of *msp3-11*, in a different reading frame and a different direction. A similar but much smaller area was seen downstream of the open reading frame of the *msp3-19* gene (Fig. 6).

DISCUSSION

Cattle which survive acute infection with *A. marginale* continue to be persistently infected and maintain a cyclic rickettsemia which fluctuates at bimonthly intervals (7, 12). Antigenic variation has been proposed as a likely mechanism by which this rickettsial organism evades the host immune system (12). Previous studies, using immunoblots of *A. marginale* proteins separated by two-dimensional gel electrophoresis, identified multiple, antigenically distinct 86-kDa polypeptides present in the FL strain (1). These polypeptides were determined to be related members of the MSP3 family by reactivity with anti-

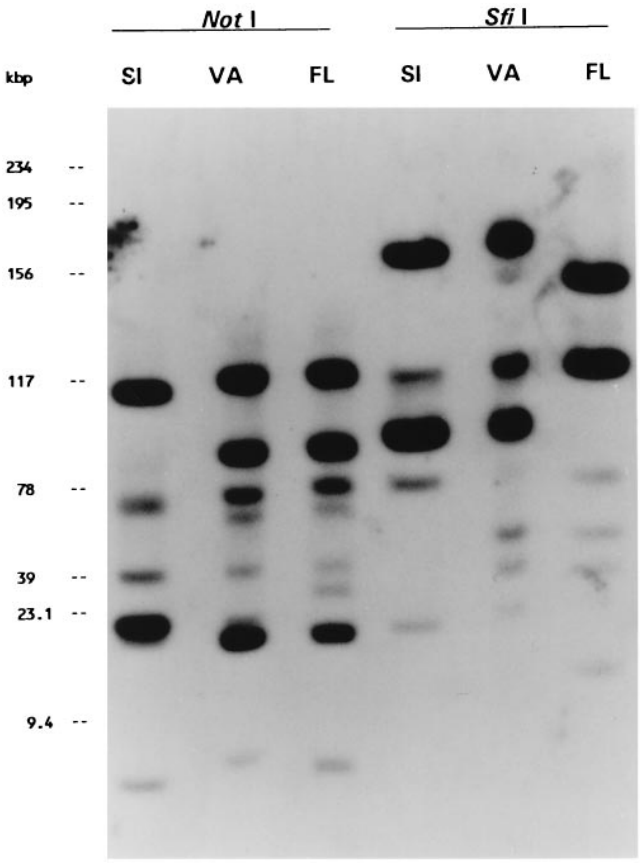


FIG. 5. *msp3* copies are widely distributed in the *A. marginale* chromosome. Genomic DNAs from SI, VA, and FL strains of *A. marginale* were digested with the restriction enzymes indicated above the lanes and separated by CHEF gel electrophoresis. The fragments were then Southern blotted and probed with digoxigenin-labeled pBluescript *msp3-12*.

MSP3 MAb (AMG75C2) and anti-MSP3 immune rabbit sera. In addition, antigenic polymorphism was identified in the MSP3 polypeptides between different *A. marginale* strains (1).

In this study, we used the *msp3* clone pBluescript *msp3-12* to identify multiple related gene copies throughout the *A. marginale* genome. Differences in size between the native (86-kDa) and the recombinant (65-kDa) molecules are expected since the cloned insert of *msp3-12* lacks the 3′ end of the native gene. However, we show that pBluescript *msp3-12* is an accurate representation of a genomic *msp3* copy by (i) expression of a recombinant protein which is bound by anti-MSP3 MAb and (ii) demonstration of comigrating bands of predetermined sizes in cloned and genomic DNA when cut with restriction enzymes *Nco*I, *Bsp*M, and *Eae*I. Digestion of *msp3-12* and genomic (FL) *A. marginale* DNA with these enzymes produces fragments which comigrate in agarose gels and hybridize with the digoxigenin-labeled *msp3-12* probe. These enzymes cut various places within *msp3-12* clone, ranging from nucleotides 12 to 2076. This range covers almost the entire 2,337-nucleotide sequence of the cloned gene. Anti-MSP3 MAb AMG75C2 binds protein expressed by *msp3-12* as well as one of the 86-kDa antigens (pI 5.6) seen on two-dimensional gel electrophoresis of *A. marginale* organisms (1). This indicates that recombinant MSP3-12 shares common epitopes with the MSP3 antigens identified by two-dimensional immunoblots.

Hybridization studies using digoxigenin-labeled *msp3-12* identified multiple copies of partially homologous *msp3* genes in the genomes of FL, SI, and VA strains of *A. marginale*. Genomic DNA was digested with restriction enzymes selected to cut outside of the *msp3-12* sequence. Hybridization of the probe with a single fragment would be seen if MSP3 was encoded by a single-copy gene. Multiple fragments homologous to the *msp3-12* sequence are identified. Although a multigene family is the most likely explanation for these results, it is also possible that within an individual strain there are nonclonal populations of organisms with polymorphic *msp3* genes. This is unlikely, however, considering that restriction endonuclease digestion of large fragments on CHEF electrophoresis consistently produces fragments of defined sizes for each strain, all of which result in a total genome size of 1,250 kbp.

The exact number of copies cannot be determined because restriction sites may be polymorphic in other copies of *msp3*, causing an exaggerated estimation of the number of gene copies. In addition, more than one gene copy may be present on large fragments of genomic DNA. Even so, these data do suggest a copy number of at least 10 to 15 *msp3* genes in the FL and SI isolates, with slightly fewer, 7 to 10, in the VA isolate. With the gene size of *msp3* being approximately 2.5 kbp, we estimate that *msp3* occupies as much as 3.0% of the 1,250-kbp genome of *A. marginale* (2).

Restriction endonuclease digestion with *Sfi*I and separation of fragments by CHEF electrophoresis allow the entire genome of *A. marginale* (FL) to be separated into 14 fragments ranging in size from 14 to 170 kbp (2). The *msp3-12* probe hybridizes to six of these fragments, as well as multiple fragments in *Not*I and *Sfi*I digests of other isolates. However, judging from the intensities of the bands, it appears that most of the copies in the FL isolate are located on two large *Sfi*I fragments of approximately 137 and 170 kbp. The smaller band has been shown to contain a doublet of comigrating fragments (2). Therefore, these two hybridizing bands could represent as much as 25 to 37% of the genome size. Because this is such a large area of the genome, conclusions regarding the proximity of the genes are difficult to reach. The *Not*I digest of the FL isolate and the *Not*I and *Sfi*I digests of the other isolates indicate a more even distribution of copy numbers throughout the genome. This suggests that *msp3* copies are widely distributed throughout the *A. marginale* genome, similar to the pattern seen with the *msp2* multigene family (19). This would indicate that any coordinated regulation of *msp3* copies would involve *trans* regulation. These copies are likely not the result of simple duplication of a single *msp3* gene, since the copies are not present in tandem along a single stretch of DNA. This assumption is supported by the heterogeneity in the partial gene sequences available for three of the *msp3* genes.

The exact function of MSP3 is unknown; however, the prevalence of its gene in the small genome of *A. marginale* suggests a need to antigenically vary this very immunogenic protein in response to stress from the host immune system. The *A. marginale* genome is also estimated to contain a minimum of 7 to 10 copies of *msp2* (19). This gene family encodes an immunoprotective, 36-kDa antigen, and multiple MSP2 polypeptides have been demonstrated by two-dimensional gel electrophoresis using immunoblots of the FL strain (1). Interestingly, we have demonstrated segments of amino acid sequence homology between MSP3 and MSP2, indicating that genetic recombination may occur between these two large, multigene families.

Antigenic polymorphism of MSP3 within the FL strain is evident in the various 86-kDa polypeptides present on two-dimensional gel electrophoresis (1). In addition, in this study we have shown genetic polymorphism between three *msp3*

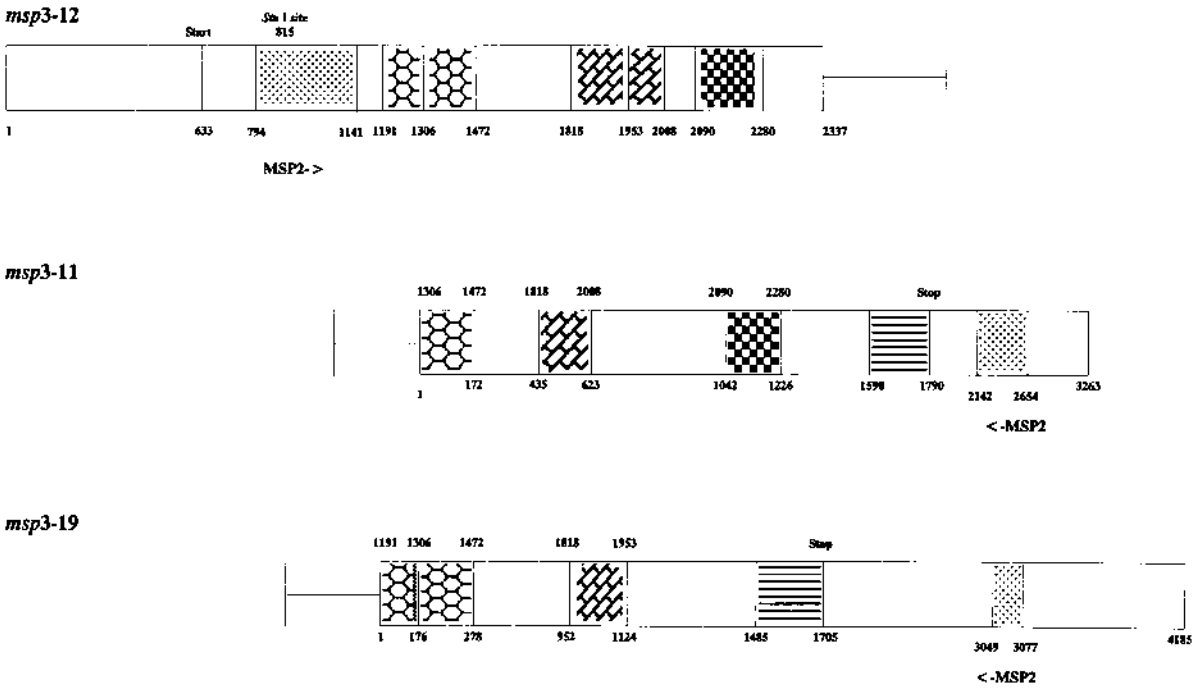


FIG. 6. Sequence heterogeneity and conservation among *msp3* gene copies: schematic representation of clones *msp3-11*, *msp3-12*, and *msp3-19*. Homologous regions are indicated by like-shaded areas. Regions homologous to the *msp2* gene are labeled MSP2->. Nucleotide numbers are indicated on the bottom. Numbers over the diagrams of clones *msp3-11* and *msp3-19* indicate corresponding nucleotides in clone *msp3-12*. The *Stu*I restriction site is illustrated on the top of *msp3-12*. Lines extending to the right of the 3′ end of *msp3-12* and to the left of the 5′ ends of *msp3-11* and *msp3-19* indicate vector sequence.

gene copies isolated from the FL strain, each of which encodes different amino acid sequences. Antigenic polymorphism between different strains was previously shown by reacting immune sera from animals infected with different strains of *A. marginale* with MSP3 polypeptides on two-dimensional immunoblots (1). In this study, genetic polymorphism between different strains is shown by variations in the length and number of restriction fragments which contain the *msp3* genes in each isolate. These results are consistent with previous experiments identifying RFLP between geographic isolates in ethidium bromide-stained gels (2).

```
55   G S F Y I G L D Y N P T F N G I K D L K I I G E T D E D E  83
     |||||||||.| | |||  |.  |
50   G S F Y I G L D Y S P A F G S I K D F K V . Q E A G G T T  77

84   M D V L T G A R G L F P M N A L A S N V T D F N S Y H F D  112
           ||.||     |.    ||  .*.||
78   . . . . . . . R G V F P Y K R D A A G R V D F K V H N F D  99

113  W S T P L P G L E F G N S T L . A L G G S I G Y R I G G A  140
     ||  |  | .  |  .|  |  ||  ||||||  ||||
100  W S A P E P K I S F K D S M L T A L E G S I G Y S I G G A  128

141  R V E V G I G H E R F V I K G G D D A A . . . . . F L L  163
     ||||  .|.|||||||||   .          |||
129  R V E V E V G Y E R F V I K G G K K S N E D T A S V F L L  157

164  G R E L A L D T A R G Q L  176
     |.|||   |||||.
158  G K E L A Y H T A R G Q V  170
```

FIG. 7. Comparison of MSP3 and MSP2 protein sequences: BestFit alignment of amino acid sequences of MSP3 (top) and MSP2 (bottom). Identical amino acids are indicated by vertical lines. Conservative substitutions are indicated by asterisks. Ellipses denote a gap used to achieve optimal alignment between the sequences. Amino acid numbers are indicated at the beginning and end of each line.

Antigenic variation may play a role in the cyclic rickettsemia and persistent infection recognized in carrier cattle infected with *A. marginale* (12). The length and consistency of the cycles suggest that recurrence is due to continual antigenic variation by the organism and development of a primary immune response by the host. Interestingly, when other *A. marginale* sequences (MSP2 and MSP4) are excluded, database searches show greatest homology of the *msp3-12* gene product with the M protein (M12) of *S. pyogenes* (regions containing from 19 to 46% identity with M protein serotype 12). Blocks of M12 with the most significant amino acid sequence homology are located within the constant regions in the carboxyl terminus of the molecule. MSP3-12 also has a small area of homology with the C-repeat region of the M12 protein. Antigenic variation is seen between various serotypes of *S. pyogenes*, and there are more than 80 serologically distinct M proteins among different strains (9). Analyses of M proteins indicate that they are all structurally related and are encoded by a family of genes (18). The mechanism of antigenic variation is not completely understood; however, nucleotide substitutions and small-scale insertions are responsible for antigenic variation in the highly variable N-terminal, nonrepeat domain of the M protein (11). Surface expression of M protein prevent phagocytosis of bacteria by polymorphonuclear leukocytes, allowing the organism to evade the host immune system.

The exact mechanism by which *A. marginale* uses multigene families is not known. However, like *S. pyogenes*, *A. marginale*

does appear to evade the host immune system and avoid complete clearance. In addition, fluctuating rickettsemia is observed in cycles consistent with the appearance of antigenic variants in response to immune pressure from the host (12). We hypothesize that *A. marginale* could use mechanisms similar to those seen in other persistent bacterial infections to vary important antigenic surface proteins such as MSP2 and MSP3 in an effort to avoid immune clearance. Further work is needed to determine if antigenic variation of MSP2 or MSP3 occurs during rickettsemia cycles in persistent carriers.

It has now been shown that two major surface antigens of *A. marginale*, MSP2 and MSP3, are actually each composed of a family of related proteins (1). Using pBluescript *msp3-12* as a probe in hybridization studies, we concluded that the multiple MSP3 antigens are the result of a complex, multigene family of partially homologous genes, similar to the MSP2 protein. The N-terminal amino acid sequence homology between MSP3 and MSP2 may also indicate that genetic recombination occurs between these two large multigene families. Surprisingly, another surface protein of *A. marginale*, MSP4, is also homologous to MSP2 (19). We estimate that a relatively large portion of this rickettsial agent's small genome is occupied by these related gene families encoding MSP2, MSP3, and MSP4. Homology between MSP2, MSP3, MSP4, and the major antigenic protein (MAP1) of *Cowdria ruminantium* (19, 23) suggests that similar mechanisms could be operative in other persistent rickettsial infections. We hypothesize that *A. marginale*, and possibly other closely related rickettsial agents, uses recombination within and among multigene families to antigenically vary these major surface proteins.

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REFERENCES

1. **Alleman, A. R., and A. F. Barbet.** 1996. Evaluation of *Anaplasma marginale* major surface protein 3 (MSP3) as a diagnostic test antigen. J. Clin. Microbiol. **34:**270–276.
2. **Alleman, A. R., S. M. Kamper, N. Viseshakul, and A. F. Barbet.** 1993. Analysis of the *Anaplasma marginale* genome by pulsed-field electrophoresis. J. Gen. Microbiol. **139:**2439–2444.
3. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignments search tool. J. Mol. Biol. **215:**403–410.
4. **Barbet, A. F., G. H. Palmer, P. J. Myler, and T. C. McGuire.** 1987. Characterization of an immunoprotective protein complex of *Anaplasma marginale* by cloning and expression of the gene coding for polypeptide AM105L. Infect. Immun. **55:**2428–2435.
5. **Barbet, A. F., and D. R. Allred.** 1991. The *msp1b* multigene family of *Anaplasma marginale*: nucleotide sequence analysis of an expressed copy. Infect. Immun. **59:**971–976.
6. **Beutler, E.** 1984. The preparation of red cells for assay, p. 8–19. *In* E. Beutler (ed.), Red cell metabolism: a manual of biochemical methods, 3rd ed. Grune and Stratton, Inc., Orlando, Fla.
7. **Eriks, I. S., G. H. Palmer, T. C. McGuire, D. R. Allred, and A. F. Barbet.** 1989. Detection and quantitation of *Anaplasma marginale* in carrier cattle by using a nucleic acid probe. J. Clin. Microbiol. **27:**279–284.
8. **Eriks, I. S., D. Stiller, and G. H. Palmer.** 1993. Impact of persistent *Anaplasma marginale* rickettsemia on tick infection and transmission. J. Clin. Microbiol. **31:**2091–2096.
9. **Fischetti, V. A., K. F. Jones, and J. R. Scott.** 1985. Size variation of the M protein of group A streptococci. J. Exp. Med. **161:**1384–1401.
10. **Goodger, W. J., T. Carpenter, and H. Reimann.** 1979. Estimation of economic loss associated with anaplasmosis in California beef cattle. J. Am. Vet. Med. Assoc. **174:**1333–1335.
11. **Harbaugh, M. P., A. Podbielski, S. Hugl, and P. P. Cleary.** 1993. Nucleotide substitutions and small-scale insertion produce size and antigenic variation in group A streptococcal M1 protein. Mol. Microbiol. **8:**981–991.
12. **Kieser, S. T., I. S. Eriks, and G. H. Palmer.** 1990. Cyclic rickettsemia during persistent *Anaplasma marginale* infection of cattle. Infect. Immun. **58:**1117–1119.
13. **Love, J. N.** 1972. Cryogenic preservation of *Anaplasma marginale* with dimethyl sulfoxide. Am. J. Vet. Res. **33:**2557–2560.
14. **McCallon, B. R.** 1973. Prevalence and economic aspects of anaplasmosis, p. 1–3. *In* E. W. Jones (ed.), Proceedings of the Sixth National Anaplasmosis Conference. Heritage Press, Stillwater, Okla.
15. **McGuire, T. C., W. C. Davis, A. L. Brassfield, T. F. McElwain, and G. H. Palmer.** 1991. Identification of *Anaplasma marginale* long-term carrier cattle by detection of serum antibody to isolated MSP-3. J. Clin. Microbiol. **29:**788–793.
16. **McGuire, T. C., G. H. Palmer, W. L. Goff, M. I. Johnson, and W. C. Davis.** 1984. Common and isolate-restricted antigens of *Anaplasma marginale* detected with monoclonal antibodies. Infect. Immun. **45:**697–700.
17. **Oberle, S. M., G. H. Palmer, and A. F. Barbet.** 1993. Expression and immune recognition of the conserved *Anaplasma marginale* surface protein MSP-4. Infect. Immun. **61:**5245–5251.
18. **O'Toole, P., L. Stenberg, M. Rissler, and G. Lindahl.** 1992. Two major classes in the M protein family in group A streptococci. Proc. Natl. Acad. Sci. USA **89:**8661–8665.
19. **Palmer, G. H., G. Eid, A. F. Barbet, T. C. McGuire, and T. F. McElwain.** The immunoprotective *Anaplasma marginale* major surface protein 2 is encoded by a polymorphic multigene family. Infect. Immun. **62:**3808–3816.
20. **Palmer, G. H., A. F. Barbet, K. L. Kuttler, and T. C. McGuire.** 1986. Detection of *Anaplasma marginale* common surface proteins present in all stages of infection. J. Clin. Microbiol. **23:**1078–1083.
21. **Richey, E. J.** 1981. Bovine anaplasmosis, p. 767–772. *In* R. J. Howard (ed.), Current veterinary therapy: food animal practice. The Sanders Co., Philadelphia, Pa.
22. **Swift, B. L., and G. M. Thomas.** 1983. Bovine anaplasmosis: elimination of the carrier state with injectable long-acting oxytetracycline. J. Am. Vet. Med. Assoc. **183:**63–65.
23. **Van Vliet, A. H. M., F. Jongejan, M. Van Kleef, and B. A. M. Van Der Zeijst.** 1994. Molecular cloning, sequence analysis, and expression of the gene encoding the immunodominant 32-kilodalton protein of *Cowdria ruminantium*. Infect. Immun. **62:**1451–1456.
24. **Von Heinje, G.** 1986. A new method for predicting signal sequence cleavage sites. Nucleic Acids Res. **14:**4683–4690.
25. **Zaugg, J. L., D. Stiller, M. E. Croan, and S. D. Lincoln.** 1986. Transmission of *Anaplasma marginale* Theiler by males of *Dermacentor andersoni* Stiles fed on an Idaho field infected, chronic carrier cow. Am. J. Vet. Res. **47:**2269–2271.

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