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Identification of *Anaplasma marginale* Proteins Specifically Upregulated during Colonization of the Tick Vector[▽]

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The transition between infection of the mammalian host and colonization of an arthropod vector is required for the 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 upregulated in the tick 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 upregulated in tick cells. All 15 had originally been annotated as hypothetical proteins. We confirmed quantitative upregulation 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 the *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 the identification of new targets for vaccine and chemotherapeutic blocking of transmission.

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 by using a small genome of <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 downregulated 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 upregulated expression of these candidate proteins in the tick cells has not been confirmed. There has been no identification of bacterial proteins specifically upregulated 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 after 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 examine 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, 16). 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. We report here 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.

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 was separated by two-dimensional gel electrophoresis

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TABLE 1. *Anaplasma marginale* proteins upregulated 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	53	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	51	6	48
15	AM1141	30	36	52
16	AM470	235	39	150

[a] An ion score greater than 23 is statistically significant with a P value of <0.05.
[b] That is, the observed molecular mass.

and stained to examine the full complement of proteins. Candidate tick-stage specific bacterial 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 resuspended in phosphate-buffered saline (PBS) 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% NP-40. 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. Isoelectric focusing (IEF) was carried out using 11-cm immobilized pH gradient strips under four conditions: a wide-range gradient (pH 3 to 10) and three narrow-range gradients (pH 3 to 6, pH 5 to 8, and pH 7 to 10). Each strip was rehydrated with a total of 150 μg of protein and focused for 35,000 V · h using a Protean IEF cell system. After 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 upregulated tick stage-specific protein expression by quantitative Western blotting. The three candidate tick-stage-specific proteins with the highest MASCOT (Matrix Science) ion scores after 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, affinity purified, and used to immunize mice to generate polyclonal and monoclonal antibodies for use in quantitative Western blot analyses. 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 1,035-bp fragment of AM410, 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAAGCCCATTTAAAAGCAGG-3′ and 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTACTATGCGGACGCTGCGGCCTG-3′; a 1,500-bp fragment of AM470, 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATAGACCCACATTGGCGA-3′ and 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTACTACATCGCCTTCCTTTGCCG-3′; and a 420-bp fragment of AM829, 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTACTGAGCAGAGTGCAGGATATTT-3′ and 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTACTACCGGCGGAACCGTC-3′. The amplicons were cloned and expressed as His-tagged fusion proteins by using a Gateway expression system (Invitrogen). The insert was sequenced using the T7 primer to ensure correct orientation, the correct protein coding sequence, and an in-frame position of the His tag. BL21-A1 *Escherichia coli* was transformed with the expression plasmid, cultured in LB broth containing 50 μg of carbenicillin/ml, and induced with 0.2% L-arabinose. His-tagged proteins were purified by using a 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 previously (32). Hybridoma supernatants were screened for reactivity by immunoblotting with *A. marginale* isolated from infected ISE6 cells. For quantitative Western blotting, *A. marginale* isolated from each host cell type were quantified by using *msp5*-based quantitative real-time PCR as previously described (5), and 10^7 bacteria were loaded per lane. Uninfected ISE6 cells and uninfected erythrocytes were used as negative controls. Electrophoresis was carried out using precast 4 to 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 by 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 upregulated 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* organisms 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 naive calf. A cohort of the transmission fed ticks was removed, and the midguts and salivary glands were 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-μm sections were deparaffinized, and immunohistochemistry was performed as previously described (29). Serial sections were reacted with 15 μg of each monoclonal antibody/ml 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.

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 upregulated 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 by 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

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FIG. 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.

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 Student *t* test) revealed statistically significant higher expression ($P = 0.01$) in the tick cell-derived *A. marginale* compared to bacteria from infected erythrocytes. The other six 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 ISE6 tick cells: AM842 (*dnaK*), AM944 (*groEL*), AM254 (*tuf*), AM666 (*atpD*), AM956 (*pepA*), AM880 (*alp2*), AM564 (*mdh*),

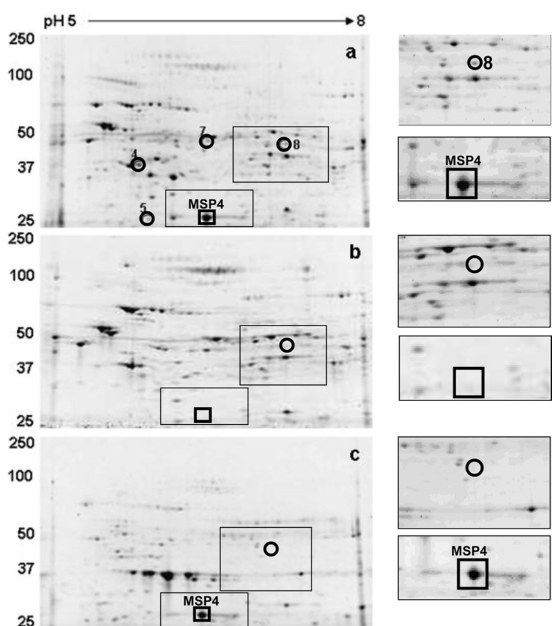

FIG. 2. Identification of *Anaplasma marginale* proteins uniquely expressed or upregulated in tick cell culture. (a) infected ISE6 cells; (b) uninfected ISE6 cells; (c) infected bovine erythrocytes. Gels were stained with SYPRO Ruby to detect total protein. Circles indicate protein spots exclusive to 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 IEF is labeled at the top of the two-dimensional images. The molecular size standards (kDa) are indicated on the left. Images on the right are enlargements of the highlighted *A. marginale* protein spots.

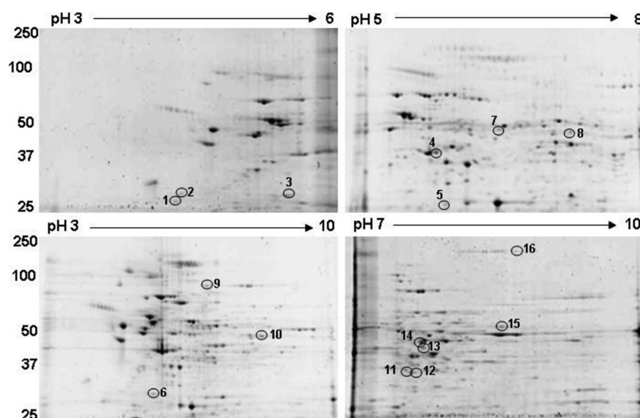

FIG. 3. *Anaplasma marginale* proteins uniquely expressed or upregulated in tick cell culture. Gels were stained with SYPRO Ruby to detect total protein. Circles represent protein spots exclusively present in *A. marginale* isolated from infected ISE6 cells; the numbers refer to the identified protein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

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

Confirmation of unique or upregulated tick stage-specific protein expression by quantitative Western blotting. 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. Am470 was only detected in the tick cell-derived *A. marginale* (Fig. 4). Am410 and Am829 were expressed at higher levels in the tick cell-derived *A. marginale* compared to bacteria isolated from infected erythrocytes (Fig. 4). Densitometric analysis of independent replicates ($n = 3$) revealed a statistically significant upregulation (unpaired Student *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 upregulated tick-stage specific proteins in *Dermacentor andersoni*.** To test whether these *A. marginale* proteins upregulated in the ISE6 cell line were actually expressed in the natural tick vector at the time of transmission, we utilized Western blots using midguts 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 or 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 midguts and salivary glands, containing respective means of $10^{5.8 \pm 0.59}$ and $10^{6.1 \pm 0.49}$ *A. marginale* per organ, revealed the expression of both AM410 and AM470

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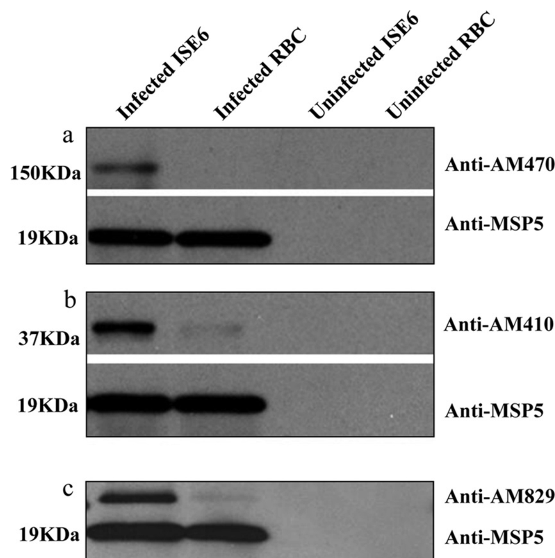

FIG. 4. Upregulated expression of AM470, AM410, and AM829 in *Anaplasma marginale* isolated from infected tick cells. *A. marginale* ($10^{7 \pm 0.05}$ organisms) isolated from infected ISE6 tick cells, *A. marginale* ($10^{7 \pm 0.05}$ organisms) isolated from infected erythrocytes, uninfected ISE6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

using monoclonal antibodies and the expression of 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 after 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 upregulated expression of individual proteins 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, 11, 29, 30). The identification of specifically upregulated *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 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 a high titer and, equally importantly, by allowing incorporation of uninfected cells of the same line as a control (1, 16, 17). 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, in contrast, *A. marginale* infects, sequentially, midgut epithelial and salivary gland acinar cells in adult ticks of several genera but not including *Ixodes* (1, 18). The demonstration that *A. marginale* proteins identified as being upregulated 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 the biological relevance of *in vitro* transcriptome and proteome analysis of other *Anaplasma* and *Ehrlichia* spp. (19, 26, 27).

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: (i) there were no comparative data available on tick-borne bacteria in closely related genera that would guide a more targeted approach, and (ii) 30% of the *A. marginale* genome 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 proteins initially annotated as hypothetical 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).

A. marginale proteins Am410, Am470, and Am829 were each expressed in both the midgut epithelium and salivary gland acinar cells of transmission fed ticks. Although 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 as 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*,

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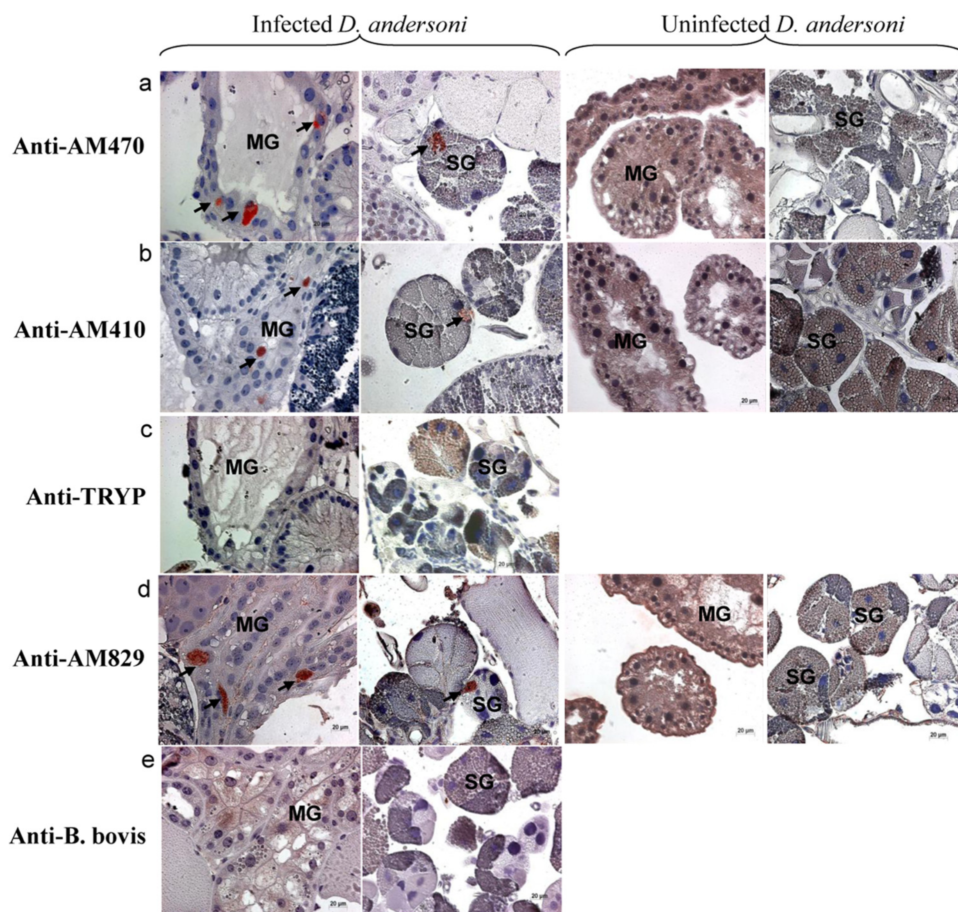

FIG. 5. Expression of AM470, AM410, and AM829 (arrows) in the midgut (MG) and salivary gland (SG) of *Anaplasma marginale*-infected *Dermacentor andersoni*. (a, b, and d) Serial sections of both infected and uninfected ticks probed with monoclonal antibody 143/694.12.11, monoclonal antibody 142/184.8,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

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 upregulated 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 but by no means definitive proof of this theory.

All prior data for *A. marginale* proteins differentially expressed between the mammalian host and tick vector was for downregulated expression (Omp1, Omp4, Omp7 to Omp9, and Omp11; Msp1a) or loss of expression (OpAG3) 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 upregulated 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.

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