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Reduced Infectivity in Cattle for an Outer Membrane Protein Mutant of *Anaplasma marginale*

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Anaplasma marginale **is the causative agent of anaplasmosis in cattle. Transposon mutagenesis of this pathogen using the *Himar1* system resulted in the isolation of an *omp10* operon insertional mutant referred to as the *omp10*::*himar1* mutant. The work presented here evaluated if this mutant had morphological and/or growth rate defects compared to wild-type *A. marginale*. Results showed that the morphology, developmental cycle, and growth in tick and mammalian cell cultures are similar for the mutant and the wild type. Tick transmission experiments established that tick infection levels with the mutant were similar to those with wild-type *A. marginale* and that infected ticks successfully infected cattle. However, this mutant exhibited reduced infectivity and growth in cattle. The possibility of transforming *A. marginale* by transposon mutagenesis coupled with *in vitro* and *in vivo* assessment of altered phenotypes can aid in the identification of genes associated with virulence. The isolation of deliberately attenuated organisms that can be evaluated in their natural biological system is an important advance for the rational design of vaccines against this species.**

Anaplasma marginale is a tick-associated bacterium and the etiologic agent of bovine anaplasmosis, a disease that causes considerable losses to both dairy and beef industries worldwide (1, 2). Although organisms of this species are principally pathogenic to cattle, they are also found in other ruminants, such as water buffalo and deer (3).

The transmission cycle of *A. marginale* has been well documented and indicates that the success of this pathogen depends on its ability to adapt to its invertebrate and vertebrate hosts. In the tick, during its transit from the midgut to the salivary glands, *A. marginale* has to overcome different tissue barriers and defense mechanisms in order to ensure its transmission to the vertebrate host (4–7). In cattle, *A. marginale* replicates within mature erythrocytes, producing an acute disease characterized by hemolytic anemia. However, one of the most important features of the biology of these bacteria is the lifelong persistent infection of its ruminant host, achieved by evasion of the immune system using a mechanism of antigenic variation in which different variants of outer membrane proteins Msp2 and Msp3 are expressed. These persistently infected cattle remain a reservoir of *A. marginale* organisms for continued tick transmission (8–11).

The ability of *A. marginale* to thrive in such diverse environments is mediated by differential gene transcription (12). Hence, the identification and characterization of these genes using recombinant DNA technologies is not only central to understanding the biology and pathogenesis of these organisms but also for the development of drug therapies and vaccines for the control of anaplasmosis. Recently, the use of transposon mutagenesis in the *A. marginale* Virginia strain to create insertional mutations was demonstrated (13). Delivery of a plasmid containing the *Himar1* transposon and the A7 transposase into host cell-free *A. marginale* resulted in the isolation of mCherry fluorescent and spectinomycin- and streptomycin-resistant bacteria. Molecular characterization of these isolated mutant organisms established that the *Himar1* transposon sequences were integrated within the *omp10* gene and that its insertion altered not only the expression of this gene but also the expression of the *omp9*, *omp8*, and *omp7* downstream genes. These recombinant *A. marginale* organisms, referred to as *omp10*::*himar1* mutants, are capable of infecting tick cell cultures, suggesting that these genes are not essential for the survival of *A. marginale* in this environment (13).

The *omp7* to *omp10* genes are members of the *msp2* superfamily and are incorporated into pfam01617, a family of bacterial surface antigens (14, 15). RNA sequencing demonstrated that in *A. marginale*-infected erythrocytes, *omp10* is transcribed as part of an operon with *AM1225* at the 5′ end and with *omp9*, *omp8*, *omp7*, and *omp6* in tandem at the 3′ end (16). Similarly, during infection of tick cells, reverse transcription (RT)-PCR experiments showed that *A. marginale* expresses these genes as a polycistronic message and that these genes are downregulated during tick cell culture relative to transcription levels during blood stage infection (12, 13). Omp6 is a truncated version of Omp10 and is thought not to be expressed as a functional protein (15). Omp7 to Omp9 appear as tandem repeats with 70 to 75% amino acid identity between paralogs, while Omp10 is more distantly related, with ~30% amino acid identity to Omp7 to Omp9. *AM1225* encodes a protein of unknown function (14). *omp7* to *omp10* are each ~1,200 bp, encoding proteins of ~400 amino acids. The protein expression of Omp10 appears to be lower than that of Omp7 to Omp9 (15).

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Omp7 to Omp9 are part of the protective outer membrane protein complexes that are capable of inducing complete protection (17). They have been identified as leading vaccine candidates, as they induce $CD4^+$ T cell responses (17–19). Given the potential role of *omp10*, *omp9*, *omp8*, and *omp7* in the pathogenesis of *A. marginale*, we hypothesized that the reduction of the expression of these outer membrane protein genes caused by insertion of the *Himar1* transposon sequences into *omp10* could result in an altered phenotype. Therefore, we wanted to determine if the *A. marginale omp10*::*himar1* mutant has morphological and/or growth rate defects compared to wild-type *A. marginale*, to determine whether the transposon insert is stable without antibiotic selection pressure, and finally to evaluate if these mutant bacteria could infect and colonize mammalian cells.

MATERIALS AND METHODS

Cell lines and *Anaplasma marginale* cultivation. For this work, two cell lines were used. ISE6 tick cells derived from embryonated eggs of the blacklegged tick, *Ixodes scapularis*, were kept at 34°C in nonvented 25-cm^2 cell culture flasks (Nunc) and maintained in tick L-15B300 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Fisher Scientific) and 5% tryptose phosphate broth (BD Diagnostics) (20). The RF/6A (ATCC CRL-1780) mammalian cells from the retina choroid endothelium of a rhesus monkey (*Macaca mulatta*) were kept at 37°C in vented 25-cm^2 cell culture flasks (Corning) and maintained in RPMI 1640 medium (HyClone) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine (Life Technologies), 0.25% $NaHCO_3$ (Sigma-Aldrich), and 25 mM HEPES (Sigma-Aldrich) (21).

The *A. marginale* Virginia wild-type parental strain and the *omp10*::*himar1* mutant transformed with a transposon bearing the *mcherry* and *aadA* genes, for mCherry fluorescent protein expression and spectinomycin/streptomycin resistance (13), were maintained in tick ISE6 cells at 34°C in tick cell medium supplemented with 0.1% Lipogro (Rocky Mountain Biologicals), 25 mM HEPES (Sigma-Aldrich), and 0.25% $NaHCO_3$ (Sigma-Aldrich) (20). Infected RF/6A endothelial cells with wild-type and *omp10*::*himar1 A. marginale* were maintained as described above.

Immunoblots. Binding pattern and specificity of the rabbit R883 antibody (monospecific antibody to affinity-purified Msp2) (22) for the *A. marginale* wild type and *omp10*::*himar1* mutant were assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting using equal amounts (10^8 cells) of host-free bacteria. Polyvinylidene difluoride (PVDF) membranes (Thermo Scientific) were incubated with polyclonal R883 anti-Msp2 antibody and normal rabbit serum (NRS). This last antibody served as a negative control. Final dilutions of each antibody were 1:10,000. Antibody binding was detected with horseradish peroxidase-conjugated protein G (Life Technologies) diluted to 1:75,000 and SuperSignal West Femto chemiluminescent substrate (Thermo Scientific) as described in the manufacturer's instructions.

Immunoelectron microscopy of the *A. marginale omp10*::*himar1* mutant in tick cell culture. Cultures of ISE6 tick cells 50% infected with wild-type or *omp10*::*himar1* mutant *A. marginale* as estimated by microscopy analysis of slides stained with Diff-Quik were fixed with electron microscopy grade 4% paraformaldehyde and 1% glutaraldehyde in 1× phosphate-buffered saline (PBS), pH 7.2. Fixed cells were processed with the aid of a Pelco BioWave Prolaboratory microwave (Ted Pella, Inc.). Samples were washed in 1× PBS (pH 7.2), washed in water, and dehydrated in a graded ethanol series (25%, 50%, 75%, 95%, 100%, 100%), infiltrated in HM-20 acrylic resin (Electron Microscopy Sciences), and UV cured at −10°C for 48 h. Cured resin blocks were trimmed, ultrathin sectioned, and collected on Ni-coated Formvar 400-mesh grids (Electron Microscopy Sciences). Ultrathin sections were immunolabeled at room temperature as follows: grids were treated with 200 mM NH_4Cl in 1× high-salt Tween (HST; 0.5 M NaCl, 0.02 M, 0.1% Tween 20 [pH 7.2]) for 20 min, rinsed in HST, incubated for 1 h with blocking solution (1.5% bovine serum albumin [BSA], 0.5% cold-water fish skin gelatin, 0.01% Tween 20 in HST [pH 7.2]), and incubated with R883/anti-Msp2 in a 1:10,000 dilution or NRS in a 1:10,000 dilution overnight at 4°C. The following day, the grids were washed in PBS 3 times for 10 min each and incubated for 1 h at 21°C in 18-nm-colloidal-gold affinity-purified goat anti-rabbit IgG (Jackson Immuno Research) diluted 1:30 in PBS solution. Subsequent washes were in PBS and distilled water, and then samples were poststained with 2% aqueous uranyl acetate and Reynolds' lead citrate. Sections were examined with a Hitachi H-7000 transmission electron microscope.

Infection of endothelial RF/6A cells with *omp10*::*himar1* mutant and wild-type *A. marginale*. For infection of RF/6A endothelial cells, the *A. marginale omp10*::*himar1* mutant and the wild type in ISE6 cells were used as the primary inoculum. ISE6 cells heavily infected (>90%) with wild-type or *omp10*::*himar1 A. marginale* were transferred into a sterile centrifuge tube and briefly vortexed for 30 s to release bacteria. One milliliter of inoculum from each strain was added to three wells (triplicate) of a six-well plate (Corning), and cultures were incubated at 37°C in a 5% CO_2 environment. Two days postinfection, the inoculum was removed and the medium was replaced with fresh supplemented RPMI 1640 medium. Medium was replaced twice a week, and infection was monitored daily by phase-contrast and fluorescence microscopy and once a week by Diff-Quik staining.

Infection of cattle and tick transmission. Successful infection of cattle by intravenous inoculation of *A. marginale* has been reported with doses ranging from 10^4 up to 10^8 organisms in *A. marginale* strain St. Maries and the attenuated *A. marginale* subsp. *centrale* (23, 24). A spleen-intact Holstein calf (number 42362) was inoculated intravenously with approximately 10^5 *A. marginale omp10*::*himar1* cells, as determined by qPCR using the single-copy *msp5* gene (25). Cells were scraped from a heavily infected 25-cm^2 cell culture flask and passed 20 times through a 20-gauge needle prior to injection. Calf number 35371 was infected with wild-type *A. marginale* using 51 infected *Dermacentor andersoni* ticks and a 7-day-transmission feed. The calves were monitored for signs of infection by Giemsa-stained blood smears to detect the percent parasitized erythrocytes (PPE), by PCR and Southern analysis to detect Msp5 (25), and by competitive enzyme-linked immunosorbent assays (cELISA) (26). The splenectomized calf (number 43817) was inoculated with 10^{10} mutant organisms and monitored as described above for 75 days. Adult male *D. andersoni* ticks from the Reynolds Creek stock were applied to calf number 43817 on day 51 and fed for 12 days, while the animal was experiencing parasitemias ranging from 0.28 to 2.5 PPE. The ticks were held for 5 days to allow for digestion of the bloodmeal, and then 100 ticks were applied to spleen-intact calf number C1417 and allowed to feed for 7 days. Ticks were dissected for collection of midguts and salivary glands, and the infection levels were quantitated using both *msp5* and *mcherry*, as described previously (25). Calf number C1417 was monitored as described above for 72 days, when the parasitemia had returned to undetectable levels.

Immunofluorescence microscopy. Salivary glands from three adult male *Dermacentor andersoni* ticks acquisition-fed on a calf infected with the mCherry fluorescent protein-transformed *A. marginale* and three control midguts and salivary glands from ticks fed on a calf infected with wild-type *A. marginale* were fixed in freshly prepared 4% paraformaldehyde (Sigma-Aldrich) for 10 min at room temperature. The tissues were incubated in 1% bovine serum albumin and 0.05% Triton X-100 in PBS for 30 min. This was followed by incubation with 2 μg/ml anti-MSP2 primary antibody overnight at 4°C, followed by 10 μg/ml of Alexa Fluor 488 goat anti-rabbit IgG-H&L secondary antibody for 2 h at room temperature. Tissues were washed five times in 1% PBS after each incubation step. Images were acquired using a Nikon Eclipse T*i* inverted microscope system using a 20× objective.

Dual immunofluorescence of RF/6A cells 10 days postinfection with the *omp10*::*himar1* mutant and wild-type *A. marginale* strains was performed using the ANAF16C1 (anti-Msp5; 2 μg/ml) (27) monoclonal an-

TABLE 1 TaqMan qPCR oligonucleotides used in this study

Oligonucleotide	Sequence (5′ to 3′)	Target	Size (bp)	Reference
AB1242	AAAACAGGCTTACCGCTCCAA	*opag2*	151	32
AB1243	GGCGTGTAGCTAGGCTCAAAGT			
AB1250[a]	CTCTCCTCTGCTCAGGGCTCTGCG			
AB1345	GGTGACCGTAAGGCTTGATG	*aadA*	279	This study
AB1346	ACCAAGGCAACGCTATGTTC			
AB1347[b]	ACCATTGTTGTGCACGACGACA			
nMSP5F	AAGTTGTAAGTGAGGGCATAGCCTCC	*msp5*	188	25
nMSP5R	AACTTATCGGCATGGTCGCCTAGT			
RFPF	TACGGTGACGCAGGATTCATCACTG	*mcherry*	227	This study
RFPR	ATGTCGTCTTGACCTCGGCATCAT			

[a] Oligonucleotide labeled with 6-carboxyfluorescein (6-FAM) at the 5′ end and tetramethylrhodamine (TAMRA) at the 3′ end.
[b] Oligonucleotide labeled with tetrachlorofluorescein (TET) at the 3′ end and black hole quencher 1 (BHQI) at the 3′ end.

tibody and the rabbit anti-human von Willebrand factor (Dako; 1:2 dilution) (28, 29) or a mixture of equal parts of both antibodies. Cells were fixed by adding 300 μl of acetone and incubated at room temperature for 10 min. Samples were blocked by adding 100 μl of 5% BSA diluted in PBS and incubated in a humid atmosphere at room temperature for 1 h and subsequently washed once with 0.05% Tween 20 (Sigma-Aldrich) in PBS.

Samples were then reacted with primary and secondary antibodies as described previously (28–30). Negative-control monoclonal Tryp1E1 antibody that exhibits specificity for a variable surface glycoprotein of *Trypanosoma brucei* was used at a concentration of 2 μg/ml (31), and NRS was used at a 1:2 dilution. Samples were then washed with 500 μl of 0.05% Tween 20 (Sigma-Aldrich) in PBS three times for 5 min at room temperature and then reacted with secondary antibodies: Alexa Fluor 488 goat anti-mouse IgG antibody (Life Technologies) to detect a reaction with the primary ANAF16C1 and Alexa Fluor 568 goat anti-rabbit IgG antibody (Life Technologies) to detect a reaction with von Willebrand factor. Both secondary antibodies were used at a 1:400 dilution. Afterward, slides were mounted using ProLong Gold antifade reagent with DAPI (4′,6-diamidino-2-phenylindole) dihydrochloride (Life Technologies).

***Anaplasma marginale* wild-type and *omp10*::*himar1* mutant growth curves in tick cell culture.** The growth kinetics of the *A. marginale* wild type and the *omp10*::*himar1* mutant were determined by TaqMan quantitative real-time PCR (qPCR) of total extracted DNA to determine genome equivalents (GE). Two groups of 12 25-cm^2 cell culture flasks, each with confluent monolayers of uninfected ISE6 cells, were inoculated with 1 ml of ISE6 cells that were >90% infected with the *A. marginale* wild type or the *omp10*::*himar1* mutant. Twenty-four hours after inoculation, the inoculum was removed and replaced with 5 ml of fresh supplemented tick cell medium, and this time was considered 0 h postinfection (p.i.). Duplicate cultures of infected cells with each *A. marginale* strain were harvested at different time points (0, 3, 5, 9, 11, and 13 days) p.i. Infected cells were scraped from the growth surface with a cell scraper into the supernatant and centrifuged at 100 × *g* for 7 min at room temperature and washed twice in 1× PBS. The final pellet was used for DNA extraction (final volume of 50 μl DNA per flask), qPCR assay of 200 ng extracted DNA, and determination of *A. marginale* GE per flask. Statistical differences in growth rates were evaluated using Student's *t* test with SigmaPlot (Systat Software).

DNA extraction and qPCR. DNA isolation from tick ISE6 cells infected with wild-type and *omp10*::*himar1* mutant *A. marginale* strains was performed using the QIAamp DNA minikit (Qiagen) per the manufacturer's instruction. DNA concentration of each sample was determined using the Qubit double-stranded DNA (dsDNA) HS assay kit (Life Technologies) on a Qubit fluorometer (Life Technologies). DNA isolation from bovine blood or tick midguts and salivary glands employed the DNeasy blood and tissue kit (Qiagen).

Quantitation of the *A. marginale* wild type and the *omp10*::*himar1* mutant in ISE6 tick cell cultures was performed by qPCR using two sets of primers and probes: the forward and reverse primers AB1242 and AB1243 and the probe AB1250, which target the single-copy gene *opag2* (32), and the forward and reverse primers AB1345 and AB1346 and the probe AB1347, which target the *aadA* or spectinomycin resistance gene found in the *omp10*::*himar1* mutant (Table 1).

Tenfold serial dilutions of the *opag2*/pCR-TOPO or the pHimarcis-A7mCherry-SS plasmids were used for standard curve preparation, and the *opag2* and *aadA* gene copy numbers were calculated based on the standard curve.

Triplicate reactions from cultures were analyzed. Samples were normalized by loading the same amount of total DNA (200 ng). Reaction mixtures of 25 μl containing 5 μl of DNA from wild-type or *omp10*::*himar1* mutant *A. marginale*, 1× QuantiTect probe PCR mix (Qiagen), 0.4 μM forward and reverse primers, and 0.2 μM probe were used for amplification in a Bio-Rad DNA Engine Opticon thermal cycler with the following conditions: 95°C for 15 min and 40 cycles of 94°C for 1 min, 54°C for 15 s, and 60°C for 1 min.

Quantitation of the *A. marginale* wild type and the *omp10*::*himar1* mutant to determine the dose of the *A. marginale omp10*::*himar1* mutant and wild-type inoculum from infected ISE6 tick cells, in blood and tick tissues, was performed by qPCR using the forward and reverse primers nMSP5F, nMSP5R, RFPF, and RFPR, targeting the *msp5* and *mcherry* genes (Table 1). Triplicate reaction mixtures from each sample were used. Reaction mixtures of 25 μl containing 1 μl of DNA from blood or tick tissue preparations, 1× Master mix for Express SYBR GreenER (Life Technologies), and 200 nM forward and reverse primers were used for amplification in a Bio-Rad C1000 Touch thermal cycler with the following conditions: 95°C for 5 min and 35 cycles of 95°C for 30s, 60°C for 30s, and 72°C for 1 min.

Tenfold serial dilutions of cloned control templates were used for standard curve preparation, and the *A. marginale* copy number was calculated based on the standard curve. The no-template control, uninfected cells, and DNA from *Anaplasma phagocytophilum* were used as negative controls.

Transposon insertion stability of the *A. marginale omp10*::*himar1* mutant in tick cell culture. The stability of the transposon insertion without antibiotic (spectinomycin/streptomycin)-selective pressure in *A. marginale omp10*::*himar1* mutants was compared by measuring the copy number of *aadA* (spectinomycin/streptomycin resistance gene-containing insert) and *opag2* using qPCR during 7 serial passages.

RESULTS

Electron microscopy of the *Anaplasma marginale omp10*::*himar1* mutant in tick cell culture. The polyclonal anti-Msp2

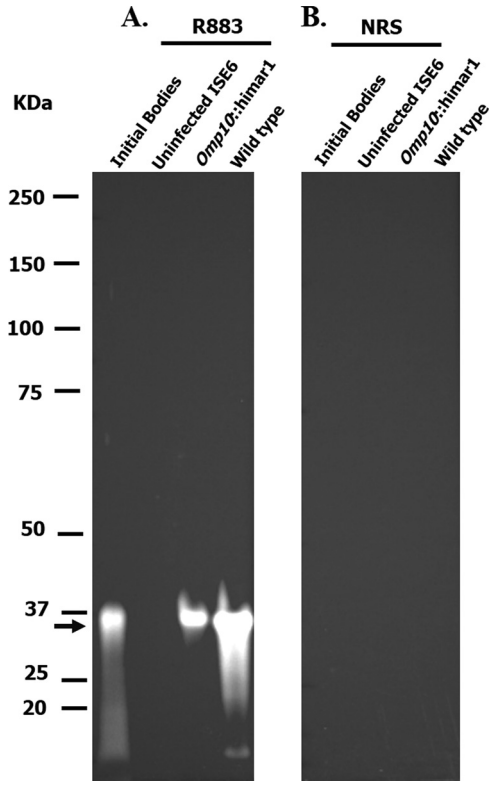

FIG 1 Immunoblotting of host cell-free *omp10*::*himar1* mutant and wild-type *A. marginale* using the specific antibody R883. Proteins from equal amounts of host cell-free wild-type and *omp10*::*himar1 A. marginale* from infected ISE6 tick cells were separated by SDS-PAGE. Immunoblotted PVDF membranes of transferred proteins reacted with specific antibodies, and reactions were visualized by chemiluminescence. (A) Polyclonal antibody R883 (1:10,000) with specificity to the Msp2 protein (36 kDa) (black arrow); (B) negative control, normal rabbit serum (1:10,000). *A. marginale* strain Virginia initial bodies and uninfected ISE6 cells were used as positive and negative controls, respectively.

R883 antibody binding pattern and specificity was evaluated by immunoblotting. *A. marginale omp10*::*himar1* mutant and wild-type copy numbers per sample were quantified by qPCR using the *opag2* single-copy gene. For this, 10^8 organisms of the *A. marginale* wild type and the *omp10*::*himar1* mutant were loaded per lane. *A. marginale* strain Virginia initial bodies and uninfected ISE6 cells were used as positive and negative controls, respectively. R883 specifically reacted with the major surface protein Msp2 (36 kDa) in *A. marginale omp10*::*himar1* mutant, wild-type, and initial bodies (Fig. 1A). A similar reaction was not detected in the uninfected ISE6 cells or by using negative-control NRS (Fig. 1B).

Transmission electron microscopy (TEM) of *omp10*::*himar1* mutant *A. marginale* immunogold labeled with polyclonal anti-Msp2 (R883) antibody was used for the localization, visualization, and analysis of these organisms to determine if their morphology was characteristic of what has been described for wild-type *A. marginale* (33, 34). Based on this analysis, morphological defects in the *omp10*::*himar1* mutant that could alter its development in infected ISE6 cells were not found. Instead, several of the characteristics common to the prototypical wild-type *A. marginale* were visualized; for example, the *A. marginale omp10*::*himar1* mutant grew within membrane-bound inclusions or intravacuolar microcolonies (morulae) (20, 33, 35) (Fig. 2A). Also, the ISE6 cells were infected with several morulae, an indication of multiple *omp10*::*himar1* mutant *A. marginale* invasion events (Fig. 2B and C) typical of *A. marginale* infection (33, 35). The two distinct morphological forms of *A. marginale* were also visualized in ISE6 cells infected with the *omp10*::*himar1* mutant: enlarged oval and electron-translucent or reticulate forms (RF) (Fig. 2D) and round or coccoid electron-dense or dense-core (DC) forms (Fig. 2E). Several RF of *omp10*::*himar1* mutant *A. marginale* were undergoing cell division by binary fission consistent with previous TEM descriptions of *A. marginale* in ISE6 cells (Fig. 2D). RF and DC forms of *omp10*::*himar1* mutant *A. marginale* have an outer and inner membrane separated by a periplasmic space (Fig. 2E). These observations suggest that the morphology and development of the *A. marginale omp10*::*himar1* mutant in tick cell culture are similar to what has been observed for the wild type (20, 35).

Growth curves of *omp10*::*himar1* mutant *Anaplasma marginale* versus the wild type in tick cell culture. The *A. marginale omp10*::*himar1* mutant was further characterized by comparing the *in vitro* growth rate to that of wild-type *A. marginale*. Duplicate cultures of ISE6 cells infected with the *A. marginale omp10*::*himar1* mutant and the wild type maintained without antibiotic selection were evaluated to determine the increase of GE by qPCR over a period of 13 days. At day 0 p.i., the mean copy numbers of GE for *omp10*::*himar1* mutant and wild-type *A. marginale* were 8.87×10^6 copies/flask (95% confidence interval [CI], 6.83×10^6 to 1.09×10^7) and 1.22×10^7 copies/flask (95% CI, 8.64×10^6 to 1.58×10^7), respectively. At day 13 p.i., the *omp10*::*himar1* and wild-type GE copy numbers were 4.70×10^8 copies/flask (95% CI, 4.551×10^8 to 4.85×10^8) and 5.01×10^8 copies/flask (95% CI, 4.26×10^8 to 5.76×10^8), respectively. The *A. marginale omp10*::*himar1* mutant and the wild type had log increases of 1.72 and 1.67 GE, respectively (Fig. 3). The growth rates of the *omp10*::*himar1* mutant and wild-type *A. marginale* in ISE6 culture were not significantly different ($P = 0.527$), and the doubling times were 54.7 h (2.3 days) for *omp10*::*himar1* and 58 h (2.4 days) for wild-type *A. marginale*. Pearson correlation coefficients for wild-type and *omp10*::*himar1* mutant *A. marginale* strains were 0.949 and 0.977, respectively.

Transposon insertion stability of the *A. marginale omp10*::*himar1* mutant in tick cell culture. Stable maintenance of the transposon insertion was evaluated by qPCR to determine the copy number of the transposon insert per *omp10*::*himar1* mutant *A. marginale* genome with primers and probes specific to the transposon-borne spectinomycin gene (*aadA*) and the single-copy *A. marginale* gene *opag2*. For this purpose, DNA samples obtained from duplicate cultures of ISE6 cells infected with the *A. marginale omp10*::*himar1* mutant maintained during seven serial passages (~3 months) with or without antibiotic selection were analyzed. Table 2 shows a comparison of the copy numbers obtained for *aadA* and *opag2* in ISE6 cells infected with the mutant with and without antibiotic selection. We used *A. marginale omp10*::*himar1* mutants growing with antibiotic selection as a positive control for the presence of the transposon insertion. Our results show that in this group, the *aadA* copy number relative to the *opag2* copy number was maintained (Table 2). During culture of these mutants without antibiotic selection pressure, loss of the transposon insert would be indicated by a decrease in *aadA* copy number with a concomitant comparative increase in *opag2* copy number. However, during the seven serial passages, a steady increase of the *opag2* copy number relative to the *aadA* gene copy

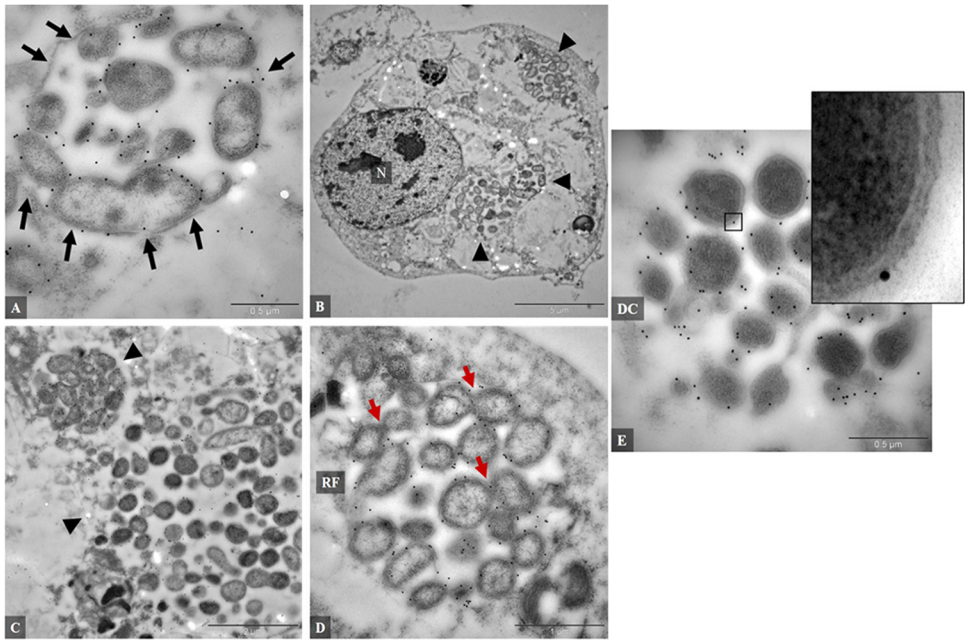

FIG 2 Transmission electron microscopy of the *A. marginale omp10*::*himar1* mutant, immunogold labeled with a polyclonal anti-Msp2 antibody (R883). (A) *Anaplasma* organisms within membrane-bound vacuole (arrows); (B and C) cell infected with multiples colonies (arrowheads); (D) colony formed by RF, reticulated forms with some organisms dividing by binary fission (red arrows); (E) colony formed by DC, dense-core organisms; inset showing double-layered membrane. N, nucleus. *A. marginale* was grown in ISE6 tick cells.

number was not observed in the *A. marginale omp10*::*himar1* mutant growing without antibiotic selection, indicating that the transposon insertion in the *A. marginale omp10*::*himar1* mutant appears to be stable without antibiotic selection for prolonged culture periods.

Infectivity of *omp10*::*himar1* mutant *A. marginale* for mammalian cells *in vitro* and *in vivo*. *A. marginale* can be propagated *in vitro* in RF/6A endothelial cells derived from the retina choroid of a rhesus monkey (21, 29). To investigate if the *A. marginale omp10*::*himar1* mutant can infect RF/6A endothelial cells, triplicate cultures of uninfected RF/6A endothelial cells were infected with *A. marginale omp10*::*himar1* mutant cells from a heavily infected ISE6 culture (>90% infected) and maintained with or without antibiotic selection. Similarly, and as a control for infection of endothelial cells, triplicate cultures of uninfected RF/6A cells were infected with wild-type *A. marginale*.

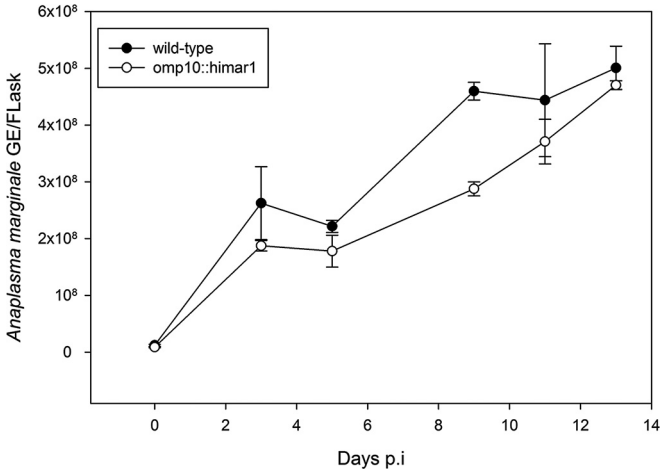

FIG 3 Growth curves for wild-type and *omp10*::*himar1* mutant *A. marginale* strains in infected ISE6 tick cells. Growth was measured by determining GE/flask based on the *A. marginale* single-copy gene *opag2*.

Infection of RF/6A cells with the *omp10*::*himar1* mutant was monitored by an increase of red fluorescence emitted by the replicating, mCherry fluorescent protein-expressing mutant. By 10 days p.i., about 50% to 60% of the cells were infected in cultures maintained with or without antibiotic selection (Fig. 4A to E). The percentage of infection was confirmed by Diff-Quik staining and was similar in RF/6A cells infected with wild-type *A. marginale* (data not shown). The cytoplasm of infected cells contained a single large or several small morulae that fluoresced red (Fig. 4A).

Figures 4B and C show the immunofluorescence results of the specific binding of the antibody ANAF16C1 (anti-Msp5) to *omp10*::*himar1* mutant *A. marginale* (green) and antibody against human von Willebrand factor to RF/6A cells (red). Juxtaposition of the green and red fluorescence signals showed colocalization of *omp10*::*himar* mutant *A. marginale* (green) within endothelial cells (red), confirming that the entire monolayer was formed by endothelial cells. Fluorescence similar to that emitted using these two specific antibodies was not detected in RF/6A endothelial cells infected with *omp10*::*himar1* mutant *A. marginale* labeled with the negative-control monoclonal antibody TrypE1 or NRS (Fig. 4D and E). Similar results were obtained from RF/6A cells infected with wild-type *A. marginale* (Fig. 4F to I).

To determine if the *A. marginale omp10*::*himar1* mutant infects cattle, we attempted infection of 2 spleen-intact calves and 1 splenectomized calf. Inoculation of a spleen-intact calf with 10^5 mutant organisms failed to establish infection, as shown by negative Giemsa-stained blood smears, PCR, Southern blotting, and com-

TABLE 2 *A. marginale omp10*::*himar1* mutant transposon stability

Passage no.	GE[a] (log_{10} ± SD)[a]			
	Treated culture		Untreated culture	
	aadA	*opag2*	*aadA*	*opag2*
1	8.33 ± 0.07	8.40 ± 0.02	8.26 ± 0.002	8.45 ± 0.05
2	10.15 ± 0.03	10.43 ± 0.05	10.26 ± 0.05	10.60 ± 0.02
3	8.09 ± 0.10	8.25 ± 0.02	9.46 ± 0.003	9.91 ± 0.07
4	9.23 ± 0.03	9.16 ± 0.35	9.15 ± 0.05	9.33 ± 0.02
5	9.34 ± 0.07	9.56 ± 0.05	9.42 ± 0.03	9.66 ± 0.01
6	9.53 ± 0.02	9.77 ± 0.04	9.42 ± 0.03	9.72 ± 0.03
7	9.54 ± 0.02	9.67 ± 0.01	9.64 ± 0.02	9.81 ± 0.04

[a] GE, genome equivalents per flask. Treated cultures were maintained with antibiotic selection.

petitive enzyme-linked immunosorbent (cELISA) assays. Because of this, a second attempt was made to infect the same calf at day 71 after the initial inoculation, delivering approximately 10^8 *omp10*::*himar1* mutant *A. marginale* organisms. This animal was monitored for a further 46 days with no signs of infection, suggesting that this mutant had a reduced infectivity in cattle compared to that of the wild type (data not shown). Splenectomized calves are known to be less resistant to *A. marginale* infection than spleen-intact calves and adults (9, 33). Typically, spleen-intact calves control the first (acute) bacteremia and then become persistently infected, with additional cyclic peaks of bacteremia (9). In contrast, splenectomized calves generally fail to control the first peak of bacteremia, which can increase to >50% PPE, and then they succumb to infection. Administration of 10^{10} organisms to a splenectomized calf resulted in a patent erythrocytic bacteremia in 34 days and a second peak on day 58 (Fig. 5). Feeding of *D. andersoni* ticks on this infected calf resulted in 100% acquisition of *omp10*::*himar1* mutant organisms in both the midgut and salivary glands, showing that although infectivity of the mutant was reduced, the mutant was acquired by ticks at rates and levels similar to those of the wild type. *Anaplasma marginale* burdens in the tick were determined by qPCR for *msp5* (on the genome backbone) and *mcherry* (on the transposed insert) and ranged for the mutant from 10^3 to 10^5 (Table 3). This is similar to tick burdens determined previously for wild-type *A. marginale* (25). The positive ticks were fed on a naive, spleen-intact calf which had a peak parasitemia of 0.5 PPE at day 62 (Fig. 5). Ticks were collected from the transmission calf, dissected to collect salivary glands, and subjected to immunofluorescence microscopy (Fig. 6). The sections were stained with antibody against Msp2. The yellow staining in Fig. 6C indicates colocalization of Msp2 and the *omp10*::*himar1* mutant.

DISCUSSION

The ability of *A. marginale* to cause disease often depends on its ability to invade and replicate within different hosts. During this work, we investigated the phenotype of *omp10*::*himar1* mutant *A. marginale* that carries the *Himar1* transposon integrated within the *omp10* gene.

The typical developmental cycle of *A. marginale* in tick cells involves two stages: a reticulate or vegetative form (RF) and the infective dense-core (DC) form. Upon internalization, *A. marginale* is enclosed within a parasitophorous vacuole, where it transitions into a reticulate form that divides by binary fission to produce multiple organisms that subsequently will change into the infective dense forms that will be released to infect adjacent cells (33–35). Electron microscopy analysis shows that *A. marginale omp10*::*himar1* mutants develop in a fashion similar to the wild type in tick cell culture (20, 34–36) and suggests that the gross structure of the outer membrane of these organisms was not affected by disruption in the expression of *omp10*, *omp9*, *omp8*, and *omp7*. Growth curves support these findings since they show that *A. marginale omp10*::*himar1* mutant growth is comparable to that of the wild type during infection of tick cell cultures. Stability of the *Himar1* insertion under non-antibiotic-selectable conditions has been demonstrated in several pathogenic bacterial species (37, 38), and this is also true for the *A. marginale omp10*::*himar1* mu-

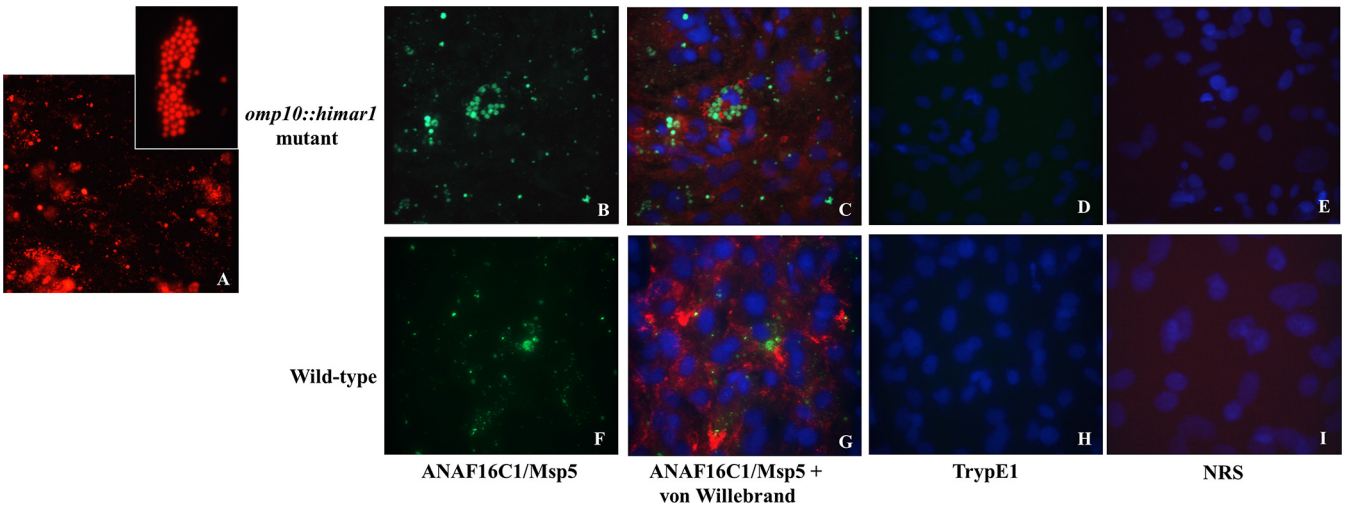

FIG 4 Infection of RF/6A endothelial cells at 10 days postinfection with the *omp10*::*himar1* mutant and wild-type *Anaplasma marginale* strains. (A) RF/6A endothelial cells infected with the *A. marginale omp10*::*himar1* mutant expressing mCherry red fluorescent protein, with the inset showing a single endothelial cell infected with multiple colonies. The *A. marginale* (green) *omp10*::*himar1* mutant (B and C) and the wild type (F and G) in endothelial cells (red). In panels C and G, host cell nuclei were counterstained with DAPI (blue). Similar fluorescence was not detected in infected cells stained with control antibodies Tryp1E1 or NRS for cells infected with the *omp10*::*himar1* mutant (D and E) or in cells infected with the wild type (H and I).

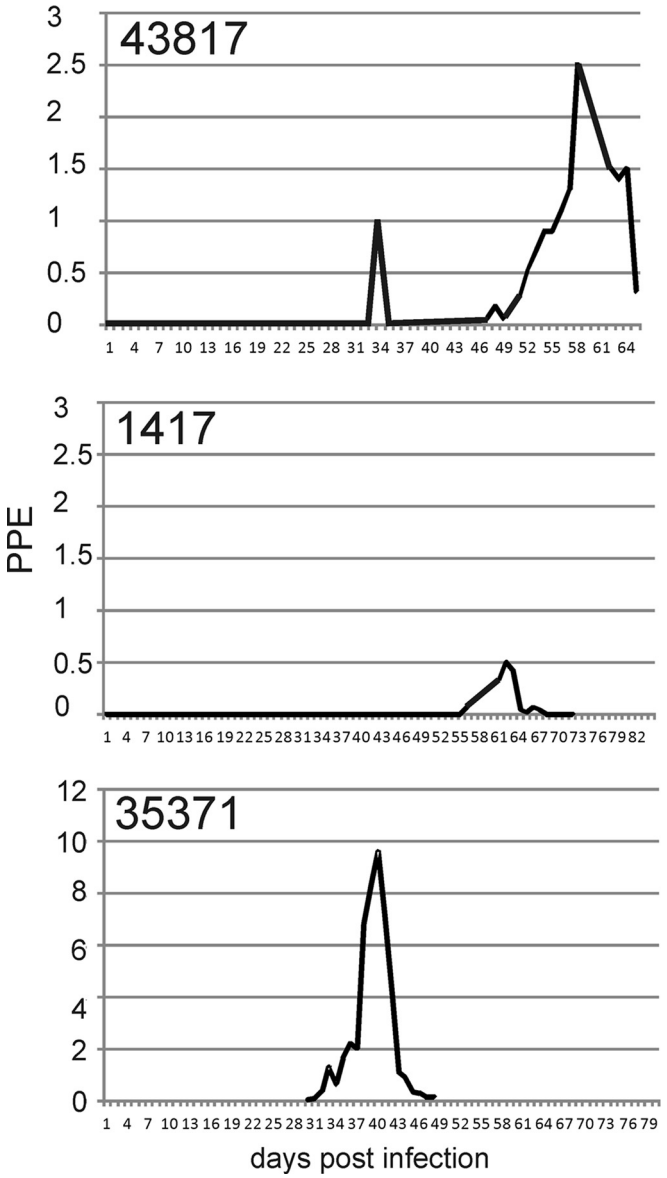

FIG 5 Infection profile of the *omp10*::*himar1* mutant strain compared to wild-type *A. marginale* in cattle. Bacteremia (left axis) is reported as percent parasitized erythrocytes (PPE) as determined by microscopic evaluation of Giemsa-stained blood smears. Animal number is indicated in the top left of each graph. Animal number 43817 was a splenectomized animal infected with the *omp10*::*himar1* mutant strain by injection of tissue culture material; animal number 1417 was a spleen-intact animal infected with the *omp10*::*himar1* mutant strain by tick inoculation, while spleen-intact animal number 35371 was infected with wild-type *A. marginale* by tick inoculation.

tant. Stability of transposon insertion in these mutants was monitored during seven serial passages over 3 months in cultures of infected ISE6 cells maintained without antibiotic selection. Quantitative PCR shows similar levels of the spectinomycin resistance gene (from the transposon) and the *opag2* gene (from the genome backbone). Maintenance of the red fluorescent phenotype for the length of the culture period confirms these results, since the *mcherry* reporter gene is also found in the transposon. Hence, loss of the red fluorescence phenotype due to the lack of antibiotic selection pressure will correlate with the loss of the transposon insertion.

Previous work has indicated a possible important role of *omp10*, *omp9*, *omp8*, and *omp7* in the pathogenesis of *A. marginale*. For example, these genes are expressed at lower levels in tick cells than in erythrocytes, and outer membrane proteome analysis and cross-linking experiments showed that although expressed, Omp9, Omp8, and Omp7 proteins are not detected at the surface of *A. marginale*-infecting tick cell culture (15, 17). On the other hand, in infected erythrocytes, these proteins are localized at the surface of *A. marginale*, suggesting a possible rearrangement in surface topology during the transition from tick to mammalian cell infection (17). Therefore, with the putative inability to upregulate the expression of these proteins on the surface of the pathogen, we hypothesized that the *A. marginale omp10*::*himar1* mutant would not be infective to mammalian cells. To test this, infectivity of the *A. marginale omp10*::*himar1* mutant in RF/6A endothelial cell cultures and in cattle was assessed. Surprisingly, the *A. marginale omp10*::*himar1* mutant invaded and replicated within RF/6A endothelial cells similar to the wild type, demonstrating that disruption of *omp10* and downstream genes did not affect infectivity of these mutants in endothelial cell cultures *in vitro*. Although it took three attempts, we were able to establish the *A. marginale omp10*::*himar1* mutant in cattle. A splenectomized calf had a detectable PPE of 1% 34 days postinoculation. However, the infection was controlled, and a subsequent peak parasitemia of 2.5% occurred at day 58 and was again controlled. Typically, wild-type *A. marginale* infections in splenectomized animals will result in consistently increasing parasitemias to high levels until the animal dies or is euthanized. Ticks were able to acquire and transmit the *omp10*::*himar1* mutant to a spleen-intact calf. *A. marginale* burden within the ticks ranged from 10^3 to 10^5 cells in the midgut and the salivary gland, which is typical for transmission-fed ticks (25).

A. marginale has been transformed only twice. The first transformant was by homologous recombination with a green fluorescent protein (GFP) marker into the St. Maries strain (32), while the second transformant is the one used in the current study (13). Interestingly, both transformed strains behave similarly when used to infect bovine hosts, producing very low PPE levels compared to those of wild-type infections (Fig. 5) (39, 40). Transcriptional profiling of the GFP mutant demonstrated alterations in

TABLE 3 Quantitation of the *omp10*::*himar1* mutant in tick tissues

	GE[a] ($\log_{10} \pm$ SD)			
	SG		MG	
Tick no.	*msp5*	*mcherry*	*msp5*	*mcherry*
1	5.12 ± 0.02	4.88 ± 0.02	5.38 ± 0.04	4.83 ± 0.02
2	5.42 ± 0.03	4.49 ± 0.01	4.33 ± 0.03	4.93 ± 0.02
3	4.36 ± 0.03	5.69 ± 0.01	4.07 ± 0.05	4.87 ± 0.01
4	4.95 ± 0.02	4.82 ± 0.03	4.74 ± 0.04	4.91 ± 0.03
5	4.09 ± 0.03	5.78 ± 0.02	4.68 ± 0.01	4.56 ± 0.03
6	4.97 ± 0.05	6.18 ± 0.01	4.51 ± 0.05	4.94 ± 0.01
7	4.92 ± 0.03	5.15 ± 0.02	5.55 ± 0.01	4.83 ± 0.03
8	5.30 ± 0.04	6.07 ± 0.02	5.66 ± 0.04	4.88 ± 0.02
9	5.02 ± 0.04	4.98 ± 0.03	4.94 ± 0.05	4.84 ± 0.02
10	5.19 ± 0.02	5.21 ± 0.05	5.40 ± 0.01	4.96 ± 0.01

[a] GE, genome equivalents per salivary gland pair (SG) or per tick midgut (MG).

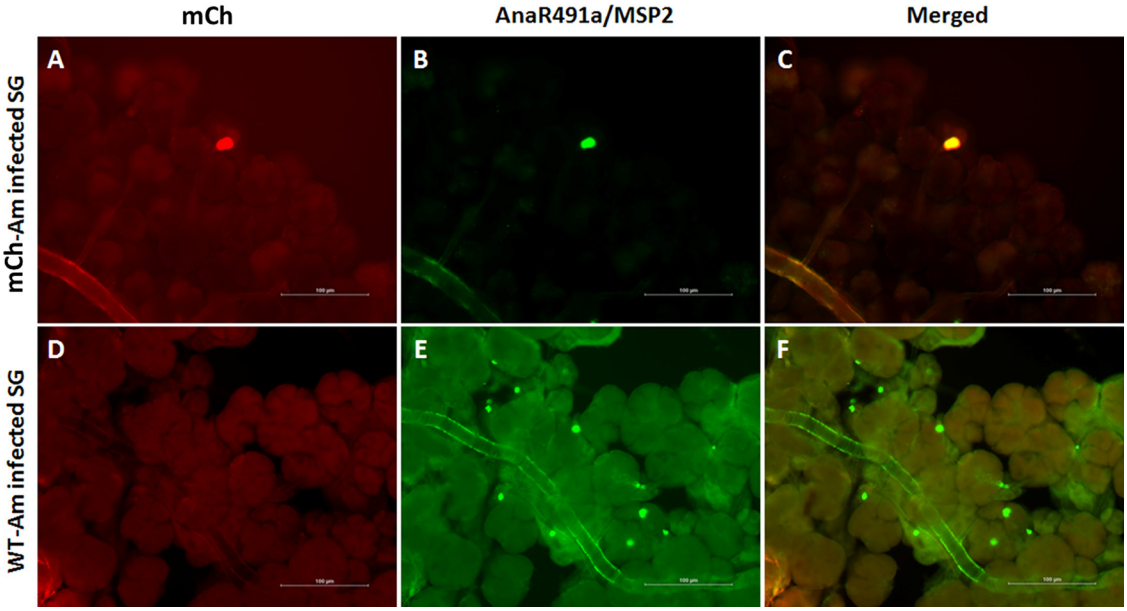

FIG 6 The *A. marginale omp10*::*himar1* mutant infects ticks. Immunofluorescence images showing infection of *D. andersoni* salivary gland (SG) acinar cells with the *omp10*::*himar1* mutant (mCh-Am) (top) and wild-type *A. marginale* (WT-Am) control (bottom). (A to C) Salivary glands from tick acquisition fed on a calf infected with mCh-Am (red) were colabeled with AnaR4901a primary antibodies against *Anaplasma marginale* MSP2 and a conjugated Alexa Fluor 488 goat anti-rabbit IgG-H&L secondary antibody (green). (D to F) Salivary glands from control ticks infected with wild-type *A. marginale* showing multiple colonies of mCherry-negative, MSP2-positive *A. marginale*.

gene transcriptional patterns for nucleotide biosynthesis, translation, and translation elongation (41). The *omp10*::*himar1* mutant was found to have altered transcription of the *omp10* operon (13). These two mutations have very different effects on the transcriptional patterns on the cell, and yet both have reduced vigor during mammalian infection. These transformed organisms are candidates for development as vaccine strains (40).

In summary, the work presented here shows that disruption of the *omp10* operon does not result in altered morphology or altered *in vitro* growth of *A. marginale*. Neither is expression of the *omp10* operon essential for the growth of *A. marginale* in ticks or cattle. However, this *A. marginale omp10*::*himar1* mutant showed reduced infectivity and growth in its bovine host. In the past, naturally attenuated strains have been used in vaccines despite their empirical derivation and drawbacks associated with live blood vaccines. These data suggest that a rational strategy employing transposon mutagenesis to attenuate *A. marginale* for growth in cattle is feasible. If the efficiency of transposon mutagenesis can be increased, this would provide a means to define virulence mechanisms and identify protective antigens in *A. marginale* and other *Anaplasmataceae*.

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