THE IMMUNOGENICITY OF THE

TYPE IV SECRETION SYSTEM IN *ANAPLASMA MARGINALE*

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of ERIC L SUTTEN find it satisfactory and recommend that it be accepted.

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Abstract

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Anaplasma marginale is an economically important tick-borne intraerythrocytic rickettsial pathogen of cattle that can cause fever, weight loss and death upon acute infection in naïve hosts. Immunization with isolated outer membrane (OM) fractions elicits protective immunity against infection, whereas immunization with individual proteins from the OM proteome fail to provide similar levels of protection. Studies have shown that type IV secretion system (T4SS) proteins of *Anaplasma marginale* elicit immune responses associated with protective immunity, which consist of IgG_2 antibody production, $CD4^+$ T-cell stimulation, as well as interferon-γ secretion.

The T4SS is a protein complex that is found in many gram-negative bacteria, and is used to actively transport effecteor molecules across the periplasmic space into the host cell. Therefore, initiating a protective response against this protein complex could produce a detrimental effect on bacterial survival and dissemination, making the T4SS a potentially good vaccine candidate.

To enhance understanding of the possible relationships among proteins within the T4SS, monoclonal antibodies (mAb) were developed, and used to determine

expression and localization of the T4SS within *A. marginale* infected ticks by immunohistochemistry. These mAb will be available for future studies designed to determine protein-protein interactions within the T4SS. Furthermore, direct immunological studies were conducted, using a high-throughput method of screening a panel of T4SS proteins expressed by *in vitro* transcription translation with T-cells from OM vaccinees. Antigens that induced significant $CD4^+$ T-cell responses were subsequently expressed as recombinant proteins in *E. coli* to verify the discovery of three novel $CD4^+$ T-cell stimulatory antigens of the T4SS: VirD4, VirB11, and VirB7. Additionally, steps were taken to further characterize a putative T4SS antigen, VirB2. Analysis of VIrB2 included T-cell epitope mapping experiments, as well as the development of multiple bioinformatic comparisons of the protein alignment across bacteria in Anaplasmataceae family. Through these studies, a better understanding of the T4SS was reached, and may help define the functional and immunological role of the T4SS in *A. marginale,* as well as in other gram-negative bacterial pathogens that have a T4SS complex.

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DEDICATION

I dedicate my thesis work to my mom and dad, as well as my sister, brother and their families.

CHAPTER 1

The development of monoclonal antibodies for characterization of the type IV secretion system (T4SS) in *Anaplasma marginale*

ABSTRACT

The outer membrane (OM) of the intraerythrocytic rickettsial pathogen, *A. marginale,* has unique components as an immunogen, providing protective immunity against challenge in bovine hosts. Understanding the protein characteristics within the OM proteome could provide information with regard to subsets of proteins that elicit responses sufficient for protective immunity upon immunization. Studies have shown that linked recognition between B-cell- and T-cell-epitopes on different, but associated, proteins may provide a possible rationale for manipulation of these proteins for vaccine development. However, protein-protein interactions must first be established before studies can focus on linked proteins. Studies have shown that the type IV secretion system (T4SS) of *A. marginale* may possess proteins that undergo linked recognition. To provide supportive evidence of possible proteins that undergo linked recognition, monoclonal antibodies (mAbs) were developed to aid in future protein-protein interaction experiments. In this study, mAb specific for native and recombinant T4SS proteins VirB9-1, VirB9-2, and VirB10 were produced and characterized for future protein complex investigations. Furthermore, the mAbs developed in this study were used in immunohistochemistry assays to localize the *A. marginale* T4SS within acquisition-fed *Dermacentor andersoni.* Preliminary results show that only the mAb

specific for VirB10 reacted with infected *D. andersoni*, and localized to *A. marginale* within the midgut epithelium, but not in the salivary glands.

INTRODUCTION

Anaplasma marginale is an economically important rickettsial pathogen of ruminants that, upon infection of immunologically naïve hosts, can cause anemia, weight loss, and death. Studies have shown that immunization with isolated *A. marginale* outer membranes (OM), or chemically cross linked OM proteins, induces a protective immune response against subsequent challenge (1, 10, 27, 30, 34, 36, 37, 40). Although a large proportion of the OM proteome is composed of a family of major surface proteins (MSP) including the well-characterized MSP1, MSP2, MSP3, MSP4, and MSP5; immunization with several of these individual proteins, (MSP1, MSP2, and MSP3, and MSP4) has resulted in incomplete or no protection against infection (10, 11, 23, 26, 32, 33, 36). Because immunization with individual proteins elicits only limited protection against infection, it is still unknown why the OM fraction can provide complete protection against infection and disease.

One possible mechanism which explains the superior protective capacity of OM in eliciting a protective immune response, compared with individual proteins, was suggested by studies performed recently by Macmillan et al. (25, 26). MSP1 is a naturally occurring heteromeric protein complex composed of MSP1a and MSP1b (25, 43). Immunization of cattle with native heteromeric MSP1 protein resulted in the development of IgG antibody to both proteins of the MSP1 heteromeric complex. However, $CD4^+$ T-cell responses were predominantly directed towards the MSP1a protein component, as T cells from two of three animals did not respond to MSP1b (8, 9). Because IgG class switching requires $CD4^+$ T cell help, this suggested that $CD4^+$ T cells specific for MSP1a were providing help, through linked recognition, to B cells

specific for MSP1b. Macmillan et al. then determined that immunizing with a recombinant fusion protein construct of the MSP1 complex, comprised of only the MSP1a T-cell epitopes and the full length MSP1b protein, elicited a significantly higher IgG antibody titer towards MSP1b, than did immunizing with an equimolar mixture of the two recombinant proteins (26). This study concluded that the protective responses derived from a vaccine could be positively manipulated by ensuring the ability of T-cell help for IgG production through linked recognition of proteins naturally associated in the bacterial OM.

The potential importance of linked recognition was also indicated in experiments performed with another protein complex in the *A. marginale* OM proteome, the type IV secretion system (T4SS). The T4SS is a multiprotein complex required for virulence and intracellular survival in many other gram-negative bacteria, including *Anaplasma phagocytophilum, Ehrlichia chaffeensis*, *Agrobacterium tumefaciens, Helicobacter pylori, Bordetella pertussis*, *Rickettsia* spp., and *Brucella suis.* Studies have shown that the T4SS complex actively transports effecter molecules, such as DNA and proteins, across the bacterial periplasmic space into host cells, making the host cell environment more suitable for bacterial survival and dissemination (3-6, 12-14, 17, 18, 20, 21, 31, 44). Proteins of the T4SS in *A. marginale* were shown to elicit immune responses in cattle either immunized with OM, or naturally infected (2, 22-24). The T4SS virulence (Vir) proteins VirB9-1 [formerly designated conjugal transfer protein (CTP)] VirB9-2 (formerly designated VirB9), and VirB10 of *A. marginale* were shown by Lopez et al. to elicit the production of IgG_2 antibodies in three OM immunized cattle (23). However, comparable to the MSP1a protein of the heteromeric MSP1 complex, one of the animals

tested in the Lopez et al. study lacked VirB9-1-specific $CD4^+$ T-cell responses (23). These additional data support the hypothesis that proteins naturally associated in the bacterial membrane, such as disulfide bonded MSP1a/MSP1b and proteins of the T4SS complex, such as VirB9-1 and other T4SS proteins to which it is naturally associated in the membrane, provide T cell-B cell linked recognition resulting in enhanced IgG responses. The novel observation that several proteins of the T4SS complex are immunogenic also suggests this complex is an excellent candidate for vaccine development.

To identify possible protein-protein interactions in the T4SS complex, and to determine the localization of specific T4SS proteins in *A. marginale*, I produced and screened monoclonal antibodies (mAb) specific for immunoreactive T4SS proteins VirB9-1, VirB9-2, and VirB10 (22-24), for use in immunoprecipitation assays and immunohistochemistry and immuno-electron microscopy studies. Although the mAb have not yet been used in immunoprecipitation or immuno-electron microscopy experiments, preliminary immunohistochemistry experiments have yielded some novel findings.

MATERIALS AND METHODS

Purification of type IV secretion system recombinant proteins. Recombinant (r)VirB9-1, rVirB9-2, and rVirB10 were expressed and purified as previously described (24). Briefly, the *virb9-1, virb9-2, and virb10* genes were amplified by PCR from *A. marginale* genomic DNA using primers specific for each gene (24). Each amplicon was constructed to contain a C-terminal FLAG epitope (DYKDDDDK) to permit affinity

purification with tag-specific mAb. All gene constructs were inserted into pBAD/TOPO ThioFusion plasmid under the manufacturer's specifications (Invitrogen). Plasmid DNA was then transformed into TOP 10 One Shot, chemically competent *E. coli* with the manufacturer's specifications (Invitrogen). Transformed *E. coli* containing the construct were then plated on LBC agar and grown overnight at 37^{o}C. Subsequent colonies were chosen and grown in 3 ml LBC broth overnight, harvested, and plasmid DNA was purified using Wizard Plus SV Minipreps DNA purification system. Each recombinant gene construct was sequenced by PCR using Big Dye kits and manufacturer supplied vector forward and reverse primers following the manufacturer's specifications (Invitrogen). Clones possessing the correct sequence of *virb9-1, virb9-2,* and *virb10* were grown in 1 ml LBC broth overnight and frozen in 50% glycerol-LBC broth. The glycerol stocks of *E. coli* containing each gene construct were used to inoculate individual 20 ml LBC broth cultures and grown overnight, then subsequently inoculated into a 400 ml LBC broth culture. After cell growth reached an OD of 0.5 at 600 nm, each batch of *E. coli* was induced with 0.2% arabinose and allowed to grow for 5 hr, after which it was harvested via centrifugation at 4,000 x *g* for 20 min, and frozen at $^{-}$20^{o}C. Recombinant *E. coli* were subjected to the Probond Purification system (Invitrogen) according to the manufacturer's specifications. A secondary immunoaffinity purification of each recombinant antigen was performed using anti-FLAG mAb, as previously described (24). The purity of each recombinant protein was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie brilliant blue staining and western blot analysis. One SDS-PAGE gel was stained using Coomassie brilliant blue stain, and a second was transferred to a nitrocellulose

membrane and blocked overnight for immunoblot analysis in I-Block (Applied Biosystems, Bedford MA) diluted to 0.2% in phosphate buffered saline (PBS) with 0.5% Tween-20, referred to as blocking reagent. The nitrocellulose membrane was probed with either mouse anti-FLAG mAb (Sigma-Aldrich) or mouse anti-Penta His mAb (Qiagen), both at a 1:10,000 dilution in blocking reagent and incubated for 1 hr. Subsequent probing was performed with alkaline phosphatase conjugated goat anti-mouse IgG antibody (Applied Biosystems), diluted at 1:10,000 in blocking reagent and incubated for 1 hr. Nitrocellulose membranes were then washed with blocking reagent for 1 hr, and developed with the Western-Star chemiluminescent immunoblot detection system (Applied Biosystems) (23). The proteins were dialyzed against PBS, pH 7.0, using Slide-A-Lyzer Dialysis Cassettes with a 10 kDa molecular weight cutoff. The concentration of each protein was determined using a Bradford assay (Bio-Rad), and the protein was then frozen at $^{-}$20ºC.

Immunization of mice with rVirB9-1, rVirB9-2, and rVirB10. Two female BALB/c mice were immunized subcutaneously with 20 µg of each protein emulsified in an equal volume of complete Freund's adjuvant for a total volume of 150 µl. Mice received 3 booster immunizations at 2-week intervals with 20 µg antigen in incomplete Freund's adjuvant. A final immunization with 10 µg protein in a total volume of 100 µl PBS was given intravenously, and mice were euthanized 3 days later. Animals were used in this study in compliance with The Washington State University Institutional Animal Care and Use Committee.

Immunoblot analysis of murine immune sera. To detect humoral responses, western blotting was performed using both pre- and post-immunization mouse sera.

Sera were acquired from mice before each immunization, and allowed to coagulate before spinning at maximum speed for 15 min. Recombinant VirB9-1, rVirB9-2, and rVirB10 (0.5 µg- 1.5 µg per well) were boiled in SDS-PAGE sample buffer for 5 min, then separated by SDS-PAGE in a 10-20% Tris precast mini gel (Bio-Rad). Isolated *A. marginale* OM (100 µg) and uninfected red blood cells (URBC, 30 µg), were also separated by SDS-PAGE in a similar fashion. Gels were then transferred to nitrocellulose membranes and incubated with blocking reagent. Transferred membranes were cut into strips or kept whole, then incubated for 1 hr with pre-immune sera or sera obtained two weeks after the third immunization and diluted to 1:200 in blocking reagent. Following incubation for 1 hr, blots were washed with blocking reagent, and subsequently incubated for 1 hr with alkaline phosphatase labeled goat anti-mouse IgG antibody diluted 1:10,000 in blocking reagent. Antibody binding was detected by the Western-Star chemiluminescent immunoblot detection system (Applied Biosystems).

Hybridoma culture and mAb screening by enzyme-linked immunosorbent assays (ELISA). Myeloma-plasma cell (hybridoma) fusions and cell cultures were performed as previously described (19, 28). Supernatants (sups) of parent hybridomas, and single, and doubly cloned hybridomas were tested by ELISA using the immunizing recombinant protein and one irrelevant recombinant protein to serve as a negative control to rule out mAb with specificity for the epitope tags (6x His and FLAG). To perform the ELISAs, recombinant proteins were diluted 2 ng/µl in sodium carbonate as a coating buffer (7.5 mM Na_2CO_3, 17 mM $NaHCO_3$), plated at 50 µl per well on Immulon 96 well plates (Thermo Fisher Scientific) and incubated overnight at 4ºC. Plates were then washed 4 times with 50 µl PBS containing 0.5% Tween-20 detergent (Bio-Rad),

and blocked for 1 hr in blocking reagent. Fifty µl of undiluted hybridoma supernatants were incubated for 30 min in duplicate wells containing either recombinant protein of interest, or a negative control recombinant protein (four wells total). The plates were then washed 4 times with 50 µl PBS containing 0.5% Tween-20. Secondary antibody, horse radish peroxidase (HRP) labeled goat-anti-mouse-immunoglobulin (KPL Inc., Gaithersburg, Maryland) was diluted to 1:5,000 in blocking reagent and 50 µl were added to each well, then incubated for 30 min. The plates were then washed a final 4 times with 50 µl PBS containing 0.5% Tween-20 detergent and developed with 100 µl SureBlue TMB peroxidase substrate (KPL) for 15 min, followed by an addition of 100 µl 1% HCl stop solution. Plates were read on a plate reader with wavelength absorbance of 450 nm. Concentrations and isotypes of positive mAb were determined by Single Radial ImmunoDiffusion assay kits following the manufacturer's specifications (The Binding Site Inc., San Diego, CA).

Hybridoma screening using immunoblotting analysis. Hybridoma supernatants that repeatedly tested positive by ELISA were then used for detection of recombinant and native protein by Western blotting. Isolated *A. marginale* OM served as a source of native protein, and URBC as a negative control antigen. SDS-PAGE was performed as described above, but with modifications to the gel, in which wells were removed to create one to two, large prep-wells. Recombinant proteins (0.5-2 µg/well), and OM and URBC (8-30 µg/well) were separated by SDS-PAGE and transferred to nitrocellulose membranes, which were cut into strips. Strips were incubated with hybridoma supernatants diluted to 1:10 in blocking reagent, washed, then incubated with alkaline phosphatase labeled goat-anti-mouse IgG antibody diluted 1:10,000 in

blocking reagent. Additional strips were also incubated with anti-FLAG mAb (1:10,000) or sera obtained two weeks after the fourth immunization from *A. marginale* OM immunized cattle (22-24). Anti-FLAG mAb was detected with alkaline phosphatase labeled goat-anti-mouse IgG antibody diluted to 1:10,000 in blocking reagent, and reactive bovine serum was detected with horseradish peroxidase labeled goat anti-bovine IgG antibody.

Detection of T4SS proteins in *A. marginale*-infected ticks by immunohistochemistry. Immunohistochemistry was performed on *A. marginale* St. Maries strain infected *Dermacentor andersoni.* Methodology and derivation of tick serial cross-sections were performed as previously described (41). Briefly, ticks that were acquisition fed for 7 days on *A. marginale* St. Maries strain infected cattle, and incubated at 26ºC for an additional 7 days, were fixed in 10% formalin and embedded in paraffin. Sections 4 µm thick were de-paraffinized in Clear-Rite, and hydrated in an ethanol gradient ranging from 100% EtOH to 70% EtOH, followed by double deionized H_20. Each section was then treated for antigen retrieval with Citrate Solution (Zymed, Carlsbad, CA). Treatment in Citrate Solution was performed in a steamer for 20 min (41). Immuno-staining of the sections was then performed using the Dako Envision IHC kit, which uses a horseradish peroxidase labeled secondary mAb specific for mouse IgG for detection of bound antibody (Dako, Carpinteria, CA). mAb against T4SS proteins were used at 5 µg/ml, and were assigned the following designations: anti-VirB10 (F138/481.3.9, IgG_1), anti-VirB9-1 (F133/248.14.1, IgG_1), and anti-VirB9-2 (F137/774.8.7, IgG_2). The positive control mAb (ANAR49 IgG_1) was used at 5 µg/ml, and has been previously characterized to specifically recognize *A. marginale* (39, 41).

Photographs were obtained using a DS-Fi1-L2 camera at 40X magnification (Nikon, Melville, NY)

RESULTS

Antibody responses in mice immunized with T4SS proteins. Seroconversion of mice immunized with recombinant T4SS proteins was determined by western blot analysis. The recombinant antigens used to immunize the mice (rVirB10, rVirB9-2, and rVirB9-1), as well as OM as the source of native protein, were used to determine serological specificity. Serum from a mouse immunized against rVirB10 reacted to the native protein within isolated OM, resulting in a band with a molecular weight of approximately 50 KDa, but did not react with the negative control URBC (Fig.1A, lanes 3 and 4). This serum also reacted with rVirB10, yielding a molecular weight of approximately 65 KDa, and no band was visible when the recombinant protein was probed with pre-immune serum from this animal (Fig. 1A, lanes 1 and 2). Similarly, sera from mice immunized with either rVirB9-2 or rVirB9-1 showed reactivity towards the respective native protein, both producing bands with molecular weights of approximately 30 kDa (FIG. 1B and C, lanes 7 and 11). The immune sera of these mice did not react with URBC (FIG. 1B and C, lanes 8 and 12), but did react specifically with each respective recombinant protein (Fig. 1B and C, lanes 5 and 9). Pre-immune sera from these mice did not react with recombinant protein (Fig. 1B and C, lanes 6 and 10). The molecular weights of the recombinant protein bands are consistent with published data using immune bovine sera (23), and the molecular weights of the native proteins in *A.*

marginale OM detected by the mAb are also consistent with the predicted molecular weights based on predicted amino acid sequences.

Identification of hybridomas specific for VirB9-1, VirB9-2, and VirB10. Western blot analysis was performed to determine the reactivity of once-cloned hybridoma supernatants with respective native protein or recombinant antigen. Numerous supernatants were screened throughout this study, and only a selected group are described here (Fig. 2-4). Of three hybridoma supernatants developed against rVirB9-1, two (no. 14 and 2) reacted with recombinant and native VirB9-1 (Fig. 2A and 2C, I,). Of nine hybridoma supernatants developed against rVirB9-2, four (no. 123, 701, 1089, and 774) reacted with recombinant and native VirB9-2 (Fig. 2B and 2C, II). The antibodies within the supernatants bound their respective native (30 kDa), and recombinant proteins (46 kDa) of the predicted size. Specificity was demonstrated by lack of reactivity with negative control recombinant protein (Fig. 2A and 2B) or URBC (Fig. 2D). Of five hybridoma supernatants developed against rVirB10, only one secreted antibody specific for rVirB10 (65 KDa) and native VirB10 (50 KDa) (FIG. 3A, II). Specificity of this hybridoma supernatant was similarly demonstrated (Fig. 3B, II).

Candidate hybridoma parent clones specific for rVirB9-1, rVirB9-2, and rVirB10 were cryopreserved. To test twice-cloned hybridomas, immunoblot analysis was again performed (Fig. 4). When testing anti-VirB9-2 (mAb F137/774.8.7), and anti-VirB9-1 (mAb F133/248.14.1) against purified OM, bands of ~30 kDa were observed for both mAb (FIG. 4. lanes 7 and 8). Similarly, specific bands of ~46 KDa for both mAbs were observed when probed against respective recombinant proteins (Fig. 4. lanes 1 and 3).

Furthermore, neither of these mAb reacted against negative control recombinant protein or URBC (FIG. 4. lanes 2, 4, 10 and 11), documenting specificity.

The twice-cloned hybridoma specific for VirB10 (mAb F138/481.3.9) reacted with a native protein band of ~50 kDa when probed against OM (Fig. 4, lane 9). This mAb also reacted with rVirB10, producing a band of ~65 kDa (Fig. 4, lane 5). The anti-VirB10 mAb did not react with negative control recombinant protein (Fig. 4, lane 6) or with URBC (Fig. 4, lane 12), documenting specificity.

Localization of T4SS proteins in tick tissues by immunohistochemistry. A preliminary experiment demonstrated mAb reactivity to *A. marginale* St. Maries strain within acquisition-fed *Dermacentor andersoni.* Ticks imbedded in paraffin were sectioned and immuno-stained with mAb specific for the VirB9-1, VirB9-2, and VirB10 proteins of the *A. marginale* shown to react to native antigen on immunoblots. Of the three mAb specific for T4SS proteins, only anti-VirB10 mAb was positive. This mAb reacted with organisms in the midgut epithelium, but not within infected salivary glands (Fig. 5A). Furthermore, positive control mAb with reactivity for the St. Maries strain of *A. marginale* produced positive results both within the midgut as well as the salivary glands of serial cross-sections (Fig. 5D). Neither the anti-VirB9-2 nor the anti-VirB9-1 mAb reacted to *A. marginale* in the tick tissues (Fig. 5B and C). Monoclonal antibody against VirB9-1 is the same isotype as anti-VirB10 (IgG_1); therefore it was considered sufficient as a negative control for this experiment.

Discussion

Bacterial OM proteomes contain a large number of protein complexes, which are used for various biological functions. Fully understanding the protein-protein interactions within the protective bacterial OM proteome of *A. marginale,* and potentially other bacterial pathogens where OMs induce protective immunity, could facilitate development of subunit vaccines. Multiprotein complexes*,* such as the T4SS, are structurally conserved and required for virulence and intracellular survival for many pathogenic gram-negative bacteria. Such protein complexes within the OM also interface with the host target cells of invasion and with the immune system. Thus, protein complexes such as the T4SS present logical targets for immune recognition and intervention. Linked recognition occurs when T-cell and B-cell epitopes, present on the same protein or on two or more proteins that physically interact, are recognized by their respective lymphocytes which are brought together during antigen recognition so that antigen-specific T cells can effectively provide help to antigen-specific B cells. Therefore, proteins that lack T-cell epitopes for a given individual MHC haplotype, may contain B-cell epitopes permitting antigen recognition, internalization, and presentation of processed peptides to T cells on MHC molecules. However, T cell help is required in the form of cell signals and cytokines produced by the T cells to effectively induce isotype switching in B cells to high affinity IgG isotypes. Isotype switching to IgG rarely occurs with most protein antigens in the absence of specific T cell recognition, but the T cell epitopes can be provided by a different protein that is physically associated with the B cell antigen, so that it too gets internalized by the B cell, processed, and presented to T cells for the antigen recognition required to activate T cell cytokine production. Linked

recognition has been previously observed between the MSP1a and MSP1b components of the MSP1 heteromeric protein complex in the *A. marginale* OM (26). Furthermore, linked recognition likely explains high titers of IgG production, but a lack of T-cell recognition, for the VirB9-1 T4SS protein of *A. marginale* observed for one OM vaccinated animal (23).

In this study we were successful in developing mAbs to three proteins of the T4SS: VirB9-1, VirB9-2, and VirB10. Although immunoprecipitation assays have not yet been performed utilizing these mAb, such studies in the future could yield important data that will aid our understanding of protein-protein interactions within the T4SS of *A. marginale.* Understanding protein-protein interactions may provide information with regard to proteins that are involved in linked recognition and those that may possess immunological significance in relation to linked protein vaccines. For example, identifying the protein(s) associated with VirB9-1 within the T4SS that results in high affinity IgG specific for VirB9-1 can now be undertaken using the VirB9-1-specific mAb. This protein complex may be useful as a vaccine to block the function of the T4SS and potentially inhibit bacterial replication. As *A. marginale* T4SS proteins are conserved within other rickettsial pathogens (23), information regarding protein-protein interactions within the T4SS of *A. marginale* will be directly applicable to understanding the structure of the T4SS in related rickettsial pathogens, and potentially, be informative as well for vaccine development.

Another area of investigation with the TFSS-specific mAb is the determination of expression of the T4SS during different life cycle stages of the bacterium. Previous studies have shown that these three T4SS proteins are expressed by *A. marginale* in

the infected erythrocyte and elicit an immune response within the bovine host (2, 23, 24, 30). However, the functional relevance of having a T4SS with erythrocytes is not clear, whereas *A. marginale* which infects nucleated midgut epithelial and salivary gland acinar cells within the tick vector might be expected to express a functional T4SS. Studies focusing on MSP2 and MSP3 of *A. marginale* have shown differences in the repertoire of MSP2 proteins expressed by isolates of *A. marginale* within the tick. MSP2 possesses plasticity due to antigenic variation upon infection of the bovine host, which, because of immune pressure, results in selection of different antigenic variants over time (7, 16, 35). Variation in MSP2 is a direct result of gene recombination of the numerous *msp2* pseudogenes into the gene's single *msp2* expression site. Initially, after infection, simple MSP2 variants of *A. marginale* are observed, but upon persistent infection complex variants develop due to constant immune pressure in the host (7, 16, 35). However, ticks that acquired infection of complex variants eventually transmit bacteria that are comprised of only simple MSP2 variant (16, 35, 38). Based on these observations, it is possible that there are factors within the tick vector that create a selective environment for the pathogen, thus affecting the type of *A. marginale* MSP2 variants that infect the bovine host. Environmental factors present within the tick vector could directly regulate the expression or utilization of the T4SS as well. However, little is known about the specific interactions that take place between *A. marginale* and the tick during transmission (15, 41, 42). The well-established importance of the T4SS in other gram-negative bacteria, suggests a potential functional significance of the *A. marginale* T4SS within the tick vector. Further information on the presence of these proteins within *A. marginale* infected ticks could also establish if vaccine development focused on the

T4SS might neutralize the pathogen upon transmission to the bovine host before erythrocyte infection.

Preliminary characterization of the expression of VirB9-1 VirB9-2, and VirB10 within *A. marginale* infected*,* acquisition-fed *Dermacentor andersoni* ticks identified VirB10 within the tick midgut epithelium. Although midgut epithelium did not react with the VirB9-1 and VirB9-2-specific mAb by immunohistochemistry, the sample size was small, and optimization of the mAb staining techniques for immunohistochemistry may be needed to improve detection. Lack of detection of any of the three proteins within salivary glands could also be due to inefficient detection, or could be a real finding. Differential expression of T4SS proteins VirB6 and VirB9 was observed during neutrophil or HL-60 cell infection by *A. phagocytophilum* (29).

Understanding the localization and regulation of expression of the proteins that form T4SS complex in *A. marginale,* as well as other rickettsial pathogens could establish a paradigm of bacterial virulence determinants and lead to preventing infection, if the TFSS proteins prove useful as a vaccine.

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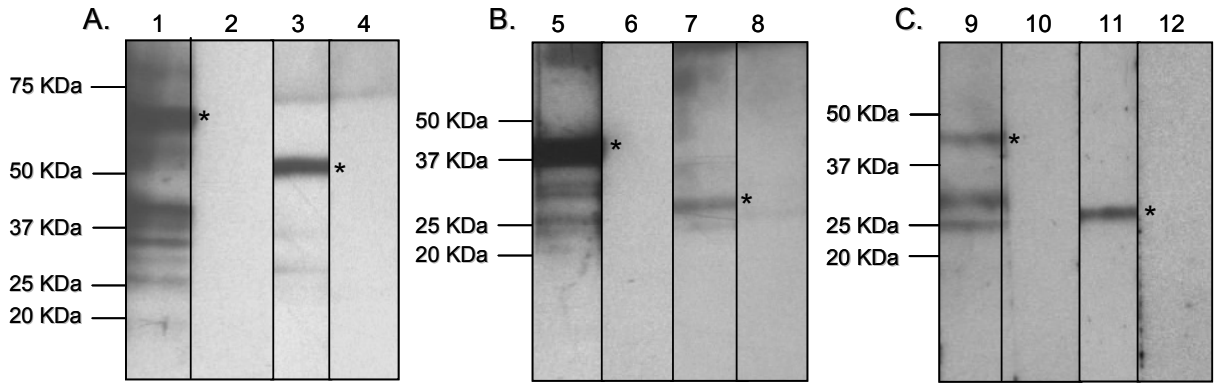

FIG. 1. Seroconversion of mice immunized with recombinant T4SS proteins. Proteins were separated by SDS-PAGE then transferred to nitrocellulose membranes. Lanes contained 1.0 µg rVirB10 (lanes 1 and 2), 1.5 µg rVirB9-2 (lanes 5 and 6), 0.5 µg rVirB9-1 (lanes 9 and 10), 100 µg OM (lanes 3, 7, and 11), or 30 µg URBC (lanes 4, 8, and 12). Blots were probed with mouse sera obtained two weeks after the third immunization (lanes 1, 3, 4, 5, 7, 8, 9, 11 and 12) or pre-immunization sera (lanes 2, 6, and 10) from mice that were immunized with either rVirB10 (A), rVirB9-2 (B), or rVirB9-1 (C). Binding was detected with alkaline phosphatase labeled goat anti-mouse IgG antibody. Asterisks mark the bands correlating to the predicted molecular weight of the proteins. Molecular weight markers are indicated in kDa.

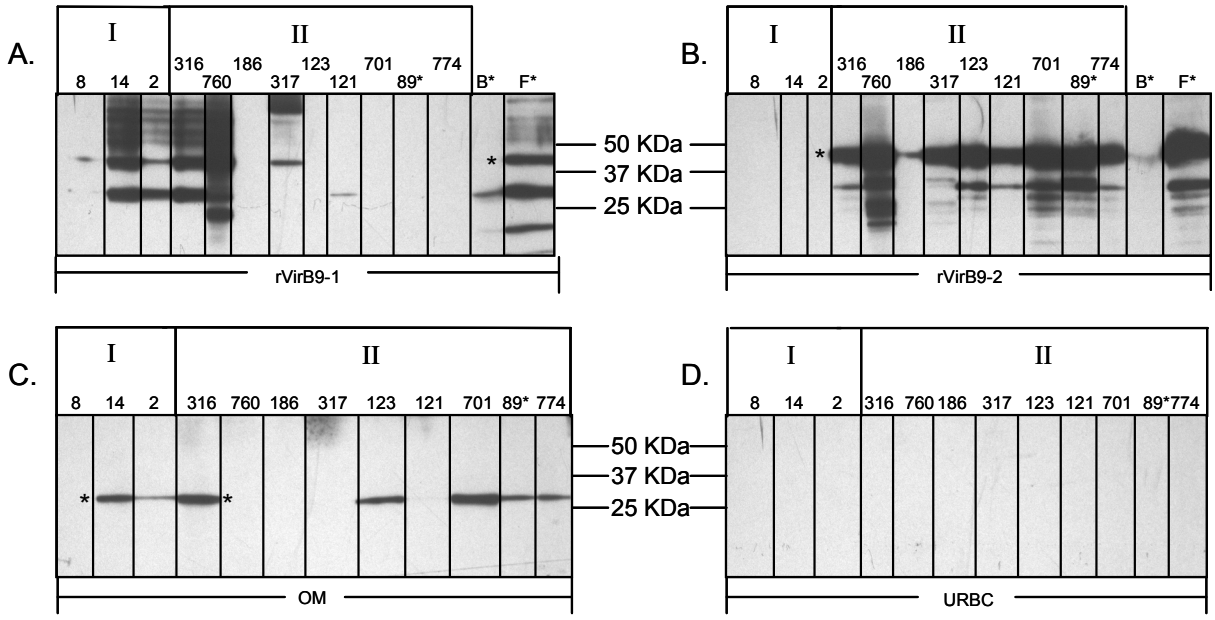

Fig. 2. Immunoblot analysis of antibody specific for VirB9-1 or VirB9-2 within the supernatants of initial and secondary clones from hybridoma fusion (F)133 and F137, respectively. Five hundred ng rVirB9-1 (A) and rVirB9-2 (B), or 30 µg OM (C), and URBC (D), were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were cut into strips and incubated with secondary clone hybridoma (F133/248.x) supernatants developed against rVirB9-1 (I) or with primary hybridoma supernatants developed against rVirB9-2 (II, 89* actual clone number is 1089) diluted 1:10, serum from an OM immunized bovine (B*) diluted 1:200, or anti-FLAG mAb (F*) diluted 1:10,000. Binding was detected with alkaline phosphatase labeled goat anti-mouse IgG antibody or horseradish peroxidase labeled anti-bovine IgG. Asterisks indicate bands of predicted molecular weights. Molecular weight markers are indicated in kDa.

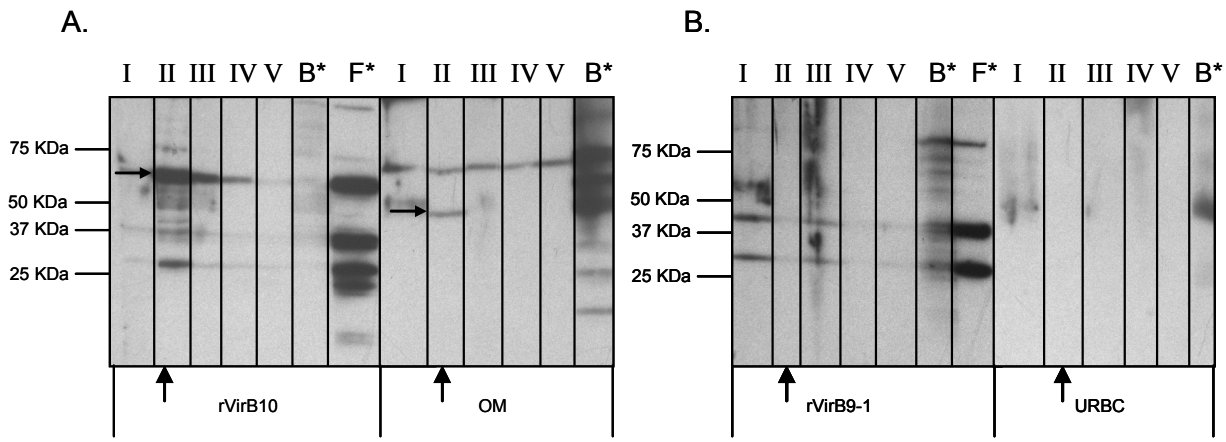

FIG. 3. Immunoblot analysis of antibody specific for VirB10 within the supernatants of parent, primary, and secondary hybridoma clones from fusion (F)138. One µg rVirB10 (A) and rVirB9-1 (B), or 25 µg OM (A) URBC (B), were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were cut into strips and incubated with hybridoma supernatant (1:10): I (504.4.4), II (481.4), III (515), IV (332.4), V (138), serum from an OM immunized bovine (B*) diluted 1:200, or anti-FLAG mAb (F*) diluted 1:10,000. Antibody binding was detected with alkaline phosphatase labeled goat anti-mouse IgG antibody or HRP labeled goat anti-bovine IgG antibody. Arrow shows a hybridoma that recognizes rVirB10 and native OM, but not rVirB9-1 or URBC. Large arrows indicate the lanes of F138/481.4, and small arrows indicate the predicted size of the protein. Molecular weight markers are indicated in kDa.

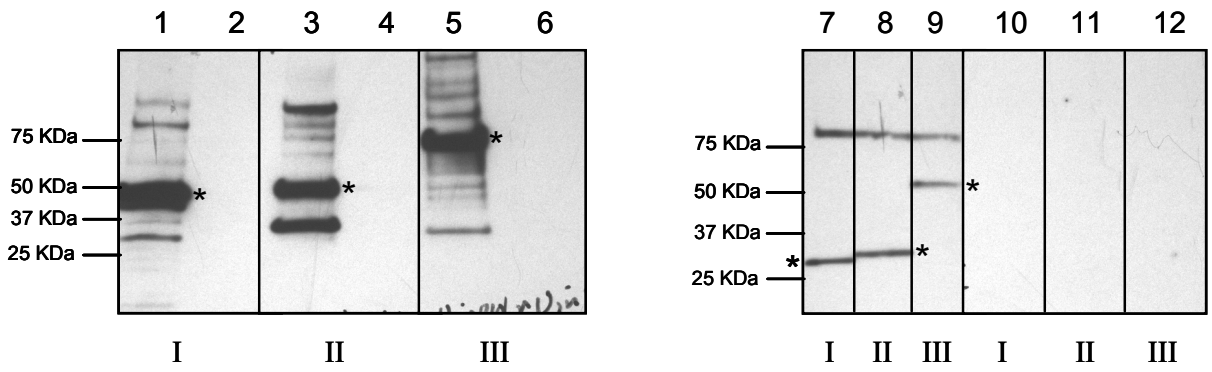

FIG. 4. Immunoblot analysis of mAb specific for VirB9-1, VirB9-2, and VirB10. Five hundred ng rVirB9-2 (lanes 1,4,6), rVirB9-1 (lanes 2,3), rVirB10 (lane 5), and 30 µg OM (lanes 7,8,9) and URBC (lanes 10,11,12) were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were cut into sections (I, II, III), and incubated with: anti-VirB9-2 (I, F137/774.8.7), anti-VirB9-1 (II, F133/248.14.1), or anti-VirB10 (III, 138/481.3.9). Antibody binding was detected with alkaline phosphatase labeled goat anti-mouse IgG antibody. Asterisks indicate the predicted molecular weights of rVirB9-2 and rVirB9-1 (46 kDa), and rVirB10 (65 kDa), or native VirB9-2 and VirB9-1 (30 kDa), and VirB10 (50 kDa). Molecular weight markers are indicated in kDa.

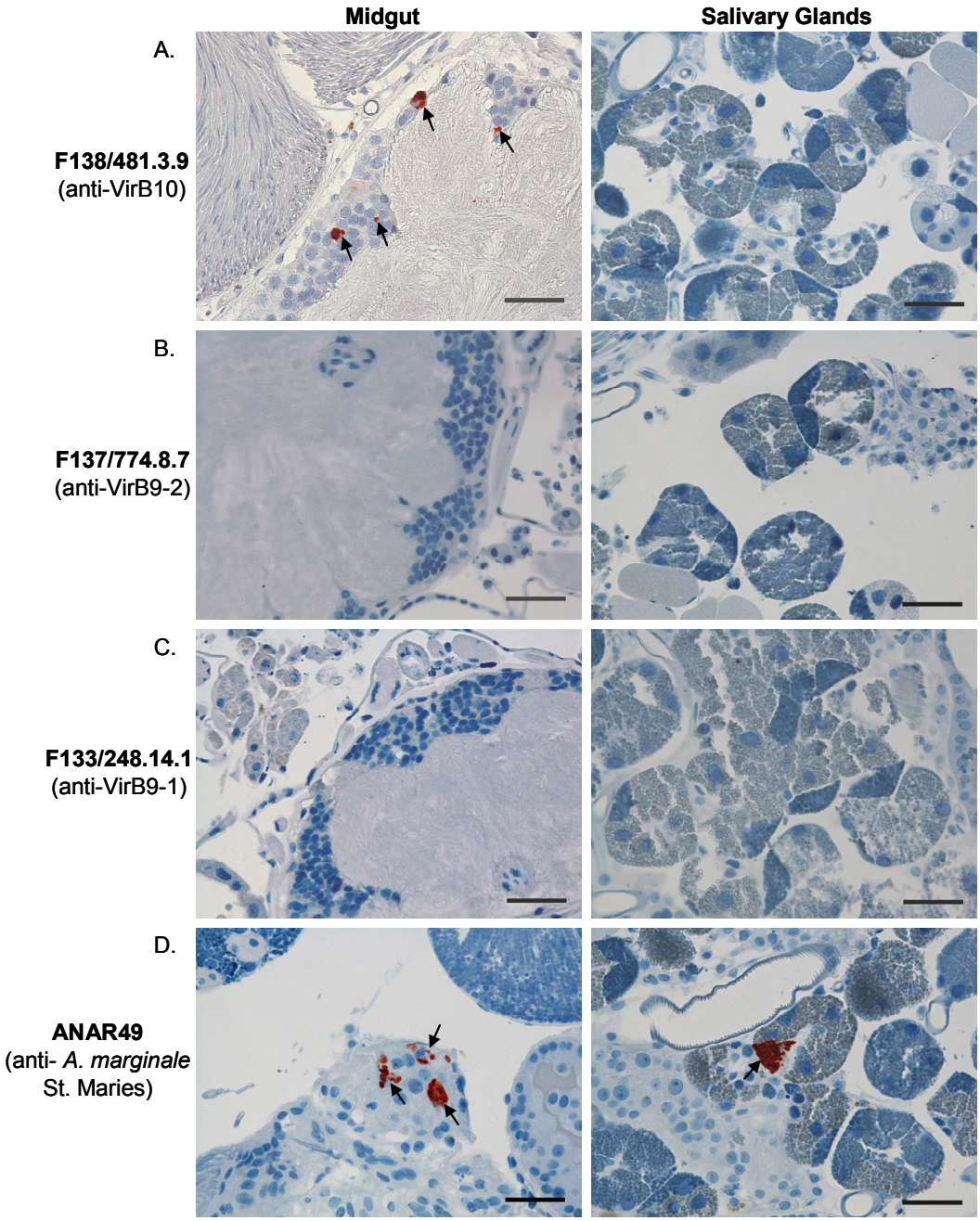

FIG. 5. Analysis of T4SS protein expression within sections of an *A. marginale* St. Maries strain acquisition-fed *Dermacentor andersoni* by immunohistochemistry. mAb specific for VirB10 (A, F137/774.8.7) binds to *A. marginale* within the midgut, but not in the salivary glands, whereas *A. marginale*-specific mAb (D, ANAR49) localizes *A. marginale* within both the midgut and the salivary glands. Neither anti-VirB9-2 (B, F137/774.8.7) nor anti-VirB9-1 (C, F133/248.14.1) mAb recognizes *A. marginale* in these tick sections. The anti-VirB9-1 mAb serves as an isotype control for anti-VirB10. Size bar is equal to 100 μm at 40X magnification.

Chapter 2

Identification of immunogenic proteins within the type IV secretion system (T4SS) of *Anaplasma marginale.*

Abstract

Anaplasma marginale is an intraerythrocytic gram-negative bacterium that causes anaplasmosis in cattle. Immunization with outer membranes (OM) can provide complete protection against infection and disease. Enhanced T-cell dependent IgG antibody responses can be elicited by immunizing with protein complexes through recognition of linked T-cell and B-cell epitopes on associated proteins. Therefore, developing a subunit vaccine for *A. marginale* would be facilitated by an immunological analysis of the protein complexes that comprise the OM proteome. The type IV secretion system (T4SS) is an essential virulence component of many gram-negative bacteria, including members of the Anaplasmataceae, and is a logical target for immunization. Screening of *A. marginale* T4SS proteins expressed by *in vitro* transcription and translation, against 2 week OM stimulated T-cell lines, identified several known and several novel proteins that stimulated antigen-specific T cell responses in OM-vaccinees. Recombinant proteins were then expressed in *E. coli* and their Immunogenicity verified. This study confirmed the immunogenicity of known T4SS antigens VirB9-1 (formerly designated conjugal transfer protein), VirB9-2 (formerly designated VirB9) and VirB10 and identified three novel immunogenic T4SS proteins, VirB7, VirB11, and VirD4. Additionally, VirB2, which was shown previously to be immunogenic in one OM-vaccinee, was also found to be immunogenic for additional

OM-immunized animals. Alignment of VirB2s from *A. marginale* and other bacteria in the family Anaplasmataceae revealed significant amino acid identity in the central conserved region of the protein. This region contained at least two T-cell epitopes, determined by testing overlapping 30-mer peptides in T-cell proliferation assays. The more complete characterization of immunogenic VirB2 and discovery of three novel immunogenic T4SS proteins further justify the T4SS as a potential vaccine candidate.

INTRODUCTION

Anaplasma marginale is a tick-borne intraerythrocytic rickettsial pathogen of cattle that is prevalent worldwide. Studies have shown that immunization with outer membranes (OM) derived from *A. marginale*, can induce complete protection against bacteremia and disease following experimental infection (1, 11, 46, 48, 53-55, 62). Protective immunity correlates with $CD4^+$ T cell responses and T-cell dependent immunoglobulin (Ig)G_2 responses to OM antigen (11). However, immunization with well-characterized major surface proteins (MSPs) 1, 2, 3, and 4, which are highly immunogenic components of the OM proteome, have elicited only limited protection against infection (11, 12, 43, 45, 51, 52, 54). This may be partially explained by extensive antigenic variation in MSP2 and MSP3, although even when controlling for the MSP2 variants used in the challenge inoculum, native MSP2 failed to confer protective immunity (1). Thus, explanations for the superior efficacy of whole OM as a vaccine include the possibility that non-variant proteins, in addition to MSP1 and MSP4, confer better protection than antigenically variant MSP2 and MSP3, the presence of multiple immunologically subdominant proteins in the immunogen provides greater protection, and the natural interaction of proteins within the OM that could increase T-cell dependent help for antibody production, thereby leading to greater neutralizing antibody responses.

Novel immunogenic proteins from the *A. marginale* St. Maries strain OM proteome have recently been identified (44). Using two-dimensional gel electrophoresis in conjunction with western blotting and liquid chromatography-tandem mass spectrometry (LC–MS-MS), Lopez et al. identified over 20 novel antigenic proteins in

the OM proteome that were recognized by immune IgG_2, including three members of the type IV secretion system (T4SS) (43, 44) . These were virulence (vir) proteins virB9 (VirB9-2), virB10, and conjugal transfer protein (CTP, now annotated as VirB9-1). Subsequent western blotting with immune serum IgG_2 and $CD4^+$ T-cell proliferation assays performed with cattle immunized with the protective OM fraction verified the ability of these proteins to stimulate an anamnestic response (43, 44). Lopez et al. later showed that OrfX, a protein encoded by multiple genes, which is now referred to as VirB2 due to conserved motifs as seen in other rickettsial bacteria (9, 10, 28), was also able to stimulate significant proliferative responses in short term 2-week T-cell lines from a single OM immunized animal (42).

The T4SS has been identified and studied in various bacteria including *Agrobacterium tumefaciens, Anaplasma phagocytophilum, Bordetella pertussis*, *Brucella suis, Ehrlichia chaffeensis*, *Helicobacter pylori,* and *Rickettsia* spp., and is associated with the transportation of effector molecules (DNA and proteins) across the periplasmic space into the host cell (4, 6-8, 13-15, 28, 32, 37, 41, 50, 64). Studies with *A. phagocytophilum* have shown that the T4SS is associated with the secretion of AnkA into host cells, and upon neutralizing the interaction of AnkA with a key co-molecule, AbI-1, there was inhibition of eukaryotic host infection (41). These studies suggest that AnkA is required for dissemination and virulence of *A. phagocytophilum* within the mammalian host (41). Virulence capabilities of the T4SS have also been demonstrated in *A. tumefaciens,* where it was shown that the T4SS secretes oncogenic T-DNA into recipient eukaryotic host cells which subsequently causes tumor (gall) formation (6, 7, 37, 64). Furthermore, studies have shown that *H. pylori* utilizes the T4SS to transport

the CagA protein into eukaryotic cells, which ultimately leads to the manifestation of stomach ulcers and tumors in patients with severe *H. pylori* infection (4). Because the T4SS is required for virulence in many gram-negative bacterial pathogens, and is a structure that is functionally conserved among many bacterial species (13, 28), the proteins within this complex are excellent candidates to target for developing vaccines against anaplasmosis and other gram-negative bacterial diseases.

The interactions of the proteins that comprise the T4SS have been best studied in *A. tumefaciens* and recently the structure of the core complex was characterized more completely (13, 16, 26). The T4SS of *A. tumefaciens* is composed of three substructures: the coupling protein homomultimer (VirD4), a transenvelope protein "core" complex, and a conjugative pilus, that are believed to span both bacterial membranes and the periplasmic space. The putative channel components are inner membrane proteins VirB6, VirB8, and VirB10 and outer membrane proteins VirB7, VirB8, VirB9, and VirB10. Cloning and expression of the VirB7-10 gene cluster revealed that the purified core complex contained VirB7, VirB9, and VirB10 but not VirB8 (26). Interestingly, this core complex was found in the outer membrane. VirB4 and VirB11 are ATPases located at the cytoplasmic face of the inner membrane and speculated to provide energy for substrate transport and biogenesis of the transfer apparatus and pilus. VirB2 is the major pilin subunit that can assemble with VirB5 to form the pilus structure. VirB1, a transglycosylase important for degrading peptidoglycan, is implicated in the biogenesis of the transfer machine. VirB4, VirB7-VirB10, and probably VirB6 form the core element of the TFSS and are responsible for transferring substrates across the cell envelope into adjacent bacteria or into the host cell (20). The coupling protein,

VirD4, forms a stable interaction with VirB10, which in *A. tumefaciens* is believed to span the inner and outer membrane within the TFSS complex. Not all components of the TFSS have been identified in all bacteria. For example, genes encoding VirB1, VirB3 and VirB5 are absent in *H. pylori* (8). Similarly, the *A. marginale* genome is lacking genes encoding VirB1 and VirB5 (10), although VirB2 was recently annotated (9).

To further investigate the immunogenic capabilities associated with the T4SS, a previously described in vitro transcription and translation (IVTT) system was used for rapid expression of the *A. marginale* T4SS proteins, which were screened for the ability to elicit memory responses of $CD4^+$ T cells obtained from OM immunized cattle (42). Proteins that induced significant T-cell proliferative responses were then expressed as recombinant proteins in *E. coli* and further tested for induction of antigen-specific proliferation. This study describes the identification of three immunogenic *A. marginale* T4SS proteins that have not previously been shown to be immunogenic in any gram-negative bacteria. Additionally, the immunogenicity of VirB2 was documented for an additional three OM-immunized cattle, and T-cell epitopes were localized to the central region of the protein that is conserved among *A. marginale* VirB2 paralogs as well as other members of the Anaplasmataceae. Discovery of these novel $CD4^+$ T-cell immunogenic proteins that comprise the T4SS further justifies the potential to target the T4SS protein complex for vaccine development against *A. marginale* and other gram-negative pathogens.

Materials and Methods

Cattle used in this study. Holstein cattle designated 04B90, 04B91, 04B92, and 4848, and 5982 were immunized with purified *A. marginale* St. Maries strain OM, as previously described (42-44, 48). Immune sera used in this study were obtained before immunization and two weeks after four immunizations (04B90, 04B91, and 04B92), two weeks after five immunizations (5982), or one week after three immunizations (4848) with 60 µg OM resuspended with PBS containing 6 mg saponin. Sera were then frozen at ¯20ºC. The DRB3 alleles were determined by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method as described (63), which was important for determining whether animals had homozygous BoLA haplotypes. Further, each PCR product from exon 2 of each *DRB3, DQA,* and *DQB* gene was sequenced as described (49, 56). The nomenclature of bovine class II genes can be found at the following websites: http://www.projects.roslin.ac.uk/bola and http://www.ebi.ac.uk/ipd/mhc/bola. BoLA-*DRB3* and *DQA* haplotypes for the cattle in this study are as follows: #04B90*, DRB3 *1101/*1501, DQA *10011/*2206, DQB *10011/*22021;* #04B91, *DRB3 *1201/*2703, DQA *12011/*2201, DQB *0101/*22031;* #04B92, *DRB3 *0201/*1201, DQA *0203, DQB *12011/*2201*; #4848, *DRB3 *1101/DQA *10011, DQB *10011;* 5982, *DRB3 *1201/*1501, DQA *12011/*2206, DQB *0101/*22021*. For simplicity, we refer to the RFLP analysis of DRB3 exon 2 designation for the corresponding allelic designations: #04B90, 22/16; #04B91, 8/23; #04B92, 8/7; #4848, 22/22; and #5982, 8/16. During the course of this study all animals maintained memory/effector T-cell responses, which enabled the development of OM-specific short-

term T-cell lines. Animals were used in this study in compliance with The Washington State University Institutional Animal Care and Use Committee.

Cloning and expression of *Anaplasma marginale St. Maries* strain T4SS genes by *in vitro* transcription and translation (IVTT). The T4SS genes including *vir* genes (AM030, AM097, AM757, AM810, AM811, AM812, AM813, AM814, AM1053, AM1312 and AM1314) were amplified by PCR with Accuprime *pfx* (Invitrogen, Carlsbad, CA) as either full-length genes for ORFs less than 1.2 kb, or as overlapping fragments of approximately 1.2 kb in size using the primers listed in Table 1. PCR primers were also designed to incorporate a C-terminal sequence encoding the FLAG epitope DYKDDDDK as described (42). PCR products were purified using a High Pure 96 UF Cleanup Plate (Roche, Indianapolis, IN) and resuspended in 60 µl of ddH_2O. One µl of each PCR product was used in a cloning reaction with pENTR-D-Topo (Invitrogen) and the mixture transformed into Top10 *E. coli* cells. pENTR-D-Topo plasmid clones were sequenced using the M13 forward and reverse sequencing primers (Invitrogen). The subsequent clones were used in a LR clonase II reaction with pEXP1-DEST (Invitrogen) to generate plasmids encoding N-terminally tagged 6X His proteins. pEXP1-DEST plasmid clones were sequenced using the T7 and pEXP1 sequencing primers. Approximately 600 ng of each pEXP1-DEST clone was used as a template in a 50 µl *in vitro* transcription/translation (IVTT) reaction using the RTS 100 *E. coli* HY kit (Roche) to achieve expression of cell-free recombinant protein.

Protein quantity was determined by dot blot analysis, as previously described (42), with slight modifications. A Hybri Dot Manifold (BRL Life Technologies, Inc. Gaithersburg, MD) was used to spot a prewetted nitrocellulose membrane with 1 µl

IVTT product diluted in 200 µl phosphate buffered saline (PBS), pH 7.0. The nitrocellulose was then flushed three times with 200 µl PBS through each well of the Hybri Dot Manifold, to enhance binding of the proteins. The membranes were washed in I-Block reagent (Applied Biosystems, Bedford MA) containing 0.05% Tween-20 (Bio-Rad), referred to hereafter as blocking reagent, for 1 hr, with a subsequent 1 hr incubation with either mouse anti-FLAG (Sigma-Aldrich, St. Louis, MO) or anti-penta His monoclonal antibody (mAb) (Qiagen) diluted 1:10,000 in blocking reagent. The membranes were then incubated for 1 hr with alkaline phosphatase goat anti-mouse IgG conjugate (Applied Biosystems, Bedford MA) diluted 1:10,000 in blocking reagent, and washed for 1 hr. Antibody was detected with the Western-Star reagent system (Applied Biosystems, Bedford MA) and exposed at different time points ranging from 1 sec to 5 min (data not shown). Dot blot densitometry was analyzed using AlphaEase FC version 4.0.0 software (Alpha Innotech, San Leandro, CA) to determine relative expression of each IVTT product. Protein expression and calculations were in performed in relation to the molarity of the reactive FLAG epitope on recombinant VirB9 (rVirB9), and ranged from 36.13 nM to 26.17 µM for IVTT-expressed proteins. This was within the range of detection of IVTT-expressed proteins in our previous study (42).

Bead-purification of IVTT proteins. IVTT protein products were purified as previously described, with slight modifications (42). Briefly, 10 µl of each IVTT reaction mixture was bound to 2.5 µg of either anti-FLAG mAb (Sigma-Aldrich) or, for VirD4 in one experiment, with anti-Penta His mAb (Qiagen) that were diluted in 440 µl A/G buffer (0.1 M Tris-HCL, 0.15 M NaCl, pH 7.4), with a final concentration of 5 µg mAb/ml. The mAb-IVTT protein mixture was incubated at 4ºC for 2 hr with continuous rocking. For

each reaction, 12.5 µl protein G carboxylate latex microspheres (Polysciences, Inc., Warrington, PA) were washed with 750 µl A/G buffer 3 times and pelleted after each wash at 16,000 x *g* for 5 min and resuspended in A/G buffer. The mixture was then incubated 2 hr at 4ºC with continuous rocking. The final bead-mAb-protein matrixes were pelleted at 16,000 x *g* for 5 min and washed three times with 750 µl A/G buffer. Based on the manufacturer's specifications, protein G beads were diluted with RPMI medium to a final concentration of 2-10 µg protein G/ml so that each well received 1-5 µg protein G beads/ml.

Recombinant protein expression and purification. Recombinant TFSS proteins used in western blotting and T-cell proliferation assays were expressed from the pEXP1-DEST plasmid obtained as described above. Plasmids containing: AM1313 (VirB11), the first (N-terminal) and second (middle) fragments of AM1312 (VirD4F1 and VirD4F2), full-length AM1312 (VirD4), and AM030 (VirB2/ORFX) were transformed, according to the manufacturer's specifications, into BL-21 DE3 LysS One Shot chemically competent *E. coli* (Invitrogen). Individual colonies were selected and grown in 3 ml LB broth with 50 µg carbenicillin (LBC)/ml. The *E. coli* were then harvested and plasmid DNA was obtained using the Wizard Plus SV Minipreps DNA purification system (Promega). Upon sequence confirmation, *E. coli* containing the particular genes of interest were grown in 1 ml LBC overnight, harvested by centrifugation at 4,000 x *g*, and frozen at $^{-}20^{o}$C as a 50% LBC broth-Glycerol stock.

Recombinant T4SS proteins VirB2, VirB11, VirD4F1, and VirD4F2 were expressed by expanding a 20 ml LBC broth overnight culture in 400 ml LBC. The cultures were induced with 1 mM IPTG upon reaching an optical density of 0.5 at 600

nm and grown for an additional 5 hr, then harvested by centrifugation at 4,000 x *g* for 20 min. The *E. coli* were lysed in 6 M guanidine hydrochloride, 20 mM dibasic sodium phosphate, and 500 mM sodium chloride, pH 7.8, for 30 min, and then sonicated 4 times at 350 Watts for 45 seconds each. Proteins were extracted from the *E. coli* lysate using nickel chelating resin from the Probond Purification System (Invitrogen). Recombinant VirD4F1 and VirB11 were purified using the Probond Purification System two consecutive times, followed by dialysis into PBS (pH 7.0) using Slide-A-Lyzer Dialysis Cassettes with 10 kDa molecular weight cutoff (Thermo Fisher Scientific, Rockford, IL). Recombinant VirD4F2 and VirB2 were purified one time using the Probond Purification System, under denaturing conditions, but were then subsequently dialyzed in 10 mM Tris buffer at pH 7.8 containing 0.1% Triton-X detergent (Bio-Rad) using Slide-A-Lyzer Dialysis Cassettes with a 10 kDa molecular weight cutoff. The rVirD4F2 and rVirB2 proteins were then purified by immunoaffinity chromatography, consisting of an anti-FLAG agarose matrix, by the manufacturer's specifications (Sigma-Aldrich). Purified rVirD4F2 and rVirB2 were then dialyzed against PBS using Slide-A-Lyzer Dialysis Cassettes with a 10 kDa molecular weight cutoff.

Protein purity was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting using 4-20% pre-cast Tris-HCL polyacrylamide Ready Gels (Bio Rad). Two sets of SDS-PAGE were run, with a different purified protein per well. The first gel was stained using Coomassie brilliant blue, and the second was transferred to a nitrocellulose membrane and blocked in blocking reagent overnight for immunoblot analysis. The nitrocellulose membrane was probed with either mouse anti-FLAG mAb (Sigma-Aldrich) or mouse anti-Penta His mAb

(Qiagen), both diluted 1:10,000 in blocking reagent, and incubated for 1 hr. After extensive washing, the blots were incubated with alkaline phosphatase conjugated goat anti-mouse IgG antibody (Applied Biosystems), diluted 1:10,000 in blocking reagent and incubated for 1 hr. The nitrocellulose membranes were then washed with blocking reagent for 1 hr, and developed with the Western-Star chemiluminescent immunoblot detection system (Applied Biosystems, Bedford, MA). The purity of the recombinant proteins was determined after the comparison of bands between the Coomassie brilliant blue stain, and western blot (Fig. 1). Protein concentrations were determined using a Bradford assay (Bio-Rad), and then the proteins were frozen at ⁻20ºC.

Positive control proteins rVirB9-1, rVirB9-2, and rVirB10, a negative control protein from unrelated *Babesia bovis,* merozoite surface antigen 1 (rMSA1), and T4SS protein rVirB7, were also expressed and purified for this study. Recombinant VirB9-1, VirB9-2, and VirB10 were shown to elicit strong immune responses in OM immunized cattle (42-44). These were expressed with a FLAG epitope and purified as previously described (44). The *msa1* gene was amplified by PCR from *B. bovis* genomic DNA using gene-specific primers (31) and Table 1, whereas AM306 (*virb7)*, was amplified from *A. marginale* St. Maries strain genomic DNA. For the *msa1* construct, a C-terminal reverse primer was constructed to contain the FLAG epitope DYKDDDDK, whereas no FLAG epitope was coded for when constructing rVirB7. Both *msa1* and *virb7* constructs were inserted into pBAD/TOPO ThioFusion plasmid under the manufacturer's specifications (Invitrogen). Plasmid DNA was then transformed into TOP 10 One Shot, chemically competent *E. coli*, following the manufacturer's specifications (Invitrogen). Transformed *E. coli* containing the construct were then plated on LBC agar and grown

overnight at 37^{o}C. Selected colonies were grown in 3 ml LBC broth overnight, harvested, and plasmid DNA was purified using Wizard Plus SV Minipreps DNA purification system. The *msa1* construct sequence was confirmed using Big Dye kits and manufacturer supplied vector forward and reverse primers (Invitrogen). A clone with the correct sequence of *msa1* or *virb7* was grown up in 1 ml LBC broth overnight and frozen in 50% glycerol-LBC broth. The glycerol stocks of *E. coli* containing *msa1* and *virb7* were each used to inoculate 20 ml LBC broth cultures and then grown overnight, and subsequently inoculated into a 400 ml LBC broth cultures. After growing to an OD of 0.5 at 600 nm, each batch of *E. coli* was induced with 0.2% arabinose and allowed to grow for 5 hr, after which the cells were harvested by centrifugation at 4,000 x *g* for 20 min, and frozen at ¯20^{o}C. Recombinant MSA1 and VirB7 were then purified from *E. coli* using the Probond Purification system. Recombinant MSA1 was further purified by immunoaffinity purification using antibody to the FLAG epitope, as described above. Purity and dialysis of rMSA1 was determined by SDS-PAGE Coomassie brilliant blue staining and western blot analysis as described above, followed by dialysis into 1X PBS, using Slide-A-Lyzer Dialysis Cassettes with a 10 kDa molecular weight cutoff. Recombinant VirB7 was also analyzed by SDS-PAGE and Coomassie brilliant blue staining as well as western blotting. The concentrations of both proteins were determined using the Bradford assay, and the proteins were frozen at ¯20ºC

VirB2 peptides and bioinformatics. Six peptides, with overlapping amino acids that span the entire sequence of VirB2 (AM030), were chosen for use in T-cell proliferation assays. The sequences of the peptides are shown in Table 4 and Fig, 2. The synthesized peptides (Laboratory for Biotechnology and Bioanalysis I, Washington

State University, Pullman, WA), were diluted in PBS (pH 7.0) with or without DMSO as needed for solubility, to a concentration of 1 mg/ml, and frozen at -20ºC. Representative VirB2 sequences were obtained from Anaplasmataceae genome sequences and aligned using ClustalW (22).The genes used to obtain the deduced amino acid sequences are as follows: *A. marginale* strain St. Maries: AM030, AM044, AM065, AM077, AM082, AM210, AM717, AM723, AM989, AM1061, AM1149, and AM1253 [CP000030; (10)], *A. phagocytophilum* strain HZ: APH_1130-1134, and APH_1144 [CP000235; (34)], *Ehrlichia canis* strain Jake: ECAJ_0842 (CP000107; Palenik et al., unpublished), *E. chaffeensis* strain Arkansas: ECH_1042 [CP000236; (34)], *E. ruminantium* strain Welgevonden: Erum7990 [CR767821; (18)], *Wolbachia pipientis* wMel: WD_0651 [AE017196; (65)], *Neorickettsia sennetsu* strain Miyayama: NSE_0770 [CP000237; (34)].

Short-term T-cell lines specific for *A. marginale* OM proteins. Two week $CD4^+$ T-cell lines were established as previously described (42), with slight modifications. Briefly, peripheral blood mononuclear cells (PBMC) were isolated from *A. marginale* OM immunized cattle (42-44, 48), and plated at 4 x 10^6 cells/well in 24-well flat-bottomed plates for 7 days with 5 µg OM and incubated at 37^o C with 5% CO_2 in air. On day 7 the cells were harvested and replated at 0.7 x 10^6 cells per well with freshly harvested irradiated (3 Krads) autologous PBMC as a source of antigen presenting cells (APC) at 2 x 10^6 cells/well (resting). The cells were harvested after 7 days, washed repeatedly in complete RPMI and tested in proliferation assays. In one experiment, $CD4^+$ T cell lines were depleted of other leukocyte subsets using monoclonal antibodies specific for bovine CD8 (mAb CACT80C), $\gamma\delta$ T cells (mAb CACT61A), $CD14^+$

monocytes (mAb CAM36A), B cells (mAb GB25A that recognizes CD21, mAb Big73A that recognizes IgM and mAb Big501E that recognizes the Ig μ chain), NK cells (NKp46-specific mAb AKS1), and dendritic cells (mAb LND41A). Cells were incubated with 15 μg mAb/ml and depleted with anti-mouse Ig coated microbeads as described by the manufacturer (Miltenyi Biotech, Bergisch Gladbach, Germany). Cell lines were tested for a proliferative response to *A. marginale* OM and T4SS antigens, expressed either by IVTT or as recombinant proteins in *E. coli.* Cells were cultured in triplicate wells of a 96-well U-bottomed plate at 0.03 x 10^6 cells per well with 0.2 x 10^6 irradiated, autologous PBMC as a source of APC and incubated at 37^{o}C in 5% CO_2 in air with medium or various concentrations of antigen. Positive controls consisted of OM antigen and 10% T-cell growth factor (TCGF) and negative control antigens consisted of uninfected red blood cell membranes (URBC), bead-incubated, no DNA control IVTT reaction, and rMSA1 of *B. bovis*. Each antigen was tested at a final concentration of 1, 2.5, or 10 μg protein/ml, and IVTT-beads were tested at 1-5 μg protein G beads/ml in 100 μl triplicate cultures. After three days, the cells were radiolabeled for 6 to 18 hr with 0.25 μCi of [^{3}H]thymidine (Dupont, New England Nuclear, Boston, MA), harvested onto glass filters, and radionucleotide incorporation was measured with a Beta-plate 1205 liquid scintillation counter (Wallac, Gaithersburg, MD). Proliferation was expressed as either mean counts per minute (CPM) +/- 1 SD of triplicate cultures or as a stimulation index (SI), determined as mean CPM of cells cultured with antigen divided by the mean CPM of cells cultured with medium or negative control antigen. Proliferative responses to antigen were considered to be statistically significant if the SI was $\geq$2.0, the mean CPM

was ≥1,000, and $P<0.05$ using a one-tailed Student t-test when antigen-induced responses were compared with medium and negative control antigen responses.

Surface phenotype analysis of T-cell lines. Flow cytometry was used to characterize the composition of the T-cell lines, as previously described (43). Briefly, mAb specific for bovine T-cell specific surface antigens CD8 (CACT80c), CD4 (CACT138), and γδ T-Cell receptor (GB21A), were used to stain 2-week OM stimulated T-cell lines. The mAbs were purchased from the Monoclonal Antibody Center located at Washington State University, Pullman, WA.

RESULTS

T cell responses to T4SS proteins expressed by IVTT. Previous studies in our laboratory determined that three TFSS proteins from *A. marginale* were recognized by both IgG and $CD4^+$ T cells from cattle immunized with the protective outer membrane vaccine (43, 44). The three proteins were identified by BLAST analysis as conjugal transfer protein (CTP) which is now designated as a homolog of VirB9-1 in *Anaplasma phagocytophilum* (accession no. YP_504712), VirB9 (now designated VirB9-2 because of greater identity to *A. phagocytophilum* VirB9-2 (accession no. YP_50587), and VirB10. These were originally identified by mass spectrometric analysis of spots excised from 2D gels that reacted with bovine IgG from outer membrane vaccinated cattle 04B90, 04B91, and 04B92 (44) and were then expressed in *E. coli* as recombinant proteins and shown to stimulate T cell responses from these cattle (43). The present study was designed to try to express and test the remaining T4SS proteins which have homology to known T4SS proteins of other gram-negative bacteria (10). In addition, the

number of cattle tested was increased to five to ensure broader coverage of MHC class II alleles.

To rapidly express and test the *A. marginale* T4SS proteins, a high-throughput IVTT expression system was employed (42). The primers used to express full-length genes or overlapping gene fragments encoding these proteins are listed in Table 1. Overlapping fragments were used for several of the larger ORFs, which were difficult to express as full-length proteins. The *A. marginale* T4SS proteins were constructed to express a C-terminal FLAG epitope and an N-terminal 6X His tag, and were affinity purified on anti-FLAG-protein G beads to ensure purification of the full-length protein. Only those proteins that expressed a C-terminal FLAG epitope, determined by dot blot analysis with mAb specific for FLAG, were bead-affinity purified and tested for T cell proliferation, with the exception of VirD4 (Table 2). In one experiment, VirD4 was not detected with anti-FLAG mAb on a dot blot, so it was affinity purified with anti-6X His-Protein G beads and protein was examined on western blots using anti-His mAb, which revealed two faint bands of approximately 40 and 50 kDa (the full-length protein is predicted to be ~ 100kDa; data not shown). Expression of three overlapping fragments of VirD4 (Table 1) was also attempted. However only the first two (F1 and F2) could be expressed by IVTT. This was true, as well, for some of the VirB4, VirB6 and VirB8 protein fragments (Tables 1 and 2).

Short term T-cell lines established from PBMC of OM immunized cattle 04B90, 04B91, 04B92, 4848, and 5982 were used to screen IVTT-expressed antigens of the T4SS (Table 2). Flow cytometry showed that in these 2-week cell lines, 94-98% of the T

cells expressed CD4, whereas only 1-6% expressed CD8 and 0.1% were γδ T cells (data not shown). Thus, the response to the T4SS proteins is mediated by $CD4^+$ T cells.

VirB9-1, VirB9-2, and VirB10 were included as positive control antigens (42, 43). We also showed previously that VirB2 expressed by IVTT weakly stimulated T cells from animal 04B90, but not animal 04B91 (42), however, additional animals had not been tested. In the present study all five animals exhibited significant proliferation against OM and VirB9-2. Other antigens that stimulated specific proliferation of short-term T-cell lines in at least one assay are the following: animal 04B90, VirB9-1, VirB2, and VirD4; animal 04B91, VirB10, VirB4-2F3, VirB11, and VirD4; animal 04B92, VirB9-1, VirB2, VirB10, and VirB11; animal 4848, VirB9-1, VirB10, VirB2, VirB3, VirD4F1, VirD4F2, and VirB6F1; and animal 5982 VirB10, VirB2, and VirB11. Screening antigens by IVTT thus verified that known immunogenic TFSS proteins could be identified by this method, but importantly showed that previously under tested VirB2 and untested VirD4 and VirB11 proteins were able to stimulate T cell responses from more than one OM immunized animal.

Expression and purification of recombinant T4SS proteins. T4SS proteins that stimulated $CD4^+$ T cell responses from more than one animal were selected for expression as recombinant proteins in *E. coli* to verify their ability to induce recall T cell proliferative responses from the OM-vaccinees. Immunogenic T4SS proteins VirB2, VirB11 and VirD4 proteins were expressed as FLAG-tagged and 6X-polyhistidine fusion proteins in *E. coli,* and isolated by affinity purification using either anti-FLAG mAb agarose and/or Ni^{2+} chelated agarose. VirB9-1, VirB9-2 and VirB10 were also expressed and purified as described previously (43). Initially, full-length VirD4 was

difficult to purify in sufficient quantities for testing due to the large size of the protein and possible conformational epitope blocking during affinity purification. Therefore the expression of overlapping VirD4 fragments 1-3 (F1-F3) that spanned the entire protein was attempted. Although VirD4 F3 was never successfully purified, we were able to obtain sufficient amounts of purified VirD4 F1 and F2 to use in T cell assays, so the initial assays measuring T-cell proliferation used these recombinant proteins. However, full-length VirD4 was eventually expressed and purified in sufficient quantity for testing. Furthermore, after we had completed testing IVTT expressed antigens, we identified a VirB7 homolog in the genome that was not originally annotated as VirB7, and expressed this as a His-tagged fusion protein (Table 1). Recombinant *B. bovis* rMSA1 was also expressed as a His- and FLAG-tagged protein for use as a negative control antigen. Newly expressed recombinant proteins VirB2, VirB7, VirB11, VirD4F1, VirD4F2, VirD4, VirB7 and control MSA1 were stained with Coomassie brilliant blue to assess relative purity (Fig. 1A). The approximate molecular weights of the recombinant fusion proteins were, for VirB2, 19 KDa; VirB7, 19 KDa; VirB11, 45 KDa; VirD4F1, 39 KDa; VirD4F2, 50 KDa; VirD4, 100 KDa; and MSA-1, 55 kDa. These bands correspond to the predicted molecular mass of each fusion protein. Immunoblots of these reacted with anti-FLAG mAb (Fig. 1B), with the exception of VirB7 lacking the FLAG epitope, and with anti-6X His mAb (Fig. 1C). VirB9-1, VirB9-2, and CTP were also of the predicted molecular weights, as previously described [(43) and data not shown].

Recognition of recombinant T4SS proteins by $CD4^+$ T-cell lines from outer membrane immunized cattle. Two-week T-cell lines derived from PBMC of OM immunized cattle were used in proliferation assays to further characterize the memory

$CD4^+$ T cell response to newly expressed T4SS proteins as well as the known immunogenic proteins VirB9-1, VirB9-2, and VirB10 (42-44). VirB9-1, VirB9-2, and VirB10 stimulated significant proliferation of T cells of all five animals, with the exception of 04B91 which does not respond to VirB9-1, and results for VirB9-2 are presented as a positive control (Fig. 2 and data not shown). Recombinant *B. bovis* MSA1 antigen, served as the negative control antigen. Four animals had significant T cell proliferative responses against rVirB2 (04B90, 04B92, 4848, and 5982) and rVirD4F1 (04B90, 04B91, 4848, and 5982), three animals had significant responses to rVirB11 (04B91, 04B92, and 5982), and two animals responded significantly to rVirD4F2 (04B91 and 4848) at one or both antigen concentrations (Fig. 2). Each antigen was tested at least three times with comparable results.

Successful purification of full-length recombinant VirD4 eventually allowed for the testing of this recombinant antigen on OM stimulated 2 week T-cell lines from each of the five animals. Animals 04B90, 04B91, 4848, and 5982 had significantly higher responses to VirD4 than to medium or negative control rMSA1 (Table 3). Furthermore, recombinant VirB7 was also found to stimulate significant responses in animals 04B90, 4848, and 5982 (Table 3). Each antigen was tested at least three times with comparable results.

In one experiment, two-week T cell lines from animals 04B92 and 4848 were enriched for $CD4^+$ T cells by depleting other subsets and tested for proliferation to the panel of recombinant proteins. These lines responded in the same way as the non-depleted T cell lines (which are already enriched for CD4+ T cells), further documenting the proliferative response is due to CD4+ T cells (data not shown).

VirB2 T-cell epitopes are located in the central conserved region. *A. marginale* has 12 copies of a gene annotated as OrFX (10) that are now believed to be orthologs of VirB2 in the Anaplasmataceae (9, 47) . Comparison of the *A. marginale* VirB2 sequences (AM030-AM1263) with representative sequences from members of the Anaplasmataceae reveals extensive conservation in the central portion of the VirB2 molecules of *Anaplasma* and *Ehrlichia* species, with less conservation when comparing *Wolbachia* and *Neorickettsia* proteins. Interestingly, there are several residues completely conserved across the family, even when more representatives are included in the alignment (data not shown), including two cysteine residues (Fig. 3). Comparison of the 12 *A. marginale* VirB2 sequences reveals two variable regions; one just after the predicted signal peptide, and the second at the carboxy-terminus of the protein. Because VirB2 is thought to be a cyclic protein with removal of the amino- and carboxy-termini (23, 28), we hypothesized that the T cell epitope(s) would localize to the central conserved region of the protein. Furthermore, if multiple copies of VirB2 are expressed during infection, this central conserved region would also serve as a common target for all protein variants. To identify the T-cell epitope-rich region, overlapping 30 amino acid peptides spanning the sequence of AM030 were synthesized (Fig. 3 and Table 4) and tested for induction of a $CD4^+$ T cell response in OM immunized cattle (Fig. 4). As expected, animal 04B91, which did not respond to rVirB2, did not respond to any of the VirB2 peptides (data not shown). However, T cell lines from animals 04B90, 04B92, and 5982 had significant proliferative responses to VirB2 peptide 3, whereas T cell lines from animal 4848 responded significantly to peptide 4. These assays were repeated two or three times with the same result.

DISCUSSION

Pathogen variability is a compromising component in the effort to globalize vaccines, and intracellular pathogens present an even greater challenge for identifying protective immunogens. For many gram-negative bacteria, the T4SS is important for intracellular survival because of its function in transporting effecter molecules into the host cell cytoplasm and nucleus (4, 6-8, 13-15, 28, 32, 37, 41, 50, 64).

Immunization of cattle with the protective *A. marginale* OM was shown previously to induce robust T-cell and antibody responses against three proteins of the T4SS (42-44). In addition, naturally or experimentally infected cattle and dogs mount significant antibody responses to a VirB9 protein of the T4SS in *A. marginale* and *E. canis,* respectively (3, 24). There is also evidence for the immunogenicity of type III secretion system (T3SS) proteins that is relevant to protective immunity. Immunization of mice with live *Burkholderia pseudomallei,* a gram-negative human class B pathogen that causes melioidosis, elicited $CD4^+$ T cell responses specific for type III secretion system proteins BopE, an effector protein, and BipD, a translocator protein. Adoptive transfer of immune $CD4^+$ T cells specific for these proteins conferred protection against challenge in mice (30)*.,* Additionally, the LcrV needle protein of the T3SS in *Yersinia pestis* was found to be a protective immunogen against infection in mouse and guinea pig models (36, 40). Similarly, immunization of mice with the Yop secretion protein F of *Y. pestis,* a component of the T3SS predicted to polymerize and puncture host cell membranes to deliver virulence factors, resulted in significant protection against subcutaneous bacterial challenge (61). Thus, the ability to elicit a protective response against T3SS proteins that share similar functions with T4SS proteins suggests that similar disruption

or blocking of the T4SS, by immunization against selected T4SS proteins could interfere with the bacteria-host cell interaction required for bacterial survival.

The objective of this study was to discover immunogenic proteins within the T4SS, in addition to the known antigens VirB9-1, VirB9-2, and VirB10, that could be possible vaccine candidates. Furthermore, we wished to better characterize the immune response to VirB2, which was previously shown to stimulate cells from a single animal (42). We did identify three new immunogenic proteins, VirD4, VirB11, and VirB7, and further showed that VirB2 was immunogenic for an additional three outer membrane immunized cattle.

The VirB11 protein of the T4SS has been characterized as an ATPase that may be responsible for the production of energy used in the facilitation of effecter molecule translocation or in the assembly of the T4SS complex (7, 16, 21, 28, 33, 38, 59). Studies focused on the localization of this protein depict an association with either the bacterial cytoplasm or periplasmic space between the inner and outer membranes (59, Fronzes, 2009 #32). Because the Holstein cattle used in this study expressed a variety of bovine leukocyte antigen (BoLA) DRB3 haplotypes (Tables 2 and 3) a relationship between T-cell response pattern and haplotype can be inferred. For the protein VirB11 tested in $CD4^+$ T-cell proliferation assays, it was observed that only cell lines derived from animals expressing DRB3 *RFLP* haplotypes of 8/23 (04B91), 8/7 (04B92), and 8/16 (5982), had a significant response (Table 2, Fig. 2 B, C, and E). This suggests that a class II molecule associated with the DRB3 RFLP 8 haplotype presented VirB11 epitope(s) to elicit significant T-cell proliferation. Although the current literature suggests

that VirB11 may not be surface exposed, this protein is still a viable vaccine candidate as it may participate in linked recognition by providing T-cell epitopes.

The VirD4 protein of the T4SS is thought to be an ATPase/coupling protein that mediates the transfer of effector proteins or DNA across the periplasmic space and into the host cell (26, 28, 29, 37). The shuttling feature of VirD4 may at some point allow the protein to be surface exposed, which coupled with its immunogenicity, further supports this protein's role as a vaccine candidate. The pattern of $CD4^+$ T-cell responses to VirD4 is also consistent with expression of a given MHC class II haplotype. Animals with haplotypes 16/22 (04B90), 8/23 (04B91), 22/22 (4848), and 8/16 (5982), had significant $CD4^+$ T-cell responses to VirD4 or VirD4F1 and F2 proteins. Because animal 04B92 with haplotype 8/7 did not respond, these results indicate that VirD4 is presented by MHC class II molecules associated with DRB3 haplotypes 22, 16, and 23.

The VirB7 protein of the T4SS has been investigated in other bacterial species such as *Agrobacterium tumefaciens,* however a *virb7* gene has yet to be annotated for any of the Anaplasmataceae genomes, including *A. marginale*. A recent study by Gillespie et al. compared annotated VirB7 amino acid sequences from *Rickettsia typhi*, to VirB7 orthologs (ComB7) from *Helicobacter pylori* and *Campylobacter jejuni* (28), and it was determined that these orthologs have conserved amino acid motifs and cysteine residues. Bioinformatic analysis yielded analogous results when the *A. marginale* gene AM306, annotated as a hypothetical protein*,* was compared to ComB7 of *H. pylori* and *C. jejuni* (data not shown)*.* The putative VirB7 stimulates significant $CD4^+$ T-cell proliferative responses in cattle possessing DRB3 haplotypes 22/16 (04B90), 22/22 (4848), and 8/16 (4848). These results are consistent with presentation of VirB7

epitope(s) by MHC class II molecules associated with haplotype 22 and 16. Although the VirB7 annotation within rickettsial pathogens in not clearly defined, it is an important component within the T4SS of other bacteria such as *A. tumefaciens* (5, 18, 27, 57, 58). Studies have shown that within *A. tumefaciens,* VirB7 forms disulfide linkages with VirB9, and heterodimers of VirB7 and VirB9 aggregate to form a 14-oligomer core channel of the T4SS (2, 26, 28). Additionally within *A. tumefaciens,* VirB7 interacts with VirB2, and VirB5 to comprise architectural support in the formation of the membrane associated pilus structure of the T4SS (6, 7, 17, 26, 28, 37, 60, 64). Localization of VirB7 on the OM of the bacteria indicates this protein could be a good target for future vaccine development where neutralizing antibody might interrupt function of the T4SS.

As mentioned above, VirB2 is also surface exposed, and is the major contributor in the formation of the pilus of the T4SS, with or without VirB5 (7, 19, 25, 28, Fronzes, 2009 #32, 35). Furthermore, studies with *A. tumefaciens* have shown that VirB2 undergoes post-translational modification, so that the full-length protein is cleaved at N- and C-termini, then made cyclic prior to pilus formation (35, 39). Testing of six overlapping peptides that span the full-length of one of the *A. marginale* VirB2 paralogs (AM030) showed that T-cell epitopes reside within the conserved central region of the protein. This region is shared by all 12 VirB2 paralogs that we predict would remain for cyclization if post-translational modification occurs with this protein as for VirB2 of *A. tumefaciens.* The rather robust response to the central conserved region of VirB2 but not to the N- or C-terminal peptides is consistent with this possibility. It is not known which of the VirB2 gene family members are expressed, but the strong responses to VirB2 (AM030) might be due to a relatively abundant amount of the conserved T-cell

epitopes if multiple copies are expressed and/or if VirB2 multimers comprise the pilus apparatus. Additional studies are needed to answer these questions.

Patterns of T-cell response to VirB2 are also consistent with haplotype expression. Animal 04B90 (22/16), 04B92 (8/7), and 5982 (8/16), recognized peptide 3, whereas animal 4848 (22/22) recognized peptide 4, and animal 04B91 (8/23) did not respond to VirB2. These data are consistent with presentation of VirB2 peptide 4 with an MHC class II molecule associated with the DRB3 22 haplotype, and with presentation of peptide 3 with a class II molecule associated with DRB3 16 and 7 haplotypes. It is not clear why animal 04B90 failed to respond as well to peptide 4, as it has the DRB3 22 haplotype. However, all T-cell responses by this animal are relatively weak because of consistently high background proliferation in the absence of antigen stimulation, so that a weaker response to peptide 4 may not have been apparent in the assays. Because VirB2 is predicted to be surface exposed and is recognized by several common haplotypes among the Holstein breed, this protein is an optimal candidate for vaccine development.

The T4SS has conserved structure and function in many gram-negative intracellular bacteria. The T4SS has not been well characterized for any of the Anaplasmataceae; however since this protein complex is likely necessary for the survival and dissemination of *A. marginale,* vaccine development targeting this protein complex to stimulate a protective immune response is warranted. Furthermore, the predicted localization of several of the T4SS protein to the outer membrane of *A. marginale* further justifies targeting this protein complex as a vaccine candidate. Investigating the specific interactions among *A. marginale* T4SS proteins within the

complex, and the immunogenic properties of T4SS proteins in relation to recognition of linked T-cell/B-cell epitopes, is needed to achieve this goal.

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Table 1. Primer and annotations for IVTT and recombinant protein expression

Genomic	Protein Annotation	Forward Primer (5'-3')	Reverse Primer[a] (5'-3')
AM097	CTP	CACCATGGGCAAAAGGCTTTCATGGTTTGTGCTG	CCCCACGTCCCCTTCTGGATG
AM757	VirB8-1	CACCATGGGCCGCGTACGGCCACAAAACAG	GTCTACGTATCTGTACGTATTGATAGTAAAC
AM1316	VirB8-2	CACCATGGGCGGTATGTTCGGTTTCGGTAAGAAG	TAGAAATTCATCGTCTGCCCTATAG
AM810	VirB6-like	CACCATGGGCACACTCCTGCAGGAGAAAAGC	TTGAGAAGACCCACCCAGACC
AM810[b]	VirB6F1	CACCATGGGCACACTCCTGCAGGAGAAAAGC	AAAGTCCGCCATGATGAGGGGC
AM810[b]	VirB6F2	CACCATGGGCCTTCTGAAGCTGTTTACAGGGGGC	TTGTATTAGTTCCTAGAGTTCTTCCCA
AM810[b]	VirB6F3	CACCATGGGCAAGGTGATCGAGGAGAGAGAG	GGGCTCCACGCCCTATCTTAT
AM810[b]	VirB6F4	CACCATGGGCGCGCAAAAGGACGATGATGCG	CTACTCCTATCCCCAGAC
AM810[b]	VirB6F5	CACCATGGGCGAGCCGCAGGAGGTTGAACCT	TTGAGAAGACCCACCCAGACC
AM811	VirB6-like	CACCATGGGCTTCAGGTTACTGCTCATTGCG	TTCCTTGTCTTGCTTTTCTCCTGC
AM811[b]	VirB6F1	CACCATGGGCTTCAGGTTACTGCTCATTGCG	AATAACGGCAACCGCACCTTC
AM811[b]	VirB6F2	CACCATGGGCGAGGACTGCACACGCTCC	CCTATCGCCATCTAGGTACCC
AM811[b]	VirB6F3	CACCATGGGCGTGATCAAAATTACCAAGTACGATCTC	AGGCCCACCGAAATCAGTCACATC
AM811[b]	VirB6F4	CACCATGGGCCCGCCAGGAGAAGAGAAGCTG	TTCCTTGTCTTGCTTTTCTCCTGC
AM812	VirB6-like	CACCATGGGCTTTTGTAACGCTTCTGCGCTGC	AAAGCTAGCCGGAGGATCAGC
AM812[b]	VirB6F1	CACCATGGGCTTTTGTAACGCTTCTGCGCTGC	GAGATGCAATGTCTGTGTGCC
AM812[b]	VirB6F2	CACCATGGGCGACGAGCTTGCAGCCAACGAA	TGCTAGCAGCTGAATCCACGTTTG
AM812[b]	VirB6F3	CACCATGGGCCCGGACAGCTGGACCTTTTTTAAC	AAAGCTAGCCGGAGGATCAGC
AM813	VirB6-like	CACCATGGGCCGTAGAGCGGTCAGGGC	CGTCCCAGACGACCCTCC
AM813[b]	VirB6F1	CACCATGGGCCGTAGAGCGGTCAGGGC	TACTGCCTTTGACTTCTCGCA
AM813[b]	VirB6F2	CACCATGGGCGCCATTTCTGGGGCTGGA	AGTAAACATCAGCGACAGGAACGA
AM813[b]	VirB6F3	CACCATGGGCCCCGCAATCCTCATAAACGCC	CGTCCCAGACGACCCTCC
AM814	VirB4-1	CACCATGGGCTTGAGACTCGGGAGAACTGCAG	CGCATTTCTAACCTTCTGGCAG
AM814[b]	VirB4-1F1	CACCATGGGCTTGAGACTCGGGAGAACTGCAG	AACCGTCAGGTGATGGAGCCC
AM814[b]	VirB4-1F2	CACCATGGGCTCTGCCGGAATGTTAGATGCG	CTCAAACCCAAAGACCCTGGA
AM814[b]	VirB4-1F3	CACCATGGGCAGCGACCTAACTCCAGATGAC	CGCATTTCTAACCTTCTGGCAG
AM1053	VirB4-2	CACCATGGGCTCTTTCATAGATAGTTTTGTGCGC	CTGCGCGAGTTTATCGCAC
AM1053[b]	VirB4-2F1	CACCGATGGGCTCTTTCATAGATAGTTTTGTGCGC	TGAGCTTTCGTCGGTGGA
AM1053[b]	VirB4-2F2	CACCATGGGCGGAGCAATTCTTGGCGTCAAG	CGAAAAAGCCCCCTGGGC
AM1053[b]	VirB4-2F3	CACCATGGGCGAGATAGCTGTTGGGGTTGTG	CTGCGCGAGTTTATCGCAC
AM815	VirB3	CACCATGGGCTCGTCCGGTAGCGTAAAGAC	CATCACATCGTAAGAATTGGCAT
AM1313	VirB11	CACCATGGGCTTGTGTGCGCATATGACAGC	ATCATTGCCTTGTGAACATTTAGTG
AM1314	VirB10	CACCATGGGCAGTTTAGGGATGTCAGACGAAACC	CCTACGCACCGCCTCCC
AM1314[b]	VirB10F1	CACCATGGGCAGTTTAGGGATGTCAGACGAAACC	CCTAGAGCCTTTCGGTATCAT
AM1314[b]	VirB10F2	CACCATGGGCACTTGGAGCACACTGGACGGC	CCTACGCACCGCCTCCC
AM1315	VirB9	CACCATGGGCAATTTCTATAAAAACTTTGCTTGCGTGC	AAGCACCGTATTCACTACTTCGAC
AM030	VirB2 (Orfx)	CACCATGGGCTTGCTAAAACTTAGATTCTTACTCATTGC	TCGGTTACTTTGGGCAGTGCC
AM1312	VirD4	CACCATGGGCCACAGTAGCTCTAATCACATACGTAAC	CGGGTTGTCATCATCACGCTG
AM1312[b]	VirD4 F1	CACCATGGGCCACAGTAGCTCTAATCACATACGTAAC	CGGGGTGGCCACAGTTC
AM 1312[b]	VirD4 F2	CACCATGGGCTATCTGCTTGCAGTGCCG	CGAGTACTTCTTTCGCGAGTC
AM1312 [b]	VirD4 F3	CACCATGGGCATCGAATCTACGTATCCGATC	TCGGTTACTTTGGGCAGTGCC
AM306	VirB7	ATGAGATTTTCTAGGGTGGTGC	CCCAAGCCACCACTGGTTTACT[c]
BBOV_I003060[d]	MSA1[d]	GCCGATACTTCAATCGTCCTTCC	TGTACCCTGTTGTCCTTGGAGG

a. Each reverse primer contains a leader sequence encoding the FLAG epitope to be expressed on the C-terminus, CTATTTGTCGTCGTCGTCTTTATAGTC.

b. Due to large gene size, fragments of AM 810, 811, 812, 813, 1053, 1314, and 1312 were constructed along with the full length counterparts.

c. Recombinant VirB7 was constructed without the FLAG epitope.

d. Recombinant MSA1 (merozoite surface antigen-1) is a *Babesia bovis* derived construct.

Table 2. Proliferative responses to *A. marginale* type IV secretion system protein antigens expressed by IVTT.

Protein[a]	Proliferation for animal number[b]:				
	04B90 (22/16)[c]	04B91 (8/23)	04B92 (8/7)	4848 (22/22)	5982 (8/16)
Expt. 1					
A. marginale OM	**44.6**	**80.7**	**740.8**	**104.1**	**409.8**
VirB9-1/CTP (AM097)	**69.9**	1.3	**207.2**	**48.8**	3.2
VirB9-2 (AM1315)	**33.3**	**69.8**	**244.6**	**9.4**	**158.9**
VirB10 (AM1314)	1.1	**23.2**	**27.7**	**4.1**	**4.1**
VirB2 (AM030)	**15.5**	0.6	**24.9**	**3.2**	**33.5**
VirB3 (AM815)	1.0	0.8	0.6	1.2	0.5
VirB4-1 (AM1053) F1	2.1	0.6	0.8	1.4	0.4
VirB4-1 (AM1053) F3	0.7	0.8	0.7	1.8	1.2
VirB4-2 (AM814) F1	1.0	1.9	1.4	1.8	0.6
VirB4-2 (AM814) F2	1.1	1.9	1.7	1.2	0.8
VirB4-2 (AM814) F3	0.9	**2.5**	1.6	1.8	0.8
VirB6 (AM810) F2	1.4	2.1	0.9	1.2	0.6
VirB6 (AM811) F1	2.1	1.6	0.6	1.4	0.7
VirB6 (AM811) F2	0.9	ND[d]	1.1	1.3	0.6
VirB6 (AM811) F3	ND	1.2	ND	ND	ND
VirB6 (AM812) F1	1.7	1.4	1.6	1.1	0.9
VirB6 (AM812) F3	1.7	1.9	1.0	1.7	1.1
VirB6 (AM813) F1	1.2	1.8	1.4	**2.3**	0.9
VirB8-2 (AM1316)	1.3	ND	2.2	1.6	0.6
VirB11 (AM1313)	1.4	**6.5**	3.7	1.4	0.5
VirD4 (AM 1312)	0.6	**3.6**	0.9	1.2	0.2
VirD4 (AM1312) F1	1.0	ND	3.3	**4.0**	0.5
VirD4 (AM1312) F2	0.7	1.1	0.9	**3.3**	0.7
Expt. 2					
A. marginale OM	**7.2**	**8.4**	**12.9**	**61.4**	**27.6**
VirB9-1/CTP (AM097)	**3.0**	1.2	**16.5**	**129.4**	1.4
VirB9-2 (AM1315)	ND	ND	**27.8**	**73.6**	**14.9**
VirB10 (AM1314)	0.9	**7.8**	**7.6**	**13.7**	1.4
VirB2/OrfX (AM030)	0.9	1.2	**7.4**	3.3	**8.1**
VirB3 (AM815)	1.2	1.4	1.3	0.7	1.9
VirB11 (AM1313)	1.4	**2.8**	**3.1**	0.7	**2.3**
VirD4 (AM1312)	**3.3**	**24.9**	1.7	0.8	1.9
VirD4 (AM1312) F1	ND	ND	1.3	**9.5**	0.9
VirD4 (AM1312) F2	ND	ND	1.2	**4.0**	0.5

a. Proteins were expressed by IVTT and dot blots were performed with anti-FLAG and anti-6x His Mab. Only proteins that expressed the C-terminal FLAG epitope were tested in proliferation assays with the exception of VirD4. The IVTT expressed VirD4, used in all but one experiment with 04B91, was truncated at the C-terminus and protein bands of ~30 and 50kDa were visualized on Western Blots with anti-His mAb. The VirD4 protein used with animal 04B91 in expt. 1 was expressed with a FLAG epitope.

b. Stimulation indices (SI) were derived by dividing the mean CPM of triplicate cultures of cells with antigen by the mean CPM of triplicate cultures of cells with medium (for *A. marginale* OM) or negative control IVTT antigen (for IVTT expressed antigens). *A. marginale* outer membranes were used at 1 µg protein/ml and bead-bound proteins from IVTT reaction mixtures were tested at a concentration of 1 or 2.5 µg Protein G/ml. Results in bold-faced type were significantly greater than either medium or negative control antigen (URBC for *A. marginale* and bead-incubated negative control no DNA IVVT reaction for other antigens) using a Student's one-tailed t-test, where $P \leq 0.05$, if the SI is ≥ 2.0, and if the mean CPM is $\geq$1,000.

c. The DRB3 RFLP haplotypes for each animal are indicated in parentheses.

d. ND, Not determined.

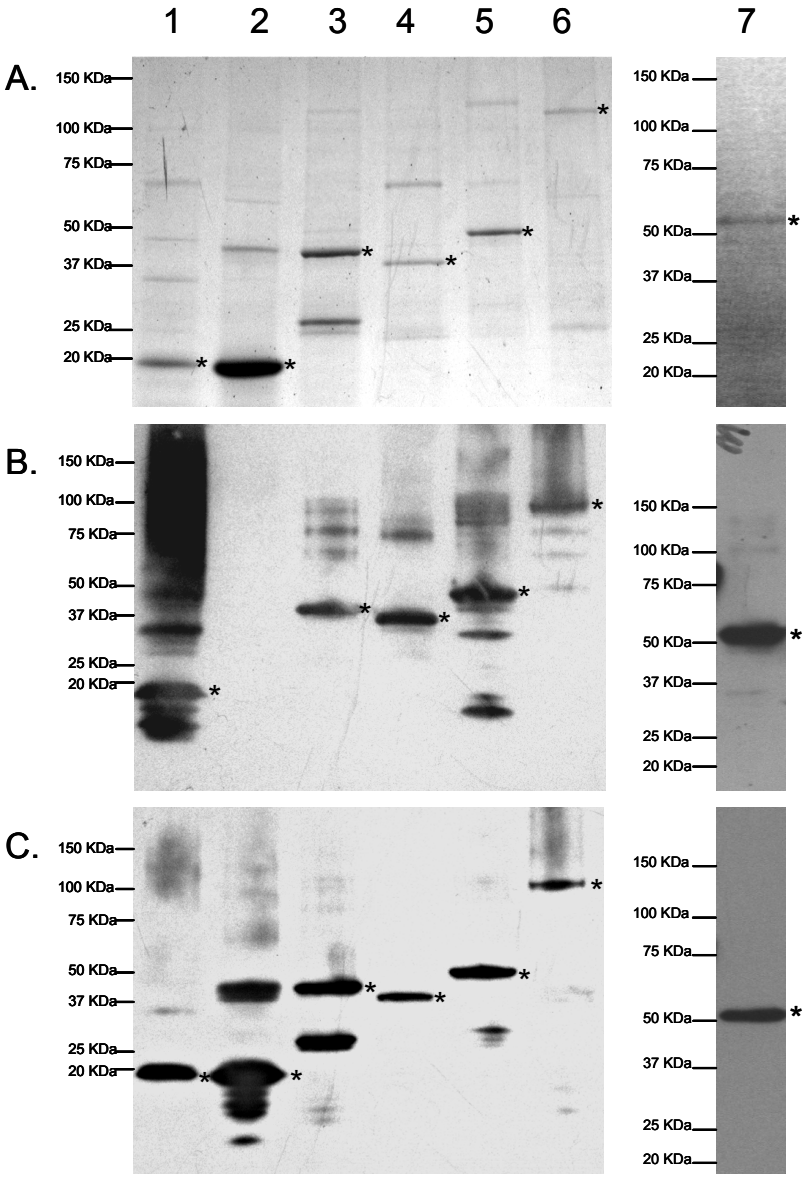

FIG. 1. Expression and relative purity of the T4SS recombinant proteins. Proteins were separated using SDS-PAGE and stained with Coomassie blue (A), or transferred to nitrocellulose membranes and probed with either anti-FLAG mAb (B), or anti-6xHis mAb (C), then detected with chemiluminescence using alkaline phosphatase labeled secondary mAb, goat-anti-mouse IgG. Wells received 1.5 µg purified rVirB2 (lane 1), rVirB7 (lane 2), rVirB11 (lane 3), rVirD4F1 (lane 4), rVirD4F2 (lane 5), rVirD4 (lane 6), and 2 µg rMSA1 (lane 7), for Coomassie blue stained gels and immunoblots. rVirB2 and rVirB7 are approximately 19 kDa, rVirB11 is approximately 45 kDa, rVirD4F1 is approximately 39 kDa, rVirD4F2 and rMSA1 are approximately 55 kDa, and VirD4 is approximately 100 kDa, as indicated by asterisks.

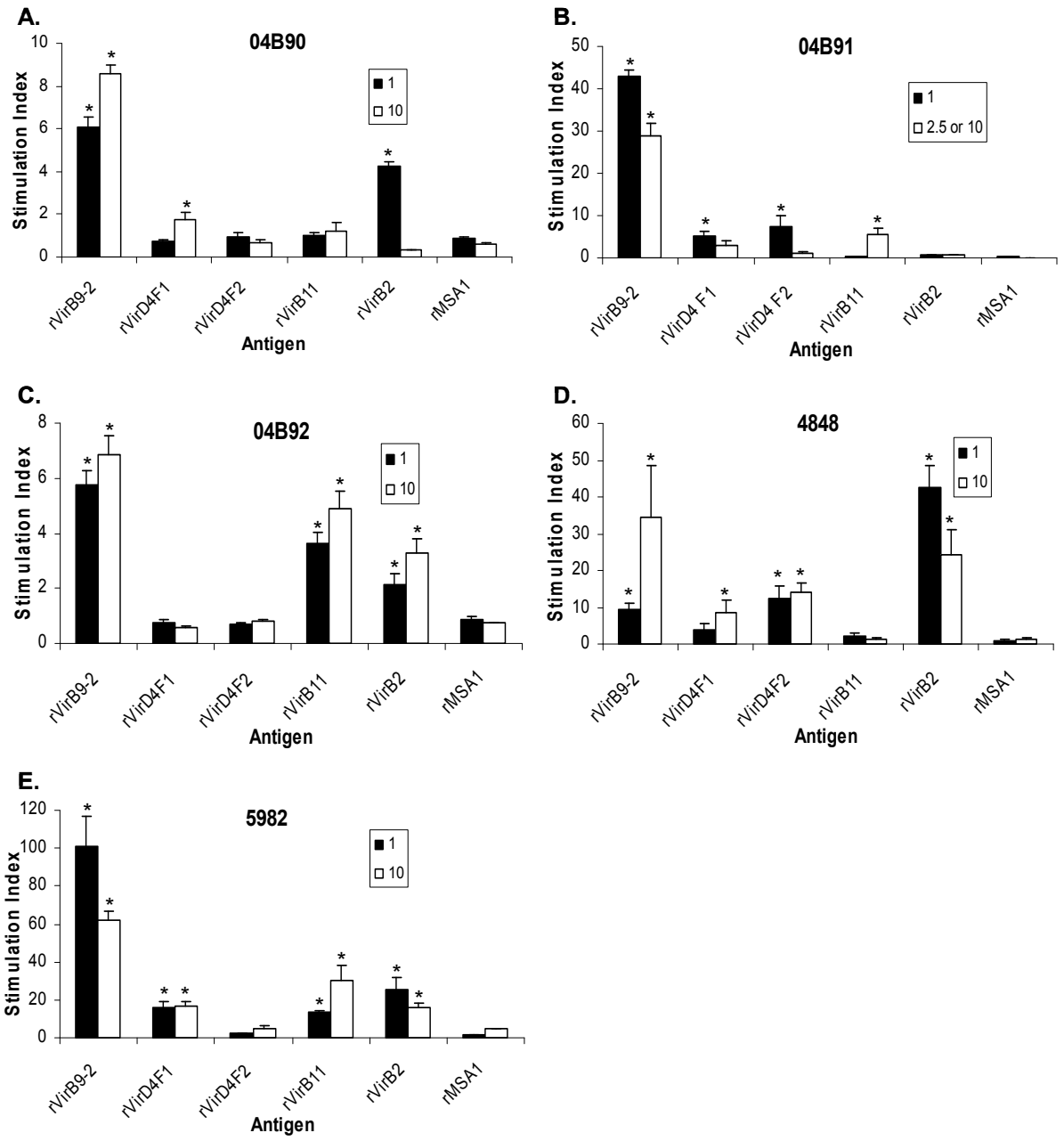

FIG. 2. Short term T-cell lines established from OM vaccinees proliferate in response to recombinant TFSS proteins. T cells from bovines 04B90 (A), 04B91 (B), 04B92 (C), 4848 (D), and 5982 (E) were tested for proliferation to the indicated recombinant proteins. T cells were stimulated with 1 and 10 µg per ml rVirB9 (positive control), rVirD4F1, rVirD4F2, rVirB11, rVirB2, and rMSA1 (negative control). Responses are presented as stimulation index compared to medium +/- 1 SD of triplicate cultures. Stimulation indexes significantly greater than those to medium or negative control MSA1 are indicated by an asterisk where $P \leq 0.05$ using a one-tailed Student's t-test, the SI ≥ 2, and the mean CPM to antigen ≥ 1000.

Table 3. Proliferative response of T cells from outer membrane vaccinees to recombinant VirD4 and VirB7

Antigen (μg/ml)	Proliferation (Mean CPM +/- 1SD) for the following animal[a]:				
	04B90 (22/16)[b]	04B91 (8/23)[b]	04B92 (8/7)[b]	4848 (22/22)[b]	5982 (8/16)[b]
Medium	8,380 +/- 2,185	401 +/- 41	35 +/- 56	468 +/- 51z	593 +/- 610
MSA-1 (1)	12,810 +/- 2,725	237 +/- 56	200+/- 117	737 +/- 276	679 +/- 289
(10)	9,788 +/- 3,264	212 +/- 27	159 +/- 177	472 +/- 120	646 +/- 280
VirB9-2 (1)	**69,657 +/- 10,281**	**45,061 +/- 6,311**	**69,088 +/- 3,789**	2,997 +/- 3,505	**60,927 +/- 26,211**
(10)	**82,108 +/- 8,962**	**62,340 +/- 1,351**	**100,566 +/- 9,559**	**3,275 +/- 1,962**	**127,695 +/- 13,393**
VirD4 (1)	**27,803 +/- 4,777**	610 +/- 389	98 +/- 21	**3,301 +/- 199**	**1,406 +/- 304**
(10)	**41,763 +/- 11,530**	**5,223 +/- 2,334**	608 +/- 532	**13,515 +/- 5,780**	**3,587 +/- 1,791**
VirB7 (1)	**28,676 +/- 5,127**	248 +/- 24	104 +/-51	**1,535 +/- 434**	668 +/-196
(10)	**45,504 +/- 7,990**	572 +/- 175	162 +/- 81	**5,134 +/- 1,866**	**5,298 +/- 596**

a. Recombinant proteins were tested in a 3-4 day proliferation assay with short-term (2-week) cell lines that had been stimulated for one week with *A. marginale* outer membranes and rested for one week. Lymphocytes were cultured at 3 x 10^4 cells per well with 2 x 10^5 irradiated autologous PBMC as a source of APC, and were radiolabeled for the last 6-18 hr of the assay with [^{3}H]thymidine, harvested, and counted. Results are presented as the mean CPM of triplicate cultures +/- 1 SD and those in bold faced type are significantly greater than those to medium or negative control MSA1 are indicated by an asterisk where $P \leq 0.05$ using a one-tailed Student's t-test, the SI ≥ 2, and the mean CPM to antigen ≥ 1000.
b. MHC class II haplotypes are indicated using the DRB3 RFLP nomenclature.

Table 4. VirB2 (AM030) peptides used in proliferation assays

Peptide	Amino acid position	Sequence[a]
1.	1-30	MLLKLRFLLIAVVLAFGILHGVPAGASKSS
2.	21-50	GVPAGASKSSEGAQTADDTATIVICNVIRF
3.	41-70	TIVICNVIRFVQKLGLPIMTGVILGSSIMA
4.	61-90	GVILGSSIMAIFGKLAWPAIVMLVVFTAIF
5.	81-110	VMLVVFTAIFFGAGKLMAKFAKGLGGEGVK
6.	101-128	AKGLGGEGVKDAGSFDCSSYKGTAQSNR

a. Thirty amino acid peptides overlapping by 10 amino acids (underlined) were synthesized.

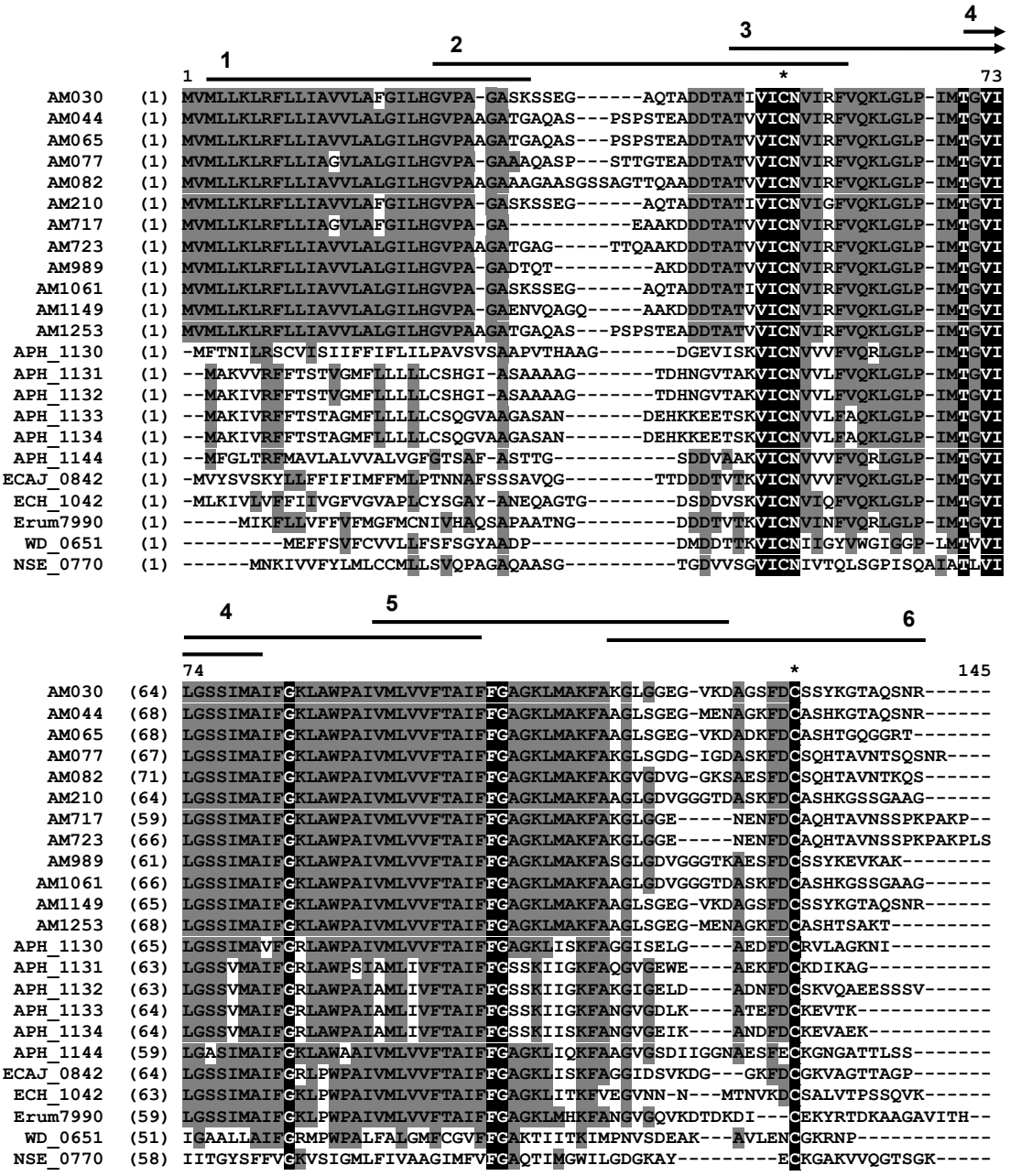

FIG. 3. Alignment of Anaplasmataceae virB2s. ClustalW alignment of 12 virB2 sequences from *A. marginale* (AM), six (of eight) sequences from *A. phagocytophilum* (APH), and representative sequences from *Ehrlichia canis* (ECAJ), *E. chaffeensis* (ECH), *E. ruminantium* (Erum), *Wolbachia pipientis* wMel (WD), and *Neorickettsia sennetsu* (NSE). Identical residues in all sequences are shown as white text on black background, while blocks of identical residues are shown as black on gray background. Conserved cysteine residues are indicated with an asterisk. The positions of *A. marginale* peptides designed against the AM030 sequence are indicated as horizontal lines above the sequence.

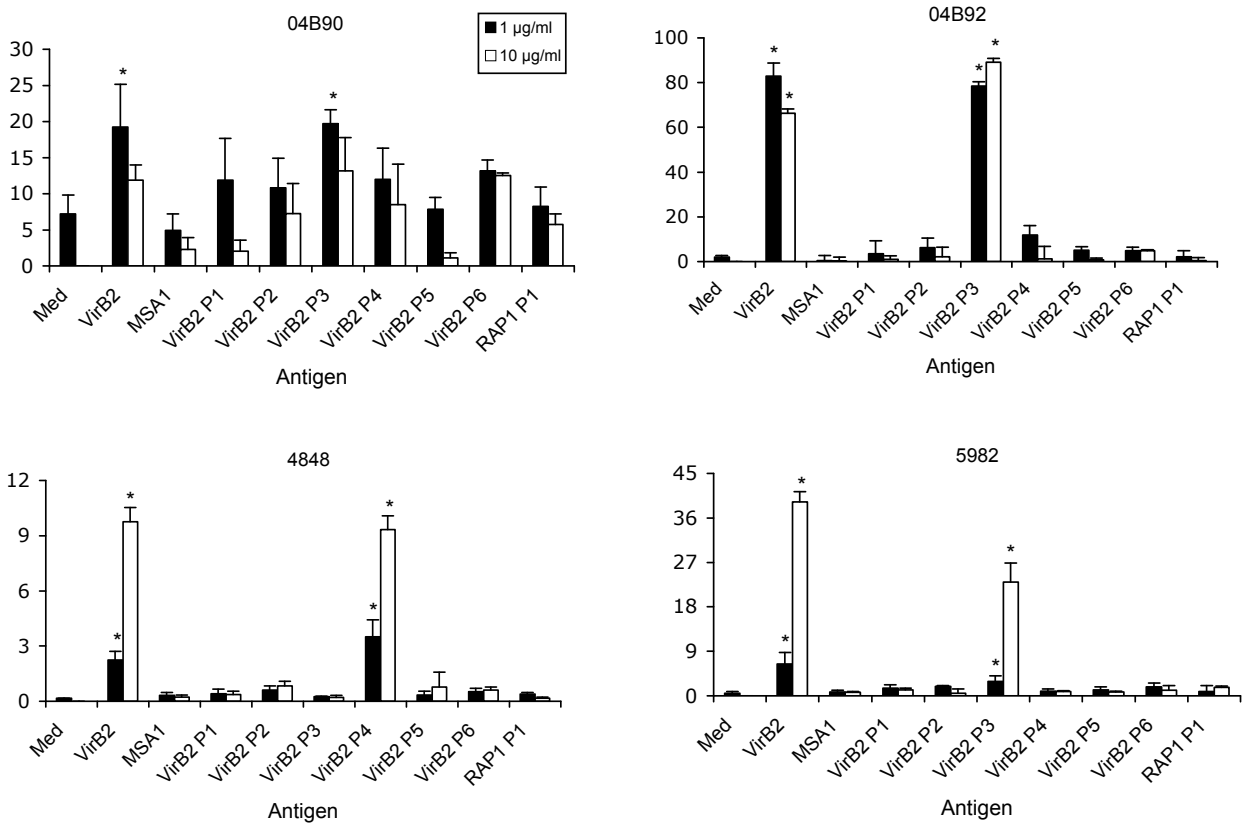

FIG. 4. Short term 2-week T-cell lines established from outer membrane vaccinees proliferate in response to specific VirB2 peptides. T-cells from bovine 04B90 (A), 04B92 (B), 4848 (C), and 5982 (D) were tested for proliferation to 1 and 10 µg per ml of rVirB2 and overlapping peptides. Responses are represented as stimulation indexes in relation to medium. Stimulation indexes significantly greater than those to medium or negative control MSA1 (for VirB2) or RAP-1 P1 (for VirB2 peptides) are indicated by an asterisk where $P \leq 0.05$ using a one-tailed Student's t-test, the SI ≥ 2, and the mean CPM to antigen ≥ 1000.