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Type IV secretion in the obligatory intracellular bacterium *Anaplasma phagocytophilum*

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Summary

Anaplasma phagocytophilum is an obligatory intracellular bacterium that infects neutrophils, the primary host defense cells. Consequent effects of infection on host cells result in a potentially fatal systemic disease called human granulocytic anaplasmosis. Despite ongoing reductive genome evolution and deletion of most genes for intermediary metabolism and amino acid biosynthesis, *Anaplasma* has also experienced expansion of genes encoding several components of the Type IV secretion (T4S) apparatus. Two *A. phagocytophilum* T4S effector molecules are currently known; *Anaplasma* translocated substrate 1 (Ats-1) and ankyrin repeat domain–containing protein A (AnkA) have C-terminal positively charged amino acid residues that are recognized by the T4S coupling protein, VirD4. AnkA and Ats-1 contain eukaryotic protein motifs and are uniquely evolved in the family *Anaplasmataceae*; Ats-1 contains a mitochondria-targeting signal. They are abundantly produced and secreted into the host cytoplasm, are not toxic to host cells, and manipulate host cell processes to aid in the infection process. At the cellular level, the two effectors have distinct subcellular localization and signaling in host cells. Thus in this obligatory intracellular pathogen, the T4S system has evolved as a host-subversive survival factor.

Introduction

Anaplasma phagocytophilum is a tick-borne gram-negative intragranulocytic bacterium in the order Rickettsiales and the class Alphaproteobacteria (Dumler, 2005). Infection of humans with *A. phagocytophilum* results in a potentially fatal acute flu-like illness called human granulocytic anaplasmosis (HGA, formerly human granulocytic ehrlichiosis). HGA is frequently accompanied by leukopenia, thrombocytopenia, anemia, and elevated levels of serum hepatic aminotransferases (Bakken *et al.*, 2008). Wild rodents are major reservoirs for *A. phagocytophilum* in the United States (Telford *et al.*, 1996). Although identified less than two decades ago, HGA is currently among the most prevalent life-threatening tick-borne zoonoses, and has been recently recognized in North America as an important and frequent cause of human fever after *Ixodes* tick bite.

A. phagocytophilum replicates in membrane-bound compartments (called inclusions or morulae) of neutrophil granulocytes, the most abundant type of white blood cells. Neutrophils are the primary immune defense cells responsible for powerful innate antimicrobial responses. Lipopolysaccharide and peptidoglycan, which activate the innate immune responses, have been eliminated from *A. phagocytophilum* at the genomic level (Lin *et al.*, 2003). To survive and replicate inside hostile neutrophils, bidirectional signals are transduced inside *A. phagocytophilum* and inside host cells upon interaction (Rikihisa, 2010b). Some of these signaling events lead to the subversion of several innate neutrophil immune responses, including inhibition of NADPH oxidase activation, lysosomal fusion

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with bacterial inclusions, autophagy, and IFN-γ signaling (Carlyon *et al.*, 2002, Mott *et al.*, 2000, IJdo *et al.*, 2004, Mott *et al.*, 1999, Pedra *et al.*, 2008, Webster *et al.*, 1998, Niu *et al.*, 2008, Rikihisa, 2010b, Rikihisa *et al.*, 2010, Rikihisa, 2010a). Furthermore, *A. phagocytophilum* inhibits spontaneous and induced human neutrophil apoptosis to maximize intracellular bacterial reproduction (Yoshiie *et al.*, 2000, Borjesson *et al.*, 2005) and hijacks host cholesterol to stabilize the bacterial membrane (Xiong *et al.*, 2009). Consequently, several non-antimicrobial compounds that block *A. phagocytophilum* or host cell signaling were shown to eliminate *A. phagocytophilum* infection in vitro without harming the host cells (Cheng *et al.*, 2006, Lin *et al.*, 2007, Lin *et al.*, 2002, Xiong *et al.*, 2009). *A. phagocytophilum* utilizes the bacterial Type IV secretion (T4S) system to subvert the host innate immune system and exploit the host cell. The T4S system is a multi-component membrane-spanning transporter machinery that translocates DNA to or from bacteria, and proteins or nucleoprotein complexes to the eukaryotic target cells (Alvarez-Martinez *et al.*, 2009).

The T4S apparatus of *A. phagocytophilum*

There are at least two ancestral lineages of the T4S effector protein or nucleoprotein delivery system: the virB/virD system of Agrobacterium tumefaciens and the dot/icm system of *Legionella pneumophila,* sometimes referred to as the T4aS and T4bS systems, respectively. *A. phagocytophilum* utilizes the T4aS system. In *A. tumefaciens*, the single *virB* operon, along with *virD4*, encodes 12 membrane-associated proteins that form a transmembrane channel (Alvarez-Martinez *et al.*, 2009). Despite the small genome size of *A. phagocytophilum*, genes encoding some *virB* orthologs, have expanded during evolution (Dunning Hotopp *et al.*, 2006). In *A. phagocytophilum, virB/D* are distributed into three major genomic islands: *sodB-virB3-virB4-virB6-1-virB6-2-virB6-3-virB6-4, virB8-1-virB9-1-virB10-virB11-virD4,* and *virB2-1- virB2-2-virB2-3-virB2-4-virB2-5-virB2-6-virB2-7-virB2-8-virB4-2*. Between these islands lie *virB8-2*, *virB9-2*, and a putative *virB7* (no open reading frame number assigned, coordinates 1033978 to 1034181). *virB7* is homologous to the *Anaplasma marginale* putative *virB7* and contains a cysteine residue and a "[I/L][K/R]SPC" motif, which are conserved among *virB7* of *Rickettsia* spp. (Sutten *et al.*, 2010, Gillespie *et al.*, 2009). A genomic map of *A. phagocytophilum virB/D* is shown in Figure 1 inset. The split T4S genomic islands and the duplicated *virB* (*A. marginale* has 22 candidates of *virB2*) are characteristics conserved among the Rickettsiaceae and Anaplasmataceae families, suggesting a common ancestral origin and a requirement for preservation of these features (Dunning Hotopp *et al.*, 2006, Gillespie *et al.*, 2009, Gillespie *et al.*, 2010). *A. phagocytophilum virB9* has been shown to be transcribed in peripheral blood leukocytes from HGA patients and from experimentally infected animals, indicating the in vivo relevance of the T4S system (Ohashi *et al.*, 2002). *A. phagocytophilum* T4S system is expressed in ISE6 tick cell culture, and in the closely related monocyte-tropic *Ehrlichia canis, virB9* is expressed in infected ticks (Felek *et al.*, 2003, Nelson *et al.*, 2008). Thus the T4S system is believed to function in the tick stages of the bacteria.

Expression of the T4S apparatus is regulated during intracellular bacterial infection. Both *virB8-virD4* and *sodB-virB6* operons are transcribed polycistronically during *A. phagocytophilum* replication in HL-60 promyelocytic leukemia cells (Ohashi *et al.*, 2002). Because *sodB* encodes an iron superoxide dismutase, a protective oxidative stress response might therefore be coupled with T4S apparatus assembly in the cytoplasm of mammalian neutrophils or blood-feeding ticks, where both activities are needed for bacterial survival and/or proliferation. In *Ehrlichia chaffeensis,* a bacterium closely related to *A. phagocytophilum,* the small DNA binding protein EcxR regulates all five *virB/D* loci (Cheng *et al.*, 2008); regulation of *virB7* has not been studied. *virB9* and *virB6,* which are present in different operons of *A. phagocytophilum,* are upregulated at the mRNA level

during infection of human neutrophils *in vitro; virB9* is upregulated at the protein level (Niu *et al.*, 2006). In contrast, in the majority of *A. phagocytophilum* spontaneously released from infected host cells, VirB9 protein expression is minimal (Niu *et al.*, 2006).

Recently, cryo-electron microscopy demonstrated that purified VirB7, VirB9, and VirB10 homologs encoded by *Escherichia coli* pKM101 assemble into a 1-MDa channel of sufficient size to span the entire gram-negative bacterial cell envelope (Fronzes *et al.*, 2009). Assembly of the T4S apparatus in *Rickettsia* is poorly understood. The *virB6* homologs in *Rickettsia* are 3- to 10-fold larger than *A. tumefaciens virB6*, and four to five tandem copies are present per genome (Dunning Hotopp *et al.*, 2006, Gillespie *et al.*, 2009). These *virB6* encodes polytopic membrane proteins with large hydrophilic domains in the N-terminal, central, or C-terminal region. VirB6-1, 6-2, 6-3, and 6-4 are coexpressed and interact with each other and with VirB9 from *E. chaffeensis* (Bao *et al.*, 2009), which is a bacterial surface–exposed protein in *A. phagocytophilum* and *E. chaffeensis* (Niu *et al.*, 2006, Ge *et al.*, 2007a, Ge *et al.*, 2007b). *E. chaffeensis* VirB6-2 undergoes proteolysis, resulting in the release of an 80-kDa fragment that accumulates in *E. chaffeensis*–containing vacuoles (Bao *et al.*, 2009). Differential transcription of several *A. phagocytophilum* T4S VirB2 pilus protein paralogs in mammalian and ISE6 tick cell cultures was recently reported (Nelson *et al.*, 2008), suggesting that this bacterium uses different sets of VirB2 proteins in different host cells. Whether tandemly expressed VirB2 molecules function in the effector transport channel and/or host cell adhesion apparatus, as reported in *Bartonella* (Dehio, 2008), remains to be determined. Some T4S apparatus proteins are immunogenic in infected or immunized animals (Felek *et al.*, 2003, Sutten *et al.*, 2010), and therefore multiple non-identical VirB proteins may provide some advantage for immune evasion.

T4S substrates

Although the total number of T4S substrates encoded by the *A. phagocytophilum* genome is unknown, two substrate proteins have been partially characterized. The coupling protein VirD4 contains docking sites for these T4S substrates. The *A. phagocytophilum* VirD4 protein has two N-terminal transmembrane domains, a P-loop and a walker-B site for nucleotide binding, and a C-terminal region that has a ~150-residue extension compared with the *Agrobacterium* or *Rickettsia* VirD4. Whether this C-terminal extension of VirD4, also present in the *E. chaffeensis* VirD4 molecule, modulates substrate transfer similar to the coupling protein TcpA, which regulates conjugative transfer of pCW3 (Steen *et al.*, 2009), remains to be determined. Using a Cre recombinase reporter assay for translocation, the *A. phagocytophilum* ankyrin repeat domain–containing protein AnkA was shown to be translocated into plant cells in an *A. tumefaciens* VirD4–dependent manner (Lin *et al.*, 2007). A hypothetical protein, later named *Anaplasma* Translocation Substrate 1 (Ats-1), was discovered by screening an *A. phagocytophilum* genomic prey library using *A. phagocytophilum* VirD4 as bait in a bacterial two-hybrid system (Niu *et al.*, 2010). Both AnkA and Ats-1 have basic C-terminal domains similar to the *A. tumefaciens* T4S substrates (Vergunst *et al.*, 2005) (Table 1). Both proteins are abundantly expressed and secreted into the host cell cytoplasm (Lin *et al.*, 2007, Niu *et al.*, 2010). AnkA and Ats-1 have distinct subcellular localization and downstream signals, and appear to be multifunctional, as summarized in Figure 1, although detailed signaling mechanisms and pathways remain to be elucidated.

AnkA

AnkA was originally discovered from a genomic DNA expression library of the *A. phagocytophilum* USG3 strain by screening with tick-challenged *A. phagocytophilum*-infected dog sera (Storey *et al.*, 1998). *A. phagocytophilum* infection of human leukocytes requires protein tyrosine kinase activity, and proteomics revealed that AnkA was the

primary phosphotyrosine protein (Lin *et al.*, 2007). This phosphotyrosine protein was also suspected as AnkA by size similarity with AnkA; tyrosine phosphorylation was later confirmed by immunoprecipitation (IJdo *et al.*, 2007). AnkA migrates between 160 and 190 kDa when subjected to SDS-PAGE, but the mass predicted from the amino acid sequence is ~ 30 kDa lower. Although some researchers have proposed that this discrepancy is due to glycosylation of AnkA, no evidence of glycosylation has yet been found. The N-terminal two-thirds of AnkA contains ~11 ankyrin repeats (Rikihisa *et al.*, 2010). The ankyrin repeat is found in a number of biologically important eukaryotic proteins. For example, IκB, the inhibitor of the inflammatory response–regulating transcription factor NF-κB, contains seven ankyrin repeats. Ankyrin is a 33-residue repeating motif, which folds into two antiparallel α-helices followed by a β-hairpin or a long loop. Similar to previously described ankyrin proteins (Mosavi *et al.*, 2004), it is predicted that the consecutive ankyrin repeats in AnkA stack together to form an L-shaped domain that mediates specific protein-protein interactions. In contrast to other protein-protein interaction domains such as SH2 (Src Homology 2) or SH3, ankyrin repeats typically do not recognize any specific amino acid sequence or structure. AnkA orthologs have been identified among members of the *Anaplasma* and *Ehrlichia* genera (Rikihisa *et al.*, 2010), but not in the trematode-borne *Neorickettsia* species, suggesting that AnkA evolved in *Anaplasma* and *Ehrlichia* during adaptation to tick and mammalian hosts, or that *Neorickettsia* spp. lost the AnkA homolog after diversification of these two genera and the genus *Neorickettsia* from the common ancestor.

Both AnkA and tyrosine phosphorylation are required for *A. phagocytophilum* infection (Lin *et al.*, 2007). AnkA contains six to seven tandem tyrosine phosphorylation sites, four to five of which can bind SH2, with a single SH3 binding site in the C-terminal one-third of the protein (Rikihisa *et al.*, 2010). In fact, in *A. phagocytophilum*–infected human promyelocytic leukemia HL-60 cells and peripheral blood neutrophils, AnkA is the predominant phosphotyrosine protein (IJdo *et al.*, 2007, Lin *et al.*, 2007). Tyrosine phosphorylation of AnkA occurs as early as 2 min after *A. phagocytophilum* binds to eukaryotic cells, and bacterial internalization is not required for this early phosphorylation event (IJdo *et al.*, 2007, Lin *et al.*, 2007). In *A. phagocytophilum*–infected HL-60 cells, tyrosine phosphorylation of AnkA occurs after secretion into the host cytoplasm by two non-receptor tyrosine kinases, Src and Abelson leukemia (Abl); specific inhibitors of these kinases reduces tyrosine phosphorylation of AnkA (IJdo *et al.*, 2007, Lin *et al.*, 2007). The Abl and Src kinase phosphorylation sites in AnkA are important for signal transduction and/or infection in host cells (IJdo *et al.*, 2007, Lin *et al.*, 2007). Following phosphorylation by Src, AnkA binds the SH2 domains of the non-receptor tyrosine phosphatase Src Homology Protein (SHP)-1 (IJdo *et al.*, 2007). SHP-1 can interact with and dephosphorylate a wide spectrum of phosphoproteins and primarily downregulates cellular activation (Poole *et al.*, 2005). The roles of AnkA recruitment and activation of SHP-1 in *A. phagocytophilum* infection remain to be determined.

The two Abl kinase phosphorylation sites in AnkA are not predicted to bind SH2 domains, indicating a distinct signaling role for AnkA following phosphorylation by the Abl and Src family kinases. Yeast two-hybrid screening has demonstrated that AnkA binds to Abl-interactor 1 (Abi-1), an adaptor protein and substrate of Abl-1 tyrosine kinase (Lin *et al.*, 2007). Abi-1 is comprised of approximately 450–500 residues (several splice variants are known) and is a proline-rich protein comprised of N-terminal WAB (Wave binding) and SNARE (syntaxin binding) domains, a homeobox homology region (HHR domain) and a C-terminal SH3 domain (Dai *et al.*, 1995). The Abi-1 SH3 domain and one of the proline-rich motifs interact with c-Abl (Shi *et al.*, 1995). Abi-1 is a trans-acting adaptor protein known to regulate Abl-1-mediated tyrosine phosphorylation of target proteins, such as Mammalian Enabled (Mena) and B-cell adaptor for phosphoinositide 3-kinase (BCAP), by direct

interaction with the target proteins (Tani *et al.*, 2003, Maruoka *et al.*, 2005). Similar to Mena and BCAP, the natural ligands of Abi-1, AnkA forms a complex with Abl-1 via Abi-1, stimulates Abl-1 kinase, and is phosphorylated by Abl-1 (Fig. 1). AnkA may pry the autoinhibitory Abl open into an active signaling-competent kinase conformation (Pluk *et al.*, 2002, Nagar *et al.*, 2003). Interestingly, AnkA, Mena, and BCAP have multiple tyrosine phosphorylation sites but do not share sequence similarity. Abl kinase was shown to be essential for *A. phagocytophilum* infection using the Abl kinase–specific inhibitor STI571 (also known as imatinib mesylate or Gleevec), which does not inhibit Src family kinases, and by knockdown using a small interfering RNA targeting Abl-1 mRNA (Lin *et al.*, 2007). STI571 has been used to treat Bcr-Abl-positive chronic myelogenous leukemia patients (Druker *et al.*, 2001, Capdeville *et al.*, 2002), suggesting a potential novel strategy for HGA treatment with STI571.

For *Listeria* and *Chlamydia* internalization, Abi-1 recruits the Wave complex in a Rac-dependent manner to reorganize the actin cytoskeleton (Bierne *et al.*, 2005, Carabeo *et al.*, 2007). However, *A. phagocytophilum* infection downregulates Rac2 in HL-60 cells (Carlyon *et al.*, 2002), indicating that this pathway may not be activated by *A. phagocytophilum*. Abl contains actin-binding repeats and phosphorylates proteins that regulate the actin cytoskeleton. Changes in subcellular localization of Abi-1 and Abl-1, and activation of Abl by AnkA, likely regulate multiple cellular processes. Further studies are needed to determine whether AnkA recruits other potential Abi-1-interacting proteins and Abl kinase substrates to facilitate *A. phagocytophilum* infection.

In *A. phagocytophilum* HZ strain, AnkA phosphorylated on tyrosine accumulates in the host cell cortical cytoplasm during infection, and very little is retained within or near the bacteria (Lin *et al.*, 2007). In the *A. phagocytophilum* Webster strain, a large proportion of AnkA is contained within the nucleus (Caturegli *et al.*, 2000, Park *et al.*, 2004, Garcia-Garcia *et al.*, 2009). In the nucleus, AnkA binds to a broad range of targets, including several nuclear proteins, the internucleosomal region of chromosomes in HL-60 cells, ATC-rich sequences, and transcriptional regulatory regions of the CYBB locus, to suppress the host cell innate immune response (Caturegli *et al.*, 2000, Park *et al.*, 2004, Garcia-Garcia *et al.*, 2009). Although the mechanisms of AnkA nuclear translocation and binding to various nuclear targets are unclear, these reports present the fascinating possibility of global transcriptional regulation of host cells by a T4S substrate. The AnkA molecule is involved in several aspects of host-pathogen interactions. AnkA is one of several *A. phagocytophilum* proteins with strain-dependent amino acid variation; phylogenic analysis of AnkA protein sequences indicates that AnkA from humans and domesticated animals (cattle and horses) are clustered together, whereas the AnkA variants of *A. phagocytophilum* isolated from field-collected *Ixodes ricinus* ticks in Germany are divided into two clusters, one close to the mammalian cluster and the other quite divergent from this cluster (Massung *et al.*, 2000, von Loewenich *et al.*, 2003) (Fig. 2). These variations may influence the function of AnkA as a T4S effector molecule. Of note, AnkA from all human isolates and from the tick isolate USG3 lacks one glycine residue at its C-terminus which may affect AnkA T4S secretion efficiency—these isolates contain the terminal sequence PKSVKGGRGR, whereas the sequence PKSVKGGGRGR is present in the German tick and in various animal isolates from the United States and Europe. Although proteins that specifically interact with the AnkA ankyrin repeats have not been identified, the presence of the ankyrin repeats and the C-terminal tyrosine phosphorylation domain in AnkA may represent an optimal adaptation to its eukaryotic host. Further studies are needed to elucidate the mechanisms of AnkA-induced signaling and cellular regulation during *A. phagocytophilum* infection.

Ats-1

Similar to AnkA, Ats-1 is abundantly expressed by *A. phagocytophilum* in mammalian cells, and secretion is readily visible by immunofluorescence microscopy. In contrast to AnkA, a large proportion of expressed Ats-1 colocalizes with the *A. phagocytophilum* inclusion; consequently, Ats-1 translocation to the host cell cytoplasm only becomes readily discernible by light microscopy at 32 h post-infection (early exponential growth stage) (Niu *et al.*, 2010). Ats-1 is predicted to be 40.5 kDa, however SDS-PAGE analysis of lysates from *A. phagocytophilum*–infected HL-60 cells reveals 48-kDa and a 35-kDa Ats-1 polypeptides (Niu *et al.*, 2010). Unlike AnkA, Ats-1 lacks any known protein motifs. However, Ats-1 contains a cleavable N-terminal mitochondria-targeting presequence, which is a hallmark of most mitochondrial matrix proteins. This sequence has been shown to direct Ats-1 localization into the mitochondrial matrix of infected human neutrophils, HL-60 cells, RF/6A monkey endothelial cells, HeLa cells, and even yeast cells via the mitochondrial protein transport system. This is an intrinsic property of Ats-1, as recombinant Ats-1 has been shown to translocate into isolated mitochondria *in vitro* in the absence of any other cellular proteins (Niu *et al.*, 2010). Ats-1 is the first example of a bacterial protein that traverses five membranes (bacterial inner and outer membrane, inclusion membrane, and outer and inner membranes of mitochondria). The presequence of Ats-1 is cleaved in the mitochondrion by a mitochondrial matrix processing peptidase, releasing the mature 35-kDa form of Ats-1, whereas the uncleaved 48-kDa form of Ats-1 stays with bacterial inclusions. The functions of inclusion-colocalized Ats-1 remain to be investigated.

A. phagocytophilum infection inhibits spontaneous and induced apoptosis of isolated peripheral blood human neutrophils for up to 48 h and of neutrophils in peripheral blood leukocyte cultures for up to 96 h (Yoshiie *et al.*, 2000). The cellular mechanisms by which *A. phagocytophilum* inhibits apoptosis of human neutrophils include inhibition of the loss of mitochondrial membrane potential, inhibition of Bax translocation to the mitochondria, and inhibition of activation of downstream caspase 3 (Ge *et al.*, 2005, Ge *et al.*, 2006). Mitochondria-translocated Ats-1 inhibits etoposide-induced apoptosis in mammalian cells (Niu *et al.*, 2010). In the absence of the Bcl-2 family of proteins in yeast, Ats-1 was shown to inhibit Bax docking to the mitochondria and subsequent apoptosis, indicating that other Bcl-2 family members are not important in this process. However, the link between Ats-1 and inhibition of apoptosis remains unclear. The *Neisseria gonorrhoeae* porin protein, PorB, induces condensation of the mitochondrial matrix and the loss of cristae structures and sensitizes cells to apoptosis (Kozjak-Pavlovic *et al.*, 2009). Therefore, a possible scenario is that Ats-1 stabilizes the mitochondrial membrane potential or even the inner-membrane cristae structure, the rearrangement of which is required for apoptosis (Scorrano *et al.*, 2002). Although cristae stabilization by Ats-1 has not been shown, overexpression of Ats-1 does not have obvious adverse effects on mitochondria in various mammalian or yeast cells (Niu *et al.*, 2010). Consistent with this, an insoluble, miscleaved Ats-1 mutant (deletion of residues 55–57) was shown to be defective for inhibition of apoptosis, suggesting that Ats-1 sub-mitochondrial routing and/or processing are critical for proper function (Niu *et al.*, 2010). The absence of similarities between the amino acid sequence or the mode of action of Ats-1 and any other known cell death suppressors suggests that Ats-1 is a member of a previously undescribed class of anti-apoptotic proteins.

Concluding remarks

AnkA and Ats-1, two originally hypothetical *A. phagocytophilum* effector molecules, are uniquely evolved in the family Anaplasmataceae; their orthologs are encoded in the genomic sequences of other members of the genera *Anaplasma* and *Ehrlichia.* Considering the great expense of energy required to produce and secrete these effectors, they likely are of fundamental importance for survival of these bacteria. Members of the Anaplasmataceae

family have a large number of hypothetical proteins, among which more effector molecules and T4S components are likely to be discovered. The identification of these molecules, and subsequent studies revealing their functions in cellular infection will be highly informative. Furthermore, future studies focusing on duplicated and modified T4S apparatus genes may uncover unusual molecular interaction and functions. Because an essential part of the life cycle of *Anaplasma* is the tick stage, it is also important to learn the function of the T4S system at this stage. Ongoing tick genome sequencing projects will facilitate this line of investigation.

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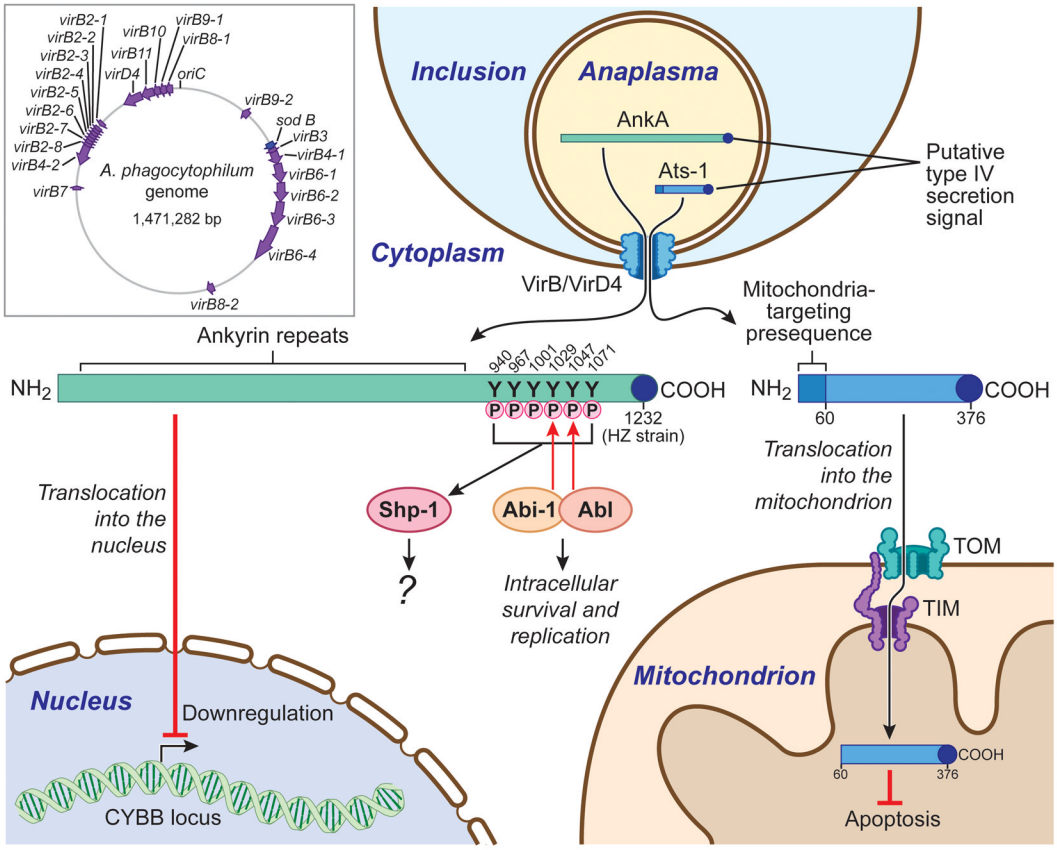

Fig. 1. *virB/D* loci on the *A. phagocytophilum* genome and subcellular location and targets of *A. phagocytophilum* AnkA and Ats-1 and host cell signaling pathways

Inset. The *virB/D* loci are shown as colored arrows on the circular genome map of *A. phagocytophilum*. Genes are not drawn to scale and arrows reflect a 10-fold expansion of the gene size relative to the genome. The putative origin of replication is indicated as *oriC*. AnkA and Ats-1 are depicted as green and blue bars, respectively, with the putative type IV secretion signal depicted as a dark blue dot. AnkA is secreted into the host cell cytoplasm and binds the adaptor protein Abi-1, which recruits and activates Abl-1 tyrosine kinase. AnkA from the *A. phagocytophilum* HZ strain contains six tyrosine phosphorylation sites, two of which (Y1029 and Y1047) are phosphorylated by Abl-1. The remaining four tyrosine phosphorylation sites are phosphorylated by Src kinases, allowing subsequent binding to the SH2 domain of Shp-1. AnkA protein translocates to the nucleus and executes gene regulation. Ats-1 lacks ankyrin repeats or tyrosine phosphorylation sites, but has a mitochondria-targeting presequence. Ats-1 binds to mitochondria via a mitochondria-targeting presequence and translocates across the outer and inner membranes. The presequence is then cleaved, and the mature Ats-l localizes in the mitochondrial matrix. Mitochondria-localized Ats-1 blocks apoptosis of eukaryotic host cells by preventing loss of mitochondrial membrane potential.

TOM: Transporter Outer Membrane complex; TIM: Transporter Inner Membrane complex

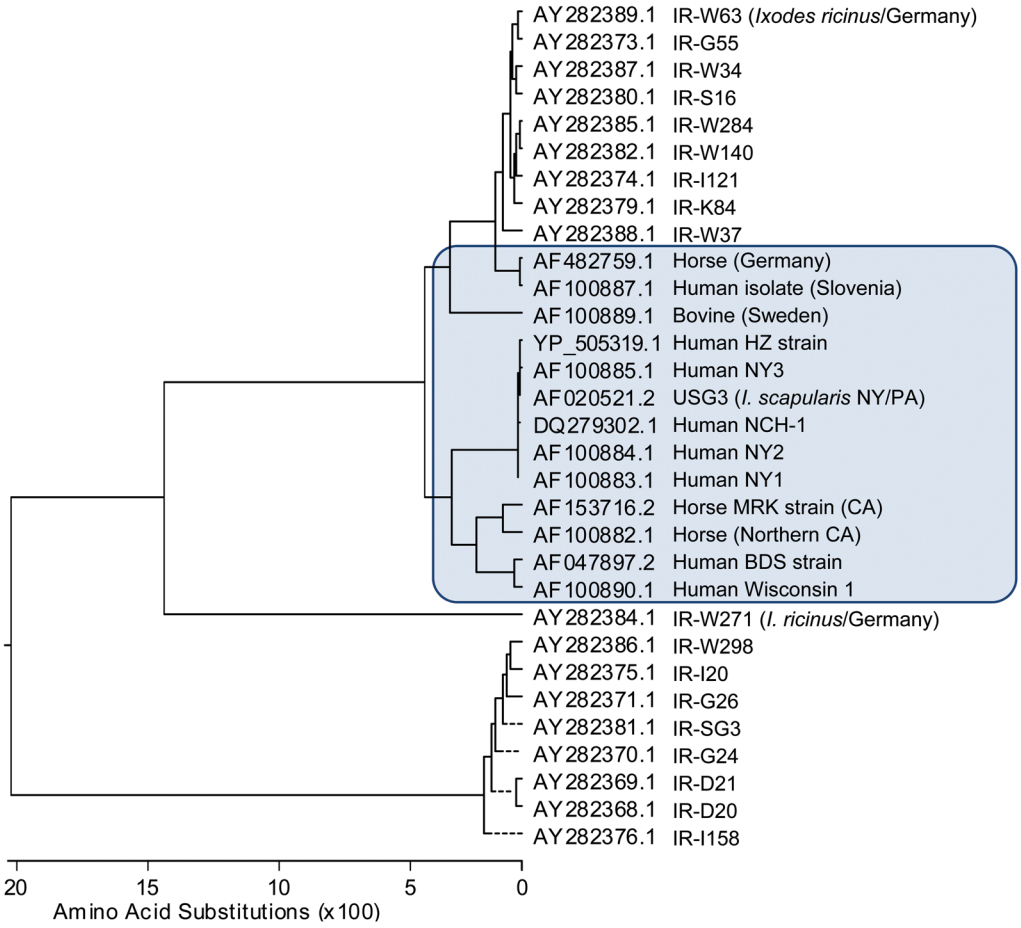

Fig. 2.

Phylogenetic analysis of *A. phagocytophilum* AnkA amino acid sequence variation. The GenBank number is indicated at left. Hosts and geographic regions from which strains were isolated are indicated at right. IR: *Ixodes ricinus* tick from Germany; modified from (von Loewenich *et al.*, 2003). Mammalian sequences (USG3 was isolated from the dog by attaching field-collected ticks) are highlighted in a blue box.

Table 1

Characteristics of Type IV secretion effectors from the *A. phagocytophilum* HZ strain

Effector	Molecular size (aa)	C-terminal residues	Protein motifs	Subcellular localization
AnkA	1232	SEGPKSVKGGRGR	Ankyrin repeats, Src/Abl tyrosine phosphorylation sites, SH2/SH3 binding motifs	Cytoplasm, nucleus
Ats-1	376	QNRGPETHGKGTR	N-terminal mitochondria localization signal	Mitochondria, bacterial inclusions