



Identification Anaplasma Marginale
Protein up-regulated

IDENTIFICATION OF *ANAPLASMA MARGINALE* PROTEINS UP-REGULATED
DURING INFECTION OF THE TICK VECTOR

By

SOLOMON STEPHEN RAMABU

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College of Veterinary Medicine

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TH
636.501
RAM



To the Faculty of Washington State University:

The members of the committee appointed to examine the dissertation of SOLOMON STEPHEN RAMABU find it satisfactory and recommend that it be accepted.

Guy H. Palmer, Ph.D., Chair

Kelly A. Brayton, Ph.D.

Wendy C. Brown, Ph.D.

Glen A. Scoles, Ph.D.

Donald P. Knowles jr, Ph.D.

| BOTSWANA COLLEGE OF AGRICULTURE SPECIAL COLLECTION | |
|---|----------------|
| CLASS: | TH 614.433 RAM |
| SUPPLIER | BCA |
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"Nama e kgolo e fetjwa ke ho ngadhw a!!".

IDENTIFICATION OF *ANAPLASMA MARGINALE* PROTEINS UP-
REGULATED DURING INFECTION OF THE TICK VECTOR

ABSTRACT

By Solomon Stephen Ramabu, BVM&S, MVSc, Ph.D.
Washington State University
August, 2010

Chair: Guy H. Palmer

The transition between infection of the mammalian host and colonization of an arthropod vector is required for ongoing transmission of a broad array of pathogens, from viruses to protozoa. Understanding how this transition is mediated provides opportunities to disrupt transmission through either chemotherapy or immunization. We used two approaches to identify *Anaplasma marginale* proteins specifically up-regulated in the tick as compared to the mammalian host. We started with an unbiased proteome-wide screen to identify a list of candidates irrespective of localization within the bacterium or presumed function. Comparative mass spectrometric analysis of proteins separated by two-dimensional gel electrophoresis of uninfected and infected ISE6 cells and infected mammalian cells identified 15 proteins exclusively expressed or up-regulated in tick cells. All 15 had originally been annotated as hypothetical proteins. We confirmed quantitative up-regulation and expression *in situ* within the midgut epithelial and salivary gland acinar cells of vector ticks during successful transmission. The results support the hypothesis that *A. marginale* gene expression is regulated by the specific host environment and, in a broader context, that the core genome evolved in the arthropod vector with differential regulation allowing adaptation to mammalian hosts.

In the second part we used a predictive approach. We predicted based on the reported nuclear translocation of *Anaplasma phagocytophilum* AnKA and *Ehrlichia chaffeensis* p200 that the expression of *A. marginale* AnKA (AM705) would either be tick stage-specific or significantly upregulated in nucleated tick cells as compared to non-nucleated erythrocytes. In addition we predicted that AM705 would translocate to the host cell nucleus. We included in the analysis the other *A. marginale* genes with the ankyrin repeat motif; AM926 and AM638. While all the proteins were expressed in the tick stage, only AM638 was tick stage-specific. Contrary to our prediction the expression of AM705 was significantly higher in erythrocytes compared to ISE6 cells. AM926 was expressed at similar levels between ISE6 cells and erythrocytes. Using monoclonal antibodies specific to each protein in double immunofluorescence labeling we found that none of the proteins translocated to the host cell nucleus. All these findings advance our understanding of pathogen mammalian host-tick vector transition.

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CHAPTER 1

Identification of *Anaplasma marginale* proteins specifically up-regulated during colonization of the tick vector

ABSTRACT

The transition between infection of the mammalian host and colonization of an arthropod vector is required for ongoing transmission of a broad array of pathogens, from viruses to protozoa. Understanding how this transition is mediated provides opportunities to disrupt transmission through either chemotherapy or immunization. We used an unbiased proteomic screen to identify *Anaplasma marginale* proteins specifically up-regulated in the tick as compared to the mammalian host. Comparative mass spectrometric analysis of proteins separated by two-dimensional gel electrophoresis of uninfected and infected ISE6 cells and infected mammalian cells identified 15 proteins exclusively expressed or up-regulated in tick cells. All 15 had originally been annotated as hypothetical proteins. We confirmed quantitative up-regulation and expression *in situ* within the midgut epithelial and salivary gland acinar cells of vector ticks during successful transmission. The results support the hypothesis that *A. marginale* gene expression is regulated by the specific host environment and, in a broader context, that the core genome evolved in the arthropod vector with differential regulation allowing adaptation to mammalian hosts. Furthermore, the confirmation of *in situ* expression of candidates identified in ISE6 cell lines indicates that this approach may be widely applicable to bacteria in the genera *Anaplasma* and *Ehrlichia*, removing a major technical impediment

to identification of new targets for vaccine and chemotherapeutic blocking of transmission.

INTRODUCTION

The transition between infection of the mammalian host and colonization of an arthropod vector is required for ongoing transmission of a broad array of pathogens, from viruses to protozoa. Understanding how this transition is mediated provides opportunities to disrupt transmission through either chemotherapy or immunization. Bacteria in the genera *Anaplasma* and *Ehrlichia* are obligate intracellular pathogens and effectively invade, survive, and replicate in markedly different cell types in the mammalian host and ixodid ticks, the arthropod vector (4). Impressively, this transition is effected using a very limited genome, <1.5 Mb (2, 3, 8, 9, 15). We and others have hypothesized that the bacterial proteome would be specifically molded for each environment, with a core set of proteins expressed universally and subsets specifically up- or down-regulated depending on the host/vector environment (6, 12, 19, 26, 27). However, there has been only minimal proteomic evidence that supports accepting this hypothesis. The best evidence comes from recent analysis of *E. chaffeensis* that detected proteins present in either *in vitro* infected tick cells or canine macrophages (26); however, unique or up-regulated expression of these candidate proteins in the tick cells has not been confirmed. There has been no identification of bacterial proteins specifically up-regulated or exclusively expressed during actual colonization in the tick.

We addressed this knowledge gap by an unbiased proteomic approach using the St. Maries strain of *A. marginale*. The St. Maries strain is naturally transmitted by *Dermacentor andersoni*, in which it colonizes the midgut

epithelium following initial acquisition feeding on an infected animal, replicates, invades the salivary gland and then undergoes a second round of replication during transmission feeding on a new mammalian host (5, 29, 30). Importantly, the complete genome of the St. Maries strain has been sequenced and annotated (2), providing a pathway to identification of expressed proteins using mass spectrometry. The strategy was to first identify the full complement of *A. marginale* proteins expressed during cultivation in the ISE6 tick cell line. Although this cell line cannot be assumed to represent the actual tick environments of either the midgut or salivary gland, the replication of *A. marginale* to high titer in ISE6 cells provided sufficient material to conduct a proteome-wide screen to generate a candidate list of proteins (1, 11, 16, 17). The expression levels of these candidate proteins were then compared to *in vivo* expression levels in the mammalian host and in the tick midgut and salivary gland using both quantitative and *in situ* localization approaches. Herein we report the testing of this approach and discuss the findings in the context of the overall hypothesis of proteome regulation at the mammalian host-tick vector interface.

Results

Proteomic screening for identification of tick stage-specific proteins.

As we were seeking to identify *A. marginale* proteins that were either uniquely expressed or with up-regulated expression in tick cells, we used three sets of controls to ensure that the number of organisms isolated from the

mammalian host (bovine erythrocytes) was greater than or equal to the number isolated from ISE6 cells. First, we determined the number of organisms isolated from each source by quantitative PCR of *msp5*, a single copy gene (2, 5, 31). Second, the quantitative PCR results were confirmed by detection of Msp5, a constitutively expressed protein, in each sample using western blotting (Fig. 1). Third, identification of Msp4, an additional constitutively expressed protein encoded by a single copy gene (2, 23), in the gels following two-dimensional electrophoresis and densitometric quantification using PD Quest image analysis software revealed no statistically significant difference between host cells (Fig. 2). Msp4 was absent in the uninfected tick cells, as expected (Fig. 2). A total of 16 spots were identified in *A. marginale* isolated from tick cells and absent in both uninfected tick cells and in *A. marginale* isolated from bovine erythrocytes (Fig. 3). Of the 16 spots, 10 were identified using the PD Quest software analysis by the overlay of gels and densitometric analysis (unpaired *t*-test) revealed statistically higher expression ($p=0.01$) in the tick cell *A. marginale* as compared to bacteria from infected erythrocytes. The other 6 spots were identified visually with no detection of a spot in the corresponding gels of *A. marginale* from infected erythrocytes. Analysis using LC-MS/MS identified 15 unique proteins from the 16 spots. All 15 proteins were mapped to the *A. marginale* genome; all had previously been annotated as hypothetical proteins (Table 1). In addition, we detected for the first time the expression of the following proteins as part of the core *A. marginale* proteome in tick cells: AM842 (*dnaK*), AM944 (*groEL*), AM254 (*tu f*), AM666 (*atpD*), AM956 (*pepA*), AM880

(aaap), AM564 (mdh), AM937 (fumC), AM326 (argD), AM887 (rpoA), AM735 (infB), AM917 (rpsA), AM418 (pbpA2), AM1313 (virB11), and AM1314 (virB10).

Confirmation of unique or up-regulated tick stage-specific protein expression by quantitative western blot.

Confirmation of differential expression was examined for the three candidate tick stage-specific proteins with the highest MASCOT ion score following LC-MS/MS analysis, AM410, AM470, and AM829 (Table 1). Equal numbers ($10^{7\pm 0.05}$) of *A. marginale* isolated from ISE6 tick cells or from infected erythrocytes were analyzed by immunoblotting with antibodies specific for each candidate protein (Fig. 4). Am470 was only detected in the tick cell *A. marginale* (Fig. 4). Am410 and Am829 were expressed at higher levels in the tick cell *A. marginale* as compared to bacteria isolated from infected erythrocytes (Fig. 4). Densitometric analysis of independent replicates (n=3) revealed a statistically significant up-regulation (unpaired *t-test*) for both Am410 (p= 0.0005) and Am829 (p=0.005) in the tick-cell *A. marginale*. As an internal control, Msp5 levels were similar among all samples (Fig. 4) with no statistically significant difference.

***In situ* expression of unique or up-regulated tick-stage specific proteins in *Dermacentor andersoni*.**

To test whether these *A. marginale* proteins up-regulated in the ISE6 cell line were actually expressed in the natural tick vector at the time of transmission, we utilized western blots using midgut and salivary glands isolated from

transmission fed ticks. Am410, Am470, and Am829 expression was detected in $10^{5.6 \pm 0.59}$ *A. marginale* isolated from infected midguts and salivary glands; there was no detection of these proteins using an equal number of *A. marginale* from infected erythrocytes nor in uninfected erythrocytes and uninfected tick cells (data not shown). To confirm the site of protein expression *in situ*, immunohistochemistry was performed on the infected, transmission fed ticks. Serial sections of midgut and salivary glands, containing respective means of $10^{5.8 \pm 0.59}$ and $10^{6.1 \pm 0.49}$ *A. marginale* per organ, respectively, revealed expression of both AM410 and AM470 using monoclonal antibodies and AM829 using a specific polyclonal antibody (Fig. 5). Serial sections of infected ticks were negative using the unrelated control monoclonal antibody TRYP1E1 or a control polyclonal antibody raised against an unrelated *B. bovis* protein (Fig. 5). Uninfected ticks were negative in immunohistochemistry with all antibodies (Fig. 5). *A. marginale* was successfully transmitted by tick feeding with microscopic detection of acute bacteremia 14 days following initiation of tick transmission feeding with confirmation by *mSP5* PCR (data not shown).

Discussion

Based on the data, we accept the hypothesis that the *A. marginale* proteome is specific to the tick vector, with unique and up-regulated expression of individual proteins as compared to expression in the mammalian host. This in itself is not surprising from either a purely theoretical framework that adaptation to markedly different environments requires a specific proteome or a comparative

perspective with other tick-borne bacterial pathogens. Both *Borrelia burgdorferi* and *B. hermsii* have been shown to have unique tick-associated gene expression with specific requirements for transmission (7, 24, 25). However, *A. marginale* differs markedly from *Borrelia* spp., including the requirement for intracellular replication and the developmental cycle within the tick (10, 29, 30). The identification of specifically up-regulated *A. marginale* proteins in the tick provides candidates for vaccine and drug development and are likely informative for other tick transmitted *Anaplasma* and *Ehrlichia* spp.

Technically, the relatively low quantity of bacterial protein relative to that of the infected cell within the tick vector has precluded broad proteomic screening. The development of tick cell lines permissive for *in vitro* growth of *Anaplasma* and *Ehrlichia* spp. have removed, in part, this impediment by supporting replication to high titer and, equally importantly, by allowing incorporation of uninfected cells of the same line as a control(1, 16). The two-dimensional gel electrophoresis approach used in the present study allowed effective discrimination between tick cell and bacterial proteins. The utility of cell lines notwithstanding, how well these cells represent the actual tick cellular environment has been a persistent question. This is illustrated by the use of the ISE6 cell line in the experiments reported here: the cells are derived from embryonic *Ixodes scapularis* while *A. marginale* infects, sequentially, midgut epithelial and salivary gland acinar cells in adult ticks of several genera but not *Ixodes* (1, 18). The demonstration that *A. marginale* proteins identified as being up-regulated or exclusively expressed in the ISE6 cell line were also expressed

in infected *D. andersoni* indicates that the cell line is a useful predictor of expression in the natural vector, at least to a first-order approximation. This supports biological relevance of *in vitro* transcriptome and proteome analysis of other *Anaplasma* and *Ehrlichia* spp (19, 26).

The proteomic approach was unbiased as to the identity, localization within the bacterium, or presumed function of the proteins. We selected this approach for two reasons: first, there was no comparative data available on tick-borne bacteria in closely related genera that would guide a more targeted approach, and second, the very high percentage of the *A. marginale* genome that is annotated as encoding hypothetical proteins (2). That all 15 proteins identified by our approach were originally annotated as hypothetical proteins supports this unbiased methodology. The addition of these 15 proteins to 39 identified in recent studies defining the *A. marginale* proteome involved in protective immunity extends linkage of the genome annotation to the proteome (14, 21, 22). The progressive confirmation that annotated hypothetical proteins are actually expressed in either the mammalian host or tick vector indicates that these proteins are unique among bacteria with unknown function rather than being erroneous identification of coding sequences. This conclusion is also supported by the linkage of proteome analysis to the genome of *E. chaffeensis* (9, 26).

Am410, Am470, and Am829 were each expressed in both the midgut epithelium and salivary gland acinar cells of transmission fed ticks. While these three identified proteins segregate by host type, tick versus mammal, we would hypothesize that there are also organ-specific expression phenotypes within the

tick. This discrimination, which requires screening of additional tick-specific proteins, may be critically important for discovery of vaccines or drugs that block acquisition (at the level of the midgut) versus transmission (at the level of the salivary gland). None of the three proteins has yet a demonstrated function in *A. marginale*. However, an Am410 ortholog has recently been identified in the closely related tick-borne pathogen *A. phagocytophilum*, APH0859 (originally also annotated as a hypothetical protein, now designated Ats-1). Ats-1 has recently been shown to traffic to the mitochondrion of *A. phagocytophilum* infected cells where it interferes with apoptosis, allowing time for intracellular bacterial replication (20). Unlike *A. phagocytophilum*, which infects neutrophils in the mammalian host and requires blockage of apoptosis to complete a replicative cycle (20), *A. marginale* infects non-nucleated mature erythrocytes and thus the need for Ats-1 would be predicted to be dispensable in the bovine host. In contrast, within the tick vector *A. marginale* must invade and replicate in phagocytic midgut epithelial cells in order to establish colonization (10). Am410 fits this prediction with expression markedly up-regulated in the tick vector and expressed in the midgut epithelium. This conservation of gene content between *A. marginale* and *A. phagocytophilum* (2, 9), which share common sites of colonization in the tick but differ in the specific hematopoietic lineage infected in the mammalian host (4), is consistent with the theory that bacteria in the Family *Anaplasmataceae* first evolved in arthropod vectors and then diverged as they infected mammals. The differential regulation of this shared gene content, as

needed for the specific host environment and cell type, exemplified by Am410 expression, is congruent with this theory.

Previously all evidence was for down-regulated expression (Omp1, 4, 7-9, 11; Msp1a) or loss of expression (OpAG3) for specific *A. marginale* proteins in tick cells (6, 13, 21, 22). Interestingly, all of these proteins are expressed on the *A. marginale* surface and exposed to the mammalian immune system. In contrast, only Am778 of the 15 proteins identified in the present study as being exclusively expressed or up-regulated in tick cells is predicted to be surface exposed (21). This suggests that interaction with the humoral immune system may be less deterministic in the tick and that evading clearance by innate mechanisms such as phagocytosis and killing or by induced apoptosis may be more important. Both the approach and the newly identified proteins provide opportunities for novel strategies to block tick colonization and subsequent transmission.

MATERIALS AND METHODS

Proteomic screening for identification of tick stage-specific proteins

The St. Maries strain of *A. marginale*, a highly tick transmissible strain for which the genome has been completely sequenced and annotated (2, 29, 30), was used in all studies. The overall approach to identify candidate *A. marginale* tick stage-specific proteins was as follows. Bacteria were isolated from infected ISE6 cells and the bacterial lysate separated by two-dimensional gel electrophoresis and stained to identify the full complement of proteins.

Candidate tick-stage specific proteins were identified by comparison to proteins separated by two-dimensional electrophoresis of uninfected ISE6 tick cells (to identify and subtract out any contaminating ISE6 cellular proteins) and *A. marginale* St. Maries strain isolated from infected bovine erythrocytes (to identify and subtract out stage-common bacterial proteins) run under identical conditions.

In detail, *A. marginale* were isolated by filtration using a 2 µm pore size filter (Whatman), as previously described (21), and the washed bacterial pellet was re-suspended in phosphate buffered saline containing Complete Mini-Protease Inhibitor (Roche). Uninfected ISE6 tick cells were handled identically as a control. Bacteria or uninfected tick cells were lysed in a buffer containing 500 mM Tris, 50 mM EDTA, and 10% NP40. The lysates were processed with a ReadyPrep 2D cleanup kit (Bio-Rad) and solubilized in 8 M urea, 2% CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate), 0.2% Bio-Lyte 3/10 ampholytes (Bio-Rad) and 0.001% Bromophenol Blue. Iso-electric focusing (IEF) was carried out using 11-cm immobilized pH gradient strips under four conditions: a wide-range gradient (pH 3-10) and three narrow range gradients (pH 3-6, pH 5-8, pH 7-10). Each strip was rehydrated with a total of 150 µg of protein and focused for 35,000 Volt hrs using a Protean IEF cell system. Following IEF, second dimension electrophoresis was performed using 10% polyacrylamide gels. The gels were stained with SYPRO Ruby (Bio-Rad) and individual gel images from infected tick cells, uninfected tick cells, and infected erythrocytes were overlaid to match spots using PD Quest image analysis software (Bio-Rad). Spots identified by either PD Quest or visual inspection as

unique to infected tick cells were excised, processed by in-gel trypsin digestion and identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Confirmation of unique or up-regulated tick stage-specific protein expression by quantitative western blot

The three candidate tick stage-specific proteins with the highest MASCOT ion score following LC-MS/MS analysis, AM410, AM470, and AM829 (Table 1), were used to confirm differential expression. Each protein was expressed as a His-tagged recombinant protein, purified with the ProBond[®] Purification System (Invitrogen), and used to immunize mice to generate polyclonal and monoclonal antibodies for use in quantitative western blots. Briefly, the following primer sets were used in PCR amplification of sequences predicted (<http://tools.immuneepitope.org>) to encode a B-cell epitope bearing region of each protein: a 1,035bp fragment of AM410, 5'-ggggacaagttgtacaaaaaagcaggcctaagccCatttaaagcagg-3' and 5'-ggggaccactttgtacaagaaagctgggtactatgCggacgctgcggcctg-3'; a 1,500bp fragment of AM470, 5'-ggggacaagttgtacaaaaaagcaggcctaataagacccacattggcga-3' and 5'-ggggaccactttgtacaagaaagctgggtactacatCgccttccttgccg-3'; and a 420 bp fragment of AM829, 5'-ggggacaagttgtacaaaaaagcaggcttactgagcagagtgaggatattt-3' and 5'-ggggaccactttgtacaagaaagctgggtactaCcggcggaaccgctc-3'. The amplicons were cloned and expressed as His-tagged fusion proteins using Gateway[®] Expression System (Invitrogen). The insert was sequenced using the T7 primer to ensure

correct orientation, the correct protein coding sequence, and in-frame position of the His-tag. BL21-A1 *E.coli* were transformed with the expression plasmid, cultured in LB broth containing 50 ug/ml carbenicillin and induced with 0.2% L-arabinose. His-tagged proteins were purified using the ProBond Purification System (Invitrogen).

To generate antibodies mice were immunized and boosted subcutaneously with 50 µg of each recombinant protein emulsified in Titermax® Gold adjuvant (CytRx). For monoclonal antibody production, mice were boosted intravenously with 50 µg of antigen without adjuvant 3 days immediately prior to hybridoma fusion. Fusion and limiting dilution cloning were performed as described (32). Hybridoma supernatants were screened for reactivity by immunoblotting using *A. marginale* isolated from infected ISE6 cells. For quantitative western blotting, *A. marginale* isolated from each host cell type were quantified using *msp5*-based quantitative real-time PCR as previously described (5) and 10⁷ bacteria were loaded per lane. Uninfected ISE6 cells and uninfected erythrocytes were used as negative controls. Electrophoresis was carried out using pre-cast 4-20% polyacrylamide gels (Bio-Rad). The proteins were transferred to nitrocellulose membrane and probed with monoclonal antibody AnaF16C1 (reactive with Msp5), as an internal control for equal loading. AM410 and AM470 expression was detected using, respectively, monoclonal antibodies 142/184.8 and 143/694.12.11 while AM829 expression was detected using a 1:500 dilution of specific polyclonal serum. Reactivity was detected using the Western Star chemiluminescence system (Applied Biosystems). An unrelated

isotype-matched monoclonal antibody TRYP1E1 (reactive with a *Trypanosoma brucei* protein) and a polyclonal serum (1:500 dilution; reactive with a *Babesia bovis* recombinant protein) were used as negative controls.

In situ* expression of unique or up-regulated tick-stage specific proteins in *Dermacentor andersoni

In situ expression of AM410, AM470, and AM829 was detected by immunohistochemistry on *A. marginale* infected male *D. andersoni* ticks. An *msp5* PCR and Msp5 C-ELISA seronegative calf (28) was infected by intravenous inoculation of the St. Maries strain. During the acute phase of infection (bacteremia $\geq 10^8$ *A. marginale* per ml), ticks were acquisition fed for 7 days. Ticks were then removed and incubated at 26° C and 96% relative humidity for 7 days to allow complete digestion of the blood-meal. Ticks were subsequently transmission fed for 7 days on a second naïve calf. A cohort of the transmission fed ticks was removed, midguts and salivary glands individually dissected and placed in PBS containing protease inhibitors for western blot analysis as described above. A second cohort was immediately fixed in 10% formaldehyde and embedded in paraffin. Serial 4- μ m sections were deparaffinized and immunohistochemistry was performed as previously described (29). Serial sections were reacted with 15 μ g/ml of each monoclonal antibody or a 1:200 dilution of anti-AM829 polyclonal serum; monoclonal antibody TRYP1E1 or a 1:200 dilution of anti- *B. bovis* polyclonal serum were used as negative antibody controls. Uninfected ticks, handled identically, were

used as a negative antigen control. Binding was detected with horseradish peroxidase-labeled anti-mouse antibody (Dako) and counterstained with Mayer's hematoxylin.

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Table 1. *Anaplasma marginale* proteins up-regulated in tick cell culture.

| Spot no. | Gene Identity | MASCOT Ion score ^a | Sequence coverage (%) | Molecular mass kDa ^b |
|----------|---------------|-------------------------------|-----------------------|---------------------------------|
| 1 | AM404 | 28 | 19 | 28 |
| 2 | AM681 | 37 | 24 | 27 |
| 3 | AM116 | 26 | 44 | 30 |
| 4 | AM268 | 35 | 54 | 43 |
| 5 | AM829 | 177 | 10 | 26 |
| 6 | AM926 | 23 | 40 | 35 |
| 7 | AM613 | 33 | 63 | 58 |
| 8 | AM410 | 139 | 8 | 48 |
| 9 | AM159 | 26 | 3 | 93 |
| 10 | AM118 | 38 | 55 | 49 |
| 11, 12 | AM414 | 33 | 28 | 40 |
| 13 | AM959 | 32 | 19 | 45 |
| 14 | AM778 | 61 | 6 | 48 |
| 15 | AM1141 | 30 | 36 | 52 |
| 16 | AM470 | 236 | 39 | 150 |

^aion score greater than 23 is statistically significant with a *p* value < 0.05

^b Observed molecular mass



Figure 1. Constitutive expression of Msp5 in *Anaplasma marginale* from infected ISE6 tick cells, bovine erythrocytes, *Dermacentor andersoni* midgut, and *D. andersoni* salivary glands. Each lane was loaded with $10^{5.43 \pm 0.59}$ bacteria and reacted with anti-Msp5 monoclonal antibody ANAF16C1. iISE6: infected ISE6 tick cells; iRBC: infected bovine erythrocytes; iMG: infected *D. andersoni* midgut; iSG: infected *D. andersoni* salivary glands.

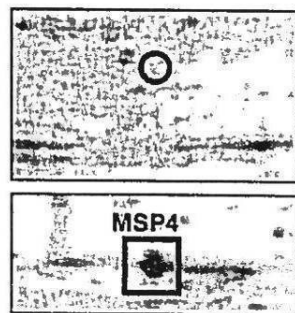
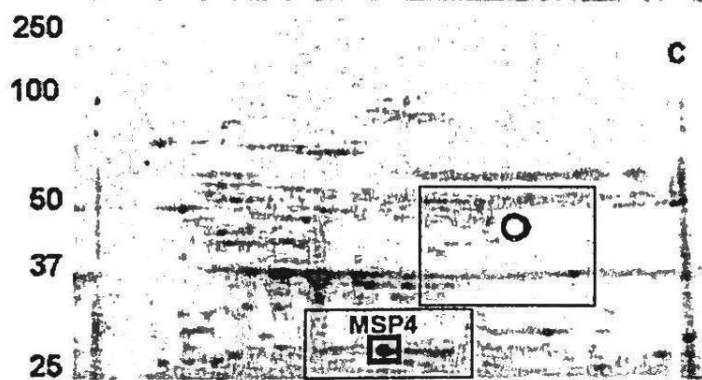
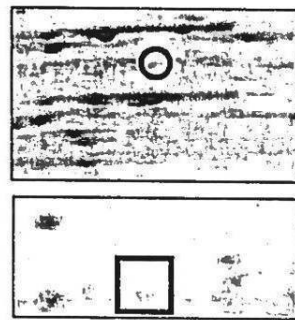
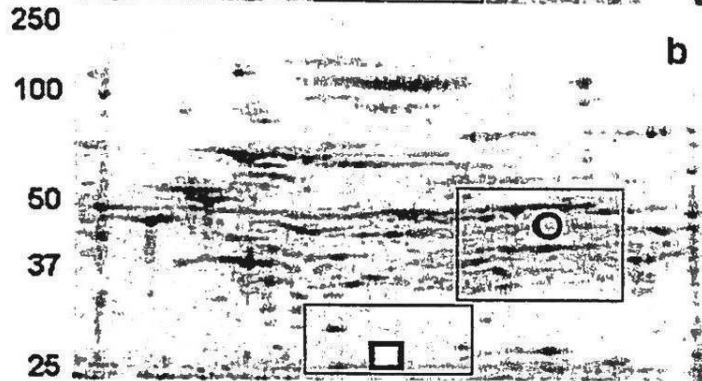
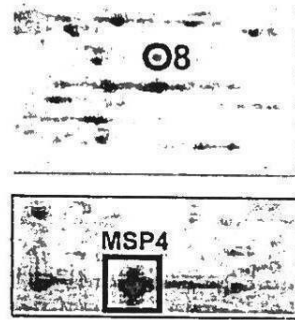
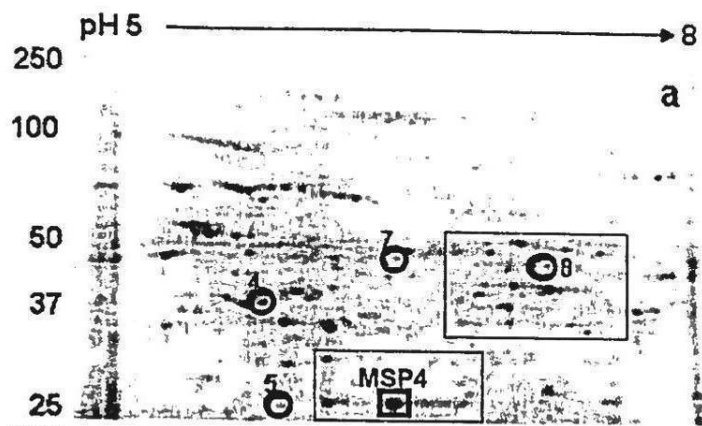


Figure 2. Identification of *Anaplasma marginale* proteins uniquely expressed or up-regulated in tick cell culture. a) infected ISE6 cells; b) uninfected ISE6 cells. c) infected bovine erythrocytes. Gels were stained with SYPRO Ruby to detect total protein. Circles indicate protein spots exclusive to infected tick cells; the numbers refer to the identified protein (Table 1). The square represents Msp4 expressed in *A. marginale* in both host cell types. The pH range for iso-electric focusing is labeled at the top of the two-dimensional images. The molecular size standards (kDa) are on the left. Images on the right are enlargements of the highlighted *A. marginale* protein spots.

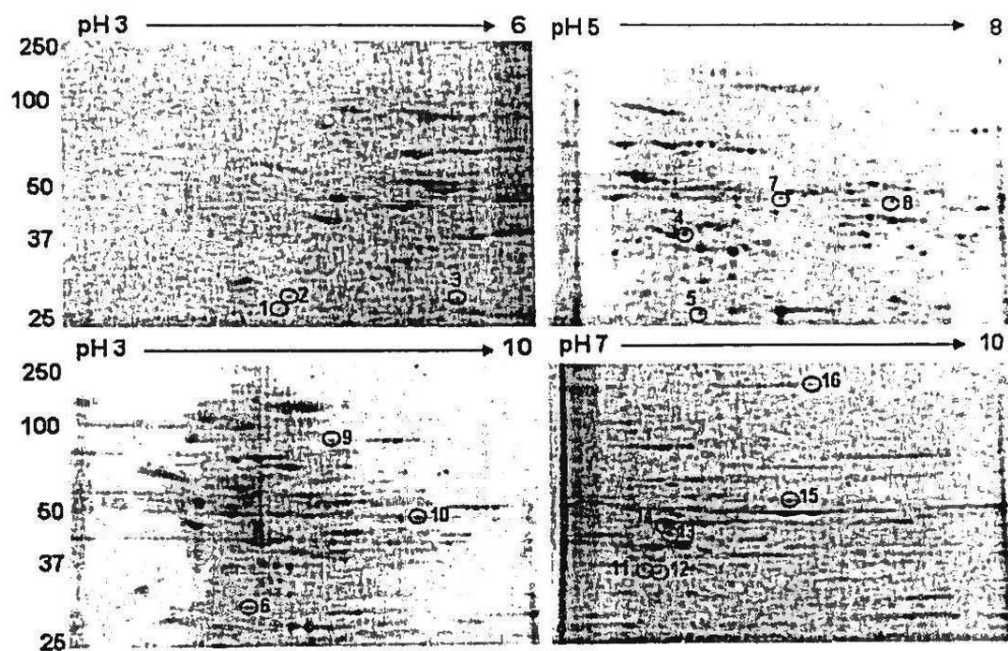


Figure 3. *Anaplasma marginale* proteins uniquely expressed or up-regulated in tick cell culture. Gels were stained with SYPRO Ruby to detect total protein. Circles represent protein spots exclusively present in *A. marginale* isolated from infected ISE6 cells; the numbers refer to the identified protein (Table 1). The pH range for iso-electric focusing is labeled at the top of each two-dimensional image. The molecular mass standards (kDa) are on the left.

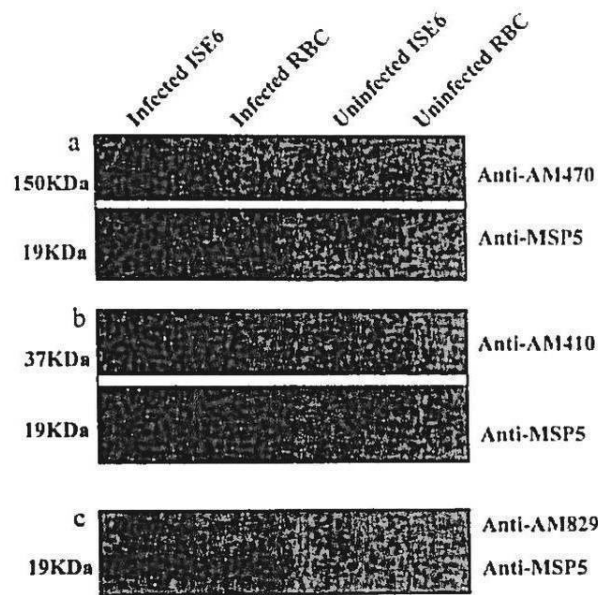


Figure 4. Up-regulated expression of AM470, AM410, and AM829 in *Anaplasma marginale* isolated from infected tick cells. *A. marginale* isolated from infected ISE6 tick cells (lane 1), *A. marginale* isolated from infected erythrocytes (lane 2), uninfected ISE6 cells (lane 3), and uninfected erythrocytes (lane 4) were probed with antibodies specific for either (a) AM470, (b) AM410, or (c) AM829 and, in the same blot, with monoclonal antibody ANAF16C1 specific for the constitutively expressed Msp5. Lanes 1 and 2 contained $10^{7\pm 0.05}$ *A. marginale*.

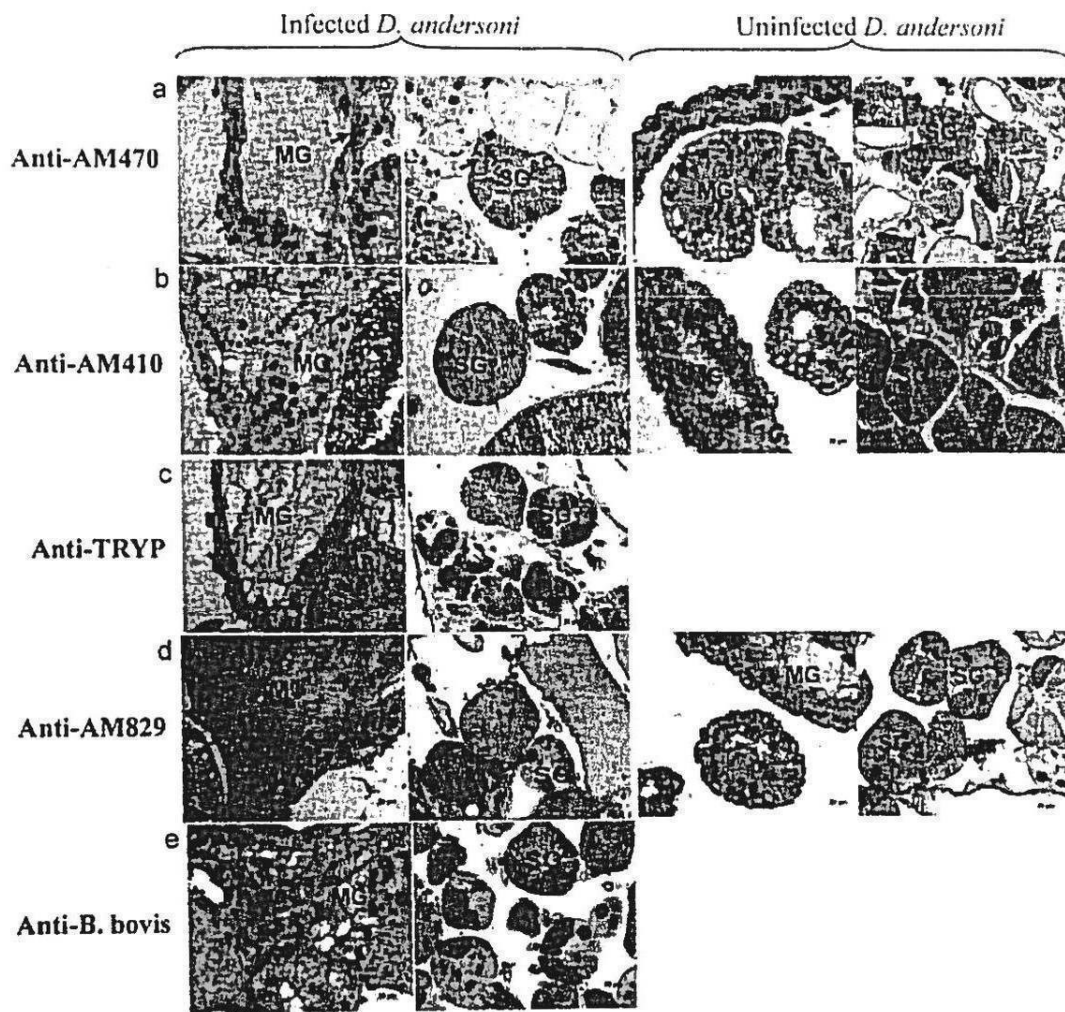


Figure 5. Expression of AM470, AM410, and AM829 (arrows) in the midgut (MG) and salivary gland (SG) of *Anaplasma marginale* infected *Dermacentor andersoni*. Panels a, b, and d: serial sections of both infected and uninfected ticks probed with monoclonal antibody 143/694.12.11, 142/184.8, or polyclonal serum specific to AM470, AM410, and AM829 respectively. Panel c and e: serial sections of infected ticks probed with monoclonal antibody TRYP1E1 and anti-*Babesia bovis* polyclonal serum specific to *Trypanosoma brucei* and *Babesia bovis*, respectively, were used as negative controls.

CHAPTER 2

Expression of *Anaplasma marginale* ankyrin repeat-containing proteins during infection of the mammalian host and tick vector

ABSTRACT

The tick-borne obligate intracellular rickettsial pathogen of ruminants *Anaplasma marginale* encodes three ankyrin repeat-containing proteins; AM705 or AnkA, AM926, and AM638. The ankyrin motif is one of the most common conserved domains in eukaryotes and is increasingly being identified in prokaryotes. It is 33 amino acids long with a basic unit comprising two anti-parallel alpha- helices and a beta-turn that projects at approximately 90° angle. We predicted based on the reported nuclear translocation of *Anaplasma phagocytophilum* AnkA and *Ehrlichia chaffeensis* p200 that the expression of *A. marginale* AnKA (AM705) would either be tick stage-specific or significantly upregulated in nucleated tick cells as compared to non-nucleated erythrocytes. In addition we predicted that this protein would translocate to the host cell nucleus. We included in the analysis the other *A. marginale* genes with the ankyrin repeat motif; AM926 and AM638. While all the proteins were expressed in the tick stage, only AM638 was tick stage-specific. Contrary to our prediction the expression of AM705 was significantly higher in erythrocytes compared to ISE6 cells. AM926 was expressed at similar levels between ISE6 cells and erythrocytes. Using monoclonal antibodies specific to each protein in double immunofluorescence labeling we found that none of the proteins translocated to the host cell nucleus. These findings should advance our understanding, at the molecular level, of how

tick-borne pathogens adapt to distinctly different two-host environments affording us opportunities to block the transmission cycle.

INTRODUCTION

Tick-borne pathogens in the genera *Anaplasma* and *Ehrlichia* must invade and replicate in two very distinct environments, hematopoietic cells within a mammalian host and both midgut and salivary gland cells within the arthropod vector (7). We, and others, have hypothesized that this transition between hosts requires expression of a unique proteome (13, 15, 16, 20, 21). This is supported by proteomic approaches, unbiased as to location or function, which identified both marked up-regulation and unique expression of bacterial proteins in the tick vector as compared to the mammalian host (18). In our recent study using *A. marginale*, all 15 proteins shown to be up-regulated in tick cells had been originally annotated as hypothetical proteins, consistent with a significant percentage of proteins of unknown function in the genera *Anaplasma* and *Ehrlichia* (4).

A second approach to discovery of proteins up-regulated or uniquely expressed in the tick vector is predictive, based on specific differences between the host environments and cell types. For *A. marginale*, a striking difference is the infection of non-nucleated cells in the mammalian host versus infection of nucleated cells in the tick vector (5). Unlike most other members of the genera *Anaplasma* and *Ehrlichia* which infect nucleated hematopoietic cells, *A. marginale* invades and replicates in mature erythrocytes in the mammalian host (22, 25). Upon acquisition by a feeding tick, *A. marginale* invades and replicates in, sequentially, midgut and salivary gland epithelial cells, a progression common among the tick-borne *Anaplasma* and *Ehrlichia* spp(7, 10). Consequently, we

proposed that while bacterial proteins that localize to the host cell nucleus during intracellular infection would be expressed in both the mammalian and tick cell environments for most bacteria in these two genera, *A. marginale* would express these proteins only in the tick vector.

Two orthologous ankyrin-repeat containing proteins have been shown to traffic to the host cell nucleus during infection: *E. chaffeensis* p200 and *A. phagocytophilum* AnkA. *E. chaffeensis* p200 localizes to the nucleus and binds Alu-Sx DNA motifs(25). *A. phagocytophilum* AnkA similarly localizes to the host cell nucleus, binds chromatin regulatory region, and down-regulates *cybb* and other host defense genes (6, 9, 17). The AnkA-mediated down-regulation of host defense genes is consistent with *A. phagocytophilum* survival not only in the mammalian neutrophil but also in the phagocytic midgut epithelial cells of the tick. In contrast, an *A. marginale* AnkA orthologue would be expected to be dispensable for survival and replication in the mature erythrocytes of the mammalian host and thus specifically expressed in the tick vector.

In the present study, we identified the *A. marginale* AnkA orthologue, tested whether AnkA is uniquely expressed or significantly up-regulated in the cells of the tick vector, and determined if AnkA localized to the nucleus of tick cells. Furthermore, we screened the *A. marginale* genome for additional ankyrin-repeat containing proteins as candidates for host cell nuclear localization and global regulators and tested whether these localized to the nucleus and were specifically expressed in the tick vector. We present the results of these studies and discuss the findings in the context of the pathogen-host-vector interaction.

RESULTS

Identification of *A. marginale* genes encoding ankyrin repeat motifs.

A genome-wide screen of the annotated St. Maries genome identified three genes with the ankyrin repeat motif (Fig. 1). AM705 is a 146 kDa protein containing 10 ankyrin repeats that is orthologous to both *A. phagocytophilum* AnkA (e value 10^{-55}) and *E. chaffeensis* p200 (e value 10^{-17}). AM926 is a 31 kDa protein with two ankyrin repeats and is orthologous to *A. phagocytophilum* APH259 (e value 10^{-33}) and *E. chaffeensis* ECH389 (e value 10^{-24}). AM638 is a 348 kDa protein with 9 ankyrin repeats and is most closely related to APH709 (e value 10^{-00}) and ECH0653 (e value 10^{-77}). The only other identified ankyrin repeat containing protein in *A. phagocytophilum*, APH928, did not have an identifiable ortholog in any of the sequenced *A. marginale* strains.

Quantitative expression levels of ankyrin repeat-containing proteins in infected mammalian and tick cells.

Using antibodies specific to each ankyrin-repeat containing protein, lysates containing 10^7 *A. marginale* isolated from either infected ISE6 cells or infected erythrocytes were probed for expression. All three proteins, AM705, AM926, and AM638, were expressed in bacteria isolated from either or both host cell types (Fig. 2). AM705 was expressed at a higher level in bacteria from infected erythrocytes than from infected ISE6 cells; densitometric analysis of three independent replicates revealed statistically significant upregulation ($p=0.001$, unpaired Student's *t*-test). In contrast, AM638 was expressed only in

bacteria isolated from ISE6 cells, with no detectable expression in 10^7 bacteria from infected erythrocytes (Fig. 2). AM926 was expressed in bacteria isolated from both cell types, with no significant difference in the levels of expression ($p=0.2$) based on densitometric analysis of three independent replicates. A *marginale* Msp5, constitutively expressed in both host cell types (2, 12, 18), was used as an internal standard for equal number of loaded bacteria (Fig. 2) and there was no significant difference in the Msp5 levels measured by densitometry.

***In situ* expression of ankyrin repeat-containing proteins in *Dermacentor andersoni*.**

The quantitative western blot analysis shown in Fig. 2 confirmed expression of AM705 and AM926 in *A. marginale* infected erythrocytes obtained from *in vivo* infection of a natural mammalian host. To confirm expression in the natural tick vector and determine whether expression occurred in the tick midgut, salivary gland, or both, sections of transmission fed adult male *D. andersoni* were probed using immunohistochemistry. AM705, AM926, and AM638 were detected both in the midgut epithelial and salivary gland acinar cells (Fig. 3). Uninfected ticks, handled identically but fed on an uninfected calf, were negative when probed with antibodies specific to each protein as were infected ticks probed with the negative control antibody TRYP1E1 or anti- *Babesia bovis* polyclonal serum (Fig. 3).

Sub-cellular localization of ankyrin repeat-containing proteins.

To test whether any of the three proteins was translocated to the host cell nucleus, as has been shown for both *A. phagocytophilum* AnkA and *E. chaffeensis* p200(6, 9, 17), infected ISE6 cells were probed with each monoclonal antibody and nuclear staining with DAPI and examined by fluorescence microscopy. Monoclonal antibody ANAR49 was used to detect Msp2, an integral outer membrane protein, as a marker for a non-nuclear translocated protein (Fig. 4). AM705, AM926, and AM638 all localized to the bacteria within the cytoplasmic vacuole, similar to Msp2, with no evidence of either nuclear translocation or translocation outside the vacuole (Fig. 4). There was no reactivity of any of the anti-*A. marginale* antibodies with uninfected ISE6 cells and no reactivity of the negative control monoclonal antibody TRYP1E1 with infected ISE6 cells (Fig. 4).

DISCUSSION

Ankyrin repeats are common in eukaryotic cells and, although initially thought to be relatively uncommon in prokaryotes, have been reported with increasing frequency in a diverse set of α -, β -, and γ -proteobacteria(14, 19, 23) Although the ankyrin domain has most commonly been linked to protein-protein interactions in the host cell cytosol, seminal studies with both *A. phagocytophilum* AnkA and *E. chaffeensis* p200 identified host cell nuclear localization with chromatin and DNA binding(9, 17). In a directed search for *A. marginale* proteins uniquely expressed or specifically up-regulated in the tick vector, we

hypothesized that an *A. marginale* AnkA orthologue would be expressed only in the tick vector, where the host cells are nucleated, and not in the mature non-nucleated erythrocyte of the mammalian host. We identified a clear AnkAp200 orthologue in *A. marginale*, AM705, as well as two additional ankyrin domain-bearing proteins, AM926 and AM638. Originally annotated as hypothetical proteins, we have now shown that these are expressed as protein during infection of either a natural mammalian host and a natural tick vector, or both. Accordingly, we now re-designate these proteins: AM705 as *A. marginale* AnkA, AM638 as AnkB, and AM926 as AnkC. All three are highly conserved among genera *Anaplasma* and *Ehrlichia*. AnkB (AM638) is conserved in the Family *Anaplasmataceae*. Based on the currently available genome sequences, the number of encoded ankyrin repeat-bearing proteins varies among the bacteria in the Family *Anaplasmataceae* from three in *A. marginale* to 60 in the wPip strain of *Wolbachia pipiens*(4, 14, 23).

The hypothesis that *A. marginale* AnkA (AM705) is expressed only in the nucleated cells of the tick has been rejected: AnkA was expressed in the mammalian erythrocyte, the tick midgut epithelium, and the tick salivary gland acinar cells. The quantitative analysis using ISE6 cells indicated that AnkA is expressed at higher levels in the erythrocyte than in the tick cells. Whether this lower level in the ISE6 cells is reflective of levels in the actual tick tissues is unknown, however our broader proteomic analysis has supported the predictive value of *A. marginale* expression in ISE6 cells for *D. andersoni*. In contrast, AnkB (AM638) was uniquely expressed in ISE6 cells and the *D. andersoni*

midgut and salivary gland. Collectively with the unbiased proteomic analysis reported previously, 15 *A. marginale* proteins have been identified as uniquely expressed or significantly up-regulated in tick cells, 9 proteins uniquely expressed or expressed at higher levels in the mammalian erythrocyte, and the majority expressed in both host cell environments, including AnkC described in this study.

Unlike either *A. phagocytophilum* AnkA or *E. chaffeensis* p200, none of the *A. marginale* Ank proteins translocated to the nucleus. None of the AnkA/p200 orthologues have a consensus nuclear localization signal, nor do the newly identified AnkB and AnkC proteins. How *A. phagocytophilum* AnkA and *E. chaffeensis* p200 are translocated remains unknown, however a requirement for an additional nuclear transporter would explain the difference between *A. marginale* AnkA and the orthologues in the other two species. *A. phagocytophilum* AnkA has also shown to be translocated to the host cell cytosol(11); in contrast, *A. marginale* AnkA, AnkB, and AnkC appear intimately associated with the bacterium itself with no evidence of secretion. This suggests that while these proteins may be derived from a common ancestor, there has been divergence to effect different functions in the specific pathogen-host cell interactions. The retention of the ankyrin repeat domains provide a structural basis for these interactions, however the trafficking of these proteins appears to be equally important in defining the interactions.

MATERIALS AND METHODS

Identification of *A. marginale* genes encoding ankyrin repeat motifs.

We identified *A. marginale* ankyrin repeat motif encoding genes in a number of ways. First, by searching the non-redundant database using Basic Local Alignment Search Tool (BLAST) (<http://www.ncbi.nlm.nih.gov/BLAST>) and *Anaplasma phagocytophilum* ankyrin repeat encoding genes APH259, APH740 (AnkA), and APH709 as query sequences. Second, the ankyrin motif was searched for in all sequenced *A. marginale* strains using the NCBI conserved domain database (<http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>). We also mined smart 00248 and pfam 0023. Combined, the searches identified three *A. marginale* genes, each containing multiple ankyrin domain repeats: AM705 with 10 ankyrin domain repeats, AM926 with 2 ankyrin repeats, and AM638 with 9 ankyrin repeats.

Quantitative expression levels of ankyrin repeat-containing proteins in infected mammalian and tick cells.

The expression of the three *A. marginale* ankyrin repeat bearing proteins in the different host environments was determined using quantitative western blots. Briefly, a large region of each open reading frame was expressed as a recombinant protein and the purified protein used to immunize mice to generate specific monoclonal antibodies for quantitative detection of each protein in infected cells. Predicted B-cell epitope-bearing regions of each gene were identified (<http://tools.immuneepitope.org>) and the encoding open reading frames

amplified using the following primer sets: for AM705 (2,100 bp fragment) 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGATGACGATACACCATTG-3' and 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTACTACCAGCCTCTGGACAGGTA-3'; for AM926 (750 bp fragment), 5'-GGGGACAAGTTTGTACAAAAA GCAGGCTTAGACTTTCTTTTGGGAGAAGTAA-3' and 5'-GGGGACCACTTTG TACAAGAAAGCTGGGTACTACCCCTCTTGTCTTCTTC-3'; and for AM638 (1,650 bp fragment), 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTATC CTC ACACTGCG ACATA-3' and 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTAC TATTCGCGTGCAGCGTCTTC-3'. The PCR cycling conditions were 10 cycles of melting at 94°C for 30s, annealing at 35°C (AM705), 41°C (AM926), or 39°C (AM638) for 30s with extension at 72°C for 2 min (AM705, AM638) or 1 min (AM926), followed by 25 cycles of melting at 94°C for 30s and annealing at 70°C for 30s and extension at 72°C for 2 min (AM705, AM638) or 1 min (AM926). Cloning and expression utilized the Gateway expression system (Invitrogen) and the recombinant His-tagged fusion proteins were affinity purified as previously described (18).

Monoclonal antibodies were generated by immunizing mice with recombinant proteins followed by hybridoma fusion and limiting dilution cloning as previously described(18, 24). Briefly, 50 µg of each recombinant protein emulsified in Titermax Gold adjuvant (CytRx) were used to immunize mice subcutaneously. Three days prior to hybridoma fusion mice were boosted intravenously with 50 µg of protein without adjuvant. Hybridoma supernatants were screened by western blotting for reactivity using the St. Maries strain of A

marginale isolated from infected ISE6 cells or infected erythrocytes. Quantitative western blotting was performed by first normalizing *A. marginale* organisms isolated from infected ISE6 cells and infected erythrocytes using two independent methods. First, the number of bacteria were quantified by real-time PCR based on the single copy *msp5* gene, as described previously (8) and equal numbers (10^7) of bacteria from each host cell were loaded. Second, the western blots were probed with monoclonal antibody ANAF16C1 reactive to Msp5 as an internal standard. Msp5 is constitutively expressed at a high level in both infected mammalian erythrocytes and infected ISE6 cells(2, 18). Uninfected ISE6 cells and uninfected erythrocytes were used as negative antigen controls. The proteins were resolved by electrophoresis using 4 to 20% precast-polyacrylamide gels (Bio-Rad). Following transfer to a nitrocellulose membrane, the expression of AM705, AM926 and AM638 were probed for with monoclonal antibodies 149/312, 148/42.17, and 150/103, respectively, and antibody binding detected by using the Western Star chemiluminescence system (Applied Biosystems). An isotype-matched monoclonal antibody TRYP1E1 (reactive with a *Trypanosoma brucei* protein) was used as a negative antibody control.

***In situ* expression of ankyrin repeat-containing proteins in *Dermacentor andersoni*.**

Ticks infected with the St. Maries strain were used to detect *in situ* expression of all three ankyrin repeat-containing proteins by immunohistochemistry. Briefly, a MSP5 C-ELISA seronegative calf was

inoculated intravenously with the St. Maries strain. Male *D. andersoni* ticks were acquisition fed on the calf for 7 days during acute infection (bacteremia $\geq 10^6$ *A. marginale* organisms per ml). The ticks were removed and incubated at 26°C and 96% relative humidity for an additional 7 days to allow complete digestion of the bloodmeal and replication in the midgut epithelium, followed by transmission feeding on a second seronegative calf for 7 days. Upon removal, the ticks were immediately fixed in 10% formaldehyde and later embedded in paraffin. Immunohistochemistry was performed as previously described (22) on serial 4- μ m deparaffinized sections of the ticks using 15 μ g of each monoclonal antibody/ml or a 1:100 dilution of polyclonal serum. Uninfected ticks treated identically were used as negative antigen controls. An isotype-matched monoclonal antibody TRYP1E1 (reactive with a *Trypanosoma brucei* protein) or a 1:100 dilution of polyclonal sera (reactive with a *Babesia bovis* protein) were used as a negative antibody controls. Binding was detected with horseradish peroxidase-labeled anti-mouse antibody (Dako) and Mayer's hematoxylin was used as a counterstain.

Sub-cellular localization of ankyrin repeat-containing proteins.

Confluent ISE6 cells were inoculated with the St. Maries strain and monitored by microscopic examination of Giemsa-stained cytopsin preparations until 30-60% of cells were infected. The cells were fixed with 10% formaldehyde and incubated overnight at room temperature. After centrifugation at 5000 x g for 2 min, half the supernatant was removed and the cells were re-suspended in

equal volume of 0.2% agarose. The cells were then paraffin embedded and serial 4- μ m sections processed as described for immunohistochemical staining with the following modifications. After antigen retrieval, the sections were blocked by applying four drops of Image-IT FX signal enhancer (Invitrogen) and incubated for 30 min at room temperature in a humid environment. Cells were individually incubated with 100 μ l of each monoclonal antibody for 30 min. Following rinsing, cells were incubated with 100 μ l Alexa Fluor 488 goat anti-mouse antibody (5 μ g/ml) for 30 min. After an additional rinse, coverslips were mounted using 4',6-diamidino-2-phenylindole (DAPI) slow fade mounting medium (Invitrogen). Uninfected ISE6 cells were treated identically and used as negative antigen controls. Monoclonal antibody ANAR49, reactive with *A. marginale* major surface protein 2 (Msp2), was used as a control for identification of a non-nuclear translocated protein. Monoclonal antibody TRYP1E1 was used as a negative antibody control. Slides were viewed and photographed using an Axio Imager.M1 microscope (Carl Zeiss Microimaging, Thornwood, NY, USA) equipped with an X-Cite 120 FI Illuminating system (EXFO Photonic Solutions, Mississauga, Ontario, Canada) for epi-fluorescence microscopy. Digital images were captured using an AxioCam MRm digital camera connected to a desktop computer running AxioVision (version 4.8.1.0). Images were processed using the ImageJ-based open source processing package Fiji (version 1.6.0_16, <http://pacific.mpi-cbg.de/>) as described previously(1)

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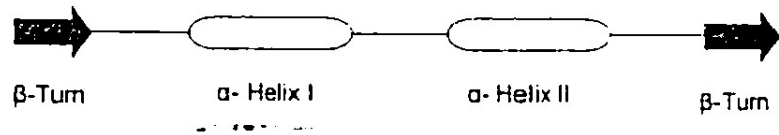


Figure 1. Schematic representation of an ankyrin repeat motif. The motif consists two anti-parallel α -helices and a β -turn that projects at an approximately 90° angle(3)

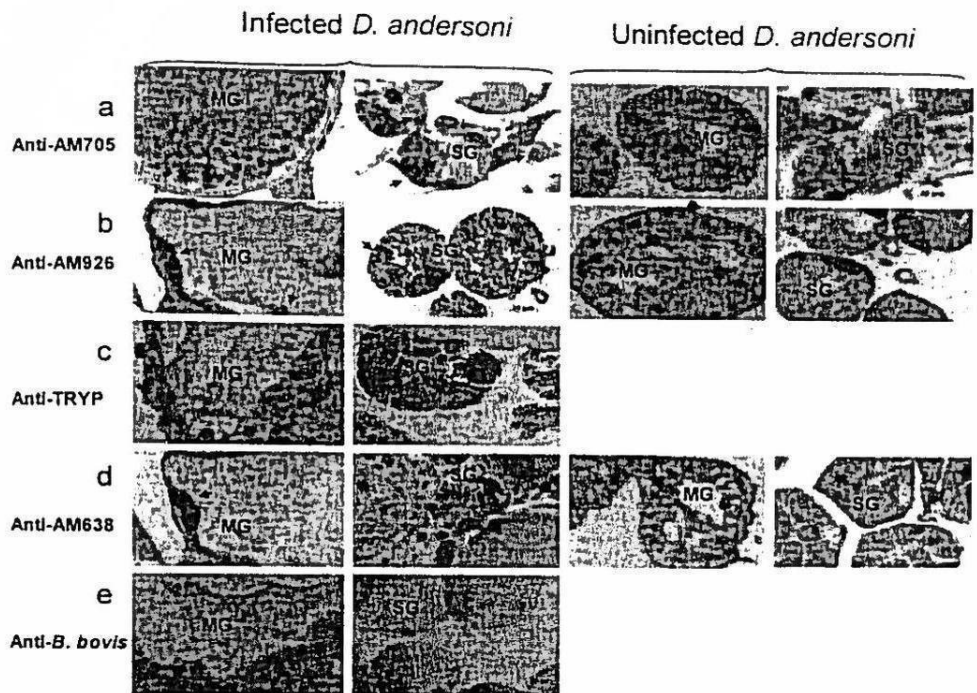


Figure 3. Expression of *Anaplasma marginale* ankyrin repeat-containing proteins in the midgut (MG) and salivary gland (SG) of St. Maries strain infected *Dermacentor andersoni*. Panels a, b, and d: serial sections of infected and uninfected ticks probed with monoclonal antibodies 149/312, 148/48.17 or polyclonal serum specific to AM705, AM926 or AM638 respectively. Panels c and e: serial sections of infected ticks probed with, respectively, monoclonal antibody TRYP1E1 and anti-*Babesia bovis* polyclonal serum as negative antibody controls.

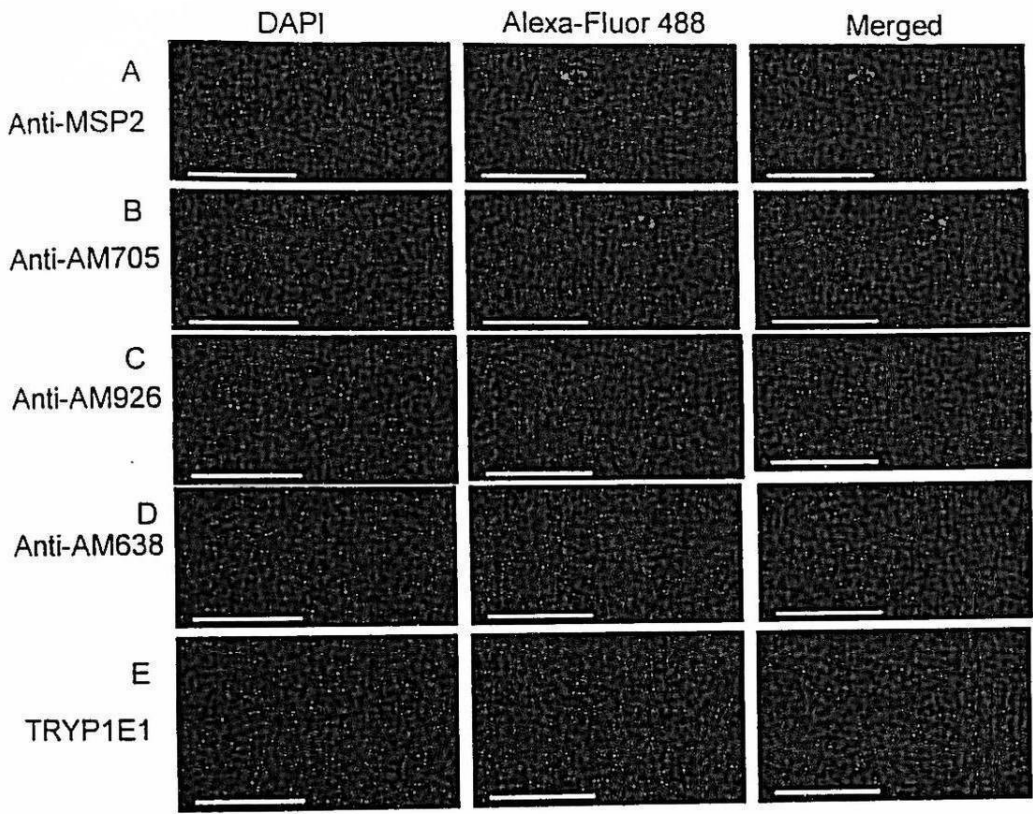


Figure 4. Sub-cellular localization of *Anaplasma marginale* ankyrin repeat-containing proteins. Panels A-D. Double immunofluorescence labeling of *A. marginale*-infected ISE6 cells. A: monoclonal antibody ANAR49 (reactive with Msp2) was used to label *A. marginale* within the intracellular vacuoles. B, C, D: monoclonal antibodies 149/312, 148/42.17, and 150/103 were used to label AM705, AM926, and AM638 respectively. Alexa Fluor 488-conjugated goat anti-mouse (green) was used as the secondary antibody. Nuclei were labeled with DAPI (blue). Images were captured using epifluorescence microscopy. Uninfected ISE6 cells treated identically (data not shown) and monoclonal antibody TRYP1E1 (E) were used as the respective negative antigen and antibody controls. Scale bar: 20 μ m.