Disruption of VirB6 paralogs in *Anaplasma phagocytophilum* attenuates its growth

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Running Head: Mutagenesis of *A. phagocytophilum* virB6 locus

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ABSTRACT

Many pathogenic bacteria translocate virulence factors into their eukaryotic hosts by means of Type 4 Secretion Systems (T4SS) spanning the inner and outer membranes. Genes encoding components of these systems have been identified within the Order Rickettsiales based upon their sequence similarities to other prototypical systems. *Anaplasma phagocytophilum* are obligate intracellular, tick-borne bacteria that are members of this Order. The organization of these components at the genomic level was determined in several *Anaplasma phagocytophilum* strains, showing overall conservation, with the exceptions of *virB2* and *virB6* genes. The *virB6* loci are characterized by the presence of four *virB6* copies (*virB6-1* through *virB6-4*) arranged in tandem within a gene cluster known as the *sodB-virB* operon. Interestingly, the *virB6-4* gene varies significantly in length among different strains due to extensive tandem repeats at the 3’ end. To gain understanding of how these enigmatic *virB6* genes function in *A. phagocytophilum* we investigated their expression in infected human and tick cells. Our results show that these genes are expressed by *A. phagocytophilum* replicating in both cell types, and that VirB6-3 and VirB6-4 proteins are surface-exposed. Analysis of an *A. phagocytophilum* mutant carrying the Himar1 transposon within the *virB6-4* gene, demonstrated that the insertion not only disrupted its expression but also exerted a polar effect in the *sodB-virBs* operon. Moreover, the altered expression of genes within this operon was associated with the attenuated *in vitro* growth of *A. phagocytophilum* in human and tick cells, indicating the importance of these genes in the physiology of this obligate intracellular bacterium in such different environments.

IMPORTANCE

Knowledge of the T4SS is derived from model systems, such as *Agrobacterium tumefaciens*. The structure of the T4SS in Rickettsiales differs from the classical arrangement. These differences include missing and duplicated components with structural alterations. Particularly, two sequenced *virB6-4* genes encode unusual C-terminal structural extensions resulting in proteins of 4322 (GenBank YYU_01815) and 9935 (P029_01630) amino acids. To understand how the T4SS is used in *A. phagocytophilum* we describe the expression of the *virB6* paralogs and explore their role as the bacteria replicate within its host cell. Conclusions about the
importance of these paralogs for colonization of human and tick cells is supported by the deficient phenotype of an *A. phagocytophilum* mutant isolated from a sequence-defined transposon insertion library.
INTRODUCTION

The type 4 secretion system (T4SS) is a macromolecular protein complex of Gram-negative and -positive bacteria that forms a channel across the cell envelope allowing the secretion of substrates. Depending on the bacterial species, this multimeric channel is a conduit for direct transfer of DNA, proteins or effectors into eukaryotic host cells, enabling virulence and survival of bacteria (1-3). In some instances, this system is also involved in DNA uptake from the extracellular milieu (4) or interbacterial killing (5, 6). The T4SS is of major medical relevance as it is used by several multidrug resistant bacterial species to spread antibiotic resistance genes (7). The VirB/VirD4 system encoded by the pTi plasmid of Agrobacterium tumefaciens represents the canonical P-T4SS, consisting of 12 subunits, VirB1 throughout VirB11 and VirD4, which serves to deliver oncogenic nucleoprotein particles into plant cells, resulting in the development of crown-gall tumors (8).

Anaplasma phagocytophilum (Order: Rickettsiales; Family: Anaplasmataceae) is an obligate intracellular tick-borne bacterium and the causative agent of human granulocytic anaplasmosis (HGA), a notifiable disease since 1998. This pathogen is of increasing concern in the United States and internationally, as an emerging agent that causes disease in both humans and animals. Members of this species have a tropism for neutrophils and granulocytes, and infection is characterized by a non-specific febrile illness with clinical manifestations that range from asymptomatic to fatal disease if left untreated (9-11). Moreover, all members of the order Rickettsiales sequenced to date encode components of a T4SS, prototypical to the VirB/VirD system of Agrobacterium tumefaciens (9, 12, 13).

Although the T4SS in A. phagocytophilum superficially resembles that of A. tumefaciens, significant differences exist. For example, in A. tumefaciens the genes that encode the transmembrane channel and coupling proteins are organized in a single locus that contains the virB and the virD operons (8, 14). However, in A. phagocytophilum these components are distributed in three distal clusters. The first includes: virB8-1, virB9-1, virB10, virB11 and virD4, the second, virB2s and virB4-2, and the third comprises virB3, virB4-1 and four virB6 paralogs (12, 15). Coinciding with the lack of genes required for peptidoglycan synthesis, A. phagocytophilum lacks virB1, a gene than encodes a murein degrading transglycosylase required for channel
assembly across the cell wall (16-19), and virB5, that encodes a pilus-associated protein, but has duplicate copies of genes encoding secretion channel-associated proteins virB4, virB8 and virB9 genes, the four copies of virB6, and multiple copies (8 to 15) of virB2, that vary in number and sequence between A. phagocytophilum strains (15). These differences in organization suggest alternate apparatus assembly, regulation and substrate transfer (12, 20) and perhaps even assembly of variable surface structures that may modulate host-pathogen interactions such as attachment to different host cells or evasion of host immune responses (21, 22).

Comparative genomics of these components in several A. phagocytophilum strains showed that they are highly conserved among strains, with the exception of virB2 and virB6. The virB6 loci, the focus of this study, are characterized by the presence of four copies in tandem (virB6-1 through virB6-4) arranged within an operon that contains sodB, virB3 and virB4-1. Interestingly, virB6-4 varies among strains due to the presence of an extensive repeat domain with varying numbers of repeats found at the 3’ end (15). Although VirB6 is essential for substrate secretion and stability of the T4SS (23-26), very little is known about the expression of the four virB6 paralogs and their role in A. phagocytophilum infection and pathogenesis.

In this study, molecular and biochemical assays lead us to conclude that the four virB6 paralogs are expressed in A. phagocytophilum during in-vitro infection of human and tick cells and that VirB6-3 and VirB6-4 proteins are surface exposed. Transposon mutagenesis of A. phagocytophilum using the Himar1 system resulted in the isolation of a mutant carrying transposon (Tn) sequences within the virB6-4 gene. Insertion of Tn sequences within this gene not only disrupted its expression but had a polar effect in the sodB-virB operon. Moreover, altered expression of genes within this operon was linked to attenuated growth of A. phagocytophilum in human and tick cells, indicating the importance of these genes during intracellular replication.
RESULTS

The virB6 loci are transcribed in A. phagocytophilum during infection of human and tick cells.

The virB6 loci are organized within an operon structure (sodB-virBs) comprised of sodB, virB3, virB4-1, virB6-1, virB6-2, virB6-3 and virB6-4 in tandem (Fig. 1A). Prior work failed to clarify the polycistronic nature or transcription status of each of these genes (27, 28) (15). Therefore, to help us understand the contribution of this locus to A. phagocytophilum virulence we determined the cistronic organization and transcription of the sodB-virBs locus in both human and tick cells. Moreover, since the peptide used to raise antibodies against VirB6-4 represents sequences from the C-terminal repeat region, we also queried whether the full length of this gene is transcribed in both cell types by targeting transcripts from the 5’ and 3’ ends. Total RNA isolated from A. phagocytophilum infected cells was reverse transcribed with random hexamer primers and the cDNA template used for PCR amplification with primers targeting sequences from intergenic regions and each gene within the operon (Fig. 1A). RT-PCR products of appropriate size from the intergenic regions between sodB-virB3, virB3-virB4-1, virB4-1-virB6-1, virB6-1-virB6-2, virB6-2-virB6-3 and virB6-3-virB6-4 genes were detected (Fig. 1B), indicating that these genes are polycistronically transcribed. Moreover, transcripts from each gene and full length virB6-4 were detected in both HL-60 and tick ISE6 cells (Fig. 1C).

The four VirB6 paralogs were detected in A. phagocytophilum infected human and tick cells.

The prototypical A. tumefaciens VirB6 is an integral membrane protein of ~300 amino acid residues and four to five transmembrane domains (TMD1 through TMD5) (26, 29). Comparison of VirB6 protein sequences from A. tumefaciens with paralogs from A. phagocytophilum, A. marginale and E. chaffeensis indicate sequence conservation restricted only towards the A. tumefaciens central DNA-transferring TrbL/VirB6 domain, specifically, three central TMDs and a cytoplasmic loop found between TMD3 and TMD4 (Fig. S1A). In A. tumefaciens, a tryptophan residue within the central cytoplasmic loop is essential for localization of this protein at the cell pole (30). This residue appears to be conserved among the VirB6 protein family (26, 30), including the five VirB6 paralogs from Rickettsia species (20, 31). Likewise, we found this residue to be invariant in VirB6 proteins from A. phagocytophilum, A. marginale and E. chaffeensis (Fig. S1A), indicating conservation...
of this residue in Rickettsiales. Relative to *A. tumefaciens*, and similar to other Rickettsiales (12, 32), *A. phagocytophilum* encodes much larger VirB6 proteins characterized by extended N- or C-terminal hydrophilic regions flanking the TrbL/VirB6 domain (Fig. S1B), that place them within the “extended VirB6-like proteins” subtype (4, 14, 16). *In silico* topology analysis indicated that in *A. phagocytophilum*, VirB6-1 and VirB6-2 have nine and seven TMDs respectively, and a putative amino-terminal cleavable signal peptide (SP) (Fig. S1B). However, it is possible that SP residues from these proteins correspond to the first TMD as it is known that prediction algorithms often confound TMDs with the SP due to similar biochemical characteristics (33). On the other hand, VirB6-3 and VirB6-4 have eight and six TMDs respectively, and are predicted to be lipoproteins as both contain highly conserved lipobox sequences (Fig. S1B). Common to these paralogs is the clustering of TMDs towards the TrbL/VirB6 hydrophobic region. VirB6-1, VirB6-2 and VirB6-3 carry the TrbL/VirB6 domain located towards the C-terminus, while in VirB6-4 this domain is found towards the N-terminus (Fig. S1B).

To determine expression of *virB6* paralogs as proteins, specific antibodies against *A. phagocytophilum* str. HZ VirB6 proteins were prepared using synthetic peptides derived from unique predicted antigenic epitopes of each protein (Fig. S1B and Table 1). An enzyme-linked immunosorbent assay (ELISA) was performed to determine the titer of rabbit polyclonal antibodies specific to VirB6-1, 6-2, 6-3 and 6-4 peptides using conjugates of the VirB6 peptides with ovalbumin, or ovalbumin alone as coating agents. This assay showed that immunized animals developed high antibody titers against the peptides. In addition, there was a lack of reactivity of the pre-immunization serum against the peptide (Fig. S2).

The expression of the individual VirB6 proteins in infected HL-60 and ISE6 cells was evaluated by immunofluorescence microscopy. As a localization control, antisera from mice infected with *A. phagocytophilum* str. HZ (with antibody responses mainly targeting the dominant surface protein MSP2/P44) (34, 35) were used. Binding of the infection sera to *A. phagocytophilum* generated a green fluorescent signal matching the known location of MSP2/P44 on the periphery of bacteria (Fig. 2A-B). This allowed us to confidently identify the specific localization of rabbit anti-VirB6 peptides to *A. phagocytophilum*. Dual
fluorescence indicated that the red fluorescent signals from VirB6-1, VirB6-2, VirB6-3 and VirB6-4 epitopes were specific to *A. phagocytophilum* organisms in infected HL-60 and ISE6 cells as shown by superimposed images and colocalization data (Fig. 2A and 2B). Similar binding was not observed in infected cells reacted with pre-immune rabbit sera. Interestingly, VirB6-3 and VirB6-4 appeared to be located predominantly at the periphery of *A. phagocytophilum* as multiple foci or punctate structures when growing in HL60 cells (Fig. 2A). In ISE6 cells, the red fluorescent signals from VirB6-3 and VirB6-4 also located at the periphery of bacteria (Fig. 2B). Conversely, the signal from VirB6-4 was additionally observed in a location consistent with it being associated with the parasitophorous vacuole (PV), and the originating signal did not overlap with the green fluorescence as indicated by colocalization data (Fig 2B). However, as we did not use antibodies specific to PV associated proteins we cannot determine this localization unambiguously.

Due to the C-terminal hydrophilic repeat domains of VirB6-3 and VirB6-4, the focus of this work, it has been proposed that these proteins or their C-terminal domains are surface exposed or proteolytically released into the host cell (15). In addition, evidence of surface exposure of the extended VirB6 proteins has been presented for other Rickettsiales (36-38). Hence, we confirmed their expression in infected HL-60 cells by Western immunoblot and determined whether these proteins are surface exposed by trypsin digestion of intact *A. phagocytophilum* organisms. Western immunoblot showed specific protein bands in samples reacted with sera from VirB6-3 and VirB6-4 peptide-immunized rabbits that were not detected in samples reacted with pre-immune sera (Fig. 3A). The sizes of the bands detected matched the predicted molecular weights of VirB6-3 (158.3 kDa) and VirB6-4 (predicted mass of 470 kDa, but can migrate anomalously because of larger repeat sequences) respectively. Additional protein bands of smaller size than predicted were detected in the sample reacted with sera against VirB6-3. It is possible that this protein undergoes proteolytic post-translational processing that results in smaller molecules, or alternatively may represent non-specific degradation products (Fig. 3A).

To confirm that VirB6-3 and VirB6-4 are surface-exposed proteins in *A. phagocytophilum*, we performed trypsin digestion of intact *A. phagocytophilum* organisms. For these experiments we used mCherry-tagged *A.
phagocytophilum organisms that carry the Himar1 Tn integrated within an intergenic region between the 165 HGE1_00520 (phosphatidate cytidylyltransferase) and the HGE1_00525 (p44-66) genes, and which growth in 166 HL-60 and ISE6 cells comparable to the parental strain A. phagocytophilum str. HGE1 (39). Intact A. 168 phagocytophilum organisms were incubated with trypsin, followed by solubilization and Western blot using 169 rabbit anti-VirB6-3 and anti-VirB6-4 sera. Mouse anti-mCherry antibody was used as a control to show that 170 trypsin would not breach the cell wall as this protein localizes to the bacterial cytosol and is not surface- 171 exposed. Treatment of bacteria with trypsin completely digested the protein band of 158 kDa and dramatically 172 reduced the detection of smaller protein bands in samples reacted with anti-VirB6-3 antisera relative to 173 untreated samples (Fig. 3B). Likewise, anti-VirB6-4 sera did not detect VirB6-4 protein in samples digested 174 with trypsin relative to untreated controls (Fig. 3C). In trypsin treated samples, mCherry was not reduced or 175 diminished in size, indicating that reduction of VirB6-3 and VirB6-4 was due to trypsin treatment of the cell 176 surface and not to damaged organisms.

Isolation of a VirB6-4 mutant generated by transposon mutagenesis

Anaplasma phagocytophilum str. HGE1, subsequently referred to here as wild-type (wt), was subject to Himar1 178 Tn mutagenesis (40, 41). Tn sequencing (Tn-seq) using the Illumina platform detected a Tn insertion into the 179 virB6-4 open reading frame. Cloning by limiting dilution allowed the isolation of a population of organisms 180 isogenic for the Tn insertion within this gene. Therefore these bacteria are designated here as virB6-4::Himar1 181 mutants.

Although the genome sequence of A. phagocytophilum str. HGE1 (GenBank accession # APHH00000000) is 182 available, the 3’ end repeats region of virB6-4 is unresolved, containing gaps. Therefore, for our analysis we 183 used the complete virB6-4 sequence from the A. phagocytophilum str. HGE1 mutant DU1 (GenBank Accession 184 # NZ_LASP01000002) as reference. Alignment of Illumina reads to the reference genome mapped the Tn 185 insertion at the TA dinucleotide coordinates (381514..381515) 5’ upstream from the extensive tandem repeat 186 domain at the 3’ end (Fig. 4A-B). This analysis indicated that the mCherry and aadA genes from the Tn are
integrated in the opposite orientation to \textit{virB6-4} (Fig. 4C). Orientation of the Himar1 transposon was also confirmed by Sanger sequencing.

**Himar1 Tn insertion within \textit{virB6-4} abolished its expression and had a polar effect in the \textit{sodB-virBs} operon.**

\textit{In silico} cloning of Tn sequences within the \textit{virB6-4} gene suggested that its insertion resulted in a shift in the reading frame that was predicted to lead to premature termination of translation and the expression of a truncated protein (Fig. 4C). To test this assumption and determine whether the inserted transposon exerted polar effects on the expression of adjacent genes, we examined the expression of \textit{virB6-4} and upstream genes. RT-qPCR was used to quantitatively determine differences in the expression of gene components of the \textit{sodB-virBs} operon in \textit{A. phagocytophilum} wt vs. the \textit{virB6-4::Himar1} mutant. For this, total RNA from HL-60 cells infected with \textit{A. phagocytophilum} wt or the \textit{virB6-4::Himar1} mutant was reverse transcribed, using random hexamer primers. The resulting cDNAs were quantified by real time PCR amplification, using primers targeting \textit{sodB, virB6-1, virB6-2, virB6-3}, and the 3' and 5' ends of \textit{virB6-4} (Fig. 5A). Relative expression levels of these genes were normalized to the geometric mean of the reference genes \textit{rpoB} and \textit{groEL}. RT-qPCR analysis revealed significantly reduced transcripts from \textit{sodB} (Mean ± SD, 69.3% ± 6.83%), \textit{virB6-1} (54.26% ± 3.67%), \textit{virB6-2} (63.96% ± 5.8%), \textit{virB6-3} (64.97% ± 3.40%), \textit{virB6-4 5'} end (70.14% ± 9.07%) and \textit{virB6-4 3'} end (14.03% ± 1.49%) in the \textit{virB6-4::Himar1} mutant relative to wt; \textit{sodB} (99.38% ± 6.02%), \textit{virB6-1} (99.79% ± 5.71%), \textit{virB6-2} (100% ± 16.45%), \textit{virB6-3} (100% ± 6.67%), \textit{virB6-4 5'} end (100% ± 14.65%) and \textit{virB6-4 3'} end (100% ± 12.79%) (Fig. 5B). Initially, for gene expression data normalization, we also evaluated transcripts from the \textit{msp5} gene. However, during this analysis, we observed decreased transcription of this gene in the \textit{virB6-4::Himar1} mutant (Mean ± SD, 78.44% ± 11.66%) relative to wt bacteria (99.91% ± 3.41%) (Fig. S3), suggesting that Himar1 insertion within \textit{virB6-4} may also have \textit{trans}-acting effects on the expression of additional, more distant, genes.

To determine whether decreased mRNA levels from \textit{virB6-3} and \textit{virB6-4} genes in the \textit{virB6-4::Himar1} mutant correlate with altered VirB6-3 and VirB6-4 protein levels, we performed Western blot analysis using
polyclonal rabbit anti-VirB6-3 and anti-VirB6-4 sera. To be sure that observed changes in VirB6-3 and VirB6-4 protein levels in the virB6-4::Himar1 mutant relative to wt bacteria are due to altered protein expression rather than differences in sample loading, we used polyclonal dog serum against A. phagocytophilum, with antibody responses mainly targeting the dominant surface protein MSP2/P44 (42-44KDa), as loading control. Western blot indicated a reduction in VirB6-3 expression in the virB6-4::Himar1 mutant when compared to wt (Fig. 6A). The ratio of the amount of VirB6-3 in the virB6-4::Himar mutant relative to the VirB6-3 amounts in the wt was determined by densitometry, normalized to the signal obtained from the MSP2/P44 band and converted to percentage. This indicated decreased levels of VirB6-3 expression in the virB6-4::Himar1 mutant (Mean ± SD, 24.27% ± 14.82%) relative to wt (100% ± 14.43%). VirB6-4 protein was detected only in protein samples form wt organisms but not in the virB6-4::Himar1 mutant (Fig. 6B). Bands of sizes corresponding to VirB6-3 and VirB6-4 proteins were not observed in samples reacted with the negative control rabbit pre-immune sera (Fig. 6A, B). Consistent with Western blot analysis, dual immunofluorescence of HL-60 infected with the virB6-4::Himar1 or wt strain indicated a dramatic reduction in the signals from VirB6-3 epitopes in the virB6-4::Himar1 mutant relative to wt-infected cells (Fig. 6C). Likewise, fluorescent signals from VirB6-4 epitopes in cells infected with the virB6-4::Himar1 were virtually absent when compared to wt-infected cells (Fig. 6D).

The virB6-4::Himar mutant has a reduced proliferation in vitro

We further evaluated the growth kinetics of the virB6-4::Himar1 mutant vs. wt by determining the numbers of A. phagocytophilum genome equivalents per cell (ApGE/cell), using duplex qPCR. For this, we used specific primers and probes targeting the A. phagocytophilum msp5 gene and the toll-like receptor 9 (tlr9) gene in HL-60, or the calreticulin (crt) gene in ISE6 cells. During the course of infection in HL-60 cells, the virB6-4::Himar1 mutant maintained a reduced growth as evidenced by significantly lower numbers of ApGE/cell (Mean ± SEM, 28.82 ± 1.870) (P= 0.0317) at day 1 p.i and (51.11 ± 5.966) (P= 0.0035) at day 6 p.i, relative to ApGE/cell in cells infected with wt bacteria at the same time points (43.26 ± 4.05 and 164.2 ± 17.28 on days 1 and 6 p.p., respectively; Fig. 7A). On the other hand, during the first 3 days p.i., there were no significant differences in the numbers of ApGE/cell in ISE6 cells infected with the virB6-4::Himar1 mutant relative to wt.
However, after day 3 p.i., the growth of the mutant declined, and by day 12 p.i., the numbers of ApGE/cell were significantly lower (Mean ± SEM, 49.68 ± 15.6) ($P = 0.0039$) than in cells infected with wt bacteria (184.35 ± 4.43) (Fig. 7B).

Given the reduced growth exhibited by the $\text{virB6-4}:\text{Himar1}$ mutant, we expected decreased levels of host cell death. To determine this, aliquots of cell culture supernatants collected from $\text{virB6-4}:\text{Himar1}$-, wt-infected HL-60 or ISE6 cells, were used to measure the levels of host-cell lactate dehydrogenase (LDH cytotoxicity assay) released into the cell medium due to the pathogenic effects exerted by the bacteria. Effectively, the reduced burden of $\text{virB6-4}:\text{Himar1}$ organisms in HL-60 cells was associated with decreased host cell death, as there was a significantly reduced level of LDH in supernatants from $\text{virB6-4}:\text{Himar1}$-infected HL-60 relative to wt-infected cells at all tested times p.i. (Fig. 7A). In contrast, the reduced infection of ISE6 cells with the $\text{virB6-4}:\text{Himar1}$ mutant did not correlate with decreased host cell death as the levels of LDH released in supernatants from infected cells were not significantly different from wt-infected cells at all tested times p.i. (Fig. 7B).
DISCUSSION

The role of the *A. phagocytophilum* T4SS during infection of human host and tick vector cells remains unclear. Here, we show that all four *virB6* paralogs of *A. phagocytophilum* are co-transcribed into a polycistronic message that includes *sodB*, *virB3* and *virB4-1* during *in vitro* infection of mammalian and tick cells. *VirB6-1*, *VirB6-2*, *VirB6-3* and strikingly, a large *VirB6-4* protein of ~470 kDa, were also detected in infected cultures.

Given the unusual C-terminal structural extensions of *VirB6-3* and *VirB6-4* in *A. phagocytophilum*, we focused most of our work on characterization of the expression and potential subcellular localization of these two proteins. Evidence obtained from this work suggests that these proteins have evolved to serve a fundamentally different function in Rickettsiales than in *A. tumefaciens*. For example, dual labeling of *A. phagocytophilum* suggests that *VirB6-3* and *VirB6-4* are distributed throughout the bacterial cell membrane in infected HL-60 and ISE6 cells, whereas in *A. tumefaciens* *VirB6* is mainly localized to a single pole (30).

Surface trypsin digestion of host-cell free intact *A. phagocytophilum* provided evidence that major C-terminal domains of *VirB6-3* and *VirB6-4* are surface-exposed. This also contrasts with *A. tumefaciens*, where *VirB6* consists of a periplasmic N-terminus, four to five transmembrane domains and a cytoplasmic C-terminus (26, 29). Topology similar to that of *A. tumefaciens* was also reported for *VirB6* in *Brucella* (42) and ComB6 (*VirB6* homolog) in *H. pylori* (43). Although further experimental evidence is required, surface trypsin digestion data suggest that the C-termini of *A. phagocytophilum* *VirB6-3* and *VirB6-4* are surface exposed on the outer membrane, as the peptide sequences used for the preparation of antibodies were derived from *VirB6-3* and *VirB6-4* C-terminal repeat sequences. It has been proposed that the extended C-terminus may be cleaved and released into the target cell to effect regulatory functions (4, 15). This localization would optimize such a transfer. Hence, these data provide initial experimental evidence for an extracytoplasmic extended C-terminus in polytopic “extended VirB6-like proteins” in bacteria of the P-T4SS group. Extended C-terminal domains have been found on the F-T4SS of *Escherichia coli* (TraG) and SXT-ICE (TraGSXT) of *Vibrio cholerae*. These C-terminal hydrophilic domains function during mating pair formation in association with TraS protein that is
found in the inner membrane of the donor of the pair in a process known as entry exclusion to block transfer of redundant DNA (14).

Conforming to other Rickettsiales (20), bioinformatics analysis indicated that sequence conservation of *A. phagocytophilum* VirB6 paralogs relative to VirB6 from *A. tumefaciens* is restricted to the TMDs and a central cytoplasmic loop. In addition, this analysis predicted SP sequences for VirB6-1 and VirB6-2 and identified VirB6-3 and VirB6-4 as putative lipoproteins given the presence of conserved lipobox signal sequences. Despite the importance of lipoproteins in physiology, virulence, and host immune evasion, very little is known about their role in *A. phagocytophilum*. In other Rickettsiales, such as *E. chaffeensis* (44) and the *Wolbachia* symbiont of filarial nematodes (38) VirB6-3, but not VirB6-4, has also been identified as a putative lipoprotein. Moreover, antibodies reacting with VirB6-3 proteins from *E. chaffeensis* and *Wolbachia* species were detected in sera from dogs infected with *E. chaffeensis* (44) and in sera from humans infected with the filarial nematode *Wuchereria bancrofti* (38). This is additional evidence that in Rickettsiales this protein is not only surface exposed, but also induces immune responses. We noted that *A. phagocytophilum* contains genes that encode proteins required for lipoprotein biosynthesis such as the prolipoprotein diacylglycerol transferase (Lgt, GenBank accession AGR79154) and the lipoprotein signal peptidase (LspA, GenBank accession AGR79052.1) but not the apolipoprotein N-acyltransferase (Lnt/CutE). It is known that *Wolbachia* (45) only synthesizes diacyl-lipoproteins which are involved in triggering of inflammatory responses (46). Collectively, the fact that VirB6-3 and VirB6-4 are surface proteins suggests that they could be targeted as potential vaccine candidates against closely related *A. phagocytophilum* strains.

Transposon mutagenesis using the Himar1 system followed by high-throughput genome sequencing to identify Tn insertion loci in these transformants, identified a population of mutants carrying the transposon sequences within the coding region of the *virB6-4* gene. Relative gene expression experiments demonstrated that insertion of transposon sequences into *virB6-4* not only resulted in the loss of its expression but also altered the expression of upstream genes, including *sodB, virB6-1, virB6-2* and *virB6-3*, suggesting that gene expression from the entire operon (including *virB3* and *virB4-1*) was disrupted. As the transposon sense strand is found in
the opposite orientation to \textit{virB6-4} it is possible that transcriptional readthrough beyond Tn sequences generated antisense transcripts that reduced the expression of sequences upstream of \textit{virB6-4}. Similar outcomes obtained by Himar1 insertion have been observed in several bacterial species including tick-borne bacteria (47-49). Consistent with gene expression data, Western blot and immunofluorescence indicated the absence of VirB6-4 and decreased levels of VirB6-3 in the \textit{virB6-4::Himar1} mutant relative to wt \textit{A. phagocytophilum}. Importantly, our data show that the disruption of \textit{virB6-4} and the altered expression of upstream genes significantly decreased \textit{A. phagocytophilum} bacterial infection in human and tick cells \textit{in vitro}, indicating the important role of these T4SS components in the intracellular survival of \textit{A. phagocytophilum} in these two different environments. Further work is required to determine whether reduced infection is due to a decreased replication or failure to evade host killing mechanisms such as avoidance of lysosomal degradation or failure to escape oxidative damage (50).

Given the polar effect exerted by the Himar1 insertion within the \textit{virB6-4} gene it is not possible to assign the mutant phenotype to this gene alone as expression of the other \textit{virB} paralogs, \textit{virB3}, \textit{virB4-1} and \textit{sodB} were also affected. However, it is possible that the altered expression levels of T4SS components present in this operon may affect the abundance of other T4SS components, impairing the assembly and function of the secretion system, and resulting in poor growth. A similar effect has been observed in other bacterial species, such as \textit{A. tunefaciens} and \textit{Brucella sp.}, carrying mutations in T4SS components, specifically \textit{virB6} mutations (25, 51). In \textit{Brucella} species, VirB3 and VirB6 are essential for persistence in mice (25). Another important consequence of disruption of \textit{virB6-4} relates to the observed reduced expression of \textit{sodB} in the \textit{virB6-4::Himar1} mutant as shown here. Expression of superoxide dismutase (SOD) in pathogenic bacteria contributes to virulence as it helps them to escape killing by reactive oxygen species released by the host defense mechanisms (52, 53). In that context, it is worth noting that the \textit{virB6-4::Himar1} mutant showed reduced expression of \textit{sodB}. It is known that \textit{A. phagocytophilum} inhibits NADPH oxidase, thus avoiding killing by toxic oxygen intermediates (50, 54-57). However, it is not known which \textit{A. phagocytophilum} effector proteins participate in this inhibitory process. It is possible that \textit{A. phagocytophilum} uses SOD to protect itself from oxidative damage,
and reduced expression of this protein as shown for the virB6-4::Himar1 mutant, may contribute to impaired proliferation in infected cells.

The isolation of the virB6-4::Himar1 mutant with an altered expression pattern in the sodB-virB5s operon supports the contribution of these proteins to A. phagocytophilum infection. Reduced transcript levels from msp5 in the virB6-4::Himar1 mutant may not only reflect modification or remodeling of membrane protein components but also that genes from the sodB-virB5s operon are possibly linked to regulation of additional genes. Therefore, comparative proteomics of virB6-4::Himar1 mutant and wt A. phagocytophilum will be of great significance to define differentially expressed proteins and pathways associated with the slow growth phenotype of this mutant. Additionally, expression of a defective T4SS will not only help to understand how the T4SS functions but also aid in the identification of T4SS substrates, and will clarify the role of this operon in persistent infection.

The Order Rickettsiales includes pathogens of high importance in human and veterinary health, and potentially, bioterrorism. Despite their genetic diversity, the different cellular niches they occupy and the variety of hosts they infect, an element common to members of this Order is the synteny of the virB3, virB4 and virB6 paralogs (4 copies in Anaplasmataceae, 5 copies in Rickettsiaceae) that are arranged in tandem within an operon unit (12). This genomic organization is most likely due to functional constrains. Hence results obtained working with A. phagocytophilum may also be applicable to other Rickettsiales. Our findings are of particular significance as they provide the first evidence for surface exposure of the intriguing C-terminal repeat extensions of VirB6-3 and VirB6-4. The characterization of a mutant with an insertion into the virB6-4 gene offers insights about the key role that these T4SS components play in infection of mammalian and tick cells, and will facilitate more detailed analysis of the T4SS structural components and the effectors they transport.
MATERIALS AND METHODS

Cultivation of *Anaplasma phagocytophilum*

The *A. phagocytophilum* strains used for this work were the wt HZ (58), wt HGE1 (39), and the HGE1 mutants *virB6-4::Himar1* and the *120879::Himar1* (used for trypsin digestion to confirm cell surface proteins). Two cell lines were used to propagate these bacteria, i.e., HL-60 human promyelocytic cells (ATCC CRL-240) and tick ISE6 cells (ATCC CRL-11974) derived from embryonated eggs of the blacklegged tick, *Ixodes scapularis*. Uninfected and wt or mutant *A. phagocytophilum* infected HL-60 cells were maintained in RPMI 1640 medium (Thermofisher Scientific) supplemented with 10% heat inactivated fetal bovine serum (FBS, BenchMark, Gemini Bio-Products), 2 mM L-Glutamine (Life technologies), 0.25% NaHCO₃ (Sigma-Aldrich) and 25mM HEPES (Sigma-Aldrich), and were kept at 37 °C in a 5% carbon dioxide (CO₂) atmosphere. Uninfected ISE6 cultures were maintained in L-15B300 medium prepared as described (59) and supplemented with 5% FBS, 5% tryptose phosphate broth (TPB, Difco, Becton Dickinson) and 0.1% bovine lipoprotein concentrate (LPC, MP-Biomedical). Infected ISE6 cells were maintained in LB300 medium additionally containing 0.25% NaHCO₃, and 25 mM HEPES buffer.

RNA extraction and Reverse transcription-PCR (RT-PCR).

Total RNA was isolated from infected HL-60 and ISE6 cells using the RNeasy kit (QIAGEN) with an added “on-column” DNase I treatment (QIAGEN) as per manufacturer’s instructions and (1µg) from each sample converted to cDNA by random hexamer priming using the Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics) as per manufacturer instructions. Fluorescence-based RNA concentrations were determined by using the Qubit RNA HS Assay kit (ThermoFisher Scientific) with a Qubit fluorometer (ThermoFisher Scientific). Specific primers (Table 2) were designed to amplify transcripts from each gene component of the *sodB-virBs* operon or intergenic regions. All reactions included primers targeting transcripts from the *msp5* and 16S rRNA genes as internal controls and to ensure integrity of RNA/cDNA. Each reaction contained 200 ng of cDNA combined with 200 µM dNTP’s, 0.25 µM of forward and reverse primers and 1.25 units of PrimeSTAR GXL DNA polymerase (Takara). PCR amplification conditions were as follows: 94° C for 2 min; 30 cycles of
denaturation 98° C for 10 sec, annealing at 55° C for 15 sec and extension at 68° C for 1 min; and a final extension step at 68° C for 7 min. PCR products were electrophoretically separated using a 2% Seakem LE (Lonza) agarose gel, and stained with SYBR Gold nucleic acid gel stain (ThermoFisher Scientific) for UV visualization. Genomic DNA and samples of reactions with no reverse transcriptase for each target were used as positive and negative controls, respectively.

**RT-qPCR.**

Transcript differences between *sodB*, *virB6-1*, *virB6-2*, *virb6-3* and *virB6-4-5’* and -3’ ends in *A. phagocytophilum* *virB6-4*:Himar1 mutant relative to wild-type were determined using RT-qPCR, and the results were based on the mean of three biological replicates (individual RNA extracts). For SYBR green quantitative PCR, cDNA obtained from HL-60 cells infected with *A. phagocytophilum* wt or the *virB6-4*:Himar1 was used with primers (Table 2) designed to amplify *sodB*, *virB6-1*, *virB6-2*, *virb6-3* and *virB6-4-5’* or 3’ ends, *msp5*, *groEL* and the *rpoB* gene sequences. Reactions were performed using the hot-start LightCycler-FastStart DNA Master SYBR Green I (Roche Diagnostics) in a LightCycler 96 instrument (Roche Diagnostics). Twenty µl reaction mixture contained 1X LightCycler FastStart DNA Master SYBR Green I, 0.4 µM of primers and 2 µl of template (~20 ng cDNA). No template (NTC) and no reverse transcriptase (-RT) control samples to assess DNA contamination were included for all reactions. The amplification program contained the following conditions: 95 °C for 10 min: 45 cycles of 95 °C for 10 sec, 60 °C for 30 sec. After amplification, a melting curve was acquired by heating of the product at 4.4 °C/s to 95 °C for 10 sec, cooling it at 2.2 °C/s to 65 °C for 60 sec, and then slowly heating at 0.1 °C/s to 97 °C). After melting curve acquisition, reactions were cooled at 37 °C for 30 sec. Significant differences between the *A. phagocytophilum* *virB6-4*:Himar1 mutant vs. wt were calculated using Student’s t test (*P* < 0.05), comparing ∆Ct values (target gene - reference gene) of the *virB6-4*:Himar1 mutant and the wild-type, and calculated based on the Pfaffl formula (60) which yields the gene expression ratio. The expression ratio was then expressed as percentage of expression by multiplying the gene expression values by 100. The geometric mean of ∆Ct values from the reference genes *rpoB*, and *groEL* was used for gene expression normalization. Transcript amplification
efficiencies for each target were sodB (87%), virB6-1 (99%), virB6-2 (97%), virB6-3 (100%), virB6-4 5’ end 
(96%), virB6-4 (100%), msp5 (93%), rpoB (100%), groEL (91%).

**VirB6 synthetic peptides and antibodies**

Specific antibodies were raised against *A. phagocytophilum* str. HZ VirB6 paralogs using synthetic peptides 
derived from predicted antigenic epitopes using the protein analysis and peptide prediction programs TMpred 
(61), Phobius (62), BcePred (B-cell epitope prediction of Continuous B-Cell Epitopes in Antigenic Sequences 
Using Physico-chemical Properties) (63), LEPS (Linear Epitope Based on Propensity Scale) (64), and the 
antigenicity predicting tools from EMBOSS (65). Peptide sequences (56-70 residues) (Table 3) were selected 
on the basis of their predicted hydrophilicity, antigenic propensity, flexibility, surface probability and lack of 
sequence identity with other *A. phagocytophilum* proteins based on BLASTP alignments (66).

VirB6-1 through VirB6-4 peptides were synthesized commercially by LifeTein, NJ. An amino-terminal cysteine 
was included for conjugation, and 4 mg of each peptide was prepared (2 mg was conjugated to Keyhole Limpet 
Hemocyanin, and 2 mg to ovalbumin). Production of polyclonal antibodies against the VirB6-1, VirB6-2, 
VirB6-3 and VirB6-4 was done by Pocono Rabbit Farm and Laboratory Inc, PA. Each rabbit was 
subcutaneously injected with 200 μg of Keyhole Limpet Hemocyanin (KLH)-conjugated peptide emulsified in 
Complete Freund’s Adjuvant. At days 14 and 28 post-immunization the rabbits were boosted by subcutaneous 
injection of 200 μg of peptide conjugates in incomplete Freund’s adjuvant. Affinity purified anti-VirB6 
bodies were prepared by immobilization of sulfhydryl-containing peptides using a commercially available 
coupling resin (SulfoLink coupling resin, ThermoFisher Scientific) as per manufacturer instructions (67). All 
animal work was performed in accordance with the standards and approval of the University of Florida 
Institutional Animal Care and Use Committee.

To determine titers and the specificity of the sera for the VirB6 paralogs we used an Enzyme-Linked 
Immunosorbent Assay (ELISA) in an antibody-capture format. For this, 96-well MaxiSorp microtiter plates 
(Nunc) were coated with 1 μg/well ovalbumin or 2 μg/well of VirB6-ovalbumin conjugated peptide. Pre- and 
post-immune serum from each rabbit was serially diluted tenfold $10^2$ to $10^5$. All assays were performed in
triplicate and antibody binding was detected with recombinant protein A/G conjugated to alkaline phosphatase. Color was developed using 4-nitrophenol phosphate and the optical densities were measured using an ELISA plate reader at 405 nm.

Detection of VirB6 paralogs by immunofluorescence

Antigen slides were prepared from *A. phagocytophilum* infected HL-60 and ISE6 cells essentially as described (68). Briefly, infected cells were washed twice in 1X PBS and collected by centrifugation at 200 x g for 5 min at room temp (RT) and fixed in 1 mL of 4% paraformaldehyde, 0.0075% glutaraldehyde in PBS for 30 min at RT, then washed in PBS, and permeabilized in ice-cold Nonidet P-40 (NP-40) 0.3% for 10 min. After washing with 1X PBS, the samples were blocked with 1 mL of blocking buffer (5% Bovine Serum Albumin, BSA, and 1% of Normal Goat Serum in PBS) and incubated for 3 h at RT. After blocking, cells were aliquoted into five 1.5 mL centrifuge tubes and collected by centrifugation at 200 x g for 5 min at RT. Dual *A. phagocytophilum* staining was done with a mixture of two primary antibodies: mouse *A. phagocytophilum* positive serum (1:320) and the appropriate anti-VirB6 serum as follows: unpurified anti-VirB6-1 serum (1:640), unpurified anti-VirB6-2 serum (1:640), affinity purified anti-VirB6-3 and anti-VirB6-4 antibodies at a concentration of 0.208 mg/mL and 0.232 mg/mL, respectively, or pre-immune rabbit serum (1:640), or protein A purified 0.768 mg/ml pre-immune serum. (negative control). All samples were incubated overnight at 4 °C. After incubation with primary antibodies, the samples were collected by centrifugation as above and washed 3 X with washing buffer (1% BSA and 0.1% Tween 20 in PBS) followed by incubation for 1.5 h at RT with goat anti-rabbit IgG-Alexa fluor 568 conjugated antibody (ThermoFisher Scientific), and goat anti-mouse IgG-Alexa fluor 488 conjugated antibody (ThermoFisher Scientific), both at a dilution of 1:800. The samples then were washed as described above followed with a final wash with 1X PBS and mounted with ProLong Gold Antifade Reagent with DAPI (4',6-diamidino-2-phenylindole dihydrochloride, (ThermoFisher Scientific).
Immunoblots.

*A. phagocytophilum* organisms were purified from infected HL-60 cells as described previously (69). Host cell-free bacterial samples used for immunoblots were resuspended in 1 X protein stabilizing cocktail (ThermoFisher Scientific), and stored at −80°C.

To determine the expression of VirB6-3 and VirB6-4 proteins in wt *A. phagocytophilum* or the *virB6-4*:Himar1 mutant, host-cell purified bacteria were solubilized with n-dodecyl-D-maltoside (DDM) lysis buffer (DDM 1.50%, 1 X Complete proteinase inhibitor (Roche Diagnostics), MgCl₂ 2mM, NaCl 150 mM, Tris 25 mM and lysozyme 1 mg/ml) for 20 min at 4 °C on a rotator, followed by sonication as described previously (60) using the Qsonica sonicator Q-125A-110 (Newtown, CT). After sonication, lysates were centrifuged for 10 min at 12,000 x g at 4 °C. Total protein concentration was determined using the Non-Interfering Protein Assay (NI Protein Assay) kit (G-Bioscience) as per manufacturer’s instructions. Equal amounts of proteins were loaded per lane and separated on NuPAGE 3-8% Tris acetate gels (ThermoFisher Scientific), then transferred to polyvinylidene difluoride (PVDF) membranes for immunoblotting. Membranes were cut into strips and probed either with pre-immune or post-immunization rabbit anti-VirB6-3, anti-VirB6-4, or *A. phagocytophilum* infected dog serum diluted 1:10³ fold, followed by incubation with 10 ng/ml of HRP conjugated goat anti-rabbit (SeraCare) or HRP conjugated anti-dog (1:150,000) antibodies. Antibody reactions against VirB6-3, VirB6-4 or *A. phagocytophilum* MSP2/p44 were visualized with the chemiluminescent substrate SuperSignal West Femto (ThermoFisher Scientific).

Surface Trypsin digestion of intact *A. phagocytophilum*.

Freshly-collected *A. phagocytophilum* str. HGE1 120879::Himar1 mutant bacteria, released from HL60 cells, were subjected to trypsinolysis (70), suspended in PBS, and split into aliquots. Sequencing Grade Modified Trypsin (Promega) and Tris 50mM pH 8, at final concentrations of 0.05 mg/ml 1.25 mM, respectively, were added to the trypsin-treated sample. A trypsin-untreated sample received Tris 50mM pH 8 at a final concentration of 1.25 mM. Both samples were incubated at 37 °C for 15 min with periodic gentle mixing. After trypsinolysis, the protease inhibitor phenylmethylsulfonyl fluoride 100 mM (PMSF) (final concentration of 6.25
mM) was added to both samples and incubated 10 min to inactivate trypsin. After incubation, bacteria from both samples were washed 3 X with 320 µl of wash solution (300 µl Tris and 20 µl PMSF) and pelleted by centrifugation at 12,000 x g for 10 min at RT. Final bacterial pellets were solubilized and proteins resolved as described above. Membranes were cut into strips and screened with anti-VirB6-3, anti-VirB6-4, and anti-mCherry antibodies (1:1000 mouse anti-mCherry, Novus Biologicals).

**Isolation of virB6-4::Himar1 mutant.**

A library of mCherry-tagged *A. phagocytophilum* mutants was generated by Tn mutagenesis using the Himar1 system. The pHimarcisA7mCherry-SS plasmid (49) was introduced into the *A. phagocytophilum* str. HGE1 by electroporation as described previously (40). Following electroporation, bacteria were incubated with HL-60 cells and diluted into three 96-well tissue culture plates and kept under spectinomycin/streptomycin antibiotic selection (40). Wells containing cells infected with red fluorescent bacteria were expanded into 12-well tissue culture plates. A total of 900 mutants were obtained and frozen stocks and DNA from each individual culture were archived. Transposon insertion sites were determined by High-throughput genome sequencing of pooled DNA. Briefly, DNA from 25 cultures was combined into one pool, for a total of 36 pools, barcoded and sequenced on a single lane of an Illumina HiSeq 2000 instrument. Raw Illumina sequencing data were processed and analyzed as described previously (49) using the open source GALAXY platform located at the University of Florida web site [http://galaxy.hpc.ufl.edu](http://galaxy.hpc.ufl.edu). A total of 35,467 reads containing *A. phagocytophilum* genome-Tn junctions were identified using the *A. phagocytophilum* str. HGE1 (GenBank accession # APHH01000000) genome and the Himar1 inverted repeat sequences as references. Through this analysis a total of 1,200 transposon insertion sites were identified.

Since the DNA used for Tn-seq corresponds to pooled DNA, a PCR screen using primer pairs that bind upstream and downstream of the Tn insertion site within the *virB6-4* gene, was used to match this particular mutant to its frozen stock. Reactions used PrimeStar GXL DNA polymerase (Takara) as per manufacturer instructions and PCR conditions were as follows: 94 °C for 2 min; 30 cycles of denaturation (98° C for 10 sec), annealing at 60 °C for 60 sec and extension at 68 °C for 3 min; and a final extension step at 72 °C for 5 min.
PCR products were run on a 1.0% agarose gel and stained with SYBR gold nucleic acid stain. Agarose gel electrophoresis analysis of PCR products showed a PCR product consistent with the size (2,202 bp) of the Tn insertion within the targeted region in the DNA from transformant stock culture #667 (data not shown). A DNA band of the size of the wild-type locus (368 bp) was also detected in the same stock indicating the presence of a background mutant population with other Tn insertions. Cloning by limiting dilution resulted in the isolation of a clonal virB6-4::Himar1 mutant population.

**Anaplasma phagocytophilum growth curves and cytotoxicity assay.**

We compared the growth rate of *A. phagocytophilum virB6-4::Himar1* mutant to wild-type in HL-60 and ISE6 cells. On the day of infection, 1 ml of uninfected HL-60 cells was seeded in two 24-well tissue culture plates at a density of 3 x 10^5 cells/ml. *A. phagocytophilum* wt and *virB6-4::Himar1* bacteria were purified from heavily infected HL-60 cells (>80% cells contained morulae) and resuspended in 1.2 ml of RPMI 1640. To quantify isolated bacteria, we performed a live/dead-staining assay (Thermofisher Scientific). As the excitation/emission wavelengths from propidium iodide (PI) dye overlap with those of mCherry, the PI was replaced with Sytox-blue (ThermoFisher Scientific), to calculate the number of dead cells. Each well was inoculated with host cell-free wt or *virB6-4::Himar1* organisms at multiplicity of infection (MOI) of 10 (~3x10^6 organisms per well) and incubated at 37 °C in the presence of CO_2_. After 24 h the inoculum was removed and replaced with 1 ml of fresh RPMI 1640 medium. Triplicate wells of infected cells with each *A. phagocytophilum* strain were harvested at different time points (1, 2, 3, 4, 5 and 6 days) post-infection (p.i.).

To evaluate growth of *virB6-4::Himar1* vs. wild-type in ISE6 cell cultures, two days before infection, two 24-well tissue culture plates were seeded with 1 ml of uninfected ISE6 cells (3x10^5 cells/ml). On the day of infection, bacteria were purified and resuspended in 1.2 ml of L-15B3SE6 cells. Each well was inoculated with host-cell free wild-type or *virB6-4::Himar1* bacteria at an MOI of 10. The next day the medium was replaced and infected cells were collected at different time points (1, 3, 6, 9 and 12 days) p.i. HL-60 and ISE6 infected with *A. phagocytophilum* wt or *virB6-4::Himar1* mutant harvested at different times p.i. were processed for DNA.
extraction, using the quick-gDNA Kit (Zymo Research) as per manufacturer’s instructions, and the supernatant used for cytotoxicity assays.

Toxic cell damage in HL-60 and ISE6 cells due to *A. phagocytophilum* infection was measured by quantitatively determining the release of lactate dehydrogenase (LDH) into the medium using the Cyto Tox 96 Non-radioactive Cytotoxicity Assay (Promega) following manufacturer’s instructions. Cell culture supernatants from infected cultures were collected at above indicated time points p.i.. All assays were performed in triplicate and LDH activity was calculated by measuring optical densities at 490 nm using the Synergy HT plate reader (BioTek Instruments). Final absorbance values were determined after subtracting background values obtained from medium only or no-cell control wells. To calculate LDH release, supernatants from wells containing uninfected cells (spontaneous LDH activity controls), and from wells containing uninfected cells lysed with 10X lysis buffer (maximum LDH activity controls) were added. Hence, cytotoxicity is expressed as 100 \( \times \) \[\frac{\text{(LDH activity from infected culture- spontaneous LDH activity controls)}}{\text{(maximum LDH activity controls - spontaneous LDH activity controls)}}\].

**Quantitative PCR (qPCR).**

Duplex qPCR was used to determine *A. phagocytophilum* GE per host cell as measure of its growth in cell culture. Quantitation of *A. phagocytophilum* GE was performed by targeting the single copy gene *msp5* (Table 2). To normalize bacteria numbers, we used primers and probes targeting the single copy gene toll like receptor 9 (*tlr9*), in HL-60 cells, and calreticulin (*crt*), in ISE6 cells. Triplicate reactions with DNA from three experimental wells were used. Reactions of 20 μL containing 2 μL of genomic DNA, 1X of LightCycler Multiplex DNA Master mix (Roche Diagnostics), 0.4 μM forward and reverse primers and 0.2 μM of probe were used for amplification in a LightCycler 96 instrument (Roche Diagnostics) with the following conditions, 95 °C for 30 sec and 45 cycles of 95 °C for 5 sec, and 60 °C for 30 sec (data collection). To allow for equimolar ratio of templates for standard curve preparation, a linear ds-DNA fragment (Eurofins Genomics) carrying *msp5*, *tlr9* and *crt* sequences was used. *A. phagocytophilum* and host cells GE number were calculated based on the standard curve.
Statistical analysis

Data are expressed as Mean ± SE values. Unpaired two-tailed t test analysis were used for comparison of growth and cytotoxicity of *A. phagocytophilum* wild-type vs. virB6-4::Himar1 mutant. Statistical comparisons and graphics were made with GraphPad Prism (GraphPad Software Inc., La Jolla, CA).
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AFB, UGM and DRA supervised this project, contributed to manuscript writing, and helped with critical revisions. MJH generated the *A. phagocytophilum* Tn mutants library and CN isolated the *virB6-4*:Himar1 mutant. AFB performed bioinformatics analysis to identify Tn insertion sites. AML performed Western blots and Y-PX provided technical assistance. FLC conceived and authored this manuscript, carried out experimental design, acquired data and interpreted results.

All authors read and approved the final manuscript.
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Figures Legends

**Fig. 1.** *A. phagocytophilum* virB6 paralogs are co-transcribed in mammalian and tick cells. A. Organization of the sodB-virBs operon in *Anaplasma phagocytophilum*. Black lines indicate location of primers designed to amplify transcripts from intergenic regions; sodB-virB3, virB3-virB4-1, virB4-1-virB6-1, virB6-1-virB6-2, virB6-2-virB6-3 and virB6-3-virB6-4. Red lines indicate binding sites for primers designed to amplify transcripts from sodB, virB3, virB4-1, virB6-1, virB6-2, virB6-3, and the 5' and 3' ends of virB6-4. Black and white boxes depict the repeats region of virB6-3 and virB6-4. The sodB-virB’s operon was drawn to scale using SnapGene software (from GSL Biotech; available at snapgene.com). B. Agarose gel analysis of complementary DNA (cDNA) products from sodB-virBs operon intergenic regions. RT-PCR was performed on total RNA isolated from *A. phagocytophilum*-infected HL-60 cells. C. Agarose gel analysis of complementary DNA (cDNA) products from sodB through virB6-4. RT-PCR was performed on total RNA isolated from *A. phagocytophilum*-infected HL-60 and ISE6 cells. Genomic DNA was used as positive control and reactions without reverse transcriptase (-RT) were used as negative controls. 100 bp/1kb DNA ladder. The msp5 and 16S rRNA genes were used as internal controls to ensure integrity of RNA/cDNA. Data obtained from two independent experiments.

**Fig. 2.** *A. phagocytophilum* (Ap) VirB6 proteins are synthesized in infected HL-60 and ISE6 cells. Representative dual immunofluorescence images of A. HL-60 and B. ISE6 cells infected with *A. phagocytophilum*. To indicate immunolabeling of VirB6-3 and VirB6-4 boxes delineated by the green dotted lines are magnified in the insets. Infected cells were fixed and viewed by indirect immunofluorescence microscopy to determine immunoreactivity with *A. phagocytophilum* infected mouse sera (green) and anti-VirB6-1, -VirB6-2, -VirB6-3 and -VirB6-4 sera (red). Host cell nuclei and bacterial DNA were stained with DAPI (blue). Pre-immune rabbit serum was used as negative control. RG2B Colocalization ImageJ plug-in (CP Mauer, Northwestern University) was used to assess the overlap between the green and red channels. Representative images are shown from two independent experiments.
Fig. 3. VirB6-3 and VirB6-4 proteins are located on the surface of *A. phagocytophilum*. A. Proteins from *A. phagocytophilum* isolated from HL-60 were separated by Tris acetate polyacrylamide gel electrophoresis. For immunoblotting, proteins were transferred to PVDF membranes and reacted with rabbit pre-immune serum (lane 1) and rabbit anti-VirB6-4 or anti-VirB6-3 serum (lanes 2 and 3). B and C. Intact *A. phagocytophilum* organisms were incubated with (+) or without (-) trypsin, solubilized, and equal amounts of proteins were separated by Tris acetate polyacrylamide gel electrophoresis. PVDF membranes with transferred proteins were processed for detection. B. Rabbit anti-VirB6-3 serum (lanes 2 and 4), rabbit pre-immune serum (lanes 1 and 3), normal mouse serum (NMS) (lanes 5 and 7) and polyclonal mouse anti-mCherry antibodies (lanes 6 and 8). C. rabbit anti-VirB6-4 serum (lanes 10 and 12), rabbit pre-immune serum (lanes 9 and 11), normal mouse serum (NMS) (lanes 13 and 15) and polyclonal mouse anti-mCherry antibodies (lanes 14 and 16). MW indicates protein standards. Immunoblots representative from three independent experiments.

Fig. 4. Himar1 Tn insertion site in the *A. phagocytophilum* virB6-4 gene. A. Artemis (genome browser and annotation tool, Wellcome Sanger Institute, UK) window showing *A. phagocytophilum* wild-type virB6-4 gene used as a reference for the location of the Himar1 insertion site. The first panel shows a “zoomed out” view of the virB6-4 DNA sequence translated into six reading frames with black vertical bars depicting stop codons. The open reading frame (ORF) for virB6-4 is shown in light blue. The black arrow indicates the Himar1 insertion location, 5’ upstream the 3’ end tandem repeats. The yellow box is “zoomed in” in the panel below to show in detail the virB6-4 sequences flanking the Himar1 Tn (yellow highlight), including the TA dinucleotide site required for successful transposition (red arrow). B. Paired-end DNA sequencing reads indicating the same *A. phagocytophilum* sequences as above (yellow highlight) and Himar1 Tn sequences (italics). C. Artemis window of in silico cloning of the Himar1 Tn in the opposite orientation to virB6-4. Black vertical bars within the virB6-4’s ORF denote putative stop codons.

Fig. 5. Deletion of virB6-4 affects transcription of upstream genes. A. Binding sites of primers designed to target transcripts from sodB, virB6-1, virB6-2, virB6-3 and the 5’ and 3’ ends of virB6-4 B. Changes in expression of these genes were calculated based on geometric mean of the ΔCT values from reference genes,
and the results were expressed as percentage of expression, with a 100% expression level being assigned to the control group, in this case, wt *A. phagocytophilum*. Transcripts from the *rpoB* and *groEL* genes were used as reference for data normalization. Bar lengths represent the percentage of expression of *sodB*, *virB6-1*, *virB6-2*, *virB6-3* and the 5’ and 3’ ends of *virB6-4* in *A. phagocytophilum* (wt) (grey bars) and *virB6-4::Himar1* mutant (white dotted bars). *, *P* = 0.01 to 0.05; **, *P* = 0.001 to 0.01; ***, *P* < 0.001. Data obtained from three independent experiments with two technical replicates.

**Fig. 6. Synthesis of VirB6-4 and VirB6-3 is disrupted in the virB6-4::Himar1 mutant.** Equal amounts of proteins from host-cell purified *virB6-4::Himar1* and wild-type (wt) *A. phagocytophilum* were separated by Tris-acetate polyacrylamide gel electrophoresis. A. PVDF membranes of transferred proteins were reacted with rabbit pre-immune serum (lanes 1 and 2), rabbit anti-VirB6-3 serum (lanes 3 and 4), dog pre-immune serum (lanes 5 and 6). Polyclonal dog serum infected with *A. phagocytophilum*, with immune responses mainly targeting the MSP2/P44 surface protein, was used as loading control to indicate equal loading of proteins from the *virB6-4::Himar1* mutant and wt *A. phagocytophilum* (lanes 7 and 8). B. PVDF membranes of transferred proteins were reacted with rabbit pre-immune serum (lanes 9 and 10), rabbit anti-VirB6-4 serum (lanes 11 and 12), dog pre-immune serum (lanes 13 and 14) and *A. phagocytophilum* infected dog serum (loading control, lanes 15 and 16). C and D. HL-60 cells infected with *A. phagocytophilum virB6-4::Himar1* or wild-type (wt) were fixed and viewed by indirect immunofluorescence microscopy to determine immunoreactivity with *A. phagocytophilum* infected mouse serum (green) and rabbit anti-VirB6-3 (panel C, red) or anti-VirB6-4 (panel D, red) serum. Representative immunoblots were obtained from three independent experiments. Representative IFA images are shown from several micrographs.

**Fig. 7. Growth and cytotoxicity of the virB6-4::Himar1 mutant.** The growth of *A. phagocytophilum virB6-4::Himar1* (dotted line) vs. wild-type (wt) (black line) in infected A. HL-60 and B. ISE6 cells was followed by determining the number of *A. phagocytophilum* genome equivalents per host cell targeting the *A. phagocytophilum* single copy gene *msp5* and the *tlr9* or *crt* genes from HL-60 and ISE6 cells, respectively. Bars
indicate *A. phagocytophilum virB6-4:* Himar1 and wild-type cytotoxicity to host cells as indicated by the levels of LDH release into the cell culture supernatants. The culture medium was not changed at any time during the course of this experiment. *, P = 0.01 to 0.05; **, P = 0.001 to 0.01. Data obtained from three independent experiments with three technical replicates.
Table 1. Peptides used to generate polyclonal antibodies against VirB6 proteins

| Peptide | Sequence                                                                 | length |
|---------|--------------------------------------------------------------------------|--------|
| VirB6-1 | C-SNYAWPKRVSRRYIEVCRYHPLGTVYMSPYVAARLGFAGRDKEVLKESKYPRE                 | 56     |
| VirB6-2 | C-YPLYFSSKLOGGSGYNYSNWYKVAKGEAPYITDGYPEYLVHSIGIVKPSMEKLDDEHE            | 58     |
| VirB6-3 | C-REYRPDDGVGEGDDRVTGSTTAGSISGDRAAGIDARGDHAEREQPADVVGEGADRVSG           | 60     |
| VirB6-4 | C-TELPREVVPEATEYGTKPDQDGDKDLPRLDPIDGDSAIDEVECVEVRSSRSESTDVSPEVTERDA     | 70     |
| RT-PCR | Sequence                        | Amplicon size | Target  |
|--------|---------------------------------|---------------|---------|
| AB1727 | TGGCAATCCATGAAGCCGAAG           | 230 bp        | sodB    |
| AB1729 | TTAACCAGGCCACCATGCT             | 130 bp        | virB3   |
| AB1730 | CGTGCATACCTGGCCGCAAA            | 347 bp        | virB4-1 |
| AB1732 | CGCACCTGCATATTCCACGC            | 414 bp        | virB6-1 |
| AB1733 | CCAGAGATCGGGTGTCAGAT            | 588 bp        | virB6-2 |
| AB1734 | TACATAGCTCCGGGTCTGG             | 512 bp        | virB6-3 |
| AB1735 | TTTGCAGGGCCTATAGGGTT            | 471 bp        | virB6-4 5' end |
| AB1736 | TGCTGTGTCATCCCCACTTCA          | 132 bp        | virB6-4 3' end |
| AB1737 | TACATAGCTCCGGGTCTGG             | 204 bp        | msp5    |
| AB1738 | TCTGCAGGTAACGCACTTCA          | 342 bp        | 16S     |
| AB1739 | AATCCGGAAGAAGAGGAGAT           | 633 bp        | sodB-virB3 |
| AB1740 | GTGCCCGAAAAGCAGCTCTAA          | 608 bp        | virB3-virB4-1 |
| AB1741 | ATGCCGCGAAGAAGAGGAGAT          | 367 bp        | virB4-1-virB6-1 |
| AB1742 | GTGCCGCGAAGAAGAGGAGAT          | 369 bp        | virB4-1-virB6-2 |
| AB1743 | TGCTGTGTCATCCCCACTTCA          | 357 bp        | virB6-2-virB6-3 |
| AB1744 | TCCAGAATGCACCCAATACG          | 362 bp        | virB6-3-virB6-4 |
| AB1745 | TCCAGAATGCACCCAATACG          | 124 bp        | virB6-3 |
| AB1746 | AAGAAGTCCGGCGCAAACTCC          | 130 bp        | virB6-1 |
| AB1747 | CCCACATTCAACGCTATCC           | 126 bp        | sodB    |
| AB1748 | CCCACATTCAACGCTATCC           | 130 bp        | virB6-1 |
| AB2053 | CGGTAGTGGAAGGTTATGTAAT         | 141 bp        | virB6-2 |
| AB2054 | GCATTCCGCTGCTCAACAA           | 124 bp        | virB6-3 |
| AB2057 | GCCGCTGATGGAAGTAAAG            | 130 bp        | virB6-1 |
| AB2058 | CCACACCCACCCAGGTATTG          | 126 bp        | sodB    |
| AB2059 | TAGGGTACCTGAGCAGCATG          | 130 bp        | virB6-1 |
| AB2060 | CTGTGTGTCATCCCCACTTCA          | 141 bp        | virB6-2 |
| AB2061 | GCACGTACTGAGGATGATTG          | 124 bp        | virB6-3 |
| Accession | Sequence                        | Length | Gene          | Position   |
|-----------|---------------------------------|--------|---------------|------------|
| AB2063    | CTCACACACAGGGGAGATT             | 108 bp | \(\text{virB6-4} \) 5\' end |
| AB2064    | AGAGCATCCAATGGCAGATTA           | 108 bp | \(\text{virB6-4} \) 3\' end |
| AB1741    | same as above                   | 132 bp | \(\text{virB6-4} \) 3\' end |
| AB1742    | same as above                   | 132 bp | \(\text{virB6-4} \) 3\' end |
| AB2065    | TCGGAAACTTGTTATGGTATC           | 118 bp | \(\text{msp5} \)         |
| AB2066    | CTCAATCACTCCCTTCAACAGGTGTA      | 118 bp | \(\text{msp5} \)         |
| AB2121    | AGGGAGGTAGTACGCATCCCTAGA        | 102 bp | \(\text{groEL} \)        |
| AB2122    | TGTTATCTCTGGGCAGCCATAATTAGA    | 102 bp | \(\text{groEL} \)        |
| AB2125    | GGCCTATGGTGCTGCTTATAC          | 115 bp | \(\text{rpoB} \)         |
| AB2126    | CCACACTGAAGTTGCTATCC           | 115 bp | \(\text{rpoB} \)         |

**qPCR**

| Accession | Sequence                        | Length | Gene          | Position   |
|-----------|---------------------------------|--------|---------------|------------|
| AB1334    | AGATGCTGACTGGGATGAG             | 125 bp | \(\text{msp5} \) (59)  |
| AB1335    | TCGGCAATCAACCAAGGTCAAGA         | 125 bp | \(\text{msp5} \) (59)  |
| **a**AB1336 | CGTATGGTGAGTGCTGATAGGAAGGG     | 111 bp | \(\text{crt} \)         |
| AB2039    | GTCAAGTCCGGCAACAATCT            | 111 bp | \(\text{crt} \)         |
| AB2040    | CATCTTCTTCTCGGCATCTCTCTCTCTATCTT | 111 bp | \(\text{crt} \)         |
| **b**AB2041 | TTGCTGACTGACGGACAGTAGGTATG     | 100 bp | \(\text{tlr9} \)         |
| AB2042    | CCCAGTCTGTGGACTCAATTAG          | 100 bp | \(\text{tlr9} \)         |
| AB2043    | GGTATAGCCAGGGATGGTTAGAAGAAGGTATGC | 100 bp | \(\text{tlr9} \)         |
| **b**AB2044 | TCTAGGTCAGTCCTGTTGGTAAGG       | 100 bp | \(\text{tlr9} \)         |

\(\text{a} \) Probe labeled with Hexachloro-fluorescein (HEX) at the 5\' end and tetramethylrhodamine (TAMRA) at the 3\' end

\(\text{b} \) Probe labeled with 6-carboxyfluorescein (6-FAM) at the 5\' end and black hole quencher-1 (BHQ-1) at the 3\' end