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Anaplasma phagocytophilum and *Ehrlichia chaffeensis* type IV secretion and Ank proteins

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Summary of recent advances

The obligatory intracellular bacterial pathogens *Anaplasma* and *Ehrlichia* infect leukocytes by hijacking host-cell components and processes. The type IV secretion system is up-regulated during infection. Among type IV secretion candidate substrates, an ankyrin repeat protein of *Anaplasma phagocytophilum*, AnkA, is delivered into the host cytoplasm via a complex that includes VirD4. AnkA is highly tyrosine-phosphorylated and binds to the Abl interactor 1, SHP-1, and nuclear DNA fragments. *Ehrlichia chaffeensis* AnkA was recently reported to be translocated into host cell nucleus. The recent discovery of several ankyrin repeat proteins secreted via the type IV secretion system of different intracellular bacteria suggests that a common strategy evolved to subvert host-cell functions.

Introduction

Anaplasma phagocytophilum and *Ehrlichia chaffeensis* are Gram-negative cocci that cause human granulocytic anaplasmosis (HGA) and human monocytic ehrlichiosis (HME), respectively. They are obligatory intracellular bacteria most closely related to *Rickettsia* spp., for example, *Rickettsia prowazekii* that causes epidemic typhus, a disease that has been responsible for the deaths of millions of people during wartime or natural disasters. Wild animals, such as white-tailed deer and white-footed mice, are reservoirs for *A. phagocytophilum* and *E. chaffeensis*, and human infection occurs through the bite of infected ticks [1,2]. HGA and HME are acute flu-like illnesses characterized by fever, headache, myalgia, anorexia, and chills, and frequently are accompanied by leukopenia with the appearance of immature cells (or rebound leukocytosis), thrombocytopenia, anemia, and elevated levels of serum hepatic aminotransferases. The severity of the disease varies from asymptomatic seroconversion to severe morbidity or death [3-5]. Although these bacteria were initially culture-isolated from patients less than two decades ago, HGA and HME are among the most prevalent tick-borne zoonoses in North America. While *A. phagocytophilum* and *E. chaffeensis* do not have genes for the biosynthesis of lipopolysaccharides and peptidoglycans that activate host leukocytes, whole genome sequence data available in 2006 has been facilitating studies to find virulence determinants of these bacteria [6]. Based on NCBI Conserved Domain searches, bacterial type II, III, V, and VI secretion components have not been detected in *A. phagocytophilum* and *E. chaffeensis*; however, the type IV secretion (T4S) system has been identified in these bacteria, and the secreted products are

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expected to function as effectors in the host to facilitate infection and disease progression. Analysis showed that components of *Agrobacterium tumefaciens* VirB/D system are conserved in *A. phagocytophilum* and *E. chaffeensis* [6,7•]. In particular, *A. phagocytophilum* VirD4 exhibits high identity with *A. tumefaciens* VirD4, a component of the T4S apparatus. VirD4 is regarded as a coupling protein because it recognizes C-terminal sequences within T4S substrate proteins prior to delivery into the VirB transmembrane channel [7•]. Targeted gene insertion, knockout, or complementation in obligatory intracellular bacteria has not been feasible yet. However, using the CRAfT (Cre recombinase reporter assay for translocation) system developed in *A. tumefaciens* [8], we demonstrated that the *A. phagocytophilum* ankyrin repeat domain–containing protein, AnkA can be translocated from *A. tumefaciens* into plant cells in a VirD4-dependent manner [9••]. Bioinformatic analysis of whole genome sequences of these bacteria showed that *A. phagocytophilum* and *E. chaffeensis* encode several proteins with eukaryotic-like domains/motifs—a common theme of bacterial effector proteins that functionally mimic host cell proteins (Table 1) [10,11•]. Additionally, based on the characteristics of *A. tumefaciens* T4S substrate motifs [12] and/or using bacterial two-hybrid system, we have identified several putative T4S candidates [6,13] (W. Bao, MS thesis, The Ohio State University, 2008). Here, we discuss host subversion and exploitation events by these bacteria and the recent progress on regulation of T4S and the secreted effector proteins, in particular highlighting Ank proteins.

Subversion and exploitation of infected host cells

A. phagocytophilum and *E. chaffeensis* replicate in the membrane-bound compartments (inclusions, also called morulae [(morulae – Latin "*morus*", mulberry)] as they look like mulberries under the light microscope) of human granulocytes and monocytes-macrophages, respectively, which are the primary immune defense cells that normally are responsible for powerful innate antimicrobial responses. These intracellular bacteria parasitize host cells by subverting their various innate immune responses, including inhibiting NADPH oxidase activation, lysosomal fusion with bacterial inclusions, and IFN-γ signaling, and by down-regulating Toll-like receptor 2/4, and CD14 expression [14]. Furthermore, this group of bacteria inhibits host cell apoptosis to maximize intracellular bacterial reproduction [15-17].

A. phagocytophilum and *E. chaffeensis* enter host cells via caveolae (lipid raft)-mediated endocytosis, which directs pathogens to an intracellular compartment secluded from late endosome or lysosome markers and NADPH oxidase components [18]. Both early and replicative *A. phagocytophilum* and *E. chaffeensis* inclusions co-localize with tyrosine-phosphorylated proteins and phospholipase C-γ2; activation of tyrosine kinases and phospholipase C-γ2 are required for infection of host cells [18]. *E. chaffeensis* inclusions retain the early endosome characteristics including Rab5 and early endosome antigen 1, and fuse with endosomes enriched with transferrin receptor [18]. In contrast, *A. phagocytophilum* inclusions are negative for these endosomal markers. Several hallmarks of early autophagosomes have been detected in *A. phagocytophilum* replicative inclusions, including a double lipid bilayer membrane, and ectopically expressed GFP-tagged LC3 and Beclin 1, the human homologs of *Saccharomyces cerevisiae* autophagy-related proteins Atg8 and Atg6, respectively [19]. Stimulation of autophagy by rapamycin favors *A. phagocytophilum* infection. Inhibition of the autophagosomal pathway by 3-methyladenine does not inhibit *A. phagocytophilum* internalization, but reversibly arrests its growth [19].

A. phagocytophilum and *E. chaffeensis* require cholesterol for survival and growth, but they lack genes for cholesterol biosynthesis or modification and thus must acquire cholesterol from host cells [20]. *A. phagocytophilum* infection significantly up-regulates host cellular cholesterol levels by enhancing low-density lipoprotein uptake through stabilization of host

LDL receptor mRNA [21]. *A. phagocytophilum* inclusions become enriched with cholesterol by hijacking non-esterified free cholesterol from the host low-density lipoprotein uptake pathway [21].

T4S apparatus

There are at least two ancestral lineages for the T4S system: the *virB/virD* system of *A. tumefaciens* and the *dot/icm* system of *Legionella pneumophila*, referred to as T4aS and T4bS systems, respectively. *A. phagocytophilum* and *E. chaffeensis* have genes encoding the T4aS system. In *A. tumefaciens*, the single *virB* operon, along with *virD4*, encodes 12 membrane-associated proteins that form a transmembrane channel complex [7•]. In the *A. phagocytophilum* and *E. chaffeensis* genomes, several *virB/D* genes are duplicated and distributed in five clusters: *sodB-virB3–virB4-virB6-1-virB6-2-virB6-3-virB6-4*, *virB8-1-virB9-1-virB10-virB11–virD4*, *virB4-2-tandem virB2*s (eight nonidentical *virB2*s in *A. phagocytophilum* and four nonidentical *virB2*s in *E. chaffeensis*), *virB8-2*, and *virB9-2* [13]. Interestingly, all five *virB/D* loci are up-regulated during the exponential growth stage of *E. chaffeensis* synchronously cultured in THP-1 human monocytic leukemia cells, and down-regulated prior to the release of *E. chaffeensis* from host THP-1 cells [22•]. Proteomic analysis identified an *E. chaffeensis* hypothetical protein, named EcxR for *E. chaffeensis* expression regulator in this study, which binds promoter regions of all five operons and genes, and transactivates them in a *lacZ* reporter assay [22•]. This may be a mechanism by which transcription of the scattered *virB/D* loci is temporarily coordinated. During the infection of human neutrophils *in vitro*, both *virB9-1* and *virB6-1* of *A. phagocytophilum*, which are present in separate genomic loci, are up-regulated at the mRNA level as is VirB9-1 at the protein level [23]. In contrast, the majority of *A. phagocytophilum* spontaneously released from infected host cells expresses only low levels of VirB9 protein [23]. This modulation of *virB/D* expression during the establishment of bacterial infection may promote intracellular survival and replication as well as resistance upon exposure to the extracellular environment. In addition to mammalian hosts, the T4S system is also expected to function during tick infection given that *virB9* is expressed by *Ehrlichia canis* in tick tissues [24], and four *E. chaffeensis virB6* paralogs and *E. chaffeensis virB9-1* are expressed in ISE6 tick cell culture [25•]. Some of the duplicated *virB* paralogs of these bacteria may be reserved to function specifically in ticks, as differential transcription of several *A. phagocytophilum virB2* paralogs in mammalian and ISE6 tick cells have been reported [26]. Interestingly, double immunofluorescence labeling of host cell–free *Neorickettsia risticii*, which is closely related to *Ehrlichia* and *Anaplasma* spp., showed bipolar surface localization of the primary T4S pilus component VirB2, despite the round shape of bacteria, suggesting an underlying subcellular structure that dictates the spatial distribution of the T4S pili [27]. VirB9 is bacterial surface-exposed in both *E. chaffeensis* and *A. phagocytophilum* [23,28]. In addition, the assembled macromolecular structure is expected to differ from the prototypical T4aS apparatus of *A. tumefaciens*, considering that all (total four) VirB6 homologs of *E. chaffeensis* are 3- to 10-fold larger than *A. tumefaciens* VirB6 and coexpressed. Coimmunoprecipitation analysis and far-western blotting of *E. chaffeensis* revealed that *E. chaffeensis* VirB9-1 and the four VirB6 proteins form a unique complex [25•].

T4S secretion substrates

The *A. tumefaciens* VirB/D T4S system secretes approximately 10 substrates into the host cell [7•]. In contrast, the *Legionella* T4S system can secrete over 100 effector molecules, and it is not yet clear whether and why so many effectors are required for the survival of this bacterium in the host cells [29]. The T4aS effector molecules, CagA of *Helicobacter pylori*, pertussis toxin, Beps of *Bartonella henselae*, and the T-DNA complex have been shown to

play a major role in disease pathogenesis [30-33]. The T4S system is essential for the biogenesis of cytoplasmic replicative compartments unique to each of several facultative intracellular bacteria, such as *Brucella*, *Bartonella*, and *Legionella* [29,34,35]. Although there are no conserved protein sequence motifs, certain C-terminal sequences are critical for secretion via T4S apparatus [7•]. In *A. tumefaciens*, basic amino acids, net positive charge, and a characteristic hydropathy profile at C-terminus are critical for recruitment and T4aS translocation of effector proteins [7•,12]. Some T4aS substrates, such as *H. pylori* CagA and *B. henselae* Beps, require positively charged C-terminal tail and certain N-terminal or internal domains for the translocation [31,36]. For RalF and several other T4bS substrates in *L. pneumophila*, a hydrophobic residue at -3 or -4 position is critical for the translocation [37]. In *A. phagocytophilum*, an ankyrin-repeats (Ank) protein, AnkA and a hypothetical protein, named *Anaplasma* translocated substrate 1 (Ats-1), are known to be secreted into the host cell cytoplasm [9••,13]. Both AnkA and Ats-1 have C-terminal positive charged residues and hydropathy profiles similar to those of T4aS substrates [9••,12,13].

A. phagocytophilum AnkA is one of strain-variable proteins [38,39]. As shown in Figure 1, different numbers of ankyrin repeats and tyrosine kinase domains are found in four *A. phagocytophilum* strains [MRK, HZ, NCH-1, and BDS (BDS strain is similar to the Webster strain for which the sequence is currently unavailable)]. *A. phagocytophilum* HZ strain AnkA is one of most abundantly expressed proteins that shows apparent molecular mass of 160 kDa, and is highly tyrosine phosphorylated after delivery into the host leukocyte cytoplasm [9••]. AnkA tyrosine phosphorylation occurs 2 min after infection, and phosphorylated AnkA accumulates during infection in the host cell cytoplasm, whereas very little AnkA is retained within the bacteria or the inclusion as demonstrated by immunofluorescence labeling [9••]. AnkA binds to Abl-interactor 1 that interacts with Abl-1 tyrosine kinase, thus mediating AnkA phosphorylation. AnkA and Abl-1 are critical for *A. phagocytophilum* infection, as infection is inhibited upon host cytoplasmic delivery of an antibody against AnkA, Abl-1 knockdown via a targeted short interfering RNA, or treatment with Gleevec, a specific pharmacological inhibitor of Abl-1, suggesting a potential supplement to the antibiotic doxycycline to enhance the efficacy of HGA therapy [9••]. This is in contrast to *Legionella* in which knockout of a single T4S substrate does not result in phenotype change due to functional redundancy of multiple T4S substrates [29, 35], suggesting that in *A. phagocytophilum* each T4S substrate has significant weight in regulating host cell functions. A study showed that the infection by *A. phagocytophilum* strain NCH-1 specifically induces tyrosine phosphorylation of a 190-kDa AnkA, which then interacts with the host tyrosine phosphatase SHP-1 through the SH2 domain [40••]. AnkA of the *A. phagocytophilum* Webster strain was reported to localize within nuclei of infected HL-60 cells and bind to the inter-nucleosomal region of chromosomes as well as transcriptional regulatory regions of the *CYBB* locus, which suppresses the host cell innate immune response [41,42••]. However, it remains to be determined whether and how AnkA can be imported topologically inside host cell nucleus. It is currently unclear whether AnkA has these multiple biological activities or these are strain-specific phenotypes. Different from AnkA, a large proportion of expressed Ats-1 colocalizes with *A. phagocytophilum* inclusions, and Ats-1 translocation to the host cell cytoplasm becomes evident 32 h post-infection (early exponential growth stage) [13]. Interestingly, Ats-1 has an N-terminal mitochondria localization signal that allows it to translocate into the mitochondria of infected-human neutrophils and HL-60 cells, and when ectopically expressed, it inhibits etoposide-induced apoptosis in mammalian cells [13] (H Niu & Y Rikihisa. Abstract D-146, 108th Amer. Soc. Microbiology General Meeting. Boston, MA. June 2008).

Ank proteins

Eukaryotic Ankyrin-repeat (Ank) proteins mediate protein-protein interactions involved in a multitude of host processes including cytoskeletal motility, tumor suppression, and transcriptional regulation [43]. Ankyrin domain contains a 33-residue repeating motif, which consists of two anti-parallel α-helices connected to the next repeat via a loop region [43]. A series of Ank-repeats are arranged in a curved structure with protein-protein interactions occurring in the loop regions. Several *ank* genes encoding heterogeneous Ank proteins are present in the facultative or obligate intracellular bacteria, including *L. pneumophila* (15 copies) [44], *Coxiella burnetii* (15 copies) [11•], *Orientia tsutsugamushi* (40 copies) [45], *Wolbachia pipientis* (23 copies) [46], *Wolbachia* from *Culex pipiens* group (60 copies) [47], and *Rickettsia prowazekii* (1 copy) [48]. The *L. pneumophila* AnkX protein prevents microtubule-dependent vesicular transport, which interferes with fusion of the *L. pneumophila*–containing vacuole with late endosomes after infection of macrophages [49••]. In *C. burnetii, ank* genes show remarkable heterogeneity [11•]. Ectopically expressed Ank proteins localize to a variety of subcellular regions in mammalian cells including microtubules, mitochondria, and the parasitophorous vacuole membrane [11•]. Using *L. pneumophila* as a surrogate host, AnkG of *C. burnetii* was recently reported to be secreted by the *L. pneumophila* T4S system. Once secreted into host cells, AnkG can bind to mammalian protein p32 (gC1qR) and interfere with the ability of p32 to modulate RNA splicing of Bcl-x, thereby preventing host cell apoptosis (A Luhrmann *et al*., abstract 137, 23rd Meeting of the American Society for Rickettsiology, Hilton Head Island, SC, August 2009).

As shown in Table 2 and Figure 2, *Anaplasma* and *Ehrlichia* spp. each have three to five Ank proteins, some of which contain multiple potential phosphorylation sites for host signaling and regulation. AnkA of *A. phagocytophilum* strain HZ has 11 ankyrin repeats, and several tyrosine phosphorylation and/or SH2 domains are concentrated near the C terminus (Figure 1). In contrast, AnkA of *E. chaffeensis* strain Arkansas (ECH0684) has 19 ankyrin repeats, and SH2 domains are concentrated near the N terminus (Figure 2). Both AnkA proteins have basic amino acids, net positive charges, and hydropathy profiles at the C terminus similar to those of *Agrobacterium* T4S substrates (Table 2) [12]. *E. chaffeensis* AnkA is also secreted and tyrosine phosphorylated in host cells (M Lin and Y Rikihisa, unpublished data). Recently, *E. chaffeensis* AnkA was reported to be translocated into host cell nucleus and bind to Alu elements, suggesting global modulation of host genes [50••]. AnkA of *Ehrlichia canis* and *Anaplasma marginale* have only two to three basic residues within the 25-residue C-terminal region (Table 2). However, net positive charge and the hydropathy profiles of these proteins suggest that they are potential T4S substrates (Table 2). The largest Ank proteins of >300-kDa are conserved among *Ehrlichia* and *Anaplasma* species. These proteins have seven to ten ankyrin repeats, several serine/threonine phosphorylation sites, and possess T4S motifs similar to those of *Agrobacterium* T4S substrates (Table 2, Figure 2). It will be interesting to learn whether these proteins are secreted by the T4S system to induce some of the above-mentioned cellular phenotypes.

Conclusions

Although much remains to be learned about the mechanistic details of how effectors are secreted by the *Anaplasma* and *Ehrlichia* T4S apparatus and their subcellular sites of action, further studies on T4S effector candidates—including Anks and cognate host cell partners—are expected to provide a molecular basis for understanding pathogen subversion of host-cell functions and disease pathogenesis.

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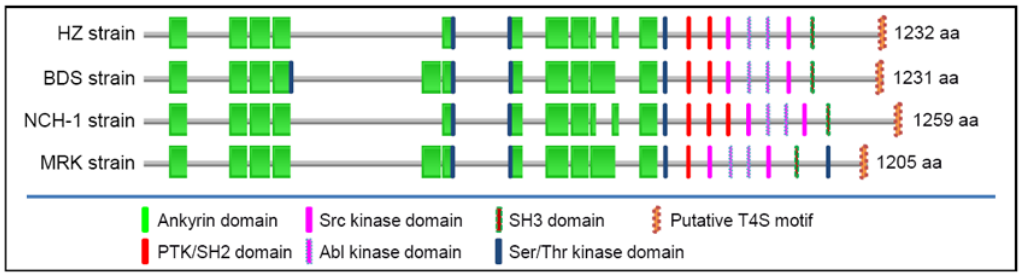

Figure 1. Domain structures of *Anaplasma phagocytophilum* AnkA proteins from various strains

Domain structures and putative motifs were predicted by searching against the Pfam database (http://pfam.sanger.ac.uk) and ScanSite (http://scansite.mit.edu), respectively, using default parameters. The presence of a T4S motif was determined as shown in Table 2. Protein lengths [amino acids (AA) numbers] are shown on the right, and domains are not drawn proportional to the actual size. All strains are human isolates with the exception of MRK, which is a horse isolate. Isolates collection locations: HZ, New York; BDS, Wisconsin; NCH-1, Massachusetts; MRK, California.

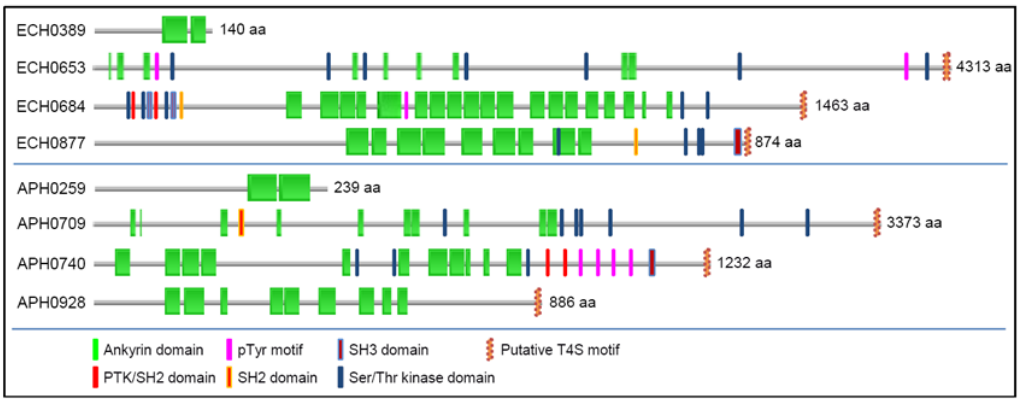

Figure 2. Domain structure of ankyrin repeat proteins in *Anaplasma phagocytophilum* HZ and *Ehrlichia chaffeensis* Arkansas

Domain structures and putative motifs were predicted by searching against the Pfam database and ScanSite, respectively, using default parameters. The presence of a T4S motif was determined as shown in Table 2. ECH: *E. chaffeensis* Arkansas gene locus; APH: *A. phagocytophilum* HZ gene locus. Protein lengths (AA numbers) are shown on the right, and domains are not drawn proportional to actual size.

Table 1

A. phagocytophilum and *E. chaffeensis* genes encoding potential eukaryotic-like motifs/domains

Motif/Domains [1]	**Predicted Roles**	**Gene Locus** [2]
Ankyrin repeats	Protein-protein interactions	APH0259, APH0709, APH0740, APH0928; ECH0389, ECH0653, ECH0684, ECH0877.
Tetratricopeptide repeat domain (TPR)	Protein-protein interactions	APH1212, APH1316, ECH0048
SNARE-associated Golgi Protein	Vesicular fusion	APH1021, ECH0947
Ras-like GTPase	Vesicular trafficking	ECH0155
Serine/Threonine protein kinases	Cellular Signaling	APH0984, ECH0840
Protein Phosphatase	Cellular Signaling	ECH0964
PKCI_related	Protein Kinase C Interacting protein related, HIT family of hydrolases	APH0860, ECH0826
WD40 domain	Signal transduction and cytoskeleton assembly	APH1037
Stomatin/prohibitin homologs, and Band 7 domain of flotillin/reggie like proteins	Lipid raft associated protein and regulated proteolysis	APH0260, APH1146, APH1147; ECH1050, ECH1051
Breast Cancer Suppressor Protein (BRCA1) C-terminal domain (BRCT)	DNA damage repair	APH0138, ECH0301

[1]The eukaryotic-like motifs/domains were determined by bioinformatic analysis of the genomes of *A. phagocytophilum* HZ strain (GenBank accession number: NC_007797) and *E. chaffeensis* Arkansas strain (NC_007799). Detailed domain information is available at GenBank Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml).

[2]Gene locus abbreviations: APH, *A. phagocytophilum*; ECH, *E. chaffeensis*.

Table 2

Ankyrin-repeats containing proteins in *Ehrlichia* and *Anaplasma* spp.

Organisms (Locus ID/GenBank #)	Protein Properties	# of Ankyrin Repeats *1*	T4S Motif (pI, Hydropathy Profile)*2* (C-Term. 25 aa)
***A. phagocytophilum* HZ:**			
APH_0259 (YP_504874)	26.4 kDa (239 aa), pI 4.2	2	- (4.2, Hydrophilic) (QRGINLEAEKGETTDDVELYTSSNK)
APH_0709 (YP_505290)	367.3 kDa (3373 aa), pI 5.4	10	± (9.9, Hydrophilic) (STTITTPGHSPIVSQASHQEGRSNR)
APH_0740 (YP_505319), **AnkA**	131.2 kDa (1232 aa), pI 6.5	11	+ (10.0, Hydrophilic) (SGAPGSQPEAPQSEGPKSVKGGRGR)
APH_0928 (YP_505501)	97.0 kDa (886 aa), pI 7.7	9	± (9.0, Neutral) (HCGVYDGIKPVYVRASSSLSSSVAK)
***A. marginale* St Maries:**			
AM638 (AAV86627)	347.8 kDa (3194 aa), pI 5.5	9	± (7.2, Hydrophilic) (DGSGLPRTPTPTPEVKQHEGRMERQ)
AM705 (AAV86672), **AnkA**	145.7 kDa (1387 aa), pI 6.3	13	+ (10.3, Hydrophilic) (AASSTSQQPAGKQPTAPTKYLSRGW)
AM926 (AAV86836)	31.0 kDa (282 aa), pI 5.0	2	- (3.9, Hydrophilic) (DKGRVDMINAISEMQVGFSEEEQEG)
***E. chaffeensis* Arkansas:**			
ECH_0389 (YP_507209)	16.0 kDa (140 aa), pI 4.7	2	- (4.0, Hydrophobic) (NNETPLSISIENGSMDIAHAILVAQ)
ECH_0653 (YP_507462)	467.8 kDa (4313 aa), pI 5.6	9	+ (9.7, Hydrophilic) (SSLDDVAPLPSPKSGDVRKKHGRFM)
ECH_0684 (YP_507490), **AnkA**	156.7 kDa (1463 aa), pI 4.6	19	+ (8.2, Hydrophilic) (DIGAQAVSPSTSQGADVKKSSCQSK)
ECH_0877 (YP_507672)	96.8 kDa (874 aa), pI 6.8	9	± (9.2, Neutral) (KPRKLTRIMSLEECCVPKPTLAFPQ)
***E. canis* Jake:**			
Ecaj_0052 (AAZ68103)	70.5 kDa (613 aa), pI 6.6	3	- (5.6, Hydrophobic) (ENIRGVLLSHLNIEAIGSTKEINIT)
Ecaj_0221 (AAZ68270)	104.4 kDa (933 aa), pI 6.9	7	- (5.4, Neutral) (IHPQVLSSTELAEQGRCDITLTSLH)
Ecaj_0365 (AAZ68408), **AnkA**	152.9 kDa (1421 aa), pI 5.3	21	± (9.0, Hydrophilic) (AVSQQQAASPSSGQAAGVQQKEMQR)
Ecaj_0387 (AAZ68430)	471.4 kDa (4245 aa), pI 5.2	7	+ (11.1, Hydrophilic) (ALDSITPLPSPGTGGTNKKQRRNSI)
Ecaj_0627 (AAZ68661)	173.4 kDa (151 aa), pI 8.6	2	± (9.0, Hydrophobic) (DGQTPLSIAIRNRSIDIAHAILAVK)
***E. ruminantium* Gardel:**			
Erga_CDS_03830 (CAI27668)	98.8 kDa (877 aa), pI 6.5	9	- (4.6, Hydrophilic) (RTVENTNNTIITQLSIEDYRTLQLT)
Erga_CDS_03830 (CAI27835), **AnkA**	175.9 kDa (1640 aa), pI 4.9	18	+ (8.2, Hydrophilic) (GMENERVSPASSQGTLQKKSSCMEK)
Erga_CDS_04060 (CAI27858)	329.1 kDa (2992 aa), pI 6.1	7	+ (9.5, Hydrophilic) (TDEKNVPLSHTPSSGKSKTGASKSM)
Erga_CDS_06440 (CAI28096)	141.5 kDa (125 aa), pI 5.1	2	+ (10.0, Hydrophilic) (NDTPISIAMKNSRYDIARAIMTMQR)

*1*The numbers of ankyrin repeats were determined by searching against PFAM domain database with E-value < 1 (http://pfam.sanger.ac.uk/).

*2*The presence of a T4S motif was determined by the characteristics of *Agrobacterium tumefaciens* T4S substrates, including three or more basic amino acids (K, H, or R shown in red), net positive charges, and hydropathy profiles at its C-terminal 25 amino acids [12].