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# Putative Adhesins of *Anaplasma marginale*: Major Surface Polypeptides 1a and 1b

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**Genes for the MSP1a and MSP1b subunits of the *Anaplasma marginale* surface antigen complex MSP1 were previously cloned and expressed in *Escherichia coli*. We report here the localization of MSP1a and MSP1b polypeptides on the surface of recombinant *E. coli* by using a live cell indirect immunofluorescent antibody assay. Recombinant *E. coli* cells expressing the *msp*1α gene or the *msp*1β gene encoding the MSP1a and MSP1b polypeptide subunits, respectively, were shown by a culture recovery adhesion assay and by direct microscopic examination to specifically adhere to bovine erythrocytes. This adhesion was more than additive when both genes were coexpressed in a single recombinant construct. Similarly, these recombinants hemagglutinated bovine erythrocytes in a microtiter hemagglutination assay. Inhibition of recombinant *E. coli* adhesion to bovine erythrocytes and hemagglutination inhibition were observed in the presence of homologous monospecific polyclonal antiserum raised against purified MSP1a or MSP1b polypeptide. These data suggest that the MSP1a and MSP1b polypeptides have functions as adhesins on *A. marginale* initial bodies, probably during erythrocyte invasion.**

Anaplasmosis, one of the most prevalent arthropod-borne hemoparasitic diseases of livestock worldwide, is caused by the obligate, intraerythrocytic rickettsia *Anaplasma marginale*. Acute anaplasmosis in cattle can result in severe anemia, weight loss, fever, abortion, lower milk production, and often death (1, 36, 38). Animals that recover from acute infection frequently act as carriers and serve as reservoirs for transmission of *A. marginale* to susceptible animals (10, 18, 36, 50). Anaplasmosis is responsible for 50,000 to 100,000 cattle deaths per year in the United States alone (12) leading to annual losses of more than 100 million dollars (22, 37). Transmission of *A. marginale* mainly occurs during feeding by arthropod vectors, usually ticks, or through contaminated fomites (19, 36). Upon entry into the bovine host, *A. marginale* initial bodies invade circulating erythrocytes and reside within a parasitophorous vacuole of host cell origin (11, 46). The organisms undergo binary fission resulting in the formation of intraerythrocytic colonies of 8 to 10 initial bodies (40). New initial bodies are released from the erythrocytes and adhere to and invade new erythrocytes by as yet unknown mechanisms.

Induction of protective immunity in cattle by immunization with a variety of nonviable *A. marginale* preparations has been reported (25, 28, 29, 34, 39, 44). Although the efficacy of these experimental vaccines is variable between and within experiments, they demonstrate the feasibility of a subunit vaccine approach to immunization for this disease. One potential class of targets for development of such a subunit vaccine would be functional factors associated with adhesion to and invasion of bovine erythrocytes, termed adhesin(s) and invasin(s), respectively. Such a vaccine could induce antibody-mediated attacks on the *A. marginale* initial body while external to the erythrocyte as well as abrogate the attachment of the initial body to the erythrocyte and its subsequent invasion of the erythrocyte by blocking the functions of these components. In this paper we report that recombinant *Escherichia coli* cells carrying cloned *msp*1α (3) and/or *msp*1β (4, 5) genes from *A. marginale* (Florida isolate) were transformed from having a nonadherent phenotype to having a phenotype adherent to bovine erythrocytes. Although it has been shown that immunization of cattle with the MSP1 surface protein complex of the Florida isolate protects against homologous and heterologous challenge (28), no biological function had previously been described for this complex. We propose that a putative function of the MSP1 surface protein complex is to mediate adhesion to bovine erythrocytes.

## MATERIALS AND METHODS

**Source of recombinant *E. coli* expressing MSP1a and/or MSP1b polypeptide.** Plasmid pAM97 contains the complete *msp*1β gene fragment, in pBR322, and encodes the MSP1b polypeptide, formerly identified as Am105L (4, 5). Plasmid pFL10 contains the complete *msp*1α gene fragment encoding the MSP1a polypeptide, formerly identified as Am105U, in pGEM4. The *msp*1α gene was subcloned from plasmid pKAna420 as described elsewhere (3). Plasmid pMSP1 contains intact copies of both genes, and it was generated by inserting the *msp*1α gene into pAM97. This was accomplished by excision of the msp1α gene from pFL10 by *Nco*I restriction endonuclease digestion, followed by its isolation from a low-melting-point agarose gel (21). The *Nco*I overhangs then were filled in with deoxynucleotide triphosphates by using the Klenow fragment, and the gene fragment was inserted into the *Sma*I site of pAM97 by blunt-end ligation. The plasmid constructs were used to transform competent *E. coli* strains DH5α and HB101 to ampicillin resistance (21). The intact MSP1a and MSP1b polypeptides were expressed from the *msp*1α and *msp*1β genes, respectively, under the control of the natural rickettsial promoters for assays of adhesion and hemagglutination.

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**Expression and purification of recombinant fusion proteins.** As a source of large quantities of MSP1a and MSP1b polypeptides for use in raising polyclonal antisera, the *msp*1α and *msp*1β genes were fused with the *Schistosoma japonicum* glutathione-*S*-transferase (GST) gene, and fusion proteins were expressed from the resulting constructs by using the vector pGEX-2T (43). The *msp*1α gene was amplified from pKAna420 (3) plasmid DNA by PCR using the primers 5′CT GTGAATTCTATCAGCAGAGTATGTGTCCAC3′ and 5′G CTAGAATTCGACTCTATCAAAGACCGGAA3′. The amplified product was digested with *Eco*RI and inserted into the *Eco*RI site of pGEX-2T. The *msp*1β gene was PCR amplified from pAM97 (4) plasmid DNA by using the primers 5′ATG GATCCATGACAGAAGACGACAAGCA3′ and 5′ATGGA TCCGTTAACAACCCTCTGAACGT3′ and then the amplified product was digested with *Bam*HI and inserted into the *Bam*HI site of pGEX-2T. Each fusion construct yields a full-length *msp*1α or *msp*1β gene product (minus the *msp*1α start methionine) fused to the carboxy terminus of *S. japonicum* GST. The fidelity and orientations of the constructs were verified by sequencing. MSP1a and MSP1b fusion polypeptides were isolated as described previously (43) by glutathione-agarose chromatography, and for MSP1b only, the GST moiety was removed by cleavage with thrombin (43). The GST fusion proteins suffered moderate to severe degradation during the purification process. It should be noted that these recombinants expressing the MSP1a:GST and MSP1b:GST fusion proteins were used solely for preparation of antisera and not for any adhesion or hemagglutination experiments.

**Preparation of antisera.** Antisera to purified recombinant MSP1a:GST and MSP1b:GST fusion peptides and antiserum to purified recombinant MSP1b polypeptide were made in New Zealand White rabbits. Rabbits initially were immunized intramuscularly and subcutaneously with 50 μg of recombinant peptides emulsified in 0.3 ml of Titer Max adjuvant (CytRx Corporation, Norcross, Ga.). A booster immunization identical to the initial immunization was administered 30 days after the initial immunization. The rabbit antisera were designated as follows: A1-aF, antiserum to the recombinant MSP1a:GST fusion polypeptide; A2-bF, antiserum to the recombinant MSP1b:GST fusion polypeptide; and A3-b, antiserum to the recombinant MSP1b polypeptide. Titers and reactivity to homologous and unrelated antigens were monitored by a dot blot assay. Other rabbit antisera used as controls included R-907, a polyclonal antiserum raised against *E. coli*/pBR322 (5); R-911, a polyclonal antiserum raised against *E. coli*/pAM25 (5); R-873, antiserum raised against MSP1 complex isolated from *A. marginale* initial bodies by immunoaffinity chromatography (29); R-783, antiserum raised against purified initial bodies of *A. marginale* (33); R-883, polyclonal antiserum raised against partially purified *A. marginale* 36-kDa surface protein (MSP2) (33, 34); and 31B, a polyclonal antiserum raised against partially purified *A. marginale* 31-kDa surface protein (MSP4) (26).

**Adsorption of anti-*E. coli* antibodies.** Parental *E. coli* strains DH5α/pBR322 and HB101/pGEM4 were used to adsorb any antibodies from preimmune and immune sera that might recognize *E. coli* surface antigens. Each strain was inoculated separately into 100 ml of Luria-Bertani broth and incubated for 16 to 18 h. The cells were collected by centrifugation (5,000 × *g*), washed three times in phosphate-buffered saline (PBS; 10 mM sodium phosphate, 145 mM sodium chloride [pH 7.2]), resuspended with PBS to a final volume of 10 ml, and then pooled. Twenty grams (wet weight) of CF11 fibrous cellulose powder (Whatman Biosystems Ltd., Kent, England) presoaked in PBS was added to the *E. coli* suspension, and the suspension was thoroughly mixed. The bacterium-cellulose slurry was added to polypropylene chromatography columns (Econo-columns; Bio-Rad, Hercules, Calif.). The columns were allowed to equilibrate at 4°C overnight and then were washed by free flow of PBS through the matrix until the PBS ran clear. The antisera were diluted 1:10, added to a column, and incubated for 3 h. Adsorbed antisera were eluted from the columns and shown to retain activity against homologous MSP1a or MSP1b recombinant polypeptide but not against *E. coli*/pBR322 or *E. coli*/pGEM4 intact cells by direct agglutination or dot blot assay.

**Dot blot assay.** Immunoreactivity of the purified recombinant MSP1a:GST and MSP1b:GST polypeptides with the various homologous and heterologous antisera was detected by dot blot assay. One microgram of each purified recombinant polypeptide was blotted onto nitrocellulose membranes, reacted with a 1:500 dilution of primary antibody, and detected by reaction with a 1:1,000 dilution of goat anti-rabbit immunoglobulin G-horseradish peroxidase conjugate (Sigma Chemical Co., St. Louis, Mo.). Bound antibodies then were detected by chemiluminescence by using the ECL Western blotting (immunoblotting) detection kit (Amersham Corporation, Arlington Heights, Ill.) according to the manufacturer's instructions.

**Live-cell IFA.** A live-cell indirect immunofluorescence assay (IFA) was used to detect surface expression of recombinant MSP1a or MSP1b polypeptide, and it was performed essentially as described by Allred et al. (2). *E. coli* cells were grown to mid-logarithmic phase, collected by centrifugation (5,000 × *g*, 10 min, 4°C), and washed three times with PBS. Approximately $10^8$ bacteria were used for each sample. Immunofluorescence was performed by incubation of the live, intact cells with preimmune or immune serum at a 1/20 dilution, amplification of the signal by incubation with goat anti-rabbit immunoglobulin G (1/100 final dilution), and visualization of bound antibody with a 1/200 final dilution of tetramethylrhodamine isothiocyanate-conjugated rabbit anti-goat immunoglobulin G (whole molecule; Sigma Chemical Co.). The cells were washed with PBS between and after incubations and then were split into two equal volumes. Cells from one sample were collected by centrifugation, resuspended in fetal calf serum, smeared onto a glass slide, and air dried. The smears were briefly fixed in methanol, and coverslips were mounted by using 90% (vol/vol) glycerol in PBS, containing 2.5% (wt/vol; final concentration) 1,4-diazabicyclo-(2,2,2)-octane (Sigma Chemical Co.), as the mounting medium (16). The smears then were examined by epifluorescence microscopy. The percentage of cells exhibiting surface fluorescence was determined by counting approximately 50 cells within a field under phase contrast and then determining the number of those cells that fluoresced. The mean and standard deviation were calculated by counting cells in two separate fields per slide for each strain, in duplicate experiments. Antiserum R-907 (anti-*E. coli*/pBR322) was used as a positive control to demonstrate surface labelling. Antisera R-883 (anti-MSP2) and 31B (anti-MSP4) were used as negative controls. To account for nonspecific labelling of dead cells, the second aliquot of cells was treated with acridine orange (Sigma Chemical Co.) to determine the percent viable cells by direct counting (20). After acridine orange treatment the cells were washed with PBS, resuspended in fetal calf serum, and examined as a wet mount by epifluorescence microscopy. The number of viable cells was determined by counting approximately 50 cells within a field under phase contrast and then counting the number of cells out of the 50 that fluoresced green under epifluorescence.

**Adhesion assay.** Adhesion of recombinant *E. coli* strains to

TABLE 1. Reactivity between purified recombinant MSP1a and MSP1b polypeptides and various antisera raised against recombinant and native *A. marginale* proteins

| Protein[a] | Result with antiserum[b]: | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | A1-aF | A2-bF | A3-b | R-873 | R-783 | R-907 | R-911 | R-883 | 31B |
| MSP1aF | +[c] | + | −[d] | + | + | − | − | − | − |
| MSP1bF | + | + | + | + | + | − | + | − | − |
| MSP1b | − | + | + | ND[e] | ND | ND | ND | ND | ND |

[a] MSP1aF, purified MSP1a:GST fusion protein; MSP1bF, purified MSP1b: GST fusion protein; MSP1b, thrombin-cleaved MSP1bF free of GST sequences.
[b] Antisera were as follows: A1-aF, polyclonal antiserum raised against recombinant MSP1a fusion protein; A2-bF, polyclonal antiserum to recombinant MSP1b fusion protein; A3-b, polyclonal antiserum to thrombin-cleaved recombinant polypeptide MSP1b; R-873, polyclonal antiserum to purified native MSP1 complex; R-783, polyclonal antiserum to purified *A. marginale* initial bodies; R-907, polyclonal antiserum to *E. coli* pBR322; R-911, polyclonal antiserum to *E. coli* pAM25 (carrying the *msp*1β gene); R-883, polyclonal antiserum to purified native MSP2; and 31B, polyclonal antiserum to purified MSP4. All sera were used at a dilution of 1/500.
[c] +, positive reaction.
[d] −, negative reaction.
[e] ND, not determined.

erythrocytes was tested by a modification of the binding assay described by Benson et al. for *Bartonella bacilliformis* (7). *E. coli* strains were grown to mid-logarithmic phase in adhesion incubation medium (AIM; 1.0% tryptone, 1.0% NaCl, 0.5% yeast extract, and 10.0% centrifuged erythrocyte lysate filter sterilized through a 0.22-μm-pore-size membrane filter). Cells were collected by centrifugation, washed three times in PBS, and resuspended in AIM. Bacteria ($10^7$) were added to 900 μl of a 0.5% (packed cell volume) suspension of washed bovine erythrocytes in AIM and were incubated for 30 min at 37°C. The erythrocytes and bacteria were collected by centrifugation (300 × *g*, 2 min, 20°C) and then were washed three times in PBS (300 × *g*, 2 min, 20°C) and resuspended in 100 μl of PBS. Elimination of unbound bacteria from erythrocytes with bound bacteria was performed by Percoll (Sigma Chemical Co.) gradient separation. The erythrocyte-bacterium suspensions were added onto the tops of 1-ml Percoll gradients (70% [vol/vol] in 154 mM NaCl) and were centrifuged at 1,500 × *g* for 5 min. Gradients were preformed by centrifugation at 10,000 × *g* for 15 min (20°C) in 1.5-ml Eppendorf tubes just prior to use. The band containing erythrocytes was removed and washed three times in PBS. The final cell pellet was lysed in 100 μl of sterile water and plated onto Luria-Bertani agar containing 100 μg of ampicillin per ml. Adhesive bacteria were quantitated as the number of CFU recovered from each test.

**Species specificity.** Recombinant *E. coli* strains were examined for species-specific adhesion by using erythrocytes from various animal sources in the adhesion assay. Blood was drawn from cows (*Bos taurus*; *n* = 4), two horses and one pony (*Equus caballus*), dogs (*Canis familiaris*; *n* = 2), a rabbit (*Oryctolagus cuniculus*; *n* = 1), and humans (*Homo sapiens*; *n* = 3) and washed three times in VYM buffer (45) with aspiration of the buffy coat, and adhesion was determined as described above.

**Adhesion inhibition assay.** The inhibitory effects of monospecific antisera to MSP1a:GST (A1-aF) and MSP1b:GST (A2-bF or A3-b) on adhesion of recombinant *E. coli* strains to bovine erythrocytes were examined by using sera preadsorbed by *E. coli* DH5α transformed with pBR322 or pGEM4 to eliminate reactivity with normal *E. coli* components. Each *E. coli* strain was preincubated separately, for 30 min at 37°C, with preimmune or immune sera at 1/20 final dilutions. Antisera R-883 and 31B were used as negative controls. The cells were collected, washed three times in PBS, and tested by the adhesion assay described above. To avoid a biased analysis of the data, the statistical significance of adhesion and inhibition was determined by analyses of variance and Scheffe's S test to determine critical differences (8, 41). Logarithmic transformation of the data prior to analysis was done to reduce the range of heterogeneity of the original data. A difference between any two means that was larger than the calculated critical difference (for an α of 0.05, the critical difference was 0.28318) was assumed to be significantly different.

**Microscopic examination of adhesion.** Visual confirmation of attachment or inhibition of attachment was performed by staining and bright-field microscopy. Instead of lysing the final pellets from adhesion or adhesion inhibition assays, we resuspended the cells in fetal calf serum, smeared them onto glass slides, and then fixed them in methanol and stained them with Giemsa. Bacterial cells were easily distinguishable by their bacillus morphology and purple coloration, whereas the discoid erythrocytes were stained light pink. Photomicrographs were made at a magnification of ×100 by using an oil immersion objective (Olympus Corporation, Lake Success, N.Y.).

**Hemagglutination assay.** Hemagglutination of bovine erythrocytes by recombinant *E. coli* strains was tested by using a microtiter hemagglutination assay performed in U-bottom Microtest III plates (Becton Dickinson, Rutherford, N.J.). Parental and recombinant *E. coli* strains were individually inoculated into 10 ml of Luria-Bertani broth containing 100 μg of ampicillin per ml and were grown to mid-logarithmic phase at 37°C. The cells were collected by centrifugation (5,000 × *g*), washed three times, and then resuspended in 0.5 ml of PBS. A volume containing $10^9$ *E. coli* cells was added to 100 μl of a 0.1% (packed cell volume) suspension of bovine erythrocytes, and the final volume was brought up to 300 μl per well. The *E.*

TABLE 2. Surface reactivity of recombinant *E. coli* with homologous and heterologous antisera as determined by a live-cell IFA[a]

| Plasmid carried by recombinant | Protein(s)[b] | Percent surface-fluorescent cells[c] with the following antiserum[d]: | | | | | | AO viability count[e] |
|---|---|---|---|---|---|---|---|---|
| | | None | R-907 | preA1-aF | preA2-bF | A1-aF | A2-bF | |
| pBR322 | None | 0 ± 0 | 100 ± 0 | 2 ± 1 | 2 ± 1 | 3 ± 1 | 3 ± 2 | 94 ± 3 |
| pMSP1 | MSP1a, MSP1b | 2 ± 2 | 100 ± 0 | 4 ± 2 | 7 ± 5 | 46 ± 3 | 70 ± 9 | 94 ± 3 |
| pAM97 | MSP1b | 3 ± 1 | 99 ± 1 | 3 ± 2 | 7 ± 3 | 13 ± 5 | 54 ± 7 | 96 ± 3 |
| pGEM4 | None | 1 ± 1 | 100 ± 0 | 2 ± 2 | 0 ± 0 | 2 ± 2 | 1 ± 1 | 96 ± 4 |
| pFL10 | MSP1a | 3 ± 2 | 99 ± 1 | 3 ± 1 | 3 ± 2 | 48 ± 6 | 9 ± 6 | 95 ± 4 |

[a] Data are means ± standard deviations.
[b] Relevant polypeptide(s) expressed by each recombinant *E. coli* strain.
[c] Percentage of cells exhibiting rhodamine fluorescence.
[d] See Table 1. pre, preimmune.
[e] AO, acridine orange. The cumulative percent viability for each strain under all treatment conditions is shown. The percent viability for the cell population was greater than 91% ± 5% for all individual treatments.

*coli*-erythrocyte suspensions were mixed and then incubated at 37°C for 1 h. The hemagglutination reactions were scored on a scale from 0 to 4, with 0 signifying no hemagglutination and 4 signifying complete hemagglutination, as described elsewhere (22a).

Inhibition of hemagglutination was performed by incubation of the *E. coli* strains ($10^9$ cells) at 37°C for 1 h prior to reaction in 1:20 dilutions of preadsorbed preimmune serum or antiserum to the homologous or unrelated MSP antigens. Antisera R-883 and 31B were used as negative controls. The cells then were washed three times in PBS, resuspended in 200 μl of PBS, and tested for hemagglutination.

## RESULTS

**Immunoreactivity of recombinant polypeptides.** Immune reactivities of the MSP1a:GST and MSP1b:GST purified recombinant polypeptides with antisera to the homologous or unrelated antigens, determined on the basis of the dot blot assay, are shown in Table 1. The assay was performed with 1/500 dilutions of primary antisera. The cross-reaction between A1-aF antiserum and both the MSP1a:GST and MSP1b:GST polypeptides that was evident was due solely to antibodies reactive with the GST portion of the fusion protein common to both antigens. No reactivity between A3-b antiserum and the MSP1a:GST recombinant polypeptide or A1-aF and the MSP1b (thrombin-cleaved) recombinant polypeptide was seen, indicating that the GST antigen was responsible for the cross-reactions. Antisera prepared against purified initial bodies or immunoaffinity-purified native MSP1 complex reacted equally with MSP1a:GST and MSP1b:GST recombinant polypeptides; however, no reaction was seen when the antiserum R-907 (anti-*E. coli*), R-883 (anti-MSP2), or 31B (anti-MSP4) was used. These data confirmed the antigenic relationships between the recombinant MSP1a:GST and MSP1b:GST polypeptides and their native or immunopurified homologs that were previously reported (3–5).

**Bacterial surface expression of recombinant MSP1a and MSP1b.** The results of a live cell IFA using immune sera prepared against purified recombinant MSP1a:GST and MSP1b:GST are shown in Table 2. The presence of MSP1a and MSP1b polypeptides on the surfaces of recombinant bacteria in which the polypeptides were expressed under the control of the rickettsial promoters was confirmed by the observation of positive fluorescence reactions when antisera to the homologous antigens were used, whereas no detectable reactions were observed when sera to unrelated MSP antigens were used. Significant fluorescence, seen as a speckled or patched pattern, was evident on *E. coli* recombinants HB101/pAM97 and HB101/pMSP1 when the homologous antiserum A2-bF (anti-MSP1b:GST) was used. Similarly, A1-aF (anti-MSP1a:GST) reacted detectably with HB101/pFL10 and HB101/pMSP1. Surface expression of the MSP1a polypeptide and the MSP1b polypeptide by the single transformants HB101/pFL10 and HB101/pAM97, respectively, appeared comparable on the basis of the apparent fluorescence intensities and the percentages of cells that were reactive during live-cell immunofluorescence. The number of cells expressing detectable levels of MSP1b polypeptide was higher for the double transformant HB101/pMSP1 than it was for the single transformant HB101/pAM97. In contrast, the numbers of cells expressing detectable levels of MSP1a polypeptide were equal for HB101/pMSP1 and HB101/pFL10.

Surface fluorescence was absent or negligible for all recombinant strains when no primary antibody or preimmune serum was used (Table 2) or when R-883 (anti-MSP2) or 31B (anti-MSP4) immune serum was used (data not shown). The control *E. coli* strains HB101/pBR322 and HB101/pGEM4 did not show significant surface labelling with immune serum. Total surface fluorescence of *E. coli* cells was seen in the positive control, for which R-907 (anti-*E. coli*) was used as the primary antibody source. Cell viability as determined by acridine orange counts was greater than 91% for all trials.

TABLE 3. Adhesion specificity of recombinant *E. coli* strains to bovine erythrocytes

| Plasmid carried by recombinant | Protein[a] | No. of CFU (mean ± standard deviation)[b] recovered with the following erythrocyte source: | | | |
|---|---|---|---|---|---|
| | | Bovine ($n$ = 4) | Dog ($n$ = 2) | Pony ($n$ = 1) | Human ($n$ = 3) |
| No plasmid | None | 20 ± 5 | 19 ± 8 | 28 ± 6 | 18 ± 8 |
| pBR322 | None | 41 ± 9 | 20 ± 5 | 42 ± 2 | 24 ± 8 |
| pMSP1 | MSP1a, MSP1b | 742 ± 71 | 26 ± 4 | 47 ± 1 | 22 ± 6 |
| pAM97 | MSP1b | 206 ± 13 | 33 ± 9 | 37 ± 1 | 20 ± 8 |
| pGEM4 | None | 38 ± 7 | 23 ± 7 | 42 ± 4 | 24 ± 8 |
| pFL10 | MSP1a | 273 ± 17 | 23 ± 7 | 50 ± 1 | 25 ± 7 |

[a] Relevant polypeptide expressed by each recombinant *E. coli* strain.
[b] Calculated from four replicate counts.

**Adhesion of recombinant bacteria to bovine erythrocytes.** The results of the adhesion and adhesion inhibition assays are presented in Tables 3 and 4, respectively. Since the numbers of adhesive cells were equivalent regardless of whether the plasmids or recombinant constructs were in an *E. coli* HB101 background or an *E. coli* DH5α background, the results for *E. coli* HB101 only were tabulated. Recombinant *E. coli* HB101/pAM97, HB101/pFL10, and HB101/pMSP1 adhered to bovine erythrocytes at levels greater than five times those of the untransformed parental type (Table 3) ($P \leq 0.05$). *E. coli* HB101/pMSP1 recombinant transformed cells, containing both the *msp*1α and *msp*1β genes, adhered to bovine erythrocytes at levels nearly 3 times greater than those at which either *E. coli* HB101/pAM97 or *E. coli* HB101/pFL10 cells did and nearly 20 times greater than those at which *E. coli* HB101/pBR322 cells did ($P \leq 0.05$). The same recombinants did not exhibit significant adhesion to the nonbovine erythrocytes tested, although nonspecific adhesion to rabbit and horse erythrocytes by parental *E. coli* HB101 and DH5α and all recombinants was seen (data not shown). Thus, the *msp*1α and *msp*1β genes transform *E. coli* to an adhesive phenotype specific for bovine erythrocytes. Adhesion to bovine erythro-

TABLE 4. Inhibition of recombinant *E. coli* adhesion to bovine erythrocytes by using polyclonal antisera to MSP1a and MSP1b recombinant polypeptides[a]

| Plasmid carried by recombinant | Protein(s)[b] | No. of adherent cells (CFU) recovered with the following antiserum or antisera[c]: | | | |
|---|---|---|---|---|---|
| | | Pre-immune | A1-aF | A2-bF | A1-aF, A2-bF |
| No plasmid | None | 12 ± 3 | 14 ± 3 | 10 ± 2 | ND[d] |
| pBR322 | None | 41 ± 3 | 35 ± 2 | 51 ± 7 | 34 ± 4 |
| pMSP1 | MSP1a, MSP1b | 594 ± 28 | 239 ± 17 | 106 ± 9 | 43 ± 3 |
| pAM97 | MSP1b | 195 ± 7 | 166 ± 4 | 26 ± 5 | ND |
| pGEM4 | None | 53 ± 5 | 44 ± 4 | 41 ± 8 | ND |
| pFL10 | MSP1a | 211 ± 7 | 34 ± 11 | 180 ± 7 | ND |

[a] Data are means ± standard deviations calculated from four replicate counts.
[b] Relevant polypeptide(s) expressed by each recombinant *E. coli* strain.
[c] See Table 1.
[d] ND, not determined.

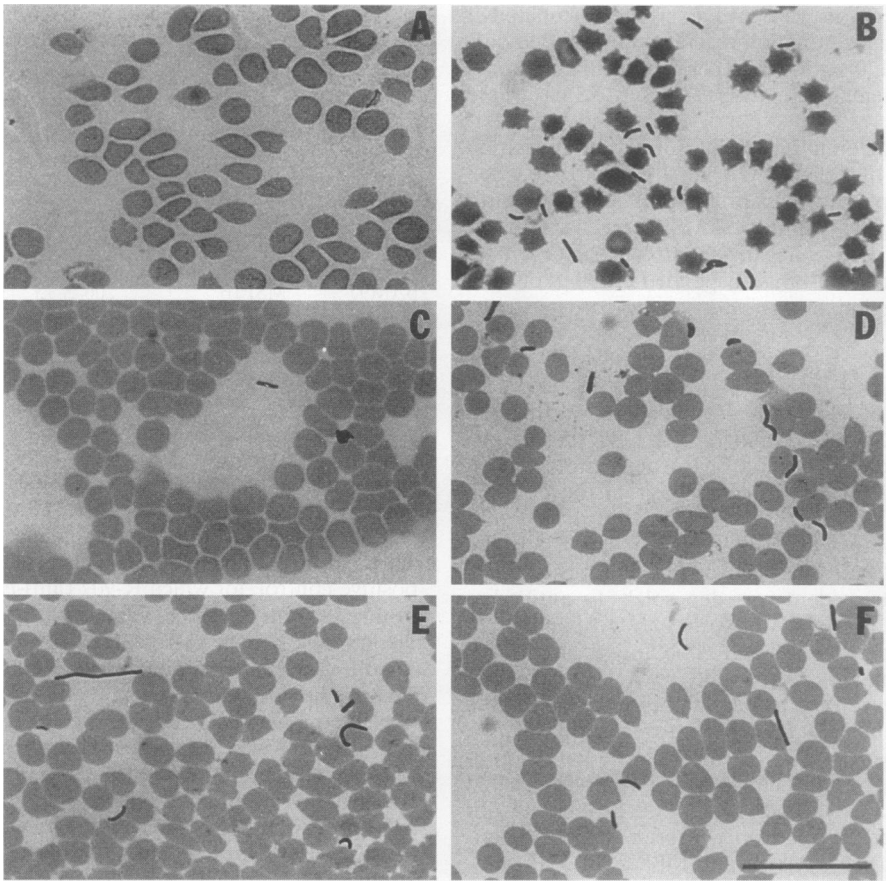


FIG. 1. Adhesion of recombinant *E. coli* to bovine erythrocytes. Recombinant *E. coli* cells were mixed with bovine erythrocytes, the resulting mixture was incubated, and then unbound bacteria were removed by washing the mixture and passaging it through Percoll density gradients. Thin smears then were made and stained with Giemsa. The recombinants used included *E. coli* DH5α transformed with pGEM4 (A); pFL10, expressing the *msp*1α gene (B); pBR322 (C); pAM97, expressing the *msp*1β gene (D); or pMSP1, expressing both the *msp*1α and *msp*1β genes (E and F). All photomicrographs are at the same magnification; bar (panel F), 25 μm.

cytes is shown clearly in Fig. 1, and it seems to occur at any point on the surface of the recombinant bacterium. A second point that is clear from Fig. 1 is that the dramatically increased numbers of adhesive recombinants are not due to the recovery of clumps of bacteria but rather to the adhesion of individual recombinant bacteria.

Inhibition of *E. coli* HB101/pMSP1, HB101/pAM97, and HB101/pFL10 adhesion to bovine erythrocytes was accomplished by using homologous antiserum (Table 4). The number of adhesive recombinant cells recovered was decreased to the level of the background when bacteria were treated with the antiserum A2-bF (cells carrying pAM97) or A1-aF (cells carrying pFL10) or the combination A2-bF–A1-aF (cells carrying pAM97, pFL10, or pMSP1). Treatment of the *E. coli* strains with preimmune serum prior to reaction had no significant effect of lowering the number of cells recovered from the adhesion assay. Additionally, treatment of the recombinants with antisera to the unrelated MSP antigens MSP4 (serum 31B) and MSP2 (serum R-883) failed to affect adhesion by the recombinants (data not shown). The absence of adhesive bacteria on bovine erythrocytes after treatment with homologous antiserum also was observed directly by the microscopic assay (data not shown). On the basis of microscopic observation, this absence was not attributable to bacterial agglutination.

**Hemagglutination by recombinant bacteria.** Hemagglutination of bovine erythrocytes occurred when they were incubated with *E. coli* HB101 transformed with pAM97, pFL10, or pMSP1 but not when they were incubated with strains which

TABLE 5. Inhibition of bovine erythrocyte hemagglutination by using polyclonal antisera to MSP1a and MSP1b recombinant polypeptides expressed by recombinant *E. coli*

| Plasmid carried by recombinant | Protein(s)[a] | Hemagglutination pattern[b] with the following antiserum or antisera[c]: | | | |
|---|---|---|---|---|---|
| | | Pre-immune | A1-aF | A2-aF | A1-aF, A2-bF |
| No plasmid | None | 0 | 0 | 0 | 0 |
| pBR322 | None | 0 | 0 | 0 | 0 |
| pMSP1 | MSP1a, MSP1b | 4 | 2 | 2 | 0 |
| pAM97 | MSP1b | 3 | 3 | 0 | ND[d] |
| pGEM4 | None | 0 | 0 | 0 | ND |
| pFL10 | MSP1a | 4 | 1 | 3 | ND |

[a] Relevant polypeptide(s) expressed by each recombinant *E. coli* strain.
[b] 0, no hemagglutination; 1, weak hemagglutination; 2, moderate hemagglutination; 3, near maximum hemagglutination; 4, maximum hemagglutination.
[c] See Table 1.
[d] ND, not determined.

carried no plasmid or plasmid pBR322 or pGEM4 (Table 5). Consistent with the results of the culture recovery adhesion assay, hemagglutination was also specific for bovine erythrocytes, since each recombinant failed to hemagglutinate dog, human, or horse-pony erythrocytes (data not shown). Hemagglutination also was inhibited when *E. coli* HB101/pFL10 and HB101/pAM97 were treated prior to reaction with the homologous antisera A1-aF and A2-bF, respectively. Significantly, hemagglutination of bovine erythrocytes by *E. coli* HB101/pMSP1 was only partially inhibited by treatment with either A1-aF or A2-bF antiserum alone, but it was fully inhibited when performed in the presence of both antisera (Table 5).

## DISCUSSION

The use of the *A. marginale* MSP1 complex in the development of a recombinant subunit vaccine against anaplasmosis has been a focus of much attention. It was shown that the MSP1 complex contains a neutralization-sensitive epitope (29, 35), which was subsequently mapped to a repeat domain within the MSP1a polypeptide (3, 5) and was found to be conserved among various geographical isolates of *A. marginale* (3, 23, 31). This epitope could be detected on 100% of the initial bodies in all stages of acute infection of the bovine host (30), and it is even expressed by *A. marginale* in midgut stages of tick vectors (32). In addition, immunization with immunoaffinity-purified MSP1 complex from the Florida isolate of *A. marginale* protected susceptible cattle against homologous and heterologous isolate challenge (28). These past studies suggest that the MSP1 polypeptides may play essential roles in the life cycle of *A. marginale*. The results presented in this study strongly suggest that the MSP1a and MSP1b polypeptides are specific cell surface adhesins enabling *A. marginale* to adhere to bovine erythrocytes, probably as part of the invasion process. This hypothesis is supported by the abilities of the antisera A1-aF and A2-bF to significantly inhibit hemagglutination of bovine erythrocytes by *A. marginale* initial bodies (22a). A similar inhibition of hemagglutination of sheep erythrocytes by *Rickettsia prowazeki* was observed in the presence of antirickettsial antiserum (47).

*E. coli* HB101 or DH5α cells were transformed from having a bovine erythrocyte-nonadherent phenotype to having an adherent, hemagglutinating phenotype by the addition of plasmid constructs carrying the *msp*1α and/or *msp*1β gene(s). It should be emphasized that these constructs encode only the rickettsial polypeptides and that they lack all fusion sequences. These genes were previously characterized and shown to express gene products in *E. coli*, driven by their own promoter sequences (3–5), but they were not previously shown to be expressed on the surface of *E. coli*. In this study we have shown by the live-cell IFA that MSP1a and MSP1b recombinant polypeptides are surface expressed at detectable levels in significant portions of the HB101/pAM97, HB101/pFL10, and HB101/pMSP1 recombinant *E. coli* populations. It is not unreasonable to expect that *A. marginale* polypeptides might be expressed, targeted properly to the surface, and folded correctly to function in adhesion. This precedent is well established, since functional adhesin or invasin gene products from a number of bacterial pathogens, including *Chlamydia trachomatis* (17, 42), *Yersinia pseudotuberculosis* (14, 15), *Yersinia enterocolitica* (24), *Salmonella typhi* (9), and nontypeable *Haemophilus influenzae* (6, 13), were produced in *E. coli*.

Both the *msp*1α gene and the *msp*1β gene individually confer upon *E. coli* HB101 and DH5α the ability to adhere specifically to and to hemagglutinate bovine red blood cells. Interestingly, when both genes are present in the same cell, a synergistic effect is seen as the number of adhesive cells recovered significantly increases. The loss of adhesion and hemagglutination through treatment of recombinant *E. coli* with antiserum homologous to the recombinant polypeptide(s), as well as the inability of antisera to unrelated MSP antigens to inhibit adhesion or hemagglutination, confirms the hypothesis that MSP1a and MSP1b polypeptides provide adhesin functions. The fact that MSP1a and MSP1b are isolated from *A. marginale* initial bodies as a complex of two noncovalently linked, antigenically distinct polypeptides which are the products of separate genes (3–5, 27) may help to explain the increased adhesion by the double transformant HB101/pMSP1. It is possible that the association between MSP1a and MSP1b is essential for complete adhesive function because of molecular conformation or stabilization of MSP1 polypeptides on the bacterial (or rickettsial) surface, increased translocation of the polypeptides to the cell surface, or the binding of distinct erythrocyte components by each polypeptide. It is not currently known whether the recombinant polypeptides form a holoprotein complex in *E. coli*; this possibility represents an area for future investigation. However, it was observed that the surface expression of recombinant polypeptide MSP1b on *E. coli* HB101/pMSP1 cells, as indicated by the proportion of cells which were reactive in the live-cell IFA, increased by approximately 30% compared with the surface expression on HB101/pAM97 cells (Table 2). In contrast, no corresponding difference between HB101/pFL10 and HB101/pMSP1 in surface expression of recombinant polypeptide MSP1a was observed, which is consistent with the possibility that the *msp*1α gene product serves to stabilize the *msp*1β gene product.

Although the MSP1a and MSP1b polypeptides were shown to have adhesin functions, it appears that *A. marginale* may express a number of different cell surface adhesins. It has been reported that at least six *A. marginale* surface proteins (with molecular masses of 105, 91, 70, 61, 38, and 29 kDa) from a Mississippi isolate were able to bind either to erythrocyte ghosts or to intact bovine erythrocytes (48, 49). We have shown through a hemagglutination assay that *A. marginale* initial bodies treated with polyclonal antiserum prepared against the 105-kDa (MSP1), 36-kDa (MSP2), or 61-kDa surface protein of an *A. marginale* Florida isolate did not hemagglutinate bovine erythrocytes. In contrast, initial bodies treated with preimmune serum, antiserum prepared against the 31-kDa (MSP4) surface protein, or antisera prepared against irrelevant non-*A. marginale* antigens retained the ability to hemagglutinate the erythrocytes (22a). These findings further support a role for the MSP1 complex in adhesion, but they also indicate that the number, association, and arrangement of *A. marginale* adhesins remain unknown. The procedures developed in this study make the identification of additional *A. marginale* adhesins possible, and they may help to lead to the development of an effective subunit vaccine to protect against bovine anaplasmosis.

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