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Knockout of an outer membrane protein operon of *Anaplasma marginale* by transposon mutagenesis

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Abstract

Background: The large amounts of data generated by genomics, transcriptomics and proteomics have increased our understanding of the biology of *Anaplasma marginale*. However, these data have also led to new assumptions that require testing, ideally through classical genetic mutation. One example is the definition of genes associated with virulence. Here we describe the molecular characterization of a red fluorescent and spectinomycin and streptomycin resistant *A. marginale* mutant generated by *Himar1* transposon mutagenesis.

Results: High throughput genome sequencing to determine the *Himar1*-*A. marginale* genome junctions established that the transposon sequences were integrated within the coding region of the *omp10* gene. This gene is arranged within an operon with *AM1225* at the 5' end and with *omp9*, *omp8*, *omp7* and *omp6* arranged in tandem at the 3' end. RNA analysis to determine the effects of the transposon insertion on the expression of *omp10* and downstream genes revealed that the *Himar1* insertion not only reduced the expression of *omp10* but also that of downstream genes. Transcript expression from *omp9*, and *omp8* dropped by more than 90% in comparison with their counterparts in wild-type *A. marginale*. Immunoblot analysis showed a reduction in the production of Omp9 protein in these mutants compared to wild-type *A. marginale*.

Conclusions: These results demonstrate that transposon mutagenesis in *A. marginale* is possible and that this technology can be used for the creation of insertional gene knockouts that can be evaluated in natural host-vector systems.

Background

Anaplasma marginale is a tick-borne and obligate intracellular bacterium that causes bovine anaplasmosis, a disease that has gained particular attention due to the considerable economic losses for the cattle industry [1-4]. Onset of clinical disease is mainly characterized by a severe hemolytic anemia [1,2]. Cattle that survive acute infection become carriers of *A. marginale* and organisms can be transmitted to susceptible cattle mechanically or by tick bite [2]. *A. marginale* persists in carrier cattle because of its capability to subvert the immune system using antigenic variation in which different variants of

outer membrane proteins such as Msp2 and Msp3 are expressed [5-8].

Work on the development of a preventive vaccine against this disease began in the early 1900's with the isolation of *A. marginale* subsp. *centrale* [9,10]. This less virulent strain, originally from South Africa, is used for immunization of cattle in Africa, Australia, South America and the Middle East and remains the most widely-used and practical vaccine against bovine anaplasmosis [9-11]. This vaccine is not approved in the United States because of the risk of transmitting contaminant blood-borne pathogens that will infect cattle [1]. Recently, comparative genomic studies demonstrated that proteins that are conserved in US strains were not conserved in *A. marginale* subsp. *centrale* [10-12].

Different vaccination methods have been developed for the control of bovine anaplasmosis that range from

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attenuated live or killed organisms, to DNA and recombinant protein vaccines [9]. But *A. marginale* derived from cell culture, killed organisms and DNA vaccines induce only partial protection [13-15]. Immunization trials using outer membrane proteins or a complex of linked or unlinked outer membrane proteins of *A. marginale* derived from erythrocytes have demonstrated good protection against high bacteremia, anemia and homologous strain challenge [16-20]. However, to promote long lasting protection, several immunization boosts may be required and in addition to this, production and purification of these components is time-consuming and expensive.

The increased use of molecular approaches such as whole genome, RNA sequencing, proteomics and comparative genomics of *A. marginale* has identified potential virulence-associated targets that can be altered or removed by reverse genetics techniques [12,21-25]. This could allow the creation of attenuated organisms that have reduced pathogenicity but still elicit cellular and antibody responses that stimulate immunity without causing disease. Consequently the development of genetic tools to transform *A. marginale* and generate *in-vitro* gene knockouts, or insertional mutants that can be tested for attenuation in their *in-vivo* environment is of great significance.

One way to create insertional mutations in pathogenic bacteria is via transposon mutagenesis, in which a library of recombinant bacteria containing different transposon insertions can be created, allowing for the screening of mutant strains with diverse phenotypes [26,27]. The *Himar1* transposon is a non-replicative class II DNA transposon that is a member of the Tc1/mariner family and is often used for the creation of insertional mutants. Since these types of transposons are horizontally transferred between species, they do not have host restricted functions, making them suitable for use in a wide-range of eukaryotic and prokaryotic hosts [27,28]. In addition to this, the *Himar1* transposon does not have DNA target specificity since it is integrated randomly in TA dinucleotide sites [28-30]. Because of these advantages, transposon mutagenesis using this system has been successfully developed in other tick-borne pathogens such as *Rickettsia rickettsii*, *Coxiella burnetii*, *Borrelia burgdorferi*, *Francisella tularensis*, *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* [31-40]. These previous results suggest that this system could be useful for the transformation of *A. marginale*.

Nevertheless, previous attempts to transform *A. marginale* by transposon mutagenesis were not successful. Previously, the *Himar1* transposon and transposase were delivered in two separate vectors into *A. marginale* which resulted in the isolation of green fluorescent and antibiotic resistant bacteria. However molecular characterization of these recombinant organisms established that the entire

plasmid carrying the transposon sequences was integrated into the *A. marginale* chromosome by a single crossover homologous recombination mechanism instead of the classical cut and paste mechanism of transposition [41]. Therefore, we wanted to evaluate first, if classical transposon mutagenesis using the *Himar1* transposon system is achievable in *A. marginale*, and second, if transposon mutagenesis using this system, is useful for the creation of insertional knockout mutations.

Results

Transformation of *Anaplasma marginale* by transposon mutagenesis

Attempts to transform *A. marginale* by transposon mutagenesis using the *Himar1* transposon/transposase system delivered in two separate plasmids were not successful. The probability that two plasmids are introduced at once into *A. marginale* organisms could be very low, especially when viability in the extracellular environment might be highly compromised, resulting in a low fraction of cells competent to take up DNA.

Therefore in order to promote transposon mutagenesis in these bacteria, the transposase was provided in *cis* with the *Himar1* transposon sequences (R. F. Felsheim unpublished data). The *pHimar1cisA7mCherry-SS* contains the hyperreactive allele *A7* transposase and the *Himar1* TIR flanking the *mCherry* reporter gene and the *aadA* gene, which confers resistance against spectinomycin and streptomycin. Expression of the transposase and the reporter and antibiotic selection genes is driven by the *A. marginale* *tr* promoter [41,42] (Figure 1A). Antibiotic selection pressure of electroporated bacteria with this construct resulted in the isolation of red fluorescent and antibiotic resistant bacteria (Figure 1B).

Mapping of transposon insertion within the *A. marginale* chromosome

We used Roche/454 and Illumina high-throughput genome sequencing to determine: 1) the location of plasmid sequences within the *A. marginale* chromosome, 2) the recombination mechanism that allowed the segregation of mutant bacteria and 3) if these recombinant organisms correspond to a population containing insertions in different genomic locations or in a single genome site.

Mutations produced by the integration of the *Himar1* transposon into the *A. marginale* chromosome will generate new junction sequences that are absent in the wild-type. These new sequences should include the *Himar1* terminal inverted repeats (TIR) followed by the sequence of the regions in which the transposon is integrated. Based on this, the strategy that we used to map the *Himar1* insertion site involved

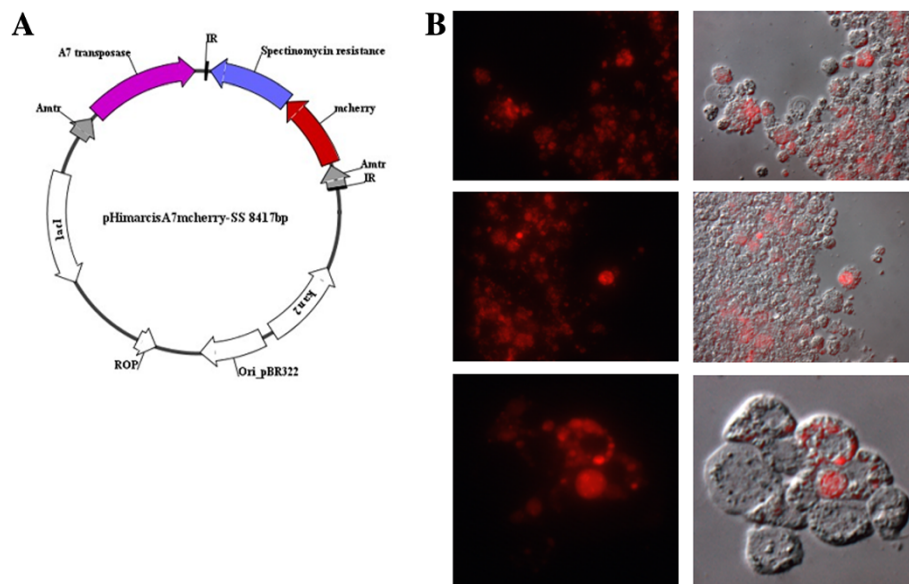


Figure 1 Red fluorescent *A. marginale*. A. Plasmid map of *pHimarcsA7mCherry-SS* used for the electroporation of *A. marginale* str. Virginia. B. Fluorescent (left) and phase contrast/fluorescence merged (right) images, of transformed *A. marginale* replicating in ISE6 tick cells.

alignment of the sequencing reads obtained by Roche/454 and Illumina methods to two reference sequences, the *A. marginale* str. St Maries genome sequence (CP000030) and the *Himar1* TIR sequence. The *Himar1* TIR-*A. marginale* genome junctions were identified by extracting reads that aligned to the *A. marginale* genome at one end and to the *Himar1* TIR at the other end.

Analysis using Illumina reads mapped the *Himar1* TIR-*A. marginale* genome junctions into a region of *omp6* and *omp10* genes. Interestingly these reads contained the same mutated sequence. The *omp6* and *omp10* genes share a large stretch of identity 456 nt/459 nt (99%) [43]. The short, 100 nt length of the Illumina reads, made it difficult to differentiate which gene contained the *Himar1* transposon. Additional analysis using longer reads obtained on the Roche/454 platform revealed that the *Himar1* transposon was integrated within the *omp10* gene. These reads contained a region of *omp10* that is not shared with *omp6*. Based on this sequencing analysis the genomic location of the *Himar1* transposon in the chromosome of the transformed *A. marginale*, is at position 245 considering 1 as the first base of the *omp10* start codon (Figure 2A).

These results were verified by PCR amplification of gDNA from ISE6 cells infected with wild-type and transformed *A. marginale* using *omp6* and *omp10* specific primers (Figure 2A-B). The size of *omp6*

amplicons (492 bp) in wild-type and transformed *A. marginale* was the same. However the size of the *omp10* amplicon in transformed *A. marginale* was increased by 1836 bp when compared to the wild-type (969 bp), indicating that the transposon was integrated within the *omp10* gene.

The genome sequence of *A. marginale* str. Virginia is available only as unannotated contigs with gaps. Therefore for our analysis we used the *A. marginale* str. St Maries genome as reference. For this reason we wanted to confirm that the transposon location in the mutated Virginia strain was the same as the one mapped in the reference genome. For this, combined Roche/454 and Illumina reads were assembled and a contig of 21,324 nucleotides identified. Alignment of this contig with the *A. marginale* str. St Maries genome showed that this sequence contained part of *omp10* and upstream genes (99% identity) (Figure 3) and that the transposon insertion site in the *A. marginale* str. Virginia matches the same region mapped using the reference genome.

Further analysis of sequencing reads determined that there is only one transposon insertion in the chromosome of recombinant *A. marginale*. The reads containing the *Himar1* TIR-*A. marginale* junctions aligned to a single genome site. Although these transformed organisms were not cloned, data suggest that they are isogenic for the transposon insertion site within the *omp10* gene.

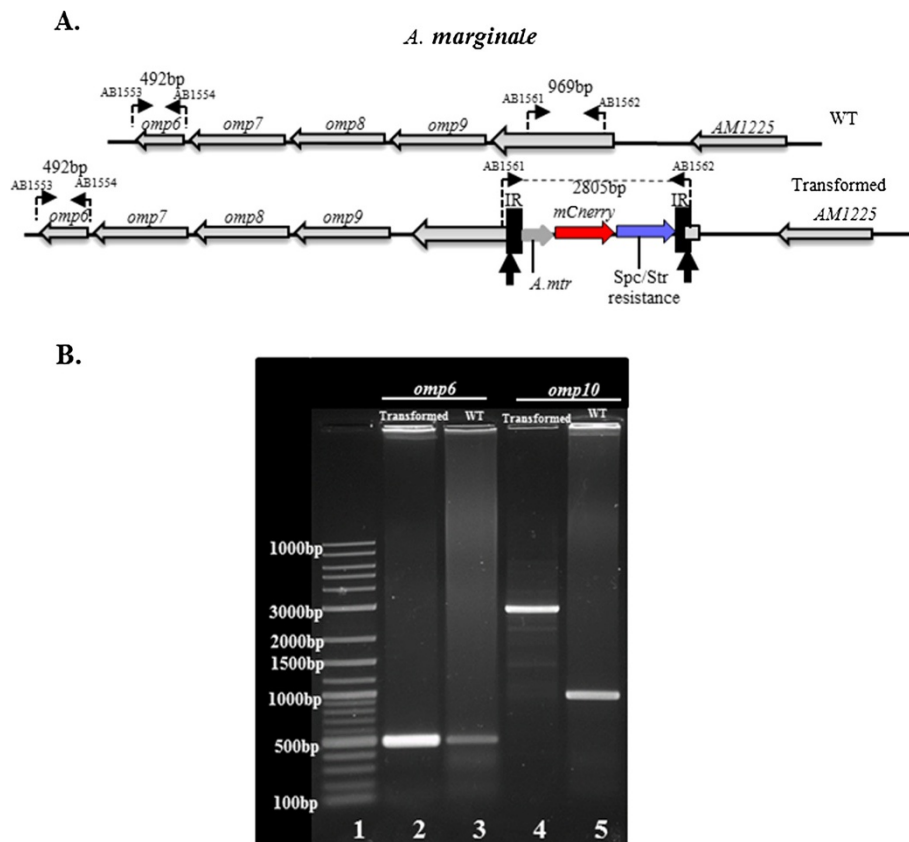


Figure 2 Mapping of *Himar1* transposon insertion site. **A.** Location of primer pairs (AB1553-AB1554) and (AB1561-AB1562) designed to target the *omp6* and *omp10* genes respectively, in wild-type (WT) and transformed *A. marginale*. Based on sequencing results the *Himar1* sequences are integrated in the chromosome of transformed *A. marginale* at nucleotide 245 after the first base of the *omp10* start codon (arrows) and *mCherry* and *aadA* (Str/Spc resistant) genes are in the opposite orientation to *omp10*. **B.** Agarose Gel electrophoresis. gDNA isolated from ISE6 tick cells infected with wild-type (WT) and transformed *A. marginale*, was used as template for PCR amplification with primers shown in A. (Lane 1) 100 bp/1Kb DNA ladder, *omp6* amplicons in transformed (lane 2) and WT (lane 3) *A. marginale* were of the same size 492 bp. The *omp10* amplicon in transformed *A. marginale* (lane 4) was 2805 bp, while in wild type was 969 bp (lane 5).

The mobilization of the *Himar1* transposon from one locus to another is mediated by a transposase using a cut and paste mechanism [27,30]. It has been shown previously in other organisms that the *Himar1* transposon integrates preferentially into a TA site and leads to duplication of this dinucleotide upon integration into the target site [30]. This was found to be true also for *A. marginale*. Sequencing analysis revealed that the *Himar1* transposon targeted a TA dinucleotide in *omp10* (Figure 4A) and upon integration it is flanked by a TA dinucleotide sequence (Figure 4B). Thus, the mobilization of the *Himar1* transposon into the *omp10* gene of *A. marginale* was mediated by means of the *A7* transposase in a cut and paste mechanism. This transformant of *A. marginale* will be referred to as *omp10::himar1* mutant.

Evidence for expression of *omp10* as part of an operon

We hypothesize that the transposon insertion could alter the expression of *omp10* and downstream genes. This

hypothesis is based on recent work in which the transcriptome profile of *A. marginale* using RNAseq indicated that *omp10* is expressed as part of a six-gene operon in erythrocytes of infected cattle [25]. This operon includes *AM1225*, *omp10*, *omp9*, *omp8*, *omp7* and *omp6* (Figure 5A).

Because of this, we wanted to determine if *omp10* is expressed within a polycistronic message in *A. marginale* replicating in ISE6 tick cells. The intergenic region between *AM1225* and *omp10* is 440 bp long, while intergenic regions between *omp10-9*, *omp9-8*, *omp8-7* and *omp7-6* are 62 bp, 63 bp, 64 bp and 36 bp respectively (Figure 5A). To test whether *AM1225* through *omp7* are expressed as a single transcriptional unit, total RNA isolated from ISE6 cells infected with wild-type *A. marginale* was reverse transcribed and template cDNA was used for amplification of intergenic regions with primers that connect neighboring genes (Figure 5A). The *omp6*

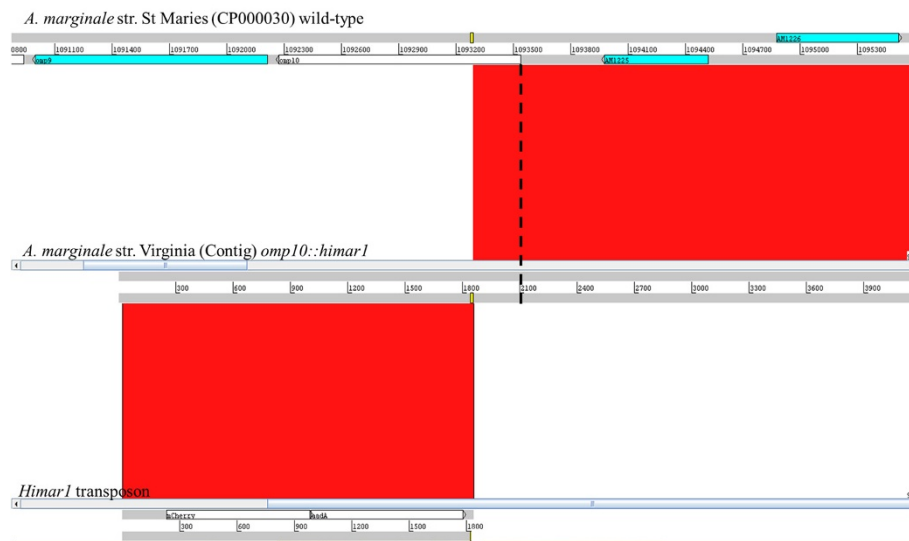


Figure 3 *Himar1* transposon insertion site in the *A. marginale* str. Virginia genome. ACT (Artemis Comparison Tool) window showing alignment between the *A. marginale* genome (CP000030) used as reference, the *A. marginale* str. Virginia *omp10::himar1* contig formed by Roche/454 and Illumina sequencing reads and the *Himar1* transposon sequences. Alignment between the *A. marginale* str St Maries and the *omp10::himar1* mutant shows that sequences flanking the transposon insertion site are highly similar sharing an identity of 99% (matching red band). This demonstrates that the transposon insertion site (yellow boxes) occurred at nucleotide 245 after the first base of the *omp10* start codon (black dotted line) in the reference strain. Alignment with the *Himar1* transposon sequences clearly show the insertion of these sequences in the *omp10::himar1* mutant which are not present in the *A. marginale* str. St Maries (absence of matching band).

gene was not included in these experiments, because previous work [43] and work in our lab showed that transcripts from this gene are not detected in *A. marginale* during infection of tick cells. Appropriate size amplicons of the intergenic regions between *omp7-8*, *omp8-9*, *omp9-10* and *omp10-AM1225* gene were detected (Figure 5B), providing evidence that these genes are transcribed as a single mRNA in *A. marginale* infected tick cells.

RNA transcript analysis

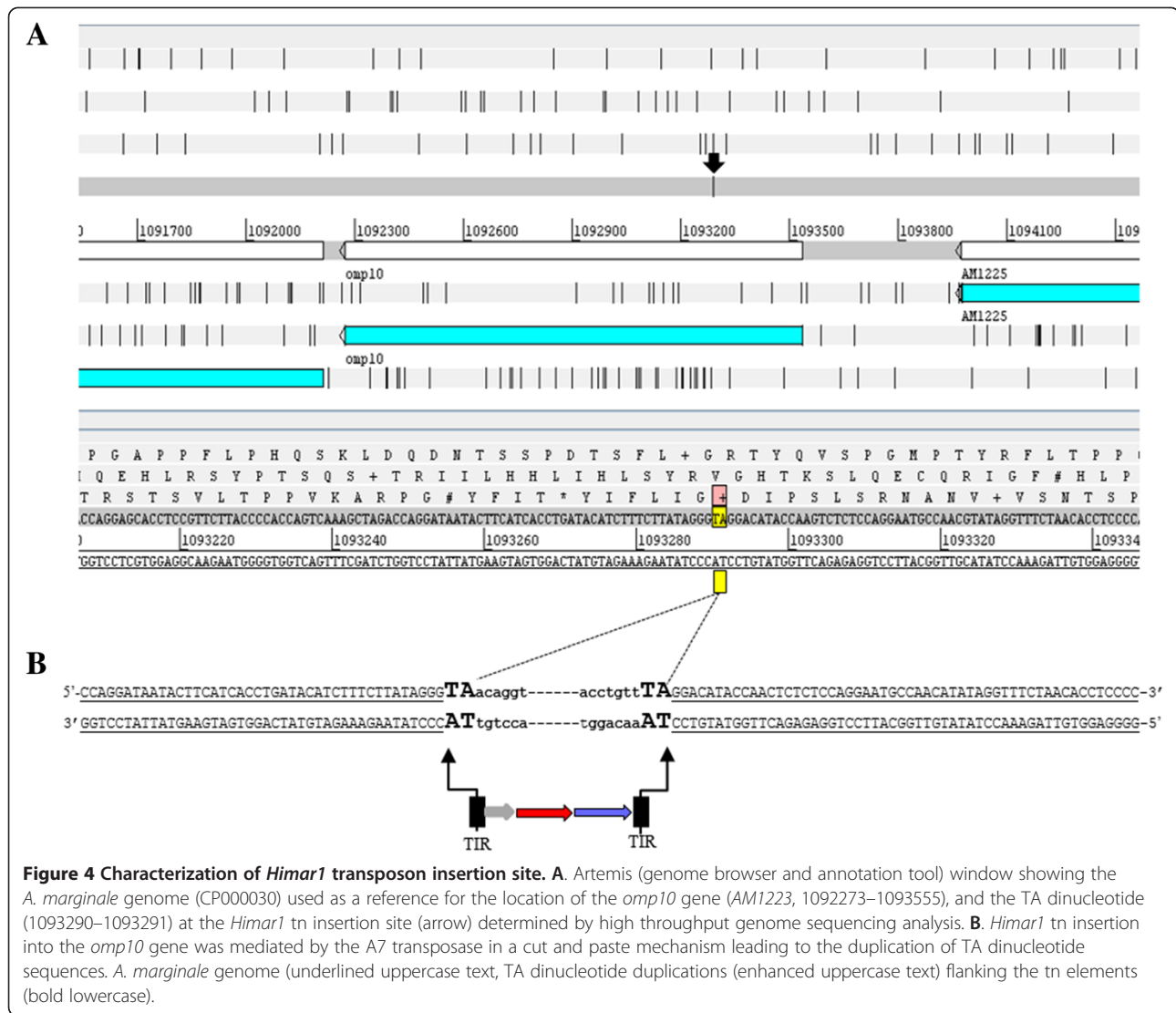
Next, we determined if insertion of the *Himar1* sequences resulted in alteration of *omp10* expression and the expression of genes downstream. For this, total RNA from ISE6 tick cells infected with *A. marginale* wild-type and *omp10::himar1* mutant was reverse transcribed and cDNA used as template for PCR amplification with specific primers that were designed to anneal to *omp6*, *omp7*, *omp8*, *omp9*, and *omp10* in wild-type and *omp10::himar1* mutant respectively (Figure 6A). The *omp10*, 9, 8, and 7 genes, but not *omp6*, are transcriptionally active in wild-type *A. marginale*, although at low levels (Figure 6B). The *Himar1* transposon insertion into the coding sequence of *omp10*, disrupted its expression and that of *omp9*, *omp8*, and *omp7* since transcripts from these genes were not detected in *omp10::himar1* mutants of

A. marginale by this method (Figure 6B). To ensure integrity, cDNA samples from *A. marginale* wild-type and *omp10::himar1* mutant were used for amplification with specific primers of a region of 131 bp of the 16S rRNA. Amplicons from this region were detected in both wild-type and *omp10::himar1* mutant. No bands were visualized in negative controls (Figure 6B).

Since *omp10* through *omp7* are expressed at low levels in ISE6 tick cells, RT-qPCR was used to quantitatively determine differences of expression between *A. marginale* wild-type and *omp10::himar1* mutant. For this, cDNA generated from ISE6 tick cells infected with *A. marginale* wild-type and *omp10::himar1* mutant was used for real time PCR amplification using primers and probes targeting *omp8*, *omp9*, and the 3' and 5' ends of *omp10* (Figure 7A).

In order to compare these gene expression results between wild-type and *omp10::himar1* *A. marginale*, Ct values were normalized to the *rpoH*, *msp5* and 16S rRNA genes. Changes in expression of these genes were calculated by the $2^{-\Delta\Delta Ct}$ method, and results were expressed as percentage of expression, with a 100% expression level being assigned to the calibrator or control group, which in this case is wild-type *A. marginale*.

Although three different reference genes were used, RT-qPCR data normalization led to similar results in



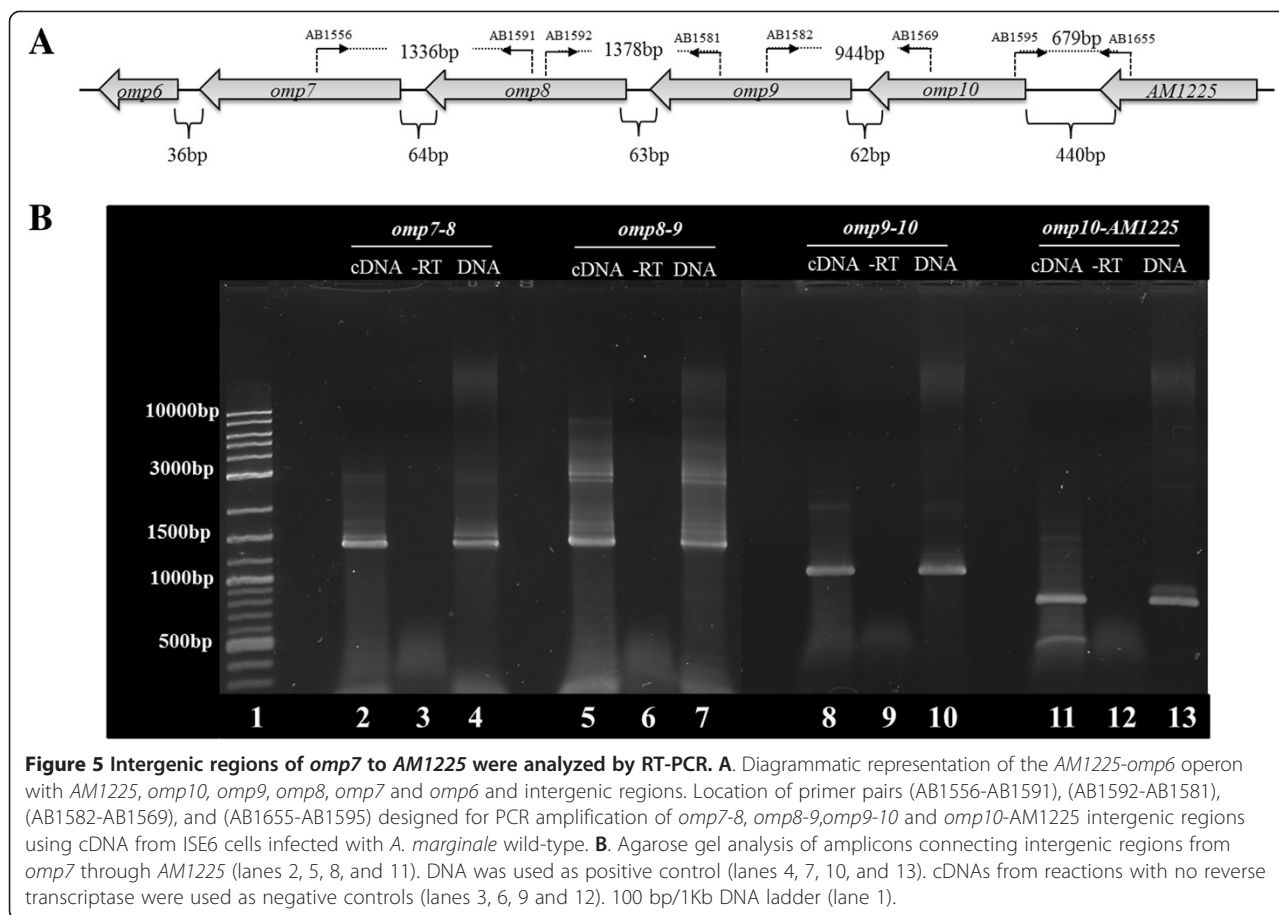
which there was a significantly reduced expression for *omp8* (97–99%), *omp9* (90–99%) and *omp10* 3' end (85–98%) relative to their counterparts in wild-type *A. marginale* (Figure 7B). These results show that *Himar1* transposon insertion into *omp10* affected its expression and the expression of genes downstream, confirming the results obtained by RT-PCR and agarose gel electrophoresis. A second experiment investigated the possibility of the same effect occurring in regions of *omp10* before the *Himar1* transposon insertion site. For this, a primer and probe set was designed to anneal with a region at the 5' end of *omp10* (Figure 7A). Even though there was a significant reduction in the detection of transcripts from this region (27–57%) relative to the 5' end of *omp10* in wild-type, this reduction was not

as great as with the sequences located in *omp10* downstream of the *Himar1* transposon insertion site.

Western immunoblot analysis

To determine if the decreased expression of mRNA in genes downstream of *omp10* correlated with protein expression a Western immunoblot analysis using anti Omp9 antibody was performed.

To compare the protein expression of *omp9* between *A. marginale omp10::himar1* and wild-type, the number of organisms per sample was quantified by qPCR using the *opag2* single copy gene to determine the copy number of *A. marginale*. Equal amounts (10^8) of organisms of *A. marginale* wild-type and *omp10::himar1* mutant were loaded per lane. *A. marginale* str. Virginia initial



bodies and uninfected ISE6 cells were used as positive and negative controls respectively.

Western immunoblot showed a reduced expression of Omp9 in *omp10::himar1 A. marginale* mutant compared with wild-type (Figure 8A). The Omp9 band of 40 kDa was present in wild-type and initial bodies but was not detected in the mutant or using negative control antibody Tryp1E1 (Figure 8B). Antibody F16C1 that reacts with major surface protein 5 (Msp5) was used as a loading control. Anti-Msp5 detected this protein (19 kDa) in wild-type and *omp10::himar1 A. marginale* (Figure 8C).

These results correlated with results obtained from the RNA transcript analysis, showing that the transposon insertion severely affected the expression of both mRNA and protein from downstream genes such as *omp9*.

Discussion

The possibility of creating insertional mutations in *A. marginale* not only could provide a broad understanding of gene products required for infectivity, growth or viability of this pathogen in the mammalian host and the tick vector, but also would allow the generation of genetically attenuated organisms that can be tested in vaccination trials.

Here we report that transposon mutagenesis using the *Himar1* transposon/transposase system for *A. marginale* is achievable and it could be useful for creating insertional mutations in these organisms. High throughput genome sequencing analysis for the characterization of these transformants established that transposon sequences are integrated within the *omp10* gene of the *A. marginale* chromosome and its mobilization within this gene was mediated by the transposase in a cut and paste mechanism, since i.) the transposon sequences were integrated within a TA dinucleotide site ii.) upon integration of the transposon, this sequence was duplicated and is found flanking the transposon TIR at the junctions with the *A. marginale* genome and iii.) sequences from the delivering vector outside the transposon were not found.

Although these *omp10::himar1* mutant organisms were not cloned, they are isogenic for the transposon insertion within the *omp10* because all the sequencing reads containing the transposon-*A. marginale* genome junctions aligned to the same genome site in the *A. marginale/St. Maries* reference genome sequence (CP000030). Possible reasons include transposon insertion into other genome regions that are essential for growth in tick cells, or

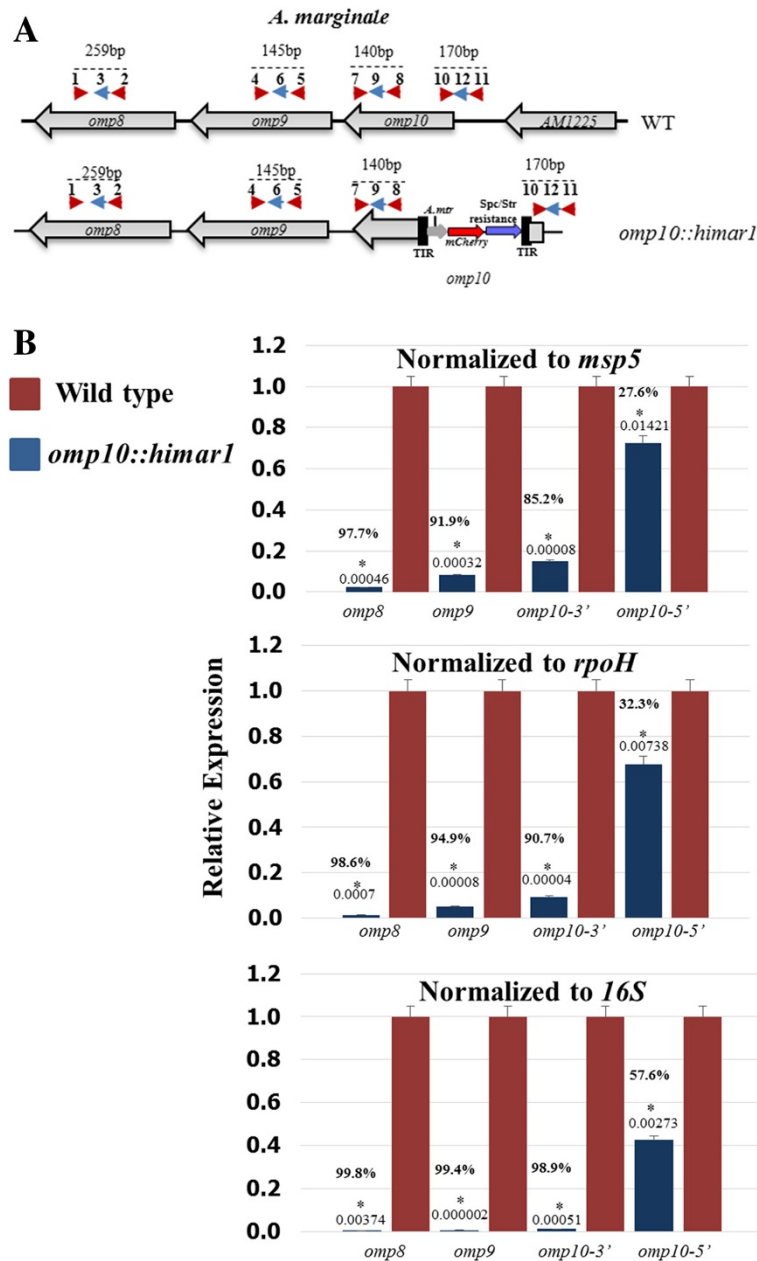


Figure 7 Relative gene expression by RT-qPCR. **A.** location of binding sites for primers and probes designed to target *omp8* (1. AB1591, 2. AB1592, 3. AB1593), *omp9* (4. AB1581, 5. AB1582, 6. AB1583), 3' end of *omp10* (7. AB1569, 8. AB1570, 9. AB1571), and the 5' end of *omp10* (10. AB1594, 11. AB1595, 12. AB1596). **B.** Bar lengths represent the percentage of expression of *omp8*, *omp9*, 3' end of *omp10* and 5' end of *omp10* in *A. marginale* wild-type (red bars) and *omp10::himar1* mutant (blue bars). *msp5*, *rpoH* and *16S* rRNA were used as reference genes for data normalization. Changes in expression of these genes were calculated using the $2^{-\Delta\Delta C_t}$ method. * Significant differences ($P < 0.05$) were calculated as described in materials and methods.

bacteria [38,39,46]. Therefore, we evaluated the effect of the *Himar1* insertion on the expression of genes downstream and upstream of *omp10* in *omp10::himar1* *A. marginale*. Results showed that the transcriptional activities of *omp9* and *omp8* were negatively influenced by the insertion of the *Himar1* within *omp10* since detection

of transcripts was significantly decreased in relation to wild-type *omp9* and *omp8*.

Although the transcription activity of regions upstream of the transposon insertion site at the 5' end of *omp10* dropped significantly in relation to wild-type *A. marginale*, it was not as severe as with genes downstream of *omp10*.

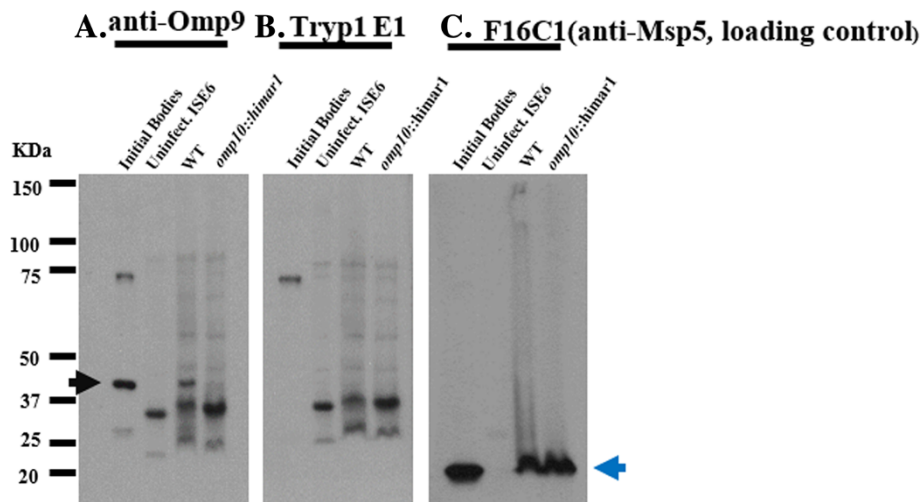


Figure 8 Immunoblotting of *omp10::himar1* mutant and WT *A. marginale* using the specific monoclonal antibody Omp9. Proteins from equal amounts of host cell-free wild-type (WT) and *omp10::himar1* *A. marginale* were separated by SDS-PAGE gel electrophoresis. Immunoblot PVDF membranes of transferred proteins were reacted with monoclonal antibodies and reactions were visualized by chemiluminescence. **A.** Monoclonal antibody Omp9 (4 µg/ml) with specificity to Omp9 protein (40 kDa) (black arrow). **B.** Negative control, monoclonal Trypan1E1 (4 µg/ml) (exhibits specificity for a variable surface glycoprotein of *Trypanosoma brucei*). **C.** Monoclonal F16C1 (2 µg/ml), reacts with the Msp5 (19 kDa) (blue arrow) protein of *A. marginale*, was used as loading control. *A. marginale* str. Virginia and uninfected ISE6 cells were used as positive and negative controls respectively.

Sequencing analysis determined that the transposon sense strand is found in the opposite orientation to *omp10*, so it might be possible for transcription to read through the *Himar1* sequences and produce anti-sense transcripts that could reduce expression of sequences upstream of *omp10*, but to demonstrate this further characterization is required.

Western immunoblot analysis showed that the transposon insertion into *omp10* markedly reduced protein expression of *omp9* in the *omp10::himar1* mutant *A. marginale* when compared to wild-type, corroborating that both mRNA and protein expression from genes downstream of *omp10* were disrupted.

The evidence presented here suggests that these genes are not essential for growth of *A. marginale* in tick cell culture. Significant work on the possible interactions between the expressed proteins in different host environments has accumulated and offers important clues about the possible phenotypic effects of the disruption of these genes in *A. marginale*. For example *omp7*, *omp8*, *omp9* and *omp10* are differentially expressed in tick and mammalian cells with lower levels in tick midgut and cultured tick cells [43]. Detection of proteins from these genes has been reported [43,47,48]. Omp7, Omp8 and Omp9 are conserved during tick transmission and in acute and persistently infected cattle [43]. Characterization of the repertoire of outer membrane surface proteins by mass spectrometry identified Omp10 and Omp7 as immunogenic in cattle [47]. Proteome analysis using crosslinking and liquid chromatography–mass spectrometry (LC-MS/MS) to determine the composition and topological organization of surface proteins in *A. marginale* in mammalian and tick cells isolated a large protein complex and analysis demonstrated that

Omp7, Omp8 and Omp9 are arranged in the outer membrane as near neighbors to Msp2, Msp3, Msp4, Omp1, Opag2, Am779, Am780, Am1011, Am854 and VirB1 in *A. marginale* isolated from erythrocytes [18]. In contrast a similar sized large protein complex in *A. marginale* isolated from tick cells was formed only by Msp2, Msp3, Msp4, Am778 and Am854. Although Omp7, Omp8 and Omp9 were expressed they did not seem to be localized to the surface, suggesting a possible re-arrangement in the topology of the surface of *A. marginale* during the transition from the tick cell into the mammalian cell [18].

Interestingly, the number of Msp2 superfamily members such as *omp1* to *omp15* in *A. marginale* subsp. *centrale*, is reduced in comparison with US *A. marginale* strains [10]. For example, closely related sequences to *omp8* and *omp6* are missing and *omp10* is found with *omp7* and a reduced *omp9* in tandem, which may indicate an important function of these genes in the pathogenicity of *A. marginale*.

Based on this, further characterization of these *omp10::himar1* mutants to understand the effects of the disruption of expression of *omp10*, 9, 8 and 7 on the phenotype of *A. marginale* is of critical importance. Phenotypic effects may include infectivity, tick transmissibility, stability under non selectable conditions, ability to induce immune responses and ability to establish persistent infection within the natural host.

Conclusions

Transposon mutagenesis is achievable for *A. marginale*. High throughput genome sequencing of recombinant bacteria electroporated with a single plasmid containing

the *Himar1* sequences and the *A7* transposase showed insertion of the *Himar1* sequences into the *omp10* gene of *A. marginale*. The insertion was mediated by the transposase in a cut and paste mechanism. In tick cells *omp10* is expressed as a polycistronic message with *AM1225* at the 5' end and *omp9*, 8 and 7 at the 3' end. Insertion of the *Himar1* transposon within *omp10* not only disrupted its expression but also the expression of genes downstream, such as *omp9*, *omp8* and *omp7*.

This work shows the utility of the *Himar1* system for the generation of insertional mutants in *A. marginale*, for the identification of genes involved in virulence and potentially for the development of attenuated organisms.

Methods

A. marginale cultivation

Cultures of *A. marginale* str. Virginia wild-type and *omp10::himar1* mutant were maintained in tick ISE6 cells derived from embryonated eggs of the blacklegged tick, *Ixodes scapularis* at 34°C in non-vented 25-cm² cell culture flasks (NUNC). *A. marginale*-infected cell cultures were maintained in L15B300 medium supplemented with 5% fetal bovine serum (FBS, BenchMark, Gemini Bio-Products), 5% tryptose phosphate broth (TPB, Difco, Becton Dickinson), 0.1% bovine lipoprotein concentrate (LPC, MP-Biomedical), 0.25% NaHCO₃, and 25 mM HEPES buffer, adjusted to pH 7.8, as previously described [49]. The cell culture medium for ISE6 cells infected with the *A. marginale omp10::himar1* mutant was supplemented with spectinomycin (Sigma Aldrich) and streptomycin (Sigma Aldrich) to a final concentration of 50 µg/ml each.

Isolation of the *A. marginale* mutant by transposon mutagenesis

To maximize chances of obtaining a transformant using transposon mutagenesis, we used a single plasmid construct that encoded both the transposon and the transposase in *cis* configuration as described [50], except that the fluorescent marker was replaced by sequences encoding a monomeric red fluorescent protein, *mCherry* [51] (Figure 1A). *A. marginale* bacteria passaged 53 times in ISE6 cells were harvested from one 25-cm² culture in 5 ml of medium when ~80% of cells were infected, and many cells were undergoing lysis. The cells were recovered in 2 ml of culture medium, and added to a 2-ml microcentrifuge tube containing 0.3 ml of sterile silicon carbide abrasive (60/90 grit; Lortone, Inc), vortexed at maximum speed for 30 sec, and the lysate transferred to a fresh 2-ml tube on ice. Bacteria were collected by centrifugation at 11,000 g for 10 min at 4°C, and washed twice in ice-cold 300 mM sucrose. They were then resuspended in 50 µl of 300 mM sucrose containing 3 µg of plasmid DNA, and incubated on ice for 15 min before being

electroporated (Biorad Gene Pulser II) at 2 kV, 400 Ohm and 25 µF in a 0.2 cm gap cuvette. The electroporation mixture was recovered in 1.5 ml of an ISE6 cell suspension (~2×10⁶ cells), and centrifuged in a microcentrifuge tube at 1,000 g for 10 min at room temperature. The tube was left undisturbed for 30 min at room temperature, and the pellet then resuspended in the supernatant medium and added to a 25-cm² flask containing ~5×10⁶ ISE6 cells in 3 ml of L15B300 medium supplemented as described for *Anaplasma*-infected cultures. The culture was incubated at 34°C in a tightly capped flask. Three days after electroporation, the culture medium was replaced with 5 ml of medium additionally containing 50 µg/ml of spectinomycin and streptomycin (selection medium). Subsequently, the culture was fed twice weekly with selection medium and examined weekly on an inverted microscope (Diaphot, Nikon) fitted for epifluorescence using a Texas Red filter. The first fluorescent colonies of bacteria were noted 6 wk following electroporation, and the culture was maintained in selection medium with twice-weekly medium changes until ~90% of cells were infected. At that time, the mutant was passaged (ten-fold dilution) to fresh cells, and the remainder was stored in liquid nitrogen.

Preparation of host cell-free *A. marginale* wild-type and *omp10::himar1* mutant from ISE6 tick cells

Isolation of *A. marginale* wild-type and *omp10::himar1* mutant was performed by disruption of ISE6 tick cells with 1 mm diameter glass beads (BioSpec Technologies) in a Minibead beater (BioSpec technologies) as described elsewhere [52], with the exception that cells were shaken only once for 10s and immediately placed on ice. Cell lysates were transferred to 1.5 ml centrifuge tubes and centrifuged at 100 g for 5 min at 4°C to pellet cell debris. The supernatant was then carefully removed and transferred to clean 1.5 ml centrifuge tubes. *A. marginale* organisms (wild-type and *omp10::himar1* mutant) were pelleted at 11,000 g for 10 min at 4°C, and stored at -20°C.

DNA isolation and Phi29 amplification of the *A. marginale omp10::himar1* mutant

Before DNA isolation, pelleted *A. marginale omp10::himar1* mutants were treated with RNaseA (QIAGEN) and DNase I (Sigma Aldrich) to remove ISE6 host cell contaminant nucleic acids. DNA isolation was performed using the QIAamp DNA Mini kit (QIAGEN) as per manufacturer's instructions, but in this case the DNA was eluted in 50 µl of 1 mM Tris pH 9.0. DNA concentration was determined using the Qubit dsDNA HS assay kit (Life technologies) on a Qubit fluorometer (Life technologies). 5 reactions of 10 ng of DNA were used for whole genome amplification using the Genomi Phi V2 DNA amplification kit (GE Healthcare) according to manufacturer's

instructions. Following amplification, aliquots were pooled together and the DNA purified with GelElute Extraction Kit (5 PRIME) by adsorption to silica particles and eluted with 10 mM Tris pH8.2.

Genome sequencing and bioinformatics

Samples from 2.0 to 3.6 μ g of amplified DNA derived from the *omp10::himar1* mutant, were provided for library construction and sequencing by the Roche/454 (GS-FLX) method to the Interdisciplinary Center for Biotechnology Research (ICBR) at the University of Florida. Also, samples of equivalent amounts were provided to the Scripps Research Institute, La Jolla, California for sequencing by the Illumina (HiSeq) method.

A total of 374,151 and 207,288,916 reads of Roche/454 and Illumina sequencing data, respectively, were obtained. The FASTQ files provided by the sequencing facilities were uploaded to the UF GALAXY web site <http://galaxy.hpc.ufl.edu>, and analyzed separately.

Uploaded Illumina FASTQ files were groomed, filtered and formatted into FASTA files using the FASTQ Groomer, Filter FASTQ and FASTQ to FASTA converter tools located in the NGS: QC and manipulation toolbox of GALAXY. FASTA files were then aligned to the *A. marginale* str. St Maries reference genome sequence (CP000030) using the Megablast alignment tool (NCBI BLAST + blastn (version 0.0.12) in GALAXY) to obtain sequencing reads that contained *A. marginale* sequences.

These *A. marginale* sequencing reads were then used for a second Megablast alignment using as a reference sequence 28 nucleotides from the *Himar1* terminal inverted repeats (TIR). The transposon insertion locus within the *A. marginale* chromosome was then determined, since the reads obtained contained the *A. marginale-Himar1* TIR junctions.

A similar strategy was used for the analysis of the Roche/454 sequencing reads. CLC genomics workbench, version 6.5 was used for assemblies of Roche/454 and Illumina reads.

RNA isolation

For RNA isolation, three samples of ISE6 cells infected with *A. marginale* wild-type and three *omp10::himar1* samples were used. Each sample derived from separate cultures grown in T-25 cell culture flasks. Samples containing approximately equal numbers of infected cells were collected in RNA stabilization reagent RNeasy lysis buffer (AMBION-Life technologies) and stored at -80°C . Total RNA was isolated using the RNeasy kit (QIAGEN) with an added "on-column" DNase I treatment (QIAGEN) according to manufacturer's instructions. Aliquots of extracted RNA were used to measure contaminant DNA concentration using the Qubit dsDNA HS assay kit (Life technologies). Additionally, RNA was treated three times

with RNase-free Dnase I (AMBION-Life technologies) to remove any trace of contaminant DNA in the sample. RNA concentration was measured with the Qubit RNA assay kit (Life technologies), and samples were stored at -80°C .

RT-PCR and RT-qPCR experiments

RNA (2 μ g) from ISE6 cells infected with *A. marginale* wild-type and *omp10::himar1* mutant was converted to cDNA by random priming using a Omniscript reverse transcriptase kit (QIAGEN) according to manufacturer's conditions. Genomic DNA and no-reverse transcriptase reactions were included as controls for each sample and each nucleic acid target. Specific primers (Table 1) were designed to amplify transcripts from intergenic regions between *omp7-omp8*, *omp8-omp9*, *omp9-omp10* and *omp10-AM1225* using cDNA from ISE6 cells infected with *A. marginale* wild-type as template. Similarly transcripts from within *omp6*, *omp7*, *omp8*, *omp9*, and *omp10* genes were detected by PCR amplification of cDNA from ISE6 cells infected with *A. marginale* wild-type and the *omp10::himar1* mutant using *omp6-10* specific primers (Table 1). PCR amplification conditions for each PCR experiment are described in Additional file 1: Tables S1 and S2 respectively.

RT-qPCR experiments

Transcript differences between *omp8*, *omp9*, *omp10-5'* end, and *omp10-3'* genes in *A. marginale* wild-type and *omp10::himar1* mutant were determined using the comparative $2^{-\Delta\Delta\text{Ct}}$ method [53,54] and the results were based on the mean of three biological samples (individual RNA extracts). For Taqman quantitative PCR, cDNA obtained from ISE6 cells infected with *A. marginale* wild-type and the *omp10::himar1* was used with primers and probes (Table 1) designed to amplify *omp8*, *omp9*, *omp10-5'* end, *omp10-3'* end, *msp5*, *rpoH* and the *16S* gene sequences. Reaction conditions are described in Additional file 1: Table S3, specificity of primers and probes is shown in Additional file 1: Figure S1 and the amplification efficiencies for each target are reported in Additional file 1: Table S4. For a valid $2^{-\Delta\Delta\text{Ct}}$ calculation, relative efficiencies of target vs. reference genes were calculated and are reported in Additional file 1: Table S5.

Significant differences between the *A. marginale* wild-type and *omp10::himar1* mutant were calculated by Student's t test ($P < 0.05$), comparing ΔCt values (target gene- reference gene) of the *omp10::himar1* mutant and the wild-type. The fold difference was based on $\Delta\Delta\text{Ct}$ (*omp10::himar1* mean ΔCt - wild-type mean ΔCt) and calculated as $2^{-\Delta\Delta\text{Ct}}$ which yields the expression ratio. The expression ratio was then expressed as percentage of expression by multiplying the $2^{-\Delta\Delta\text{Ct}}$ values by 100. For normalization of relative gene expression data *msp5* [55], *rpoH*, and *16S* were used as reference genes.

Table 1 PCR and Taqman qPCR oligonucleotides used in this study

Oligonucleotide sequence (5' to 3')	Target	Size	Reference
PCR			
AB1553	<i>omp6</i>	492bp	[43]
AB1554			
AB1555	<i>omp7</i>	399bp	[43]
AB1556			
AB1591	<i>omp8</i>	259bp	This study
AB1592			
AB1559	<i>omp9</i>	1096bp	[43]
AB1560			
AB1561	<i>omp10</i>	969bp	[43]
AB1562			
AB1572	<i>16S</i>	131bp	This study
AB1573			
**qPCR			
AB1591	<i>omp8</i>	259bp	This study
AB1592			
AB1593			
AB1581			
AB1582	<i>omp9</i>	145bp	[43]
AB1583			
AB1594			
AB1595	<i>omp10-5'</i>	170bp	This study
AB1596			
AB1569			
AB1570	<i>omp10-3'</i>	140bp	[43]
AB1571			
AB1572			
AB1573	<i>16S</i>	131bp	[42]
AB1574			
AB1606			
AB1607	<i>msp5</i>	145bp	[55]
AB1610			
AB1608			
AB1607	<i>rpoH</i>	116bp	This study
AB1611			
AB1242			
AB1243	<i>opag2</i>	151bp	[41]
AB1250			

*Primers and TaqMan probes used were manufactured at Eurofins MGM Operon.
 Oligonucleotides are labeled with 6-Carboxyfluorescein 6-FAM at the 5' end and Tetramethylrhodamine TAMRA at the 3' end.

Western immunoblots

Expression of the Omp9 protein in *A. marginale* wild-type and *omp10::himar1* mutant was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting using equal amounts (10⁸) of host-free

bacteria. Membranes were incubated with three different antibodies; the anti-Omp9 monoclonal antibody (121/1055) [43], the monoclonal antibody F16C1 (reacts with the Msp5 protein and served as a loading control) [56] and the monoclonal antibody Tryp1E1 (exhibits specificity for a

variable surface glycoprotein of *Trypanosoma brucei* [56]. This last antibody served as a negative control. Final concentrations of each antibody used were 4 µg/ml, 2 µg/ml and 4 µg/ml. Antibody binding was detected with the secondary antibody goat anti-mouse IgG, horseradish peroxidase labeled and diluted to 1:10,000 using the Pierce ECL Western blotting substrate (Thermo scientific) as described in manufacturer's instructions.

Quantification of the number of *A. marginale* wild-type and *omp10::himar1* organisms was performed as described elsewhere [41].

GenBank accession numbers

for assembled contigs containing the *Himar1* transposon sequences integrated within *omp10* and upstream genes (KJ567138) and *omp10* (partial 3' end) and *omp9* genes (KJ567139).

Additional file

Additional file 1: RT-PCR and RT-qPCR experiments Figure and Tables.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

FLC designed and carried out experiments, data analysis and authored this manuscript. HLW and MGP carried out tick cell media preparation, maintenance of uninfected tick cell cultures and co-authored the manuscript. AML performed Western blot experiments and co-authored the manuscript. AFB advised on experiments, genome sequencing analysis, critically evaluated and co-authored the manuscript. SMN provided monoclonal antibodies for Western blot experiments, co-authored and critically evaluated the manuscript. UM kindly provided transformed *A. marginale* organisms co-authored and critically evaluated the manuscript. All authors read and approved the final manuscript.

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