



Ehrlichia chaffeensis tandem repeat proteins and Ank200 are type 1 secretion system substrates related to the repeats-in-toxin exoprotein family

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Ehrlichia chaffeensis has type 1 and 4 secretion systems (T1SS and T4SS), but the substrates have not been identified. Potential substrates include secreted tandem repeat protein (TRP) 47, TRP120, and TRP32, and the ankyrin repeat protein, Ank200, that are involved in molecular host–pathogen interactions including DNA binding and a network of protein–protein interactions with host targets associated with signaling, transcriptional regulation, vesicle trafficking, and apoptosis. In this study we report that *E. chaffeensis* TRP47, TRP32, TRP120, and Ank200 were not secreted in the *Agrobacterium tumefaciens* Cre recombinase reporter assay routinely used to identify T4SS substrates. In contrast, all TRPs and the Ank200 proteins were secreted by the *Escherichia coli* complemented with the hemolysin secretion system (T1SS), and secretion was reduced in a T1SS mutant (Δ TolC), demonstrating that these proteins are T1SS substrates. Moreover, T1SS secretion signals were identified in the C-terminal domains of the TRPs and Ank200, and a detailed bioinformatic analysis of *E. chaffeensis* TRPs and Ank200 revealed features consistent with those described in the repeats-in-toxins (RTX) family of exoproteins, including glycine- and aspartate-rich tandem repeats, homology with ATP-transporters, a non-cleavable C-terminal T1SS signal, acidic pls, and functions consistent with other T1SS substrates. Using a heterologous *E. coli* T1SS, this investigation has identified the first *Ehrlichia* T1SS substrates supporting the conclusion that the T1SS and corresponding substrates are involved in molecular host–pathogen interactions that contribute to *Ehrlichia* pathobiology. Further investigation of the relationship between *Ehrlichia* TRPs, Ank200, and the RTX exoprotein family may lead to a greater understanding of the importance of T1SS substrates and specific functions of T1SS in the pathobiology of obligately intracellular bacteria.

Keywords: *Ehrlichia*, tandem repeat protein, ankyrin repeat protein, type 1 and 4 secretion systems, RTX family, tyrosine phosphorylation, exoproteins

INTRODUCTION

Members of the family *Anaplasmataceae* consist of a group of Gram-negative obligately intracellular alphaproteobacteria belonging to the order Rickettsiales, and are responsible for various arthropod-borne diseases of mammalian hosts including ehrlichiosis and anaplasmoses. Human monocytotropic ehrlichiosis (HME) is an emerging life-threatening tick-borne zoonosis caused by *Ehrlichia chaffeensis*, which exhibits tropism for mononuclear phagocytes, and survives by evading the innate host defenses, most likely by secreting multiple effectors into the host cell (Barnewall et al., 1997; Lee and Rikihisa, 1998; Lin and Rikihisa,

2004). Genes encoding Sec-dependent and Sec-independent Tat, TRAP-T (tripartite ATP-independent periplasmic transporters), type 1 and 4 secretion systems have been identified in *E. chaffeensis* genome; however, genes representing components of other secretion systems (type 2, 3, 5, 6) are not present (Hotopp et al., 2006).

Recent studies have reported an increasing number of tyrosine phosphorylated bacterial effector proteins translocated into host cells by type 3 or type 4 secretion systems (T3SS or T4SS; Deibel et al., 1998; Stein et al., 2000; Clifton et al., 2004; Backert and Selbach, 2005; Selbach et al., 2009). Several important human pathogens such as *Helicobacter pylori*, *Legionella pneumophila*, *Coxiella burnetii*, *Bordetella pertussis*, *Brucella melitensis*, and *Bartonella henselae* utilize T4SS for the delivery of bacterial effector proteins into the cytoplasm of mammalian host cells in order to manipulate host cell functions (Christie et al., 2005;

Abbreviations: Ank, ankyrin repeat protein; CRAfT, Cre recombinase reporter assay for translocation; HME, human monocytotropic ehrlichiosis; RTX, repeats-in-toxins; T1SS, type 1 secretion system; T3SS, type 3 secretion system; T4SS, type 4 secretion system; TRs, tandem repeats; TRP, tandem repeat protein.

Backert and Meyer, 2006; Alvarez-Martinez and Christie, 2009). The T4SS consists of a substrate translocation channel that spans the periplasm and both membranes of Gram-negative bacteria. The archetypal *Agrobacterium tumefaciens* T4SS comprises 12 proteins named VirB1 through 11 (clustered in a single *virB* locus) and VirD4 (VirB/D4). T4SS gene clusters (generally clustered into two to five groups) have been identified in the members of family *Anaplasmataceae* (Ohashi et al., 2002; Collins et al., 2005; Hotopp et al., 2006; Mavromatis et al., 2006; Alvarez-Martinez and Christie, 2009), and expression of *Ehrlichia* T4SS genes is upregulated during infection (Cheng et al., 2008). Furthermore, using the Cre recombinase reporter assay for translocation (CRAfT) system developed in *A. tumefaciens* (Vergunst et al., 2000), translocation of an ankyrin repeat protein of *Anaplasma phagocytophilum*, AnkA, was recently reported (Lin et al., 2007).

The type 1 secretion system (T1SS) is widespread among Gram-negative bacteria and directs the translocation of a variety of proteins of various sizes and diverse functions from the cytoplasm to the extracellular medium (Delepelaire, 2004). The common substrates for T1SS include pore-forming hemolysins (HlyA), adenylate cyclases, lipases, proteases, surface layers, and hemophores (Delepelaire, 2004). T1SS consists of three proteins; an inner membrane protein with a cytoplasmic ATPase domain operating as an ATP-binding cassette (ABC) transporter (*Escherichia coli* HlyB), a periplasmic adaptor (also known as membrane fusion protein, MFP; *E. coli* HlyD), and an outer membrane channel protein of the TolC family (*E. coli* TolC). The interaction of the T1SS substrate with HlyB and HlyD triggers recruitment of TolC, thereby creating a continuous, but transient channel-tunnel from the cytosol directly into the extracellular medium (Thanabalu et al., 1998; Benabdelhak et al., 2003). Previous studies have shown that *E. coli* hemolysin transporter promotes secretion of heterologous T1SS substrates expressed in *E. coli*, including exotoxins Cya of *B. pertussis* (Sebo and Ladant, 1993), LtkA of *Aggregatibacter actinomycetemcomitans* (Lally et al., 1989), PaxA of *Pasteurella aerogenes* (Kuhnert et al., 2000), and FrpA of *Neisseria meningitidis* (Thompson and Sparling, 1993). Maintenance of genes encoding the type 1 and 4 secretion system components in the small *Ehrlichia* spp. genome that has evolved through reductive evolution is indicative of the importance of these secretion systems for survival, yet knowledge regarding *Ehrlichia* spp. secreted effectors and secretion mechanisms remain undefined.

Major immunoreactive proteins of *E. chaffeensis* have been molecularly characterized and many are members of a tandem repeat protein (TRP) family that includes TRP120 (ECH_0039), TRP47 (ECH_0166), TRP32 (ECH_0170), and one member of an ankyrin repeat protein (Ank) family, Ank200 (ECH_0684; Yu et al., 1996; Doyle et al., 2006; Luo et al., 2008, 2010). Microscopic, proteomic, and ultrastructural evidence indicates that these proteins are secreted and exposed on the surface of the bacterium and extracellularly associated with the morula matrix and membrane (Popov et al., 2000; Doyle et al., 2006; Luo et al., 2008; Wakeel et al., 2009, 2010a; Zhu et al., 2009). Several functions have been associated with TRPs in pathogenic bacteria, including immune evasion, adhesion, actin nucleation, and other host–pathogen interactions (Gaillard et al., 1991; Wren, 1991; Kling et al., 1997; Jordan et al.,

2003; Clifton et al., 2005; Palmer and Brayton, 2007; Shak et al., 2009; Wang et al., 2009). Recent studies have demonstrated that *E. chaffeensis* TRP47 interacts with a network of host cell proteins involved in signaling, modulation of gene expression, and intracellular vesicle trafficking (Wakeel et al., 2009) and is tyrosine phosphorylated (Wakeel et al., 2010a). TRP120 is a novel DNA binding protein that binds a G + C-rich DNA motif and is involved in host gene transcriptional regulation (Zhu et al., 2011). *E. chaffeensis* TRP120 also interacts with a diverse group of host cell proteins associated with major biological processes including transcription and regulation, cell signaling, protein trafficking, and actin cytoskeleton organization (Luo et al., 2011). TRP47 and TRP120 are differentially expressed on the surface of *E. chaffeensis* (Popov et al., 2000; Doyle et al., 2006), and degradation of surface exposed TRP120 by endogenous serine protease HtrA been linked to reduced bacterial aggregation and internalization into THP-1 cells (Kumagai et al., 2010, 2011). *E. chaffeensis* Ank200 is translocated to the host cell nucleus and interacts with *Alu-Sx* element motifs located in promoters and introns of various host cell genes involved in transcription, apoptosis, ATPase activity, and structural proteins associated with the nucleus and membrane-bound organelles (Zhu et al., 2009). Similarly, AnkA (APH_0740), an ankyrin repeat-containing protein of *A. phagocytophilum* that is a T4SS substrate (Lin et al., 2007), is also translocated to the nucleus of infected neutrophils (Garcia-Garcia et al., 2009) where it appears to bind regulatory regions of the *CYBB* gene and interacts with gene regulatory regions containing high AT content (Garcia-Garcia et al., 2009). Others have shown that AnkA of *A. phagocytophilum* binds to Abi-1 and is tyrosine phosphorylated by Abl-1 host cell tyrosine kinase (Lin et al., 2007).

The objective of this study was to determine the secretion mechanism of *E. chaffeensis* tandem repeat (TR) and Ank proteins (effectors) using well established T4SS (Vergunst et al., 2000) and T1SS models (Masure et al., 1990; Thompson and Sparling, 1993). We determined that the TRP120, TRP47, TRP32, and Ank200 are not translocated by a VirB/D4-dependent T4SS. Conversely, we demonstrated that secretion of *E. chaffeensis* TR and Ank proteins is mediated by the hemolysin secretion apparatus in *E. coli*. We demonstrated that the C-terminus is essential and required for the secretion of *E. chaffeensis* TRPs and Ank200. Collectively, this work demonstrated that *E. chaffeensis* TRP120, TRP47, TRP32, and Ank200 are *E. chaffeensis* T1SS substrates, and like other bacterial effector proteins, the Ank200 protein is tyrosine phosphorylated by a host cell tyrosine kinase. These findings provide new insights into the *E. chaffeensis* TRPs and Ank200 secretion mechanisms, substrates, and demonstrate the importance of the T1SS in ehrlichial pathobiology.

RESULTS

EXAMINATION OF *E. CHAFFEENSIS*-SECRETED TRP AND ANK PROTEINS IN T4SS

Expression of E. chaffeensis-secreted TRP and Ank proteins in *A. tumefaciens*

We have previously demonstrated that TRP120, TRP47, TRP32, and Ank200 are secreted in *E. chaffeensis*-infected cells (Popov et al., 2000; Doyle et al., 2006; Luo et al., 2008; Zhu et al., 2009). However, the secretion mechanism of TRP120, TRP47, TRP32, and

Ank200 are still unknown. Interestingly, the C-terminal 20 amino acids of Ank200 contains a potential VirB/D4 T4SS recognition motif (AVSPSTSQGADVKKSSCQSK) that is positively charged (pI 9.2), and has a hydrophathy profile similar to the consensus secretory motif R-X(7)-R-X-R-X-R of *A. tumefaciens* effectors, where replacement of the Arg residues by Lys has negligible effect on substrate translocation efficiency (Vergunst et al., 2005). To investigate whether *E. chaffeensis* TRP120, TRP47, TRP32, and Ank200 are T4SS substrates, we used the previously developed CRAFT system, a surrogate system that has been used successfully to identify or verify the translocation of several substrates including Anka of *A. phagocytophilum* from *A. tumefaciens* into plant cells (Vergunst et al., 2000, 2005; Lin et al., 2007). To demonstrate *E. chaffeensis* protein transport in a VirB/D4-dependent manner, the C-terminal (320 amino acids) of Ank200, near full length TRP120 (99%), and full length TRP47 and TRP32 were translationally fused to the C-terminus of the Cre protein (Cre::Ank200-C, Cre::TRP120, Cre::TRP47, Cre::TRP32; **Figure 1A**; **Tables A1** and **A2** in Appendix). The expression of the fusion proteins was brought under the control of the *vir* induction system in *A. tumefaciens* and confirmed by Western blot analysis with anti-Cre antibody (**Figure 1B**). Visualization of the large Cre::TRP120 was difficult, which may be due inefficient transfer of this large size protein. But after long exposure of the film a faint band was visible at 175 kDa (**Figure 1B**, lane 4).

Cre recombinase activity of Cre::Ehrlichia fusion proteins in *A. tumefaciens*

As the detection of protein translocation relies on Cre activity of the fusion proteins in the host cells we examined fusion protein Cre activity. A Cre recombinase activity assay was performed with Cre::Ehrlichia fusion proteins in *A. tumefaciens* strain LBA1100 containing the plasmid pSDM3043. Digestion of pSDM3043 by *Bam*HI gives two fragments, but after deletion of a small floxed fragment by Cre recombination one of the *Bam*HI sites is lost and only one fragment becomes visible after digestion with *Bam*HI. The results showed that Cre is active in the Cre::Ehrlichia fusion proteins (Cre::Ank200-C, Cre::TRP120, Cre::TRP47, and Cre::TRP32) in *A. tumefaciens* strain LBA1100 as demonstrated by loss of the *Bam*HI restriction site in the presence of these fusion proteins (**Figure 1C**, lanes 3, 4, 5, and 6). In contrast, two DNA fragments were detected in plasmid pSDM3043 isolated from *A. tumefaciens* strain LBA1100 lacking any Cre::Ehrlichia fusion protein, thus demonstrating the absence of Cre activity and served as a control (**Figure 1C**, lane 2).

Detection of protein translocation using CRAFT assay

Transformation of CB1 roots with *A. tumefaciens* strain LBA1100 with pSDM3155 expressing Cre–VirF fusion proteins (Cre::VirFΔ42N; *A. tumefaciens* fusion protein that serves as positive control) resulted in high numbers of CB1 cells expressing GFP 3 days after cocultivation (**Figure 2B**). Cocultivation with the negative control strain expressing Cre alone from the *A. tumefaciens* *virF* promoter, pSDM3197, rarely resulted in any GFP expression (**Figure 2A**). In contrast to the positive control, but similar to the negative control CB1 root explants cocultivated with *A. tumefaciens* strain LBA1100 transformed with the Cre::Ank200-C,

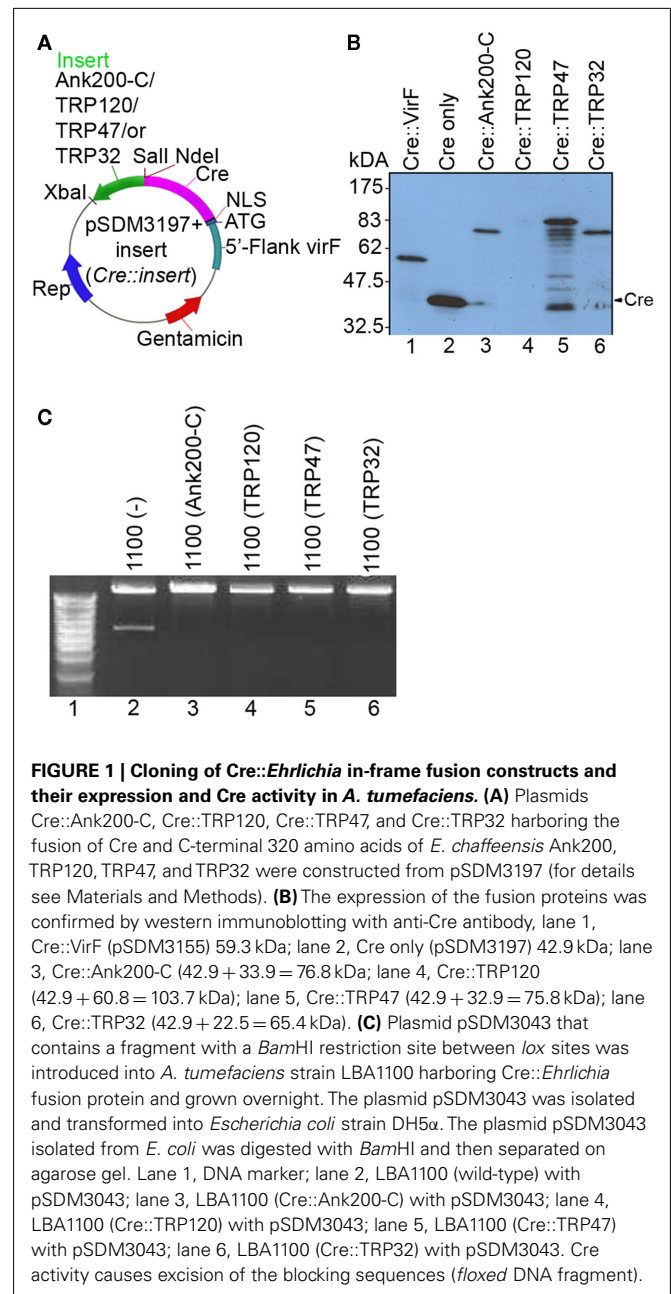


FIGURE 1 | Cloning of Cre::Ehrlichia in-frame fusion constructs and their expression and Cre activity in *A. tumefaciens*. (A) Plasmids Cre::Ank200-C, Cre::TRP120, Cre::TRP47, and Cre::TRP32 harboring the fusion of Cre and C-terminal 320 amino acids of *E. chaffeensis* Ank200, TRP120, TRP47, and TRP32 were constructed from pSDM3197 (for details see Materials and Methods). (B) The expression of the fusion proteins was confirmed by western immunoblotting with anti-Cre antibody, lane 1, Cre::VirF (pSDM3155) 59.3 kDa; lane 2, Cre only (pSDM3197) 42.9 kDa; lane 3, Cre::Ank200-C (42.9 + 33.9 = 76.8 kDa); lane 4, Cre::TRP120 (42.9 + 60.8 = 103.7 kDa); lane 5, Cre::TRP47 (42.9 + 32.9 = 75.8 kDa); lane 6, Cre::TRP32 (42.9 + 22.5 = 65.4 kDa). (C) Plasmid pSDM3043 that contains a fragment with a *Bam*HI restriction site between *lox* sites was introduced into *A. tumefaciens* strain LBA1100 harboring Cre::Ehrlichia fusion protein and grown overnight. The plasmid pSDM3043 was isolated and transformed into *Escherichia coli* strain DH5α. The plasmid pSDM3043 isolated from *E. coli* was digested with *Bam*HI and then separated on agarose gel. Lane 1, DNA marker; lane 2, LBA1100 (wild-type) with pSDM3043; lane 3, LBA1100 (Cre::Ank200-C) with pSDM3043; lane 4, LBA1100 (Cre::TRP120) with pSDM3043; lane 5, LBA1100 (Cre::TRP47) with pSDM3043; lane 6, LBA1100 (Cre::TRP32) with pSDM3043. Cre activity causes excision of the blocking sequences (floxed DNA fragment).

Cre::TRP120, Cre::TRP47, and Cre::TRP32 fusion protein constructs, did not or only rarely result in any GFP expression (**Figures 2C–F**).

Ehrlichia VirD4 as coupling factor for translocation

The coupling factor VirD4 forms the interface between the translocated substrates and the VirB translocation channel. We hypothesized that in an effort to gain access to the VirB translocation channel Ehrlichia protein substrates may require their own cognate VirD4. To determine whether this is the case, *E. chaffeensis* *virD4* with N-terminal c-Myc tag was cloned behind the *virD* promoter of *A. tumefaciens* into an incP plasmid (pSDM3668) so that protein transfer could be checked in the presence of *E.*

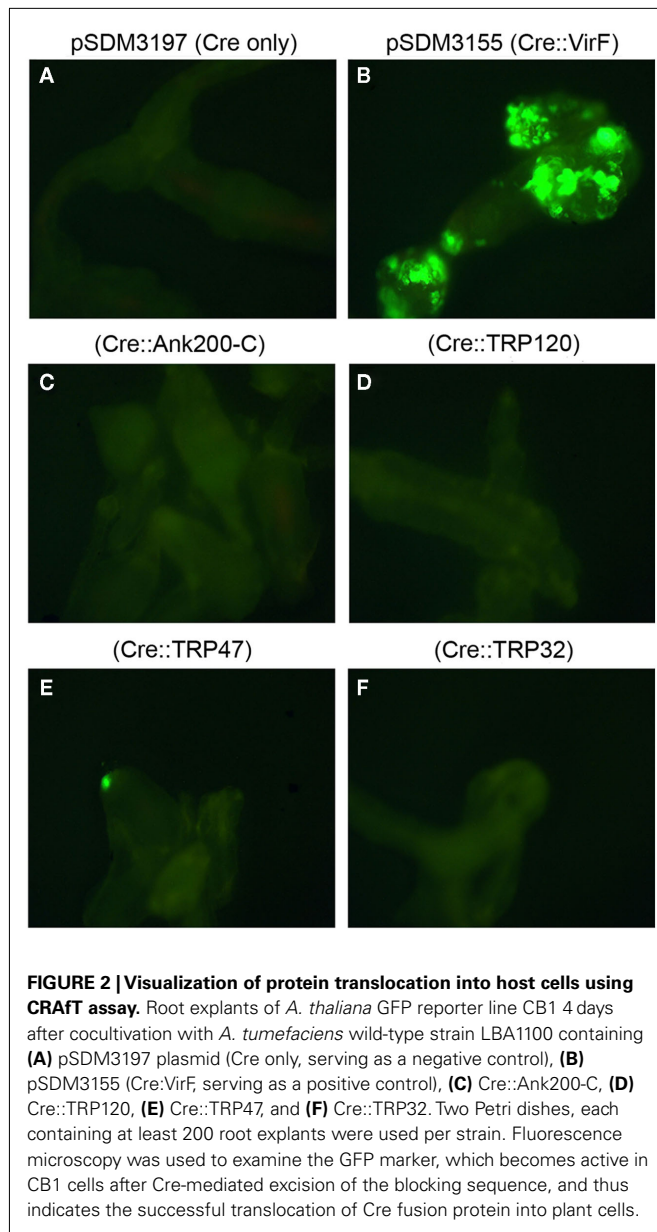


FIGURE 2 | Visualization of protein translocation into host cells using CRAFT assay. Root explants of *A. thaliana* GFP reporter line CB1 4 days after cocultivation with *A. tumefaciens* wild-type strain LBA1100 containing (A) pSDM3197 plasmid (Cre only, serving as a negative control), (B) pSDM3155 (Cre:VirF, serving as a positive control), (C) Cre::Ank200-C, (D) Cre::TRP120, (E) Cre::TRP47, and (F) Cre::TRP32. Two Petri dishes, each containing at least 200 root explants were used per strain. Fluorescence microscopy was used to examine the GFP marker, which becomes active in CB1 cells after Cre-mediated excision of the blocking sequence, and thus indicates the successful translocation of Cre fusion protein into plant cells.

chaffeensis VirD4. The expression of *E. chaffeensis* VirD4–c-Myc fusion protein in *A. tumefaciens* was confirmed by immunoblot using c-Myc specific antibodies (data not shown). Functional replacement of *A. tumefaciens* VirD4 by *E. chaffeensis* VirD4 was evaluated in a tumor assay on *Nicotiana glauca*. Strains with the wild-type *A. tumefaciens* Ti-plasmid, LBA1010 (octopine pTiB6; Beijersbergen et al., 1992), and strain LBA1010 Δ VirD4 (LBA2586) complemented by *A. tumefaciens* VirD4 protein, caused similar levels of tumor formation. In contrast, strain LBA2586 complemented by the *E. chaffeensis* VirD4 protein induced no or much smaller overgrowths, hardly better than LBA2586 in *N. glauca* (Figure 3), corroborating that *A. tumefaciens* VirD4 is essential for virulence and that *E. chaffeensis* VirD4 cannot complement LBA2586 in the tumor assay on *N. glauca*. Thus, it is possible that the *E. chaffeensis* VirD4 cannot function as an intermediate

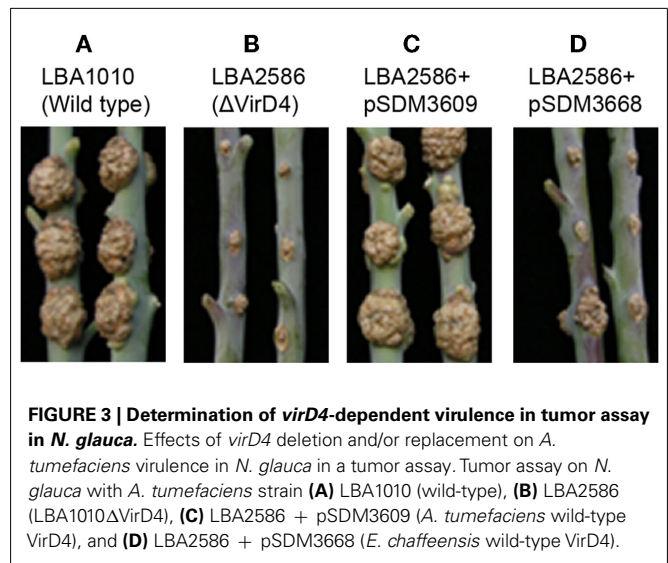


FIGURE 3 | Determination of *virD4*-dependent virulence in tumor assay in *N. glauca*. Effects of *virD4* deletion and/or replacement on *A. tumefaciens* virulence in *N. glauca* in a tumor assay. Tumor assay on *N. glauca* with *A. tumefaciens* strain (A) LBA1010 (wild-type), (B) LBA2586 (LBA1010 Δ VirD4), (C) LBA2586 + pSDM3609 (*A. tumefaciens* wild-type VirD4), and (D) LBA2586 + pSDM3668 (*E. chaffeensis* wild-type VirD4).

in the transfer of the *A. tumefaciens* translocation substrates to the VirB channel. In the following step, protein translocation was tested in the CRAFT assay on *A. thaliana* CB1. In this assay, derivatives of the non-tumorigenic (oncogenic T-DNA lacking) helper strain LBA1100 and LBA2587, LBA1100 with the same *virD4* deletion as in LBA2586, were used. A large number of CB1 cells expressing GFP were observed 3 days post cocultivation with *A. tumefaciens* strain LBA1100 [45] containing Cre:VirF (positive control), whereas no GFP expressing cells were seen after cocultivation with the *virD4* mutant LBA2587 containing Cre:VirF (negative control). Complementation of the *virD4* mutant by a plasmid containing *A. tumefaciens* *virD4* restored its ability for Cre:VirF translocation, but introduction of the *E. chaffeensis* *virD4* did not lead to translocation of the Cre:VirF protein. This further confirms that the *E. chaffeensis* VirD4 cannot mediate the translocation of the *A. tumefaciens* T4SS substrates to the VirB channel. In order to test whether *E. chaffeensis* VirD4 could mediate translocation of the *E. chaffeensis* substrates, the above strains (LBA1100, 2587, 2587/3609, 2587/3668, 1100/3668) were tested for translocation of Cre:TRP32, Cre:TRP47, Cre:TRP120, and Cre:Ank200-C. However, also in the presence of *E. chaffeensis* VirD4 no or only rarely GFP expressing cells were seen in the CRAFT assays, indicating that even in the presence of *E. chaffeensis* VirD4 no clear indication for translocation of Cre:TRP32, Cre:TRP47, Cre:TRP120, and Cre:Ank200-C by the T4SS was obtained (Table A3 in Appendix). These findings demonstrate that *E. chaffeensis* TRP32, TRP47, TRP120, and Ank200 are not translocated to host cells by the T4SS and suggest that their translocation is mediated by another secretion system.

***E. chaffeensis* Ank200 is a tyrosine phosphorylated effector protein**
Ank200 is the largest immunoreactive protein identified in *E. chaffeensis* and is translocated to the nuclei of the infected monocytes, where it interacts with the mid-A-stretch of host promoter and intronic *Alu* elements (Zhu et al., 2009; Luo et al., 2010). It contains 11 potential tyrosine phosphorylation sites as predicted by NetPhos 2.0. In order to identify the *E. chaffeensis* tyrosine

phosphorylated proteins we performed Western blotting analysis of uninfected and *E. chaffeensis*-infected THP-1 cell lysates with anti-pTyr monoclonal antibody (PY99). The Western blot analysis showed that *E. chaffeensis* infection of THP-1 cells led to a major tyrosine phosphorylated protein at ~200 kDa (Figure 4A). To confirm the protein identity, an Ank200 specific antibody was used (Figure 4B). This 200 kDa protein was further detected by Western blot analysis using anti-Ank200 antibody in lysates of *E. chaffeensis*-infected THP-1 cells immunoprecipitated with anti-pTyr antibody and not in lysates of *E. chaffeensis*-infected THP-1 cells immunoprecipitated with normal mouse IgG confirming that the 200-kDa protein is tyrosine phosphorylated Ank200 (Figure 4C).

Comparative biophysical and domain analysis of tyrosine phosphorylated Ank proteins

The *E. chaffeensis* Ank200 and *A. phagocytophilum* AnkA proteins have recently been the focus of the several studies (McBride et al., 2003; Park et al., 2004; IJdo et al., 2007; Lin et al., 2007; Thomas and Fikrig, 2007; Garcia-Garcia et al., 2009; Zhu et al., 2009; Luo et al., 2010). The *E. chaffeensis* Ank200 and *A. phagocytophilum* AnkA proteins both contain Ank repeats and both are tyrosine phosphorylated (this study, IJdo et al., 2007; Lin et al., 2007). Some functional similarities have been reported between *E. chaffeensis* Ank200 and *A. phagocytophilum* AnkA, including translocation to the host cell nucleus and DNA interactions (Park et al., 2004; Garcia-Garcia et al., 2009; Zhu et al., 2009). Using the Cre recombinase reporter assay of *A. tumefaciens* a recent study reported that AnkA is translocated by the VirB/D4-dependent T4SS into the host cells (Lin et al., 2007). However, using the same Cre recombinase reporter assay, we found that Ank200 was not translocated by the VirB/D4-dependent T4SS, suggesting that Ank200 is translocated by another mechanism.

Although Ank200 and AnkA appear functionally similar, they have no significant sequence homology as demonstrated by their sequence alignment (BLASTN), and also have different biophysical properties, and thus, appear to be different in nature (Figure A1 in Appendix; Altschul et al., 1997). However, a search of *E. chaffeensis* Ank200 orthologs in the Integrated Microbial Genomes database identified *A. phagocytophilum* AnkA as an ortholog of Ank200, but with a limited (22%) sequence similarity that is primarily located in the Ank domain-containing regions of both the proteins. Ank200 (1463 amino acids) is more acidic (pI 4.9) with

the majority of Ank motifs localized to the central region, while the tyrosine kinase, Src homology 2 (SH2), and Src homology 3 (SH3) domains are located in the N-terminus of the protein, which is more hydrophilic (Figure A1A in Appendix). In contrast, AnkA (1232 amino acids) is less acidic (pI 6.1), the Ank domains are localized to two distinct domains (N-terminus and central region) while the majority of tyrosine kinase, SH2, and SH3 domains were in the hydrophilic C-terminus of the protein (Figure A1B in Appendix). Moreover, the AnkA C-terminal 20 amino acids (SQPEAPQSEGPKSVKGGGRGR) are more hydrophilic (grand average of hydropathy, -1.68 , ExPasy Proteomic Server) and in agreement with the requirements of the C-terminal T4SS signal [R-X(7)-R-X-R-X-R] (Vergunst et al., 2005) while the Ank200-C-terminal 20 amino acids (AVSPSTS QGADVKKSSCQSK) are less hydrophilic (grand average of hydropathy, -0.76) and do not have a prototypical T4SS signal (Figure A1C in Appendix).

EXAMINATION OF *E. CHAFFEENSIS*-SECRETED TRPs AND Ank PROTEINS IN T1SS

E. chaffeensis TRP47 TRP120, TRP32, and Ank200 amino acid composition and characteristics

The *E. chaffeensis* genome (NCBI accession number NC_007799) encodes T1SS genes (Hotopp et al., 2006). The *E. coli* hemolysin secretion system considered to be the prototype T1SS and is composed of the HlyB and HlyD proteins encoded by genes normally cotranscribed with *hlyC* and *hlyA*, while the outer membrane protein is encoded outside of the *hly* operon on the chromosome (Welch and Pellett, 1988; Wandersman and Delepeleire, 1990). We performed a BLASTP search for *E. chaffeensis* T1SS component genes (ECH_0383, ECH_0970, ECH_1020), and BLASTP identified a closest match for *E. coli hlyB* (YP_308793.1), *hlyD* (ZP_08360101.1), and *tolC* (EGB61997.1) genes with 27% ($P = 5 \times 10^{-56}$), 28% ($P = 10^{-42}$), and 26% ($P = 10^{-26}$) identity, respectively (Altschul et al., 1997). Although the similarity was low, the BLASTP results indicated that *E. coli*-like T1SS components exist in *E. chaffeensis*. Previous complementation studies have shown that the gene products of *hlyB*, *hlyD*, and *tolC* are required for the secretion of *E. coli* hemolysin (Mackman et al., 1985a,b; Wandersman and Delepeleire, 1990). The last 27 amino acids of the C-terminal region of hemolysin contain a specific signal sequence required for secretion (Nicaud et al., 1986; Mackman et al., 1987; Koronakis et al., 1989). The examination of the last 27 amino acids of the C-terminal region of the *E. chaffeensis* TRP47 and TRP120 proteins in a blast (BLASTP) search identified homology to several type 1 secretion substrates including ABC superfamily ABC transporter binding protein (*Achromobacter piechaudii*), ABC transporter periplasmic-binding protein (*Bordetella petrii*), and hemolysin (*Sphingobacterium spiritivorum*), and hemolysin A (*S. spiritivorum*; Table 1). A BLASTP search of the Ank200-C-terminal (last 27 amino acids) identified 69 and 89% homology to putative ABC transporter permease protein (*Streptomyces cattleya*) and nitrate/sulfonate/bicarbonate ABC transporter periplasmic protein (*Starkeya novella*), respectively (Table 1). Moreover, the *E. chaffeensis* TRP47 seven 19-mer TRs (ASVSEG-DAVVNAVVSQETPA, each repeat) covering a major part of the C-terminal region (42% of the full length protein) is glycine- and aspartate-rich and exhibits homology to adhesin (*Staphylococcus*

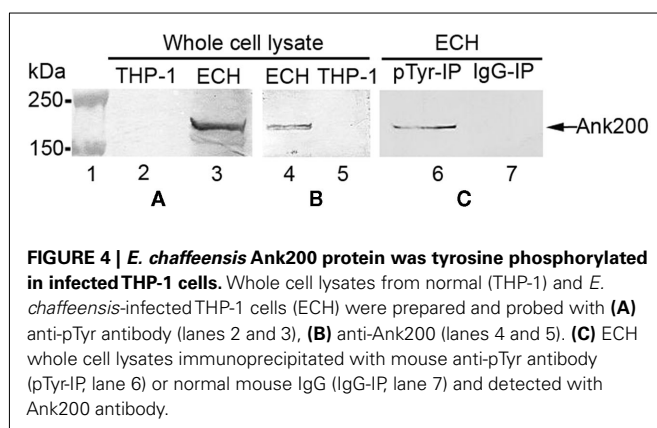


Table 1 | NCBI BLASTP analysis result of C-terminal 27 amino acids of (A) TRP47, (B) TRP120, (C) TRP32, and (D) Ank200 identified homology to type 1 secretion substrates.

Accession	Description	Total score	% Similarity	% Coverage
(A)				
ABA39260.1	Immunodominant surface protein gp47 (<i>Ehrlichia chaffeensis</i>)	92.7	100	100
YP_004041752.1	DNA replication and repair protein recf (<i>Paludibacter propionigenes</i>)	35.8	67	70
YP_003977269.1	Extracellular solute-binding protein, family 5 middle family protein 5	34.1	67	59
EFV82729.1	ABC transporter (<i>Achromobacter xylosoxidans</i> C54)	33.7	72	59
ZP_06686212.1	ABC superfamily ATP-binding cassette transporter, binding protein (<i>Achromobacter piechaudii</i> ATCC 43553)	33.7	72	59
YP_001630192.1	ABC transporter periplasmic-binding protein (<i>Bordetella petrii</i> DSM 12804)	33.7	72	59
(B)				
AAM00417.1	120-kDa antigen (<i>Ehrlichia chaffeensis</i>)	96.5	100	100
ACO11363.1	Ras-related GTP-binding protein A (<i>Caligus rogercresseyi</i>)	33.3	67	62
XP_001348074.1	transcription factor with AP2 domain(s), putative (<i>Plasmodium falciparum</i>)	94.6	33	81
ZP_07081229.1	Hemolysin (<i>Sphingobacterium spiritivorum</i> ATCC 33861)	31.6	64	51
ZP_03970623.1	Hemolysin A (<i>Sphingobacterium spiritivorum</i> ATCC 33300)	31.6	64	51
(C)				
AAD38131.1	Variable length PCR target protein (<i>Ehrlichia chaffeensis</i>)	101	100	100
YP_001956809.1	Hypothetical protein 201phi2-1p084 (<i>Pseudomonas phage</i> 201phi2-1)	34.1	63	59
ABR15596.1	Immunoglobulin mu heavy chain (<i>Oncorhynchus mykiss</i>)	34.1	75	44
NP_811832.1	Hypothetical protein BT_2920 (<i>Bacteroides thetaiotaomicron</i> VPI-5482)	32.9	48	59
NP_001023194.1	Cyclic Nucleotide Gated channel family member (cng-3) (<i>Caenorhabditis elegans</i>)	32	78	33
XP_002422896.1	Voltage and ligand gated potassium channel, putative (<i>Pediculus humanus corporis</i>)	30.8	73	40
(D)				
AEG67297.1	Ankyrin repeat-containing protein (<i>Ehrlichia chaffeensis</i>)	82.9	100	100
XP_4187072	PREDICTED: similar to multidrug resistance protein 1a (<i>Gallus gallus</i>)	32.0	65	62
XP_001489965.3	PREDICTED: zinc finger protein 709-like (<i>Equus caballus</i>)	30.3	75	48
AEJ44582.1	NADH:flavin oxidoreductase/NADH oxidase (<i>Alicyclobacillus acidocaldarius</i> subsp. <i>acidocaldarius</i> Tc-4-1)	29.1	75	44
CCB76597.1	Putative ABC transporter permease protein (<i>Streptomyces cattleya</i> NRRL 8057)	26.9	69	48
ZP_06190193.1	Hydrophobe/amphiphile efflux-1 (HAE1) family protein (<i>Serratia odorifera</i>)	26.9	63	74
YP_003696031.1	Nitrate/sulfonate/bicarbonate ABC transporter periplasmic protein (<i>Starkeya novella</i> DSM 506)	26.5	89	33

Most of the type 1 secretion system substrates of Gram-negative bacteria contain a translocation signal at the carboxyl terminus. The complete signal is contained within the HlyA C-terminal 113 residues, and it has been suggested that it may be located entirely within the extreme terminal (27 amino acid) sequence. We analyzed the extreme C-terminal 27 amino acids of TRP47 and TRP120 using NCBI BLASTP; the results reveal that TRP47 and TRP120 have homology to other type 1 secretion substrates.

epidermidis, SdrE), which is consistent with the common attributes of T1SS substrates (Delepelaire, 2004). A TRP32 C-terminal BLASTP search determined no substantial homology to other type 1 substrates; however, it identified homology to a Cyclic Nucleotide Gated Channel family member (*Caenorhabditis elegans*), an ion transport protein related to voltage and ligand gated potassium channel.

The T1SS translocates proteins to the extracellular environment via a C-terminal uncleaved secretion signal. We analyzed the last 50 C-terminal residues of TRP47, TRP120, TRP32, and Ank200 for the presence of LDAVTSIF amino acids and found that LDAVTSIF constitutes 54, 46, 38, and 56% of TRP47, TRP120, TRP32, and Ank200, respectively (Table 2). A previous study based on alignment and statistical analysis of the last 50 C-terminal residues of putative type 1 secreted proteins identified LDAVTSIF-enriched and KHPMWC-poor amino acids (Delepelaire, 2004).

Almost all the T1SS secreted proteins that have been characterized, including HlyA, LktA, CyaA, share a common domain structure and a secretion signal in the C-terminal domain of the protein (Delepelaire, 2004; Holland et al., 2005; Linhartova et al., 2010). *E. chaffeensis* TRPs and Ank200 exhibited a domain structure similar to repeats-in-toxin (RTX) exoprotein family such as HlyA, LktA, and CyaA (Figures 5A–C). Although the TRP47 19 amino acid TR sequence (ASVSEGDVVNAVSQETPA) was not identical to RTX consensus sequence, it exhibited 69% similarity to S-layer protein in *Methanoterris igneus* (YP_004485351.1), 56% similarity to hemagglutinin in *Stenotrophomonas* sp. (ZP_05134659.1), 55% similarity to ABC transporter ATP-binding protein in *Alteromonas* sp. (YP_004469594.1) and 100% similarity to ABC superfamily ABC transporter, ABC protein in *Kingella denitrificans* (ZP_08132666.1), and metalloprotease, hemolysin-type calcium-binding region in *Cupriavidus taiwanensis* (YP_002008092.1).

Table 2 | Analysis of 50 C-terminal residues for occurrence of type 1 secretion signal.

Protein	Occurrences of L DA VTSIF rich amino acids in the 50 C-terminal residues of type 1 secretion signal (Delepelaire, 2004) marked with underline
TRP47	Q <u>ET</u> PAASVSEGD <u>AVV</u> NAV <u>SO</u> ETPATQPOS <u>R</u> D <u>S</u> LLNEEDMAAQ <u>F</u> GNRY <u>E</u> Y <u>F</u> (27/50 = 54%)
TRP120	YMYGFQDV <u>K</u> D <u>L</u> LG <u>L</u> LSNV <u>P</u> CCNV <u>S</u> LY <u>F</u> MEHNY <u>F</u> TNHNEN <u>I</u> N <u>H</u> N <u>V</u> N <u>D</u> I <u>V</u> (23/50 = 46%)
TRP32	<u>L</u> LLGGV <u>F</u> STMN <u>Y</u> LSG <u>Y</u> TP <u>Y</u> Y <u>Y</u> HHYCCYN <u>P</u> Y <u>Y</u> Y <u>F</u> D <u>V</u> TP <u>D</u> Y <u>C</u> H <u>H</u> C <u>S</u> ESS <u>L</u> E (19/50 = 38%)
Ank200	<u>S</u> EEQL <u>Q</u> EL <u>S</u> EEITD <u>I</u> V <u>Q</u> GL <u>P</u> PI <u>S</u> ED <u>I</u> GA <u>Q</u> AV <u>S</u> P <u>S</u> T <u>S</u> OG <u>A</u> D <u>V</u> K <u>K</u> <u>S</u> SC <u>Q</u> <u>S</u> K (28/50 = 56%)
<i>E. coli</i> HlyA	<u>P</u> LINE <u>S</u> K <u>I</u> SAAGN <u>F</u> D <u>V</u> KEERA <u>A</u> AS <u>L</u> L <u>Q</u> LSGN <u>A</u> S <u>D</u> FS <u>Y</u> GRNS <u>I</u> TL <u>T</u> ASA (33/50 = 66%)

Type 1 secretion system secretes proteins to the extracellular environment via a C-terminal uncleaved secretion signal. The elements important for C-terminal secretion signal activity are still poorly understood. Alignment and statistical analysis of the last 50 C-terminal residues of all the putative type 1 secreted proteins (Delepelaire, 2004) exhibited higher frequency of L~~DA~~VTSIF amino acids. Although it is difficult to extend this gross analysis as different secretion systems are likely to have different requirements for their cognate C-terminal signals, we analyzed the last 50 C-terminal residues of TRP47, TRP120, TRP32, and Ank200 for presence of L~~DA~~VTSIF amino acids and found that L~~DA~~VTSIF constitutes 54, 46, 38, and 56% of TRP47, TRP120, TRP32, and Ank200, respectively (% values for each protein are shown in parentheses).

Furthermore, BLASTP identified amino acid sequence GDAVVN in each of the seven 19 amino acids TR sequences, which showed 100% similarity to ABC transporter ATP-binding protein in *Gluconacetobacter hansenii* (ZP_06834421.1) and *Acetobacter pasteurianus* (YP_003188074.1). An identical consensus sequence (GDAXN) predicted to bind calcium ions has been identified in RTX proteins (Linhartova et al., 2010; Figure 5D).

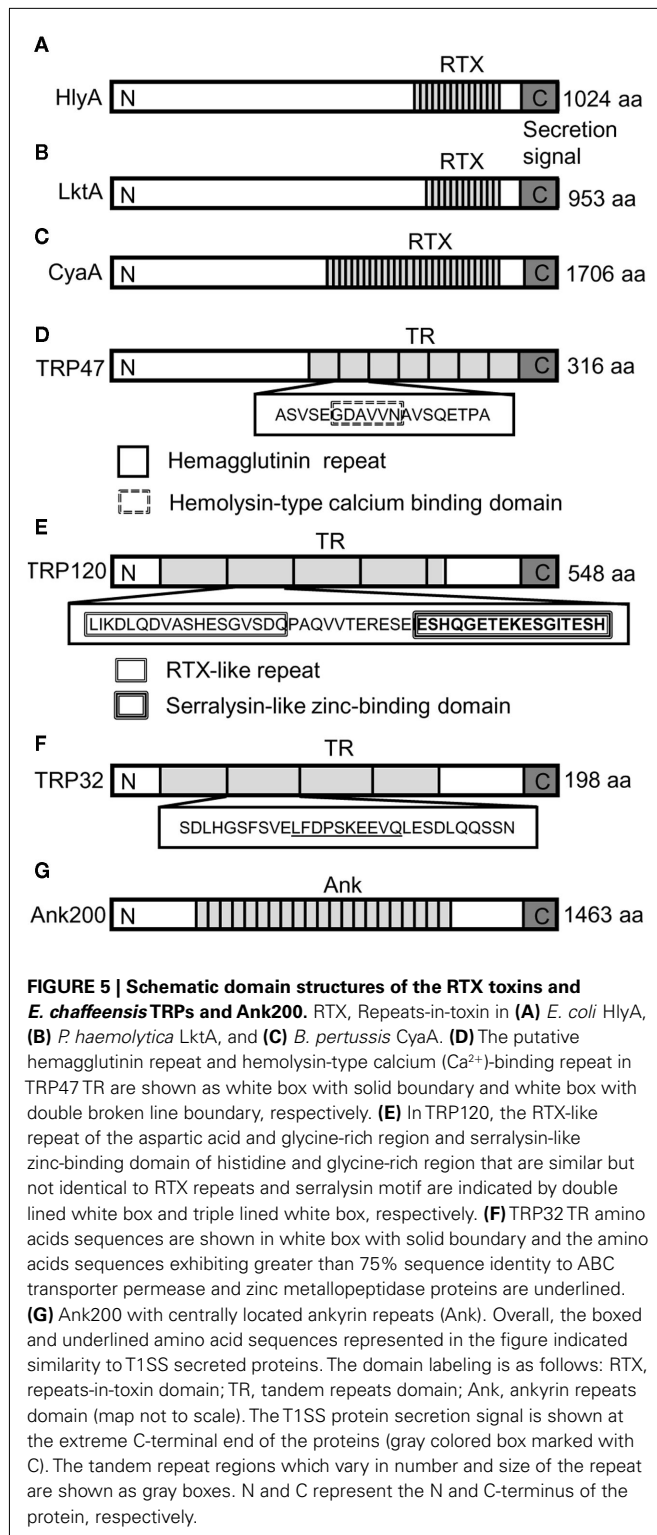
Although the consensus sequence of RTX toxin (L/I/F-X-G-G-X-G-N/D-X, where X represents any amino acid) was not identified in the TRPs, but an RTX-like sequence (L/I/K-D-L-Q-D-VASHESGVSDQ) was found three times within the 80 amino acids long TRP120 TRs that exhibited 45% similarity with the ABC transporter, ATP-binding protein in *Bacteroides clarus* (ZP_08297392.1; Figure 5E). A part of the RTX-like sequence VASHESGVSDQ exhibited 64% similarity with putative ABC transporter ATP-binding protein in *Marine actinobacterium* (ZP_01129295.1) and ABC transporter ATP-binding protein in *B. vulgatus* (YP_001297542.1), *B. fluxus* (ZP_08301787.1), and *B. clarus* (ZP_08297392.1). In addition, a unique TRP120 amino acid sequence (SEPFVAESEVSKVE) found within the TRs was similar to type 1 secretion membrane fusion protein, HlyD in *Pectobacterium wasabiae* and *Pseudomonas mendocina*, indicating that these regions may be required for TRP120 extracellular secretion by T1SS. Another unique glutamic acid- and histidine-rich amino acid sequence (ESHQGETEKESGITESH) was detected within the TRP120 TRs that exhibited similarity to zinc finger protein in *Ailuropoda melanoleuca* and *Canis familiaris* reminiscent of zinc-binding motif (HEXXHXXGXXH) analogous to that of the serralsin motif reported in *P. pneumotropica* RTX toxin PnxIIA (Sasaki et al., 2009; Figure 5E). Interestingly, the TRP32 TR showed homology to ATPase in *Archaeoglobus profundus* (YP_003400909.1) and beta-lactamase in *Bacteroides vulgatus* (ZP_06741900.1). Beta-lactamases were previously identified and predicted among the computationally detected RTX proteins (Linhartova et al., 2010). Moreover, TRP32 TR amino acid sequence (LFDPSKEEVQ) showed 80% identity to putative ABC transporter permease protein in *Desulfovibrio magneticus* (YP_002953007.1) and 75% identity to zinc metallopeptidase in *Segniliparus rotundus* (YP_003658757.1; Figure 5F). Although, we did not observe

any homology of Ank200 to RTX proteins, a search for the RTX repeat structure GGXGXG using PATTINPROT software program set to find regions with 50 and 75% identity to the consensus RTX sequence identified a total of 27 and 4 repeat domains in Ank200. Moreover, the histidine-rich ankyrin repeat domain in Ank200 showed homology to zinc finger proteins that are involved in protein-protein and protein-DNA interactions (Figure 5G).

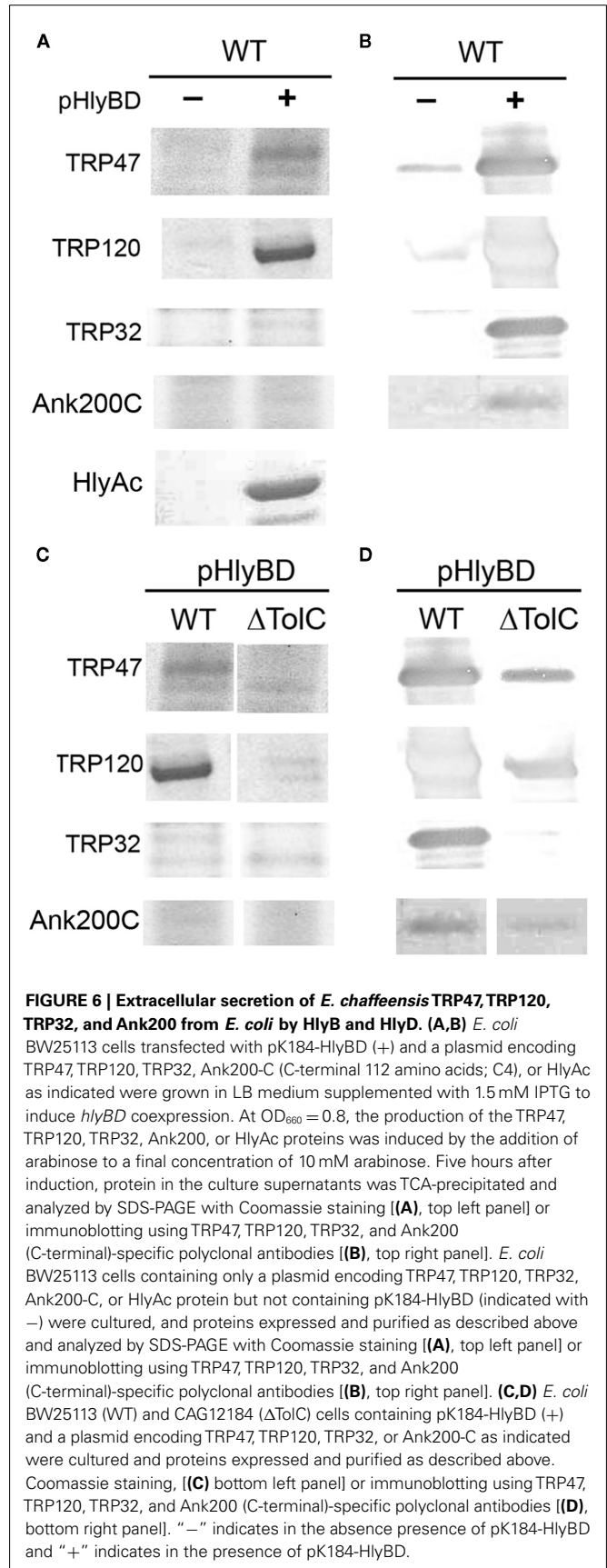
Secretion of *E. chaffeensis* TRPs and Ank200 by *E. coli* expressing HlyB and HlyD

Although, many previous studies using biochemical and molecular cellular imaging such as immunofluorescence and immunoelectron microscopy have clearly provided evidence of extracellular secretion of *E. chaffeensis* TRPs and Ank200 in infected mammalian cells, the secretion mechanism is unknown (Popov et al., 2000; Doyle et al., 2006; Luo et al., 2008; Zhu et al., 2009). TRP domain homology to RTX toxins and recent reviews of RTX toxins (Delepelaire, 2004; Linhartova et al., 2010) were supportive of *E. chaffeensis* TRPs as T1SS substrates. Thus, we investigated the ability of the *E. coli* HlyB and HlyD proteins to directly secrete *E. chaffeensis* TRPs and Ank200 into the extracellular medium. To this end, *E. coli* K-12 strain BW25113 that contains *tolC*, but does not contain the *hlyCABD* genes required for secretion of hemolysin was complemented with a dual vector, where vector pK184-HlyBD encodes inner membrane components HlyB and HlyD under the control of a *lacZ* promoter reconstituting the type 1 secretion apparatus and another vector pTRP/Ank200 encodes either *E. chaffeensis* TRP47, TRP120, TRP32, Ank200C4, or pHlyAc was used in the secretion assay.

In the type 1 secretion assay, large amounts of TRP47, TRP120, and TRP32 were secreted into the extracellular medium only in the presence of vector pK184-HlyBD compared to *E. coli* strain WM25113 harboring the single vector pTRP (Figures 6A,B). Although the expression levels of the TRPs were similar in *E. coli* lysates (data not shown), a higher concentration of *E. chaffeensis* TRP120 was detected in the supernatant compared to TRP47 and TRP32, and similar to that of HlyAc. Secretion of 23 kDa HlyAc into the medium was observed in the presence of the dual vector, pK184-HlyBD and pHlyAc, indicating that the HlyBD transport



components were functional as previously demonstrated (Bakkes et al., 2010) and served as a positive control (Figure 6A). The size of the secreted TRP47, TRP120, and TRP32 was consistent with the sizes of the native proteins which migrate at larger than the actual molecular masses in SDS-PAGE (Wakeel et al., 2010a)

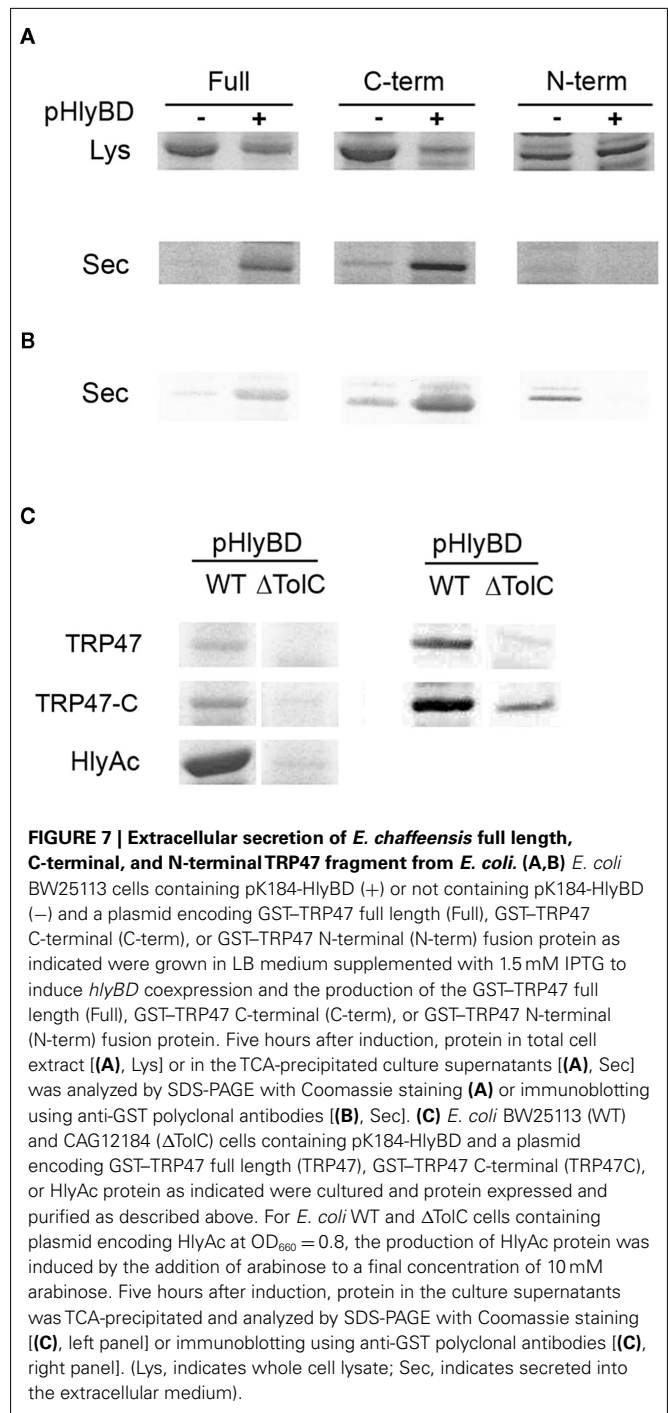


as was demonstrated by Coomassie stain and confirmed by western immunoblotting using TRP47, TRP120, and TRP32 specific antibodies (Figures 6A,B). Ank200-C-terminus contains a type 1 secretion-like signal sequence as indicated by its similarity to ABC transporter permease and ABC transporter periplasmic proteins (Table 1). The Ank200C4 (Ank200-C-terminal 112 amino acids) secretion was detected in the extracellular medium only in the presence of HlyBD demonstrating that Ank200C4 is secreted by the functional T1SS system (Figures 6A,B).

The structures at the C termini of RTX toxins that serve as secretion signals as well as the proteins required for their secretion are conserved among the bacteria secreting RTX toxins. This conservation is demonstrated by the ability of some of the transport proteins to mediate secretion of heterologous RTX toxins (Chang et al., 1989; Masure et al., 1990). In order to further define the domain necessary for secretion, we selected TRP47 as a model *E. chaffeensis* TRP and performed the secretion assay as described above for full length TRP47 using a dual vector system where type 1 secretion components HlyB and HlyD expressed by one vector and the substrate expressed by another vector in *E. coli* exhibited an increased level of secretion of C-terminal and full length GST-TRP47 fusion proteins in the presence of pHlyBD (Figures 7A,B). The N-terminal region of TRP47 was not secreted by itself to the extracellular medium. These results are consistent with the previous reports emphasizing the importance of the C-terminal domain of hemolysin which contains a secretion signal sequence that is required for secretion of the protein (Nicaud et al., 1986; Mackman et al., 1987; Koronakis et al., 1989) and demonstrating that secretion of TRP47 into the extracellular medium is dependent on type 1 secretion components similar to hemolysin.

Extracellular secretion of *Ehrlichia chaffeensis* TRPs and Ank200 is reduced in the absence of *Escherichia coli* TolC protein

The type 1 secretion apparatus always includes a specific outer membrane protein, and in case of *E. coli* hemolysin secretion, this protein is TolC (Wandersman and Deleplaire, 1990). The TolC protein is an essential *E. coli* outer membrane protein that is required for hemolysin secretion (Wandersman and Deleplaire, 1990). In this study, we used a *tolC210::Tn10*, an insertional mutant derivative of *E. coli* K-12 strain CAG12184 (Singer et al., 1989). We cotransformed *tolC210* with vector pK184-HlyBD and vector pTRP/Ank200C4 or pHlyAc to examine the extracellular secretion of *E. chaffeensis* TRPs, Ank200C4, and HlyAc. This *tolC* mutant strain containing pK184-HlyBD exhibited a reduced level of *E. chaffeensis* TRP47, TRP120, TRP32, Ank200C4, and HlyAc secretion into the extracellular medium compared to wild-type *E. coli* (Figures 6C,D and 7C). Moreover, secretion of full length and C-terminal of GST-TRP47 fusion proteins was reduced in the *tolC* mutant compared to wild-type *E. coli* (Figure 7C). A small amount of protein (TRP47, TRP120, Ank200) was detected in supernatants of *tolC* mutant by western immunoblot, but no extracellular protein was detected for TRP32, which may be due to minimal lysis from overexpression or inefficient secretion due to the fact that HlyBD are expressed and functional (through complementation; Figures 6D and 7C). These results demonstrate that the outer membrane component, TolC, is important for translocation of the *E. chaffeensis* proteins from *E. coli*.



DISCUSSION

In bacteria, secretion is essential for virulence and survival, and it is well established that TRPs and Ank200 proteins of *Ehrlichia* spp. are secreted and are involved in complex protein-protein and protein-DNA interactions with a diverse group of host cell targets and genes and are protective primary targets of the host humoral immune response (Yu et al., 1997; Sumner et al., 1999; McBride et al., 2003, 2007; Doyle et al., 2006; Nethery et al., 2007; Luo et al., 2009, 2010). *E. chaffeensis*, an obligately intracellular

bacterium, resides and proliferates inside mononuclear phagocytes by manipulating host cell processes that affect cell signaling, transcription, and vesicle trafficking through specific interactions of its surface-expressed and secreted effector proteins (Popov et al., 2000; Doyle et al., 2006; Luo et al., 2008, 2009, 2011; Wakeel et al., 2010b; Zhu et al., 2011). Immunoelectron microscopy has identified TRP47 and TRP120 as differentially expressed proteins on the surface of dense-cored (DC) ehrlichiae, in addition to a non-differentially expressed TRP32, all of which are extracellularly associated with morular fibrillar matrix and the morula membrane, indicating that these proteins are secreted (Popov et al., 2000; Doyle et al., 2006; Luo et al., 2008). We have recently demonstrated that TRP47 interacts with multiple host proteins associated with cell signaling, transcriptional regulation, and vesicle trafficking and that TRP120 binds a G + C-rich motif in host cell DNA and exhibits eukaryotic transcriptional activator function and interacts with a diverse array of host proteins involved in transcription, signaling, and cytoskeleton organization similar to TRP47 (Wakeel et al., 2009; Luo et al., 2011; Zhu et al., 2011). Ank200 is translocated to the host cell nucleus where it binds with a specific adenine-rich motif of host promoter and intronic *Alu* elements (Zhu et al., 2009).

In general T1SS substrates are acidic proteins that contain TRs and a C-terminal secretion signal that is not cleaved during secretion. Protein BLAST (BLASTP) search of C-terminal amino acid sequence of TRP47, TRP120, TRP32, and Ank200 identified homology with type 1 secretion substrates (Altschul et al., 1997). Moreover, *E. chaffeensis* TRPs are acidic (pI ~ 4) similar to type 1 substrates of other Gram-negative pathogens. A consensus T4SS substrate signal [R-X(7)-R-X-R-X-R] (Vergunst et al., 2005) is not present in TRPs. However, Ank200 contains a putative T4SS substrate motif, which is not similar to the prototypical T4SS signal. Although, previous studies have suggested secretion of the TRPs and Ank200 to be Sec-independent as they lack a classical signal peptide (SecretomeP 2.0), the secretion mechanisms of these *E. chaffeensis* effectors have remained undetermined. In this study we examined secretion of *E. chaffeensis* TRPs and Ank200 in T1SS and T4SS models and determined that TRPs and Ank200 are secreted into to the extracellular medium by T1SS similar to *E. coli* hemolysin and consistent with other RTX family exoproteins.

Recently, the use of a surrogate host enabled the identification of secretion substrates of a T4SS functioning in the obligate intracellular pathogen *C. burnetii*, which phylogenetically closely related to *L. pneumophila*. Both contain a Dot/Icm-like T4SS (Voth and Heinzen, 2009). Eleven *C. burnetii* Ank proteins expressed in *L. pneumophila* were found to be translocated through the *L. pneumophila* Dot/Icm system (Voth and Heinzen, 2009; Voth et al., 2009). In order to identify the substrates of the *E. chaffeensis* T4SS machinery, we investigated the secretion of *E. chaffeensis* Ank200, TRP32, TRP47, and TRP120 by using a previously developed CRAfT assay, which was used for the identification of T4SS translocation substrates from *A. tumefaciens* (Vergunst et al., 2000, 2005). The data obtained from the CRAfT assays demonstrated that translocation of Cre:: *Ehrlichia* Ank200, TRP32, TRP47, and TRP120 fusion proteins to *A. thaliana* CB1 plant cells by the T4SS does not occur. Although, the use of this heterologous T4SS system

has provided insights into the translocation of many effector proteins, this study indicated that the *E. chaffeensis* TRPs and Ank200 were not translocated by the T4SS, underscoring the likelihood that another secretion mechanism may be involved in their secretion from *E. chaffeensis* into infected host cell (Doyle et al., 2006; Hotopp et al., 2006; Luo et al., 2008; Wakeel et al., 2009; Zhu et al., 2009).

Although the T4SS has been reported to be responsible for substrate translocation by *Anaplasmataceae*, only two T4SS substrates have been identified so far, one (AnkA) by the CRAfT assay and another (Ats-1) by using the bacterial two-hybrid assay (Lin et al., 2007; Niu et al., 2010; Rikihisa and Lin, 2010). Contrary to *A. tumefaciens*, in the *E. chaffeensis* genome the T4SS genes are spread over five groups, and several *virB* genes are duplicated (Hotopp et al., 2006; Cheng et al., 2008; Alvarez-Martinez and Christie, 2009). Although, *trp120* is in the opposite orientation relative to the *virB8-virD4* cluster (Yu et al., 1997), the close proximity of these genes is suggestive of a coordinated expression and function among T4SS and surface constituents (Alvarez-Martinez and Christie, 2009). Interestingly, although TRP120, which is located downstream of *virD4* (*ribA-virB8-virB9-virB10-virB11-virD4-trp120*), it is not a T4SS substrate in contrast to other Gram-negative bacteria (Schulein et al., 2005; Hotopp et al., 2006; Alvarez-Martinez and Christie, 2009).

The results of this study are particularly important in the light of a previous report (Lin et al., 2007) and highlight our conclusion that Ank200 of *E. chaffeensis* is distinct from *A. phagocytophilum* AnkA in many respects. For instance, they have dissimilar nucleic acid sequences and exhibit a minimal (22%) amino acid identity limited to conserved Ank repeats. In Ank200 there are centralized Ank domains, and a majority of motifs including tyrosine kinase motif are localized in the N-terminus compared to AnkA where the Ank domains are spread over two major loci in the N-terminus and the central region, respectively, and the majority of motifs are in the C-terminus of the protein. However, most importantly, the C-terminal 20 amino acids of Ank200 and AnkA are clearly different, whereby the C-terminus of AnkA has more amino acids sequence similarity to the T4SS substrate signal [R-X(7)-R-X-R-X-R] (Vergunst et al., 2005) than that of Ank200, and thus AnkA, but not Ank200 is secreted by the T4SS machinery. Similarity of Ank200 domain structure and homology to TRPs and other T1SS substrates suggested that Ank200 is a T1SS substrate. Indeed, in this study, we demonstrated that Ank200-C-terminal (112 amino acids) peptide is secreted by T1SS.

Several previous studies reported that infection with *Ehrlichia* or *Anaplasma* induces tyrosine phosphorylation which is required for bacterial entry and proliferation (Zhang and Rikihisa, 1997; Lin et al., 2002, 2007; IJdo et al., 2007; Thomas and Fikrig, 2007). Tyrosine phosphorylation of the effector AnkA of *A. phagocytophilum* was reported recently (IJdo et al., 2007; Lin et al., 2007). However, no tyrosine phosphorylated effectors of *E. chaffeensis* were known until recently (Wakeel et al., 2010a; McBride et al., 2011). In this present study, we demonstrated that the strongly tyrosine phosphorylated 200 kDa protein in the *E. chaffeensis*-infected cell, is DNA binding protein Ank200, the largest major immunoreactive protein identified thus far in *E. chaffeensis* and *E. canis*

(Nethery et al., 2007; Luo et al., 2010). Using mass spectrometry and immunoprecipitation, we have previously reported that *E. chaffeensis* TRP47, TRP75, and *E. canis* TRP95 are tyrosine phosphorylated (Wakeel et al., 2010a; McBride et al., 2011). Recent studies have shown that AnkA of *A. phagocytophilum* is tyrosine phosphorylated by host Abl-1 and Src tyrosine kinases and plays an important role in bacterial infection (Ijdo et al., 2007; Lin et al., 2007). The *E. chaffeensis* effectors TRP47 (Wakeel et al., 2010a) and Ank200 (this study) are tyrosine phosphorylated; however, the host tyrosine kinases involved have not been identified. A recent study suggests that TRP47 physically interacts with Src family tyrosine kinase, Fyn, a key component of the $\alpha\beta$ TCR-coupled signaling pathway, and thus may be involved in tyrosine phosphorylation of TRP47 (Wakeel et al., 2009). The tyrosine kinase involved in Ank200 phosphorylation is unknown; however, Motif Scan prediction suggests that Abl and Lck tyrosine kinases may be involved.

T1SS in Gram-negative bacteria is dependent upon an ABC transporter but is Sec-independent, bypasses the periplasm and allows secretion of proteins of diverse sizes (19–800 kDa) and functions (proteases, adhesins or S-layer proteins, hemophores, hydrolases, lipases, toxins, or hemolytic enzymes) from the cytoplasm into the extracellular medium in a single step via a C-terminal uncleaved secretion signal (Delepelaire, 2004; Holland et al., 2005; Linhartova et al., 2010). Several unique features identified using bioinformatics in *E. chaffeensis* TRPs including glycine and aspartic acid-rich RTX-like repeats that specifically bind calcium ions in RTX proteins, are very acidic ($pI \sim 4$), and a non-cleavable C-terminal secretion signal and exhibit homology with adhesins, are hallmarks of the T1SS substrates (Delepelaire, 2004; Linhartova et al., 2010). Alpha hemolysin (HlyA) of some uropathogenic *E. coli* isolates, leukotoxin (LktA) of *Mannheimia haemolytica*, bifunctional adenylate cyclase hemolysin (CyaA) of *B. pertussis*, metalloprotease PrtA and PrtB of *Erwinia chrysanthemi*, hemophore (HasA) and lipase (LipA) of *Serratia marcescens*, and FrpA and FrpC of *N. meningitidis* are some of the well characterized T1SS secreted proteins (Thompson and Sparling, 1993; Delepelaire, 2004; Linhartova et al., 2010). Although normally associated with the secretion of toxins or hydrolytic enzymes, the T1SS is essentially promiscuous and efficiently secretes a wide range of proteins carrying a type 1 secretion signal (Delepelaire, 2004; Linhartova et al., 2010).

The *E. chaffeensis* T1SS apparatus exhibits close similarity to the protease secretion apparatus in other bacteria. *E. chaffeensis* T1SS ATPase (ECH_0383) predicted to code for the T1SS ABC protein exhibited similarity to *S. proteamaculans*, *E. amylovora*, *P. fluorescens*, and *Photobacterium luminescens* T1SS ABC transporter of the PrtD family. The type 1 secretion membrane fusion protein of the HlyD family is encoded by ECH_0970 showed homology with the HlyD family secretion proteins in *Rhodospirillum centum*, *Marinomonas* sp., and *Pseudomonas syringae*. The third component of the T1SS, the outer membrane protein TolC encoded by *E. chaffeensis* tolC (ECH_1020), exhibited similarity to type 1 secretion outer membrane protein, TolC in *R. centenum* and *Parvibaculum lavamentivorans*. *E. coli* hemolysin secretion system is considered to be the prototype T1SS and is composed of the HlyB and HlyD proteins encoded by genes normally cotranscribed

with *hlyC* and *hlyA*, whereas the outer membrane protein TolC is encoded outside of the *hly* operon on the chromosome (Welch and Pellett, 1988; Wandersman and Delepelaire, 1990). Although the *E. coli* hemolysin secretion apparatus showed low homology to the *E. chaffeensis* T1SS apparatus, *E. coli* complemented with *hlyB* and *hlyD* efficiently secreted *E. chaffeensis* TRPs and Ank200 similar to FrpA of *N. meningitidis* (Thompson and Sparling, 1993). In contrast to the cistronic organization of the secretion genes, the *E. chaffeensis* genome encodes genes with similarity to *E. coli* *hlyB* and *hlyD* in two non-contiguous sequences like *N. meningitidis* where scattered genes encode a functional T1SS required for the secretion of meningococcal RTX proteins (Thompson and Sparling, 1993; Wooldridge et al., 2005).

The fact that the last 50 C-terminal residues of TRP47, TRP120, TRP32, and Ank200 contain a higher percentage of LDAVTSIF residues similar as previously reported to be present in T1SS secretion signals. This observation is consistent with and supports the concept that *E. chaffeensis* TRPs and Ank200 are typical T1SS substrates (Delepelaire, 2004). It is interesting to note that a LDAVTSIF residues-rich C-terminal secretion signal has been recently reported in *A. phagocytophilum* APH_0032 and APH_7378, which are proposed to be secreted by T1SS (Huang et al., 2010). Substantial similarity of seven *E. chaffeensis* TRP47 TRs to S-layer protein in *M. igneus*, hemagglutinin in *Stenotrophomonas* sp., ABC transporter ATP-binding protein in *Alteromonas* sp., and *K. denitrificans*, and metalloprotease, hemolysin-type calcium-binding region in *C. taiwanensis* is not only indicative of TRP47 being a T1SS secreted protein, but also points to its role as an *E. chaffeensis* effector. Furthermore, the presence of a consensus sequence (GDAXXN) seven times within TRP47 TRs predicted to bind calcium ions in RTX proteins and its similarity to ABC transporter ATP-binding protein in *G. hansenii* and *A. pasteurianus* provide additional evidence of the similarity of TRP47 to other T1SS substrates (Linhartova et al., 2010). Although the significance of a domain in *E. chaffeensis* TRP47 similar to hemagglutinin and hemolysin-type calcium-binding repeat domain is unknown, a recent study identified these repeat domains in RTX PnxIIIA of *P. pneumotropica* localized on the bacterial surface and associated with bacterial adherence and invasion of the host cell (Sasaki et al., 2011). The presence of multiple copies of RTX-like sequence (L/I/K-D-L-Q-D-VASHESGVSQ) in TRP120 TRs showing similarity with the ABC transporter, ATP-binding protein in *B. clarus*, putative ABC transporter ATP-binding protein in *Marine actinobacterium*, ABC transporter ATP-binding protein in *B. vulgates*, *B. fluxus*, and *B. clarus* provides strong evidence that TRP120 is an RTX-like secreted protein. Furthermore, a glutamic acid and histidine-rich TRP120 amino acid sequence (ESHQGETEKESGITESH) exhibiting similarity to zinc finger protein in *A. melanoleuca* and *C. familiaris* and zinc-binding motif (HEXXHXXGXXH) reported in the major zinc-dependent metalloprotease secreted by *S. marcescens* serralyisin and PnxIIA in *P. pneumotropica* supporting the conclusion that TRP120 is also secreted by T1SS (Sasaki et al., 2009).

Thus, overall the putative domains and repeat sequence in the primary structure of *E. chaffeensis* TRPs and Ank200 appear to be similar to those previously reported in *E. coli* HlyA, *P. haemolytica* LktA, *B. pertussis* CyaA and recently identified *P. pneumotropica*

PnxIA, PnxIIA, and PnxIIIA RTX toxins (Sasaki et al., 2009, 2011; Linhartova et al., 2010). In addition, a unique TRP120 TR amino acid sequence (SEPFVAESEVSKVE) that showed similarity to type 1 secretion membrane fusion protein, HlyD in *P. wasabiae* and *P. mendocina*, indicates that these regions may be essential for TRP120 extracellular secretion by T1SS. HlyD forms a substrate-specific complex with inner membrane protein HlyB and subsequently recognizes the C-terminal signal peptide of HlyA. Upon binding of HlyA, the HlyD trimer interacts with the trimeric TolC protein inducing conformational change and extracellular export of HlyA (Andersen et al., 2001). Others have reported ABC transporter and MFP association only after substrate binding in *S. marcescens* hemophore HasA and *E. chrysanthemi* metalloprotease B and C (Letoffe et al., 1996).

The paradigm of type 1 secretion pathway is based on the analysis of the mechanism of secretion of the *E. coli* hemolysin. Complementation studies have shown that the gene products of *hlyB*, *hlyD*, and *tolC* are required for the secretion of hemolysin, the gene product *hlyA* of *E. coli*. The *E. coli* hemolysin secretion apparatus was shown promote secretion of heterologous RTX proteins expressed in *E. coli*, including the LktA of *M. haemolytica*, Cya of *B. pertussis*, HlyIA of *Actinobacillus pleuropneumoniae*, and FrpA of *N. meningitidis* (Chang et al., 1989; Gygi et al., 1990; Masure et al., 1990; Thompson and Sparling, 1993). In this study we demonstrated that *E. chaffeensis* TRP47, TRP120, TRP32, and Ank200 possess features of T1SS substrates and are secreted by the *E. coli* T1SS secretion apparatus (HlyB, HlyD, and TolC). Secretion was exclusively dependent on *E. coli* HlyB and HlyD proteins, as secretion was significantly reduced in their absence. In the present work we also report that the TolC protein is required for *E. chaffeensis* TRP47, TRP120, TRP32, and Ank200 secretion. Previous studies have demonstrated the specific requirement of TolC for hemolysin and FrpC secretion, and that TolC is able to form an active T1SS transporter system in the presence of HlyB, HlyD to transport CyaA of *B. pertussis*, LtkA of *A. actinomycetemcomitans*, Pax of *P. aerogenes*, and FrpA and FrpC of *N. meningitidis* (Lally et al., 1989; Masure et al., 1990; Wandersman and Delepelaire, 1990; Thompson and Sparling, 1993; Kuhnert et al., 2000; Vakharia et al., 2001; Kamal et al., 2007). The small amount of extracellular TRPs and Ank200 detected in the supernatant of the TolC mutant in this study is consistent with similar TolC experiments reported previously (Thomas et al., 1992; Vakharia et al., 2001). Thus, *E. chaffeensis* TRPs and Ank200 are secreted only in the presence of a functional hemolysin secretion apparatus suggesting that TolC has a direct effect on TRPs and Ank200 secretion as reported for hemolysin (Wandersman and Delepelaire, 1990).

Using the heterologous type 1 secretion apparatus of *E. coli*, we demonstrated in this study that *E. chaffeensis* TR and Ank200 proteins/fragments are secreted into the extracellular medium by the T1SS dependent on HlyB, HlyD, and TolC indicating that these proteins may be transferred to the host cell by T1SS. This study increases our knowledge of an *E. chaffeensis* secretion mechanism for the effectors to host cells. Our present study demonstrated for the first time the *E. chaffeensis* TRPs and Ank200 secretion by an ABC transporter dependent T1SS similar to RTX proteins. This knowledge may be useful for targeting and inhibiting *Ehrlichia* proliferation in future.

MATERIALS AND METHODS

PROPAGATION AND PURIFICATION OF *E. CHAFFEENSIS*

Propagation of uninfected and *E. chaffeensis*-infected THP-1 cells was performed as previously described (Wakeel et al., 2009).

PREPARATION OF WHOLE CELL LYSATES

Whole cell lysates were prepared as described previously (Wakeel et al., 2009) with some modifications. Briefly, 10^7 of uninfected and *E. chaffeensis*-infected (3 days post-infection) THP-1 cells were collected ($500 \times g$, 5 min), washed twice in ice-cold phosphate buffered saline (PBS), resuspended in 1 ml of ice-cold RIPA lysis buffer (Pierce, Rockford, IL, USA) that contained complete Mini protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA), phosphatase inhibitors cocktail (Pierce), 5 mM EDTA, and 1 mM of phenylmethylsulfonyl fluoride, sodium fluoride, sodium orthovanadate, and incubated for 20 min on ice. Cell lysates were prepared by sonication of cells for 1 min on ice. Lysates were collected by centrifugation at $12,000 \times g$ for 10 min at 4°C .

CLONING AND EXPRESSION OF RECOMBINANT *E. CHAFFEENSIS*

Ank200-C, TRP120, TRP47, AND TRP32

For protein translocation study using T4SS model, in-frame fusions between the 3' region of *ank200* encoding the C-terminal 320 amino acids (Ank200₃₄₂₉₋₄₃₉₂), nearly full length *trp120* (*trp120*₁₇₋₁₆₄₇), *trp47* (*trp47*₂₋₉₅₁), *trp32* (*trp32*₂₋₅₉₇) and the *cre* coding region resulting in Cre::Ank200-C, Cre::TRP120, Cre::TRP47, Cre::TRP32 fusion proteins were generated by PCR, amplifying the corresponding coding regions from *E. chaffeensis* Arkansas strain genomic DNA using custom synthesized oligonucleotide primers (Table A1 in Appendix) in plasmid pSDM3197 (Schrammeijer et al., 2003). *SallI/XbaI* or *SallI/NdeI*-digested PCR product was translationally fused to *cre* via *SallI/XbaI* or *SallI/NdeI*-digested plasmid pSDM3197 (Schrammeijer et al., 2003). All *cre* control and *cre-vir* genes used in this study were expressed from the *A. tumefaciens virF* promoter sequence, and the chimeric proteins contained an N-terminally located simian virus 40 nuclear localization signal sequence to ensure nuclear targeting after Vir-mediated translocation into host cells. All plasmids were introduced into *A. tumefaciens* by electroporation (den Dulk-Ras and Hooykaas, 1995), and expression was confirmed by Western blot analysis as described (Vergunst et al., 2003). Briefly, the transformed *A. tumefaciens* strains including the control lines LBA1100 with pSDM3197 (Cre only) and pSDM3155 (Cre::VirF Δ 42N of *A. tumefaciens* expressing Cre-VirF fusion proteins; Vergunst et al., 2000; Schrammeijer et al., 2003) were induced overnight with acetosyringone (Sigma). The pellets of the induced culture were boiled for 10 min and separated on SDS-PAGE gel prior to Western blot analysis using anti-Cre antibody.

For T1SS assay, the coding regions of the *E. chaffeensis* TRPs were amplified by PCR from *E. chaffeensis* genomic DNA using a forward primer that included a 5' *NcoI* site and reverse primer with a 5' *HindIII* site and stop codon, and ligated into the complementary sites of pBAD/Thio plasmid resulting in in-frame cloning of *E. chaffeensis* TRPs without thioredoxin fusion under the control of arabinose promoter and generation of plasmids pTRP47,

pTRP120, pTRP32, and pAnk200C4 (see **Tables A1** and **A2** in Appendix for details). *E. coli* Top 10 (Invitrogen) was used for cloning procedures. *E. coli* K-12 strain BW25113 (wild-type) and *tolC::Tn10* insertional mutant in *E. coli* K-12 strain CAG12184 (*tolC* mutant; Singer et al., 1989; Baba et al., 2006) were obtained from the Coli Genetic Resource Center (CGSC, *E. coli* Genetic resources at Yale University), and these cells were used for expression and secretion analysis in this study. The cloning and expression of the recombinant GST-TRP47 (full length, GST-TRP47; N-terminal, GST-NterTRP47; and C-terminal, GST-CterTRP47) fusion proteins have been described previously (Wakeel et al., 2010a). The plasmids pTRP47, pTRP120, pTRP32, pAnk200C4, pGEX-TRP47 (full length), pGEX-TRP47C-term (C-terminal), pGEX-TRP47N-term (N-terminal), and pHlyAC compatible with plasmid pK184-HlyBD were transformed into *E. coli* strains BW25113, CAG12184 (**Tables 1** and **2**) and selected on LB media containing appropriate antibiotics. The fusion proteins were expressed from an arabinose-inducible promoter (pBAD-Thio derivative) and isopropyl 1-thio- β -D-galactopyranoside (IPTG)-inducible promoter (pK184- and pGEX-derivative). *E. coli* (strains BW25113) cells harboring both compatible plasmids (pBAD-derived and pK184-HlyBD) were grown in the presence of ampicillin (100 μ g/ml) and kanamycin (30 μ g/ml). Secretion experiments in the absence of TolC were performed with *E. coli* CAG12184 *tolC210::Tn10* (tetracycline resistant). Cells harboring both compatible plasmids (pBAD-derived and pK184-HlyBD) were grown in the presence of ampicillin (100 μ g/ml), kanamycin (30 μ g/ml), and tetracycline (10 μ g/ml).

EXPRESSION AND SECRETION OF RECOMBINANT *E. CHAFFEENSIS* TRP AND Ank PROTEINS BY WILD-TYPE AND *tolC* mutant (*tolC210::Tn10*) *E. COLI* STRAINS

Secretion experiments were performed as previously described with some modifications (Bakkes et al., 2010). Briefly, overnight cultures of *E. coli* strains (wild-type and *tolC* mutant) harboring the appropriate recombinant plasmids were diluted 1:20 into fresh LB supplemented with antibiotics. Cells were grown in LB medium containing isopropyl 1-thio- β -D-galactopyranoside at a final concentration of 1.5 mM for the production of the inner membrane proteins HlyB and HlyD with agitation at 30°C to an optical density at 600 nm (OD₆₀₀) of 0.8, and then production of the TRP47, TRP120, TRP32, Ank200C4, and HlyAc fusion proteins was induced for 5 h by the addition of arabinose to a final concentration of 10 mM. Total cell extracts or culture supernatants were collected by centrifugation (12,000 \times g, 10 min, 4°C). Proteins in the supernatants were concentrated by precipitation with 10% (v/v) trichloroacetic acid for 1 h at 4°C. The precipitated proteins were collected by centrifugation (16,000 \times g, 30 min, 4°C) and washed in 80% acetone. Total cell extracts and precipitated proteins were resuspended in 1x sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer, and total cell extract from 0.025 ml of culture pellet or protein precipitated from 1.0 ml of culture supernatant was analyzed by SDS-PAGE followed by staining with Coomassie brilliant blue (CBB) or probing by immunoblotting using anti-GST or anti-TRP47, -TRP120, -TRP32, and Ank200 specific antibodies.

DETECTION OF PROTEIN TRANSLOCATION BY CRAFT ASSAY

Translocation of Ank200, TRP120, TRP47, and TRP32 was performed as described previously by CRAFT assay (Vergunst et al., 2005). This system uses the site-specific recombinase Cre translationally fused to transport signals of the effector proteins (T4SS substrates). In brief, the seedlings from *A. thaliana* CB1 were grown for 10 days. Roots were collected and precultured for 3 days, followed by a 3-day cocultivation period with *A. tumefaciens*. Two Petri dishes, each containing at least 200 root explants, were used per strain. The GFP marker, which becomes active in CB1 cells only after Cre-mediated excision of the blocking sequence [*lox*-flanked (*floxed*) DNA sequence], allowed assaying for translocation directly after cocultivation by fluorescence microscopy (Leica MZ FLIII microscope and a Sony 3CCD color video camera).

Cre RECOMBINASE ACTIVITY ASSAY OF Cre::EHRlichia FUSION PROTEINS IN *A. TUMEFACIENS*

To assay Cre activity in Cre::*Ehrlichia* fusion proteins, Cre::Ank200-C, Cre::TRP120, Cre::TRP47, and Cre::TRP32 containing *A. tumefaciens* strain LBA1100 further transformed with pSDM3043 (Vergunst and Hooykaas, 1998; Vergunst et al., 2000) and grown overnight. The plasmid pSDM3043 that contains a single *Bam*HI restriction site between the *floxed* DNA fragments was rescued and transformed into *E. coli* strain DH5 α , which was then grown overnight before isolation of plasmid pSDM3043. The plasmid pSDM3043 isolated from *E. coli* was digested with *Bam*HI and then separated on an agarose gel.

ANTIBODIES

Rabbit anti-*E. chaffeensis* Ank200 antiserum was generated against synthetic keyhole limpet hemocyanin-conjugated 25-mer C-terminus Ank200 peptide (1439-DIGAQAVSPSTSQGADVKKSSC QSK-1463) by a commercial vendor (Bio-Synthesis, Lewisville, TX, USA). Normal mouse IgG and other antibodies used in this study were mouse monoclonal anti-pTyr (PY99; Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat anti-GST (GE Healthcare, Bio-Sciences Corp., Piscataway, NJ, USA) and anti-Cre (Eurogentec, Seraing, Belgium), and anti-TRP47, anti-TRP120, and anti-TRP32 described previously (Wakeel et al., 2010a).

COIMMUNOPRECIPITATION

Immunoprecipitation was performed as described (Wakeel et al., 2010a) except the nitrocellulose membranes were incubated with horseradish peroxidase-conjugated anti-pTyr or rabbit anti-Ank200 antibody.

WESTERN IMMUNOBLOTTING

Following electrophoresis, proteins were transferred to nitrocellulose membrane and detected with antibodies specific for TRP47, TRP120, TRP32, Ank200, *E. chaffeensis*, Cre, GST, or phosphotyrosine (pTyr; PY99; Santa Cruz Biotech) as previously described (Vergunst et al., 2003; Wakeel et al., 2010a).

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APPENDIX

Table A1 | The list of bacterial strains and plasmids used in this study.

Strain or plasmid	Genotype or relevant characteristics	Source or reference
BACTERIAL STRAINS		
<i>A. tumefaciens</i> LBA1100	C58 containing disarmed Ti-plasmid pTiB6Δ (TDNA, occ, tra), Rif ^r , Spc ^r	Vergunst et al. (2003)
<i>A. tumefaciens</i> LBA1010	C58 containing oncogenic wild-type pTiB6, Rif ^r	Vergunst et al. (2003)
<i>A. tumefaciens</i> LBA2587	LBA1100 Δ <i>virD4</i> , non-polar in-frame deletion	Hubber et al. (2004)
<i>A. tumefaciens</i> LBA2586	LBA1010 Δ <i>virD4</i> , non-polar in-frame deletion	This study
<i>E. coli</i> BW25113	F ⁻ , Δ(<i>araD-araB</i>)567, Δ <i>lacZ4787</i> ::(rrnB-3), λ ⁻ , <i>rph-1</i> , Δ(<i>rhaD-rhaB</i>)568, <i>hsdR514</i>	Baba et al. (2006)
<i>E. coli</i> CAG12184	F ⁻ , λ ⁻ , <i>tolC210::Tn10</i> , <i>rph-1</i>	Singer et al. (1989)
<i>E. coli</i> Top10	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>lacZ</i> ΔM15 Δ <i>lacX74</i> <i>recA1</i> <i>araD139</i> Δ(<i>ara-leu</i>) 7697 <i>galU galK rpsL</i> (Str ^R) <i>endA1 nupG</i> λ ⁻	Invitrogen
<i>E. chaffeensis</i> Arkansas	Isolated from patient on an army base in Arkansas in 1990	Hotopp et al. (2006)
PLASMIDS		
pK184-HlyBD	Contains the <i>lac</i> promoter and the kanamycin resistance gene modified containing <i>Bsp</i> HI restriction site next to the rbs and the genes <i>hlyB</i> and <i>hlyD</i>	Bakkes et al. (2010)
pHlyAc	pBADHisB containing His6-HlyAc (encoding amino acids 805–1023 of HlyA)	Bakkes et al. (2010)
pGEX-TRP47	pGEX-6P-1 containing TRP47 (in-frame GST fusion protein)	Wakeel et al. (2010a)
pGEX-TRP47C	pGEX-6P-1 containing TRP47C (in-frame GST fusion protein)	Wakeel et al. (2010a)
pGEX-TRP47N	pGEX-6P-1 containing TRP47N (in-frame GST fusion protein)	Wakeel et al. (2010a)
pBAD/Thio	pBAD/Thio is a 4,436-bp control vector expressing a 16-kDa HP-thioredoxin fusion protein under the control of <i>pBAD</i> promoter and the ampicillin resistance gene. The whole fusion gene may be excised with <i>Nco</i> I and <i>Pme</i> I.	Invitrogen
pTRP47	pBAD/Thio containing TRP47 (the whole HP-thioredoxin fusion gene was excised out using <i>Nco</i> I and <i>Hind</i> III)	This study
pTRP120	pBAD/Thio containing TRP120 (the whole HP-thioredoxin fusion gene was excised out using <i>Nco</i> I and <i>Hind</i> III)	This study
pTRP32	pBAD/Thio containing TRP32 (the whole HP-thioredoxin fusion gene was excised out using <i>Nco</i> I and <i>Hind</i> III)	This study
pAnk200C4	pBAD/Thio containing Ank200C4 encoding amino acids 1352–1463 of Ank200 (the whole HP-thioredoxin fusion gene was excised out using <i>Nco</i> I and <i>Hind</i> III)	This study
pSDM3197	pRL662 containing <i>pvirF-NLS::cre</i> Δ <i>STOP</i> cassette, gentamicin resistant (Gm ^R)	Vergunst et al. (2000)
pSDM3155	pRL662 containing <i>pvirF-NLS::cre::virF</i> Δ1-126, Gm ^r	Vergunst et al. (2000)
pSDM3043	In which a <i>lox</i> -flanked (<i>flox</i> ed) DNA segment prevents expression of a neomycin phosphotransferase (<i>nptII</i>) marker gene	Vergunst et al. (2000)
pSDM3609	Inc P plasmid with <i>A. tumefaciens</i> wild-type <i>VirD4</i> under <i>A. tumefaciens</i> <i>virD</i> promoter	This study
pSDM3668	Inc P plasmid with <i>E. chaffeensis</i> wild-type <i>VirD4</i> with N-terminal c-Myc epitope tag (36 nucleotides) under <i>A. tumefaciens</i> <i>virD</i> promoter	This study

Table A2 | List of oligonucleotide primers used for (A) T4SS and (B) T1SS study.

Fragment	Name	Primer (5' to 3')
(A)		
Ank200-C	<i>Sall</i> -Ank200-F	Sense, GCGCTCGACAGACCATTTCAGCAAGG
	<i>NdeI</i> -Ank200-R	Antisense, GGCCATATGTTATTTGCTCTGGCAAGA
TRP120	<i>Sall</i> -TRP120-F	Sense, GCGCTCGACGTAACATAAGTACAGCCGATATAC
	<i>XbaI</i> -TRP120-R	Antisense, TCTCTAGATTATACAATATCATTACTACATTGTG
TRP47	<i>Sall</i> -TRP47-F	Sense, GCGCTCGACTGCTTCATTTAACAACAGAA
	<i>NdeI</i> -TRP47-R	Antisense, GGCCATATGTTAGAAATAAAAGTATCTATTACCAA
TRP32	<i>Sall</i> -TRP32-F	Sense, GCGCTCGACTGTCACAATTCTCTGAAGAT
	<i>XbaI</i> -TRP32-R	Antisense, GGCTCTAGACTACTCTAAACTACTTTCACTACAG
Ech-VirD4	<i>HindIII</i> -VirD4-F	Sense, GGCAGCTTATGGAGGAGCAGAAGCTGAT
	<i>NdeI</i> -VirD4-F	Sense, GGCCATATGATGGATAGTGTAAAGTGCAAACC
	<i>EcoRI</i> -VirD4-R	Antisense, GCGAATCTTATATATCTTGATCCTTGACAGGTT
(B)		
TRP120	<i>NcoI</i> -TRP120-F	Sense, GACCATGGTGGATATTGATAATAGTAACATAAGTAC
	<i>HindIII</i> -TRP120-R	Antisense, TGCAAGCTTATACAATATCATTACTACATTGTG
TRP47	<i>NcoI</i> -TRP47-F	Sense, GACCATGGTGGTTCATTTAACAACAGAA
	<i>HindIII</i> -TRP47-R	Antisense, TGCAAGCTTAGAAATAAAAGTATCTATTACCAA
TRP32	<i>NcoI</i> -TRP32-F	Sense, GACCATGGTGTGACAATTCTCTGAAGATAA
	<i>HindIII</i> -TRP32-R	Antisense, TGCAAGCTACTCTAAACTACTTTCACTACAGTGAT
Ank200C4	<i>NcoI</i> -Ank200C4-F	Sense, GACCATGGCTCTAGGTGCATCAGCG
	<i>HindIII</i> -Ank200C4-R	Antisense, TGCAAGCTTATTTGCTCTGGCAAGA
HlyBD	HlyB_for	Sense, GCTATCCATGGCGAATTCTGATTCTTGTACATAAAATTGATTATGGG
	HlyD_rev	Antisense, CGTTGTCTAGATTCTTAACGCTCATGTAACCTTTCTGTTACAGACT

The underline indicates the restriction site; the stop codon marked as bold; Ech, *Ehrlichia chaffeensis*.

Table A3 | Cre::Vir and Cre::Ehrlichia protein translocation from *A. tumefaciens* strain LBA1100 into *A. thaliana* (CB1), a reporter plant line that allows expression of GFP as a reporter for translocation upon Cre activity.

Protein	LBA1100 (<i>A. tum</i> D4)	LBA2587 (1100Δ D4)	2587(3609) (<i>A. tum</i> D4)	2587(3668) (<i>E. chaf</i> D4)	1100(3668) (Both D4's)
Cre::VirF	++++	–	++++	–	++++
Cre::TRP32	–	–	–	–	–
Cre::TRP47	–	–	–	–	–
Cre::TRP120	–	–	–	–	–
Cre::Ank200–C	–	–	–	–	–

Root explants of *A. thaliana*, a GFP reporter line cell, CB1, 4 days after cocultivation with *A. tumefaciens* strain LBA1100 were visualized for protein translocation resulting in a GFP fluorescing host cell and scored as “++++” (high numbers of GFP fluorescing host cells observed) or “–” (no/rare GFP fluorescing host cells observed). *A. tumefaciens* strain LBA1100, containing octopine type pTiB. *A. tum* D4 = *A. tumefaciens* with wild-type *virD4*; *E. chaf* D4 = *E. chaffeensis* wild-type *virD4*.

