IMMUNOGENICITY AND LINKED RECOGNITION OF ANAPLASMA MARGINALE TYPE IV SECRETION SYSTEM PROTEINS

By

KAITLYN MORSE

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WASHINGTON STATE UNIVERSITY
Department of Veterinary Microbiology and Pathology

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To the Faculty of Washington State University:

The members of the committee appointed to examine the dissertation of KAITLYN MORSE find it satisfactory and recommend that it be accepted.

_________________________________
Wendy C. Brown, Ph.D., Chair

_________________________________
Douglas R. Call, Ph.D.

_________________________________
James E. Bruce, Ph.D.

_________________________________
Junzo Norimine, D.V.M., Ph.D.

_________________________________
Kevin K. Lahmers, D.V.M., Ph.D.
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Abstract

By Kaitlyn Morse, Ph.D.
Washington State University
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Chair: Wendy C. Brown

Like several other bacterial pathogens, Anaplasma marginale has an outer membrane (OM) that induces protection from infection and disease. However proteins that confer protective immunity and whether the protection requires linked T-cell and immunoglobulin G epitopes and/or interacting proteins are not known. Our goal was to target the conserved type IV secretion system (T4SS) to identify immunogenic membrane proteins that are interacting and linked recognition candidates. Linked recognition is a process by which B cells are optimally activated by helper T cells responding to the same or physically associated antigen. The T4SS is a membrane complex within many bacterial pathogens secreting virulence factors and promoting host cell invasion and intracellular survival. A. marginale T4SS proteins VirB2, VirB4-1, VirB4-2, VirB6-1, VirB7, VirB8-2, VirB9-1, VirB9-2, VirB10, VirB11 and VirD4 were screened for their ability to induce IgG and stimulate CD4+ T cells from OM vaccinated cattle. VirB9-1, VirB9-2, and VirB10 induced the strongest IgG and T cell responses in the majority of cattle, although animals with major histocompatibility complex class II (MHC class II) DRB3 RFLP types 3/16, 8/23, and 16/27 lacked T-cell responses to VirB9-1, VirB9-1 and VirB9-2, or VirB9-2 and VirB10, respectively. For these animals-specific IgG production may result from T cell help provided by responses to an interacting protein partner(s). Interacting partners were
determined by far western blotting and confirmed by immunoprecipitation assays, and revealed, for the first time, specific interactions of VirB9-1 with VirB9-2 and VirB10. Since, MHC class II molecules influence antigen-specific CD4$^+$ T-lymphocyte responses, specific bovine leukocyte antigens required for presentation of peptides from VirB9-1, VirB9-2, and VirB10 were also determined. Overlapping peptides spanning each protein were tested in T cell assays with autologous antigen presenting cells (APC) and artificial APC expressing combinations of bovine DR and DQ molecules. Twenty immunostimulatory peptides were identified, of which, four DRA/DRB3 presented fifteen peptides, four DQA/DQB presented seven peptides, and three functional mixed isotype (DQA/DRB3) were identified. The immunogenicity, interactions, and broad MHC class II presentation of VirB9-1, VirB9-2, and VirB10 justify their testing as a linked multi-epitope vaccine against A. marginale.
TABLE OF CONTENTS

Acknowledgements........................................................................................................................................ iii
Abstract............................................................................................................................................................ iv
List of Tables .................................................................................................................................................. xii
List of Figures ................................................................................................................................................ xiv

Chapter 1: Significance and Background

1.1 *Anaplasma marginale* ............................................................................................................................. 1

1.2 OM from various pathogens are protective and individual proteins are not......................... 6

1.3 Linked proteins from the OM are protective ..................................................................................... 7

1.4 What is the Type IV Secretion System? ......................................................................................... 10

1.5 Type III Secretion System proteins are protective .......................................................................... 14

1.6 Major Histocompatibility Complex class II .................................................................................. 15

1.7 Case for T4SS proteins as a vaccine against *A. marginale* ......................................................... 18

1.8 Hypothesis and specific aims ............................................................................................................ 20

1.9 Work Cited........................................................................................................................................... 20

Chapter 2: Preliminary Information

2.1 Cattle Haplotypes ............................................................................................................................... 31

2.2 Type IV Secretion System Protein Alignments ............................................................................... 31

2.2.1 VirB6-1 ........................................................................................................................................ 32

2.2.2 VirB7 .......................................................................................................................................... 32

2.2.3 VirB8-2 ...................................................................................................................................... 33

2.2.4 VirB9-1 and VirB9-2 ............................................................................................................. 33
2.2.5 VirB10........................................................................................................36
2.3 Predicted Type IV Secretion System Localizations...............................37
2.4 Predicted Interacting A. marginale Proteins .........................................37
2.5 Predicted three dimensional Models ....................................................39
   2.5.1 VirB9-1, VirB9-2, and VirB10 .........................................................39
   2.5.2 MSP5................................................................................................40
   2.5.3 MPS2................................................................................................41
2.6 Work Cited...............................................................................................42
2.7 Tables.........................................................................................................45
2.8 Figures and Figure Legends.................................................................47

Chapter 3: Characterize A. marginale T4SS proteins for their recognition by T cells
   and IgG from OM vaccinated cattle with diverse MHC class II haplotypes
3.1 Brief introduction.......................................................................................63
3.2 Materials and Methods..........................................................................65
   3.2.1 Expression of the recombinant T4SS proteins and MSA1 ..............65
   3.2.2 Purification of recombinant proteins ..............................................66
   3.2.3 Isolation of A. marginale OM..........................................................67
   3.2.4 Immunization of six haplotype-diverse cattle with A. marginale OM ...68
   3.2.5 Determination of serological responses to T4SS proteins
      by immunoblotting.................................................................................68
   3.2.6 T cell proliferation assays to recombinant T4SS proteins ..............70
   3.2.7 Detection of Interferon gamma (IFN- γ) in supernatants..................71
3.3 Results.......................................................................................................72
3.3.1 Recombinant T4SS proteins ................................................................. 72
3.3.2 Cattle Immunizations ........................................................................ 72
3.3.3 IgG responses to T4SS proteins in OM-vaccinees .............................. 73
3.3.4 T lymphocyte responses to T4SS proteins in OM-vaccinees .............. 73
3.3.5 Quantity of IFN-γ in supernatants ..................................................... 74
3.3.6 Selection of linked recognition candidates ......................................... 74

3.4 Discussion ............................................................................................. 75

3.5 Work Cited ........................................................................................... 77

3.6 Tables .................................................................................................... 80

3.7 Figures and Figure legends .................................................................. 84

Chapter 4: Identify the immunogenic A. marginale T4SS proteins that are surface exposed and naturally associated with other antigenic T4SS proteins

4.1 Brief introduction .................................................................................. 87

4.2 Materials and Methods ........................................................................ 88

4.2.1 Preparation of antibodies specific for VirB7, VirB9-1, VirB9-2, VirB10, MSA1, ACP, and normal rabbit ................................................................. 88

4.2.2 Immunohistochemistry of intact and potentially live A. marginale ...... 90

4.2.3 Gold labeling of iRBC and transmission electron-microscopy .......... 92

4.2.4 Gold labeling of isolated A. marginale ................................................ 93

4.2.5 Tandem Mass Spectrometric Analysis of rVirB7, rVirB9-1, rVirB9-2, and rVirB10 ......................................................................................... 93

4.2.6 Far western blotting ............................................................................ 94

4.2.7 Determining the binding coefficients of interacting VirB9-1, VirB9-2
and VirB10.................................................................95

4.2.8 Immunoprecipitation..............................................96

4.3 Results........................................................................99

4.3.1 Specificity of polyclonal antibodies against rVirB7, VirB9-1, VirB9-2, VirB10, and MSA1 .................................................................99

4.3.2 Experimental determination of Surface localization..........................101

4.3.3 Evidence for dimerization of VirB7, VirB9-1, VirB9-2, and VirB10..102

4.3.4 Detecting the interactions of VirB7, VirB9-1, VirB9-2, and VirB10 with far western blots.................................................................103

4.3.5 Binding coefficients for VirB9-1 interactions ...............................104

4.3.6 Native VirB9-1, VirB9-2, VirB10 interactions determined by
    immunoprecipitation................................................104

4.4 Discussion......................................................................106

4.5 Work Cited......................................................................110

4.6 Tables............................................................................113

4.7 Figures and Figure legends .............................................115

Chapter 5: Determine T cell and MHC class II epitopes on the interacting
    immunogenic T4SS proteins.

5.1 Brief Introduction..........................................................126

5.2 Materials and Methods...................................................138

5.2.1 Synthetic peptides ...................................................128

5.2.2 Cattle...................................................................129

5.2.3 Two week T cell lines used for proliferation assays.................129
5.2.4 MHC class II expression.................................................................130
5.2.5 Flow cytometric analysis of transfected 293-F cells...............131
5.2.6 T cell proliferation assays with transfected 293-F cells as APCs......131

5.3 Results
5.3.1 Breadth of CD4+ T cell responses to peptides on VirB9-1, VirB9-2,
and VirB10..............................................................................................131
5.3.2 DR and DQ expression and antigen presentation .......................133

5.4 Discussion.........................................................................................136
5.5 Work Cited........................................................................................140
5.6 Tables................................................................................................143
5.7 Figures and Figure legends ..............................................................146

Chapter 6: Determine if immunization of peptides containing T cell and MHC class II
epitopes linked to B cell antigens will undergo linked recognition and
induce a stronger protective immune response as compared to
individual T4SS epitopes

6.1 Brief Introduction..............................................................................153
6.2 Strategy ............................................................................................154
6.3 Work Cited .......................................................................................158
6.4 Tables ................................................................................................159

Chapter 7: Other research Projects

7.1 Implications of MSP2 Function ....................................................160
7.1.1 Protein-protein interactions with MSP2 .....................................160
7.1.2 MSP2 erythrocyte attachment ...................................................162
7.1.3 Conclusions about MSP2........................................................................164
7.2 Formaldehyde crosslinking of recombinant VirB9-1 to native OM proteins ..... 166
7.3 Co-expression of VirB9-1, VirB9-2, VirB10, VirB7 and MSA1
   in 293 F cells ................................................................................................. 167
7.4 Work Cited .................................................................................................. 169
7.5 Tables .......................................................................................................... 170
7.6 Figures and Figure legends ......................................................................... 171
Chapter 8: Conclusions ................................................................................... 176
  8.1 Work Cited .................................................................................................. 178
LIST OF TABLES

Chapter 1
None

Chapter 2
1. DRB3, DQA, and DQB alleles identified in the cattle used in this study. .........................45
2. Results from Surface Localized Extracellular Protein (SLEP) Algorithm Predictions .........46

Chapter 3
1. IgG responses to recombinant T4SS proteins in cattle immunized with OM.....................80
2. T cell responses to recombinant A. marginale T4SS antigens in Holstein cattle
   with different MHC class II haplotypes ..............................................................................81
3. IFN-γ quantity within supernatants of three day cultured T cells with antigen and
   its correlation with T-cell proliferation stimulation index ..................................................82
4. Summary of IgG and T cell responses to A. marginale T4SS antigens in six
   Holstein cattle ..................................................................................................................83

Chapter 4
1. Evidence for dimerization of VirB9-1, VirB9-2, and VirB10 .............................................113
2. Mass spectrometric analysis of immunoprecipitated A. marginale OM .........................114

Chapter 5
1. Peptide sequences used for T cell epitope mapping .......................................................143
2. Expressed BoLA class II α/β- combinations ....................................................................144
3. Summary of CD4+ T cell epitopes and their BoLA-class II restriction elements .............145

Chapter 6
1. Full length protein Immunogens ......................................................................................159
2. T cell epitope linked to B cell Ag Immunogens .................................................................159

Chapter 7

1. Bicistronic vector made for expression.................................................................170

Chapter 8

None
LIST OF FIGURES

Chapter 1

None

Chapter 2

1. Images of RFLP analysis of exon 2 of the DRB3 gene ..................................................47
2. MHC class II allelic frequency of Holstein Cattle in WA. .................................................47
3. Multiple sequence alignment of five VirB7 sequences .......................................................48
4. *A. marginale* VirB9s sequence alignment with *A. phagocytophilum* VirB9s ..................49
5. Alignment of VirB9-1 and VirB9-2 amino acid sequences from *A. marginale* and *A. centrale* strains .......................................................................................................50
6. VirB9 sequence alignment across species ............................................................................52
7. Alignment of VirB10 amino acid sequences from *A. marginale* and *A. centrale* strains ....54
8. VirB10 sequence alignment across species ...........................................................................56
9. Bioinformatic predictions for the *A. marginale* surface localization of VirB2 (ORF X), VirB4-1, VirB4-2, VirB6-1, VirB7, VirB8-2, VirB9-1, VirB9-2, VirB10, VirB11 and VirD4. .........................................................................................................................58
10. Predictions for interacting proteins .....................................................................................61
11. Predictions for three dimensional structures .....................................................................62

Chapter 3

1. Purity of recombinant T4SS proteins ..................................................................................84
2. Characterization of the *A. marginale* before cattle immunizations. ...............................85
3. Successful immunization of six MHC-diverse cattle ..........................................................86

Chapter 4
1. Purity of polyclonal immunoglobulins ................................................................. 115
2. Specificity of rabbit polyclonal antibodies against VirB7, VirB9-1, VirB9-2, VirB10,
   and MSA1. .................................................................................................................. 116
3. Polyclonal antibodies anti-VirB9-1, anti-VirB9-2, and anti-VirB10 detect native protein
   within intact A. marginale .......................................................................................... 117
4. Surface localization experiment with infected erythrocytes ....................................... 118
5. Surface localization experiment with erythrocyte-free intact A. marginale ................. 119
6. Evidence for dimerization of rVirB9-1, rVirB9-2, and rVirB10 ................................. 119
7. Far western blotting to detect interacting recombinant T4SS proteins with specific
targets ............................................................................................................................ 120
8. Single site saturation binding curves for the interactions of rVirB9-1, rVirB9-2, and
   rVirB10. ....................................................................................................................... 122
9. Detection of native protein interactions of VirB9-1, VirB9-2, and VirB10 .................... 124
10. Detection of native protein interactions of VirB9-1, VirB9-2, and VirB10 are not
dependent on disulfide linkages ..................................................................................... 125

Chapter 5
1. Stimulation of CD4⁺ T-cell lines from A. marginale OM-immunized cattle against
   synthetic peptides from VirB9-1, VirB9-2, and VirB10 ............................................. 146
2. Dose-dependent proliferative response from each animal to selected peptides .......... 148
3. Identification of BoLA- class II restriction elements for VirB9-1, VirB9-2, and VirB10
   epitopes using 293-F cells expressing DR and DQ molecules ................................. 150
4. Expression of peptide presenting mixed isotype pairs ............................................. 151
5. Identification of BoLA-class II restriction elements for VirB9-1, VirB9-2, and VirB10 epitopes using 293-F cells expressing bovine class II mixed isotypes..........................152

Chapter 6
None

Chapter 7
1. Far western blot indicating native MSP2 binding to recombinant proteins.........................171
2. MSP2 native interactions determined by immunoprecipitation........................................171
3. FACS analysis to determine if MSP2 is an erythrocyte adhesive protein ......................172
4. MSP2 binds to a protein located on erythrocytes. .................................................................173
5. Formaldehyde crosslinking of rVirB9-1 to native proteins in A. marginale OM fraction.174
6. Bi-cistronic expression of recombinant T4SS proteins .......................................................175

Chapter 8
None
Chapter 1: Significance and Background

1.1 *Anaplasma marginale*

*Anaplasma marginale* was first identified in 1909 by Theiler (1). The name “*Anaplasma*” is based on the inability of the cytoplasm to be stained and “*marginale*” refers to the peripheral location of the organism within the host erythrocyte. Anaplasma has been reclassified numerous times since its first description, and it is now a gram-negative α-proteobacterium in the order Rickettsiales and family Anaplasmataceae (2). All members of Anaplasmataceae (*Anaplasma, Ehrlichia, Neorickettsia, Wolbachia*) reside and replicate while enclosed in a cytoplasmic vacuole inside eukaryotic mature and immature haemotopoetic host cells and invertebrate (or helminth) vector hosts (2). Anaplasmosis is caused by the obligate intra-erythrocyte gram negative rickettsia, *Anaplasma marginale*. *A. marginale* enters erythrocytes by endocytosis, however, erythrocyte adhesive protein(s) has not been thoroughly examined. Once in the erythrocyte, *A. marginale* replicates intracellularly by binary fission (2, 3) in a vacuole derived from erythrocyte membranes usually containing 3-8 pathogens (4, 5). During acute infection, the destruction of red blood cells causes anemia, with other clinical signs of infection including fever, weight loss, respiratory distress, lower milk production, abortion, and often death. Animals that recover from acute infection are lifelong carriers and serve as reservoirs for the tick transmitted disease. Levels of rickettsemia in persistently infected cattle roughly fluctuate in 6-8 week submicroscopic peaks of rickettsemia rising to $10^6$-$10^7$ organisms/ ml of blood and falling back to $10^2$ organisms/ml of blood, regardless of the strain of *A. marginale* (6-8). Anaplasmosis is one of the most prevalent tick-borne diseases of cattle worldwide and it is both deadly and an economic hardship in temperate, subtropical and tropical regions (9, 10). The pathogen is known to occur in Africa, Asia, Australia, South Europe, South America, Russia, and major areas of the United
States. The losses attributed to bovine anaplasmosis in the continental US are over $300 million (11).

The disease is efficiently transmitted to feeding ticks during low ($10^2$) or high ($10^7$) levels of persistent rickettsemia. *Dermacentor andersoni, D. occidentalis,* and *D. variables* have been identified as the main tick vectors in the United States that transmit *A. marginale* (7-10). Ticks become infected by feeding on an infected host, the bacteria multiply in the gut epithelium, move to the gut muscle and hemolymph, and finally replicate in the salivary glands (10-15). *A. marginale* can also be mechanically transmitted via contaminated needles, dehorning saws, nose tongs, tattooing instruments, and castration instruments (16).

Species within *Anaplasma* genus have several well-characterized immunodominant major surface proteins (MSP), MSP2 (17-19), MSP3 (20, 21), MSP4 (22, 23), MSP5 (23-28), and p44 in *A. phagocytophilum* (29-33). MSP1 (105kDa), MSP2 (36kDa), MSP3 (86kDa), MSP4 (31kDa), and MSP5 (19kDa) from different isolates Israel, Kenya, Zimbabwe, and the United States have conserved epitopes (24, 34-36). MSP2, MSP3 and p44 are encoded by polymorphic multigene families; continually undergo antigenic variation, contributing to immune evasion and persistent infection (7, 10, 20, 29-33, 37-39). MSP2 antigenic variation has been most widely studied, as there are multiple msp2 gene-related copies widely distributed throughout the *A. marginale* genome (18, 40). Expressed msp2 genes are characterized by a large central region of amino acid polymorphisms, called the hyper variable region, flanked by the highly conserved N- and C- terminus (41). The hyper variable region contains epitopes that are recognized by antibodies subsequent to the rickettsemia cycle, followed by repeated emergence of MSP2 variants, thus true antigenic variation (7). MSP2 undergoes antigenic variation by segmental gene conversion in which expression site mosaics are generated (42). At least four different
variants of the central hyper variable region are found in each rickettsemia cycle during persistent infection (39). Although there have been multiple advancements in understanding the mechanism for MSP2 antigenic variation, little progress has been made towards the function of MSP2. Protein function is discovered by identifying the interactions and protein localization.

Typically most immune systems can control the acute phase of *A. marginale* infection but when the surface coat of *A. marginale* changes, infection persists. *A. marginale* is distinctive because it infects erythrocytes, which having no nucleus are incapable of making antigen presenting molecules such as major histocompatibility complexes needed to induce CD8+ cytotoxic T cells to destroy infected cells. Th1-mediated responses have been implicated in protection against pathogens infecting erythrocytes, however initial CD4+ T cell-responses often evolve towards a Th2 profile during persistent infection. For example, mice recover from acute stage-malaria infection through Th1-mediated responses, while clearance of chronic infection is Th2 regulated and antibody dependent (43). Cytokine profiling of *Babesia microti* CBA infected mice via reverse transcriptase polymerase chain reaction revealed dominant Th1 cytokines IL-2 and IFN-γ during acute infection and during persistent infection Th2 cytokines IL-4 and IL-10 were expressed (44). The switch from Th1 to Th2 immune responses may also be happening with *A. marginale* persistently infected cattle, which have dysfunctional memory CD4+ T cell responses (45, 46). However, the role of CD8+ T cells during *A. marginale* infection has received little attention. The Th1 response is influenced by (i) cytokine environment, (ii) antigen dose and/or antigen affinity to the T cell receptor, and (iii) the timing and level of co-stimulatory molecules between the antigen presenting cell (APC) and T cell (43, 47). Therefore, for *A. marginale*, APC are extremely important for inducing the immune responses, and the majority of antigen specific-T cells co-express IL-4 and IFN-γ (47, 48). The immune response during natural
infect ed cattle compared to OM vaccinated cattle is different even though both partially protect (49-51).

There are inconsistencies in the literature whether or not antibodies play a role in immunity against A. marginale. The examples of antibody independent mechanisms and evidence for cell mediated control are as follows. Immune carrier animals relapse severely when splenectomized; this is how multiple studies acquired A. marginale for challenge (17, 52-55). Supernatants from mononuclear cell cultures isolated from infected calves during acute anaplasmosis reduced the amount of erythrocytes containing viable A. marginale in vitro (56). High levels of antibodies in naïve calves alter neither the course of the infection nor the outcome of infection (57), and in the absence of high IgG1 and/or IgG2 titers, calves are able to control acute anaplasmosis (58). A. marginale MSPs were originally identified with neutralizing polyclonal antibody (19); however, immunizations with MSP1-5 do not induce protection from homologous and heterologous challenge (8, 34, 48, 54, 55, 59), negating the importance of neutralizing antibody. Lastly, bovines immunized with a surface protein complex containing MSP2 were protected against challenge. However, there were no correlations between the breadth and magnitude of the anti-MSP2 antibody responses with protection from infection or control of bacteremia (17). The examples that support antibodies playing a role in controlling anaplasmosis are as follows. Immune sera against A. marginale generated in rabbits were able to neutralize infection in splenectomized calves, whereas normal rabbit serum did not alter infectivity (19). Protection against A. marginale infection has been shown to correlate with antibody titers against membrane proteins (35) and adaptive immunity correlates closely with IgG2 (60). Lastly, infected cattle that lost their CD4+ T cell response during persistent infection still developed and maintained high A. marginale-specific IgG titers (46). These inconsistencies
in the literature is likely due to the differences in experimental procedures and genetic footprint of the animals (especially haplotypes), and of course it is probable that more than one mechanism is involved in control of *A. marginale* infections. It is likely that the “right” immune response will be delicate balance between protection and pathology. A bias towards interferon (IFN)-γ and immunoglobulin (Ig)G2 production directed against the pathogenic surface is present in cattle that have been immunized with *A. marginale* OM (60). IFN-γ production enhances the expression of IgG2 and activates macrophages to release toxic chemicals such as nitric oxide, thus IFN-γ producing CD4⁺ T lymphocytes is central to immunity against *A. marginale* (59). Thus, a combination of cellular and humoral immunity is likely important for the control of anaplasmosis.

Vaccination strategies and control methods for anaplasmosis include the live attenuated sub-species, *A. centrale* (50, 61-64), killed whole organisms (51, 65), and outer membrane (OM) fractions (35, 36, 53). These strategies have established protective immunity; however, the bacteria must be purified, a high cost procedure, from infected erythrocytes because they are difficult to culture. Also blood derived vaccines must be standardized for killed organisms and monitored for newly emerging or unknown pathogen transmission (59). Since most strains used for vaccination are found in different geographic regions, they are not protective in all locations and for all isolates (9, 35, 66-68). The inconsistent protection is attributed to antigenic variation between strains (9, 10, 59, 69), morphological differences (70), different restriction endonuclease patterns or other genetic markers (71, 72), polyclonal bovine serum (73), and monoclonal antibodies (74, 75) within a single *A. marginale* bacterium. The use of antibiotics such as oxytetracycline and imidocarb has been proven to be effective treatment against clinical anaplasmosis (9, 76, 77).
Some research has been done to identify the proteins within the OM that confer protective immunity. When cattle were vaccinated with immunodominant MSP1, MSP2, MSP3, MSP4, none were completely protected against *A. marginale* infection (8, 34, 48, 54, 55, 59). In 1998, Brown *et al.* showed that IFN-γ secreting CD4+ T cell clones with a specific response to OM did not recognize MSP1-5 indicating that there are other T cell epitopes to subdominant/unidentified OM proteins (60). Subdominant, non-variable OM proteins were identified by two-dimensional electrophoresis and membrane impermeable crosslinking both coupled with tandem mass spectrometry (53, 78). Importantly, among these identified proteins were some type IV secretion system proteins which have also been shown to elicit CD4+, IgG, and IFN-γ responses (79-81). A successful new vaccine for anaplasmosis will depend on its ability to cross protect among genotypes, its ability to prevent infection in cattle and in ticks, and most likely contain a subdominant and conserved OM protein (82).

1.2 OM from various Pathogens are Protective but Individual OM Proteins are Not

Membranes play a critical role in a bacteria’s life. They form a physical barrier between the cell and the environment, and cell membranes mediate the exchange of information from cell to cell or to the environment by transporting ions, DNA, and proteins. The basic structure of a biological membrane is made up of a phospholipid bilayer and membrane-spanning proteins. The bacterial membrane proteome consists of proteins involved in cell signaling (G-protein coupled receptors), cell-cell interactions (integrins and adhesions), energy generation (ATP synthases), and unique proteins specific to pathogens. Pathogen outer membranes are important for their growth, host cell invasion, intracellular survival, and are the primary target for immune response. For these reasons OM and outer membrane proteins form the basis of vaccine research.
Treponema palladium (83), Burkholderia multivorans (84), Neisseria meningitides (85, 86), N. gonorrhoeae (87, 88), Haemophilus influenzae (89), Chlamydia trachomatis (90, 91), Francisella tularensis (92-98), and Anaplasma marginale (35, 53, 66) are all examples of pathogens that have a protective OM or OM vesicles. There are also examples of OM vesicle-immunized mice that were protected from different bacteria species. OM vesicles or OM protein pools from N. lactamia protected mice against challenge with N. meningitides B and C serogroups (85). As well as, immunization with Salmonella-derived OM vesicles completely protected mice against a 10 x LD50 challenge of Streptococcus pneumoniae (99).

However, there are also multiple examples of individual proteins within the protective OM that did not confer protective immunity. For example, H. influenzae challenged chinchillas were protected against infection and disease if they were immunized with whole bacteria or OM prior to challenge; but not with immunization using proteins P6, rPCP, P4, or a mixture (89). Also F. tularensis OM associated lipoprotein TUL4 elicited a T cell response, but this protein did not protect BALB/cJ mice like immunization with the OM (98). For A. marginale, immunization with OM or crosslinked OM induced protection in cattle against bacteremia and anemia (35, 53, 66). However Anaplasma major surface proteins MSP1, MSP2, MSP3, MSP4 and MSP5 are not protective (8, 34, 48, 54, 55, 59).

1.3 Linked Proteins from the OM are Protective

Activated T cells secrete cytokines such as IL-2, IL-4, and IL-5 that along with cognate interactions drive somatic hypermutation and isotype switching of B cells for the production of high-affinity immunoglobulins. This feature of the T- and B-cell interaction in the humoral response is called linked recognition. Linked recognition has been studied since the 1920s by
Landsteiner’s research on haptens and later in the 1970s by Mitchison. Mitchison revealed that both T cells and B cells must recognize antigenic determinants on the same molecule for B-cell activation to occur (100-102). However, linked recognition can also occur with two associated proteins, where one has B cell epitopes and a second provides T cell epitopes. For *A. marginale*, this was shown in a study with MSP1, a heterodimer consisting of covalently associated MSP1a and MSP1b (52, 103, 104). MSP1a-specific T cells provided help to B cells specific for MSP1b to promote increased IgG production (52, 105). Thus, it is required that the epitope recognized by the CD4⁺ T cell is physically associated with the epitope that is recognized by the B cell, so the B cell can phagocytose the foreign molecule(s) and present an epitope to the CD4⁺ T cell through the major histocompatibility complex (MHC) class II pathway. The process is as follows, when a membrane bound B cell receptor recognizes a protein, the protein is internalized, processed, and presented as a linear peptide on MHC class II molecule to CD4⁺ T cells. Peptides are presented on bovine leukocyte antigens (BoLA) DRA/DRB or DQA/DQB class II molecules and recognized by T-cell receptors (TCR). Some TCR recognize some epitopes better than others and some epitopes not at all. Linked recognition also indicates that individual proteins associated with each other have a better chance of providing T cell help and consequently inducing higher IgG titers.

There are multiple examples of linked proteins conferring protection, likely through linked recognition, but many studies fall short of determining the immunological basis. Many studies have been focused on T-independent antigens, mostly capsular polysaccharide antigens and lipopolysaccharides (LPS). However, both of these antigens are capable of eliciting an adjuvant effect, tricking the immune system into responding as though there were an active infection, usually causing inflammation. LPS has pathogen associated molecular patterns that
initiate strong B cell responses without the help from any other cell and also interacts with Toll-like receptor 4 on dendritic cells. Responses to LPS alone stimulate low affinity IgM and IgA antibodies but no memory, IL-1, IL-2 nor IL-10 were produced because linked recognition did not occur (94, 97). However, these immune responses were enough for LPS to induce protective immunity against *F. tularensis* (92, 93, 106), *B. pseudomallei* (107), *N. gonorrhoeae* (108), and *Pseudomonas aeruginosa* (109). To induce immunological memory and develop neutralizing antibody responses T-dependent antigens are linked to the T-independent polysaccharides. Examples of capsular polysaccharide-based vaccines for *Haemophilus influenzae* and *Streptococcus pneumoniae*, T-dependent protein antigens were linked to the T-independent polysaccharide to achieve neutralizing antibody responses directed against the polysaccharide (110, 111). The following are examples using LPS fusion complexes to stimulate helper T-cell responses and long lasting protective immunity (112, 113).

Immunization studies using two or more associated proteins are also suggestive that linked recognition enhances protection against bacterial pathogens. For example, protective immunity was induced with bacterial membrane protein complexes or fusion proteins of surface exposed type III secretion system proteins of *Yersenia pestis* (114, 115). Association of proteins within the context of the membrane is also important for leptospiral outer membrane porin OmpL1 and lipoprotein LipL41. In a gerbil model, co-expression of OmpL1 and LipL41 in *E. coli* membranes was required for the vaccine to protect against challenge. The expression of either protein alone was not effective, nor was immunization with the recombinant proteins alone or in a mixture (116). There are three examples of linked recognition of proteins during *A. marginale* infection. (i) One explanation for the protection induced by immunization with OM or crosslinked OM versus incomplete protection with individual MSPs, is that linked recognition of
epitopes on more than one surface protein is required for protective immunity. (ii) The linked recognition of MSP1a and MSP1b lead to enhanced IgG responses to MSP1b via T cell help from MSP1a (52). (iii) Lastly, cattle with an 8/23 haplotype immunized with \textit{A. marginale} OM had very strong antibody against type IV secretion system (T4SS) proteins VirB9-1, VirB9-2 and VirB10 (80). However, that animal did not have T-cell responses to VirB9-1 (80, 81). This could indicate, in a haplotype specific response, that VirB9-1 is associated with another protein(s) that have T-cell epitopes and provides T-cell help. T4SS proteins are not antigenically variable and may form a complex within the \textit{A. marginale} OM targeting them against neutralizing antibody. Having many proteins in a vaccine that are naturally associated in the membrane of \textit{A. marginale} will not only consist of more than one characteristic protein but have a better chance of possessing effective B- and T-cell epitopes.

1.4 What is the Type IV Secretion System?

Targeting protein-protein interactions is an important approach for drug discovery and vaccine development. Protein complexes are involved in all cellular processes and are more likely to undergo linked recognition, enhancing immune responses. Type IV secretion system (T4SS) proteins are ancestrally related to both the bacterial conjugal system and the F-plasmid in \textit{Escherichia coli} and are considered to have evolved for intracellular survival (117). The versatile T4SS translocation family has three subfamilies grouped by function: conjugation, DNA uptake and release, and most interesting effector translocation (118). The T4SS within outer membranes of bacteria bind to the host cell with the pilus or conjugation junction, through juxtaposition or fusion (119). The conjugation system enables bacteria to adapt to changing environments and it is the dominant mechanism for transmission of antibody resistance genes and virulence factors.
T4SS proteins are not antigenically variable because variation of these proteins would be too much of a fitness burden on the organisms that harbor this secretion system (117, 118). Likewise, the function and structure for *A. marginale* T4SS are likely conserved between strains and necessary for survival because the genes remained after reductive evolution (117, 118, 120, 121).

Host cell interactions mediated by T4SS proteins have been identified many other gram negative disease causing bacteria such as *Bordetella pertussis* (whooping cough, ref. 122), *Brucella* spp. (brucellosis, ref. 123, 124), *Helicobacter pylori* (gastric ulcers, ref. 119, 125), *Campylobacter jejuni* (gastroenteritis), *Legionella pneumophila* (Legionnaires’ disease, ref. 126, 127), *Rickettsia prowazekii* (typhus), *Ehrlichia chaffeensis* (human monocytic ehrlichioses), *Anaplasma phagocytophila* (human granulocytic ehrlichioses, ref. 128), *Bartonella henselae* (cat-scratch disease), *Coxiella burnetii* (Q fever), *Actinobacillus* (endocarditis and meningitis), and mostly studied within *Agrobacterium tumefaciens* (causing crown gall disease in plants, ref. 6, 118, 129). T4SS proteins have been classified into two types IVA and IVB, where *A. tumefaciens* is type IVA with the archetypal VirB/D4 (129) and *L. pneumophila* is type IVB with archetypal Dot/Icm (127). The vir transport system of *A. tumefaciens* mediates the transfer of T-DNA on the Ti plasmid into the nuclei of host cells. The *virB/D4* genes encode for 12 proteins called virulence B (VirB)1-11 and VirD4, that make up a 1.1 megadalton complex spanning the inner and outer membrane (130). The core complex is made up of VirB7, VirB9, and VirB10 (130). VirB7 is the smallest component, usually a lipoprotein, it interacts and stabilizes VirB9 via disulphide bonds (131, 132), and also interacts with VirB2, VirB5, VirB6, and VirB10 (133-135). VirB9 is located in the OM and it interacts with VirB10 (136-138) only after VirB10 had bound to nucleotide triphosphate (NTP) binding VirD4 and VirB11, these are an essential set of
interactions for substrate transfer (139, 140). VirB10 forms a homodimer that bridges the entire periplasm and anchors the OM and VirB9 (137). VirB10 has energy sensing ability and interacts with NTPases VirB4, VirB11, and VirD4 (139, 141). VirB6 is a polytopic inner membrane protein with multiple transmembrane spanning regions (142). VirB6 stabilizes VirB3 and VirB5 (143), facilitates VirB7 dimerization and VirB7-VirB9 interactions (133). VirB6 also arranges the export of T-DNA through the channel (144). The majority of VirB8 is located in the perisplasmic space with some inner membrane localization (145-147). VirB8 dimerizes (148) and interacts with half of the Vir components: VirB1, VirB4, VirB5, VirB9, VirB10, and VirB11 (137, 149, 150). Thus, VirB8s role as a scaffold for T4SS pilus, connection to the core complex, and VirB8 has some regions of substrate interaction (146, 148, 151). VirB1, VirB2, VirB3 and VirB5 make up the pilus, are transiently associated, and could participate solely in stabilizing the complex organelle (117, 134, 152, 153). VirB2 comprises the major pilin of the T pilus and are essential components of the mating channel (154, 155). A. tumefaciens VirB2 is made circular through cyclization and it interacts with VirB5 and VirB7 (152, 156, 157). However, VirB2 interaction with VirB5 is dependent on interactions with VirB4 and VirB8 (150). Polymers of VirB2 are critical for substrate transfer and possibly span the entire periplasm (129). VirB4, VirB11, and VirD4 are the primary NTPases (129, 158-160). VirB4 is typically the largest Vir component and yeast two hybrid showed that it interacts with VirB1, VirB8, VirB10, and VirB11 (161). VirD4 is primarily located in the cytosol with two transmembrane regions (162) and a short periplasmic section that interacts with VirB10 (141). VirD4 contains a NTP binding domain and forms a gate that controls substrate translocation through the T4SS apparatus (163, 164). All Rickettsiales have the type IVA archetype VirB/D4 T4SS with genes that are typically organized in two operons (40, 120, 121, 128). For Anaplasma, the first operon has virB3, virB4-1,
followed by four paralogs of virB6 and the second operon includes virB8-2, virB9-2, virB10, virB11, and virD4 (128). Throughout the genome are additional copies of virB4-2, virB8-1, and virB9-1 as well as virB7 and multiple copies of virB2 (40, 120, 121). Genes that encode for proteins similar to VirB1 and VirB5 have not been located (121). The finding that virB and virD genes of Anaplasma were found in two separate loci suggests these genes are independently controlled and two different conditions must be met for the complete T4SS assembly (128). sodB activation may be required for the construction of the loci containing virB3, virB4-2, and virB6-1-4 genes (128), however it has also been found that E. chaffeensis protein EcXR regulates all of the vir islets (165). Another candidate T4SS regulator was identified with bioinformatics gppA (120).

Historically the T4SS has been used by bacteria to transfer effector DNA and proteins into host cells to facilitate infection (6, 118, 158). The effector molecules (DNA or protein) ejected into host cells have a broad range of host-altering functions such as cytoskeletal modifications (166), genome introgression (167), and highjacking of vesicular trafficking (168). For example, the proteins that make up the T4SS of L. pneumophila and Brucella spp, are required for their infection, replication, and survival within host cells (126, 127, 169, 170). The T4SS substrates of the vascular tumor-inducing pathogen Bartonella henselae mediate all cellular changes and subversion of human endothelial cell including invasion into the cell, suppression of apoptosis, and pro-inflammatory activation (171, 172). H. pylori T4SS proteins mediate host-cell invasion (119) and substrate CagA are responsible for lumen-inflammation, ulcers, adenocarcinoma, and mucosal-associated lymphomas (119, 125). The role of the T4SS in A. marginale has not been determined, but it may be required for invasion and survival within erythrocytes and/or tick cells. Using a cre recombinase reporter assay, the T4SS in A.
*phagocytophilum* was shown to secrete a protein called AnkA (173). After secretion, AnkA binds to a tyrosine kinase (Abl-1) via Abi-1. Blocking AnkA or Abl-1 with monoclonal antibodies inhibited *A. phagocytophilum* infection, suggesting AnkA and Abl-1 are required for host cell infection (173). Using VirD4 as bait in a bacterial-two-hybrid assay, Ats-1 was found as another substrate (174). Ats-1 is imported into host neutrophil mitochondria by *A. phagocytophilum* T4SS where it inhibits apoptosis of the host cell (174). It has also been shown for *A. phagocytophilum* that the T4SS is expressed at various stages of infection, including within ticks and mammalian neutrophils (128, 165, 175). Because *Anaplasmataceae* lack lipopolysaccharides (40, 121) several T4SS proteins may be surface exposed where they could be targeted by neutralizing antibody. Furthermore, T4SS proteins interact within the bacterial membrane so associated proteins could provide linked recognition for T cell-B cell interactions.

T4SS proteins are essential for a large number of processes determining bacterial virulence, including auto-aggregation, adhesion, twitching motility, biofilm formation, DNA uptake, and cellular invasion (118, 176). This diverse array of functions makes these proteins attractive candidates for a vaccine, not only against *A. marginale* but also against many other gram negative pathogens. Furthermore, the naturally associated T4SS protein complex that spans the inner and outer membrane provides a model system to examine the importance of membrane protein-protein interactions in stimulating protective immunity and facilitating linked recognition. It is also a new target that has not yet been explored as a vaccine.

### 1.5 Type III Secretion System Proteins are Protective

Even though there is a gap of knowledge for protective efficacy of T4SS proteins, there are several examples showing that the type III secretion system (T3SS) proteins when used as
vaccines protect against the intracellular pathogens. Recombinant membrane secreting protein F (YscF) from *Y. pestis* is a T3SS protein that punctures the host cell membrane, elicits high specific antibody titers, and protection with subcutaneous and intravenous challenge (177). Another virulence factor from *Y. pestis*, the V-antigen substrate is secreted through the T3SS and helps translocate *Yersinia* outer proteins (Yops) into the host. Mice immunized intraperitoneally with recombinant V-antigen had high antibody titers (1: 128,000), specific T-cell response, and upon subcutaneous challenge all rV-antigen immunized mice were protected from *Y. pestis* infection (178, 179). Also, mice immunized with *Y. pestis* T3SS proteins YopB, YopD, YopE and LcrV; were protected against lethal F(-) *Y. pestis* challenges (115, 180). More recently, Tarp a T3SS protein from *C. trachomatis* induced protective Th1 responses that reduced bacterial shedding in mice (181), and immunization with Bsp22, a substrate of the Bordatella T3SS, protected mice against *Bordatella bronchiseptica* infection (182). These studies confirm that immunizations with T3SS proteins are protective, and since they are the similar, the T4SS proteins may also elicit protective immune responses.

1.6 Major Histocompability Complex Class II

Cell-cell interactions of the adaptive immune response are critically important in protection from pathogens. These interactions are orchestrated by the immunological synapse whose primary components are the T cell antigen receptor (TCR) and major histocompatibility complex (MHC) molecules. Cytotoxic T cells (CD8+) recognize peptides bound to class I MHC molecules and helper T cells (CD4+) recognize peptides bound to class II MHC molecules, the latter being the primary focus for *A. marginale* infections. Before T cell recognition of foreign antigens via TCR, CD4+ T lymphocytes must bind to the trimeric complex consisting of
antigenic peptide and MHC class II α- and β-chains located on professional antigen presenting cells (APC), and T cells must also recognize the co-stimulatory molecule(s). The α- and β-polypeptides are assembled in the lumen of the endoplasmic reticulum. For a peptide to be presented on a MHC class II molecule, an APC must first internalize the exogenous antigen bound to its membrane receptor through endocytosis or phagocytosis. The antigen traverses increasingly acidic vesicles containing proteases where it is denatured and processed into peptides. At the final compartment, MIIC, the peptide is loaded onto a generated MHC class II molecule and finally this trimeric complex is re-expressed on the cell surface. B cells that bind to a particular antigen are 10,000 times more efficient at displaying peptide fragments of that antigen on their MHC class II molecules than B cells that do not bind to the antigen. The TCR determines specificity of an interaction based on antigen and MHC molecule and this interaction is activated and stabilized by the T cell accessory molecule CD28 that interacts with CD80 (B7-1) or CD86 (B7-2) on an APC.

Class II MHC molecules are composed of two equal length polypeptide chains (α and β), each chain containing: a cytoplasmic tail for phosphorylation and binding to other cytoskeletal elements, a transmembrane region which anchors the molecules to the cell membrane, conserved α2 and β2 domains that bind to CD4, and a highly polymorphic peptide binding groove formed by α1 and β1 domains. In cattle, the bovine leukocyte antigen (BoLA) locus is located on chromosome 23 (183), and has 2 regions one for DR gene(s) and one for DQ genes (184), and both DR and DQ molecules are involved in antigen presentation. Also, the class II region in cattle is unique from humans as it has a separation of the DR and DQ genes from DO genes, a region also including ruminant-unique non-polymorphic DY genes (183). Bovine DR has one monomorphics chain (DRA) and one polymorphic chain encoded by DRB3, DQ is made up by
two polymorphic chains encoded by *DQA* and *DQB* genes, which are usually duplicated (185, 186). Epitopes bound to the MHC class II molecules range from 11 to greater than 25 residues in length which are buried in the antigen binding groove formed by the MHC molecule, leaving only a few side chains available for direct TCR contact (187). Specific interactions of certain MHC molecules with peptides come from the interaction of peptide side chain moieties with pockets in the floor and ridges on the sides of the binding groove. Whether a particular peptide will bind to the groove depends on the amino acids that line the groove, and because class II molecules are polymorphic different class II molecules will bind to different peptides.

The polymorphism in MHC molecules is determined by an individual’s germline and is important for the survival of the species. BoLA class II molecules are highly polymorphic, there are 104 alleles and 50 types of DRB3, 47 alleles and 20 types of DQA, 52 alleles and 30 types of DQB that have been identified (www.projects.roslin.ac.uk/bola/). Genetic diversity of cattle is permitted through polymorphisms in *DRB3* gene as well as *DQA* and *DQB* gene pairing which can occur within a locus or between duplicated gene loci. This diversity within a single haplotype is used by the immune system to increase antigen epitope-binding repertoire (183, 185, 188). Majority of cattle have heterozygous haplotypes, and some peptides presented to T cells may be restricted to the intra- or interhaplotype pairing of *DQA* and *DQB* genes. Intrahaplotype pairing refers to the combination of DQA and DQB gene products on the same chromosome within the same haplotype. Interhaplotype pairing refers to the combination of DQA and DQB gene products on different chromosomes between haplotypes. DQ restricted epitope pairing has been shown with mice using T cells specific to hen egg lysozyme (189) and later with cattle T cell responses to MSP1a F3-3, MSP2 P25 and MSP2 P12-AM5 peptides (190). Many studies are focused on DR restricted T cell responses, thus the role of DQ in cattle is not well documented.
The importance of both DR and DQ was shown when both anti-DR and anti-DQ antibodies inhibit T cell proliferation as well as many peptide specific CD4+ T cell clones were restricted to DQ molecules (186, 190). Human DQ alleles are associated with auto-immunity and suppressive immune responses. If some DR and DQ alleles are more important than others for antigen presentation, then protective immune responses against a bovine pathogen such as A. marginale are strongly influenced by expressed MHC class II DR and DQ molecules and their characterization is extremely important for effective vaccine development.

Increased protection for pathogens, especially for bacteria that have variable OMVs can be established through immunization with synthetic peptides (191). MHC class II epitope-based vaccines can circumvent evolutionary events favoring immune escape present with native protein vaccines. Inclusion of appropriate (highly antigenic) CD4+ T cell epitopes is essential for vaccine efficacy, as well as the excluding the epitopes that yield a detrimental effect (191). For example, rhesus macaques immunized with recombinant adenovirus 5 vector encoding for Gag, Pol, and Nef from human immunodeficiency virus recognized on average a single epitope per protein (192). T cell specificity may be restricted by how the antigen is processed in the endosomal compartment, MHC class II molecule binding to a limited number of peptides, and the TCR repertoire may be limited to both the peptide and MHC molecule. Therefore, choosing the “right” peptides for a vaccine still takes careful planning. Also for consideration, the identification of MHC allele-specific T cell epitopes may not be enough; vaccine epitopes should effectively cover the BoLA and HLA allelic diversity in both the cattle and human populations.

1.7 Case for T4SS proteins as a vaccine against A. marginale
The ability of bacterial OM to stimulate effective, and sometimes complete, protection against antigenically variable bacteria that are difficult to treat with antibiotics, provides a rationale for identifying the protective components of the membrane for vaccine development. *A. marginale* is a good model organism to study adaptive immune responses directed towards its OM proteins because it doesn’t have a complete biosynthetic pathway for LPS (121), or peptidoglycans (40), and it can escape the immune response through antigenic variation. The paradigm that whole OM from *A. marginale* protect from infection and disease while individual immunodominant proteins do not provide equivalent protection has lead to the discovery of immunogenic T4SS proteins within the OM. Not only are T4SS proteins evolutionally conserved among gram negative obligate intracellular pathogens, they are surface exposed, and shown to be required for intracellular survival and virulence. Inclusion in a vaccine of multiple proteins that are naturally associated in the membrane of *A. marginale*, will not only provide more than one immunogenic protein, but will provide an opportunity for linked recognition; which can increase T-cell dependent IgG responses in out-bred populations that express a broad repertoire of MHC class II molecules. Protecting a large population of genetically heterogeneous individuals requires understanding of MHC class II-restricted epitope presentation. Furthermore, the naturally associated T4SS protein complex that spans the inner and outer membrane provides a model system to examine the importance of membrane protein-protein interactions in stimulating protective immunity and facilitating linked recognition. By crosslinking T4SS proteins that are recognized by T cells and presented by MHC class II to other T4SS proteins that have IgG epitopes, the humoral and cellular immune response can be assessed through linked recognition, thus providing a more “complete” immune response against infection and disease. It has never been assessed if T4SS proteins are a good vaccine against *A. marginale* or any other T4SS-
containing bacteria. These findings direct the research to determine whether T4SS proteins are protective antigens and undergo linked recognition. We hope to show that immunogenic epitopes from naturally associated proteins originating from the T4SS complex used as immunogens will induce stronger protective immune response than immunization with individual T4SS epitopes. The result of this project is a generally applicable vaccine development for many human diseases.

1.8 Hypothesis and Specific aims:

Naturally associated epitopes from T4SS proteins used as immunogens will undergo linked recognition and induce stronger protective immune responses than immunization with individual T4SS epitopes.

1. Characterize A. marginale T4SS proteins for their recognition by T cells and IgG from OM vaccinated cattle with diverse MHC class II haplotypes

2. Identify the immunogenic A. marginale T4SS proteins that are surface exposed and naturally associated with other antigenic T4SS proteins

3. Determine T cell and MHC class II epitopes on the interacting immunogenic T4SS proteins

4. Determine if immunization of peptides containing T cell and MHC class II epitopes linked to B cell antigens will undergo linked recognition and induce a stronger protective immune response as compared to individual T4SS epitopes

1.9 Work Cited


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Chapter 2: Preliminary Information

Some of the information presented in chapter 2 is Copyright © protected by American Society for Microbiology, Infection and Immunity, 2011, Oct. 28. Title: Association and evidence for linked recognition of type IV secretion system proteins VirB9-1, VirB9-2, and VirB10 in *Anaplasma marginale*

2.1 Cattle Haplotypes

Five age-matched Holstein steers and one Holstein cow were purchased from Washington State University (WSU) dairy with varying MHC class II haplotypes. Bovine lymphocyte antigen (BoLA) MHC class II DRB3 alleles were determined by restriction fragment length polymorphism (RFLP) analysis of exon 2 of the DRB3 gene and the alleles were confirmed with cDNA sequencing (Fig. 1, and ref. 1-6). Animals used in this study were cared for according to an approved Institutional Animal Care and Use protocol for WSU and were as follows: 35113, 35141, 35160, 35280, 35287, and 583. Please refer to table 1 for DRB3 RFLP haplotype, DRB3 alleles, and DQ alleles. Our lab is constantly getting new cattle for experiments or blood samples from Washington Holsteins for haplotyping. This RFLP data was compiled to determine the allelic frequencies. We discovered that the haplotypes of all six of the cattle used in this research are the most abundant in Holsteins from Washington (Fig. 2) and Canada (3). Because linked recognition is MHC class II restricted, screening animals with multiple MHC class II alleles will identify which animal(s)/haplotype(s) lack a T-cell response to individual proteins and require protein-protein interaction to gain T-cell help.

2.2 Type IV Secretion System Protein Alignments
Most alignments described below were performed with the Vector NTI 11.5 software, unless otherwise specified. Names of proteins are given in terms of locus name and NCBI reference sequence.

2.2.1 VirB6-1

*A. marginale* VirB6-1 (AM813, YP_153995) was aligned with the VirB6s from* A. phagocytophila *APH0374 (YP_504980), APH0375 (YP_504981), APH0376 (YP_504982), and APH0377 (YP_504983) (alignments not shown). Am813 was most similar with APH0374 at 61%, and was therefore designated as VirB6-1. Four to five copies of VirB6 are found in most *Rickettsia* genomes, making them the most variable and unique components of the *Rickettsia vir* system. The four VirB6 proteins are remarkably different from each other and the only similarity that the proteins have with other bacterial VirB6s is a transmembrane spanning-cytoplasmic region involved in substrate transfer to VirB8 (7). Only the first fragment of VirB6-1 was cloned into pEXP1-DEST vector for expression in *E. coli* because full length VirB6s did not express and made huge constructs (Chapter 3 section 3.2.1).

2.2.2 VirB7

Five VirB7 sequences were aligned with other VirB7 homologues, accession numbers are as follows: Am= *A. marginale* AM306, YP_153652.1; Hpa= *H. pylori* VirB7-1 HPP12_0465, YP_002301101.1; Hpb= *H. pylori* VirB7-2 HPP12_1327, YP_002301958.1; Rt = *R. typhi* hypothetical protein, YP_067241; Cj= *C. jejuni* VirB7, NP_863349. Previously determined structural and/or functional information was superimposed over all alignments (7, 8). The VirB7 alignments were made with ClustalW and adjusted around the conserved cysteine bridge site and the NMR [KI]KSP (7, 9). A leucine-rich repeat region was identified in the *A. marginale* VirB7
sequence (Fig. 3). This motif is present in a number of proteins with diverse functions and cellular locations, and all proteins containing leucine repeats are thought to be involved with protein-protein interactions (10). These alignments revealed amino acid characteristics of *A. marginale* putative VirB7 that are conserved with *H. pylori*, *R. typhi*, *C. jejuni*, and *A. tumefaciens* (Fig. 3).

2.2.3 VirB8-2

An alignment was performed with *A. marginale* Am1316 (YP_154363) and Am747 (YP_153956) and indicated that the similarity of Am1316 with Am747 was only 20%. An alignment with Am1316 and *A. phagocytophila* VirB8s: VirB8-1 (APH0430, YP_505033) and VirB8-2 (APH1406, YP_505898) revealed that Am1316 had 84% similarity with VirB8-2 and only 22% with VirB8-1, thus Am1316 was designated as VirB8-2. Am747 was 63% similar with VirB8-1 and 19% with VirB8-2, thus AM757 was designated as VirB8-1. Gillespie *et al.* reported that VirB8-2 is more conserved across *Rickettsia* genomes than VirB8-1, but both proteins are similar in size, composition, and structure to other bacteria VirB8s (7).

2.2.4 VirB9-1 and VirB9-2

Am097 (YP_153506.1) and Am1315 (YP_154362.1) sequences were aligned with *A. phagocytophilum* VirB9-1 (APH0081, YP_504712) and VirB9-2 (APH1405, YP_505897) (Fig. 4). *A. marginale* Am097 (conjugal transfer protein trbG) was 74% similar to APH0081 and 27% with APH1405, and Am1315 was 30% similar with APH0081 and 57% with APH1405 (Fig. 4). This alignment indicates that the *A. marginale* St. Maries protein annotated as Am097 conjugal transfer protein trbG is homologous to the *A. phagocytophilum* APH0081 VirB9-1, thus Am097
was designated as VirB9-1. Am1315 VirB9 protein was designated VirB9-2 because there is more homology to APH1405 as compared to its homology with APH0081. Gillespie et al. reported that VirB9-1 is more conserved across *Rickettsia* genomes than VirB9-2, but both proteins are comparable to VirB9 from other bacteria. VirB9-2 lacks the necessary cysteine residue to interact with VirB7, and both proteins contain signal peptides (7). *A. marginale* VirB9-1 is only 30% similar to VirB9-2, indicating that VirB9-1 and VirB9-2 are paralogs of each other but also two different proteins (Fig. 4, same as above).

An alignment of VirB9-1 and VirB9-2 amino acid sequences from different strains of *A. marginale* (Puerto Rico, Florida, Virginia, Mississippi) including *A. centrale* was completed to justify the use of these proteins as a vaccine against all strains of *A. marginale* (Fig. 5 A and B). Sequences used for the alignment across different Anaplasma strains are given in terms of locus name (NCBI reference sequence) and are as follows: *A. marginale* St. Maries Am097 (YP_153506.1), Florida (AMF) 070 (YP_002563217.1), Mississippi (AmarM) 010100000373 (ZP_05276855.1), Puerto Rico (AmarPR) 010100000358 (ZP_05277735.1), Virginia (AmarV) 010100000383 (ZP_05278707.1), and the *A. centrale Israel* strain (ACIS) 01168 (YP_003328941.1). Sequences for VirB9-2 alignments were obtained for St. Maries Am1315 (YP_154362.1), AMF_995 (YP_002564069.1), AmarM_010100006032 (ZP_05277643.1), AmarPR_010100005483 (ZP_05278608.1), and AmarV_010100005878 (ZP_05279570.1), and *A. centrale* ACIS_00091 (YP_003328109.1). These alignments were performed with BOXSHADE 3.21 available at [http://www.ch.embnet.org/software/BOX_form.html](http://www.ch.embnet.org/software/BOX_form.html). These alignments revealed that both VirB9-1 and VirB9-2 proteins are highly conserved across *A. marginale* strains. For VirB9-1, amino acid identity is 97-100% (with the exception of the Mississippi strain for which the VirB9-1 sequence is incomplete), and for VirB9-2 the sequence
identity is 100% (Fig. 5 A and B, respectively). The comparison of sequence identities of these proteins with those of the vaccine strain, *A. centrale*, also revealed a high degree of conservation, which was 98-99% identity for VirB9-1 with all *A. marginale* strains (except Mississippi), 92% for VirB9-2. Because of this high degree of amino acid sequence identity, VirB9-1 and VirB9-2 would be a good candidate for inclusion in a cross-protective vaccine such as, with a heterologous challenge, or in a vaccine against a superinfected animal.

Lastly, an alignment of VirB9s with other species harboring T4SS was completed to justify the use of certain peptides within the *A. marginale* VirB9-1 and VirB9-2 proteins to be used as cross-protective vaccines in other species (Fig. 6). The sequences used for these alignments are indicated as the locus name and NCBI reference and as follows: *Ehrlichia chaffeensis* (Ech) Ech0439 (ZP_00544847.1) *E. chaffeensis* Ech0043 (YP_50875.1), *A. tumefaciens* (Agt) AvhB9 (NP_396100.2), Agt VirB9 (NP_396496.1), *Bukholderia sp.* (Bu) Bu6852 (ZP_0645585.1), Bu6564 (ZP_06845297.1), *Brucella sp.* (Br) (ZP_07477893.1), *Bartonella henselae* (Ba) TrwF (YP_034270.1), *E. coli* (Ec) TrwF (emb_CAA57030.1), Ec TraO (AAA86458.1), *Legionella pneumophila* (L) lpa00807 (YP_001252054.1), and L LvhB9 (YP_095279.1). There are certain regions of VirB9 that are highly conserved among other bacterial strains but the similarity is relatively low 20-34% (Fig. 6). VirB9-1 was more conserved to other bacterial VirB9s than VirB9-2, which is the same as Gillespie *et al.* (7) found for rickettsial genes. Both VirB9-1 and VirB9-2 are more similar to the *L. pneumophila* VirB9 than the *A. tumefaciens* VirB9, suggesting that the *A. marginale* T4SS may belong to the dot/icm group IVb T4SS (Fig. 6, and ref. 7). A similar alignment was performed by Ohashi *et al.* in 2002 with *A. phagocytophila* T4SS proteins and the authors suggest that the Vir proteins among obligate intracellular bacteria play a different role than the Vir proteins among facultative
intracellular bacteria (11). In terms of conserved peptides, there are certain regions in each protein that look conserved across VirB9 proteins from different organisms (see black highlighted regions on Fig. 6)

2.2.5 VirB10

For the VirB10 (Am1314, YP_154361.1) alignments across different strains of A. marginale included: ACIS_00092 (YP_003328110.1), AMF_994 (YP_002564068.1), AmarM_010100006027 (ZP_05277642.1), AmarPR_010100005478 (ZP_05278607.1), and AmarV_010100005873 (ZP_05279569.1). Predicted amino acid alignments revealed that VirB10 proteins are highly conserved across A. marginale strains, a VirB10 sequence identity is 100%. (Fig. 7). The comparison of sequence identities of these proteins with those of the vaccine strain, A. centrale was 93% for all VirB10 A. marginale strains. Like, VirB9, VirB10 would be a good candidate for inclusion in a cross-protective vaccine.

The alignment of VirB10 sequence from A. marginale with VirB10 from different species was also performed: APH1404, Ech0042, Agt AvhB10 (NP_396101.1), Agt on plasmid pTiBo142 (YP_001967540.1), Bu Trb1 (ZP_06845586.1), Bu VirB10 (ZP_07344134.1), Br VirB10 (YP_003104865.1), N VirB10 (ZP_06981737.1), L LvhB10 (YP_095278.1), Ba VirB10 (YP_034059.1), Ba TrwE (YP_034271.1), Ec TrwE (emb_CAA5703.1), and Ec TraL on plasmid pSB102 (NP_361040.1). VirB10 was conserved when compared to both Rickettsia and other pathogenic bacteria VirB10s ranging from 18-60% sequence similarity (Fig. 8), particularly within the C-terminal domain and proline-rich N-terminal domain (7). Thus, immunogenic peptides from A. marginale VirB10 could be used a vaccine against A. marginale, B. suis, A. tumefaciens, L. pneumophila, B. henselea, etc. infections.
2.3 Predicted Type IV Secretion System Protein Localizations

Protein localization was predicted using the full length amino acid sequences for *A. marginale* T4SS proteins VirB2, VirB4-1, VirB4-2, VirB6-1, VirB7, VirB8-2, VirB9-1, VirB9-2, VirB10, VirB11, and VirD4. Sequences were put into the following bioinformatic programs: Transmembrane (TMHMM, Fig. 9A), Hydropathy (GREASE, Fig. 9B), and Surface Localization Extracellular Proteins (SLEP) which incorporates Glimmer, TMHMM, PRODIV-TMHMM, LipoP, PSortB (12-21) to predict localization for bacterial proteins (Table 2). A combination of the above programs was necessary to predict the *A. marginale* surface localization of the proteins. Taken all three of these predictions together, VirB2, VirB3, VirB6-1-4, VirB7, VirB8-1, VirB8-2, VirB9-1-2, and VirB10 are likely to be located on the exterior surface of *A. marginale* OM. Surface localized T4SS proteins of *A. tumefaciens* include VirB2, VirB3, VirB7, VirB9, and portions of VirB10 (22). Surface localized proteins are important in the *A. marginale* system because they are targets for neutralizing antibodies.

2.4 Predicted Interacting *A. marginale* proteins

Protein interaction was predicted with a Search Tool for the Retrieval of Interacting Genes (STRING), which quantitatively integrates interaction data from genomic context, high-throughput experiments, co-expression, and previous knowledge to reveal physical and functional associations (23). Predictions for the interactions of VirB9-1, VirB9-2, VirB10, VirB7, MSP5, and MSP2 were assessed (Fig. 11 A-F). The images for VirB9-1 (Fig. 11A), VirB9-2 (Fig. 11B), and VirB10 (Fig. 11C) interactions look very similar which means they are interconnected, and they are all part of the T4SS. VirB9-1 (trbG) is strongly associated with VirB9-2, VirB10, VirD4, VirB4-1 (Am814), VirB8-2, predictions that were based on database searches, related
proteins and their interactions from other species, literature search, and nearest neighbor in the genome. VirB9-1 is also weakly associated with VirB3, VirB4-2 (Am1053), VirB11, thioredoxin (trxA), and Am846 because less evidence in the database search was found. Similarly, VirB9-2 is strongly associated with VirB9-1 (trbG), VirB10, VirB4-1, and VirB8-2 and weaker with VirB11, VirB4-2, VirD4, VirB6-2 (Am812), and GTP cyclohydrolase II (Am1317). VirB10 interacts strongly with VirB9-1, VirB9-2, VirB8-2, VirB4-1, and VirD4 and weaker with VirB11, VirB3, VirB4-2, and GTP cyclohydrolase II. It is not surprising that VirB10 is predicted to interact with VirB4s, VirB11, VirD4 and GTP cyclohydrolase II because in *A. tumefaciens* VirB10 crosses the periplasmic space coupling the core-pilus made up of VirB2, VirB7, VirB8, and VirB9 with NTPases such as VirB11, VirB4 and VirD4 (24).

VirB7 (Am306) is weird and it only appears to have weak associations with Am302, Am303, and phosphatidylglycerophosphatase A (pgpA) (Fig. 11D). These interactions were identified using only the intergenic distance from Am306 and the results are not surprising because VirB7 is only putative.

MSP5 had three strong interactions (based on database, text, genome mining, and the identification of fusion) with protoheme IX farnesyltransferase (ctaB), cytochrome C oxidase subunit 2 (coxB), and Heme A synthase (ctaA) (Fig. 11E). The weaker MSP5 interactions are with similar proteins cytochrome C oxidase subunit I (coxA), cytochrome C oxidase assembly protein (cox11), cytochrome C oxidase subunit III (coxC), surfet locus protein (surf1), phosphate ABC transporter (pstS), secD, and preprotein translocase subunit (yajC). All of the STRING-predicted associations with MSP5 are heme-related and suggest that it may be involved in *A. marginale* erythrocyte adhesion; further evidence for this association is also described in section 2.5.2 below.
MSP2 (Am1144) is only associated moderately with Opag1 and Opag2, and less with Am1141 (Fig. 11F), all of these interactions were based solely on the intergenic distance from MSP2 in the genome. The very little interactions predicted to bind with MSP2 is surprising, as MSP2 is an integral part in the *A. marginale* escape from bovine immune responses (25).

2.5 Predicted Three-Dimensional Models

The three-dimensional structure and function of VirB9-1, VirB9-2, VirB10, MSP5, and MSP2 were also predicted using I-TASSER (Iterative Threading ASSEmbly Refinement), which uses amino acid sequence alignments to generate three-dimensional models and iterative structural assembly simulations where the function can be inferred with known proteins (26, 27). An estimate of accuracy of the predictions is provided based on the confidence score of the modeling. The following scores will be given for each 3D structure: C-score (confidence of structure, ranging from 2 to -5, greater than 1.5 being the best), Tm-score (accuracy of the template model, ranging from 0 to 1, the best scores are greater than 0.17) RMSD (root mean squared deviation, ranges from 0-30Å), and Z-scores (threading alignment, a Z-score >1 represents a confident alignment).

2.5.1 VirB9-1, VirB9-2, and VirB10

Three-dimensional models for VirB9-1, VirB9-2, and VirB10 are in figure 12 A-C. The overall scores for VirB9-1 were as follows, C= -3.22, Tm= 0.35 ±0.12, RMSD= 13.7 ±4.0Å (Fig. 12A); for VirB9-2 was C= -3.5, Tm= 0.35 ±0.11, RMSD= 14.6. ±3.7Å (Fig. 12B); and for VirB10 was C= -1.628, Tm= 0.52 ± 0.15, RMSD= 10.9 ± 4.6Å (Fig. 12C). Even though the confidence scores were low for VirB9-1, VirB9-2, and VirB10, the Tm scores and RMSD are
okay. Yet, a high Z-score around 7 and was fit for all three proteins to the crystal structure of the core T4SS from Chandran et al. (reference picture 3jqoB) for all three proteins (28). This indicates that the 3D structure of the A. marginale VirB9-1, VirB9-2, and VirB10 are likely associated and form a complex similar to the A. tumefaciens core complex.

2.5.2 MSP5

The three dimensional model for MSP5 had a C-score= -1.37, Tm= 0.55 ±0.15, and RMSD 8.4 ±4.5Å (Fig. 12 D). The MSP5 protein 3D structure is mostly similar to a family of proteins belonging to Sco. For example MSP5 predicted structure was similar to the pathogenic mutant (P174L) of human Sco1 with copper bound (Cu(I) P174L-HSco1, PDB ref. picture 2HRN) with a Z-score= 6.62, yeast Sco1 (Cu-ySco1, PDB ref. pic 2B7J) with a Z-score= 5.29, and the Bacillus subtilis Sco1 (PDB ref. pic. 1ON4) with a Z-score= 4.17. The Sco family of proteins is involved with aerobic respiration, specifically in the assembly of the enzyme cytochrome c oxidase (29). These proteins are characterized by a conserved CXXXC sequence motif (located at amino acids 82-86 of MSP5) that binds to copper ions a site also linked to thiol-disulfide oxidoreductase function. Sco1 proteins have thioredoxin-like folds. This also explains the interactions observed with the STRING predictions for MSP5 (above section 2.4). Which, were interactions with cytochrome C oxidases and other enzymes involved in aerobic respiration, also seen to interact with the above listed Sco1 proteins. Also, later in this dissertation one will observe that VirB9-1 interacts with MSP5, demonstrated through far western blotting and immunoprecipitation. This interaction is likely due to MSP5 thioredoxin fold and potential cysteine-thiol reduction properties. Above VirB9-1 was predicted to interact with A. marginale trxA a thioredoxin protein (section 2.4) that may have a role in disulfide bond breakages.
2.5.3 MSP2

The scores for the MSP2 3D model were C-score= -1.84, Tm-score= 0.49 ±0.15, and RMSD= 11.2 ±4.6Å (Fig. 12E). The most accurate model of MSP2 structure in Fig. 12E indicates that MSP2 forms a β-barrel. The predicted β-barrel model for MSP2 was confirmed using TMBETA-GENOME available at http://tmbeta-genome.cbrc.jp/annotation/ (30, 31). β-barrels are well characterized structures found exclusively in the outer membrane of gram-negative prokaryotes (32). β-barrel-forming transmembrane proteins are imperative to a cell's survival owing to their function in controlling the transport of solutes in and out of a cell. They have been classified into six families according to their functions: (i) porins, (ii) passive transporters, (iii), active transporters, (iv) enzymes, (v) defensive proteins, and (vi) structural proteins (30, 33). The model of MSP2 is similar to Escherichia coli outer membrane long-chain fatty acid transporter, FadL (Tm= 0.9), crystallized using Pseudomonas aeruginosa FadL (34), and FadL subfamily including Ralstonia picketti TbuX and Pseudomonas putida TodX (Tm-score= 0.7) (35). Each of these proteins have β-barrel structures and are thought to transport hydrophobic substrates from extracellular environment into the periplasm, and these transporters have implications for roles in multi-drug resistance, combating bacterial infections, and bioremediation xenobiotics. Of similar function E. coli OmpG (Tm= 0.5, BS= 0.96) (36, 37), Neisseria meningitides NaIP (Tm= 0.45, BS= 1.16) (38), E. coli FhuA (BS= 0.8) (39), E. coli FecA (BS= 1.62) (40), and E. coli BtuB (BS= 0.91) (41) were also detected, placing MSP2 in the same category, active transporters, specifically of siderophores. Outer membrane proteins like OmpC, OmpF, and OmpG are non-selective in what they transport through their pores, others like LamB, FhuA, BtuB, are solute specific. One other protein that MSP2 model was similar to,
that is not part of the same transporter family, was *Neisseria meningitides* surface protein A (NspA) (*Z*-score= 3.55), which was crystallized in 2003 (42) forming an eight-stranded anti-parallel β-barrel with four putative surface exposed loops. Later, it was found that NspA binds to a complement inhibitory protein, factor H, enabling meningococcus to resist complement-dependent killing in human host (43). NspA is a vaccine candidate for *N. meningitides* because it is highly conserved among meningococcal strains and induces bactericidal antibodies (44, 45).

2.6 Work Cited


2.7 Tables

TABLE 1: *DRB3*, *DQA*, and *DQB* alleles identified in the cattle used in this study.

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TABLE 2. Results from Surface Localized Extracellular Protein (SLEP) Algorithm Predictions

<table>
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| Surface exposed (exported) | Am030 (VirB2)  
                          | Am097 (VirB9-1)  
                          | Am1315 (VirB9-2)  |
| Membrane-associated   | Am306 (VirB7)  
                          | Am1316 (VirB8-2)  
                          | Am1312 (VirD4)  
                          | Am813 (VirB6-1)  
                          | Am1314 (VirB10)  |
| Lipo Proteins          | None                                               |
| Not in output          | Am814 (VirB4-1)  
                          | Am1053 (VirB4-2)  
                          | Am1313 (VirB11)  |

\(^a\) The indicated T4SS proteins were subjected to analysis using the SLEP algorithm specifically designed for gram-negative bacteria. Predicted exported, lipoproteins, inner membrane, outer membrane proteins are listed. Those not in the output are not predicted to be membrane-associated proteins.
2.8 Figures and Figure legends

**Figure 1.** Images of RFLP analysis of exon 2 of the DRB3 gene. Digested cDNA was run on 12% polyacrylamide gels and incubated with ethidium bromide for analysis. Each animal number is indicated at the bottom of each panel, cDNA loaded in lane 1 was digested with RsaI, lane 2 digested with BstYI, and lane 3 was digested with HaeIII. Also indicated above each lane is the observed splicing pattern used to determine the RFLP DRB3 haplotype from known patterns schemes available at [http://www.projects.roslin.ac.uk/bola/drb3pcr.html#rsa](http://www.projects.roslin.ac.uk/bola/drb3pcr.html#rsa).

**Figure 2.** MHC class II allelic frequency of Holstein Cattle in WA. Y-axis is the frequency the MHC class II allele occurs independent of the heterozygous pair, N=878.
Figure 3. Multiple sequence alignment of five VirB7 sequences. Coordinates for each sequence are shown to the right, with numbers in parentheses depicting flanking residues of the alignment not shown. Strains are represented as Am= *A. marginale* AM306, Hpa= *H. pylori* VirB7-1, Hpb=*H. pylori* VirB7-2, Rt = *R. typhi* hypothetical protein, and Cj= *C. jejuni* VirB7. The orange C, is the cysteine residue that makes up the disulphide bridge with VirB9/VirB9-like proteins. The shared "[KI]KSP" in a black region indicates the motif involved with VirB9 (TraO)/VirB7 (TraN) interaction in *E. coli* (IncN plasmid R46) determined with NMR. All alignments were adjusted around the C and [KI]KSP regions. Identical amino acids are shown as white text on a black background, conserved amino acids are shown as black letters on a gray background, and non-conserved amino acids are shown as black letters on a white background. Dashes indicate gaps in the sequence. The highlighted yellow region for Am306 indicates the leucine repeats involved in protein-protein interaction.
Figure 4. *A. marginale* VirB9s sequence alignment with *A. phagocytophilum* VirB9s. CTP (Am097) and Am1315 (VirB9) sequences were aligned with *A. phagocytophilum* VirB9-1 (APH0081) and VirB9-2 (APH1405). Percent identity is located within the text. Identical amino acids are shown as white text on a black background, as well as with black text and dark gray background, conserved amino acids are shown as black letters on a light gray background, and non-conserved amino acids are shown as black letters on a white background. Dashes indicate gaps in the sequence.
Figure 5. Alignment of VirB9-1, and VirB9-2 amino acid sequences from *A. marginale* and *A. centrale* strains. The BOXSHADE 3.21 program was used to align sequences for A) VirB9-1 and B) VirB9-2. Strains are represented as AmarPR: *A. marginale* Puerto Rico, AmarV: *A. marginale* Virginia, AmarM: *A. marginale* Mississippi, AMF: *A. marginale* Florida, AM: *A. marginale* St. Maries, and ACIS: *A. centrale* Israel. Each sequence is followed by the locus number for the given sequence. Identical amino acids are shown as white text on a black background, conserved amino acids are shown as white letters on a gray background, and non-conserved amino acids are shown as black letters on a white background. Dashes indicate gaps in the sequence.
Figure 6. VirB9 sequence alignment across species. Strains are represented as follows: *A. marginale* (Am), *Ehrlichia chaffeensis* (Ech), *A. tumefaciens* (Agt), *Bukholderia sp.* (Bu), *Brucella sp.* (Br), *Bartonella henselae* (Ba), *E. coli* (Ec), and *Legionella pneumophila* (L). Each strain is followed by one or two different locus name for one or two different VirB9 proteins. Identical amino acids are shown as white text on a black background for both VirB9-1 and VirB9-2, identity to VirB9-1 is also shown with white text on blue background, and identity to VirB9-2 is also shown with white text on red background. Conserved amino acids are shown as white letters on a gray background and non-conserved amino acids are shown as black letters on a white background. Dashes indicate gaps in the sequence. At the end of the sequence alignments is the percent identity of that species VirB9 sequence to VirB9-1 (Am097) followed by VirB9-2 (Am1315).
| Aml314 | 1 | SKEPVIEKQVDKKLKESEAPQETAPRILTPPPKLPDLPLPLVMPTAPELPTLARIK  |
| AmaFR_010100005470 | 1 | SKEPVIEKQVDKKLKESEAPQETAPRILTPPPKLPDLPLPLVMPTAPELPTLARIK  |
| AmaV_010100005873 | 1 | SKEPVIEKQVDKKLKESEAPQETAPRILTPPPKLPDLPLPLVMPTAPELPTLARIK  |
| AmaMr_010100006027 | 1 | SKEPVIEKQVDKKLKESEAPQETAPRILTPPPKLPDLPLPLVMPTAPELPTLARIK  |
| AMF_994 | 1 | SKEPVIEKQVDKKLKESEAPQETAPRILTPPPKLPDLPLPLVMPTAPELPTLARIK  |
| ACIS_00092 | 1 | SKEPVIEKQVDKKLKESEAPQETAPRILTPPPKLPDLPLPLVMPTAPELPTLARIK  |
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| AmaFR_010100005470 | 61 | KKKKEEVVSETKEILPAAASFEPEELQRRPMEDGDPQHPIMMPYRPGGGAIPFPVPSF  |
| AmaV_010100005873 | 61 | KKKKEEVVSETKEILPAAASFEPEELQRRPMEDGDPQHPIMMPYRPGGGAIPFPVPSF  |
| AmaMr_010100006027 | 61 | KKKKEEVVSETKEILPAAASFEPEELQRRPMEDGDPQHPIMMPYRPGGGAIPFPVPSF  |
| AMF_994 | 61 | KKKKEEVVSETKEILPAAASFEPEELQRRPMEDGDPQHPIMMPYRPGGGAIPFPVPSF  |
| ACIS_00092 | 61 | KKKKEEVVSETKEILPAAASFEPEELQRRPMEDGDPQHPIMMPYRPGGGAIPFPVPSF  |
| Aml314 | 120 | LGYDREKGRTPMLVLGGGCPDGSPEDGQGQDTSDFSWSTLDTDGSTSSSSEVAKRAGDDPG  |
| AmaFR_010100005470 | 120 | LGYDREKGRTPMLVLGGGCPDGSPEDGQGQDTSDFSWSTLDTDGSTSSSSEVAKRAGDDPG  |
| AmaV_010100005873 | 120 | LGYDREKGRTPMLVLGGGCPDGSPEDGQGQDTSDFSWSTLDTDGSTSSSSEVAKRAGDDPG  |
| AmaMr_010100006027 | 120 | LGYDREKGRTPMLVLGGGCPDGSPEDGQGQDTSDFSWSTLDTDGSTSSSSEVAKRAGDDPG  |
| AMF_994 | 120 | LGYDREKGRTPMLVLGGGCPDGSPEDGQGQDTSDFSWSTLDTDGSTSSSSEVAKRAGDDPG  |
| ACIS_00092 | 120 | LGYDREKGRTPMLVLGGGCPDGSPEDGQGQDTSDFSWSTLDTDGSTSSSSEVAKRAGDDPG  |
| Aml314 | 179 | YVILQGHMDAVLETAINSDITPGVLRAIVSDVAYEACRGVMIPKGSRSLGYSFTASSGN  |
| AmaFR_010100005470 | 179 | YVILQGHMDAVLETAINSDITPGVLRAIVSDVAYEACRGVMIPKGSRSLGYSFTASSGN  |
| AmaV_010100005873 | 179 | YVILQGHMDAVLETAINSDITPGVLRAIVSDVAYEACRGVMIPKGSRSLGYSFTASSGN  |
| AmaMr_010100006027 | 179 | YVILQGHMDAVLETAINSDITPGVLRAIVSDVAYEACRGVMIPKGSRSLGYSFTASSGN  |
| AMF_994 | 179 | YVILQGHMDAVLETAINSDITPGVLRAIVSDVAYEACRGVMIPKGSRSLGYSFTASSGN  |
| ACIS_00092 | 179 | YVILQGHMDAVLETAINSDITPGVLRAIVSDVAYEACRGVMIPKGSRSLGYSFTASSGN  |
| Aml314 | 239 | NTGVTSWRSVILPHIGDIQINSAGTDELRGSAGFDITKCMNVLSTLLAGVSMGTA  |
| AmaFR_010100005470 | 239 | NTGVTSWRSVILPHIGDIQINSAGTDELRGSAGFDITKCMNVLSTLLAGVSMGTA  |
| AmaV_010100005873 | 239 | NTGVTSWRSVILPHIGDIQINSAGTDELRGSAGFDITKCMNVLSTLLAGVSMGTA  |
| AmaMr_010100006027 | 239 | NTGVTSWRSVILPHIGDIQINSAGTDELRGSAGFDITKCMNVLSTLLAGVSMGTA  |
| AMF_994 | 239 | NTGVTSWRSVILPHIGDIQINSAGTDELRGSAGFDITKCMNVLSTLLAGVSMGTA  |
| ACIS_00092 | 239 | NTGVTSWRSVILPHIGDIQINSAGTDELRGSAGFDITKCMNVLSTLLAGVSMGTA  |
| Aml314 | 299 | FTVSICPOQSEIKDTTREGEKKEEKKEQLTVKIVSDAVKFDESMEKMKIRKYDTS  |
| AmaFR_010100005470 | 299 | FTVSICPOQSEIKDTTREGEKKEEKKEQLTVKIVSDAVKFDESMEKMKIRKYDTS  |
| AmaV_010100005873 | 299 | FTVSICPOQSEIKDTTREGEKKEEKKEQLTVKIVSDAVKFDESMEKMKIRKYDTS  |
| AmaMr_010100006027 | 299 | FTVSICPOQSEIKDTTREGEKKEEKKEQLTVKIVSDAVKFDESMEKMKIRKYDTS  |
| AMF_994 | 299 | FTVSICPOQSEIKDTTREGEKKEEKKEQLTVKIVSDAVKFDESMEKMKIRKYDTS  |
| ACIS_00092 | 299 | FTVSICPOQSEIKDTTREGEKKEEKKEQLTVKIVSDAVKFDESMEKMKIRKYDTS  |
| Aml314 | 358 | KPTIYIDQGTVMKVFVQNDIVFPREAVRR  |
| AmaFR_010100005470 | 358 | KPTIYIDQGTVMKVFVQNDIVFPREAVRR  |
| AmaV_010100005873 | 358 | KPTIYIDQGTVMKVFVQNDIVFPREAVRR  |
| AmaMr_010100006027 | 358 | KPTIYIDQGTVMKVFVQNDIVFPREAVRR  |
| AMF_994 | 358 | KPTIYIDQGTVMKVFVQNDIVFPREAVRR  |
| ACIS_00092 | 358 | KPTIYIDQGTVMKVFVQNDIVFPREAVRR  |
Figure 7. Alignment of VirB10 amino acid sequences from *A. marginale* and *A. centrale* strains. The BOXSHADE 3.21 program was used to align sequences. Strains are represented as AmarPR: *A. marginale* Puerto Rico, AmarV: *A. marginale* Virginia, AmarM: *A. marginale* Mississippi, AMF: *A. marginale* Florida, AM: *A. marginale* St. Maries, and ACIS: *A. centrale* Israel. Each sequence is followed by the locus number for the given sequence. Identical amino acids are shown as white text on a black background, conserved amino acids are shown as white letters on a gray background, and non-conserved amino acids are shown as black letters on a white background. Dashes indicate gaps in the sequence.
Figure 8. VirB10 sequence alignment across species. Strains are represented as follows: *A. marginale* (Am), *Ehrlichia chaffeensis* (Ech), *A. tumefaciens* (Agt), *Bukholderia sp.* (Bu), *Brucella sp* (Br), *Bartonella henselae* (Ba), *E. coli* (Ec), and *Legionella pneumophila* (L). Each strain is followed by a locus name VirB10 protein. Identical amino acids are shown as white text on a black background, conserved amino acids are shown as black letters on a gray background, and non-conserved amino acids are shown as black letters on a white background. Dashes indicate gaps in the sequence. At the end of the sequence alignments is the percent identity of that species VirB10 sequence to *A. marginale* VirB10 Am1314.
Figure 9. Bioinformatic predictions for the *A. marginale* surface localization of VirB2 (ORF X), VirB4-1, VirB4-2, VirB6-1, VirB7, VirB8-2, VirB9-1, VirB9-2, VirB10, VirB11 and VirD4. (A) Transmembrane hidden Markov model (TMHMM) prediction for the T4SS proteins showing transmembrane spanning regions (red), exposed regions to outside of the cell or not predicted to span the membrane (pink), and periplasmic regions (blue). Individual proteins are indicated at the top of each graph. (B) Hydropathic profiles of the indicated full-length *A. marginale* T4SS proteins are presented. Amino acid position is indicated on the x-axis and the hydropathy index is indicated on the y-axis. Individual proteins are indicated at the bottom of each graph. Hydrophobic moieties are indicated above the line, and hydrophilic moieties are indicated below the line.
Figure 10. Predictions for interacting proteins. Full length amino acid sequences were analyzed using STRING for A) VirB9-1 (trbG), B) VirB9-2, C) VirB10, D) VirB7, E) MSP5, and F) MSP2 (Am1144) each indicated by the red ball. Predictions were based on database searches, related proteins and their interactions from other species, literature search, and nearest neighbor in the genome. Tightly associated proteins are indicted by more lines connecting the two proteins, as stronger search evidence was available. Size of the ball is not relative to its interactions, the size indicates if that protein has a homologous crystal structure.

![Predictions for interacting proteins](image1.png)

Figure 11. Predictions for three dimensional structures. Full length proteins were subjected to I-Tasser which generated three-dimensional models and iterative structural assemblies simulations inferred with known proteins for A) VirB9-1, B) VirB9-2, C) VirB10, D) MSP5, and E) MSP2. Scores of the model are indicated in the text.

![Predictions for three dimensional structures](image2.png)
Chapter 3: Characterize *A. marginale* T4SS Proteins for their Recognition by T cells and IgG from OM Vaccinated Cattle with Diverse MHC class II Haplotypes

Some of the information presented in chapter 3 is Copyright © protected by American Society for Microbiology, Infection and Immunity, 2011, Oct. 28. Title: Association and evidence for linked recognition of type IV secretion system proteins VirB9-1, VirB9-2, and VirB10 in *Anaplasma marginale*

3.1 Brief Introduction

*Anaplasma marginale* is an obligate intra-erythrocyte gram negative bacterium causing bovine anaplasmosis. The pathogen invades erythrocytes, replicates intracellularly, and causes acute anemia, which when controlled, results in lifelong persistent infection. *A. marginale* has several well characterized immunodominant major surface proteins (MSP): MSP1, MSP2, MSP3, MSP4, and MSP5 (1-6). When used as immunogens, none of the MSPs induce complete protection against *A. marginale* infection (7-11), whereas immunization with outer membranes (OM) and crosslinked OM can (12, 13). Because immunodominant MSPs are not protective, we have focused on identifying subdominant and conserved surface proteins, including those that associate within the membrane that may be important components of the protective OM vaccine. Among these subdominant antigens are type IV secretion system (T4SS) proteins. Several T4SS proteins induced CD4+ T cell responses, including IFN-γ production, and were recognized by IgG2 in cattle immunized with the OM vaccine (14-16). These type 1 immune responses are associated with protection against *A. marginale* infection in cattle immunized with OM (10, 17, 18).
The T4SS is a 1.1 megadalton protein complex that spans the outer and inner bacterial membrane and has been most widely studied in *Agrobacterium tumefaciens* (19, 20). Typically the T4SS is made up of 12 interacting VirB/D membrane proteins, several of which are likely to be surface exposed (21, 22) and are thus targets for neutralizing and protective immune responses. The T4SS proteins harbored by *A. marginale* and other members of the family *Anaplasmataceae* are unique because they are encoded by two copies of *virB4*, *virB8*, and *virB9*, four or more copies of *virB6*, and multiple copies of *virB2* split into multiple islets across the genomes. There are only single copies of *virB3*, *virB7*, *virB10*, *virB11*, and *virD4* whereas homologues to *virB1* and *virB5* have not been identified (16, 23-25). Furthermore, T4SS proteins interact within the bacterial membrane so associated proteins could provide linked recognition for T cell-B cell interactions.

Chapter 3 reports the immunogenicity of eleven *A. marginale* type IV secretion system proteins: VirB2, VirB4-1, VirB4-2, VirB6-1, VirB7, VirB8-2, VirB9-1, VirB9-2, VirB10, VirB11, and VirD4 for six outer membrane immunized cattle expressing diverse major histocompatibility complex class II molecules. Identifying the T cell and B cell antigens helps narrow down the candidate T4SS proteins that could undergo linked recognition and be involved in native protein-protein interactions (presented in chapter 4). Linked recognition has been studied since the 1970’s by Mitchison, who revealed that both T cells and B cells must recognize antigenic determinants on the same molecule for B-cell activation to occur (26-28). T cells help B cells to undergo somatic hypermutation and isotype switching through cognate interaction and cytokine secretion. However, linked recognition can also occur with two associated proteins, where one has B cell epitopes and a second provides T cell epitopes. For *A. marginale*, this was shown in a study with MSP1, a heterodimer consisting of covalently associated MSP1a and
MSP1b (29-31). In these studies, MSP1a-specific T cells provided help to B cells specific for MSP1b to promote increased IgG production (30, 32). Immunization with physically associated B- and T-cell antigens achieves long lasting immunological memory to the B-cell antigen, and is a common strategy used for vaccination against bacterial polysaccharides that lack T cell epitopes (33, 34).

3.2 Materials and Methods

3.2.1 Expression of recombinant T4SS proteins and MSA1

The negative control protein Babesia bovis merozoite surface antigen-1 (MSA1) (35) was expressed and purified using the amplified gene from Mo7 genomic DNA (gDNA) with the forward primer (GCCGATACCTTCAATCGTCCTTCC) and reverse primer without a stop codon (TTTGTCGTCGTCGTCTTTATAGTCTGTACCCGTGTTGCTCTTGAGG) encoding a C-terminal FLAG epitope (DYKDDK) as indicated in bold letters. Amplification from gDNA consisted of 40 cycles of melting at 94°C for 30 sec, annealing at 58°C for 30 sec, and elongation at 72°C for 2 min. The plasmid DNA was excised from agarose gels and purified using a Genelute agarose column (Sigma-Aldrich). Amplicons were cloned into the pBAD/TOPO ThioFusion expression vector (Invitrogen) following the manufacturer's protocol, and plasmid DNA was extracted from E. coli using the Wizard Plus SV Miniprep DNA purification system (Promega) and sequenced.

Full length recombinant T4SS proteins were expressed from the A. marginale St. Maries strain. Genes encoding virB9-1 (AM097), virB9-2 (AM1315), and virB10 (AM1314) were cloned into pBAD/TOPO ThioFusion vector and expressed as previously described with C-terminal FLAG and 6x His tags (15) and purified to maintain native conformation of the protein, described below. Recombinant VirB7 (AM306) with just a C-terminal 6x His tag was also
cloned into the pBAD-Topo/Thio fusion vector and expressed and purified as previously described (16). Genes encoding for \textit{virB2} (AM030), \textit{virB4-1} (AM814), \textit{virB4-2} (AM1053), the first fragment of \textit{virB6-1} (AM813 F1), \textit{virB8-2} (AM1316), \textit{virB11} (AM1313), and \textit{virD4} (AM1312) were cloned into the pEXP1-DEST vector (Invitrogen), with N-terminal 6x His and C-terminal FLAG tags, expressed in BL-21 DE3 pLysS One Shot chemically competent \textit{E.coli} (Invitrogen), and purified to maintain native conformation of the protein. Primers used for all of these proteins were listed previously (16). Plasmid DNA was extracted from \textit{E. coli} and sequencing was performed using the Big Dye kit and ABI PRISM automated sequence (Applied Biosystems).

Colonies with the correct sequence were grown to mid log phase in LB broth containing 50 μg/ml carbenicillin and induced using 0.2% arabinose for pBAD/TOPO ThioFusion constructs or 1-3 mM isopropyl β-D-1-thiogalactopyranoside for the pEXP1-DEST constructs. LB broth used for the expression of rVirB8-2 contained both 50 μg/ml carbenicillin and 34 μg/ml chloramphenicol. Induced \textit{E. coli} were collected by centrifugation at 3800 x g, and pellets were resuspended in denaturing lysis buffer (6 M guanidine HCl, 20 mM sodium phosphate and 500 mM NaCl at pH 7.8), sonicated twice at maximum strength for 3 min, and frozen overnight at -80°C.

3.2.2 Purification of recombinant proteins

All proteins, except rVirB7, were purified using the hybrid conditions following the Probond Nickel Purification Kit instructions (Invitrogen). Briefly, the denatured and sonicated \textit{E. coli} lysate containing the expressed recombinant T4SS proteins were passed through a prepared nickel column several times. Recombinant T4SS proteins, now attached by their affinity of 6x
His tag to the nickel resin were washed with at least 2 column volumes of lysis buffer, and increasing concentrations of imidizole up to 200 mM. The recombinant proteins were eluted with 5-10 ml of 1 M imidazole and immediately dialyzed first against double distilled deionized water then against phosphate buffered saline (PBS), pH 7.2. The concentrations of the recombinant T4SS proteins were determined with Quick Start Bradford Protein Assay (Bio-Rad), and the purity was assessed using 1 μg/well protein separated on Coomassie-stained pre-cast sodium doedecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) 4-20% gradient gels (Bio-Rad). Western blotting was also performed using electrophoretically transferred proteins and bands detected with horseradish peroxidase (HRP) conjugated anti-His (Qiagen) or alkaline phosphatase (AP) conjugated anti-FLAG (Invitrogen) monoclonal antibodies (MAb), diluted to 1: 2,000, and appropriate substrates.

3.2.3 Isolation of *A. marginale* OM

Intact St. Maries *A. marginale* were isolated as previously described (12) from splenectomized bovine C31919 blood with 40.4% parasitemia. Intact Anaplasma was embedded in epoxy resin, sections cut and visualized on the JOEL transmitting electron microscope to ensure the bacteria were intact and to assess degree of purification from erythrocytes. OM were isolated from the intact *A. marginale* as previously described (36, 37). To confirm the presence of *A. marginale* major surface proteins and absence of erythrocyte membranes 10 μg per well of OM were loaded on 4-20% gradient gels, separated by SDS-PAGE, and analyzed with several monoclonal antibodies (mAb) against *Anaplasma* outer membrane proteins used at 2 μg/ml: anti-mAb MSP2 115/362.17.19, mAb anti-MSP5 AnaF16C1 (WSU monoclonal antibody center), polyclonal anti-VirB9-1, polyclonal anti-VirB9-2, polyclonal anti-VirB10 (made in house see
chapter 4 section 2.1), and Ana8a an anti-erythrocyte mAb (WSU monoclonal antibody center). A dilution of 1:10,000 was used for the secondary antibody Alkaline phosphatase (AP) conjugated goat anti-mouse IgG+IgM (Invitrogen) and AP conjugated goat anti-rabbit IgG (H+L) (Invitrogen) with the substrate Western-Star chemiluminescence (Applied Biosystems). The isolated OM were used for immunization of cattle, as a positive control in T cell proliferation assays, in immunoblotting assays, and later for experiments outlined in chapter 4.

3.2.4 Immunization of six haplotype-diverse cattle with A. marginale OM

Five age-matched Holstein steers and one Holstein cow were purchased from the Washington State University (WSU) dairy with varying major histocompatibility complex class II (MHC class II) haplotypes, and typed as described in the preliminary data section 2.1. Animals (DRB3 RFLP types) used in this study were cared for according to an approved Institutional Animal Care and Use Center (IACUC) Protocol for WSU and were designated as follows: 35113 (11/22), 35141 (22/24), 35160 (3/16), 35280 (16/27), 35287 (16/22), and 583 (8/23). It is important to note that the haplotypes represented by the six OM-immunized cattle are the most abundant haplotypes of Holsteins in the Washington and Canada (preliminary data section 2.1 and ref. 38). Cattle were immunized 4 times subcutaneously with 60 μg of OM emulsified in saponin at 0, 2, 4, and 8 weeks, as previously described (36). All six cattle produced specific antibody, determined by immunoblotting, and had statistically significant peripheral blood mononuclear cell (PBMC) proliferative responses to A. marginale OM, as compared to pre-immunization responses.

3.2.5 Determination of serological responses to T4SS proteins by immunoblotting
Two different immunoblots were performed to determine if the six OM-immunized cattle produced antibodies that recognize the purified rT4SS proteins. Purified recombinant T4SS proteins (1-2 μg) were loaded onto a 4-20% precast gradient gel (Bio-Rad) with denaturing sample buffer (8.5 mM Tris, 15% SDS, 50% glycerol, 12% β-mercaptoethanol, 0.1% bromophenol blue), electrophoresed at 100 V for 1.5 h, and transferred to a nitrocellulose membrane (Bio-Rad) at 100 V for 1 h. The proteins were blocked on the membrane overnight with blocking buffer (10 mM Tris-HCl, 150 mM NaCl, 0.2% Tween-20, 1% PVP40) or with I-block (PBS containing 0.2% I-block (Thermo Scientific) and 0.1% Tween-20). OM-immunized bovine sera that had been extensively adsorbed with protease-inhibited and sonicated BL21 or Top10 *E. coli* (Invitrogen) cells that contained either no vector or a vector of MSA1 were diluted 1:100 and added to the membrane for 1 h. The membrane was washed three times with either wash buffer (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20, 1% PVP40) or with I-block. For membranes blocked with blocking buffer and washed with wash buffer the secondary antibody was 1:20,000 dilution of horse radish peroxidase (HRP) conjugated recombinant protein G (Zymed) or for membranes incubated in I-block the secondary antibody was goat anti-bovine IgG (H+L) conjugated to HRP (Kirkegaard & Perry Laboratories) applied to the corresponding membrane for 1 hr. It was necessary with both protocols to wash extensively with washing buffer or I-block after the secondary antibody. The substrates used were a 1:1 diluted ECL western blotting reagent (Thermo Scientific) applied for 2 min for both protocols. Absorbed- pre-immune sera from all the animals were also used at 1:100 dilution on the western blots containing all the T4SS proteins to rule out non-specific IgG binding.

We used both above protocols in trying to reduce the background, and we determined that the best protocol was when using protein G-HRP (Zymed) as the secondary antibody and
corresponding buffers, listed above. Goat anti-bovine IgG (H+L) bound non-specifically to a 100 kDa band of *A. marginale* OM. The 100kDa background band was also excised from a Coomassie gel and its identity was determined by tandem MS (method described in chapter 4.2.5) to be either MSP3 (Mascot score 274) or bovine band 3 protein (Mascot score 168), each protein having a mass around 100 kDa.

After positive IgG responses were identified for a given animal and protein, positive proteins were separated by SDS-PAGE, transferred to nitrocellulose, blocked with blocking buffer, and developed with absorbed sera diluted 1:300- 1:10,000 and HRP conjugated recombinant protein G.

3.2.6 T cell proliferation assays to recombinant T4SS proteins

T cell lines specific for *A. marginale* OM were used in proliferation assays to measure T4SS protein-specific responses. PBMC were collected from the six haplotype-diverse OM-immunized cattle using standard Ficoll overlay method and then depleted of CD8+ cells and γδ T-cells. For the depletion, each of the following sodium azide free- mAb were diluted to 3 μg/ml: anti-CD8α IgG2a (7C2B), anti-CD8α IgM (BAQ111A), anti-TCRδ IgG2b (GB21A), and anti-TCRδ IgM (CACT61A) obtained from WSU Monoclonal antibody Center. The above antibody cocktail was added to 1 x 10^8 PBMC for 20 min on ice followed by 30 min of complement lysis with 1:10 HLA-ABS freeze dried rabbit complement (Dynal Biotech) at 37°C. The efficency of the depletion was confirmed by FACS analysis, which showed 93.4-96.7% CD4+ T cells, 1.1-2.4% CD8+ T cells, and 2.2-2.4% γδ T cells. Two-week cell lines were generated from 4 x10^6 CD8- and γδ- T cell-depleted PBMC stimulating for one week with 5 μg/ml St. Maries OM cultured in complete (c) RPMI-1640 medium (Gibco) 1.5 ml-wells in a 37°C 5% CO₂ incubator.
The stimulated CD4+ T cells were washed and “rested” during the second week in a culture with 7 x 10⁵ T cells and 2 x 10⁶ irradiated, autologous antigen presenting cells (APCs) in a 24-well plate. APCs were made by irradiating autologous PBMC with ⁶⁰Co at 6 inches for 3,000 rads. After one week of “rest” the CD4+ T cells from the individual steers were used in a proliferation assay with the purified recombinant T4SS proteins and appropriate controls. Proliferation assays were performed in triplicate wells with 100 μl culture in 96-well round bottom plates for 3-4 days, using 3 x 10⁴ T cells and 2 x 10⁵ irradiated autologous PBMC as a source of APCs per well. The rT4SS proteins were diluted to 1 and 10 μg/ml in cRPMI-1640 medium. Positive controls include A. marginale OM and 10% T cell growth factor and negative controls included uninfected red blood cell membranes (URBC) and B. bovis MSA1 also used at 1 and 10 μg/ml. T-cell proliferation was quantified by incorporation of 0.25 μCi/well ³H-thymidine (Dupont, New England Nuclear) during the last 6-18 hours of culture. The radiolabeled DNA was harvested (Tomtec Cell harvester) on glass filters and the emitted β-particles were counted with liquid scintillation. All assays were repeated at least five times on different days. Results are presented as stimulation index (SI), calculated by dividing the mean counts per minute (cpm) of cell cultured with antigen divided by the mean cpm of cells cultured with medium. The SI of the different T4SS antigens were compared to the SI for the matching concentration of MSA1 with Dunnett’s test, a one-way multiple comparisons. Statistically significant T cell stimulation by an antigen was set at a P-value < 0.05.

3.2.7 Detection of Interferon gamma (IFN-γ) in supernatants

Supernatants were harvested from the third day of cultured T cell lines and APCs stimulated with 1 μg of OM, VirB9-1, VirB9-2, VirB10 or MSA1. Cell supernatants were diluted
1:20 if they had strong T cell proliferation and diluted 1:4 if the T cell proliferation was weak or insignificant. The levels of IFN-γ in supernatants were determined by a commercial ELISA kit (Bovigam, Prionics AG, Parkville, Victoria, Australia) according to manufacture’s protocol. The IFN-γ activity for each supernatant was determined by comparison with a standard curve obtained from supernatant from Mycobacterium bovis- purified protein derivative-specific T cell clone, which contained 440 U IFN-γ/ml (previously determined by the neutralization of vesicular stomatitis virus) (39, 40). The results are presented as units of activity of IFN-γ per ml supernatant. The quantities of IFN-γ produced by each animal in response to the antigens were correlated with the stimulation index of T cell responses from the same culture.

3.3 Results

3.3.1 Recombinant T4SS proteins

All proteins were expressed with their full length, with the exception of VirB6-1 F1, and all proteins except VirB7 were purified under conditions to maintain native conformation (Fig. 1). The first fragment of VirB6-1 was used because the full-length protein did not express. Some additional weak bands were observed (i.e. for VirB4-2, VirB8-2, and VirB11). For VirB4-2 these reacted with anti-HIS mAb on immunoblotting, suggesting they are degraded protein fragments (see Fig. 7 in chapter 4).

3.3.2 Cattle immunizations

_A. marginale_ isolated from the infected erythrocyte was intact and very little erythrocyte membrane contamination was present (Fig. 2A). Furthermore, the isolated _A. marginale_ OM contained MSP2, MSP5, VirB9-1, VirB9-2, and VirB10, and no detectable erythrocyte
membranes (Fig. 2B). After four immunizations all six cattle had antibody responses to *A. marginale* OM (Fig. 3A), and PBMC proliferative responses to OM that were statistically significant as compared to pre-immunization response (Fig. 3B).

3.3.3 *IgG responses to T4SS proteins in OM-vaccinees*

All six OM-immunized cattle produced IgG against VirB9-1, VirB9-2, and VirB10 (Table 1), with VirB9-1 stimulating the highest titers of at least 10,000. Cattle 35113, 35141, 35160, 35280, and 35287 produced strong IgG responses directed at VirB7 with titers of at least 10,000. Cattle 35280 and 35287 also made IgG against VirB4-2. However, none of the animals made detectable IgG responses against VirB2, VirB4-1, VirB6-1 F1, VirB8-2, VirB11, or VirD4 when compared with pre-immunization sera.

3.3.4 *T lymphocyte responses to T4SS proteins in OM-vaccinees*

Two-week T cell lines enriched for CD4\(^+\) T cells from the six OM-immunized cattle were tested for antigen specific proliferation to recombinant T4SS proteins (Table 2). Differential responses among cattle with different MHC class II haplotypes were observed. VirB9-1, VirB9-2, and VirB10 were highly immunogenic for the majority of the animals. However, animals 35160 and 583 did not respond to VirB9-1, animals 35160 and 35280 did not respond to VirB9-2, and animal 35280 did not respond to VirB10. Animals 35160 and 35287 had significant T cell responses to VirB2, and animal 35160 had a significant T cell response to VirD4. Interestingly, 35160 was the only animal that had T cell responses to VirB6-1, and 35287 was the only animal that responded to VirB4-2. Lastly, there was no detectable T cell response from any animal to VirB4-1, VirB7, VirB8-2 and VirB11 antigens. These results are representative of three or more
experiments and false negative T cell responses were decreased by using the Dunnett’s test rather
than Bonferroni correction for multiple comparisons during statistical analysis.

3.3.5 Quantity of IFN-γ in supernatants

IFN-γ was detected in the supernatants of all the cell cultures responding to VirB9-1, VirB9-2, and VirB10, but not detected in supernatants cultured with media, MSA1, nor cell cultures that did not have T cell responses to VirB9-1, VirB9-2, and VirB10 (Table 3). The activity of IFN-γ (U/ml) correlated with proliferation (SI) with correlation coefficient of at least 0.8 (Table 3).

3.3.6 Selection of linked recognition candidates

In the context of selecting candidates for future vaccine development, three criteria were met for an antigen to be considered a candidate for linked recognition and to identify its interacting protein partner(s). First, the antigen induced insignificant CD4+ T cell proliferation. Second, the antigen induced IgG in the same individual. Finally, the antigen was predicted to be surface exposed so that it would be accessible during infection to neutralizing antibody. Based on previous research with *A. phagocytophilum* and *A. marginale* (12, 41-44) and bioinformatic predictions for *A. marginale* (see preliminary data section 2.3), VirB2, VirB6-1, VirB7, VirB8-2, VirB9-1, VirB9-2, and VirB10 are predicted to be localized on the exterior surface of *A. marginale*. (Surface exposed experiments are also elaborated in chapter 4.3.2, yet no conclusions were made.) Based on these criteria, the candidates for linked recognition, as summarized in Table 4, are VirB7 for all animals except 583, VirB9-1 and VirB9-2 for animal 35160 (DRB3 RFLP type 3/16), VirB9-1 for animal 583 (8/23), and VirB9-2 and VirB10 for animal 35280.
(16/27). These proteins were therefore selected to identify protein partners within the T4SS that had T cell epitopes recognized by these animals, and the interactions are described in chapter 4.

3.4 Discussion

This study sought to identify *A. marginale* T4SS protein candidates for linked recognition that could be incorporated into a vaccine. Therefore we first identified proteins that were recognized by IgG in cattle immunized with the protective OM vaccine, and not recognized by T cells from the same animal. Consistent with previous studies (14-16), VirB9-1, VirB9-2, and VirB10 are highly immunogenic and in this study are the most immunogenic of the eleven *A. marginale* T4SS proteins examined for cattle with diverse MHC class II haplotypes. We also confirmed that cattle with the DRB3 RFLP type 8/23 (animal 583 in this study and animal 04B91, ref. 15 and 16) have an IgG response, but no T-cell response to VirB9-1. This suggests that for animals with this haplotype, T cell help for IgG production is provided by a different, but associated protein. In addition, we have new evidence that linked recognition occurs through cattle with the DRB3 RFLP type 3/16 to produce IgG to VirB9-1 and VirB9-2 (animal 35160), DRB3 RFLP type 16/27 to produce IgG to VirB9-2 and VirB10 (animal 35280), and DRB3 RFLP types 11/22, 22/24, 3/16, 16/27, and 16/22 to produce IgG to VirB7 (all animals except 583, respectively). All of these proteins are likely to be surface exposed as predicted by bioinformatics. In addition, VirB9-2 and VirB10 are recognized by immune sera of infected animals (41, 42), and VirB10 was identified in outer membrane complexes following crosslinking with non-membrane permeable crosslinkers (45). Based on the criteria of predicted surface localization and pattern of immune recognition by three animals/haplotypes that had IgG but undetectable T cell responses to one or two of these proteins, VirB7, VirB9-1, VirB9-2, and
VirB10 were designated candidates for linked recognition. We then focused on identifying the interacting protein partners of VirB9 and VirB10 proteins within the T4SS, described in chapter 4.

Linked recognition is a well-known rationale for designing vaccines against pathogens with capsular polysaccharide antigens, which on their own, are poorly antigenic. Examples include vaccines for *Haemophilus influenzae* and *Streptococcus pneumoniae*, where T-dependent protein antigens are linked to the T-independent polysaccharide to achieve neutralizing antibody responses directed against the polysaccharide (33, 34). Immunization studies using two or more associated proteins are also suggestive that linked recognition enhances protection against bacterial pathogens. For example, protective immunity was induced with bacterial membrane protein complexes or fusion proteins of surface exposed type III secretion system proteins of *Yersenia pestis* (46, 47). Association of proteins within the context of the membrane is also important for leptospiral outer membrane porin OmpL1 and lipoprotein LipL41 (48).

A combination of cellular and humoral immunity is likely important for the control of anaplasmosis and vaccine antigens should be highly conserved across multiple strains to insure cross-protective immunity. Including multiple proteins in a vaccine that are naturally associated in the membrane of *A. marginale* will not only provide more than one immunogenic protein, but provide an opportunity for linked recognition. Immunization with several naturally associated, linked proteins could increase T-cell dependent IgG responses upon infection in out-bred populations that express a broad repertoire of MHC class II molecules. Protecting a large population of genetically heterogeneous individuals requires understanding of the MHC class II-restricted epitope presentation (discussed in chapter 5). The T4SS proteins provide a model system to examine the importance of membrane protein-protein interactions in stimulating
protective immunity and facilitating linked recognition. The interactions of the T4SS proteins will be described in chapter 4.

3.5 Work Cited


### TABLE 1. IgG responses to recombinant T4SS proteins in cattle immunized with OM

<table>
<thead>
<tr>
<th>Protein</th>
<th>IgG titers from each animal&lt;sup&gt;b&lt;/sup&gt;</th>
<th>35113&lt;sup&gt;c&lt;/sup&gt; (11/22)</th>
<th>35114 (22/24)</th>
<th>35160 (3/16)</th>
<th>35280 (16/27)</th>
<th>35287 (16/22)</th>
<th>583 (8/23)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VirB2</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>&lt;100</td>
</tr>
<tr>
<td>VirB4-1</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>&lt;100</td>
</tr>
<tr>
<td>VirB4-2</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>1,000</td>
<td>3,000</td>
<td>&lt;100</td>
<td>&lt;100</td>
</tr>
<tr>
<td>VirB6-1 F1</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>&lt;100</td>
</tr>
<tr>
<td>VirB7</td>
<td>≥10,000</td>
<td>≥10,000</td>
<td>≥10,000</td>
<td>≥10,000</td>
<td>≥10,000</td>
<td>≥10,000</td>
<td>&lt;100</td>
</tr>
<tr>
<td>VirB8-2</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>&lt;100</td>
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<td>≥10,000</td>
<td>≥10,000</td>
<td>≥10,000</td>
<td>≥10,000</td>
<td>≥10,000</td>
</tr>
<tr>
<td>VirB9-2</td>
<td>3,000</td>
<td>1,000</td>
<td>≥10,000</td>
<td>≥10,000</td>
<td>3,000</td>
<td>≥10,000</td>
<td>≥10,000</td>
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<td>1,000</td>
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<td>3,000</td>
<td>3,000</td>
<td>≥10,000</td>
<td>≥10,000</td>
</tr>
<tr>
<td>VirB11</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>&lt;100</td>
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<td>VirB4</td>
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<td>&lt;100</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>&lt;100</td>
</tr>
</tbody>
</table>

<sup>a</sup> T4SS proteins were expressed in *E. coli* and purified. Each protein was used at 1 or 2 μg/ well and transferred.<br><sup>b</sup> Sera were diluted and tested for reactivity to recombinant proteins on immunoblots. <100 indicates that no IgG response was detected at a 1:100 dilution. Positive sera recognizing the antigen at the predicted molecular weight and when pre-immune sera were negative were diluted 1:300 to 1:10,000. The titer is defined as the reciprocal of the highest serum dilution giving a positive signal.<br><sup>c</sup> Animal numbers (DRB3 RFLP type) are indicated.
### TABLE 2. T cell responses to recombinant *A. marginale* T4SS antigens in Holstein cattle with different MHC class II haplotypes

<table>
<thead>
<tr>
<th>Protein</th>
<th>Proliferation of T cells from each animal&lt;br&gt;(% of maximum)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>35113</td>
</tr>
<tr>
<td>OM</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>351.1±5.7</td>
</tr>
<tr>
<td>10</td>
<td>941.1±52.8</td>
</tr>
<tr>
<td>URBC</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.2±0.1</td>
</tr>
<tr>
<td>10</td>
<td>0.9±0.7</td>
</tr>
<tr>
<td>MSA1</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1±1.3</td>
</tr>
<tr>
<td>10</td>
<td>0.3±0.1</td>
</tr>
<tr>
<td>VirB2</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.4±0.1</td>
</tr>
<tr>
<td>10</td>
<td>1.5±0.3</td>
</tr>
<tr>
<td>VirB4-1</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.6±0.7</td>
</tr>
<tr>
<td>10</td>
<td>1.0±0.5</td>
</tr>
<tr>
<td>VirB4-2</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.3±0.1</td>
</tr>
<tr>
<td>10</td>
<td>1.1±0.1</td>
</tr>
<tr>
<td>VirB6-1 F1</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3.0±0.7</td>
</tr>
<tr>
<td>10</td>
<td>0.7±0.2</td>
</tr>
<tr>
<td>VirB7</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.5±0.2</td>
</tr>
<tr>
<td>10</td>
<td>2.0±0.4</td>
</tr>
<tr>
<td>VirB8-2</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.3±0.1</td>
</tr>
<tr>
<td>10</td>
<td>0.9±0.1</td>
</tr>
<tr>
<td>VirB9-1</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>385.7±36.5</td>
</tr>
<tr>
<td>10</td>
<td>779.3±71.4</td>
</tr>
<tr>
<td>VirB9-2</td>
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</tr>
<tr>
<td>1</td>
<td>139.2±11.7</td>
</tr>
<tr>
<td>10</td>
<td>201.5±27.1</td>
</tr>
<tr>
<td>VirB10</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>35.5±3.6</td>
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<tr>
<td>10</td>
<td>381.2±39.0</td>
</tr>
<tr>
<td>VirB11</td>
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</tr>
<tr>
<td>1</td>
<td>1.8±0.1</td>
</tr>
<tr>
<td>10</td>
<td>3.2±1.4</td>
</tr>
<tr>
<td>VirD4</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.4±0.1</td>
</tr>
<tr>
<td>10</td>
<td>0.5±0.0</td>
</tr>
</tbody>
</table>

\[a\] Recombinant T4SS proteins were used at 1 and 10 μg/ml in the T cell assays, as indicated. *A. marginale* OM and URBC were positive and negative controls, respectively.
A stimulation index (SI) was calculated as the mean cpm of a two-week CD4 T cell line to an individual protein/the mean cpm to medium. Results in bold indicate significantly greater T cell responses to a T4SS protein compared to the response to the same concentration of MSA1, where \( P<0.05 \) using the Dunnett’s test.

Animal numbers (DRB3 RFLP type) are indicated.

### TABLE 3: IFN-γ quantity within supernatants of three day cultured T cells with antigen and its correlation with T-cell proliferation stimulation index.

<table>
<thead>
<tr>
<th>Ag(^a)</th>
<th>IFN-γ quantity in Units/ml from each animal(^b)</th>
<th>35113</th>
<th>35141</th>
<th>35160</th>
<th>35280</th>
<th>35287</th>
<th>583</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>OM</td>
<td></td>
<td>21</td>
<td>28.3</td>
<td>20.9</td>
<td>25.2</td>
<td>40.7</td>
<td>15.8</td>
</tr>
<tr>
<td>VirB9-1</td>
<td></td>
<td>6.8</td>
<td>38.8</td>
<td>0</td>
<td>5.5</td>
<td>35.3</td>
<td>0</td>
</tr>
<tr>
<td>VirB9-2</td>
<td></td>
<td>8.1</td>
<td>15.9</td>
<td>0</td>
<td>0</td>
<td>34.2</td>
<td>51.3</td>
</tr>
<tr>
<td>VirB10</td>
<td></td>
<td>10.3</td>
<td>18.1</td>
<td>16.3</td>
<td>0</td>
<td>39.7</td>
<td>20.1</td>
</tr>
<tr>
<td>MSA1</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.46</td>
<td></td>
</tr>
</tbody>
</table>

Correlation Coefficient\(^c\)

| \( R^2 \) | 0.898 | 0.859 | 0.823 | 0.812 | 0.862 | 0.842 |

\( a\) Recombinant T4SS antigens (Ag) were expressed in *E. coli* and purified. Each antigen was used at 1 μg/ml in T cell assays and supernatants were harvested after 3 days. *A. marginale* OM was the positive control and *B. bovis* MSA1 was the negative control.

\( b\) Activity of IFN-γ was determined by comparison with a standard curve made from *M. bovis* PPD T cell clone supernatants at 440 U/ml.

\( c\) Correlation of the quantity of IFN-γ (U/ml) with T cell proliferation (SI) using data from the same assay.
TABLE 4. Summary of IgG and T cell responses to *A. marginale* T4SS antigens in six Holstein cattle\(^a\)

<table>
<thead>
<tr>
<th>Ag</th>
<th>OM-immunized animal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>35113 (11/22)(^b)</td>
</tr>
<tr>
<td>VirB2</td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>-</td>
</tr>
<tr>
<td>T cells</td>
<td>-</td>
</tr>
<tr>
<td>VirB4-1</td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>-</td>
</tr>
<tr>
<td>T cells</td>
<td>-</td>
</tr>
<tr>
<td>VirB4-2</td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>-</td>
</tr>
<tr>
<td>T cells</td>
<td>-</td>
</tr>
<tr>
<td>VirB7</td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>+(^c)</td>
</tr>
<tr>
<td>T cells</td>
<td>+(^c)</td>
</tr>
<tr>
<td>VirB6-1 F1</td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>-</td>
</tr>
<tr>
<td>T cells</td>
<td>-</td>
</tr>
<tr>
<td>VirB8-2</td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>-</td>
</tr>
<tr>
<td>T cells</td>
<td>-</td>
</tr>
<tr>
<td>VirB9-1</td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>+</td>
</tr>
<tr>
<td>T cells</td>
<td>+</td>
</tr>
<tr>
<td>VirB9-2</td>
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<td>IgG</td>
<td>-</td>
</tr>
<tr>
<td>T cells</td>
<td>+</td>
</tr>
<tr>
<td>VirB10</td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>+</td>
</tr>
<tr>
<td>T cells</td>
<td>+</td>
</tr>
<tr>
<td>VirB11</td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>-</td>
</tr>
<tr>
<td>T cells</td>
<td>-</td>
</tr>
<tr>
<td>VirD4</td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>-</td>
</tr>
<tr>
<td>T cells</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\) IgG responses from Table 2 and T cell responses from Table 3 are summarized. Negative (-) indicates that IgG titers were <100 or the T cell response was insignificant. Positive (+) indicates an IgG titer was $\geq 300$, or the T-cell response was significant.

\(^b\) DRB3 RFLP types are indicated

\(^c\) Candidates for linked recognition
3.7 Figures and Figure Legends

**Figure 1.** Purity of recombinant T4SS proteins. Recombinant T4SS proteins were loaded at 1 μg/well, separated on 4 to 20% gradient SDS-PAGE gels and stained with Coomassie blue dye. The proteins were electrophoresed on separate gels, and scanned images were arranged as presented. An asterisk indicates the predicted molecular weight of the recombinant protein, indicated in parentheses as follows: rVirB2 (20 kDa), rVirB4-1 (90 kDa), rVirB4-2 (91 kDa), rVirB6-1 F1 (90 kDa), rVirB7 (20 kDa), rVirB8-2 (23 kDa), rVirB9-1 (46 kDa), rVirB9-2 (46 kDa), rVirB10 (65 kDa), rVirB11 (38 kDa), rVirD4 (93 kDa), and control rMSA1 (58 kDa).
Figure 2. Characterization of the *A. marginale* before cattle immunizations. (A) Electron Microscopic image of intact *A. marginale* isolated from infected erythrocytes. Relative size is indicated on the images. (B) *A. marginale* OM (10 μg) were separated by SDS-PAGE and transferred to nitrocellulose for western blotting. Each lane indicates the primary antibody (used at 2 μg/ml) to develop a strip on the immunoblot. Native molecular weights as follows: MSP2 (32kDa), MSP5 (19kDa), ViB9-1 (30kDa), VirB9-2 (30kDa), and VirB10 (50kDa). Ana8a was the anti-erythrocyte mAb that would label an erythrocyte membrane protein around 150kDa.
**Figure 3.** Successful immunization of six MHC-diverse cattle. (A) A western blot containing 10 μg of St. Maries OM was developed with 1:100 bovine sera before immunization (pre) and after 4 immunizations (post). Each lane designates the animal number pre and post immunization. (B) Stimulation of PBMC from each animal with 10 μg of *A. marginale* OM before and after immunization. Statistically significant responses were detected for each animal for post-immunization as compared to pre-immunization using homoscedastic one-tailed T-test, p<0.0001.
Chapter 4: Identify the Immunogenic A. marginale T4SS Proteins that are Surface Exposed and Naturally Associated with other Antigenic T4SS Proteins

Some of the information presented in chapter 4 is Copyright © protected by American Society for Microbiology, Infection and Immunity, 2011, Oct. 28. Title: Association and evidence for linked recognition of type IV secretion system proteins VirB9-1, VirB9-2, and VirB10 in Anaplasma marginale

4.1 Brief Introduction

The type IV secretion system (T4SS) has been most widely studied in Agrobacterium tumefaciens and it is typically made up of 12 interacting VirB/D membrane proteins. The core T4SS complex is made up of 14 copies of interacting VirB7, VirB9, and VirB10 which form a complex that was crystallized and studied with Escherichia coli VirB homologues TraN (VirB7), TraO (VirB9), and TraF (VirB10) (1, 2). The Agrobacterium VirB proteins assemble into a pilus made up of VirB2, VirB5, and VirB7 (3-6). There are three nucleotide triphosphate (NTP)-utilizing T4SS proteins; VirB4, VirB11, and VirD4 that supply energy for substrate translocation, and VirD4 specifically acts as the substrate-coupling protein (7, 8). VirB6 and VirB8 assemble the T4SS apparatus at the cell pole and also make up the core complex (9-11). Several of T4SS proteins are likely to be surface exposed (12, 13) and are thus targets for neutralizing and protective immune responses. The T4SS proteins harbored by A. marginale and other members of the family Anaplasmataceae are unique because they are encoded by two copies of virB4, virB8, and virB9, four or more copies of virB6, and multiple copies of virB2. There are single copies of virB3, virB7, virB10, virB11, and virD4, whereas homologues of virB1 and virB5 have
not been identified (14-18). Because Anaplasmataceae lack lipopolysaccharides (14, 16), several T4SS proteins may be surface exposed where they could be targeted by neutralizing antibody. The function of the T4SS in A. marginale has not been determined, but in a pathogen that has undergone reductive evolution, retention of these genes indicates their requirement for invasion and survival within erythrocytes and/or tick cells (15, 16). Furthermore, T4SS proteins interact within the bacterial membrane so associated proteins could provide linked recognition for T cell-B cell interactions.

In this study linked recognition candidates determined in chapter 3, VirB7, VirB9-1, VirB9-2, and VirB10 were selected to identify localizations within A. marginale cellular membranes and their protein partners. The results presented in chapter 4 will provide further evidence for linked recognition of these proteins and thus provide a basis for testing these proteins as a vaccine for anaplasmosis.

4.2 Materials and Methods

4.2.1 Preparation of antibodies specific for VirB7, VirB9-1, VirB9-2, VirB10, MSA1, ACP, and normal rabbit

Purified recombinant proteins were expressed and purified as detailed in chapter 3 sections 3.2.1 and 3.2.2, respectively. VirB7, VirB9-1, VirB9-2, VirB10, and MSA1 were separated on a 4-20% gradient gel (Bio-Rad) by sodium doedecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and bands excised, and trypsin-digested for Q-TOF MS analysis (see protocol below) to confirm that the purified protein was the protein of interest. Rabbits were used in this study in compliance with the WSU IUCAC. Before protein injections pre-immune sera were collected from all animals and protein A purified for use as a negative control and
normal rabbit IgG. Recombinant VirB7, rVirB9-1, rVirB9-2, rVirB10, and rMSA1 were emulsified with 50% phosphate buffered saline, pH 7.2 (PBS, Fisher) and 50% TiterMax® Gold adjuvant (Sigma-Aldrich) to make a final concentration of 200 μg protein /ml; and 100 μg protein was inoculated subcutaneously into rabbits at 0, 3, 5, 7, 9, and 11 weeks (4-6 times) until the immunization was successful, which was determined by western blotting with pre- and post-immunization sera diluted 1:500. Anti-sera to VirB7, VirB9-1, VirB9-2, and VirB10 were absorbed with crude Top10 E. coli lysate expressing MSA1 for removal of non-specific antibodies. A negative control rabbit antiserum was made by Pacific Immunology, using Freund’s complete and incomplete adjuvants and a fragment of a B. bovis nuclear-encoded protein, acyl carrier protein (ACP). The ACP fragment from amino acids 68-148 was expressed in pTrcHIS TOPO TA expression vector (Invitrogen) and purified using denaturing conditions on a nickel column followed by electro-elution of the gel-embedded protein.

Prior to purification of rabbit IgG, 5 ml of rabbit serum was absorbed with 3 ml of crude Top10 E. coli lysate expressing MSA1 (or BL21 E. coli expressing Am813) for 1 h in binding buffer (20 mM sodium phosphate, pH 7.0) at room temperature and centrifuged to remove non-specific antibodies. The absorbed antisera were buffer-exchanged twice by diluting with binding buffer and centrifuging using 15 ml Amicon 10k filters (Millipore). A HiTrap Protein A column (GE Healthcare Biosciences) was used to purify the buffer-exchanged and absorbed antisera following the manufactures protocol. Eluted and purified IgG was buffer exchanged with sterile PBS, concentrated with the Amicon 10k filters, and the final concentration of IgG was determined with the Bio-Rad Bradford assay. Separate protein A columns were used to purify anti-VirB7, anti-VirB9-1, anti-VirB9-2, anti-VirB10, anti-MSA1, and anti-ACP IgG. The purity of the antibodies were assessed by SDS-PAGE and specificity was determined on immunoblots.
using 1 μg of each the recombinant T4SS protein, 10 μg of A. marginale OM, iISE6 tick cells, or B. bovis Merozoite. Blots were developed with 2 μg/ml purified IgG, the secondary antibody was alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG (H+L) (Invitrogen) diluted 1:10,000, and blots were developed with Western Star chemiluminescence kit (Applied Biosystems).

4.2.2 Immunohistochemistry of intact and potentially live A. marginale

Calf C31024 was splenectomized and inoculated with the St. Maries strain of A. marginale. When parasitemia reached 46% and packed cell volume was 25% the calf was exsanguinated. The buffy coat and plasma were removed and discarded. The erythrocytes were repeatedly washed using PBS and centrifuged at 2,000 rpm for 30 min until only infected erythrocytes remain. Fresh infected erythrocytes (iRBC) were required to maintain the integrity of the Anaplasma for the labeling experiments; stabilate was not used. Infected red blood cells (200 μl) were either mixed with agarose and embeded in paraffin for the immunoelectron microscopy or processed further to isolate intact A. marginale from the erythrocytes (also described briefly in chapter 3 section 3.2.3).

To isolate intact A. marginale from infected erythrocytes the red blood cells were lysed by adding an equal volume of 18MΩ de-ionized water followed by immediately doubling the volume with 2X PBS. The volume was brought up to 50 ml with 1X PBS and centrifuged at 30,000 x g for 20 min at 4°C. The red supernatant containing hemoglobin was aspirated and the white floccular pellet was resuspend for a series of 5-7 washes with 1X PBS until the supernatant was clear. These washed hemoglobin-free erythrocytes were combined and brought to 20 ml with 1X PBS, and sonicated for 4 min with 30 sec intervals at 40%, approximately 60 watts, with an additional 30 sec at 70% or 120 watts (Branson digital sonifier). After sonication the samples
were brought up to 50 ml with 1X PBS and centrifuged at 30,000 x g for 20 min at 4°C. The pellets were collected and placed in 1.5 ml eppendorf tubes and centrifuged at 15,800 x g for 15 min at room temp. The tubes were inverted to remove the part of the pellet that is easily resuspendend and washed with 500 μl of 1X PBS. The pellet (~50μl) was brought up to a final volume of 200 μl with 1X PBS and resuspended completely. The quality of the A. marginale isolation was checked microscopically with Giemsa staining and confirmed 80% or more enrichment of A. marginale. The A. marginale isolated from erythrocytes using the same procedure above were also visualized by the JOEL TEM in Chapter 3, Figure 2 A.

Isolated A. marginale (200 μl) or iRBC (200 μl) were combined with an equal amount of 10% neutral buffered-formalin (Thermo Scientific) and fixed overnight at room temp. Fixed Anaplasma was split into two tubes, quickly centrifuged to pellet, 50 μl was removed, and the remaining A. marginale was vortexed. Two percent of agarose (Fisher) was added at 50 μl and 100 μl to each tube containing the fixed isolated A. marginale and iRBC to solidify. Two volumes of agarose were used to ensure a pellet with uniform thickness was obtained. The Anaplasma pellet was put in a histology cassette and submersed in 10% formalin. The WSU histology lab did the processing of the Anaplasma and/or iRBC pellets to remove the plastic eppendorf, dehydration, clearing, and infiltration into paraffin.

A microtome was used to cut 4 μm sections of the paraffin embedded intact A. marginale. Just the sections containing intact A. marginale separated from the erythrocyte were processed to verify the antibody binds to native and fixed Anaplasma before immunoelectron microscopy, the procedure followed was previously published (19-21). Briefly, paraffin was removed by incubation at 56°C for 1 h, incubated 3 times in Clear-Rite for 5 mins, and hydrated with a decreasing ethanol gradient. Sections were treated with pH 6.0 citrate buffer (Zymed) for 20 min
while steaming for antigen retrieval. After blocking with protein block serum-free (Dako Cytomation) for 1 h and mixing isolated *Anaplasma* sections with 3% hydrogen-peroxide for 5 min the primary antibodies were added. The protein-A purified polyclonal rabbit antibodies against VirB9-1, VirB9-2, VirB10, and normal rabbit were incubated on the isolated *Anaplasma* slides using 20 μg/ml antibodies diluted in the blocking buffer for 30 min. For a positive control 1 μg/ml of AnaR49a monoclonal antibody (mAb) specific for MSP2 was also added to a slide containing intact *A. marginale* (22). Two milliliters of 1x tris-buffered saline with 0.5% Tween-20 (TBST) was used to wash and 3 drops of horse radish peroxidase (HRP) labeled anti-rabbit or anti-mouse (Dako Cytomation) was added for 30 min as the secondary antibody. After washing the labeled sections with 4 ml of TBST, 3 drops of 3-amino-9-ethylcarbazole containing hydrogen peroxide substrate was used to detect antibody binding and sections were counterstained with Meyer’s hematoxilin. Red stain under light microscopy indicated that the antibody binds to the native and fixed *Anaplasma*, and thus the protein was still available for visualization.

4.2.3 Gold labeling of iRBC and transmission electron-microscopy

Once the antibody recognition of intact *A. marginale* was verified by IHC, paraffin containing iRBC were cut into 4 μm sections and processed for gold labeling. After paraffin removal and antigen retrieval iRBC sections were blocked and 1 μg/ml mAb anti-MSP2, polyclonal anti-MSA1, and polyclonal anti-VirB9-1 antibodies were incubated overnight. After washing the sections with TBST, a 1:50 dilution of 1.4 nm-gold conjugated anti-mouse or anti-rabbit IgG (Nanoprobes) was applied for 2 h at 4°C. The antibody reactions were enhanced with gold enhance-EM following manufactures specifications (Nanoprobes). Sections were washed
with 0.1 M cacodylate buffer, fixed in 2% OsO₄ overnight at room temp and rinsed with 0.1 M cacodylate buffer. Following dehydration in an ethanol gradient, samples were infiltrated with acetone and embedded in Spurr’s resin. The cover slip was removed, and thin 70-90 nm sections were placed on nickel grids and stained with 4% uranyl acetate for 10 min and in Reynolds lead for 3 min. Sections were examined on a JOEL TEM 1200 EX transmission electron microscope for visualization of the surface exposed T4SS proteins.

4.2.4 Gold labeling of isolated Anaplasma marginale

Intact *A. marginale* was also labeled with gold conjugated antibodies to detect surface localized proteins, but following a very different procedure. Intact *A. marginale* was put directly on 200-mesh nickel formavar, so no resin or section was required. The primary antibody labeling was performed the same as the above procedure with iRBC immunoelectron microscopy, but washed with blocking buffer (Dako Cytomation). The secondary antibody was 15 nm gold labeled goat anti-mouse or anti-rabbit antibody (BBInternational) used at 1:50 dilution to by-pass the enhancement step. Before the formvar was viewed in the JOEL TEM the final wash was with water then with 1% phosphotungstic acid pH 7.0.

4.2.5 Tandem Mass Spectrometric Analysis of *rVirB7, rVirB9-1, rVirB9-2, and rVirB10*

Bands corresponding to 20 and 40 kDa for VirB7, 50 and 100 kDa for VirB9-1 and VirB9-2, and 65 and 130 kDa for VirB10 were excised from the Coomassie stained gels, destained (50% methanol, 5% acetic acid), dehydrated with 100% acetonitrile, reduced with 10 mM DTT, quenched with 50 mM iodoacetemide, and digested with 20 ng/μl of trypsin (Promega). The peptides were subjected to tandem mass spectrometric (MS) fragmentation on a
high performance liquid chromatography (HPLC) coupled quadruple-time of flight (Q-TOF) MS instrument located at University of Idaho, Environmental Biotechnology Institute. Fragment ion lists and the identified peptide sequences were searched against the MASCOT database that contain *A. marginale*, *E. coli*, or NCBI all entries. Identification of the protein was based upon the score given, probability, and by mass. One missed trypsin cleavage, fixed carbamidomethyl modifications, and variable oxidation was allowed during the search. A probability of 95% or greater showed that the peptide match was not a random occurrence, and the individual ion score is reported as $-10 \log_{10} P$, where $P$ is the probability. An ion score greater than 19 has significant identity.

### 4.2.6 Far western blotting

Far western blotting was performed as previously described (23-26). SDS-PAGE was performed with 1 µg of each recombinant protein loaded per well on 4-20% gradient gels and electrophoresis at 100V for 1.5 h. Following transfer to nitrocellulose membranes at 100 volts for 1 h, the nitrocellulose membrane with recombinant T4SS protein was first incubated with 6 M guanidino-hydrochloride, 20 mM HEPES pH 7.5, 50 mM KCl, 10 mM MgCl$_2$, 1 mM DTT, 0.1% Nonidet P40, 2% milk (GuHCl buffer) for 30 min at room temp, then successively for 30 min each with 3 M and 1.5 M GuHCl buffer at room temp, then with 0.6 M GuHCl buffer at 4°C for 30 min, and finally with buffer lacking GuHCl overnight at 4°C. This ensured that all of the T4SS proteins on the membrane were in native conformation. The membrane was blocked with 20 mM Tris-HCl pH 7.5, 150 mM NaCl (TBS) with 5% milk for 3 h. Two far western membranes were run in tandem. One membrane was incubated with a 1:2,000 dilution of HRP-conjugated anti-HIS mAb (Qiagen) to confirm proteins on the membrane after denaturing and re-
The second membrane was used to identify a target recombinant protein interacting with another recombinant protein. To identify if the target recombinant protein (VirB7, VirB9-1, VirB9-2, or VirB10) bound to a prey recombinant T4SS proteins, the membrane containing 1 μg of each prey protein was incubated with 10 μg of target protein in 5 ml of TBS with 5% milk overnight at 4°C. The membrane was then washed three times with TBS containing 0.05% Tween 20 (TBST). Polyclonal rabbit antisera specific for the target recombinant protein were absorbed with crude Top10 E. coli lysate expressing MSA1 to eliminate non-specific binding to recombinant tags and E. coli contamination. The primary antibody directed against the target protein (anti-VirB7, anti-VirB9-1, anti-VirB9-2, or anti-VirB10) was added to the membrane at a 1:500 dilution in TBS plus 5% milk and excess was washed off. The membrane was incubated with a 1:10,000 dilution of AP-conjugated goat anti-rabbit IgG (H+L) (Applied Biosystems) for 1 h, then washed extensively with TBST, and developed using the Western Star chemiluminescent substrate.

4.2.7 Determining the binding coefficients of interacting VirB9-1, VirB9-2 and VirB10

Serial dilutions (0.05-5.0 μg) of recombinant VirB9-2 and rVirB10 were applied to a dot blot apparatus and used to find the binding coefficient to rVirB9-1 (26). Recombinant VirB9-1 was also applied to the dot blot as a positive control, and higher concentrations of (0.5-10.0 μg) rMSA1 was used as a negative control for background subtraction. The dot blot was developed using the same procedure as far western blots described above using 2 μg/ml of rVirB9-1 to probe and a 1:500 dilution of absorbed polyclonal anti-VirB9-1 rabbit sera. Densitometry analysis of the spots containing interacting proteins and controls was performed with ImageJ 1.43 from National Institutes of Health. Data was fit to a standard one-site saturation binding
curve $Y = \frac{Y_{\text{max}}(X)}{(K_D + X)}$, where $Y$ is the intensity of the spot, $X$ is concentration of the protein on the membrane, and $K_D$ is the binding association constant using SigmaPlot 10.0. From this equation of the line the $K_D$ was found for VirB9-1 binding to VirB9-2 and VirB10. The reverse of the reactions were also performed using VirB9-2 and then VirB10 as the target protein.

4.2.8 Immunoprecipitation

*A. marginale* OM (2.5 mg/ml) were solubilized in 2% n-dodecyl β-D maltoside (DM; Sigma-Aldrich) for at least 4 h at 4°C, and cell debris was removed by centrifugation at 10,000 x g for 10 min at 4°C. A high concentration of OM was used for immunoprecipitation because the T4SS proteins are not abundant (27). Many different non-ionic detergents including but not limited to sodium deoxycholate, tween-20, triton X-100, nonidet-40, and LDAO were tried at varying salt concentrations and percentages of detergent. DM was the most successful detergent that maintained protein-protein interaction and solubilized the membrane T4SS proteins (data not shown). A non-ionic detergent, DM, was used for solubilization to help maintain native protein conformation and the natural hydrophobic interaction or hydrogen bonding of the membrane proteins. The supernatant was collected and split into equal halves to use for two different immunoprecipitation (IP) reactions. Protein-A purified polyclonal IgG (2 μg) specific for VirB9-1, VirB9-2, VirB10, or *B. bovis* ACP (negative control) were incubated with the solubilized OM proteins for 2 h at 4°C. Protein A agarose (20 μl, Santa Cruz Biotechnology) was added and incubated for 1 h at 4°C. Monoclonal antibodies anti-MSP5 AnaF16C1 were also incubated with solubilized OM and pulled down with protein A/G agarose (ThermoScientific) as an additional specificity control. The native protein complexes were centrifuged at 660 x g (2500 rpm) for 5 min and the pellet was washed 4 times with 400 μl of 50 mM Tris-HCl (pH 8), 150 mM NaCl,
0.1% Nonidet P40 and once with sterile PBS. For analysis, the pellet was immediately resuspended in 0.3 M Tris-HCl, 5% SDS, 50% glycerol, 100 mM dithiothreitol (DTT), and tracking dye; pH 6.8 (Lane Marker Sample Buffer, Thermo Scientific) to yield a total volume of 95 µl in 1X sample buffer, and 10 µl were loaded per well on 4-20% gradient gels and electrophoresed at 100 V for 1.5 h. Following transfer to nitrocellulose membranes at 100 volts for 1 h, the interacting proteins were determined on western blots probed with 1 µg/ml of polyclonal rabbit IgG specific for a candidate interacting protein partner or for the target protein. MAbs used at 2 µg/ml specific for MSP5 (AnaF16C1) and MSP2 (AnaR49a) were also used to probe the blots for additional specificity controls (22). The blots probed with anti-MSP5 were incubated with HRP-Clean-Blot and developed as below, and the anti-MSP2 probed blots were incubated with AP-conjugated goat anti-mouse IgG + IgM (Invitrogen) diluted 1:10,000, and developed with the Western-Star chemiluminescent substrate. Following washing in I-block containing 0.1% Tween-20, blots were incubated with HRP conjugated Clean-Blot (Thermo Scientific) that only binds to intact antibodies, according to the manufacture’s protocol. After incubation with the secondary antibody blots were extensively washed with I-block containing 0.3% Tween-20, and developed with ECL Western Blotting Reagent (Thermo Scientific). Each polyclonal antibody and anti-MSP5 used for immunoprecipitation pulled down the respective target protein.

Interacting proteins were also identified by Q-TOF MS. The immunoprecipitated pellets were run in SDS-PAGE gel for less than 5 min until visually separated as compared to the molecular weight ladder and enough to leave the agarose beads in the top of the well. Without staining, the bands were cut and processed as above method (section 4.2.5). Peptide signal from tandem MS of the immunoprecipitated pellet was masked by heavy and light chain of IgG used
to pull down the interacting proteins, so very little results were obtained. The MS results that were considered significant hits from peptide searches also contained the target protein in the same MS-analysis, thus if a Mascot score greater than 19 was detected for a protein in an IP pellet but the target protein was not then that protein was not considered to be interacting.

In addition, immunoprecipitation reactions were performed with rabbit anti-VirB9-1 IgG as described above, and the IPs were electrophoresed in a single large well, transferred to nitrocellulose, and strips were cut and probed with 2 µg/ml mouse mAb specific for VirB9-1, VirB9-2, VirB10, MSP2, MSP5, or B. bovis MSA1 (23/10.41, IgG2b) (28). Secondary antibody was AP conjugated goat anti-mouse IgG + IgM, diluted 1:10,000. Blots were developed with Western-Star chemiluminescent substrate for 30 min or longer. However, this procedure did not work for immunoprecipitations performed with polyclonal anti-VirB9-2 and anti-VirB10. mAb against VirB9-1, VirB9-2, and VirB10 when incubated with solubilized OM failed to pull down their specific and native protein. Another observation was mAb anti-VirB9-1, anti-VirB9-2, and anti-VirB10 did not detect native proteins during IHC and gold IEM. Thus, the mAb may have been directed at a small epitope on the protein close to the recombinant tag on the recombinant proteins that may be hidden with native protein confirmation. Another difficulty is that VirB9-1, VirB9-2, and VirB10 bind to themselves creating homodimers. This feature made it NOT useful to use monoclonal antibodies to develop the western blot because they failed to recognize the homodimer of all three proteins. Therefore, no data was shown using the mAb against VirB9-1, VirB9-2, and VirB10.

Crosslinking the polyclonal antibodies to protein A beads before immunoprecipitation with solubilized OM was also tried using the Thermo Scientific Crosslinking IP Kit. However, crosslinking over modified the polyclonal antibodies so much that the native target protein was
not pulled down (data not shown). Therefore, the only successful procedure was to use the polyclonal antibodies to pull down native complexes and using the polyclonal antibodies again to develop the western blot.

4.3 Results

4.3.1 Specificity of polyclonal antibodies against rVirB7, VirB9-1, VirB9-2, VirB10, and MSA1

Rabbits that were immunized with rVirB7 produced severe abscessing lesions, but all other rabbits immunized with rVirB9-1, rVirB9-2, rVirB10, and rMSA1 were fine (data not shown). This reaction may be evidence for rVirB7 virulence effector function that has not been thoroughly studied. After the rabbit’s produced sera that responded to both the recombinant protein and native the animals were exsanguinated. Immunoglobulin G was purified from sera with protein A columns and the purity was assessed with SDS-PAGE (Fig. 1). The SDS-PAGE separation of these antibodies revealed bands corresponding to heavy chain (50 kDa) and light chain (25 kDa) after Coomassie staining the gel. Protein A purified rabbit IgG raised against rVirB7, rVirB9-1, rVirB9-2, rVirB10, and rMSA1 were tested for antigen specificity by immunoblotting with native protein and recombinant proteins (Fig. 2A-E). In repeated assays, anti-VirB7 detected a band in the OM that was greater than the predicted native mass of ~6 kDa (Fig. 2A). Full length Am306 (ref. YP_153652.1) has the molecular weight 9.5 kDa, however Gillespie et al. proposed an alterative start cite making the predicted protein mass 6 kDa (16). Both A. marginale OM and infected ISE6 (iISE6) cells were used as a source of native VirB7. ISE6 cells are A. marginale infected embryonic tick cells that can be cultured. Bands in the OM were detected approximately at 10 kDa and 15 kDa, yet this was not consistent, and interestingly a single band in iISE6 cells was detected approximately at 37 kDa but nothing was detected in
uninfected OM or uninfected ISE6 cells (Fig 2A). The discrepancy in size of native VirB7 makes this antibody not useful for immunoprecipitation, but was used to develop western blots. In contrast, IgG against rVirB9-1, rVirB9-2, and rVirB10 recognized the native OM protein at the predicted molecular weight and at a two-fold higher molecular weight (Fig. 2B-D). In addition, anti-VirB9-2 detected a fainter, narrow band at approximately 140 kDa, which may represent a multimer of the native protein or a multimer of VirB9-2 and another protein(s). When recombinant proteins were used, antibody against rVirB7 reacted with 20 and 40 kDa bands in rVirB7, but not with the other T4SS proteins (Fig. 2A). For rVirB9-1 (Fig. 2B), rVirB9-2 (Fig. 2C), and rVirB10 (Fig. 2D) IgG bound specifically to their target proteins and at a band of approximately twice the molecular weight; which we showed are dimers (Fig. 6 and Table 1). Bands larger than the dimer that are present in the immunizing protein detected by the IgGs against VirB9-1 and VirB9-2 may represent multimers of the VirB9 protein with other T4SS proteins, as it is known that the A. tumefaciens core-complex has 14 copies of VirB9 that interact with 14 copies of VirB7 and 14 copies of VirB10 (1, 2). Importantly, the IgGs did not bind to any other T4SS protein, showing that the IgGs were specific for the immunizing protein. An artifact band often observed in immunoblots with polyclonal rabbit antisera that was determined to be keratin was observed with the anti-VirB9-2 antibody, which detects a diffuse band at ~68 kDa and another at ~140 kDa (Fig. 2C). Because of the characteristic size and width of these bands, they are assumed to be contaminating keratin monomers and dimers. These bands were only observed with this antibody and detected in only certain recombinant proteins, but not in native OM. We were not able to eliminate this non-specific keratin band by lowering the amount of reducing agent in the sample buffer as suggested by others (29). IgG against MSA1 recognized the native protein located in B. bovis merozoites, nothing was detected within A.
*marginale* OM and a negative control antibody, anti-MSA1, predominantly recognized rMSA1 and its native protein in *B. bovis* merozoites (Fig. 2E).

Immunohistochemistry (IHC) was also performed with isolated intact *A. marginale* (fixed with 10% formaldehyde) to indicate if the polyclonal antibodies anti-VirB9-1, anti-VirB9-2, anti-VirB10, and monoclonal anti-MSP2 (AnaR49a) bind to intact native protein, and they all did with no non-specific detection with normal rabbit IgG (Fig. 3A-E, respectively). Therefore, these polyclonal antibodies were the best possible choice for immuno-electron microscopy with native antigen and for studying protein-protein interaction of VirB9-1, VirB9-2, and VirB10 using the techniques far western blotting and immunoprecipitation.

### 4.3.2 Experimental determination of Surface localization

To determine if VirB9-1 and MSP2 are surface localized *A. marginale* infected erythrocytes were examined with polyclonal antibody specific for VirB9-1 or monoclonal antibodies against MSP2 and gold conjugated secondary antibody. The colonies of *A. marginale* and their inner and outer membranes were clearly visualized at 3.8k on a transmitting electron microscope (Fig. 4A), thus a conclusion about protein localization could be made. There was background staining present on the sections labeled with both the negative control antibody anti-MSA1 and the secondary antibody alone. This is not surprising because western blots of *A. marginale* OM developed with just a secondary antibody (anti-mouse, anti-bovine, or anti-rabbit) have a non-specific band at 100 kDa (discussed in Chapter 3 section 3.2.5). Similar background was also visualized by Giardina et al. using uninfected erythrocytes labeled with *A. marginale* immune sera and gold conjugated secondary antibodies (30). There were more electron dense areas on the *A. marginale* colonies when incubated with the anti-MSP2 antibody as compared to
the negative control. And it appeared the signal was strongest on the surface of *A. marginale*, but it was also present inside the *A. marginale* and throughout the infected erythrocyte (Fig. 4B). A conclusion could not be made about the surface localization of VirB9-1 from Figure 4C. The gold labeling of iRBC with anti-VirB9-1 looked the same as the negative control anti-MSA1 (Fig. 4D). Immuno-electron microscopy was also performed with intact and not fixed *A. marginale* isolated from the erythrocytes. These images in figure 5 were difficult to interpret because of formvar cracking that caused high background and no point of reference that made it problematical to identify *A. marginale* as a bacterium. However, using bioinformatic predictions, we successfully predicted that VirB2, VirB3, VirB6-1-4, VirB7, VirB8-1-2, VirB9-1-2, and VirB10 are predicted to be localized on the exterior surface of *A. marginale* (preliminary data section 2.3).

### 4.3.3 Evidence for dimerization of VirB7, VirB9-1, VirB9-2, and VirB10

Visualization of immunoblots of rVirB9-1, rVirB9-2, and rVirB10 probed with anti-FLAG mAb indicated that the purified proteins migrated at 50, 50, and 65 kDa as well as 100, 100, and 130 kDa, respectively (Fig. 6A). This pattern was also detected with western blotting using anti-HIS-HRP (Fig.7A), and/or autologous polyclonal antibodies (Fig. 2B-D). The corresponding weak bands on Coomassie stained gels (Fig. 6B) were excised for tandem MS analysis, and rVirB9-1 and rVirB9-2 were each identified in their respective 50 and 100 kDa bands, and VirB10 was identified in both 65 and 130 kDa bands (Table 1). Because other *A. marginale* proteins were not identified, this is consistent with the formation of homodimers by rVirB9-1, rVirB9-2, and rVirB10. Although faint bands migrating at ~140 kDa were observed for VirB9-1 and VirB9-2 on some immunoblots (Fig. 6A), there were no corresponding protein
bands visible on the gel, even when ten time more protein was loaded (Fig. 6B), so these were not analyzed by tandem MS. These may represent protein trimers or multimers with other T4SS proteins (1, 2). In addition to the protein bands that were identified as dimers, products of VirB9-1, VirB9-2, and VirB10 that migrated below the dimeric and monomeric forms were visualized on both the gels and western blots. Because these bands react with anti-FLAG mAb, which is highly specific for this epitope, these bands likely represent degradation products of the individual proteins resulting in bands smaller than the monomers and dimers, as previously observed for these proteins (17, 31).

4.3.4 Detecting the interactions of VirB7, VirB9-1, VirB9-2, and VirB10 with far western blots

Far western blotting is a technique that has been widely used to screen protein-protein interactions (24). It also was used to identify the interactions of VirB6 with VirB9-1 in *Ehrlichia chaffeensis* (23). The far western blotting strategy was performed as a screening tool to determine which rT4SS proteins bind to rVirB7, rVirB9-1, rVirB9-2, and rVirB10 that could enable them to undergo linked recognition (Fig. 7). Prior to the far western blotting with a candidate protein, membranes that were denatured and re-natured were developed with anti-HIS mAb, and all the proteins were maintained on the membrane (Fig. 7A). It was found that rVirB9-1 interacted with rVirB8-2 at the correct molecular mass of 23 kDa; with rVirB9-2 at the predicted mass of 46 kDa as well as with a predicted dimer and with apparent degradation products; with rVirB10 at the predicted mass of 65 kDa as well as with apparent degradation products; and with rMSP5 at the predicted mass of 28 kDa (Fig. 7B). Recombinant VirB9-2 interacted with only rVirB9-1 at the predicted mass of 46 kDa as well as with a predicted dimer and apparent degradation products (Fig. 7C). Recombinant VirB10 interacted with rVirB4-1 at
the predicted mass of 91 kDa; with rVirB8-2 at the predicted mass of 23 kDa; with rVirB9-1 at the predicted mass of 46 kDa; and with rVirD4 at the predicted mass of 93 kDa and two smaller bands (Fig. 7D). Far western blotting with rVirB7 was unsuccessful, as there were no other bands on the far western blot in figure 7E other than VirB7 itself, this failure is likely due to the purification using non-native conditions.

4.3.5 Binding coefficients for VirB9-1 interactions

The binding coefficients for VirB9-1 interaction with VirB9-2 was $0.0173 \pm 0.0034 \mu M$ and for VirB9-1 interaction with VirB10 was $0.0109 \pm 0.0094 \mu M$ (Fig. 8 A and B). The binding coefficients for the reverse of the interactions were also determined and found to be similar, VirB9-2 with VirB9-1 $K_D = 0.0205 \mu M \pm 0.0173$ (Fig. 8C), and VirB10 with VirB9-1 $K_D = 0.0135 \mu M \pm 0.0080$ (Fig. 8E). The graphed intensities of the dots that yielded the above $K_D$–values all had a goodness of fit to the binding curve equation with at least 80% confidence (Fig. 8). The binding coefficient for the interactions of VirB9-2 with VirB10 (and visa versa) could not be determined because they do not interact and the intensities of the dots did not fit to the binding curve equation (Fig. 8 D and F). From the binding coefficients listed above VirB9-1 has stronger binding to VirB10 than to VirB9-2.

4.3.6 Native VirB9-1, VirB9-2, VirB10 interactions determined by immunoprecipitation

We focused on identifying native interacting protein partners by using antibodies specific to VirB9-1, VirB9-2, and VirB10 and immunoprecipitation of solubilized OM (Fig. 9). The rabbit IgG or mouse MSP5-specific mAb used for immunoprecipitation is shown at the top of each lane; the antibody used for detection is shown under each panel. For anti-VirB9-1 and anti-
VirB9-2 immunoprecipitations, bands of the predicted size for VirB9-1 (29.7 kDa) and for VirB9-2 (30.6 kDa) were detected in both IPs and OM (Fig. 9A lanes 1 and 6, Fig. 9B, lanes 2 and 6, respectively). However, an additional band at ~60 kDa was also detected with these antibodies. The 60 kDa bands could represent homodimers of these proteins, a heterodimer of VirB9-1 and VirB9-2, or binding to another 30 kDa protein. VirB9-2 (Fig. 9B, lane 1), VirB10 (Fig. 9C, lane 1), and 19 kDa MSP5 (Fig. 9D, lane 1), were also detected in the VirB9-1 IP, but the ~37 kDa MSP2 was not (Fig. 9E, lane 1). The anti-VirB9-2 IP probed with anti-VirB9-1 IgG also showed ~30 and ~60 kDa bands (Fig. 9A, lane 2). VirB10 (Fig. 9C, lane 2), MSP5 (Fig. 9D, lane 2) and MSP2 (Fig. 9E, lane 2) were not detected in the anti-VirB9-2 IP. For the anti-VirB10 IP a band with the predicted mass for VirB10 (49 kDa) was detected along with an additional band at approximately 100 kDa (Fig. 9C, lane 3 and 6). This 100 kDa band could be a homodimer of VirB10, or an interaction of VirB10 with another protein(s). VirB9-1 was detected in this IP (Fig. 9A, lane 3), but VirB9-2 (Fig. 9B, lane 3), MSP5 (Fig. 9D, lane 3), and MSP2 (Fig. 9E, lane 3) were not. Immunoprecipitation reactions were also alkylated to prevent reformation of disulfide bonds, electrophoresed, and probed with anti-VirB9-1, anti-VirB9-2, anti-VirB10, and anti-ACP. However, reduction and alkylation had little effect on the interactions or apparent homo-dimerization (Fig. 10), suggesting that the observed T4SS interactions are not formed with disulfide linkages. In all three IP using anti-VirB9-1, anti-VirB9-2 and anti-VirB10, VirB7 was detected at approximately 25, 60 and 90 kDa (Fig. 9F lanes 1-3 and 5). However, none of these bands were predicted to be the correct molecular weight of VirB7. It is possible that VirB7 is only stable in formation of hetero-protein complexes with these proteins, thus its interaction cannot be negated, but a definitive conclusion can not be made.
The negative control rabbit anti-ACP IgG immunoprecipitation did not pull down any T4SS protein, MSP5, or MSP2 (Fig. 9A-F, lane 4).

To confirm the interaction of native VirB9-1 with MSP5, immunoprecipitation was performed with mAb against MSP5 and it was determined that MSP5 does interact with VirB9-1 (Fig. 9A, lane 5), but not with VirB9-2 or VirB10 (Fig. 9B and C, lane 5, respectively). Furthermore, the highly abundant MSP2 (27) was never identified in the T4SS IPs (Fig. 9E, lanes 1-5), supporting the specificity of the detected T4SS interactions. Taken together, our results show that VirB9-1 binds to VirB9-2, VirB10, and MSP5, VirB9-2 only interacts with VirB9-1, VirB10 only interacts with VirB9-1, and native MSP5 interacts only with VirB9-1.

There was very little success with identifying interacting proteins present in the IP pellet by tandem MS analysis. It was only identified that VirB9-1 interacts with VirB9-2 and two hypothetical proteins Am1254 and Am692 (Table 2). It is possible that these hypothetical proteins are substrates to the A. marginale T4SS.

4.4 Discussion

This study sought to identify the surface exposed A. marginale T4SS interacting proteins that are candidates for linked recognition. Therefore we first tried experiments to identify surface exposed proteins, and then focused on identifying the interacting proteins using far western blotting and immunoprecipitation.

The immuno-electron microscopy of iRBC with anti-MSP2 indicates that the MSP2 is mostly surface exposed but could have some protein located in the cytoplasm and on the iRBC surface. The only evidence to date that would suggest MSP2 localization is that MSP2 is recognized by sera from persistently infected (32). The electron dense labeling of the anti-MSP2
antibody around the erythrocyte that was different from the negative control background labeling posed some interesting questions. Is MSP2 shuttled to the infected erythrocyte membrane after infection? Or is MSP2 involved in *A. marginale* erythrocyte adhesion and left behind? The later question was answered in Chapter 7.1.2 with fluorescence activated cell sorting on uninfected erythrocytes, as well as in McGarey *et al.* with a hemagglutination assay (33). The iRBC developed with anti-VirB9-1 and gold labeled secondary antibody in figure 4C may indicate that the VirB9-1 is not present, which could be because VirB9-1 is not expressed, at too low in abundance to detect, or it is secreted out of the erythrocyte. Also these images may suggest that the polyclonal antibody does not bind to fixed tissues very well, however the same antibody worked with IHC (Fig. 3A). To solve these questions about why the labeling didn’t work with anti-VirB9-1, I would have liked to use a higher concentration of primary polyclonal antibody and other antibodies specific for VirB9-2 and VirB10. There is no experimental evidence that indicates that VirB9-1 would be surface exposed, just bioinformatics, however there is evidence for the exposure of VirB10 and VirB9-2. VirB9-2 and VirB10 are recognized by antibodies from *A. marginale*-infected animals (34, 35). VirB10 was also identified in intact *A. marginale* crosslinked with membrane-impermeable crosslinkers (36). VirB8 was also detected on the surface of *A. phagocytophilum* (37) and a putative VirB9 was co-localized with lysosome associated membrane protein during neutrophil expression (38). We predicted with bioinformatics that VirB2, VirB3, VirB6-1-4, VirB7, VirB8-1-2, VirB9-1-2, and VirB10 are localized on the exterior of *A. marginale* OM (preliminary data section 2.3). Thus, VirB2, VirB3, VirB6-1-4, VirB7, VirB8-1-2, VirB9-1-2, and VirB10 are targets for neutralizing antibody during infection.
To narrow down the proteins of interest for determining their interactions we focused on the most immunogenic T4SS proteins and proteins that would undergo linked recognition (see Chapter 3 section 3.3.6). Piecing together the pattern of immune recognition from chapter 3 and surface localization predictions, VirB7, VirB9-1, VirB9-2, and VirB10 were the proteins under consideration. Therefore, far western blotting was initially used to screen for interactions of rVirB7, rVirB9-1, rVirB9-2, and rVirB10 with each other and other recombinant T4SS proteins. And the interactions with VirB9s and VirB10 were confirmed with immunoprecipitation. All of the observed recombinant protein interactions in *A. marginale* identified with far western blotting (with exceptions of VirB9-1 interactions with VirB9-2 and MSP5 because these proteins are only located in Anaplasmataceae) have also been observed with homologs from *A. tumefaciens* (1, 2, 7, 8, 39-41). The *A. tumefaciens* VirB7 is a small lipoprotein that binds and stabilizes VirB9 through a di-sulphide bond and it also forms the core-complex (42, 43). Figure 7 and 9 shows that *A. marginale* VirB9-1, VirB9-2, and VirB10 interacts with VirB7, however the native size of VirB7 is problematic. Recombinant VirB10 was found to interact with rVirB9-1, rVirB4-1, VirB8-2, and rVirD4, interactions that have been observed with homologous proteins of *A. tumefaciens* (7, 39, 40). The interactions of rVirB10 with rVirB4-1 and rVirD4, two putative NTPases, have also been observed for *Helicobacter pylori* and *Rickettsia sibirica* (44, 45), and implicates VirB10 in substrate shuttling (8). VirB4-1 is more conserved across Rickettsiales genomes than VirB4-2 and VirB4-2 has more insertions and deletions, including in the NTP-binding cleft, relative to other bacteria (15), which is consistent with the observed interaction of VirB10 with VirB4-1 and not with VirB4-2.

Recombinant VirB10 and rVirB9-1 both interact with rVirB8-2, indicates that VirB8-2 may also be part of the core complex of the T4SS apparatus (11, 40). However, VirB8-2 was not
of particular interest for pursuing because it was not antigenic for the OM vaccinees used in chapter 3. The interaction of rVirB9-1 and rVirB9-2 had not been previously reported, because only bacteria in the family Anaplasmatacae are known to express two full-length VirB9 proteins (15, 16). The evidence of their interaction is novel and has never been shown in any other bacteria with a T4SS. Within the A. tumefaciens T4SS, VirB9 is thought to be part of the OM channel, be surface exposed, and span the entire outer gram negative bacterial envelope. The interaction of rVirB9-1 with rMSP5 is interesting as none of the other proteins interacted with MSP5. However it is unknown if MSP5 has T cell epitopes to stimulate T cell help to VirB9-1 in 3/16 and 8/23 DRB3 RFLP typed animals.

Immunoprecipitation of native proteins in solubilized A. marginale OM confirmed that the VirB9 proteins bind to each other and VirB9-1 binds to VirB10. These interactions and the potential formation of homodimers are consistent with what is known about the A. tumefaciens T4SS core complex structure (1, 2). Recently, cryo-EM structures of the A. tumefaciens core T4SS complex was found to contain 14 interacting copies of each VirB7, VirB9, and VirB10 that span the inner and outer membrane (1, 2). In our study the binding coefficients were determined for interactions of VirB9-1 with VirB9-2 and VirB10. VirB9-1 has higher affinity for VirB10 than for VirB9-2, and VirB9-2 does not interact with VirB10. This may be evidence for the requirement of a stabilizer protein, such as VirB7, that could “glue” the A. marginale T4SS core complex together like the disulfide linkage between VirB7 and VirB9 in A. tumefaciens (42, 43). Or it may indicate that the T4SS assembly within A. marginale consists of VirB9-1 sandwiched between VirB10 on the outside and VirB9-2 on the innermost portion of the complex.

It is anticipated that in animals that have the DRB3 RFLP type 8/23, VirB9-1 receives T cell help from its interacting protein partners VirB10 and VirB9-2 to produce IgG directed
against VirB9-1 through linked recognition (immune recognition was described in chapter 3 section 3.3.6 and 3.4). Likewise, for animals with the 3/16 haplotype, B-cells specific for VirB9-1 could receive T cell help from its interacting protein partner VirB10, and for animals with the 16/27 haplotype, VirB9-2 and VirB10 could receive T cell help from VirB9-1. It also could happen that VirB7 receives T cell help from VirB9-1, VirB9-2, and VirB10 in animals with the haplotypes 11/22, 22/24, 3/16, 16/27, and 16/22. We previously reported evidence for linked recognition of A. marginale MSP1a and MSP1b which lead to enhanced IgG responses to MSP1b via T cell help from MSP1a (46). Therefore, a protein complex made up of VirB7, VirB9-1, VirB9-2, and VirB10 used as a vaccine may produce an enhanced IgG responses and T cell proliferation in cattle with the RFLP alleles 3, 8, 11, 16, 22, 23, 24, and 27.

4.5 Work Cited


4.6 Tables

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

<table>
<thead>
<tr>
<th>Protein (Mass, kDa)(^a)</th>
<th>Mascot scores(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rVirB7 (20)</td>
<td>40</td>
</tr>
<tr>
<td>rVirB7 (40)</td>
<td>172(^c)</td>
</tr>
<tr>
<td>rVirB9-1 (50)</td>
<td>249</td>
</tr>
<tr>
<td>rVirB9-1 (100)</td>
<td>210</td>
</tr>
<tr>
<td>rVirB9-2 (50)</td>
<td>243</td>
</tr>
<tr>
<td>rVirB9-2 (100)</td>
<td>62</td>
</tr>
<tr>
<td>rVirB10 (65)</td>
<td>99</td>
</tr>
<tr>
<td>rVirB10 (130)</td>
<td>223</td>
</tr>
</tbody>
</table>

\(^a\) Molecular mass of protein monomers and dimers are indicated in parentheses.

\(^b\) Using an *A. marginale* specific search, MASCOT ion scores greater than 19 are significant at *P*<0.05

\(^c\) MASCOT ion score is an *E. coli* specific search and identified as thioredoxin 1, no significant hits for *A. marginale* were detected.
TABLE 2. Mass spectrometric analysis of immunoprecipitated *A. marginale* OM

<table>
<thead>
<tr>
<th>IP reaction&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Identified Protein</th>
<th>Mascot Scores&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-VirB9-1</td>
<td>VirB9-1</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>VirB9-2</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Am1254</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>Am692</td>
<td>22</td>
</tr>
<tr>
<td>Anti-VirB9-2</td>
<td>VirB9-2</td>
<td>31</td>
</tr>
<tr>
<td>Anti-VirB10</td>
<td>ns&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Anti-ACP</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>Anti-MSP5</td>
<td>ns</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Solublized *A. marginale* OM was immunoprecipitated with the indicated antibodies

<sup>b</sup> Using an *A. marginale* specific MASCOT search, ion scores greater than 19 and searches including the target protein were significant

<sup>c</sup> No significant hits were identified
4.7 Figures and Figure Legends

**Figure 1.** Purity of polyclonal immunoglobulins. Protein A-purified IgG (1 μg) were separated on a 4-20% gradient polyacrylamide gel and stained with Coomassie blue as follows: anti-VirB7 (lane 1), anti-VirB9-1 (lane 2), anti-VirB9-2 (lane 3), anti-VirB10 (lane 4), anti-ACP (lane 5), anti-MSA1 (lane 6), and normal rabbit IgG (lane 7). The IgG heavy and light chain can be visualized at 50 and 25 kDa, respectively.
Figure 2. Specificity of rabbit polyclonal antibodies against VirB7, VirB9-1, VirB9-2, VirB10, and MSA1. Purified recombinant T4SS proteins (1 μg), 10 μg of *A. marginale* OM, iSE6 cells, and *B. bovis* merozoites (native proteins) were separated on a 4 to 20% gradient SDS-PAGE gel, transferred to a nitrocellulose membrane, and detected by western blotting with 2 μg/ml of protein A-purified and *E. coli*-absorbed polyclonal rabbit IgG (A) anti-VirB7, (B) anti-VirB9-1, (C) anti-VirB9-2, (D) anti-VirB10, and (E) anti-MSA1. Predicted molecular weights for recombinant (r) and native (n) proteins are in parentheses: rVirB7 (20kDa), nVirB7 (~6kDa), rVirB9-1 (46kDa), nVirB9-1 (30kDa), rVirB9-2 (46kDa), nVirB9-2 (30kDa), rVirB10 (65kDa), nVirB10 (49kDa), rMSA1 (52kDa), and nMSA1 (35kDa). The bands with the predicted size of monomeric and dimeric (VirB9-1, VirB9-2, and VirB10) proteins are indicated by asterisks.

Figure 3. Polyclonal antibodies anti-VirB9-1, anti-VirB9-2, and anti-VirB10 detect native protein within intact *A. marginale*. Intact *A. marginale* was isolated from infected erythrocytes and embedded in agarose for immunohistochemistry. Sections were labeled with antibodies specific for A) anti-VirB9-1, B) anti-VirB9-2, C) anti-ViB10, D) anti-MSP2 as a positive control, and E) normal rabbit as a negative control. Sections were developed with HRP conjugated secondary anti-mouse or anti-rabbit and 3-amino-9-ethylcarbazole containing hydrogen peroxide substrate counterstained with Meyer’s hematoxilin. Bar indicates 10 μM.
Figure 4. Surface localization experiment with infected erythrocytes. Immunoelectron microscopy of sections including agarose-embedded *A. marginale* infected erythrocytes labeled with (A) no antibody, (B) mAb anti-MSP2, (C) pAb anti-VirB9-1, (D) pAb anti-MSA1, and (E) just secondary antibody 1.4nm AU-conjugated anti-rabbit. Secondary 1.4nm AU-conjugated anti-mouse was used on the anti-MSP2 section. Bar indicated 500 nm. *A. marginale* is approximately 0.5 μm and bovine erythrocytes are approximately 5.5 μm.
Figure 5. Surface localization experiment with erythrocyte-free intact *A. marginale*. Erythrocyte-free *A. marginale* were embedded in agarose and sectioned. Antibody reactions and washes were performed directly on the formvar and were as follows: (A) mAb anti-MSP2, (B) pAb anti-VirB9-1, (C) pAb anti-VirB9-2, and (D) pAb anti-VirB10. Bar indicates 200 nm.

Figure 6. Evidence for dimerization of rVirB9-1, rVirB9-2, and rVirB10. (A) Recombinant proteins (1 μg) were transferred from a 4 to 20% gradient SDS-PAGE gel to a nitrocellulose membrane that was probed with anti-HIS mAb (rVirB7) or anti-FLAG mAb (rVirB9-1, rVirB9-2, and rVirB10) and developed. (B) Coomassie stained SDS-PAGE gel corresponding to the western blot in panel A, with the exception that 10 μg protein was loaded in each lane to visualize the faint bands. Asterisks denote bands where the *A. marginale* T4SS protein was identified by tandem mass spectrometric analysis.
**Figure 7.** Far western blotting to detect interacting recombinant T4SS proteins with specific targets. The indicated purified recombinant T4SS proteins (1 μg) were separated on 4 to 20% gradient SDS-PAGE gels, transferred to a nitrocellulose membrane, and denatured and re-natured with decreasing concentrations of guanidine HCl. Membranes were incubated with (A) 1:2,000 dilution of HRP conjugated anti-HIS mAb to ensure presence of the bait proteins or 10 μg of target protein (B) rVirB9-1, (C) rVirB9-2, (D) rVirB10, and (E) rVirB7. The interactions were detected with *E. coli*-adsorbed rabbit antisera (1:500) specific for (B) VirB9-1, (C) VirB9-2, (D) VirB10, and (E) VirB7. Blots were developed with goat-anti-rabbit IgG (H+L). An asterisk indicates an interacting protein partner to the target protein with the following predicted molecular masses: rVirB4-1 (90 kDa), rVirB4-2 (91 kDa), rVirB6-1 F1 (90 kDa), VirB7 (20 kDa), rVirB8-2 (23 kDa), rVirB9-1 (46 kDa), rVirB9-2(46 kDa), rVirB10 (65 kDa), rVirB11 (38 kDa), rVirD4 (93 kDa), and MSP5 (28 kDa).
VirB9-1 interactions with VirB9-2

\[ y = \frac{13115x}{0.02 + x} \]
\[ R^2 = 0.91 \]

VirB9-1 interactions with VirB10

\[ y = \frac{83677x}{0.004 + x} \]
\[ R^2 = 0.92 \]

VirB9-2 interactions with VirB9-1

\[ y = \frac{43099x}{0.036 + x} \]
\[ R^2 = 0.82 \]

VirB9-2 interactions with VirB10

\[ y = \frac{31460x}{x} \]
\[ R^2 = 0.00 \]

VirB10 interactions with VirB9-1

\[ y = \frac{119915x}{0.008 + x} \]
\[ R^2 = 0.8 \]

VirB10 interactions with VirB9-2

\[ y = \frac{92264x}{0.004 + x} \]
\[ R^2 = 0.14 \]
Figure 8. Single site saturation binding curves for the interactions of rVirB9-1, rVirB9-2, and rVirB10. Change in densitometry was evaluated for the interactions of (A) rVirB9-1 with rVirB9-2, (B) rVirB9-1 with rVirB10, (C) VirB9-2 with VirB9-1, (D) VirB9-2 with VirB10, (E) VirB10 with VirB9-1, and (F) VirB10 with VirB9-2. Bait proteins were applied to dot blots at amounts up to 5 μg, allowed to interact with 10 μg of (A and B) rVirB9-1, (C and D) rVirB9-2, and (E and F) rVirB10, and developed with rabbit (A and B) anti-VirB9-1, (C and D) anti-VirB9-2, and (E and F) anti-VirB10 antiserum. Each blot was then incubated with AP-conjugated goat anti-rabbit IgG (H+L) and developed using the Western-Star chemiluminescent substrate. Reactions were compared with the density of the reaction to negative control rMSA1. Data are graphically represented as concentration of the protein on the spot vs. spot intensity and the curve was fit to a single site binding curve, to yield the binding association constant. Graphs are representative of three independent experiments.
Figure 9. Detection of native protein interactions of VirB9-1, VirB9-2, and VirB10. Solubilized *A. marginale* OM proteins were immunoprecipitated with purified rabbit IgG against VirB9-1 (lane 1), VirB9-2 (lane 2), VirB10 (lane 3), negative control *B. bovis* ACP (lane 4), and mAb specific for MSP5 (lane 5) as indicated for each lane. Solubilized OM were included (lane 6) for a size comparison. Immunoprecipitated pellets and OM were electrophoresed and transferred to nitrocellulose membranes. Detection of the interacting proteins was performed by western blotting of individual strips with purified rabbit IgG against (A) VirB9-1, (B) VirB9-2, (C) VirB10, and (F) VirB7, and mAb specific for (D) MSP5, (E) MSP2, as indicated under each
panel. Secondary antibody was clear blot-HRP (A-D, F and E, lane 5) or goat anti-mouse IgG + IgM (E, lanes 1-4 and 6). Scanned images were rearranged and presented as shown. Predicted molecular weights for native proteins are indicated in parentheses: VirB9-1 (30 kDa), VirB9-2 (30 kDa), VirB10 (49 kDa), B. bovis ACP (15 kDa), MSP5 (19 kDa), MSP2 (37 kDa), and VirB7 (~6 kDa).

**Figure 10.** Detection of native protein interactions of VirB9-1, VirB9-2, and VirB10 are not dependent on disulfide linkages. Solubilized *A. marginale* OM proteins were immunoprecipitated with purified rabbit IgG against VirB9-1 (lane 1), VirB9-2 (lane 2), VirB10 (lane 3), and negative control ACP (lane 4). Solubilized OM were included (lane 5) for a size comparison. Immunoprecipitated pellets were reduced with DTT and treated with 0.5 M iodoacetamide for 15 min, electrophoresed and transferred to nitrocellulose membranes. Detection of the interacting proteins was performed by immunoblotting individual strips with purified rabbit IgG against (A) VirB9-1, (B) VirB9-2, or (C) VirB10 as indicated under each panel. Secondary antibody was Clean Blot-HRP. Predicted molecular weights for native proteins are indicated in parentheses: VirB9-1 (30 kDa), VirB9-2 (30 kDa), VirB10 (49 kDa), and *B. bovis* ACP (15 kDa).
Chapter 5: Determine T cell and MHC class II Epitopes on the Interacting Immunogenic T4SS Proteins

5.1 Brief Introduction

Cell to cell interactions of the adaptive immune response are critically important in protection from pathogens. These interactions are orchestrated by the immunological synapse whose primary components are the T cell receptor (TCR) on CD4+ T helper cells and major histocompatibility complex class II (MHC class II) molecules on antigen presenting cells (APC). The primary role of the MHC class II molecules is to display extracellular-derived peptides in a multivariant form on the surface of the APC (1, 2) so they are available to interact with the TCR and initiate an antigen-specific T cell response. MHC class II molecules comprise a set of peptide binding proteins of varying specificities (3). This diversity largely reflects the extensive allelic polymorphism used by the immune system to increase the antigen epitope-binding repertoire (4-6).

Cattle express two MHC class II proteins DR and DQ, yet half of the haplotypes also have duplicated DQ regions (7, 8). Mice express two class II proteins (H2-A and –E), and humans express three (HLA-DR, -DQ, and –DP). Genetic diversity of cattle is permitted through polymorphisms in DRB3, DQA, and DQB genes as well as through gene pairing, which can occur for any combination of α- and β-chains. Many antigenic peptides are presented by bovine DR molecules to T cells (7-10), however antigenic peptides are also presented by intra- or interhaplotype pairing of DQA and DQB molecules (7-9, 11, 12). Intrahaplotype pairing refers to the combination of DQA and DQB gene products on the same chromosome within the same haplotype. Interhaplotype pairing refers to the combination of DQA and DQB gene products on different chromosomes between haplotypes. It has also been noted that isotype-mismatched
combinations of DQ gene products with DR gene products, denoted mixed isotype, can be expressed with human HLA-DR and -DQ (13, 14) and murine H2-A and -E (15, 16). However, this has not been shown for cattle. If some alleles are more active than others for antigen presentation, then protective immune responses against a bovine pathogen such as Anaplasma marginale will be strongly influenced by certain MHC class II alleles, and their characterization is therefore extremely important for effective vaccine development.

The tick-borne pathogen, A. marginale, causes a persistent infection of cattle characterized by acute and chronic high-load bacteremia. Control measures against anaplasmosis are largely inadequate and the lack of a safe and effective vaccine results in large economic losses (17). CD4\(^+\) T lymphocytes and APC are important for inducing protective immune responses following immunization with outer membranes (OM) of A. marginale (18). Effective vaccines against A. marginale will likely require the inclusion of multiple T-lymphocyte epitopes derived from conserved antigens, as immunodominant, antigenically variant surface proteins such as major surface protein (MSP)2 have failed to elicit protective immunity (19, 20). Because MHC class II molecules influence antigen-specific CD4\(^+\) T-lymphocyte responses, characterization of MHC class II molecules and the identification of immunogenic T-cell epitopes are also important considerations for vaccine development. Immunogenic proteins comprising the protective OM include the subdominant and conserved type IV secretion system (T4SS) proteins. The T4SS is a membrane protein complex of many bacterial pathogens which secretes virulence factors and promotes host cell invasion and intracellular survival (21). It was previously shown that A. marginale T4SS proteins VirB9-1, VirB9-2, and VirB10 are strongly immunogenic for CD4\(^+\) T lymphocytes from A. marginale OM- immunized cattle and that these proteins are highly conserved across A. marginale strains and A. centrale (Chapter 3 and 2 and
ref. 22-24). Determining the breadth of the response to T-cell epitopes on VirB9-1, VirB9-2, and VirB10 in outbred cattle is important to develop a multiple-antigen and peptide-based vaccine construct.

In order to gain further insights into the antigenic structure of T4SS proteins VirB9-1, VirB9-2, and VirB10, we have investigated the proliferative responses of CD4\(^+\) T lymphocytes from MHC class II-defined cattle to a series of peptides spanning the length of each of these proteins. In addition, bovine MHC class II DR- and DQ- transfected 293-F cells were used to determine MHC class II restriction elements. Our study highlights the importance of MHC class II DQ in addition to DR molecules in presenting pathogen derived peptides to CD4\(^+\) T cells.

5.2 Materials and Methods

5.2.1 Synthetic peptides

Full length protein sequences for VirB9-1, VirB9-2, and VirB10 of the St. Maries strain of *A. marginale* were used to synthesize peptides thirty amino acids in length overlapping by ten amino acids. This yeilded 13 peptides for VirB9-1, 14 peptides for VirB9-2, and 22 peptides for VirB10 (Table 1). Peptides with 75% purity were manufactured. VirB9-1 peptides 8-13 were synthesized and purified by Gerhard Munske, Biotechnology and Biochemistry Laboratories, Washington State University (WSU). VirB9-1 peptides 1-7, VirB9-2 peptides 1-14, and VirB10 peptides 1-22 were synthesized and purified by NeoPeptides (Cambridge, MA). The 30-mer VirB10 peptide 2 was difficult to synthesize, and was divided into two 20-mer peptides with 10 overlapping amino acids and re-named VirB10 P2.1 and P2.2 (Table 1). Peptides were solubilized in 10% DMSO in PBS and diluted to a stock concentration of 1 mg/ml.
5.2.2 Cattle

Five age-matched steers and one cow (animal numbers: 35113, 35141, 35160, 35280, 35287, and 583) were purchased from the WSU dairy with varying and heterozygous MHC class II. Bovine lymphocyte antigen (BoLA) MHC class II DRB3 types were determined by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method described previously (22, 25 and chapter 2 section 1) and DQA1, DQA2, DQB1, DQB2 alleles were determined by sequencing entire cDNA using primer sets previously described (6, 7). The class II haplotypes of all cattle are shown in Table 1 of Chapter 2. Cattle were immunized with A. marginale OM as previously described (22 and Chapter 3.2.3).

5.2.3 Two week T cell lines used for proliferation assays

Peripheral blood mononuclear cells (PBMC) from six A. marginale OM-immunized cattle were collected, depleted of CD8+ and γδ T cells using monoclonal antibodies (mAb) and complement lysis (22 and Chapter 3.2.6). Two-week cell lines from CD4+ T cell-enriched PBMC were stimulated with A. marginale OM as described (22 and Chapter 3.2.6). Proliferation assays were performed with 3 x 10^4 T cells and 2 x 10^5 irradiated autologous PBMC diluted in complete RPMI medium (cRPMI, Gibco) as a source of antigen presenting cells (APC) per well for 3-4 days. Positive control antigens included A. marginale OM and full length recombinant VirB9-1, VirB9-2, and VirB10, expressed as described (22 and Chapter 3.2.6). These were used at a final concentration of 1 μg/ml and peptides were used at 0.1-20 μg/ml. The N-terminal peptide P1 from B. bovis rhoptry associated protein 1 (Rap1 P1) was used as a negative control (26). The cells were radiolabeled during the last 18 h of culture with ³H-thymidine and the results are reported as stimulation index (SI). The SI was calculated by dividing the mean counts per minute
(cpm) of replicate cultures with a test antigen by the mean cpm of cells cultured with medium. The SI for the different peptides peptides were compared to the SI for Rap1 P1 using a one-way ANOVA corrected for multiple comparisons with the Dunnett’s test. Statistically significant T cell stimulation by an antigen was set at $P < 0.05$ and SI > 3.

5.2.4 MHC class II expression

Amplification of full-length cDNA encoding sequences for DRA, DRB3, DQA, DQB, and CD80 was performed by PCR and the PCR product was clonted into the eukaryotic expression vector pCR3.1 (Invitrogen) as previously described (7). Human embryonic kidney 293-F cells (Invitrogen) with 90% confluence on a six-well plate were transfected with 3 μg DNA mixture (CD80, α-, and β-chains) added to 200 μl OptiMem (Gibco) plus 6 μl GeneJuice (Novagen) that was incubated at room temperature for 10-15 min. After transfection, the 293-F cells were cultured for 2 days in a 37°C incubator with 5% CO$_2$. Medium was removed and 50 μg/ml mitomycin C (Sigma-Aldrich) in cRPMI was added to each well and incubated for 2-2.5 h. The transfected cells were then harvested with HBSS containing 2 mM EDTA, washed, and used as APC to the OM-specific two week cell lines.

5.2.5 Flow cytometric analysis of transfected 293-F cells

Expression of BoLA-class II and co-stimulatory molecules on transfected 293-F cells was verified by flow cytometry using 15 μg/ml bovine DR-specific mAb TH14B (IgG2a), DQ-specific mAb CC158 (IgG2a), and CD80 specific mAb IL-A159 (IgG1). TH14B was purchased from the WSU mAb center and mAbs CC158 and IL-A159 were received from the Institute of Animal Health, Compton, UK (7). For the secondary antibodies, R-phycoerythrin (R-PE)-
conjugated goat anti-mouse IgG2a or R-PE conjugated goat anti-mouse IgG1 (Caltag Laboratories) were used at a 1:200 dilution.

5.2.6 T cell proliferation assays with transfected 293-F cells as APC

To determine DR and DQ molecules that present immunogenic peptides, transfected 293-F cells treated with mitomycin C were plated in 96 well round-bottomed plates at 5 x 10⁴ cells/well and used as APC with the OM-stimulated two-week T cell lines. The transfected 293-F cells were loaded with 20 μg/ml individual peptide in triplicate wells, incubated at 37°C for 1h, washed three times, and bovine T cells from two-week cell lines were added and proliferation was measured after 3-4 days as previously described (7). For each peptide, T-cell proliferation to a peptide presented by 293-F cells transfected with a pair of class II α and β chains was compared to non-transfected 293-F cells, and significance was evaluated by the Student’s one-tailed t-test where P< 0.05

5.3 Results

5.3.1 Breadth of CD4⁺ T cell response to peptides of VirB9-1, VirB9-2, and VirB10

To assess the position and number of T-cell epitopes, 30-mer peptides overlapping by 10 amino acids and spanning the complete sequences of VirB9-1, VirB9-2, and VirB10 were synthesized (Table 1). The peptides were tested for T cell stimulation using OM-specific T cell lines derived from animals 35113, 35141, 35160, 35280, 35287, and 583 with varying MHC class II haplotypes, specified in Table 1 of Chapter 2. VirB9-1 peptide specific responses were detected with T cell lines derived from animals 35113, 35141, 35280, and 35287. Animal 35113 had significant T cell proliferation for peptides VirB9-1 P5, P6, and P10 (Fig. 1A). These
peptides included T cell responses with as little as 0.1 µg/ml for VirB9-1 P6, 1 µg/ml for VirB9-1 P10, and 5 µg/ml for VirB9-1 P5 (Fig. 2A). Animal 35287 also responded to VirB9-1 P5, P6, and P10 although the response to P5 was not significant using a multiple comparison correction (Fig. 1A), and P6 and P10 induced significant proliferation with ≥ 1 µg/ml peptide (Fig. 2E). T cells from animals 35141 and 35280 also recognized VirB9-1 P6 and P10 (Fig. 1A) with as little as 0.1 µg/ml peptide (Fig. 2C and J, respectively). T cells from animals 35160 and 583 did not recognize the full-length recombinant VirB9-1 and thus responses to VirB9-1 peptides were not observed (Fig. 1A).

VirB9-2 P5 was recognized by T cells from animals 35113, 35141, 35280, and 35287 (Fig. 1B). Animal 35113 also responded to VirB9-2 P11 and P12 (Fig. 1B). The T cell responses from 35113 to the three VirB9-2 peptides were significant when using 20 µg/ml peptide (Fig. 2B). The T cell responses to VirB9-2 P5 from 35141 and 35280 were significant using peptide concentrations ≥ 0.1 µg/ml (Fig. 2C and J, respectively). In addition, VirB9-2 P5, animal 35287 also recognized VirB9-2 P8 (Fig. 1B), and each was significant with as little as 0.1 µg/ml of peptide (Fig. 2E and F). Animal 35160 did not recognize the full length VirB9-2 and, thus, no responses to peptides from VirB9-2 were observed (Fig. 1B). Animal 583 recognized eight peptides spanning VirB9-2: peptides 2, 3, 4, 9, 10, 11, 12 and 13 (Fig. 1B). T cell responses to VirB9-2 P2, P3, P10, P11, P12, and P13 were significant with as little as 0.1 µg/ml, and responses to VirB9-2 P4 and P9 were significant using 20 µg/ml of peptide (Fig. 2G and H).

VirB10 P21 was recognized by T cells from animals 35113, 35280, and 35287 (Fig. 1C) and the responses were significant with 10 µg/ml, 1 µg/ml, and 10 µg/ml of peptide, respectively (Fig. 2B, F, and J). Animal 35287 also recognized VirB10 P6 (Fig. 1C) using 1 µg/ml (Fig. 2F). T cell lines derived from animal 35160, which responded to VirB10, only recognized peptides 13
and 14 from VirB10 (Fig. 1C) but not any peptides from VirB9-1 and VirB9-2. This response was significant with as little as 0.1 μg/ml of peptide (Fig. 2D). T cells from animal 583 recognized VirB10 peptides 2.1, 3, 13, and 22 (Fig. 1C) and these were significant with 10 and 20 μg/ml of peptide (Fig. 2I).

Dose response curves diagramed in Fig. 2 yielded interesting information about the strength of the T cell response for specific peptides from each animal. For animals 35113, 35141, and 35287 the strongest T cell responses were induced using VirB9-1 P6 and VirB9-1 P10 at all the concentrations tested (Fig. 2A, C, and E). This may suggest a high avidity interaction between the TCR, MHC class II presenting molecule, and these peptides. Likewise, animal 35280 also had a strong T cell response to VirB9-1 P10 and the second strongest response from this animal was induced by VirB9-2 P5 (Fig. 2J). For animal 35160, each VirB10 peptide 13 and 14 induced a similar strength of T cell response (Fig. 2D). Yet, considering that contiguous peptides have an overlapping sequence by ten amino acids, the T cell response from 35160 may be represented by a minimum of one epitope. VirB9-2 peptides 2, 3, and 10-13 induced the strongest T cell responses from 583, and they could represent a minimum of one and three epitopes, respectively (Fig. 2G and H). There is not a single peptide that induced T cell responses from every animal, yet four of the six animals had strong T cell response to VirB9-1 P10. Peptides that induced the strongest T cell responses within each animal may have indications of the avidity of their interactions, and these are the peptides that would be best for inclusion in a vaccine. For all six MHC class II- diverse animals together that would be VirB9-1 P6 and 10, VirB9-2 P2, 3, 5 and P10-13, and VirB10 P13 and 14.

5.3.2 DR and DQ expression and antigen presentation
293-F cells were transfected with all the possible combinations of DRA and DRB3, DQA and DQB, DRA and DQB, and DQA and DRB3 alleles known for each animal. Not all of the possible DR and DQ combinations available for each animal were expressed within transfected 293-F cells. The combinations that did express are listed in Table 2. Each of the expressed MHC class II combinations were tested for their ability to present peptides using T cells from a corresponding animal. T cells from animals 35113, 35141, and 35287 were stimulated with DRA/DRB3*1101 presenting peptides VirB9-1 P6, P10, and VirB9-2 P5 (Fig. 3). Also, DRA/DRB3*1101 presented VirB9-1 P5 to T cells from animals 35113 and 35287. T cells from 35280 also responded to VirB9-1 P6, P10, and VirB9-2 P5, but unlike animals 35113, 35141, and 35287, does not have a DRA/DRB3*1101 combination (Table 1 of Chapter 2). The class II molecules presenting these peptides to T cells derived from animal 35280 were identified as DRA/DRB3*1401 for VirB9-1 P10, DQA*2202/DQB*1301 for VirB9-1 P6, and VirB9-2 P5 (Fig. 3). Thus, VirB9-1 P6, and P10, and VirB9-2 P5 may contain two core epitopes that are presented and recognized by the TCR. The presentation of VirB9-1 P6 by DQA*2202/DQB*1301 was also observed using T cells from animal 35287. VirB10 P21 was presented by DQA*1001/DQB*1002 to T cells from animals 35113 and 35287 (Fig. 3). Animals 35141, 35160, and 35280 also had this DQ combination, but significant responses to VirB10 P21 were not detected for these animals using this DQ pair. For animal 35113, we were unable to identify a restriction element for VirB9-2 P11 and P12, possible because the response to this peptide was relatively weak, and only detected with 20 µg/ml peptide (Fig. 2B).

T cells from animal 35160 only responded to P13 and P14 from VirB10, and these peptides were presented by DQA*2202/DQB*0901 (Fig. 3). This combination was clearly an interhaplotype DQ molecule since the DQA*2202 allele comes from the DH16A haplotype and
DQB*0901 allele comes from the DH03A haplotype. Therefore, in order to express DQA*2202/DQB*0901, bovine must be heterozygous. Another interhaplotype combination available only for 35287 was DQA*2206/DQB*1301, which presented VirB9-2 P8 and VirB10 P6 (Fig. 3). This explains why 35287 was the only animal to respond significantly to these peptides.

Animal 583 has unique DR and DQ combinations when compared to the other five animals (Table 1 of Chapter 2), and unique responses to VirB9-2 and VirB10 peptides were observed. DRA/DRB3*1201 presented the majority of the peptides that 583 responded to: VirB9-2 P2, P3, P9, P10, P11, and P12, as well as VirB10 P2.1, P3, P13, and P22. We were unable to identify a restriction element for VirB9-2 P4, possible because the response to this peptide was relatively weak, and only detected with 20 µg/ml peptide (Fig. 2G). VirB9-2 P13 was presented by DRA/DRB3*2703.

MHC class II molecules shown to present immunostimulatory VirB9-1, VirB9-2, and VirB10 peptides to CD4⁺ T cells are summarized in Table 3. We identified four DRA/DRB3 molecules that presented the majority of peptides (15). Two intrahaplotype DQ combinations presented three peptides; DQA*1001/DQB*1002 presented VirB10 P21, and DQA*2202/DQB*1301 presented VirB9-1 P6 and VirB9-2 P5. We also identified two interhaplotype DQ combinations that presented four peptides; DQA*2206/DQB*1301 presented VirB9-2 P8 and VirB10 P6 and DQA*2202/DQB*0901 presented VirB10 P13 and P14. Five of these peptides were not presented by DRA/DRB3 molecules. The MHC class II DQ interhaplotype- pair DQA*2202/DQB*0901 was the only functional class II molecule for animal 35160, and it presented the only T cell epitope identified on VirB10. Thus, interhaplotype pairing was extremely important in this animal for recognition of VirB10.
Mixed isotype pairs containing DQA and DRB3 alleles were also examined. Each combination that was expressed, listed in Table 2, was tested with T cells from the corresponding animals. We identified that mixed isotypes DQA*2206/DRB3*1101, DQA*1201/DRB*1201, and DQA*2201/DRB3*1201-presented peptides. Thus, their expression as determined by fluorescence activated cell sorting analysis using mAb TH14B is shown in Figure 4, which also includes the negative controls DQA*1001/DRB3*1101 (which did not express) and non-transfected 293-F cells. We discovered that VirB9-2 P5 was presented by DQA*2206/DRB3*1101 to T cells derived from 35141 and 35287 (Fig. 5). In addition, T cell lines from animal 583 responded to the mixed isotype combinations DQA*1201/DRB*1201 and DQA*2201/DRB3*1201 presenting VirB9-2 P12 (Fig. 5). A residual response to this peptide was also still present in non-transfected 293-F cells. This can be explained by residual peptide remaining in the well despite repeated washing, and residual autologous APC in the two-week cell lines from this animal, but the response to VirB9-2 P12 presented by DQA*1201/DRB*1201 and DQA*2201/DRB3*1201 was nevertheless significantly greater than the response with 293-F cells alone.

5.4 Discussion

*Anaplasmaphagocytophilum* T4SS proteins VirB9-1, VirB9-2, and VirB10 are strongly immunogenic for CD4+ T lymphocytes from OM-immunized cattle (35113, 35141, 35160, 35280, 35287 and 583, (22 and Chapter 3). Animals 35113, 35141, and 35287, which are half-matched with the DRB3*1101 allele, recognized all three of these proteins. However, animals 35160, 35280, and 35287, which are half-matched with DRB3*1501, had differential responses to each protein. In order to understand the differences in T cell responses observed for these animals to VirB9-1,
VirB9-2, and VirB10 we sought to identify MHC class II-allele specific T cell epitopes. Furthermore, the identification of T-cell epitopes on VirB9-1, VirB9-2, and VirB10 is needed to develop a multiple-antigen, epitope-based vaccine construct. Summarizing all the information for each cattle in this study, we identified 11 peptide presenting elements including four DRA/DRB3, two intrahaplootype-DQA/DQB pairs, two interhaplootype-DQA/DQB pairs, and three DQA/DRB3 mixed isotype pairs. Thus, consistent with previous studies, it was very important to identify DQ-restriction elements that uniquely present pathogen peptide epitopes to CD4+ T cells (7-9). Notably, we now understand the differential T cell responses within our animals and we can potentially predict T cell responses for haplotype-defined cattle.

The four DRA/DRB3 combinations presenting peptides were DRA/DRB3*1101, DRA/DRB3*1401, DRA/DRB3*1201, and DRA/DRB3*2703. The function of DRA/DRB3*1201 in presenting peptides to T cells from animal 583 was important, presenting a minimum of four epitopes from VirB9-2 and four from VirB10, when considering that contiguous overlapping peptides contain a minimum of one epitope. BoLA DR genes have been associated with the resistance and susceptibility to several viral, bacterial, and parasitic infections, including, bovine leukemia virus (27-29), foot and mouth disease virus (30, 31), Staphylococcus aureus-associated bovine mastitis (6, 32, 33), Neospora caninum (34), and Theileria parva (35). However, despite the indication of the role that BoLA-DR molecules play in protective immunity, very few studies have performed functional assays with BoLA class II molecules. Among these studies pathogen epitope restriction elements have been identified for DRA/DRB3*1201 presenting A. marginale peptides MSP2 P16-7 (7) as well as MSP1a peptide F2-1-1b (10). DRB3*1101 presented MSP1a F2-B to T cells (7). Several foot and mouth viral peptides were presented by DRA/DRB3*1101, DRA/DRB3*1201, and DRA/DRB3*2703 as well (30, 31).
In cattle, duplication of DQ molecules amplifies the opportunity for intra- and interhaplotype pairing to form functional heterodimers, thereby increasing the complexity of restriction elements and providing more dynamic immune responses \((7, 8)\). We identified the products of two interhaplotype DQ molecules, DQA*2202/DQB*0901 and DQA*2206/DQB*1301 that presented peptides from VirB9-2 and VirB10. This is the first study to report a functional DQA*2202/DQB*0901 molecule that presents VirB10 P13 and 14. This combination was the only presenting element identified for animal 35160, explaining its T cell response to VirB10. Interhaplotype DQA*2206/DQB*1301 was previously reported to present \(A.\ marginale\) MSP1a peptide B \((7)\) and it is apparently not- rare because cattle heterozygous for the RFLP type 16/22 are common in Washington state Holstein herds (unpublished observations). The \(\alpha\)- and \(\beta\)-chains of this combination may also have some flexibility as to which protein they partner with as seen with (Tables 3, 4) and ref. \((7)\). Furthermore, even though not all possible DQ combinations were expressed on the cell surface, we have observed that there is preferential pairing and that many combinations are conserved within this and previous studies from our lab \((7, 9, 10)\).

Three mixed isotype combinations that presented peptides were also identified, i.e. DQA*2206/DRB3*1101 presented VirB9-1 P5, and DQA*1201/DRB3*1201 and DQA*2201/DRB3*1201 presented VirB9-2 P12. However, the corresponding DRA/DRB3 combination for these mixed isotypes also presented the same peptide. This indicates that the \(\beta\)-chain may be more important for presentation of the two peptides and the \(\alpha\)-chain is interchangeable. This is the first documentation that bovine mixed isotypes have the capability to present peptides; however there is precedence from studies with mice and humans \((13-16)\). Despite mixed isotype combinations being a relatively unexplored area, there is a possibility that
this may be a mechanism for the immune response to further increase diversity, but further analyses are required. We cannot rule out that the bovine mixed isotype peptide presentation is an \textit{in vitro} artifact; we currently do not have the means to confirm that this presentation occurs \textit{in vivo}, because there is no mAb specific to detect mixed isotypes.

During the making of two week cell lines, populations of T cells that are more numerous compete for limiting MHC class II peptide complexes, therefore, resulting in an early cessation of T cell responses (36-39). This process may explain why we observed that some DR and DQ combinations do not present peptides for every animal. There are several examples in the literature indicating varying CD4\(^+\) T lymphocyte populations due to properties involving TCR contact with peptide-MHC class II complex(es) or due to differences in naïve T cell populations in mice (36, 38, 40). Similarly, peptide-MHC class II complex-dependent variations in CD4\(^+\) T cell population may also occur at the peak of immune response during infection (38). Thus, choosing high avidity interactions of the peptide MHC class II complex with the TCR and inclusion of appropriate (highly antigenic) CD4\(^+\) T cell epitopes is an essential step for the construct of a multiple-antigen and peptide-based vaccine. Also for consideration, the identification of MHC allele-specific T cell epitopes may not be enough; vaccine epitopes should effectively cover the MHC class II allelic diversity in both the cattle and human populations. In the case of \textit{A. marginale} and the T4SS proteins, the suggested epitopes that have strong affinity with high frequency MHC class II alleles for use in a bovine vaccine would be VirB9-1 P6 and 10, VirB9-2 P2, 3, 5, and P10-12, and VirB10 P13 and 14.

The identification of MHC class II restricted antigenic peptides for the inclusion into bovine vaccines is a high research priority. However, unlike mice and humans, bioinformatic tools have not been developed to predict whether a given peptide will interact with BoLA class II
molecules (41, 42). The prediction accuracy is dependent on the experimental datasets used, for the bovine system this is severely lacking. Within this study, we used six cattle with different haplotypes to identify the MHC class II restriction elements for the immunogenic peptides spanning VirB9-1, VirB9-2, and VirB10 proteins. We hope our results will help formulate a prediction model for peptide binding to bovine MHC class II DR and importantly DQ combinations. This information could help expand our knowledge about DQ restriction elements and assist with future bovine vaccine developments.

Different combinations of heterozygous MHC class II cattle increases the range and identification of pathogen-derived peptides bound. We have identified highly antigenic CD4+ T cell epitopes on T4SS proteins VirB9-1, VirB9-2, and VirB10 that are also presented by BoLA class II DR and DQ molecules prevalent for Holstein cattle in the U.S. Inclusion of these peptides linked together in a multiple-antigen and peptide-based vaccine will not only provide more than one immunogenic peptide, but will offer the opportunity for increased T cell dependent IgG responses upon infection. Thus, a vaccine made up of highly conserved VirB9-1, VirB9-2, and VirB10 peptides will be tested in a future study to protect cattle against A. marginale.

5.5 Work Cited


5.6 Tables

TABLE 1: Peptide sequences used for T cell epitope mapping
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TABLE 2: Expressed BoLA class II α/β- combinations

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\( ^a \) The expression of all the combinations containing DR were detected using Th14B mAb.

\( ^b \) The expression of all the intra- and interhaplotype DQ combinations was detected using CC158 mAb.

\( ^c \) Combinations with DRA and DQB were not detected, only DQA and DRB combinations are shown.
TABLE 3: Summary of CD4<sup>+</sup> T cell epitopes and their BoLA-class II restriction elements

<table>
<thead>
<tr>
<th>BoLA-class II restriction element</th>
<th>Presented peptide</th>
<th>Animal(s) #</th>
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<td><strong>Isotype</strong></td>
<td><strong>Allele</strong></td>
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<tr>
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<tr>
<td>DRA/DRB3*1101</td>
<td>VirB9-1 P5</td>
<td>35113, 35287</td>
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<tr>
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5.7 Figures and Figure legends
Figure 1. Stimulation of CD4+ T-cell lines from *A. marginale* OM-immunized cattle against synthetic peptides from VirB9-1, VirB9-2, and VirB10. Short term T-cell lines from animals 35113, 35141, 35160, 35280, 35287, and 583 (animal number and RFLP type in parenthesis is indicated on the top of each panel) were stimulated with 10 or 20 μg/ml of peptides from (A) VirB9-1 peptides P1-P13, (B) VirB9-2 peptides P1-P14, and (C) VirB10 peptides P1, P2.1, P2.2, and P3-P22. As a negative control, a 30-mer peptide derived from *B. bovis* Rap1 was used at an equal concentration. Additional controls were *A. marginale* OM and the full length recombinant protein used at 1 μg/ml. Results are presented as stimulation indices (SI), and responses significantly higher than those for *B. bovis* Rap1 P1 are indicated with asterisks, where *P <0.05.*
Figure 2. Dose-dependent proliferative responses to stimulatory peptides. Short term T-cell lines from animals 35113, 35141, 35160, 35280, 35287, and 583 (animal number is indicated on the top of each panel) were stimulated with varying concentrations of peptides and cultured for three
days. As a negative control, *B. bovis* Rap1 P1 was used at an equivalent concentration. Results are indicated as mean counts per minute (CPM).
**Figure 3.** Identification of BoLA class II restriction elements for VirB9-1, VirB9-2, and VirB10 epitopes using 293-F cells expressing bovine DR and DQ molecules. Short term T-cell lines from animals 35113, 35141, 35160, 35280, 35287, and 583 (animal number is indicated on the top of each panel) were cultured for three days with the indicated DR- or DQ- and CD80-tranfected human embryonic kidney 293-F cells that were loaded with 10 μg/ml of the indicated peptide. Results are presented as stimulation index (SI) and responses from each artificial APC with an individual peptide were compared to non-transfected 293-F cells with the same peptide using a one-tailed homoscedastic t-test. Significant responses are indicated with asterisks where $P < 0.05$.

**Figure 4.** Expression of peptide presenting mixed isotype pairs. Human embryonic kidney 293-F cells transfected with DNA encoding the indicated mixed isotype BoLA class II and CD80 were subjected to fluorescent activated cell sorting analysis using mAb TH14B and secondary antibody R-PE conjugated goat anti-mouse IgG2a. Positive controls were TH14B labeled DRA/DRB3 counterparts and negative controls were TH14B labeled untransfected 293-F cells and DQA*1001/DRB3*1101 which failed to express.
Figure 5. Identification of BoLA class II restriction elements for VirB9-1, VirB9-2, and VirB10 epitopes 293-F cells expressing bovine class II mixed isotypes. Short term T-cell lines from animals 35113, 35141, 35160, 35280, 35287, and 583 (animal number is indicated on the top of each panel) were cultured for three days with human embryonic kidney 293-F cells transfected with DNA encoding the indicated mixed isotype BoLA class II and CD80 that were loaded with 10 μg/ml of the indicated peptide. Results are presented as stimulation index (SI) and responses were compared to non-transfected 293-F cells using a one-tailed homoscedastic t-test. Significant responses are indicated with asterisks where *P < 0.05.
Chapter 6: Determine if Immunization of Peptides Containing T cell and MHC class II Epitopes Linked to B cell Antigens will Undergo Linked Recognition and Induce a Stronger Protective Immune Response as Compared to Individual T4SS Epitopes

6.1 Brief Introduction

We have classified the T4SS proteins that stimulate strong IgG responses but lack T cell epitopes in the context of an individual MHC class II haplotype. We have also identified the associated T4SS partner proteins by far western blotting and immunoprecipitation; that are stimulatory for CD4+ T cells within the same individual. Furthermore, we have detected the specific epitope(s) on the protein(s) that the T cell recognizes and how it is presented by MHC class II on the antigen presenting cell (APC).

T4SS proteins are essential for a large number of processes determining bacterial virulence, survival and cellular invasion. This diverse array of functions makes these proteins attractive candidates for a vaccine, not only against *A. marginale* but also against many other gram negative pathogens. Furthermore, the naturally associated T4SS protein complex that spans the inner and outer membrane provides a model system to examine the importance of membrane protein-protein interactions in stimulating protective immunity and facilitating linked recognition. However the T4SS has not been studied as a vaccine candidate.

Protecting a large population of genetically heterogeneous individuals requires understanding of MHC class II-restricted epitope presentation. By crosslinking T4SS proteins that are recognized by T cells and presented by MHC class II molecules to other T4SS proteins that are recognized by B cells, the humoral and cellular immune response can be assessed through linked recognition; thus, providing a more “complete” immune response against
infection and disease. This outlook directs the research to determine whether T4SS proteins are protective antigens and undergo linked recognition. The B- and T-cell responses to the intermolecularly associated proteins within the T4SS and their role in protective immunity will be evaluated.

6.2 Strategy

There are two ways to test the hypothesis that T4SS proteins provide protection against *A. marginale* infection and disease via linked recognition. The first method includes proteins that elicited an IgG response, the B cell antigen (Ag) will be crosslinked to the proteins that elicited T cell responses, the T cell Ag, and used in an immunization and challenge experiment. The full length proteins and their pair that were B- and T-cell Ags are shown in Table 1. The protein pairs selected for vaccination are VirB9-1 linked to VirB9-2 and to VirB10 because they are surface exposed and susceptible to neutralizing antibody. We also know that VirB9-1, VirB9-2, VirB10, as well as VirB7 are B cell Ags for animals with the RFLP type 3/16, 8/23, and 16/27. VirB9-1 is a T cell Ag for 16/27 animals, VirB9-2 is a T cell Ag for 8/23 animals, and VirB10 is a T cell Ag for 3/16 and 8/23 animals. The interactions of VirB9-1 with VirB9-2 and VirB10 were definitively characterized in chapter 4 and shown to interact with each other. Therefore, as table 1 indicates a combination of interacting proteins for each of these RFLP typed animals would include both a B cell and T cell Ag, and thus the necessary epitopes are present to obtain T cell help and undergo linked recognition.

The RFLP types 3/16, 8/23, and 16/27 are examples of animals required for the immunization and challenge experiment. VirB9-1, VirB9-2, and VirB10 also induced IgG and T cell responses in animals with the RFLP types 11/22, 22/24, and 16/22. The specific T cell
importantly the MHC class II epitopes for VirB9-1, VirB9-2, and VirB10 was identified in Chapter 5. We identified that the DR molecule corresponding to the RFLP type 22 presented peptides from VirB9-1, DR molecule corresponding to the RFLP type 8 presented VirB9-2 and VirB10 peptides, DR molecule corresponding to the RFLP type 23 presented a VirB9-2 peptide, and the DR molecule corresponding to the RFLP type 27 presented a VirB9-1 peptide. We also identified several DQ restriction elements that presented peptides derived from VirB9-1, VirB9-2, and VirB10. The data described in Chapter 5 helps us to potentially predict T cell responses to each of these proteins in haplotype defined cattle. Therefore, the other possibility to this immunization and challenge experiment is to use the T cell epitopes that were shown to be presented by DR and DQ combinations of MHC class II molecules and link them to the B cell Ag (Table 2). Animals that are half matched could be used because we know what MHC class II molecule presents the peptide to the T cell receptor. This will also allow for the use of constructed DRA/DRB3 tetramers loaded with the immunogenic peptides to evaluate the CD4\(^+\) T cell response in the animals immunized with the crosslinked complexes and un-linked individual proteins (6).

In order to complete the final specific aim we need to purchase four sets of four cattle with defined haplotypes. We need four sets of cattle to allow for one group of cattle for a positive control which would be immunized with \textit{A. marginale} OM, a negative control group immunized with adjuvant alone. The selected peptides or protein pairs will be chemically crosslinked and animals immunized with this complex will be compared with animals immunized with an equivalent amount of individually administered peptides/proteins administered at separate sites. As an example immunogen for cattle receiving the complexed proteins, VirB9-1 will be artificially crosslinked with VirB9-2 and VirB10 and used to immunize
4-previous naive cattle with 8/23 RFLP type. Ideally, four cattle with these RFLP types will also be immunized with the un-linked VirB9-1 and VirB9-2 and VirB10 individual proteins administered at separate sites. There are more examples of protein and peptide complexes that could be used in table 1 and 2, respectively. Each immunized group should contain four cattle because this many animals allowed for the detection of significant differences in the induction of MSP1b-specific IgG2 responses by the MSP1 chimeric protein versus the unlinked mixture of MSP1a and MSP1b1 proteins (I). This makes a total of a minimum of sixteen cattle to complete the goal for the finally objective.

The protein complex made up of VirB9-1, VirB9-2, and VirB10 proteins can be chemically crosslinked using the Bioconjugate Toolkit (ThermoScientific), or similar kit, which uses heterobifunctional crosslinking reagents. The crosslinking reagents will be used separately to modify each purified protein separately, and then the two protein solutions are mixed to be crosslinked together. The Bioconjugate Toolkit is capable of producing inter-crosslinked proteins and dead end products, but will not produce intra-crosslinked proteins and crosslinking of the same protein in solution. In vivo, a chemical crosslinker-specific antibody could be generated. In order to control for antibody targeting the crosslinking moiety, the individual protein immunogens will be also mixed independently with their half of the Bioconjugate Toolkit heterobifunctional moiety. Interacting proteins can be detected with electrophoresis and the molecular weight will correspond to the mass of the recombinant protein plus the weight of the crosslinking reagent. Alternatively, for the peptides from VirB9-1, VirB9-2, and VirB10 that contain T cell epitopes, each peptide can be artificially linked together either by crosslinking or by synthesizing a synthetic protein containing each epitope. The chemical crosslinker will not
disrupt the relevant immune response because the epitope that the T-cell recognizes is linear and has been processed to be presented on MHC class II in this way.

Animals will be immunized subcutaneously four times at three-week intervals with 60 μg of crosslinked proteins emulsified in Quil A-saponin, each interacting pair administered at separate sites. For animals receiving the individual proteins, 30 μg of each protein will be administered subcutaneously in separate sites, to avoid potential folding of the proteins together. For immunization with OM, four subcutaneous injections 60 μg of saponin-emulsified OM will be used. Finally, for the negative control animals each will receive an injection of just saponin. All animals will be bled before immunization, and two weeks after each immunization. Sera will be stored at -20°, and PBMC cryopreserved in liquid nitrogen. One month after the last immunization, calves will be challenged with the St. Maries strain of A. marginale.

Before challenge, the animals will be evaluated for successful immunization by western blot and T lymphocyte proliferation assays. Each of these assays will be repeated several times in order to obtain a baseline response. The cattle will be intravenously challenged with 1 x 10^4 St. Maries A. marginale and monitored for infection as previously performed (2). Sera and PBMC will also be collected from the challenged animals to access IgG (specifically IgG2) production and CD4^+ T cell responses to VirB9-1, VirB9-2, and VirB10. The negative control antigen will be B. bovis MSA1 and the positive control will be A. marginale OM.

The crosslinked protein pair should produce a higher IgG2 response as compared to the un-crosslinked pair. This is because, the presence of IgG2, will indicate that T cell-dependent class-switching has occurred. IgG2 is also associated with enhanced opsonizing activity in cattle (3) and has been shown to correlate with complete protection in outer membrane-immunized calves (4). Therefore, animals that are immunized with OM will produce high levels of IgG2,
and we expect that similar levels will occur within animals immunized with the protein/peptide complex. Immunization with either OM or the crosslinked proteins is expected to induce CD4\(^+\) T cell and corresponding antibody responses previously demonstrated in outer membrane vaccinates (1, 2, 4, 5). These immunogens are also expected to reduce parasite loads and have less severe anemia compared to animals immunized with adjuvant and individual proteins. In contrast to the crosslinked protein pairs, immunization with individual recombinant proteins/peptides can stimulate T cell proliferative responses to the T cell Ag(s), but weak or absent T cell responses and IgG production to the B cell Ag. In the absence of physical linkage of T cell epitopes to the B cell Ag, the necessary cognate T cell-B cell interaction required for IgG class switching should be absent and less protective immunity will be observed as well.

We expect the results from the above immunization and challenge experiment would give more insight into the requirements of linked recognition and protein interaction for vaccine development and protective immunity. This experiment would also be the first experiment that could provide evidence for the protective efficacy of T4SS proteins. This is an important step to identifying a vaccine that could be protective against \textit{A. marginale} and potentially multiple pathogens infecting humans.

6.3 Work Cited


### 6.4 Tables

**Table 1.** Full length protein Immunogens.

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<thead>
<tr>
<th>RFLP type</th>
<th>T cell Ag</th>
<th>B cell Ag</th>
<th>Interacting pairs&lt;sup&gt;a&lt;/sup&gt;</th>
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<td>3/16</td>
<td>VirB10</td>
<td>VirB7 VirB9-1 VirB9-2 VirB10</td>
<td>VirB9-1—VirB10 VirB9-1—VirB9-2</td>
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<td>VirB9-1 VirB9-2 VirB10</td>
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<tr>
<td>16/27</td>
<td>VirB9-1</td>
<td>VirB7 VirB9-1 VirB9-2 VirB10</td>
<td>VirB9-1—VirB9-2 VirB9-1—VirB10</td>
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<sup>a</sup> VirB7 was not considered in the interacting protein pairs.

**Table 2.** T cell epitope linked to B cell Ag Immunogens

<table>
<thead>
<tr>
<th>RFLP type&lt;sup&gt;a&lt;/sup&gt;</th>
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<th>B cell Ags</th>
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<td>8</td>
<td>VirB9-2 P2-3 VirB9-2 P9-12 VirB10 P2.1, P3, P13, P22</td>
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</table>

<sup>a</sup> The animals used in the peptide immunization challenge study could be homozygous or heterozygous with 11 and 24.
Chapter 7: Other research

7.1 Implications of MSP2 function

*Anaplasma marginale* is an obligate intracellular gram negative pathogen that infects bovine erythrocytes causing persistent rickettsemia. *A. marginale* can escape the immune response by undergoing antigenic variation by segmental gene conversion (1-3). One of the primary proteins responsible for *Anaplasma* escape from the immune system is major surface protein 2 (MSP2), where several variants are present in each rickettsemia cycle (4, 5). Expressed msp2 genes are characterized by a large central region of amino acid polymorphisms, called the hyper variable region, flanked by the highly conserved N- and C- terminus (5, 6). The hyper variable region contains epitopes that are recognized by antibodies subsequent to the rickettsemia cycle, followed by repeated emergence of MSP2 variants (6, 7). At least four different variants of the central hyper variable region are found in each rickettsemia cycle during persistent infection (4).

Although there have been multiple advancements in understanding the mechanism for MSP2 antigenic variation, little progress has been made towards the actual function of MSP2. One method of understanding protein function is to identify MSP2 protein partners. The goal of the present study was to understand more about the function(s) of MSP2 by looking for its protein partners with other MSPs and erythrocytes.

7.1.1 Protein-protein interactions with MSP2

To research the role of MSP2 during *A. marginale* infection, the protein binding partners of MSP2 were identified with far western blotting and immunoprecipitation (IP). Far western blotting was performed as previously described (Chapter 4 section 4.2.6). Briefly, 1 μg of native
MSP2 purified from *A. marginale* outer membrane (OM) (4), and 1 μg of recombinant MSP1a, MSP1b, (8) and MSP5 (9) were suspending in sample buffer containing 100 mM DTT (Thermo Scientific) and separated by sodium doedecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 4-20% gradient gels. These proteins were transferred to a nitrocellulose membrane and blocked with 20 mM Tris-HCl pH 7.5, 150 mM NaCl (TBS) with 5% milk for 3 h. To identify if the native MSP2 binds to rMSP1a or rMSP5 the membrane was incubated with 10 μg of nMSP2 in 5 ml of TBS with 5% milk overnight at 4°C, followed by 1 μg/ml monoclonal antibody (mAB) AnaR49a IgG2a against MSP2. For a negative control the membrane containing MSP2, MSP1a and MSP5 proteins was incubated with just AnaR49a. The membrane was washed with TBS containing 0.05% Tween 20 (TBST) and detected using a 1:10,000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (H+L) (Kirkgaard & Perry Laboratories) for 1 h, then washed extensively with TBST and developed using ECL detection substrate (Thermo Scientific).

To confirm that the detected recombinant protein interactions was also present in with native proteins, IP was performed as previously described (Chapter 4 section 4.2.8) using 2 mg/ml n-dodecyl β-D maltoside-solubilized *A. marginale*. Monoclonal antibody AnaR49a (2 μg/ml) was used to pull down interactions specific to MSP2 and the negative control was anti-MSA1 23/10.41 mAB. Candidate interacting protein partners were identified by immunoblotting assays using antibodies directed against the protein partner. The immunoprecipitated pellet containing MSP2, interacting proteins, anti-MSP2 antibody, and protein G sepharose (as well as the negative control IP pellet) was separated with SDS-PAGE on a 4-20% pre-cast gel (Bio-Rad) and then transferred to a nitrocellulose membrane. The proteins on the membrane were blocked with I-block (Applied Biosystems) containing 0.1% Tween-20 which was also used as the wash
buffer. Strips were cut and incubated with 1 μg/ml mAb against MSP1a (Ana22B1), MSP3 (115/152.20.19), and MSP5 (AnaF16C1). The secondary antibody was either HRP conjugated clean-blot (ThermoScientific) or 1:10,000 dilution of HRP-conjugated goat anti-mouse IgG (H+L) and substrate was ECL.

It was determined that native MSP2 binds to rMSP1a and MSP1b but not rMSP5 by far western blotting (Fig. 1), and native MSP2 interactions determined by IP were with MSP1a, MSP1b, and MSP3 (Fig. 2 B, C, and D, lane 1) but not MSP5 or VirB9-1 (Fig. 2 E and F, lane 1). Native MSP2 does not interact with MSP5 nor with VirB9-1 and the reverse has been confirmed (Chapter 4, Section 4.3.6 and Fig. 9). The negative control mAb anti-MSA1 IP did not pull down any *A. marginale* proteins; and only heavy and light chain were detected with the secondary antibody (Fig. 2 A-F, lane 2). Protein interactions were also predicted with a Search Tool for the Retrieval of Interacting Genes (STRING) which quantitatively integrates interaction data from genomic context, high-throughput experiments, co-expression, and previous knowledge to reveal physical and functional associations (10). MSP2 (Am1144) was predicted to only associated moderately with Opag1 and Opag2, and less with Am1141 (Preliminary data section 2.4 and Fig. 11), all of these interactions were based solely on the intergenic distance from MSP2 in the genome.

### 7.1.2 MSP2 erythrocyte attachment

After performing immunoelectron microscopy with MSP2 there was some question whether or not MSP2 binds to host erythrocytes, as there were residual gold labeling all over the erythrocytes (Chapter 4 section 4.3.2 and Fig. 4 and ref. 11). Thus, fluorescence-activated cell sorting (FACS) analysis was performed using native MPS2 and it’s monoclonal antibody
(AnaR49a) was used to detect if MSP2 bound to the erythrocyte. The positive control was anti-bovine erythrocyte monoclonal IgG1 (Ana8a). Bovine erythrocytes were isolated from an uninfected steer and separated from plasma and the buffy coat by Ficoll gradient centrifugation. Uninfected erythrocytes (0.35 x10⁶) were put in V-bottom 96-well plate and washed three times with cRPMI and 0.2% sodium azide (FACS media) after each step. Recombinant or native protein was added first to the erythrocytes and then the primary antibody each added at 100 μg/well. Native MSP2 from Florida strain, recombinant VirB9-2, and recombinant leukotoxin were used with their respected antibodies mouse anti-MSP2 IgG2a (AnaR49a), rabbit anti-VirB9-2 IgG, and mouse anti-leukotoxin mm605 supernatant IgG2a. The secondary antibody was an isotype-matched anti-mouse IgG1-FITC (for Ana8a, Southern Biotech) or anti-mouse IgG2a-PE (Caltrac) used at 1:200 titer. For the polyclonal antibodies against VirB9 the secondary antibody was donkey anti-rabbit F(ab’)2–PE (ImmunoResearch). Leukotoxin and anti-leukotoxin were used as a negative control because it has been shown that this protein specifically binds to leukocytes and polymorphonuclear cells (12-14). Additional negative controls were, secondary antibody alone, AnaR49a alone, and mm605 alone. Results are representative of three independent experiments.

The majorities of the cells were intact erythrocytes and labeled with Ana8a (Fig. 3B), but without any staining or just the secondary antibody alone virtually no cells were labeled (Fig 3A, C respectively). Likewise, the negative control leukotoxin protein or mm605 alone did not bind erythrocytes, only 3% and 2% was detected (Fig. 3G and H, respectively). When the uninfected erythrocytes were incubated with native MSP2 and AnaR49a 26% of the cells were labeled (Fig. 3D) and without the native MSP2 only 3% cells were stained (Fig. 2E), indicating that MSP2 does bind to uninfected erythrocytes. A previous study using hemagglutination assays by
McGarey et al. also indicated that MSP2 and MSP1 is involved with erythrocyte adhesion (15). This experiment also was performed with rVirB9-2 and anti-VirB9-2. Only 7% of VirB9-2 bound to erythrocytes which may or may not be significant enough to indicate that it is an adhesion protein (Fig. 3F). Yet, homologous proteins to VirB9 from Bartonella henselae, TrwL and TrwJ, act as erythrocyte adhesion molecules in a variety of different mammals (16).

The interaction of MSP2 binding to intact uninfected erythrocytes was visualized further using far western blotting. Two sources of uninfected erythrocytes were separated by SDS-PAGE on 4 to 20% gradient gels and transferred to nitrocellulose and developed as described above to determine what proteins MSP2 binds to on the erythrocyte (Fig. 4). In Figure 4A the uninfected erythrocytes were directly loaded on the gel and in figure 4B the uninfected erythrocytes were washed free of hemoglobulin before electrophoresis. Two dominant proteins in the unwashed erythrocytes were detected one ~10 and the other ~25 kDa, as well as significant smearing down the lanes most likely due to the sticky nature of hemoglobulin (Fig. 4A, lane 1). Lysed uninfected erythrocytes were also washed with phosphate buffered saline, pH 7.2 with centrifugation at 30,000 x g to remove hemoglobulin, platelets, and iron. When 20 μg of electrophoretically separated and transferred uninfected erythrocyte membranes were probed with first native MSP2 then AnaR49a, no significant interactions were detected (Fig. 4B lane 1). Nothing was detected with washed or not washed uninfected erythrocytes incubated with just AnaR49a (Fig. 4A and B lane 2). This suggests MSP2 binds with iron containing proteins or iron itself, and not directly with erythrocyte membrane proteins.

7.1.3 Conclusions about MSP2
The observation that MSP2 is recognized by sera from persistently infected cattle is the only evidence that would suggest its localization on the surface of *A. marginale* (7). One goal of this study was to further characterize the localization of MSP2 during bovine infection by labeling infected erythrocytes and visualizing them with an electron microscope. Protein localization is an important piece of evidence that is necessary to understand protein function. The image in figure 4B from Chapter 4 show more electron dense areas on the *A. marginale* colonies when incubated with the anti-MSP2 antibody as compared to the negative control. And it appears the signal is strongest on the surface of *A. marginale*, but some electron dense signal is also present inside the *A. marginale* and throughout the infected erythrocyte. This indicates that the MSP2 is mostly surface exposed but could have some expression in the cytoplasm. The electron dense labeling of the anti-MSP2 antibody around the erythrocyte that was different from the negative control background labeling posed interesting questions. Is MSP2 involved in *A. marginale* erythrocyte adhesion? This question was answered when we directly measuring of MSP2 interactions with intact uninfected erythrocytes. Furthermore, we also found via far western blotting that MSP2 is likely to interact with iron containing proteins present on uninfected erythrocytes, as supposed to erythrocyte membrane proteins.

MSP2 is an integral part in the *A. marginale* escape from bovine immune responses (17). MSP2 is likely to aid *A. marginale* with host cell infection as it is an adhesive to uninfected bovine erythrocytes and is surface exposed. It also interacts with MSP1a within intact *A. marginale*. Like MSP2, MSP1a and MSP1b are both bovine erythrocyte adhesive proteins (15). The model of MSP2 is predicted to be a β-barrel, a structure that is restricted to gram negative bacteria outer membranes and puts MSP2 in the category as active siderophore transporters which has implication for MSP2 adhesion to iron or heme (Preliminary data Section 2.5, Fig. 12).
The breadth and magnitude of immunoglobulin G against the conserved and hypervariable regions of MSP2 produced by infected cattle were not significant correlates to control of bacteremia or protection from infection (18). Therefore, MSP2 function could be to aid *A. marginale* entrance into host erythrocytes during colonization, being left behind to camouflage the infected erythrocyte from the immune system. Thus, MSP2 may enable *A. marginale* entry into the erythrocyte and protect infected erythrocytes from ingestion by phagocytes by means of antigenic variation.

7.2 Formaldehyde crosslinking of recombinant VirB9-1 to native OM proteins

The idea for the formaldehyde crosslinking experiment was to force native proteins present in solubilized OM to interact with recombinant VirB9-1, but provided at a much higher concentration. This interaction would be crosslinked and then detected by a molecular weight shift of the recombinant protein with western blotting.

Approximately 650 μg of *A. marginale* OM were solubilized in 800 μl of 2% N-dodecyl maltoside for 4 h at 4°C with orbital shaking. Debris was removed by centrifugation at 10,000 xG for 20 min at 4°C. Twenty-five ug/ml of rVirB9-1 was added to the solubilized OM overnight with rotation at 4°C. Using 100 μl of solubilized OM mixed with rVirB9-1 for each reaction different percentages of paraformaldehyde (0%, 0.25%, 0.5%, 1%, 1.5%, 2% and 3%) were added in a 20 μl volume similar to Chowdhury *et al.* 2009 (19). The recombinant protein was crosslinked to the native proteins for 10 min at RT and quenched with 1.25M Glycine pH 6.3 (final concentration). SDS-PAGE and western blotting was performed on all of the crosslinked samples, soluble OM, insoluble OM and rVirB9-1. The western blotting was
performed twice on two separate membranes, developed with 1:2000 anti-flag-AP (Invitrogen) (Fig. 5B) and 2 μg/ml polyclonal anti-VirB9-1 (Fig. 5C) as the primary antibodies.

Each figure is set up the same one is Coomassie (Fig. 5A) with varying concentrations of crosslinking reagent and the controls which are solubilized OM, rVirB9-1 alone, and OM mixed with rVirB9-1, or no crosslinking labeled as 0%. Ideally I would see a molecular weight shift of both 50 kDa and 100 kDa size of VirB9-1 at higher concentrations of formaldehyde indicating interactions with native proteins. As you can see from both western blots developed with anti-VirB9-1 and anti-FLAG molecular weight shifts did not occur (Fig. 5B and C, respectively). As expected the proteins crosslinked with higher concentration of formaldehyde were stuck at the top of the gel and not separated. When we added more solutions (both to crosslink and quench) to the reaction I diluted the abundance of the interactions so much so I can not detect it with western blotting (different between lanes labeled with OM + rVirB9-1 as compared to 0% in Fig. 5A). Even if the reaction worked as supposed to and I saw a molecular weight shift all this would tell me is that VirB9-1 has a partner, not what it is, therefore this experiment was abandoned.

**7.3 Co-expression of VirB7, VirB9-1, VirB9-2, VirB10 and MSA1 into 293-F cells**

Another method to identity recombinant protein interaction with another protein is co-expression. Sequences for two different proteins are put on the same expression vector, expressed, and purified with chromatography directed against one of the proteins that was co-expressed to see if the other protein co-purifies. To try this we purchased the bicistronic expression vector, pCMV-BICEP-4 (Sigma-Aldrich). pCMV-BICEP-4 is a mammalian expression vector that is used for the co-expression of two genes resulting in one product with an N-terminal FLAG epitope and N-terminal c-myc protein, allowing for the study of:protein
interaction candidates through the capture and detection of fusion-tagged complexes via the FLAG and/or the \textit{c-myc} epitopes. It has two multiple cloning sites (MCS) separated by an internal ribosome entry site (IRES) derived from the encephalomyo-carditis virus (EMCV) allowing for translation of the second MCS. The initial promoter comes from the cytomegalovirus, directing both MCS sites. While translation of the upstream FLAG fusion sequences occurs via normal cap-dependent translational processes, the EMCV IRES region controls translation of the downstream \textit{c-myc} fusion by recruiting the ribosomal subunits for cap-independent translational initiation. The pCMV-BICEP-4 expression vector is a shuttle vector containing both bacterial and SV40 origins of replication for propagation in both \textit{E.coli} and mammalian cells. Efficiency of replication in mammalian cells is optimal when using host cells that express the SV40 Large T antigen, thus we selected the human embryonic kidney 293-F cells. The provided control vector was pCMV-BICEP-4+p53/LTA contained the interacting p53, gene, inserted into MCS1, and the SV40 Large T Ag, inserted into MCS2.

Before the expression, we designed forward and reverse primers for the expression of full length VirB7, VirB9-1, VirB9-2, VirB10, and negative control MSA1 (sequences not shown). On the primers we put restriction enzyme cut sites that would allow for the entry of any one of these proteins into either the MCS1 or MCS2 on the bi-cistronic vector. We ensured that the enzyme cut sites used did not have native cut sites within the protein sequence. After amplifying the genes for VirB7, VirB9-1, VirB9-2, VirB10, and MSA1 and sequence the genes to ensure the engineered cut sites were present, we cloned the genes into two separate vectors, as outlined in table 1. We then transfected our 293-F cells using the method described in chapter 5 section 5.2.4. For the detection of protein expression we separated whole 293-F cells (without protein purification) by SDS-PAGE on 4-20% pre-cast gels (Bio-Rad) and transferred to nitrocellulose
membranes for western blotting. We performed two western blots in tandem, one detected with AP conjugated anti-FLAG and the other detecting with AP conjugated anti-c-myc mAbs (Fig. 6). None of our proteins expressed in the bi-cistronic vector (Fig. 6). Within the control vector only p53 expressed as detected with anti-FLAG (Fig. 6A), however LTA expression was not detected (Fig.6B). There is no evidence in the literature that would support the expression of two proteins in this manner, i.e. using an IRES to control downstream translation. Sigma-Aldrich also did not have any supporting information on this vector that could support their experiments and selling of it. We identified that the second MCS did not express the protein, even within the control vector, and thus the bi-cistronic expression was not acceptable for our use.

7.4 Work Cited


7.5 Tables

**Table 1**: Bicistronic vector made for expression

<table>
<thead>
<tr>
<th>Vector #</th>
<th>MCS1 gene</th>
<th>MCS2 gene</th>
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<tbody>
<tr>
<td>1</td>
<td><em>virB10</em></td>
<td><em>msa1</em></td>
</tr>
<tr>
<td>2</td>
<td><em>virB10</em></td>
<td><em>virB9-2</em></td>
</tr>
<tr>
<td>3</td>
<td><em>virB9-1</em></td>
<td><em>virB9-2</em></td>
</tr>
<tr>
<td>Control</td>
<td><em>p53</em></td>
<td><em>LTA</em></td>
</tr>
</tbody>
</table>
7.6 Figures and Figure Legends

**Figure 1.** Far western blot indicating native MSP2 binding to recombinant proteins. Purified native MSP2 (lane 1) and purified recombinant MSP1a (lane 2), MSP1b (lane 3), and MSP5 (lane 4) (1 μg) were separated on a 4 to 20% gradient SDS-PAGE gel, transferred to a nitrocellulose membrane. (A) Membrane was incubated with 10 μg native MSP2 and 1 μg/ml AnaR49a anti-MSP2 mAB. (B) Membrane was incubated with 1 μg/ml AnaR49a mAB as the negative control. The interactions were detected with goat anti-mouse IgG (H+L) and ECL substrate.

**Figure 2.** MSP2 native interactions determined by immunoprecipitation. Solubilized *A. marginale* were either immunoprecipitated with 2 μg AnaR49a (lane 1) or with a control mAb
anti-MSA1 (lane 2), then separated by 4-20% SDS-PAGE, and transferred to nitrocellulose membrane. Lane 3 indicates solubilized A. marginale for a size comparison. Each membrane was developed with 1 μg/ml antibody against candidate interacting proteins (A) anti-MSP2, (B) anti-MSP1a Ana22B1, (C) anti-MSP1b AnaR38 (D) anti-MSP3 115/152.20.19, (E) anti-MSP5 AnaF16C1, and (F) a negative control affinity purified anti-VirB9-1 polyclonal antibody. A, C, E, and F used clean blot-HRP and B and D used HRP conjugated 1:10,000 goat anti-mouse IgG (H+L) as secondary antibodies.

Figure 3. FACS analysis to determine if MSP2 is an erythrocyte adhesive protein. Uninfected erythrocytes were labeled with (A) nothing (B) anti-erythrocyte monoclonal antibody (Ana8a) as a positive control or (C) just secondary antibody goat anti-Mouse IgG2-PE as a negative control. Erythrocytes were labeled with (D) native MSP2 and anti-MSP2 mAB (AnaR49a) to determine binding of MSP2, and (F) rVirB9-1 and anti-VirB9-1 to determine VirB9-1 erythrocyte adhesion.
For additional negative controls erythrocytes were labeled with (E) just AnaR49a (G) 
recombinant leukotoxin and anti-leukotoxin mAB and (H) anti-leukotoxin mAB alone.

**Figure 4.** MSP2 binds to a protein located on erythrocytes. Not washed uninfected erythrocytes 
(A) and 20 μg erythrocyte membranes (B) were separated on a 4 to 20% gradient SDS-PAGE gel, 
transferred to a nitrocellulose membrane. Membranes were incubated with 10 μg native MSP2 
and 1 μg/ml AnaR49a anti-MSP2 mAb (lane 1), or with 1 μg/ml AnaR49a mAb as the negative 
control (lane 2). The interactions were detected with goat anti-mouse IgG (H+L) and ECL 
substrate.
Figure 5. Formaldehyde crosslinking of rVirB9-1 to native proteins in *A. marginale* OM fraction. OM was solubilized and incubated with an abundance of rVirB9-1. The proteins in the sample were then crosslinked at varying percentages of formaldehyde. An aliquot of each crosslinked proteins and control was separated on a 4-20% gel and stained with (A) Coomassie stained gel or transferred to nitrocellulose membrane and developed with either (B) anti-VirB9-1 or (C) anti-FLAG. Percentage is percent of formaldehyde used for crosslinking. Recombinant VirB9-1 is 46 kDa and native is 25 kDa.
Figure 6. Bi-cistronic expression of recombinant T4SS proteins. 293-F cells expressing two proteins within the bi-cistronic vector pCMV-BICEP-4 were separated by SDS-PAGE on a 4-20% gradient gel and transferred to nitrocellulose membrane for western blotting. Proteins in the vector are labeled on each lane. Identical western blots were developed with (A) anti-FLAG or (B) anti-c-myc, as indicated at the bottom of each panel.
Chapter 8: Conclusions

In this study, we tested the hypothesis that naturally associated T4SS proteins used as immunogens will undergo linked recognition and induce stronger protective immune responses than immunization with individual T4SS epitopes. We identified the subdominant T4SS proteins of *A. marginale* that induce an IgG response but no CD4+ T cell response in OM-vaccinated individuals, these proteins and animals were VirB7 (35113, 35141, 35160, 35280, and 35287), VirB9-1 (35160 and 583) and VirB9-2 (35160). The T cell responses for VirB9-1, VirB9-2, and VirB10 specific responses were later confirmed with T cell assays using overlapping 30-mer peptides spanning each of these proteins. Next, we identified which T4SS protein(s) that stimulated T cell proliferation could provide T-cell help through a cognate interaction. Interacting protein partners were indentified by far western blotting and confirmed by immunoprecipitation assays, and revealed, for the first time, specific interactions of VirB9-1 with VirB9-2 and VirB10. Also, we identified the T cell epitopes on VirB9-1, VirB9-2, and VirB10 that were restricted by DR and DQ MHC class II presentation. The peptides best for a vaccine would be VirB9-1 P6, and 10, VirB9-2 P2, 3, 5, and P10-12, and VirB10 P13 and 14 as these peptides induced a T cell response using a low concentration and presented by MHC class II proficiently. We present evidence for the linked recognition of VirB9-1, VirB9-2, and VirB10, which are naturally associated in *A. marginale*, and a basis for testing multiple-antigen and peptide-based vaccine containing VirB9-1, VirB9-2 and VirB10 proteins/epitopes for the use against anaplasmosis.

In order to induce a strong immune response in a vaccine, one has to consider the use of haptens with carriers, the production of high affinity antibodies, the capture of antigen by professional antigen presenting cells, targeting specific helper T cells, and the production of
cytokines such as IFN-γ (2). We have essentially addressed all of these topics in our research with VirB9-1, VirB9-2, and VirB10. Each of these proteins acts like a hapten-carrier complex because they contain CD4+ T-cell epitopes on one protein and on a second-linked protein contain B-cell epitopes. A complex made up of VirB9-1, VirB9-2, and VirB10 would produce high affinity antibodies directed at all three of these proteins, stimulate strong T cell responses which produce high levels of IFN-γ in cattle that harbor the RFLP typed alleles 3, 8, 11, 16, 22, 23, 24, and 27. These alleles are the most prevalent within the cattle population in the U.S. Finally, we have also addressed the capture and more importantly the presentation of VirB9-1, VirB9-2, and VirB10 by professional antigen presenting cells by determining the MHC class II restriction elements. The identification of MHC class II restriction elements allows us to potentially predict T cell responses to VirB9-1, VirB9-2, and VirB10. By crosslinking T4SS proteins that are recognized by B-cells to other T4SS proteins that are recognized by T-cells, the humoral and cellular immune response can be assessed through linked recognition, thus providing a more “complete” immune response against infection and disease caused by A. marginale. The T4SS is a model to study if interacting proteins and linked recognition will provide protective immunity. Our strategy behind vaccine development is novel and can be used to make a vaccine against A. marginale as well as to identify vaccine candidates using immunogenic proteins within any pathogen.

Since A. marginale has the ability to adapt to their environment and continuously change there is no single characteristic will adequately reflect on the epidemiological complexity of infection or even predict its outcome. Profiles need to be created to resemble many different markers including the genome, transcriptome, proteome, metabolome, etc., and with this information at hand we could have a more successful surveillance and disease management (1).
For *A. marginale* the type IV secretion system proteins are a great marker for infection because they are not antigenically variable, could be important for host cell survival, and are recognized by the humoral and cellular immunity in protected cattle. We have shown that sequences of VirB9-1, VirB9-2, and VirB10 are highly conserved across all strains of *A. marginale* and *A. centrale*, and fairly conserved (~20%) to sequences from other organisms that harbor a T4SS. We hope to show that cattle immunized with a complex containing VirB9-1, VirB9-2, and VirB10 proteins and/or T cell epitopes will protect cattle from *A. marginale*. In the future, the same vaccine could be used against multiple strains of *A. marginale* and other gram negative pathogens harboring a T4SS. This will be the first time that T4SS proteins have been assessed for their ability to protect against any infection or disease.

8.1 Work Cited
