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Association and Evidence for Linked Recognition of Type IV Secretion System Proteins VirB9-1, VirB9-2, and VirB10 in *Anaplasma marginale*

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Like several other bacterial pathogens, *Anaplasma marginale* has an outer membrane that induces complete protection from infection and disease. However, the proteins that confer protective immunity and whether protection requires interacting proteins and/or linked T-cell and immunoglobulin G epitopes are not known. Our goal is to target the conserved type IV secretion system (T4SS) to identify conserved, immunogenic membrane proteins that are interacting and linked recognition candidates. Linked recognition is a process by which a B cell is optimally activated by a helper T cell that responds to the same, or physically associated, antigen. *A. marginale* T4SS proteins VirB2, VirB4-1, VirB4-2, VirB6-1, VirB7, VirB8-2, VirB9-1, VirB9-2, VirB10, VirB11, and VirD4 were screened for their ability to induce IgG and to stimulate CD4⁺ T cells from outer membrane-vaccinated cattle. VirB9-1, VirB9-2, and VirB10 induced the strongest IgG and T-cell responses in the majority of cattle, although three animals with major histocompatibility complex class II DRB3 restriction fragment length polymorphism types 8/23, 3/16, and 16/27 lacked T-cell responses to VirB9-1, VirB9-1 and VirB9-2, or VirB9-2 and VirB10, respectively. For these animals, VirB9-1-, VirB9-2-, and VirB10-specific IgG production may be associated with T-cell help provided by responses to an interacting protein partner(s). Interacting protein partners indicated by far-Western blotting were confirmed by immunoprecipitation assays and revealed, for the first time, specific interactions of VirB9-1 with VirB9-2 and VirB10. The immunogenicity and interactions of VirB9-1, VirB9-2, and VirB10 justify their testing as a linked protein vaccine against *A. marginale*.

Anaplasma marginale is an obligate intraerythrocytic Gram-negative bacterium causing bovine anaplasmosis. Immunization of cattle with *A. marginale* outer membranes (OM) and cross-linked OM proteins can induce complete protection against disease and infection (10, 43, 57). However, the well-characterized immunodominant major surface proteins 1 to 5 (MSP1 to MSP5), which include antigenically variant MSP2 and MSP3, do not induce equivalent protection against *A. marginale* infection (1, 46, 47, 49, 50). Because immunodominant MSPs are not protective, we have focused on identifying subdominant and conserved surface proteins, including those that associate within the membrane that may be important components of the protective OM vaccine. Among the subdominant antigens identified in the OM are type IV secretion system (T4SS) proteins. Several T4SS proteins induced CD4 T cell responses, including gamma interferon (IFN- γ) production, and were recognized by IgG2 in cattle immunized with the OM vaccine (32–34, 56). These type 1 immune responses are associated with protection against *A. marginale* infection in cattle immunized with OM (9, 10, 50).

The T4SS is a 1.1-MDa protein complex that spans the outer and inner bacterial membranes and has been most widely studied in *Agrobacterium tumefaciens* (13, 17). Typically, the T4SS is made up of 12 interacting VirB/D membrane proteins, several of which are likely to be surface exposed (14) and are thus targets for neutralizing and protective immune responses. The T4SS core complex is made up of 14 copies of interacting VirB7, VirB9, and VirB10, which form a complex that was crystallized and studied with *Escherichia coli* VirB homologues TraN (VirB7), TraO (VirB9), and TraF (VirB10) (13, 17). The *Agrobacterium* VirB proteins also assemble into a pilus made up of VirB2, VirB5, and VirB7 (28, 29, 53, 54). There are three nucleotide triphosphate

(NTP)-utilizing T4SS proteins—VirB4, VirB11, and VirD4—that supply energy for substrate translocation, and VirD4 specifically acts as the substrate-coupling protein (3, 11). VirB6 and VirB8 assemble the T4SS apparatus at the cell pole and also make up the core complex (24, 25, 27). The T4SS proteins harbored by *A. marginale* and other members of the family *Anaplasmataceae* are unique because they are encoded by two copies of *virB4*, *virB8*, and *virB9*, four or more copies of *virB6*, and multiple copies of *virB2*. There are single copies of *virB3*, *virB7*, *virB10*, *virB11*, and *virD4*, whereas homologues of *virB1* and *virB5* have not been identified (7, 19, 20). The function of the T4SS in *A. marginale* has not been determined, but in a pathogen that has undergone reductive evolution, retention of these genes indicates their requirement for invasion and survival within erythrocytes and/or tick cells (19, 20). Because *Anaplasmataceae* lack lipopolysaccharides (7, 20), several T4SS proteins may be surface exposed where they could be targeted by neutralizing antibody. Furthermore, T4SS proteins interact within the bacterial membrane so associated proteins could provide linked recognition for T-cell–B-cell interactions.

Linked recognition was described in the 1970s by Mitchison,

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who revealed that both T cells and B cells must recognize antigenic determinants on the same molecule for B-cell activation to occur (39–41). T cells help B cells to undergo somatic hypermutation and isotype switching through cognate interaction and cytokine secretion. However, linked recognition can also occur with two associated proteins, where one has B cell epitopes and a second provides T-cell epitopes. For *A. marginale*, this was suggested in studies with MSP1, a heteromer consisting of covalently associated MSP1a and MSP1b (35, 60). In these studies, MSP1a-specific T cells provided help to B cells specific for MSP1b to promote increased IgG production (8, 36). Immunization with physically associated B- and T-cell antigens achieves long-lasting immunological memory to the B-cell antigen and is a common strategy used for vaccination against bacterial polysaccharides that lack T-cell epitopes (26, 52).

In the present study, we test the hypothesis that subdominant T4SS proteins of *A. marginale* that induce an IgG response but no CD4⁺ T-cell response in an OM-vaccinated individual interact with a T4SS protein(s) that does stimulate T-cell proliferation which could provide T-cell help. We present evidence for the linked recognition of VirB9-1, VirB9-2, and VirB10, which are naturally associated in *A. marginale*, and a basis for testing these linked T4SS proteins as a vaccine for anaplasmosis.

MATERIALS AND METHODS

Immunization of six haplotype-diverse cattle with *A. marginale* OM. St. Maries strain *A. marginale* OM were isolated as previously described from splenectomized bovine C31919 blood with 40.4% bacteremia (34, 43, 48). Five age-matched Holstein steers and one Holstein cow with various major histocompatibility complex (MHC) class II haplotypes were purchased from the Washington State University (WSU) dairy. Bovine lymphocyte antigen (BoLA) MHC class II DRB3 types were determined by restriction fragment length polymorphism (RFLP) analysis of exon 2 of the DRB3 gene (44, 51, 55, 59). Animals (DRB3 RFLP types) used in the present study were cared for according to an approved Institutional Animal Care and Use Center (IUCAC) protocol for WSU and were designated by number (type) as follows: 35113 (11/22), 35141 (22/24), 35160 (3/16), 35280 (16/27), 35287 (16/22), and 583 (8/23). It is important to note that the haplotypes represented by the six OM-immunized cattle are the most abundant haplotypes of Holsteins in Washington and Canada (unpublished data and reference 55). Cattle were immunized four times subcutaneously with 60 μ g of OM emulsified in saponin, at 0, 2, 4, and 8 weeks as previously described (34). Four immunizations were used to effectively prime cattle to these subdominant antigens so that T-cell and antibody responses would be detected (33, 34). All six cattle produced specific antibody, determined by immunoblotting, and had statistically significant peripheral blood mononuclear cell (PBMC) proliferative responses to *A. marginale* OM, compared to preimmunization responses (see Fig. S1 in the supplemental material).

Expression of recombinant proteins. Full-length recombinant T4SS proteins were expressed from the *A. marginale* St. Maries strain. Genes encoding VirB9-1 (AM097), VirB9-2 (AM1315), and VirB10 (AM1314) were cloned into pBAD/TOPO ThioFusion vector and expressed as previously described with C-terminal FLAG and 6 \times His tags (33). Recombinant VirB7 (AM306) with just a C-terminal 6 \times His tag was also cloned into the pBAD/TOPO ThioFusion vector and expressed and purified as previously described (56). Genes encoding VirB2 (AM030), VirB4-1 (AM814), VirB4-2 (AM1053), the first fragment of VirB6-1 (AM813 F1), VirB8-2 (AM1316), VirB11 (AM1313), and VirD4 (AM1312) were cloned into the pEXP1-DEST vector (Invitrogen), with N-terminal 6 \times His and C-terminal FLAG tags, and expressed in BL-21 DE3 pLysS One Shot *E. coli* (Invitrogen). Primers used for all of these proteins were described previously (56). Plasmid DNA was extracted from *E. coli* and sequencing was

performed using the BigDye kit and ABI Prism automated sequence (Applied Biosystems) as described previously (56).

The negative control protein *Babesia bovis* merozoite surface antigen 1 (MSA1) (31) was expressed using the amplified gene from Mo7 genomic DNA with the forward primer (GCCGATACTTCAATCGTCCTTCC) and reverse primer without a stop codon (TTTGTCGTCGTCGTCTTA TAGTCTGTACCCTGTTGTCCTTGGAGG) encoding a C-terminal FLAG epitope (DYKDDDDK). The thermal cycling parameters consisted of 40 cycles of melting at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 2 min. The plasmid DNA was excised from agarose gels and purified using a Genelute agarose column (Sigma-Aldrich). Amplicons were cloned into the pBAD/TOPO ThioFusion expression vector (Invitrogen) according to the manufacturer's protocol, and plasmid DNA was extracted from *E. coli* using the Wizard Plus SV Miniprep DNA purification system (Promega) and sequenced.

Colonies with the correct sequence were grown to mid log phase in LB broth containing 50 μ g of carbenicillin/ml and induced using 0.2% arabinose for pBAD/TOPO ThioFusion constructs or 1 to 3 mM IPTG (isopropyl- β -D-thiogalactopyranoside) for the pEXP1-DEST constructs. LB broth used for the expression of rVirB8-2 contained 50 μ g of carbenicillin/ml and 34 μ g of chloramphenicol/ml. Induced *E. coli* was centrifuged at 3,800 \times g, and the pellets were resuspended in denaturing lysis buffer (6 M guanidine HCl, 20 mM sodium phosphate, 500 mM NaCl [pH 7.8]), sonicated twice on maximum strength for 3 min, and frozen overnight at -80°C.

Purification of recombinant proteins. All proteins were purified using the hybrid conditions according to the ProBond nickel purification kit instructions (Invitrogen). Protein concentrations were determined with the Quick Start Bradford protein assay (Bio-Rad), and purity was assessed using 1 or 10 μ g/well separated by using Coomassie blue-stained precast sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) 4 to 20% gradient gels (Bio-Rad).

MS/MS analysis of rVirB9-1, rVirB9-2, and rVirB10. Bands corresponding to 20 and 40 kDa for rVirB7, 50 and 100 kDa for rVirB9-1 and rVirB9-2, and 65 and 130 kDa for rVirB10 were excised from Coomassie blue-stained gels, destained (50% methanol, 5% acetic acid), dehydrated with 100% acetonitrile, reduced with 10 mM dithiothreitol (DTT), quenched with 50 mM iodoacetamide, and digested with 20 ng of trypsin (Promega)/ μ l. The peptides were subjected to tandem mass spectrometric (MS/MS) fragmentation on a high-performance liquid chromatography (HPLC)-coupled quadrupole-time of flight (Q-TOF) MS instrument located at University of Idaho, Environmental Biotechnology Institute. Fragment ion lists and the identified peptide sequences were searched against the Mascot databases that contain *A. marginale*, *E. coli*, or National Center for Biotechnology Information all entries. Identification of the protein was based upon the Mascot score, the probability, and the mass. One missed trypsin cleavage, fixed carbamidomethyl modifications, and variable oxidation were allowed during the search. A probability of $\geq 95\%$ showed the peptide match was not a random occurrence, and the individual ion score is reported as $-10 \log_{10} P$, where P is the probability. An ion score greater than 19 has significant identity.

Determination of serological responses to T4SS proteins by immunoblotting. Purified recombinant T4SS proteins (1 to 2 μ g) were loaded onto a 4 to 20% precast gradient SDS-polyacrylamide gel (Bio-Rad) with denaturing sample buffer (8.5 mM Tris, 15% SDS, 50% glycerol, 12% β -mercaptoethanol, 0.1% bromophenol blue), electrophoresed at 100 V for 1.5 h, and transferred to a nitrocellulose membrane (Bio-Rad) at 100 V for 1 h. The membrane was blocked overnight with blocking buffer (10 mM Tris-HCl, 150 mM NaCl, 0.2% Tween 20, 1% PVP40). OM-immune bovine sera that had been extensively adsorbed with protease-inhibited and sonicated BL21 or Top10 *E. coli* (Invitrogen) cells that contained either no vector or a vector encoding MSA1 were diluted 1:100 and added to the membrane for 1 h. The membrane was washed three times with wash buffer (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, 1% PVP40). A 1:20,000 dilution of horseradish peroxidase (HRP)-labeled

recombinant protein G (Zymed) was added for 1 h, and blots were then washed extensively with wash buffer. ECL Western blotting reagent substrate (Thermo Scientific) was applied for 2 min. Preimmune, adsorbed sera from all animals were also used at a 1:100 dilution on the blots containing all of the T4SS proteins to rule out nonspecific IgG binding. After positive IgG responses were identified for a given animal and protein, the same procedure was followed as described above, except sera were diluted 1:300 to 1:10,000.

T-cell proliferation assays. To establish T-cell lines, PBMC were depleted of CD8⁺ cells and $\gamma\delta$ T cells prior to antigen stimulation by incubation with sodium azide-free monoclonal antibodies (MAbs; 3 μ g/ml), followed by complement lysis (33). The antibodies were anti-CD8 α IgG2a (7C2B), anti-CD8 α IgM (BAQ111A), anti-TCR δ IgG2b (GB21A), and anti-TCR δ IgM (CACT61A) obtained from the WSU Monoclonal Antibody Center. Two-week cell lines were generated from CD8- and $\gamma\delta$ -T-cell-depleted PBMC by stimulating for 1 week with 5 μ g of OM/ml and rested for 1 week as previously described (33). The efficiency of depletion was confirmed by fluorescence-activated cell sorting analysis, which showed the T cells were composed of 93.4 to 96.7% CD4⁺ T cells, 1.1 to 2.4% CD8⁺ T cells, and 2.2 to 2.4% $\gamma\delta$ T cells. Proliferation assays were performed in triplicate with 1 and 10 μ g of recombinant T4SS proteins/ml, as described previously (33, 56). Positive controls included *A. marginale* OM and 10% T-cell growth factor and negative controls included uninfected red blood cell membranes (URBC) and *B. bovis* MSA1 also used at 1 and 10 μ g/ml. T-cell proliferation was quantified by incorporation of 0.25 μ Ci/well [³H]thymidine. The results are presented as stimulation indices (SI), calculated as the mean counts per minute (cpm) of cells cultured with antigen/mean cpm of cells cultured with medium. The SI for the different T4SS antigens were compared to the SI for the matching concentration of MSA1 using Dunnett's test, a one-way multiple-comparison test. Statistically significant T-cell stimulation by an antigen was set at a *P* value of <0.05.

Preparation of antibodies specific for VirB7, VirB9-1, VirB9-2, and VirB10. Rabbits were used in the present study in compliance with the WSU IUCAC. Purified recombinant proteins were excised from the SDS-PAGE gels and trypsin-digested for Q-TOF MS analysis to confirm that the purified protein was the protein of interest. Recombinant VirB7, rVirB9-1, rVirB9-2, and rVirB10 were emulsified with 50% phosphate-buffered saline (PBS) and 50% TiterMax Gold adjuvant (Sigma-Aldrich) to make a final concentration of 200 μ g of protein/ml, and 100 μ g of protein was inoculated subcutaneously into rabbits at 0, 3, 5, 7, 9, and 11 weeks (4 to 6 times) until the immunization was successful, which was determined by Western blotting with pre- and postimmunization sera diluted 1:500. A negative control rabbit antiserum was made by Pacific Immunology, using Freund complete and incomplete adjuvants and a fragment of a *B. bovis* nuclear-encoded protein, acyl carrier protein (ACP). The ACP fragment from amino acids 68 to 148 was expressed in pTrcHis TOPO TA expression vector (Invitrogen) and purified using denaturing conditions on a nickel column, followed by electroelution of the gel-embedded protein.

Prior to purification of rabbit IgG, 5 ml of each rabbit serum was incubated with 3 ml of crude Top10 *E. coli* lysate expressing MSA1 for 1 h in binding buffer (20 mM sodium phosphate [pH 7.0]) at room temperature and centrifuged to remove nonspecific antibodies. The adsorbed antisera were buffer-exchanged twice by diluting with binding buffer and centrifuging using 15-ml Amicon 10k filters (Millipore). Subsequently, a HiTrap protein A column (GE Healthcare Biosciences) was used to purify IgG from the sera according to the manufacturer's protocol. Purified IgG was buffer exchanged with sterile PBS and concentrated with an Amicon 10-kDa cutoff filter, and the final concentration of IgG was determined with a Bio-Rad Bradford assay. IgGs were purified on protein A columns, the purity was assessed by SDS-PAGE, and the specificity was determined on immunoblots with 1 μ g of each recombinant T4SS protein, 10 μ g of *A. marginale* OM, and 2 μ g of adsorbed and purified IgG/ml. Secondary antibody was alkaline phosphatase (AP)-conjugated goat anti-rabbit

IgG(H+L) (Invitrogen) diluted 1:10,000, and blots were developed with the Western Star chemiluminescence kit (Applied Biosystems).

Far-Western blotting. Far-Western blotting was performed as previously described (4, 12, 62) with the following modifications and under conditions of protein denaturation and renaturation. SDS-PAGE was performed with 1 μ g of each recombinant protein loaded per well on 4 to 20% gradient gels and electrophoresis at 100 V for 1.5 h. After transfer to nitrocellulose membranes at 100 V for 1 h, the nitrocellulose membrane with recombinant T4SS protein was first incubated with 6 M guanidine hydrochloride–20 mM HEPES (pH 7.5)–50 mM KCl–10 mM MgCl₂–1 mM DTT–0.1% Nonidet P40–2% milk (GuHCl buffer) for 30 min at room temperature, then successively incubated for 30 min each with 3 M and 1.5 M GuHCl buffer at room temperature, then incubated with 0.6 M GuHCl buffer at 4°C for 30 min, and finally incubated with buffer lacking GuHCl overnight at 4°C. The membrane was blocked with 20 mM Tris-HCl (pH 7.5)–150 mM NaCl (TBS) with 5% milk for 3 h. Two far-Western membranes were run in tandem. One membrane was incubated with a 1:2,000 dilution of HRP-conjugated anti-His MAb (Qiagen) to confirm proteins on the membrane after denaturing (see Fig. S2 in the supplemental material). The second membrane was used to identify a target recombinant protein interacting with another recombinant protein. To determine whether the target recombinant protein (VirB9-1, VirB9-2, or VirB10) bound to a prey recombinant T4SS protein(s), the membrane containing 1 μ g of each transferred prey protein was incubated with 10 μ g of target protein in 5 ml of TBS with 5% milk overnight at 4°C. The membrane was then washed three times with TBS containing 0.05% Tween 20 (TBST). Rabbit antiserum, adsorbed with Top10 *E. coli* lysate expressing MSA1 and directed against the target protein (rVirB9-1, rVirB9-2, or rVirB10), was added to the membrane at a 1:500 dilution in TBS plus 5% milk, and the excess was washed off. The membrane was incubated with a 1:10,000 dilution of AP-conjugated goat anti-rabbit IgG(H+L) for 1 h and then washed extensively with TBST and developed using the Western-Star chemiluminescent substrate.

Determining the binding coefficients of interacting rVirB9-1, rVirB9-2, and rVirB10. Serial dilutions (0.5 to 5.0 μ g) of rVirB9-2 and rVirB10 were applied to a dot blot apparatus and used to find the binding coefficient to rVirB9-1 (12). Recombinant VirB9-1 was also applied to the dot blot as a positive control, and a higher concentration of (0.5 to 10.0 μ g) rMSA1 was used as a negative control for background subtraction. The dot blot was developed using the same procedure as for far-Western blotting described above using 10 μ g of rVirB9-1 to probe the blot and a 1:500 dilution of adsorbed polyclonal anti-VirB9-1 rabbit serum to detect rVirB9-1. Densitometry analysis of the spots containing interacting proteins or controls was performed with ImageJ 1.43 software from National Institutes of Health. The data were fit to a standard one-site saturation binding curve $Y = Y_{\max}(X)/(K_D + X)$, where *Y* is the intensity of the spot, *X* is concentration of the protein on the membrane, and *K_D* is the binding association constant using SigmaPlot 10.0. From this equation of the line the binding association constants were found for rVirB9-1 binding to rVirB9-2 and rVirB10.

Production of MAbs. Mice were used in the present study in compliance with the WSU IUCAC. Two female BALB/c mice were immunized subcutaneously with 20 μ g of rVirB9-1, rVirB9-2, or rVirB10 emulsified in an equal volume of complete Freund adjuvant for a total volume of 150 μ l. Mice received three booster immunizations at 2-week intervals with 20 μ g of antigen in incomplete Freund adjuvant. Pre- and postimmunization sera were used to probe blots to detect specific protein in native OM. A final immunization with 10 μ g of protein in 100 μ l of PBS was given intravenously, and spleens were removed 3 days later for hybridoma fusion. Fusion and limiting dilution cloning were performed as described previously (63). Hybridomas were screened for a positive reaction on immunoblots of all three recombinant proteins, as well as the OM. Hybridomas that reacted specifically with the recombinant protein of interest, as well as native protein, were selected for cloning. Supernatants from clones were similarly screened, and three clones were selected for MAb

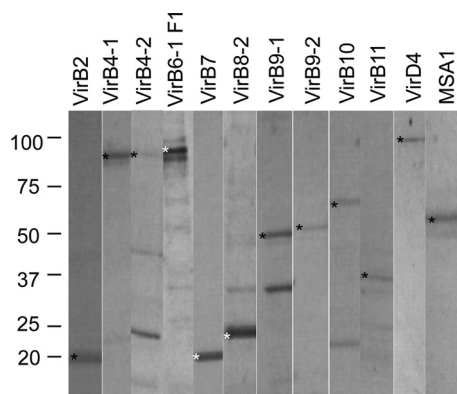


FIG 1 Purity of recombinant T4SS proteins. Recombinant T4SS proteins were loaded at 1 μ g/well, separated on 4 to 20% gradient SDS-PAGE gels, and stained with Coomassie blue dye. The proteins were electrophoresed on separate gels, and scanned images were arranged as presented. An asterisk indicates the predicted molecular mass of the recombinant protein, indicated in parentheses as follows: rVirB2 (20 kDa), rVirB4-1 (90 kDa), rVirB4-2 (91 kDa), rVirB6-1 F1 (90 kDa), rVirB7 (20 kDa), rVirB8-2 (23 kDa), rVirB9-1 (46 kDa), rVirB9-2 (46 kDa), rVirB10 (65 kDa), rVirB11 (38 kDa), rVirD4 (93 kDa), and control rMSA1 (58 kDa).

production. These are 133/248.14.1.28 specific for VirB9-1 (IgG1), 137/774.8.7 specific for VirB9-2 (IgG2b), and 138/481.3.9 specific for VirB10 (IgG1).

Immunoprecipitation. A high concentration of OM was used for immunoprecipitation because the T4SS proteins are not abundant (34). The nonionic detergent *n*-dodecyl β -D-maltoside (DM) was used for solubilization to help maintain native protein conformation and the natural hydrophobic interaction or hydrogen bonding of the membrane proteins. *A. marginale* OM were solubilized for at least 4 h in DM (Sigma-Aldrich) at 4°C with rotation to yield a final concentration of 2.5 mg/ml in 2% DM, and cell debris was removed by centrifugation at 10,000 \times g for 10 min at 4°C. A 350- μ l aliquot of supernatant was used for each immunoprecipitation reaction. Protein A-purified polyclonal rabbit IgG (2 μ g) specific for VirB9-1, VirB9-2, VirB10, or *B. bovis* ACP (negative control) was incubated with the solubilized OM proteins for 2 h at 4°C with rocking. Protein A-agarose (20 μ l; Santa Cruz Biotechnology) was added, followed by incubation for 1 h at 4°C, with rocking. The native protein complexes were centrifuged at 660 \times g for 5 min, and the pellet was washed four times with 400 μ l of 50 mM Tris-HCl (pH 8)–150 mM NaCl–0.1% Nonidet P40 and once with sterile PBS. For analysis, the pellet was immediately resuspended in 0.3 M Tris-HCl–5% SDS–50% glycerol–100 mM dithiothreitol–tracking dye (pH 6.8 [Lane Marker Sample Buffer; Thermo Scientific]) to yield a total volume of 95 μ l in 1 \times sample buffer, and 10 μ l was loaded per well on 4 to 20% gradient gels and electrophoresed at 100 V for 1.5 h. After transfer to nitrocellulose membranes at 100 V for 1 h, the interacting proteins were determined on Western blots probed with 1 μ g/ml of polyclonal rabbit IgG specific for a candidate interacting protein partner or for the target protein. After extensive washing in I-block containing 0.3% Tween 20, blots were incubated with HRP-conjugated Clean-Blot (Thermo Scientific) that only binds to intact antibodies, according to the manufacturer's protocol, washed with I-block containing 0.3% Tween 20, and developed with the ECL Western blotting reagent (Thermo Scientific). MAbs used at 2 μ g/ml specific for MSP5 (AnaF16C1) and MSP2 (AnaR49a) (38) were also used to probe the blots for additional specificity controls. The blots probed with anti-MSP5 were incubated with HRP-Clean-Blot and developed as described above, and the anti-MSP2 probed blots were incubated with AP-conjugated goat anti-mouse IgG + IgM (Invitrogen) diluted 1:10,000 and developed with the Western-Star chemiluminescent substrate. Immunoprecipitation was also performed with 2 μ g of MSP5-specific MAb/ml and protein A/G agarose

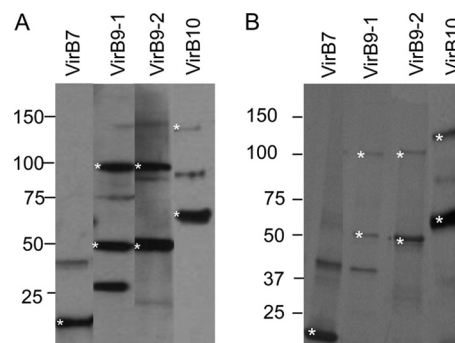


FIG 2 Evidence for dimerization of rVirB9-1, rVirB9-2, and rVirB10. (A) Recombinant proteins (1 μ g) were transferred from a 4 to 20% gradient SDS-PAGE gel to a nitrocellulose membrane that was probed with anti-His MAb (rVirB7) or anti-FLAG MAb (rVirB9-1, rVirB9-2, and rVirB10) and developed. Scanned images were rearranged and presented as shown. The results for VirB7 were obtained in a separate experiment. (B) Coomassie blue-stained SDS-PAGE gel corresponding to the Western blot in panel A, with the exception that 10 μ g of protein was loaded in each lane to visualize the faint bands. Asterisks denote bands where the *A. marginale* T4SS protein was identified by MS/MS analysis.

(Thermo Scientific), and the immunoprecipitate (IP) was electrophoresed, transferred to nitrocellulose, probed with antibodies, and developed as described above. Each polyclonal antibody and anti-MSP5 used for immunoprecipitation pulled down the respective target protein.

In addition, immunoprecipitation reactions were performed with rabbit anti-VirB9-1 IgG as described above, and the IPs were electrophoresed in a single large well, transferred to nitrocellulose, and strips were cut and probed with 2 μ g of mouse MAb specific for VirB9-1, VirB9-2, VirB10, MSP2, MSP5, or *B. bovis* MSA1 (23/10.41, IgG2b) (22)/ml. Secondary antibody was AP-conjugated goat anti-mouse IgG + IgM diluted 1:10,000. Blots were developed with Western-Star chemiluminescent substrate for 30 min or longer.

RESULTS

Recombinant T4SS proteins. All proteins were expressed with their full-length, with the exception of VirB6-1 F1, for which the first fragment was used because the full-length protein did not express (Fig. 1). Some additional weak bands were observed (i.e., for VirB4-2, VirB8-2, and VirB11). For VirB4-2, these reacted with anti-HIS MAb on immunoblotting, suggesting they are degraded protein fragments (see Fig. S2 in the supplemental material). Visualization of immunoblots of rVirB9-1, rVirB9-2, and rVirB10 probed with anti-FLAG MAb indicated that the purified proteins migrated at 50, 50, and 65 kDa, as well as at 100, 100, and 130 kDa, respectively (Fig. 2A). The corresponding weak bands on Coomassie blue-stained gels (Fig. 2B) were excised for MS/MS analysis, rVirB9-1 and rVirB9-2 were each identified in their respective 50- and 100-kDa bands, and VirB10 was identified in both 65- and 130-kDa bands (Table 1). Because other *A. marginale* proteins were not identified, this is consistent with the formation of homodimers by rVirB9-1, rVirB9-2, and rVirB10. The same procedure was followed with rVirB7 bands at 20 and 40 kDa (Fig. 2); however, there was no evidence that rVirB7 dimerized. The 20-kDa band was identified as VirB7, and the 40-kDa band was identified as *E. coli* thioredoxin 1 (Table 1). Although faint bands migrating at ~140 kDa were observed for VirB9-1 and VirB9-2 on some immunoblots (Fig. 2A), there were no corresponding protein bands visible on the gel, even when 10 times more protein was loaded (Fig. 2B), so these were not analyzed by MS/MS. These may

TABLE 1 Evidence for dimerization of VirB9-1, VirB9-2, and VirB10

Protein (mass [kDa]) ^a	Mascot score ^b
rVirB7 (20)	40
rVirB7 (40)	172 ^c
rVirB9-1 (50)	249
rVirB9-1 (100)	210
rVirB9-2 (50)	243
rVirB9-2 (100)	62
rVirB10 (65)	99
rVirB10 (130)	223

^a The molecular masses of protein monomers and dimers are indicated in parentheses.

^b Using an *A. marginale*-specific search, Mascot ion scores greater than 19 are significant at $P < 0.05$.

^c The Mascot ion score is an *E. coli*-specific search and identified as thioredoxin 1; no significant hits for *A. marginale* were detected.

represent protein trimers or multimers with other T4SS proteins (13, 17). In addition to the protein bands that were identified as dimers, products of VirB9-1, VirB9-2, and VirB10 that migrated below the dimeric and monomeric forms were visualized on both the gels and Western blots. Because these bands react with anti-FLAG MAb, which is highly specific for this epitope, these bands likely represent degradation products of the individual proteins resulting in bands smaller than the monomers and dimers, as previously observed for these proteins (33).

IgG responses to T4SS proteins in OM vaccinees. All six OM-immunized cattle produced IgG against VirB9-1, VirB9-2, and VirB10, with VirB9-1 inducing the highest titer tested of at least 10,000 (Table 2). Cattle 35113, 35141, 35160, 35280, and 35287 produced strong IgG responses directed at VirB7 with titers of at least 10,000. Cattle 35280 and 35287 also produced IgG against VirB4-2. However, none of the animals produced detectable IgG responses against VirB2, VirB4-1, VirB6-1 F1, VirB8-2, VirB11, or VirD4 compared to preimmunization sera.

T-lymphocyte responses to T4SS proteins in OM vaccinees. Two-week T-cell lines enriched for CD4⁺ T cells from the six OM-immunized cattle were tested for antigen specific proliferation to recombinant T4SS proteins (Table 3). VirB9-1, VirB9-2, and VirB10 were highly immunogenic for the majority of the animals. However, animals 35160 and 583 did not respond to

VirB9-1, animals 35160 and 35280 did not respond to VirB9-2, and animal 35280 did not respond to VirB10. Animals 35160 and 35287 had significant T-cell responses to VirB2, and animal 35160 had a significant T-cell response to VirD4. Interestingly, 35160 was the only animal that had T-cell responses to VirB6-1, and 35287 was the only animal that responded to VirB4-2. Lastly, there was no detectable T-cell response from any animal to VirB4-1, VirB7, VirB8-2 and VirB11 antigens. These results are representative of three or more experiments.

Selection of linked recognition candidates. Selection of T4SS protein candidates that could undergo linked recognition was based on predicted surface localization, as well as induction of an IgG response, but not a significant CD4 T-cell response, in the same individual. Based on previous results with *A. phagocytophilum* and *A. marginale* (2, 18, 42, 43, 61) and bioinformatic predictions for *A. marginale* (see Fig. S3 and Table S1 in the supplemental material), VirB2, VirB6-1, VirB7, VirB8-2, VirB9-1, VirB9-2, and VirB10 are predicted to be localized on the exterior surface of *A. marginale*. Therefore, the ability of any of these proteins to induce IgG without a significant CD4 T-cell response would make it a candidate for linked recognition and inclusion in future vaccine trials, as surface exposed proteins could be blocked by neutralizing antibody. These candidates are VirB9-1 for animal 583 (DRB3 RFLP type 8/23), VirB9-1, and VirB9-2 for animal 35160 (3/16), VirB9-2 and VirB10 for animal 35280 (16/27), and VirB7 for all animals except 583 (summarized in Table 4). These proteins were therefore selected to identify protein partners within the T4SS that had T-cell epitopes recognized by these animals.

Detecting the interactions of VirB9-1, VirB9-2, and VirB10 by far-Western blotting. Protein A-purified rabbit IgG against rVirB7, rVirB9-1, rVirB9-2, and rVirB10 was tested for antigen specificity by immunoblotting with native OM and recombinant T4SS proteins (Fig. 3). In repeated assays, anti-VirB7 detected a band in the OM that was greater than the predicted native mass of ~6 kDa (Fig. 3A). In contrast, IgG against rVirB9-1, rVirB9-2, and rVirB10 recognized the native OM protein at the predicted molecular weight and at a 2-fold-higher molecular weight (Fig. 3B to D). In addition, anti-VirB9-2 detected a fainter, narrow band at ~140 kDa, which may represent a multimer of the native protein or a multimer of VirB9-2 and another protein(s). When recombi-

TABLE 2 IgG responses to recombinant T4SS proteins in cattle immunized with outer membranes

Protein ^a	IgG titers from animal ^b :					
	35113 (11/22)	35114 (22/24)	35160 (3/16)	35280 (16/27)	35287 (16/22)	583 (8/23)
VirB2	<100	<100	<100	<100	<100	<100
VirB4-1	<100	<100	<100	<100	<100	<100
VirB4-2	<100	<100	<100	1,000	3,000	<100
VirB6-1 F1	<100	<100	<100	<100	<100	<100
VirB7	≥10,000	≥10,000	≥10,000	≥10,000	≥10,000	<100
VirB8-2	<100	<100	<100	<100	<100	<100
VirB9-1	≥10,000	≥10,000	≥10,000	≥10,000	≥10,000	≥10,000
VirB9-2	3,000	1,000	≥10,000	≥10,000	3,000	≥10,000
VirB10	3,000	1,000	≥10,000	3,000	3,000	≥10,000
VirB11	<100	<100	<100	<100	<100	<100
VirD4	<100	<100	<100	<100	<100	<100

^a T4SS proteins were expressed in *E. coli* and purified. Each protein was used at 1 or 2 μg/well and transferred.

^b Sera were diluted and tested for reactivity to recombinant proteins on immunoblots. A value of <100 indicates that no IgG response was detected at a 1:100 dilution. Positive sera recognizing the antigen at the predicted molecular weight and when preimmune sera were negative were diluted 1:300 to 1:10,000. The titer is defined as the reciprocal of the highest serum dilution giving a positive signal. Animal numbers (DRB3 RFLP types) are indicated in the column subheadings.

TABLE 3 T-cell responses to recombinant *A. marginale* T4SS proteins in Holstein cattle with different MHC class II haplotypes

Antigen ^a	Proliferation of T cells from animal ^b :					
	35113 (11/22)	35141 (22/24)	35160 (3/16)	35280 (16/27)	35287 (16/22)	583 (8/23)
OM						
1	736.5 ± 5.7	21.9 ± 4.2	250.1 ± 13.1	50.2 ± 1.8	259.4 ± 9.2	220.0 ± 13.4
10	941.1 ± 52.8	36.7 ± 9.7	252.2 ± 9.2	44.0 ± 2.1	251.1 ± 9.2	312.1 ± 2.1
URBC						
1	0.2 ± 0.1	0.8 ± 0.3	0.6 ± 0.1	0.3 ± 0.1	0.6 ± 0.2	0.4 ± 0.3
10	0.9 ± 0.7	1.6 ± 0.6	0.3 ± 0.1	0.8 ± 0.4	2.0 ± 2.6	0.9 ± 0.9
MSA1						
1	1 ± 1.3	0.9 ± 0.1	0.9 ± 0.1	0.4 ± 0.3	0.3 ± 0.2	0.1 ± 0.0
10	0.3 ± 0.1	0.8 ± 0.4	1.4 ± 0.2	0.4 ± 0.1	0.9 ± 0.5	0.1 ± 0.0
VirB2						
1	0.4 ± 0.1	2.0 ± 0.2	191.7 ± 33.6	9.4 ± 4.4	62.7 ± 17.1	3.4 ± 0.5
10	1.5 ± 0.3	2.8 ± 0.2	46.7 ± 2.2	3.3 ± 1.4	155.0 ± 2.5	7.9 ± 2.0
VirB4-1						
1	1.6 ± 0.7	0.5 ± 0.1	0.1 ± 0.0	0.2 ± 0.0	1.0 ± 0.3	1.0 ± 0.2
10	1.0 ± 0.5	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.7 ± 0.2	2.6 ± 1.0
VirB4-2						
1	1.3 ± 0.1	0.3 ± 0.0	0.2 ± 0.2	0.2 ± 0.1	2.4 ± 1.9	1.3 ± 1.1
10	1.1 ± 0.1	0.1 ± 0.0	0.2 ± 0.2	0.1 ± 0.0	22.8 ± 19.6	1.8 ± 0.0
VirB6-1 F1						
1	3.0 ± 0.7	2.6 ± 0.7	8.2 ± 3.2	0.9 ± 0.3	0.9 ± 0.3	1.5 ± 1.1
10	0.7 ± 0.2	2.1 ± 0.4	78.4 ± 4.7	1.0 ± 0.2	1.5 ± 0.8	0.8 ± 0.1
VirB7						
1	0.5 ± 0.2	0.5 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	1.2 ± 1.0	0.7 ± 0.1
10	2.0 ± 0.4	1.1 ± 0.3	2.2 ± 0.4	2.3 ± 2.1	1.8 ± 0.7	2.0 ± 0.8
VirB8-2						
1	1.3 ± 0.1	0.2 ± 0.0	0.7 ± 0.9	0.0 ± 0.0	0.9 ± 0.1	1.6 ± 0.5
10	0.9 ± 0.1	0.1 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	1.2 ± 0.6	1.7 ± 0.6
VirB9-1						
1	385.7 ± 36.5	22.5 ± 4.2	0.7 ± 0.1	46.8 ± 11.2	264.7 ± 10.6	0.1 ± 0.1
10	779.3 ± 71.4	8.0 ± 4.6	0.9 ± 0.2	103.7 ± 16.4	239.8 ± 11.5	0.1 ± 0.0
VirB9-2						
1	139.2 ± 11.7	35.3 ± 16.0	0.8 ± 0.1	8.0 ± 2.8	32.8 ± 4.1	25.9 ± 4.4
10	201.5 ± 27.1	32.8 ± 0.7	1.7 ± 0.8	11.5 ± 2.1	109.6 ± 4.3	303.7 ± 19.5
VirB10						
1	35.5 ± 3.6	3.7 ± 0.5	90.6 ± 9.4	0.1 ± 0.0	96.2 ± 11.6	6.7 ± 2.7
10	381.2 ± 39.0	5.8 ± 0.3	126.2 ± 5.6	0.3 ± 0.0	162.5 ± 2.3	72.9 ± 8.6
VirB11						
1	1.8 ± 0.1	0.6 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	1.0 ± 0.4	0.0 ± 0.0
10	3.2 ± 1.4	0.7 ± 0.2	0.0 ± 0.0	0.1 ± 0.0	0.8 ± 0.2	0.1 ± 0.0
VirD4						
1	0.4 ± 0.1	2.7 ± 0.4	30.4 ± 6.2	0.3 ± 0.1	0.4 ± 0.1	2.8 ± 1.2
10	0.5 ± 0.0	1.7 ± 0.1	99.7 ± 6.4	0.5 ± 0.1	0.5 ± 0.0	6.3 ± 4.0

^a Recombinant T4SS proteins were used at 1 and 10 µg/ml in the T-cell assays, as indicated. *A. marginale* OM and URBC were positive and negative controls, respectively.

^b A stimulation index (SI) was calculated as the mean cpm of a 2-week CD4 T-cell line to an individual protein/the mean cpm to medium. Results in boldface indicate significantly greater T-cell responses to a T4SS protein compared to the response to the same concentration of MSA1, where *P* was <0.05 using the Dunnett's test. Animal numbers (DRB3 RFLP types) are indicated in the column subheadings.

TABLE 4 Summary of IgG and T-cell responses to *A. marginale* T4SS proteins in six Holstein cattle^a

Antigen	OM-immunized animal ^b :					
	35113 (11/22)	35141 (22/24)	35160 (3/16)	35280 (16/27)	35287 (16/22)	583 (8/23)
VirB2						
IgG	–	–	–	–	–	–
T cells	–	–	+	–	+	–
VirB4-1						
IgG	–	–	–	–	–	–
T cells	–	–	–	–	–	–
VirB4-2						
IgG	–	–	–	+	+	–
T cells	–	–	–	–	+	–
VirB7						
IgG	+*	+*	+*	+*	+*	–
T cells	–*	–*	–*	–*	–*	–
VirB6-1 F1						
IgG	–	–	–	–	–	–
T cells	–	–	+	–	–	–
VirB8-2						
IgG	–	–	–	–	–	–
T cells	–	–	–	–	–	–
VirB9-1						
IgG	+	+	+*	+	+	+*
T cells	+	+	–*	+	+	–*
VirB9-2						
IgG	–	–	+*	+*	–	+
T cells	+	+	–*	–*	+	+
VirB10						
IgG	+	+	+	+*	+	+
T cells	+	+	+	–*	+	+
VirB11						
IgG	–	–	–	–	–	–
T cells	–	–	–	–	–	–
VirD4						
IgG	–	+	–	–	–	–
T cells	–	–	+	–	–	–

^a The IgG responses from Table 2 and the T-cell responses from Table 3 are summarized.^b DRB3 RFLP types are indicated in parentheses in the column subheadings. A negative symbol (–) indicates that IgG titers were <100 or that the T-cell response was insignificant. A positive symbol (+) indicates an IgG titer was ≥300 or that the T-cell response was significant. *, Candidates for linked recognition.

nant proteins were used, antibody against rVirB7 reacted with 20- and 40-kDa bands in rVirB7, but not with the other T4SS proteins (Fig. 3A). For rVirB9-1 (Fig. 3B), rVirB9-2 (Fig. 3C), and rVirB10 (Fig. 3D) IgG bound specifically to its target protein and at a band of approximately twice the molecular weight, which we showed are dimers (Fig. 2 and Table 1). Bands larger than the dimer that are present in the immunizing protein detected by the IgGs against VirB9-1 and VirB9-2 may represent multimers of the VirB9 protein with other T4SS proteins, since it is known that the *A. tumefaciens* core-complex has 14 copies of VirB9 that interact with 14 copies of VirB7 and 14 copies of VirB10 (17). Importantly, the IgGs did not bind to any other T4SS protein, showing that the IgGs were specific for the immunizing protein. An artifactual band of-

ten observed in immunoblots with polyclonal rabbit antisera that was determined to be keratin (45) was observed with the anti-VirB9-2 antibody, which detects a diffuse band at ~68 kDa and another at ~140 kDa (Fig. 3C). Because of the characteristic sizes and widths of these bands, they are assumed to be contaminating keratin monomers and dimers. These bands were only observed with this antibody and detected in only certain recombinant proteins, but not in native OM. We were not able to eliminate this nonspecific keratin band by lowering the amount of reducing agent in the sample buffer as suggested by others (30).

The far-Western blotting strategy was performed as a screening tool to determine which rT4SS proteins bind to rVirB7, rVirB9-1, rVirB9-2, and rVirB10 that could enable them to undergo linked

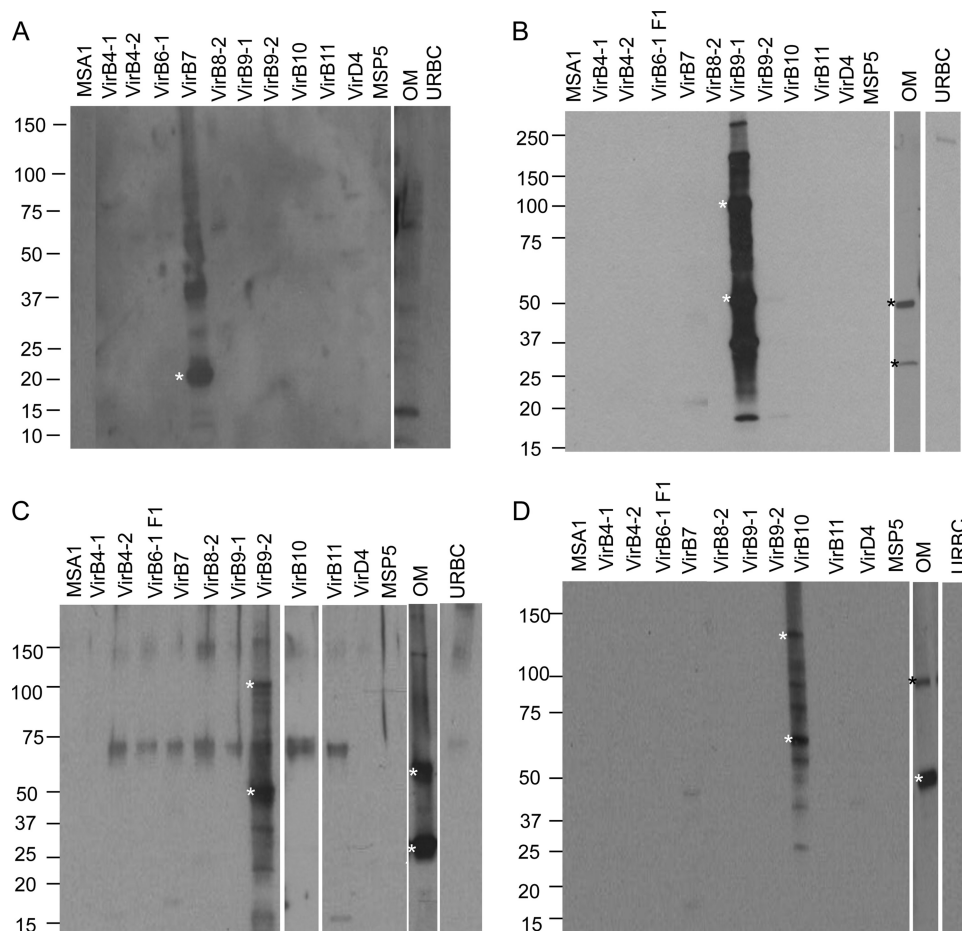


FIG 3 Specificity of rabbit polyclonal antibodies against VirB7, VirB9-1, VirB9-2, and VirB10. Purified recombinant T4SS proteins (1 μ g) and 10 μ g of *A. marginale* OM (native proteins) were separated on a 4 to 20% gradient SDS-PAGE gel, transferred to a nitrocellulose membrane, and detected by Western blotting with 2 μ g of purified, *E. coli*-adsorbed polyclonal rabbit IgG/ml. (A) anti-VirB7; (B) anti-VirB9-1; (C) anti-VirB9-2; (D) anti-VirB10. Scanned images were rearranged and presented as shown in the figure. The predicted molecular masses for recombinant (r) and native (n) proteins are indicated in parentheses: rVirB7 (20 kDa), nVirB7 (6 kDa), rVirB9-1 (46 kDa), nVirB9-1 (30 kDa), rVirB9-2 (46 kDa), nVirB9-2 (30 kDa), rVirB10 (65 kDa), and nVirB10 (49 kDa). The bands with the predicted sizes of monomeric and dimeric (VirB9-1, VirB9-2, and VirB10) proteins are indicated by asterisks.

recognition (Fig. 4). The far-Western blots developed with anti-VirB7 failed to detect potential interacting proteins (data not shown), so proteins interacting with VirB7 were not pursued further. However, the results indicate several interactions among T4SS proteins. Recombinant VirB9-1 interacted with rVirB8-2 at the correct molecular mass of 23 kDa; with rVirB9-2 at the predicted mass of 46 kDa, as well as with a predicted dimer and with apparent degradation products; with rVirB10 at the predicted mass of 65 kDa, as well as with apparent degradation products; and with rMSP5 at the predicted mass of 28 kDa (Fig. 4A). Recombinant VirB9-2 interacted with rVirB9-1 at the predicted mass of 46 kDa, as well as with a predicted dimer and apparent degradation products (Fig. 4B). Recombinant VirB10 interacted with rVirB4-1 at the predicted mass of 91 kDa, with rVirB8-2 at the predicted mass of 23 kDa, with rVirB9-1 at the predicted mass of 46 kDa, and with rVirD4 at the predicted mass of 93 kDa and two smaller bands (Fig. 4C). The reactivity of degradation products of these proteins with anti-His MAb can be seen in Fig. S2 in the supplemental material.

Binding coefficients for VirB9-1 interactions. The binding coefficient for rVirB9-1 interaction with rVirB9-2 was $0.0173 \pm$

$0.0034 \mu\text{M}$ and that for rVirB9-1 interaction with rVirB10 was $0.0109 \pm 0.0094 \mu\text{M}$ (Fig. 5). The binding coefficients for the reverse of the interactions were also determined and found to be similar (rVirB9-2 with rVirB9-1, $K_D = 0.0205 \pm 0.0173 \mu\text{M}$; and rVirB10 with rVirB9-1, $K_D = 0.0135 \pm 0.0080 \mu\text{M}$; see Fig. S4 in the supplemental material), whereas there was no binding detected between VirB9-2 and VirB10 (see Fig. S4 in the supplemental material). The graphed intensities of the spots that yielded the above K_D values all had a goodness of fit to the binding curve equation with at least 80% confidence (Fig. 5). These results indicate that rVirB9-1 has stronger binding to rVirB10 than to rVirB9-2.

Native VirB9-1, VirB9-2, and VirB10 interactions determined by immunoprecipitation. Because the anti-VirB7 antibody did not consistently bind to a native protein of the predicted molecular weight, we focused on using antibodies to VirB9-1, VirB9-2, and VirB10 to identify interacting native protein partners using immunoprecipitation of solubilized OM (Fig. 6). The rabbit IgG or mouse MSP5-specific MAb used for immunoprecipitation is shown at the top of each lane; the antibody used for detection is shown under each panel. For anti-VirB9-1 and anti-

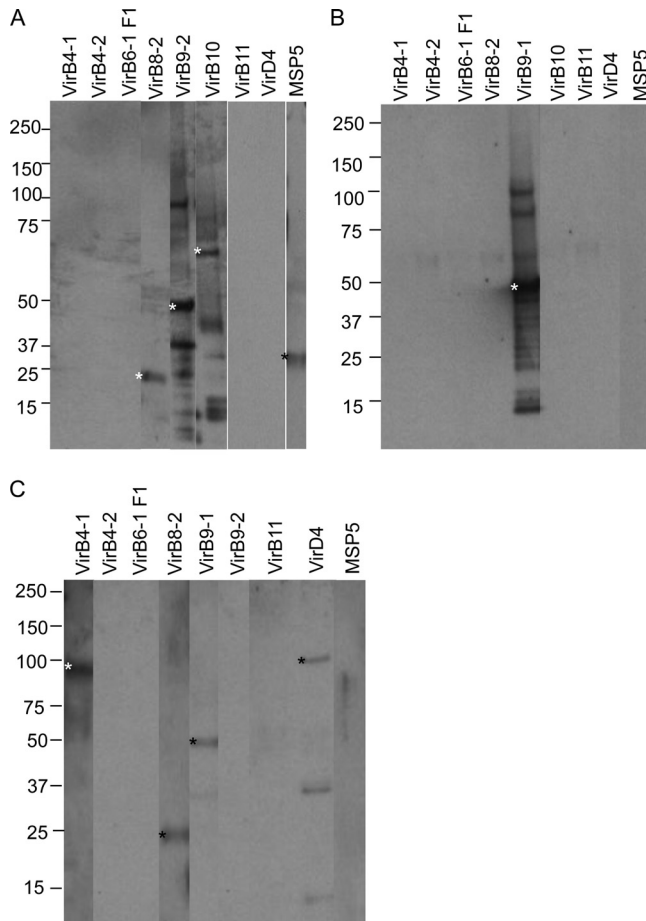


FIG 4 Far-Western blotting to detect interacting recombinant T4SS proteins with specific targets. The indicated purified recombinant T4SS proteins (1 μ g) were separated on 4 to 20% gradient SDS-PAGE gels, transferred to a nitrocellulose membrane, and incubated with 10 μ g of target protein rVirB9-1 (A), rVirB9-2 (B), and rVirB10 (C). The interactions were detected with *E. coli*-adsorbed rabbit antisera (1:500) specific for VirB9-1, VirB9-2, and VirB10. Blots were developed with goat anti-rabbit IgG(H+L). Scanned images were rearranged and presented as shown. In panel A, the results for VirB11 and VirD4 were obtained from a separate experiment. An asterisk indicates an interacting protein partner to the target protein with the following predicted molecular masses: rVirB4-1 (90 kDa), rVirB4-2 (91 kDa), rVirB6-1 F1 (90 kDa), rVirB8-2 (23 kDa), rVirB9-1 (46 kDa), rVirB9-2 (46 kDa), rVirB10 (65 kDa), rVirB11 (38 kDa), rVirD4 (93 kDa), and MSP5 (28 kDa).

VirB9-2 immunoprecipitations, bands of the predicted size for VirB9-1 (29.7 kDa) and for VirB9-2 (30.6 kDa) were detected in both the IPs and the OM (Fig. 6A, lanes 1 and 6, and Fig. 6B, lanes 2 and 6, respectively). However, an additional band at \sim 60 kDa was also detected with these antibodies. The 60-kDa bands could represent homodimers of these proteins, a heterodimer of VirB9-1 and VirB9-2, or binding to another 30-kDa protein. VirB9-2 (Fig. 6B, lane 1), VirB10 (Fig. 6C, lane 1), and 19-kDa MSP5 (Fig. 6D, lane 1) were also detected in the VirB9-1 IP, but the \sim 37-kDa MSP2 was not (Fig. 6E, lane 1). The anti-VirB9-2 IP probed with anti-VirB9-1 IgG also showed \sim 30- and \sim 60-kDa bands (Fig. 6A, lane 2). VirB10 (Fig. 6C, lane 2), MSP5 (Fig. 6D, lane 2), and MSP2 (Fig. 6E, lane 2) were not detected in the anti-VirB9-2 IP. For the anti-VirB10 IP, a band with the predicted mass for VirB10 (49 kDa) was detected, along with an additional band

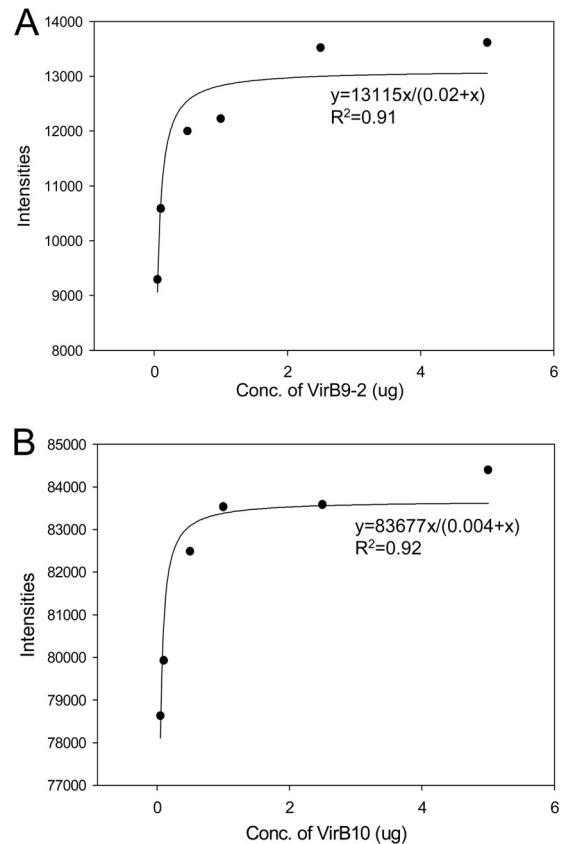


FIG 5 Single site saturation binding curves for the interactions of rVirB9-1 with rVirB9-2 and rVirB10. Densitometry analysis was performed to evaluate the interactions of rVirB9-1 with rVirB9-2 (A) and rVirB9-1 with rVirB10 (B) that were applied to dot blots at amounts up to 5 μ g, allowed to interact with 10 μ g of rVirB9-1, probed with rabbit anti-VirB9-1 antiserum, and developed. Reactions were compared to the density of the reaction of rVirB9-1 to negative control rMSA1. The data are graphically represented as concentration of the protein on the spot versus spot intensity, and the curve was fit to a single site binding curve to yield the binding association constant. The graphs are representative of three independent experiments.

at \sim 100 kDa (Fig. 6C, lanes 3 and 6). This 100-kDa band could be a homodimer of VirB10 or an interaction of VirB10 with another protein(s). VirB9-1 was detected in this IP (Fig. 6A, lane 3), but VirB9-2 (Fig. 6B, lane 3), MSP5 (Fig. 6D, lane 3), and MSP2 (Fig. 6E, lane 3) were not. Immunoprecipitation reactions were also alkylated to prevent reformation of disulfide bonds, electrophoresed, and probed with anti-VirB9-1, anti-VirB9-2, anti-VirB10, and anti-ACP. However, reduction and alkylation had little effect on the interactions or apparent homodimerization (see Fig. S5 in the supplemental material), suggesting that the observed T4SS interactions are not formed with disulfide linkages. The negative control rabbit anti-ACP IgG immunoprecipitation did not pull down any T4SS protein, MSP5, or MSP2 (Fig. 6A to E, lane 4).

To confirm the interaction of native VirB9-1 with MSP5, immunoprecipitation was performed with MAb against MSP5, and it was determined that MSP5 does interact with VirB9-1 (Fig. 6A, lane 5), but not with VirB9-2 or VirB10 (Fig. 6B and C, lane 5, respectively). Furthermore, the highly abundant MSP2 (34) was never identified in the T4SS IPs (Fig. 6E, lanes 1 to 5), supporting the specificity of the detected T4SS interactions. Taken together,

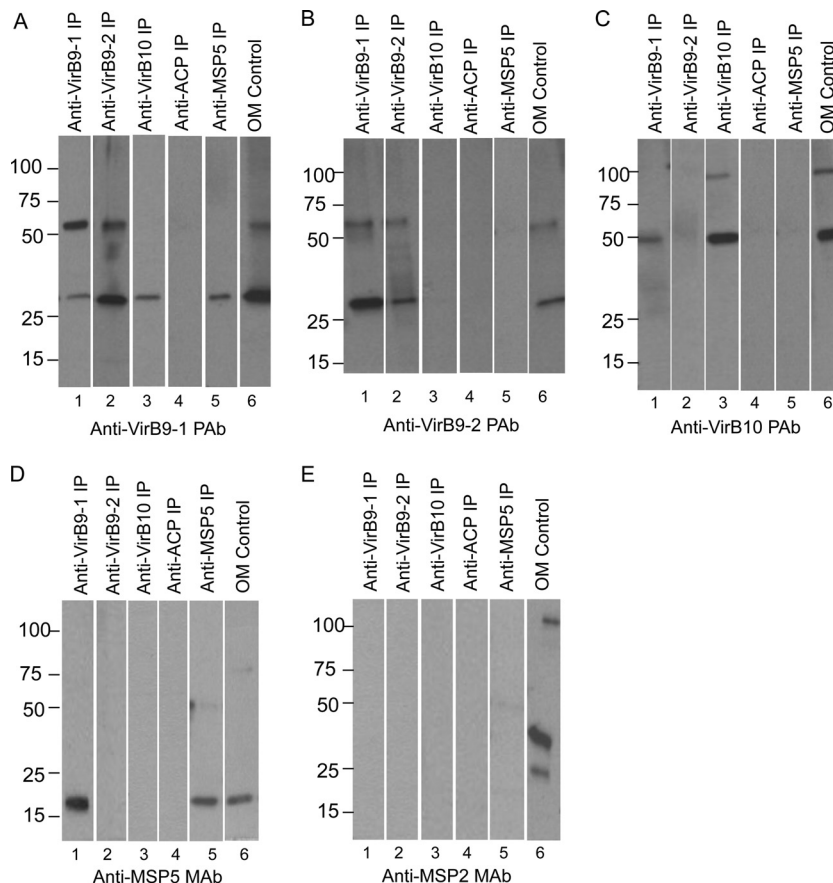


FIG 6 Detection of native protein interactions of VirB9-1, VirB9-2, and VirB10. Solubilized *A. marginale* OM proteins were immunoprecipitated with purified rabbit IgG against VirB9-1 (lane 1), VirB9-2 (lane 2), VirB10 (lane 3), negative control *B. bovis* ACP (lane 4), and an MAb specific for MSP5 (lane 5) as indicated for each lane. Solubilized OM were included (lane 6) for a size comparison. Immunoprecipitated pellets and OM were electrophoresed and transferred to nitrocellulose membranes. Detection of the interacting proteins was performed by Western blotting individual strips with purified rabbit IgG against VirB9-1 (A), VirB9-2 (B), and VirB10 (C) and MAbs specific for MSP5 (D) and MSP2 (E), as indicated under each panel. The secondary antibody was Clear Blot-HRP (panels A to D and panel E, lane 5) or goat anti-mouse IgG+IgM (panel E, lanes 1 to 4 and 6). Scanned images were rearranged and presented as shown. The predicted molecular masses for native proteins are indicated in parentheses: VirB9-1 (30 kDa), VirB9-2 (30 kDa), VirB10 (49 kDa), *B. bovis* ACP (15 kDa), MSP5 (19 kDa), and MSP2 (37 kDa).

our results show that VirB9-1 binds to VirB9-2, VirB10, and MSP5, VirB9-2 only interacts with VirB9-1, VirB10 only interacts with VirB9-1, and native MSP5 interacts only with VirB9-1.

An additional confirmation to detect interactions was performed by following the same immunoprecipitation procedure with rabbit anti-VirB9-1 IgG but using MAbs against VirB9-1, VirB9-2, VirB10, MSP2, MSP5, and MSA1 to probe the Western blot (Fig. 7). The anti-VirB9-1 IP contained 30-kDa VirB9-1, 30-kDa VirB9-2, 49-kDa VirB10, and 19-kDa MSP5 but not negative control MSP2 or *B. bovis* MSA1 (Fig. 7A). There was no antibody reactivity with IPs using preimmune rabbit IgG (Fig. 7B). This result confirms that VirB9-1 interacts with VirB9-2, VirB10 and MSP5.

Comparison of amino acid sequences for VirB9-1, VirB9-2, and VirB10 among *A. marginale* and *A. centrale* strains. Predicted amino acid sequence alignments revealed that VirB9-1, VirB9-2, and VirB10 proteins are highly conserved across *A. marginale* strains. For VirB9-1 the amino acid identity is 97 to 100% (with the exception of the Mississippi strain for which the VirB9-1 sequence is incomplete), and for VirB9-2 and VirB10 the sequence identity is 100% (see Fig. S6 in the supplemental material). Fur-

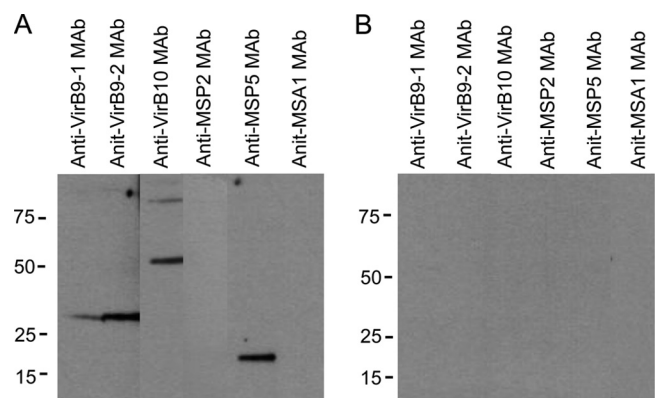


FIG 7 MAb detection of interacting T4SS proteins immunoprecipitated by rabbit anti-VirB9-1 IgG. Solubilized OM proteins were immunoprecipitated with rabbit anti-VirB9-1 (A) or normal rabbit (B) IgG, and the pellets were electrophoresed and transferred to nitrocellulose membranes. Strips were cut and probed with MAbs specific for VirB9-1, VirB9-2, VirB10, MSP2, MSP5, and *B. bovis* MSA1, as indicated at the top of each lane. The secondary antibody was goat anti-mouse IgG+IgM. Scanned images were rearranged and are presented as shown. The predicted molecular masses for native proteins are indicated in parentheses: VirB9-1 (30 kDa), VirB9-2 (30 kDa), VirB10 (49 kDa), MSP2 (37 kDa), MSP5 (19 kDa), and *B. bovis* MSA1 (42 kDa).

thermore, comparison of sequence identities of these proteins to those of the vaccine strain, *A. centrale*, also revealed a high degree of conservation, which was 98 to 99% identity for VirB9-1 with all *A. marginale* strains (except Mississippi), 92% for VirB9-2, and 93% for VirB10 for all *A. marginale* strains (see Fig. S6 in the supplemental material). Because of this high degree of amino acid sequence identity, these T4SS proteins would be good candidates for inclusion in a cross-protective vaccine.

DISCUSSION

This study sought to identify *A. marginale* T4SS protein candidates for linked recognition that could be incorporated into a vaccine. Linked recognition is a well-known rationale for designing vaccines against pathogens with capsular polysaccharide antigens, which on their own are poorly antigenic. Examples include vaccines for *Haemophilus influenzae* and *Streptococcus pneumoniae*, where T-dependent protein antigens are linked to the T-independent polysaccharide to achieve neutralizing antibody responses directed against the polysaccharide (26, 52). Immunization studies using two or more associated proteins are also suggestive that linked recognition enhances protection against bacterial pathogens. For example, protective immunity was induced with bacterial membrane protein complexes or fusion proteins of surface exposed type III secretion system proteins of *Yersinia pestis* (6, 23). Association of proteins within the context of the membrane was also important for leptospiral outer membrane porin OmpL1 and lipoprotein LipL41 in protection studies (21).

Consistent with previous results (32–34, 56) VirB9-1, VirB9-2, and VirB10 are highly immunogenic and in the present study were the most immunogenic of the 11 *A. marginale* T4SS proteins examined for cattle with diverse MHC class II haplotypes. We confirmed that cattle with the DRB3 RFLP type 8/23 (animal 583 in the present study and animal 04B91 [33, 56]) have an IgG response, but no T-cell response, to VirB9-1. This suggests that for animals with this haplotype, T-cell help for IgG production is provided through linked recognition by a different, but associated protein. In addition, we have new evidence that linked recognition occurs for cattle with the DRB3 RFLP type 3/16 to produce IgG to VirB9-1 and VirB9-2 (animal 35160), DRB3 RFLP type 16/27 to produce IgG to VirB9-2 and VirB10 (animal 35280), and DRB3 RFLP types 11/22, 22/24, 3/16, 16/27, and 16/22 to produce IgG to VirB7 (animals 35113, 35141, 35160, 35280, and 35287, respectively). All of these proteins are likely to be surface exposed, as predicted by bioinformatics. Furthermore, these proteins were recognized by the immune sera of infected animals (2, 56, 61), and VirB10 was identified in outer membrane complexes following cross-linking with membrane-impermeable cross-linkers (43). Based on the criteria of predicted surface localization and pattern of immune recognition by three animals and haplotypes that had IgG but undetectable T-cell responses to one or two of these proteins, VirB7, VirB9-1, VirB9-2, and VirB10 were designated candidates for linked recognition. We then focused on identifying the interacting protein partners of VirB9 and VirB10 proteins within the T4SS.

Far-Western blotting was used as an initial screening tool to identify potential interacting T4SS proteins. The data suggest that recombinant VirB10 interacts with rVirB4-1, rVirB8-2, VirB9-1, and rVirD4, interactions that have also been observed with homologous proteins of *Agrobacterium tumefaciens* (3, 5, 15). The interactions of rVirB10 with rVirB4-1 and rVirD4, two putative

NTPases, have also been observed for *Helicobacter pylori* and *Rickettsia sibirica* (37, 58) and implicate VirB10 in substrate shuttling (11). The VirB4-1 gene is more conserved across the *Rickettsiales* than VirB4-2, and VirB4-2 has more insertions and deletions, including those in the NTP-binding cleft, relative to other bacteria (19). This is consistent with the observed interaction of VirB10 with VirB4-1 and not with VirB4-2. Recombinant VirB10 and rVirB9-1 both appear to interact with rVirB8-2, indicating that VirB8-2 may also be part of the core complex of the T4SS apparatus (15, 27). However, VirB8-2 was not of particular interest to pursue because it was not antigenic for the OM vaccinees used in the present study. The interaction of rVirB9-1 and rVirB9-2 had not been previously reported, because only bacteria in the family *Anaplasmataceae* are known to express two full-length VirB9 proteins (19, 20). The interaction of rVirB9-1 with rMSP5 is interesting since none of the other proteins interacted with MSP5. However, it is unknown if MSP5 has T-cell epitopes to stimulate T-cell help to VirB9-1 in 3/16 and 8/23 DRB3 RFLP-typed animals. The interactions of rVirB9 and rVirB10 proteins were confirmed for native proteins in solubilized *A. marginale* OM using pulldown assays with polyclonal, monospecific antibodies and detection with both polyclonal and monoclonal antibodies. These interactions and the potential formation of homodimers are consistent with what is known about the *A. tumefaciens* T4SS core complex structure (13, 17). However, this is the first study to report interacting T4SS proteins of the *A. marginale*, as well as the first to document interactions of the VirB9 and VirB10 proteins in the *Anaplasmataceae*.

The immunogenicity of VirB9 and VirB10 proteins may be explained by the lack of surface lipopolysaccharide (LPS) leading to increased surface exposure of these proteins that form the outer cap of the T4SS core complex (7, 17, 19, 20). Although *Rickettsia* spp. synthesize LPS, the *Anaplasmataceae* do not (20). In agreement with our results and the observation that cattle infected with *A. marginale* have antibody to VirB9 and VirB10 proteins (2, 56, 61), dogs infected with *Ehrlichia canis* were also serologically positive for VirB9 (16). Thus, the T4SS VirB9 and VirB10 proteins may generally be good vaccine targets for the *Anaplasmataceae*.

A combination of cellular and humoral immunity is likely important for the control of anaplasmosis, and vaccine antigens should be highly conserved across multiple strains to ensure cross-protective immunity. Furthermore, inclusion in a vaccine of multiple linked proteins that are naturally associated in the membrane of *A. marginale* will not only provide more than one immunogenic protein but will offer the opportunity for linked recognition. Immunization with several naturally associated, linked proteins could increase T-cell-dependent IgG responses upon infection in outbred populations that express a broad repertoire of MHC class II molecules. Protecting a large population of genetically heterogeneous individuals requires understanding of MHC class II-restricted epitope presentation. The naturally associated T4SS protein complex that spans the inner and outer membrane provides a model system to examine the importance of membrane protein-protein interactions in stimulating protective immunity. Thus, a protein complex made up of highly conserved VirB9-1, VirB9-2, and VirB10 will be tested as a vaccine in a future study to protect MHC haplotype-defined cattle against *A. marginale*.

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