THE ROLE OF CELLULAR AUTOPHAGY AND TYPE IV SECRETION SYSTEM IN ANAPLASMA PHAGOCYTOPHILUM INFECTION

DISSERTATION

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ABSTRACT

Human granulocytic anaplasmosis (HGA), an emerging tick-borne zoonosis is caused by a gram-negative, obligatory intracellular bacterium, *Anaplasma phagocytophilum*. *A. phagocytophilum* has the remarkable ability to inhibit the spontaneous apoptosis of neutrophils, block the production of reactive oxygen intermediates, and replicate in membrane-bound inclusions in the cytoplasm of neutrophils. However, the *A. phagocytophilum* inclusions have not been fully characterized, and bacterial factors contributing to these phenomena remain unknown. In this study, we studied several molecular aspects of *A. phagocytophilum* pathogenesis.

(1) Characterization of *A. phagocytophilum* replicative inclusions. We demonstrated that *A. phagocytophilum* replicative inclusions had the characteristic of early autophagosomes, as shown by the presence of autophagosome markers, LC3 and double lipid bilayer membrane in the *A. phagocytophilum* inclusions. Furthermore our data suggested that autophagy enhanced *A. phagocytophilum* replication instead of inhibiting its growth.

(2) Investigation of the expression of genes encoding type IV secretion system apparatus in *A. phagocytophilum*. We found the expression of *virB6* and *virB9* was up-regulated during the bacterial growth in human neutrophils. Furthermore, differential
VirB9 expression was shown to associate with the binding of *A. phagocytophilum* to neutrophils, and prevention of internalized bacteria from being delivered to lysosomes.

(3) Identification of a novel substrate of type IV secretion system in *A. phagocytophilum*. A novel substrate, Ats-1 was identified by bacterial two-hybrid screening. We demonstrated that Ats-1 targeted mitochondria of host cells, and inhibited cell apoptosis.

(4) Development of a reporter system in *A. phagocytophilum* to detect substrates of type IV secretion system. We made some progress to achieve the goal by constructing plasmid harboring reporter-substrate fusion gene.
Dedicated to my family whom I honor and love
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CHAPTER 1

INTRODUCTION

Human Granulocytic Anaplasmosis

Human granulocytic anaplasmosis (HGA) is a tick-borne disease caused by infection with *Anaplasma phagocytophilum*. It was first identified in a Wisconsin patient in 1990, and increasingly recognized in many regions of United States, and other places in the world, including Upper Midwest, New England, parts of the Mid-Atlantic States, northern California, and Europe (Bakken et al., 1994; Chen et al.1994; Brouqui et al., 1995). Since the day HGA was first described, more than 2,871 cases had been reported to the Centers for Disease Control and Prevention (CDC) by the end of 2004 (Bakken and Dumler, 2006). The average annual incidence of HGA was 1.4 cases per million people nationwide during 2001–2002, and the number in endemic regions was much higher, such as 33.34 in Rhode Island (Demma et al., 2005). However, the disease was still underreported. Seroepidemiologic studies demonstrated that as much as 15–36% of the population in some endemic areas has been exposed to *A. phagocytophilum* (Bakken et al., 1998; Aguero-Rosenfeld et al., 2002). HGA is a potentially fatal systemic disease. The symptom of HGA includes fever, headache, myalgia, and malaise. Laboratory testing
showed the occurrence of thrombocytopenia, leukopenia, anemia, and elevated hepatic transaminase levels in majority of HGA patients (Dumler et al., 2005).

**Anaplasma phagocytophilum**

*A. phagocytophilum*, the etiologic agent of HGA, is a gram-negative, pleomorphic, obligatory intracellular bacterium. Taxonomically, it belongs to the genus *Anaplasma*, the family *Anaplasmataceae*, the order *Rickettsiales* in *Alphaproteobacteria* based on genetic analyses of 16S rRNA genes (Dumler et al., 2001). *A. phagocytophilum* can infect myeloid or granulocytic cells, tick and endothelial cells, and has been propagated in human HL-60 and NB4 promyelocytic leukemia cells, endothelial cell cultures, tick cell cultures, and megakaryocytic lineage (Goodman et al., 1996; Munderloh et al., 2003; Munderloh et al., 2004; Herron et al., 2005; Pedra et al., 2005; Granick et al., 2008). The genome of *A. phagocytophilum* was sequenced and annotated. It is 1.4 million base-pair long, encoding 1,369 open reading frames. *A. phagocytophilum* lacks the genes for biosynthesis of most amino acids. It only can make glycine, glutamine, glutamate, and aspartate (Hotopp et al., 2006). It is expected that *A. phagocytophilum* has to rely on transporting other amino acids from the host cell for survival. *A. phagocytophilum* also lacks lipopolysaccharide biosynthetic genes; instead it incorporates cholesterol into its membrane (Lin and Rikihisa, 2003). The genes encoding bacterial signal transduction (two-component) system, and type IV secretion system are present in the *A. phagocytophilum* genome. These two systems are expected to play an important role in *A. phagocytophilum* growth and pathogenesis.
Transmission of *A. phagocytophilum* between mammalian hosts and ticks

*Ixodes* ticks are biological vectors of *A. phagocytophilum*. Species of *Ixodes* ticks prevalent in each geographic region are responsible for *A. phagocytophilum* transmission in endemic regions. *I. scapularis* is in the Eastern and Midwestern United States, *I. pacificus* in the Western United States, *I. ricinus* in Europe, and probably *I. persulcatus* in parts of Asia (Dumler et al., 2005). As *A. phagocytophilum* can not pass to larvae from adult female ticks by transovarial passage, progeny of ticks must acquire the infection in a subsequent blood meal from infected mammalian hosts. Small rodents, such as *Peromyscus leucopus* (the white-footed mouse) can maintain persistent infection as reservoir hosts (Telford et al., 1996). Human is an incidental host for *A. phagocytophilum*. It was shown that uninfected *I. scapularis* larvae acquire *A. phagocytophilum* within 24 h of attachment on *A. phagocytophilum*-infected mice (Hodzic et al., 1998). After acquisition by ticks, *A. phagocytophilum* travels through the gut and infect the salivary glands. Tick salivary protein Salp16 is required to establish infection in salivary glands (Sukumaran et al., 2006). Infected *Ixodes* ticks maintain infection transstadially and then transmit infection to mammalian hosts as nymphs or adults. It was shown that transmission of *A. phagocytophilum* from tick salivary glands to mammalian host occurs between 24 and 48 h post attachment (Katavolos et al., 1998; Vignes et al., 2001). Tick feeding stimulates the replication and migration of the bacteria from the salivary glands to the mammalian host (Hodzic et al., 2001). Once in the mammalian tissue, *A. phagocytophilum* infects microvascular endothelial cells and neutrophils (Herron et al., 2005).
**Binding and internalization of *A. phagocytophilum* into host cells**

Binding to host cells is the initial step for *A. phagocytophilum* infection. In human myeloid cells, platelet selectin glycoprotein ligand-1 (PSGL-1) and the α2, 3-sialylated and α1, 3-fucosylated moiety sialyl Lewis x (sLex6), which modifies the PSGL-1 N-terminus, are important for adhesion and invasion of *A. phagocytophilum* (Herron *et al.*, 2005). For infection of mouse neutrophils with *A. phagocytophilum*, PSGL-1 is not required, but α1, 3-fucosylation is (Carlyon *et al.*, 2003). However some subpopulation of *A. phagocytophilum* can bind and infect host cells independent of these molecules, suggesting there is another receptor for *A. phagocytophilum* (Sarkar *et al.*, 2007). It was reported that P44, the major outer membrane protein of *A. phagocytophilum*, is involved in bacterial adhesion to PSGL-1 on host myeloid cells (Park *et al.*, 2003). It was also demonstrated that the cholesterol-rich lipid rafts or caveolae and glycosylphosphatidylinositol (GPI)-anchored proteins are involved in the entry and intracellular infection of *A. phagocytophilum* (Lin and Rikihisa, 2003). Entry into host cells through caveolae helps intracellular pathogens avoid fusion with lysosomes (Duncan *et al.*, 2002).

**Nature of *A. phagocytophilum* inclusions**

After internalization into host cells, *A. phagocytophilum* replicates in the cytoplasm of host cells, forming a membrane-bound inclusion. The inclusion is not acidic, and it is surrounded by lysosomes, but not fused with them (Webster *et al.*, 1998; Mott *et al.*, 1999). *A. phagocytophilum* inclusion lacks the major protein markers for endocytic pathway. The inclusion was not labeled with anti-lysosome-associated
membrane protein 1 (LAMP 1), anti-CD63, anti-Rab5, anti-EEA 1, anti-clathrin heavy chain, anti-alpha adaptin, anti-transferrin receptor, anti- beta-coatomer protein, and anti-annexins I, II, IV, and VI, and anti-vacuole-type H1-ATPase, although cation-dependent MPR, major histocompatibility complex class I and II antigens, and caveolin-1 were found in some of inclusions, and vesicle-associated membrane protein 2 (a SNARE protein, involving in vesicle fusion) were found in majority of inclusions (Webster et al., 1998; Mott et al., 1999; Lin and Rikihisa, 2003). Additionally, sphingomyelin from C6-NBD-ceramide is not incorporated into *A. phagocytophilum* inclusions, and *A. phagocytophilum* replication is not affected by Brefeldin A (Mott et al., 1999). These results suggest *A. phagocytophilum* inclusions are neither early nor late endosomes, and do not fuse with lysosomes or Golgi-derived vesicles (Webster et al., 1998; Mott et al., 1999). The nature of *A. phagocytophilum* inclusions is still mysterious.

**Inhibition of spontaneous apoptosis of neutrophils by *A. phagocytophilum* infection**

Neutrophils have a short half-life (6–12 h) after their release from the bone marrow (Akgul et al., 2001), and life cycle of *A. phagocytophilum* is 2 or 3 days. It is important for *A. phagocytophilum* to inhibit the apoptosis of neutrophils, so that it can complete its life cycle in life-extended neutrophils. Yoshiie et al. found that *A. phagocytophilum* delays the spontaneous apoptosis of human neutrophils in vitro (Yoshiie et al., 2000). This anti-apoptotic phenomenon has been confirmed by studying ovine neutrophils infected in vivo with a sheep isolate (Scaife et al., 2003). The mechanisms by which *A. phagocytophilum* inhibits the spontaneous apoptosis of human neutrophils have been partially elucidated. Ge et al. found that *A. phagocytophilum*
infection inhibits human neutrophil apoptosis by blocking extrinsic and intrinsic pathways. Cell surface Fas clustering, cleavage of pro-caspase 8 and Bid, and translocation of Bax into mitochondria were found to be blocked (Ge et al., 2005; Ge et al., 2006). Additionally it was also shown that the transcription of the gene encoding Bfl-1, an anti-apoptotic Bcl-2 family member is upregulated, mitochondrial membrane potential is maintained, and caspase 3 activation is prevented in *A. phagocytophilum*-infected neutrophils (Borjesson et al., 2005; Ge et al., 2005; Ge et al., 2006). It was also reported that p38 MAPK signal transduction pathway is involved in the inhibition of neutrophil apoptosis. p38 MAPK is continuously phosphorylated and activated in *A. phagocytophilum*-infected neutrophils, and inactivation of this pathway by an inhibitor abrogated delayed neutrophil apoptosis (Choi et al., 2005) However, this is in disagreement with previous report that p38 MAPK was not activated within 1 h in neutrophils incubated with *A. phagocytophilum* (Kim and Rikihisa, 2002). The bacterial factors contributing to this apoptosis inhibition remain unknown.

**Blockage of reactive oxygen species (ROS) production by *A. phagocytophilum* infection**

Production of ROS is a powerful mechanism by which neutrophils kill ingested pathogens. Ingestion of *A. phagocytophilum* failed to trigger the ROS production by neutrophils (Mott and Rikihisa, 2000). The production of superoxide (O$_2^-$), the starting molecule of all ROS is catalyzed by NADPH oxidase, which consists of a cytochrome b$_{558}$ (heterodimer of integral membrane proteins gp91$^{phox}$ and p22$^{phox}$) and several cytosolic components (p67$^{phox}$, p47$^{phox}$, and p40$^{phox}$). In resting neutrophils, the inactive
oxidase components remain unassembled and segregated. Cytochrome \textit{b}_{558} \text{resides in the membranes of secretory vesicles, and p67}_{phox}, \textit{p47}_{phox}, \text{and p40}_{phox} \text{are in cytosol. Upon activation, secretory vesicles rapidly fuse with plasma or the phagosomal membrane, and a complex of p47}_{phox}, \textit{p67}_{phox}, \text{and possibly p40}_{phox} \text{translocates and associates with cytochrome \textit{b}_{558}} \text{to assemble functional NADPH oxidase enzyme, allowing exertion of the lethal effects of } \text{O}_2^- \text{and its derivatives on extracellular or ingested bacteria in close proximity. The small GTP-binding protein, Rac2, a major Rac protein expressed in neutrophils, is required for oxidase activity through direct interaction with p67}_{phox} \text{and cytochrome \textit{b}_{558}} \text{(Babior, 1999). It was reported that reduction of p22}_{phox} \text{protein level, or transcription downregulation of gp91}_{phox} \text{gene and Rac2 gene was responsible for the inhibition of ROS production in } \textit{A. phagocytophilum}-\text{infected cells (Banerjee et al., 2000; Carlyon et al., 2002; Mott et al., 2002). However, none of these genes was significantly down-regulated after } \textit{A. phagocytophilum} \text{infection of human neutrophils in a microarray study performed later (Borjesson et al., 2005).}

**Outer membrane proteins of \textit{Anaplasma phagocytophilum}**

Outer membrane proteins of \textit{Anaplasma phagocytophilum} are expected to play an important role in the interaction between the bacterium and host cells. The surface proteins were found to be involved in the inhibition of neutrophil apoptosis (Yoshiie et al., 2000; Borjesson et al., 2005). Several outer membrane proteins were identified by capillary liquid chromatography-nanospray tandem mass spectrometry analysis, following biotinylation and affinity purification (Ge and Rikihisa, 2007). Among them were P44 family proteins, Omp85, Omp-1A, and hypothetical proteins APH0404
(designated Asp62) and APH0405 (designated Asp55). P44 is the major outer membrane protein of *A. phagocytophilum*. Multiple copies encoding P44 were found in the *A. phagocytophilum* genome (Hotopp et al., 2006). P44 proteins consist of a single central hypervariable region flanking by N-terminal and C-terminal conserved regions. The N- and C-terminal regions flanking the central hypervariable region contain a signature of four conserved amino acid regions (C, C, WP, A) (Lin et al., 2002; Zhi et al., 1999). P44 can act as adhesin, facilitating the bacterium binding to host cells (Park et al., 2003, Wang et al., 2006). Recently P44 was found to have porin activity (Huang et al., 2007).

Additionally, P44 undergo antigenic variation during infection in infected HL-60 cells, experimentally infected mice and horses, and human granulocytic anaplasmosis patients, suggesting that the bacterium evades host cell immunity surveillance by antigenic variation of the P44 (Barbet et al., 2003; Wang et al., 2004; Lin and Rikihisa, 2005).

**Bacterial two-component system**

*A. phagocytophilum* lives in dichotomous environment, ticks and mammalian hosts. To cope with diverse environment, *A. phagocytophilum* may use bacterial two-component systems, as other bacteria do. Bacterial two-component systems are the signal transduction system in bacteria used to sense environment changes and respond to these changes by regulating specific gene expression. This system generally is composed of a sensor kinase and a response regulator. Sensor kinases reside in the integral inner membrane with a periplasmic sensor domain and a cytoplasmic catalytic kinase domain. Environmental stimuli lead to the autophosphorylation on a conserved histidine residue in the sensor kinase. The phosphoryl group is then transferred to a conserved aspartate
residue in a cognate response regulator. Acting as transcription factor, phosphorylated response regulator binds to the promoter region of specific genes and regulates the expression of these genes to mediate adaptation of the bacterium to the new environment (Cheng et al., 2006). The genes encoding three pairs of sensor kinases and response regulators, CckA and CtrA, NtrY and NtrX, and PleC and PleD, were annotated in the *A. phagocytophilum* genome. Treatment of HL-60 cells with closantel, an inhibitor of histidine kinases, blocked *A. phagocytophilum* infection, suggesting that a bacterial two component system plays an important role in *A. phagocytophilum* infection (Cheng et al., 2006).

**Type IV secretion system**

Type IV secretion system (TFSS) is a translocation apparatus used by some gram-negative bacteria to transport macromolecules (DNA, proteins) from bacterial cytoplasm to eukaryotic cells, other bacteria, or the extracellular milieu. Generally this apparatus spans inner- and outer-membrane, directly transporting substrates from cytoplasm to the outside of the bacteria. The VirB/VirD4 system of *Agrobacterium tumefaciens* has served as a prototype for TFSSs. *A. tumefaciens* is a plant pathogen, causing tumors by transporting oncogenic T-DNA and accessory proteins into plant cells through TFSS. The VirA/VirG bacterial two-component system activates the transcription of TFSS genes in *A. tumefaciens* after detecting phenolic signals released from wounded plant cells. TFSS of *A. tumefaciens* is composed of 11 VirB proteins, and one VirD4 protein. Except for VirB1, all of VirBs and VirD4 are required for the functional TFSS. Based on distinct functions, TFSS apparatus is divided into three complexes, pilus, core complex, and
coupling protein VirD4. Pilus is composed of VirB2, VirB3 and VirB5; core complex composed of VirB4, VirB6, VirB7, VirB8, VirB9, VirB10, and VirB11 for transfer channel (Christie et al., 2005). Coupling protein VirD4 recruits the substrates and delivers them to transfer channel. By using transfer DNA immunoprecipitation method, it was showed that T-DNA is transported outside by contact VirB/D4 proteins in this order, from VirD4, VirB11, VirB6/VirB8, to VirB2/VirB9 (Cascales and Christie, 2004). The signal of substrates for being recognized by TFSS machinery lies on their C-termini. Analysis of sequences of known substrates for *A. tumefaciens* revealed that all these substrates carry a consensus Arg motif and a similar hydropathy profile at their C termini. The importance of the Arg residues at C-termini was confirmed by mutational analysis (Vergunst et al., 2005). The translocated substrates play an important role in bacterial pathogenesis. For extracellular bacteria, the substrates can act as exotoxins, such as pertussis toxin of *Bordetella pertussis*. For intracellular bacteria, the substrates can regulate multiple cell activities, including vesicle trafficking, apoptosis, or others, as showed by the study of effectors of *Legionella pneumophila* and *Bartonella henselae*). SidM/DrrA of *L. pneumophila* specifically intercepts host cell vesicles of early secretory pathway by acting as both guanine nucleotide exchange factor and GDI-displacement factor for Rab1 (Ingmundson et al., 2007; Machner and Isberg, 2007). SidF of *L. pneumophila* inhibits macrophage apoptosis by directly interacting with and neutralizing the activities of two pro-apoptotic members of the Bcl2 protein family, BNIP3 and Bcl-rambo (Banga et al., 2007). BepA of *B. henselae* inhibits apoptosis in vascular endothelial cells through BepA-mediating cAMP increase (Schmid et al., 2006).
The genes encoding TFSS apparatus was identified in the *A. phagocytophilum* genome (Ohashi *et al.*, 2002; Hotopp *et al.*, 2006), although *virB/D* composition and genomic loci are different from those of *A. tumefaciens*. AnkA was identified as the first substrate of TFSS in *A. phagocytophilum* (Lin *et al.*, 2007). AnkA is phosphorylated by Abl-1 tyrosine kinase after translocation from *A. phagocytophilum* into the cytoplasm of host cells. Both AnkA and Abl-1 are critical for bacterial infection (Lin *et al.*, 2007).

**Autophagy**

Autophagy is an evolutionary conserved and regulated intracellular catabolic mechanism that mediates the degradation of cytosolic components, including proteins, large protein complexes and damaged organelles in a lysosome-dependent fashion (Levine and Klionsky, 2004). The hallmark of autophagy is the formation of a double membrane cytosolic vesicle, the autophagosome, which sequesters cytoplasm and delivers it to the lysosome for degradation (Levine and Klionsky, 2004). Multiple steps are involved in the formation and maturation of autophagosomes: autophagy induction, vesicle nucleation, vesicle expansion and completion, retrieval of autophagosome-forming proteins for recycling, and fusion with lysosomes to form autolysosomes (Klionsky, 2005; Levine and Yuan, 2005). Many proteins participate in this process. Genetic analysis in yeast has identified 27 autophagy-related (Atg) genes that encode components of the autophagic machinery (Yorimitsu and Klionsky, 2005). Homologs of many of them have also been identified in mammalian cells, such as Atg6 (Beclin 1), Atg7, and Atg8 (microtubule-associated protein light chain 3; LC3) (Klionsky and Emr, 2000). Most of the Atg proteins are soluble and transiently associate with the vesicle,
cycling off the vesicle after completion of its function (Klionsky, 2005). Of these proteins, only two, Atg8, when conjugated to phosphatidylethanolamine, and Atg19 are known to remain associated with the autophagosomes in yeast (Kabeya et al., 2000; Scott et al., 2001).

Autophagy serves to help clear intracellular infection and process non-self and self antigens in the host cytoplasm as part of the innate and adaptive immune system (Amano et al., 2006; Ling et al., 2006; Schmid et al., 2006). Autophagosome formation facilitates killing of several intracellular bacteria, including Rickettsia conorii, Mycobacterium tuberculosis, and Shigella flexneri (Walker et al., 1997; Gutierrez et al., 2004; Ogawa and Sasakawa, 2006). However, diversion of phagosomes containing bacteria to autophagosomes favors survival of other intracellular bacteria such as Legionella pneumophila and Coxiella burnetii, perhaps by delaying the fusion of pathogen-containing vacuoles with lysosomes, thereby giving them time to develop into a more acid and protease-resistant stage (Kirkegaard et al., 2004; Amer et al., 2005; Colombo, 2005; Gutierrez et al., 2005). For Francisella tularensis, autophagy induction helps the bacterium to re-enter a membrane-bound compartment after replication in the cytoplasm (Checroun et al., 2006).

Objectives of this study

1. **Subversion of cellular autophagy by Anaplasma phagocytophilum**

To investigate the presence of autophagosome markers in A. phagocytophilum inclusions in infected HL-60 cells.
To determine the effect of autophagy on the growth of *A. phagocytophilum* in HL-60 cells.

2. **Differential expression of VirB9 and VirB6 during the life cycle of *Anaplasma phagocytophilum* in human leucocytes is associated with differential binding and avoidance of lysosome pathway**

To determine the transcription level of genes encoding type IV secretion system apparatus during *A. phagocytophilum* infection in neutrophils by semi-quantitative RT-PCR.

To determine when VirB9 was detectable by immunofluorescence-labeling during *A. phagocytophilum* infection in neutrophils.

To determine the colocalization of VirB9-detectable and -undetectable *A. phagocytophilum* with LAMP-1.

3. **Anaplasma phagocytophilum** Ats-1 targets mitochondria and inhibits host cell apoptosis

To identify the substrate of type IV secretion system of *A. phagocytophilum* by bacterial two-hybrid system.

To characterize this substrate and study its function.

4. **Development of a reporter system for identification of secreted proteins from *A. phagocytophilum* to HL-60 cells**

To construct a reporter plasmid that allows us to screen secreted proteins from *A. phagocytophilum*. 
Our results suggest that *A. phagocytophilum* takes advantage of host cell autophagy for its own benefit, and the expression of type IV secretion system of *A. phagocytophilum* is upregulated during its infection. Furthermore, we identified a novel mitochondria-targeting substrate of TFSS of *A. phagocytophilum* which is indicated to inhibit host cell apoptosis. Lastly we tried to develop a reporter system to facilitate the TFSS research of *A. phagocytophilum*.

Hereby, the new knowledge generated in my Ph.D. study helps understanding of the molecular pathogenicity of *A. phagocytophilum*, and the interaction between the bacterium and its host cells that permits the intracellular parasitism. The results are expected to facilitate a new approach in treatment and designing vaccine candidates against HGA.
CHAPTER 2

SUBVERSION OF CELLULAR AUTOPHAGY BY ANAPLASMA PHAGOCYTOPHILUM

2.1 Abstract

*Anaplasma phagocytophilum*, the causative agent of human granulocytic anaplasmosis, is an obligatory intracellular pathogen. After entry into host cells, the bacterium is diverted from the endosomal pathway and replicates in a membrane-bound compartment devoid of endosomal or lysosomal markers. Here, we show that several hallmarks of early autophagosomes can be identified in *A. phagocytophilum* replicative inclusions, including a double lipid bilayer membrane and colocalization with GFP-tagged LC3 and Beclin 1, the human homologs of *Saccharomyces cerevisiae* autophagy-related proteins Atg8 and Atg6, respectively. While the membrane-associated form of LC3, LC3-II, increased during *A. phagocytophilum* infection, *A. phagocytophilum*-containing inclusions enveloped with punctate GFP-LC3 did not colocalize with a lysosomal marker. Stimulation of autophagy by rapamycin favored *A. phagocytophilum* infection. Inhibition of the autophagosomal pathway by 3-methyladenine did not inhibit *A. phagocytophilum* internalization, but reversibly arrested its growth. Although
autophagosomes are considered part of the innate immune system that clears a variety of intracellular pathogens, our study implies that *A. phagocytophilum* subverts this system to establish itself in an early autophagosome-like compartment segregated from lysosomes to facilitate its proliferation.

### 2.2 Introduction

Human granulocytic anaplasmosis, an emerging zoonosis in the United States and other parts of the world, is caused by *Anaplasma phagocytophilum*, a Gram-negative obligate intracellular bacterium in the order Rickettsiales (Goodman *et al.*, 1996; Dumler and Bakken, 1998; Demma *et al.*, 2005). This pathogen infects and replicates in granulocytes and endothelial cells (Goodman *et al.*, 1996; Munderloh *et al.*, 2004; Herron *et al.*, 2005). A key feature in the pathogenesis of granulocytic anaplasmosis is the ability of *A. phagocytophilum* to avoid being processed by the endocytic machinery of the host cell. After endocytosis, the pH of the membrane-bound vacuole in which *A. phagocytophilum* resides remains neutral and this vacuole is not stainable with endosomal or lysosomal markers (Webster *et al.*, 1998; Mott *et al.*, 1999). Webster *et al.* (Webster *et al.*, 1998) reported that some inclusions are mannose-6-phosphate receptor-positive. Mannose-6-phosphate receptor is considered as a late endosome marker, but it may be also present in autophagosomes (Dunn, 1990; Dorn *et al.*, 2002; Eskelinen *et al.*, 2002). In order to replicate within the vacuole, *A. phagocytophilum* is obliged to usurp and acquire various components from its host cytoplasm, as it has a limited number of genes for de novo amino acid biosynthesis and central intermediary metabolism (Hotopp *et al.*, 2006). Despite the importance of modulation of vesicular
traffic and access to host cell metabolites in the course of *A. phagocytophilum* infection, very little is known about the biogenesis of the *A. phagocytophilum* replicative vacuole.

Autophagy is an evolutionary conserved and regulated intracellular catabolic mechanism that mediates the degradation of cytosolic components, including proteins, large protein complexes and damaged organelles in a lysosome-dependent fashion (Levine and Klionsky, 2004). The hallmark of autophagy is the formation of a double membrane cytosolic vesicle, the autophagosome, which sequesters cytoplasm and delivers it to the lysosome for degradation (Levine and Klionsky, 2004). Multiple steps are involved in the formation and maturation of autophagosomes: autophagy induction, vesicle nucleation, vesicle expansion and completion, retrieval of autophagosome-forming proteins for recycling, and fusion with lysosomes to form autolysosomes (Klionsky, 2005; Levine and Yuan, 2005). Many proteins participate in this process. Genetic analysis in yeast has identified 27 autophagy-related (Atg) genes that encode components of the autophagic machinery (Yorimitsu and Klionsky, 2005). Homologs of many of them have also been identified in mammalian cells, such as Atg6 (Beclin 1), Atg7, and Atg8 (microtubule-associated protein light chain 3; LC3) (Klionsky and Emr, 2000). Most of the Atg proteins are soluble and transiently associate with the vesicle, cycling off the vesicle after completion of its function (Klionsky, 2005). Of these proteins, only two, Atg8, when conjugated to phosphatidylethanolamine, and Atg19 are known to remain associated with the autophagosomes in yeast (Kabeya *et al.*, 2000; Scott *et al.*, 2001).

Autophagy serves to help clear intracellular infection and process non-self and self antigens in the host cytoplasm as part of the innate and adaptive immune system.
(Amano et al., 2006; Ling et al., 2006; Schmid et al., 2006). Autophagosome formation facilitates killing of several intracellular bacteria, including *Rickettsia conorii*, *Mycobacterium tuberculosis*, and *Shigella flexneri* (Walker et al., 1997; Gutierrez et al., 2004; Ogawa and Sasakawa, 2006). However, diversion of phagosomes containing bacteria to autophagosomes favors survival of other intracellular bacteria such as *Legionella pneumophila* and *Coxiella burnetii*, perhaps by delaying the fusion of pathogen-containing vacuoles with lysosomes, thereby giving them time to develop into a more acid and protease-resistant stage (Kirkegaard et al., 2004; Amer et al., 2005; Colombo, 2005; Gutierrez et al., 2005). For *Francisella tularensis*, autophagy induction helps the bacterium to re-enter a membrane-bound compartment after replication in the cytoplasm (Checroun et al., 2006). However, the role of autophagosomes in *A. phagocytophilum* infection is unknown.

Here, we explore the possibility of involvement of autophagosomes in *A. phagocytophilum* infection. Our results demonstrate a new cellular process by which this intracellular bacterium creates a replicative compartment, which does not mature into an autolysosome.

### 2.3 Materials and Methods

**Cell culture and transmission electron microscopy**

*A. phagocytophilum* HZ strain was propagated in HL-60 cells (ATCC, Manassas, VA) in RPMI 1640 medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine. Monkey endothelial cell line RF/6A (ATCC) was cultured in advanced MEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 2 mM L-
glutamine for infection with *A. phagocytophilum*. Cultures were incubated at 37°C in a humidified 5% CO$_2$/95% air atmosphere. The degree of bacterial infection in host cells was assessed by Diff-Quik staining (Baxter Scientific Products, Obetz, OH) of cytocentrifuged preparations. No antibiotic was used throughout the study. Host cell-free *A. phagocytophilum* was prepared by sonication, as described elsewhere (Mott *et al.*, 2002).

*A. phagocytophilum*-infected HL-60 cells were processed for TEM as described (Rikihisa, 1991). As a control, the pellet of 1 ml of overnight culture of *E. coli* DH5α was incubated with 1 x 10$^7$ HL-60 cells for 2 h, and processed for TEM as *A. phagocytophilum*-infected HL-60 cells.

**LC3 localization and conversion**

GFP-LC3 and GFP-LC3$^{\Delta C22, G120A}$ plasmids were kindly provided by Dr. Tamotsu Yoshimori at The Research Institute for Microbial Diseases, Osaka University, Japan, through Dr. Jean Celli at the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, MT. Plasmids were purified using the EndoFree Plasmid Maxi Kit (Qiagen, Valencia, CA) according to the manufacturer’s instruction. Transfection of RF/6A endothelial cells with these plasmids was performed using electroporation. Briefly, RF/6A cells were washed once using PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, and 2 mM KH$_2$PO$_4$, pH 7.4) and resuspended in RPMI 1640 medium (no serum) at a final density of 2 x 10$^7$ cells ml$^{-1}$. RF/6A cells (80 µl) were mixed with 5 µg plasmid and subjected to a pulse from a Gene Pulser Xcell System (Bio-Rad, Hercules, CA) in a 0.2-cm cuvette. The setting was 100 V, 1000 µF, which resulted in a pulse time of 50 ms. For localization of GFP-LC3, 1.6 x 10$^6$ RF/6A cells transfected
with GFP-LC3 or GFP-LC3△C22 plasmid were cultured on cover slips in a 6-well plate for 1 d post-transfection, and host cell-free *A. phagocytophilum* in 2 ml of advanced MEM with serum was added at the approximate ratio of host cell to bacteria of 1:100. Infected cells were washed twice using advanced MEM with serum (3 ml each time) after 4 h incubation and continued to incubate for the designated time periods. Cover slips were washed three times with PBS, fixed with 2% paraformaldehyde and subjected to immunofluorescence labeling after saponin permeabilization using horse anti-*A. phagocytophilum* (1:200 dilution) as primary antibody and Cy3-conjugated goat anti-horse IgG (diluted 1:100, Jackson ImmunoResearch Laboratories) as secondary antibody as described (Niu *et al.*, 2006). For triple labeling GFP-LC3-transfected RF/6A cells were infected with *A. phagocytophilum* for 48 h and fixed with 2% paraformaldehyde on the coverslip. Cells were subjected to immunofluorescence labeling after permeabilization with cold methanol for 3 min, using mouse monoclonal anti-LAMP-3 conjugated with PE (BioLegend, San Diego, CA). Immediately prior to viewing under fluorescence microscope, the cells were counterstained for the nucleus of RF/6A cells and *A. phagocytophilum* with 300 nM 4′,6-diamidino-2-phenylindole, dilactate (DAPI, Molecular Probes) for 5 min. Fluorescence images were analyzed by Nikon Eclipse E400 fluorescence microscope with a xenon-mercury light source (Nikon Instruments, Melville, NY) or LSM 510 laser-scanning confocal microscope (Carl Zeiss, Thornwood, NY). The original color emitted by excited DAPI (blue) was transformed to gray pseudocolour for clear viewing with Photoshop 7.0 software (Adobe, San Jose, CA).
**Real-time PCR analysis**

The RF/6A cells were transfected with GFP-LC3 plasmid, and infected with *A. phagocytophilum* as described above. The cells were harvested with the cell scraper after incubation for designated time periods, and homogeneously dispersed by pipetting using 1 ml pipette tip for 20 times. The pellets from 1 ml culture of each sample were subjected to genomic DNA extraction (QIAamp DNA Blood Mini Kit, Qiagen) after centrifugation at 12,000 g, 4°C for 10 min. The genomic DNA was eluted in 50 µl of elution buffer (10 mM Tris-HCl, 0.5 mM EDTA, pH 9.0). *pleD* copy number in each sample was quantified by a real-time, quantitative PCR instrument, the Stratagene Mx3000P (Stratagene, La Jolla, CA). The quantitative PCR was performed in the presence of SYBR Green, using a pair of primer (Forward primer: 5’-ACATGCGTACAAACCCTGCCATTG-3’; and Reverse primer: 5’-AAATCATCTGCACCAGCACTCAGC-3’). The data analysis was performed using the integrated analysis software (Stratagene). A standard curve was made by amplification of a serial 10-fold dilution of pET-33b (+) plasmid containing *pleD* (T-H. Lai and Y. Rikihiisa, unpublished data).

**Western blot analysis**

To examine LC3 forms I and II, protein was extracted from *A. phagocytophilum*-infected or uninfected GFP-LC3-transfected RF/6A cells using cold lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% (v/v) Triton X-100) containing protease inhibitors (Cocktail Set III, 1:50 dilution, EMD Chemicals, San Diego, CA). Western blot analysis was performed using anti-GFP (Santa Cruz Biotechnology, Clone B-2, diluted 1:1,000), and peroxidase-conjugated goat anti-mouse IgG (KPL, Gaithersburg, MD, diluted in 1:1,000). The bound antibody was detected by ECL chemiluminescence (Pierce
Biotechnology, Rockford, IL). The images were captured by a CCD camera (Fuji LAS-3000 imaging system), and band density was measured by Fujifilm MultiGauge software.

**Beclin 1 localization**

HL-60 cells (1.5 \( \times \) 10^7) were incubated with *A. phagocytophilum* derived from 2 \( \times \) 10^7 infected HL-60 cells in 15 ml of RPMI medium with serum (the approximate ratio of host cell to bacteria was 1:100). After 2 h of incubation at 37°C, infected cells were washed twice with PBS to remove bacteria not associated with host cells, and then incubated at 37°C to allow *A. phagocytophilum* to replicate for the designated time periods. *A. phagocytophilum*-infected HL-60 cells were fixed with 2% paraformaldehyde and subjected to double immunofluorescence labeling after saponin permeabilization. Horse anti-*A. phagocytophilum* or pre-immune horse serum (1:200 dilution) and mouse monoclonal anti-Beclin 1 (Santa Cruz Biotechnology, clone E-8, 1:50 dilution) were used as primary antibodies, and FITC-conjugated goat anti-horse IgG (diluted 1:50, Jackson ImmunoResearch) and Cy3-conjugated goat anti-mouse IgG (diluted 1:100, Jackson ImmunoResearch) were used as secondary antibodies. An unrelated mouse monoclonal antibody of the same isotype (IgG2a) as anti-Beclin 1 was used as a negative control.

**3-MA and rapamycin treatment**

For the study of the dose-response effect of 3-MA, HL-60 cells (1.5 \( \times \) 10^5 per well) in 12-well plates were incubated with host cell-free *A. phagocytophilum* at the approximate ratio of host cell to bacteria of 1:100 in 1 ml of RPMI 1640 medium with serum containing the indicated concentration of 3-MA. 3-MA (Sigma, St. Louis, MO) was prepared from a 20 mM stock solution in RPMI 1640 medium, and added to the HL-60 cell culture at 2 h pre-infection. For the time course study, 3-MA at a final
concentration of 2 mM was added to *A. phagocytophilum*-infected HL-60 cells at 5, 10 and 20 h p.i. and *A. phagocytophilum* infection was determined 2 d p.i. To study the effect of rapamycin, 1.5 x 10⁵ HL-60 cells at the logarithmic stage of growth were pre-incubated with 50 ng ml⁻¹ of rapamycin (Sigma) for 3 h, and then host cell-free *A. phagocytophilum* was added at the approximate ratio of host cell to bacteria of 1:2. Rapamycin was added from a stock solution of 50 µg ml⁻¹ in DMSO. At 2 d p.i., the degree of bacterial infection in host cells was assessed by Diff-Quik staining and by Western blot analysis using anti-P44 (mAb5C11, diluted at 1:2,000) and anti-α-tubulin (Santa Cruz Biotechnology, clone B-7, diluted at 1:2,000). To determine the toxicity of 3-MA and rapamycin to HL-60 cells, 1.0 x 10⁶ HL-60 cells in 2 ml complete RPMI 1640 medium were incubated at 37°C for 2 d in the presence of 10 mM 3-MA or 50 ng ml⁻¹ of rapamycin. The cell number was scored, morphology was observed by phase contrast microscopy, and the cell viability was determined with trypan blue dye exclusion test (Altman *et al.*, 1993).

To determine the toxicity of 3-MA, host cell-free *A. phagocytophilum* from 1.0 x 10⁷ infected HL-60 cells was first incubated with 10 mM 3-MA or culture medium for 1.5 h on ice. After washing to remove 3-MA, *A. phagocytophilum* was incubated with 1.0 x 10⁶ HL-60 cells in 2 ml complete RPMI 1640 medium. Bacterial infection was determined at 48 h p.i. by Diff-Quik staining. For determination of the effect of 3-MA on the *A. phagocytophilum* internalization into HL-60 cells, host cell-free *A. phagocytophilum* from 1.0 x 10⁷ infected HL-60 cells was incubated with 1.0 x 10⁶ HL-60 cells for 2 h in the presence of 10 mM 3-MA. The cells were washed to remove unbound bacteria, and fixed with 2% paraformaldehyde. The extracellular and
intracellular bacteria were distinguished by immuno-staining using horse anti-*A. phagocytophilum* and Cy3-conjugated goat anti-horse IgG before permeabilization, and horse anti-*A. phagocytophilum* and FITC-conjugated goat anti-horse IgG after permeabilization (Niu *et al*., 2006). To determine whether *A. phagocytophilum* colocalizes with LAMP-1 after the treatment of 3-MA, HL-60 cells infected with host cell-free *A. phagocytophilum* were incubated for 20 h to allow the appearance of small inclusions. 3-MA was added into cell culture to final concentration of 10 mM, and continued to incubate for additional 5 h. HL-60 cells were fixed and immuno-stained with horse anti-*A. phagocytophilum* and mouse monoclonal anti-LAMP-1 (1D4B; Developmental Hybridoma Bank), and the appropriate secondary fluorochrome-conjugated antibody and observed under LSM 510 laser-scanning confocal microscope. To determine reversibility of 3-MA inhibition of the *A. phagocytophilum* replication, *A. phagocytophilum*-infected HL-60 cells were first incubated at 37°C for 20 h, then a portion of cells were incubated in the presence of 10 mM 3-MA for 5 h or 24 h. The cells, which were treated with 3-MA for 5 h, were washed to remove 3-MA, and continued to incubate for additional 19 h. The *A. phagocytophilum* inclusions in HL-60 cells were observed under light microscope after Diff-Quik staining.

2.4 Results

*A. phagocytophilum* inclusions are enveloped by double lipid bilayer membranes

The hallmark of early autophagy is the formation of double lipid bilayer membrane autophagosomes (Kirkegaard *et al*., 2004). After fusion with lysosomes, autolysosomes have a single lipid bilayer membrane because the inner membrane
Transmission electron microscopy (TEM) revealed that many of inclusions containing replicating *A. phagocytophilum* in human myelocytic HL-60 cells are at least partially enveloped with double lipid bilayer membranes (Fig. 2.1A and B), indicating that the inclusions have a defining property of autophagosome. The percentage of inclusions with double lipid bilayer membrane increased from 50% at 40 h post infection (p.i.) to 78% at 60 h p.i. (Fig. 2.1D). As a control, the membrane of phagosomes harboring *Escherichia coli* in HL-60 cells was a single lipid bilayer (Fig. 2.1C).

**LC3, an essential component of cellular autophagosomes, colocalizes with *A. phagocytophilum* replicative inclusions**

Upon induction of autophagy, cytosolic LC3, a protein essential for autophagosome formation, is conjugated to phosphatidylethanolamine through a C-terminal glycine and associates with the membrane (Ichimura *et al*., 2000; Kabeya *et al*., 2000). To determine whether *A. phagocytophilum* replicative inclusions are autophagosomes, we examined the localization of LC3, throughout the time course of infection. A plasmid encoding GFP-tagged LC3 has been developed and widely used to study LC3 processing and distribution, as cellular amount of native LC3 is too low (Kabeya *et al*., 2000). Because HL-60 cells are hard to transfet, and *A. phagocytophilum* infection progresses in a similar fashion in both human HL-60 cells and monkey endothelial RF/6A cells (Munderloh *et al*., 2004), RF/6A cells were used for the study of LC3. RF/6A cells were transfected either with a plasmid encoding GFP-LC3 or a plasmid encoding the nonfunctional mutant GFP-LC3ΔC22, G120A (GFP-LC3ΔC22), which is unable to be lipidaded at the C terminus as a control. By fluorescence
microscopy, a cytosolic LC3 has a diffuse distribution pattern, whereas membrane-associated LC3 has a punctate pattern corresponding to nascent autophagosomes (Mizushima et al., 2004). Uninfected transfected RF/6A cells showed predominantly diffuse GFP-LC3 fluorescence distribution throughout the cytoplasm, indicating little autophagosome formation (Fig. 2.2G). In contrast, during infection GFP-LC3 became increasingly punctated, and most of the punctate GFP-LC3 was associated with *A. phagocytophilum* inclusions at 48 and 72 h p.i. (Fig. 2.2B, C and H). Especially at 48 h p.i., *A. phagocytophilum* inclusions were individually encased by the punctuate GFP-LC3 green circle (Fig. 2.2B). Neither GFP-LC3 punctation nor colocalization with the inclusion was evident at 14 h p.i. (Fig. 2.2A and H). GFP-LC3ΔC22 did not make punctate pattern during infection and was not specifically concentrated on *A. phagocytophilum*-containing vacuoles, although it was squeezed in between growing inclusions, and thus appeared colocalized with some inclusions at 48 and 72 h p.i. (Fig. 2.2D, E, and F). Infectivity did not differ between RF/6A cells transfected with the wild-type or mutant LC3 constructs.

A cytosolic LC3 form is called LC3-I, and its membrane-associated lipidated form is called LC3-II (Kabeya et al., 2000). By SDS-PAGE followed by immunoblotting, GFP-LC3-II with an apparent molecular mass (Mr) of 43 kDa can be distinguished from GFP-LC3-I with the Mr of 45 kDa. To confirm modification of LC3-I to LC3-II during *A. phagocytophilum* infection, Western blot analysis was performed on *A. phagocytophilum*-infected RF/6A cells transfected with GFP-LC3. A weak GFP-LC3-II band was detected in *A. phagocytophilum*-infected RF/6A cells at 2 d p.i., and the amount of GFP-LC3-II increased at 3 d p.i. (Fig. 2.2I). The ratios of GFP-LC3-II to GFP-LC3-I were 1:33 at 2 d,
and 1:10 at 3 d p.i. Thus, *A. phagocytophilum* infection induced the conversion of LC3-I to LC3-II, a signature of autophagosome formation (Kabeya *et al.*, 2000).

To examine whether autophagosome formation in RF/6A cells was positively associated with bacterial load, real-time PCR analysis targeting *A. phagocytophilum* pleD (a single copy gene) was performed to determine bacterial numbers in the infected cells. While during the lag phase (up to 24 h p.i) of *A. phagocytophilum* growth, autophagosome formation was undetectable, during the rapid growth (from 24 to 72 h p.i.) autophagosomes were formed (Fig. 2.2J). Thus the increasing bacterial load was associated with the induction of autophagosome formation, suggesting its induction in response to infection.

Because autophagy formation leads to destruction of several bacterial pathogens in autolysosomes, we examined whether *A. phagocytophilum* inclusions surrounded with punctate LC3 were undergoing lysosomal fusion by triple fluorescence labeling. These *A. phagocytophilum* inclusions did not fuse with lysosomes as revealed by the absence of LAMP-3 (lysosome-associated membrane protein 3), a late endosomal and lysosomal marker (Fig. 2.2K). Furthermore, the *A. phagocytophilum* inclusions surrounded with GFP-LC3-II increased in number and in size during logarithmic growth (Fig. 2.2H). Taken together, these results imply that *A. phagocytophilum* infection induces autophagy and *A. phagocytophilum* in the autophagosome is not on the way to destruction.

**Bclin 1, another essential component of cellular autophagosomes, colocalizes with**

*A. phagocytophilum* **replicative inclusions**

Bclin 1, a tumor suppressor, forms a complex with the mammalian class III phosphatidylinositol 3-kinase, Vps34 that is essential for induction of autophagosome

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formation (Liang et al., 1999). Beclin 1, which is not required for the conventional endosomal/lysosomal pathway (Zeng et al., 2006), is only present on autophagosomes during formation, as it dissociates from the membrane prior to autophagosome maturation (Klionsky, 2005). To further assess the ability of *A. phagocytophilum* to induce autophagosomes and whether *A. phagocytophilum* inclusions are autophagosomes, Beclin 1 localization was examined in infected HL-60 cells by confocal double immunofluorescence labeling using mouse monoclonal anti-human Beclin 1 and horse anti-*A. phagocytophilum* antiserum. The horse antibody does not react with uninfected HL-60 cells, as shown in previous studies (Niu et al., 2006), and the normal horse serum control and mouse isotype control did not label infected or uninfected HL-60 cells (data not shown). Beclin 1 did not colocalize with the internalized individual bacteria at 3 h p.i., or inclusions of the early replication stage at 20 h p.i. (Fig. 2.3A, B, and E), but did colocalize with approximately 15% of *A. phagocytophilum* replicative inclusions at 32 h p.i and with 5% *A. phagocytophilum* replicative inclusions at 50 h p.i. (Fig. 2.3C, D and E). This result corroborates with the GFP-LC3 and TEM results that *A. phagocytophilum* induces autophagosome formation and that the replicative inclusion has the properties of an early autophagosome.

**Influence of an inhibitor and a stimulator of cellular autophagy on *A. phagocytophilum* infection**

Because *A. phagocytophilum* infection induces autophagosome formation, but lysosomal fusion with the autophagosome containing *A. phagocytophilum* was not detected, we further examined whether autophagy hinders or facilitates *A. phagocytophilum* replication. 3-methyladenine (3-MA) is a widely used pharmacological
inhibitor of autophagy, which inhibits the activity of class III phosphatidylinositol 3-kinase (Seglen and Gordon, 1982; Lindmo and Stenmark, 2006). A dose response study showed that 2 mM 3-MA significantly inhibited *A. phagocytophilum* infection, and 3-MA at 5 mM and 10 mM completely blocked *A. phagocytophilum* infection in HL-60 cells (Fig. 2.4A). 3-MA at 10 mM concentration was widely used for inhibition of autophagy in many types of cells (Amer *et al.*, 2005). At 10 mM 3-MA was not toxic to the HL-60 cells, as no morphologic and viability changes were seen in HL-60 cells and cells treated at this concentration maintained the ability to replicate at a rate similar to non-treated cells (data not shown). When *A. phagocytophilum* was pretreated with 3-MA and then incubated with HL-60 cells in the absence of 3-MA, there was no inhibitory effect on its infection of HL-60 cells: with 3-MA pretreated *A. phagocytophilum* % infected cells was $80 \pm 5\%$, and $82 \pm 6\%$ with untreated *A. phagocytophilum* control at 2 day post incubation ($n = 3$ independent experiments). Thus 3-MA likely inhibited *A. phagocytophilum* infection by preventing cellular autophagy induction, rather than by a direct toxic effect on the bacteria or host cells. This result also implies that autophagy is required for *A. phagocytophilum* infection. To further investigate the effect of 3-MA on the binding, internalization or growth of *A. phagocytophilum*, 3-MA was added to infected HL-60 cells at different time p.i. When 2 mM 3-MA was added to HL-60 cells at 5, 10, or 20 h p.i., *A. phagocytophilum* infection was significantly inhibited as determined at 2 d p.i. (Fig. 2.4B). 3-MA did not inhibit binding or internalization (Fig. 2.5B and E). These results suggest that internalized bacteria could not replicate when autophagy was blocked by 3-MA. In fact, *A. phagocytophilum* inclusions did not enlarge in 3-MA-treated cells, but bacterial growth and expansion of the inclusion size resumed after

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removal of 3-MA from the culture medium (Fig. 2.5 G-L). Furthermore, lysosome-associated membrane protein-1 (LAMP-1) did not colocalize with growth-arrested inclusions in 3-MA-treated cells (Fig. 2.5D and F). Taken together, these results support autophagy facilitates the *A. phagocytophilum* replication.

In yeast, target of rapamycin (TOR), a serine/threonine phosphatidylinositol 3-kinase-related kinase, controls autophagy as a negative regulator of Atg1 (Noda and Ohsumi, 1998). Inhibition of mammalian TOR (mTOR) by rapamycin stimulates autophagy (Klionsky and Emr, 2000). Therefore, we tested whether TOR was involved in *A. phagocytophilum* infection and found that infection by the bacteria increased approximately two-fold when cells were pretreated with 50 ng ml\(^{-1}\) rapamycin (Fig. 2.6A). At this concentration, rapamycin was not toxic to HL-60 cells (data not shown). The increase in infectivity by rapamycin treatment was also supported by data from Western blot analysis (Fig. 2.6B). Compared to the DMSO solvent control, the band density of the major outer membrane protein of *A. phagocytophilum*, P44, as detected with P44-specific monoclonal antibody 5C11 (Kim and Rikihisa, 1998), increased by 2.2-fold in rapamycin-treated cells at 2 d p. i. after normalization to the \(\alpha\)-tubulin band density. Infection rates of control cultures with/without DMSO were almost identical (data not shown). Therefore, during *A. phagocytophilum* infection, the activity of the autophagic pathway correlates with productive *A. phagocytophilum* replication, not with *A. phagocytophilum* destruction.
2.5 Discussion

In this work, we have shown that several hallmarks of autophagosomes, including a double lipid bilayer membrane and colocalization with GFP-tagged LC3 and Beclin 1 can be observed in *A. phagocytophilum* inclusions. Does the autophagosome formation help *A. phagocytophilum* infection or is it a part of anti-rickettsial response? Based on observation of the absence of lysosome fusion with *A. phagocytophilum* inclusions surrounded with GFP-LC3-II, increase in the number and the size of GFP-LC3-II-labeled inclusions during rapid bacterial growth, and the effects of pharmacologically disrupted or induced autophagy on infection of *A. phagocytophilum*, we argue that the autophagosome formation favors *A. phagocytophilum* infection. Taken together with previous studies that showed an absence of endosomal and lysosomal markers, and lack of acidification in *A. phagocytophilum* inclusions (Webster *et al.*, 1998; Mott *et al.*, 1999), the present study indicates that the maturation of autophagosomes harboring *A. phagocytophilum* to late autophagosomes and the fusion with lysosomes are arrested. These early autophagosomes, therefore, can provide a safe haven for intracellular *A. phagocytophilum* to survive and replicate. This mechanism is distinct from other intracellular bacteria for which autophagosome induction favors their infection (Colombo, 2005), as these pathogens replicate in acidic LAMP-1-positive late autophagosomes (*Porphyromonas gingivalis*, and *Brucella abortus*) or autolysosomes that are positive for the lysosomal marker cathepsin D (*L. pneumophila, C. burnetii* and *F. tularensis*) (Pizarro-Cerda *et al.*, 1998; Sturgill-Koszycki and Swanson, 2000; Dorn *et al.*, 2001; Checroun *et al.*, 2006; Voth and Heinzen, 2007).
*A. phagocytophilum* is the first bacterium colocalized with Beclin 1, which acts favorably for its growth. There is a limited literature describing percentage of Beclin 1 colocalization with intracellular pathogens. Induction of autophagy inhibits mycobacterial survival in infected macrophages. Upon induction of autophagy by starvation, 51.4% ± 8.3% of Mycobacterium–containing phagosome colocalizes with LysoTracker, versus 26.5% ± 6.3% in the full nutrient condition control samples. Upon induction of autophagy, colocalization of Beclin 1 with Mycobacterium–containing phagosome increases from 37.6% to 57.9% (Gutierrez *et al.*, 2004). In this paper macrophage transiently transfected with FLAG epitope-tagged human Beclin 1 was used. The 37.6% of Beclin 1 colocalization under non-starved condition is higher % than the result of our study, which used the specific antibody to detect native Beclin 1. The different colocalization rates between the studies of Gutierrez *et al.* (Gutierrez *et al.*, 2004) and ours may be in part due to the technique used. Liang *et al.* (Liang *et al.*, 1999) showed that Beclin 1 gene transfer in MCF7 cells increases the basal level of the autophagy. In addition, compared to LC3 colocalization, the lower % of colocalization of Beclin 1 with replicative inclusions of *A. phagocytophilum* may be due to the transient nature of the interaction of Beclin 1 with the autophagosome (Klionsky, 2005). Approximately 15 and 5% *A. phagocytophilum* inclusion was Beclin 1-positive at 32 h p.i., and 50 h p.i., respectively. The decreasing colocalization of Beclin 1 may also be due to its transient nature. The similar phenomenon was observed for Atg7, another transient component in autophagosome formation, in *L. pneumophila*-infected macrophage. About 50% of phagosome containing *L. pneumophila* was Atg7-positive immediately after
internalization. However, Atg7 was rarely detected near vacuoles at 3 h p.i. (Amer and Swanson, 2005).

The timing of autophagy in *A. phagocytophilum* inclusion biogenesis is unique compared with other intracellular bacteria. Vacuoles containing *L. pneumophila* colocalize with LC3 by 4 h p.i. (Amer *et al.*, 2005). *C. burnetii* inclusions acquire LC3 at 5 min p.i. (Romano *et al.*, 2007). In contrast, *A. phagocytophilum* inclusions did not colocalize with LC3 and Beclin 1 at 14 and 20 h p.i., respectively. This relatively slow induction of autophagy during exponential growth is in agreement with the fact that 3-MA, which inhibits autophagy at the induction stage, was effective in inhibiting *A. phagocytophilum* infection, even when added at 20 h p.i. Autophagy is mainly regulated at the post-translation level (Klionsky, 2005). In fact the amount of Beclin 1 is not different between uninfected and *A. phagocytophilum*-infected HL-60 cells (unpublished data). This result is consistent with the finding by de la Fuente *et al.* (de la Fuente *et al.*, 2005) that mRNAs of Beclin 1 and LC3 are unchanged in *A. phagocytophilum*-infected HL-60 cells.

The present data led to a new question: are autophagosomes recruited to the *A. phagocytophilum*–containing vacuole, or some other mechanism leads to recruitment of autophagosome-inducing proteins or autophagosome components to generate an early autophagosome enclosing the bacteria? Punctate staining of LC3-II is currently viewed as a definitive marker for activation of the autophagic pathway (Kirkegaard *et al.*, 2004). Since numerous LC3 and Beclin-1-positive punctate structures were observed in the infected host cytoplasm, we propose that small autophagosomes are induced by *A. phagocytophilum* and recruited to the vacuole enclosing the bacteria. Another
fundamental question is: is autophagy a host response specific to a bacterial product or a host starvation response due to deprivation of nutrients by *A. phagocytophilum*? In this regard, recently several bacterial products have been shown to induce autophagosome formation, such as *Vibrio cholerae* cytolsin, SipB, an effector protein of the type III secretion system of *Salmonella enterica*, and VirG of *S. flexneri* (Hernandez *et al.*, 2003; Ogawa *et al.*, 2005; Gutierrez *et al.*, 2007). Induction of autophagy in *L. pneumophila*-infected macrophages also depends on its type IV secretion system (Amer and Swanson, 2005). *A. phagocytophilum* has a functional type IV secretion system that can deliver a protein substrate into the host cytoplasm (Niu *et al.*, 2006; Lin *et al.*, 2007). Thus, we anticipate that *A. phagocytophilum* might also induce autophagy by secreted products.

Our study showed that induction of autophagy with rapamycin facilitates *A. phagocytophilum* infection, suggesting that autophagy not only shields the bacterium from endosomal and lysosomal pathway, but also enhances replication of the bacterium. In fact 3-MA did not inhibit binding or internalization, but internalized bacteria could not replicate. The Lamp-1 did not colocalize with *A. phagocytophilum* inclusions in 3-MA-treated cells. The lack of colocalization may be due to the incomplete inhibition of autophagosome formation by 3-MA. 3-MA at 5 mM suppresses the autophagosome formation by 80% in hepatocytes (Seglen and Gordon, 1982). The remaining activity of autophagosome formation may be sufficient for *A. phagocytophilum* survival and preventing lysosomal fusion, but may be insufficient for its replication. The result of reversible inhibition of *A. phagocytophilum* replication by 3-MA supports this assumption. *A. phagocytophilum* has a limited number of genes for biosynthesis and central intermediary metabolism (Hotopp *et al.*, 2006). Because autophagy engulfs
cytosolic components (Klionsky and Emr, 2000), we speculate that the autophagosome could provide *A. phagocytophilum* with the direct access to host cytosolic nutrients without the need for transport across the inclusion membrane.
Figure 2.1. Transmission electron micrograph of an *A. phagocytophilum* replicative inclusion. **A.** An *A. phagocytophilum* replicative inclusion in the HL-60 cell at 40 h p.i. Double lipid bilayer is highlighted with arrows. **B.** An enlarged part of a replicative inclusion. Double lipid bilayer is indicated with arrows. **C.** A phagosome containing *E. coli* in HL-60 cells. Scale bar = 0.25 µm in A and C, and = 0.125 µm in B. **D.** Percentages of replicative inclusions containing double lipid bilayer membranes at three time-points p.i. One hundred inclusions were scored at each time point.
Figure 2.1
Figure 2.2. Colocalization of *A. phagocytophilum* inclusions with GFP-LC3 and conversion of LC3-I to LC3-II. Immunofluorescence micrographs of RF/6A cells transfected with GFP-LC3 (A, B and C) or GFP-LC3Δ<sup>C22,G120A</sup> (D, E and F) and infected with *A. phagocytophilum* for 14 h (A and D), 48 h (B and E), and 72 h (C and F). Infected cells were fixed and stained with horse anti-*A. phagocytophilum* and Cy3-conjugated goat anti-horse IgG. Scale bar = 10 µm. Insets are images taken under a confocal microscope. Typical *A. phagocytophilum* inclusions were circled with dashed white line.

**G.** Uninfected RF/6A cells transfected with GFP-LC3 as a control. Scale bar = 10 µm

**H.** The percentage of *A. phagocytophilum* inclusions that were colocalized with punctate GFP in RF/6A cells transfected with GFP-LC3 or GFP-LC3Δ<sup>C22,G120A</sup> at three time-points p.i. One hundred GFP-positive and infected RF/6A cells in each group were scored and the percentage of punctate GFP colocalization with *A. phagocytophilum* inclusions was determined. Data are presented as the means and standard deviations of triplicate samples. * indicates a significant difference compared to GFP-LC3Δ<sup>C22,G120A</sup> by Student’s *t*-test (*P* < 0.01) within each group at each time-point.

**I.** *A. phagocytophilum* infection induces LC3-I processing to LC3-II. GFP-LC3-transfected RF/6A cells were infected with *A. phagocytophilum*. Cells were harvested at 2 and 3 d p.i. for Western blot analysis using mouse monoclonal anti-GFP. Uninfected RF/6A cells transfected with GFP-LC3 were used as a control. The data are the representative of three independent experiments.

**J.** *A. phagocytophilum* growth curve in transfected RF/6A cells as determined by real-time PCR. The genomic DNA extracted from infected RF/6A transfected with GFP-LC3
plasmid during different time-points p.i., was subjected to real-time PCR analysis. Data are presented as the means and standard deviations of triplicate samples.

K. Lack of fusion of *A. phagocytophilum* autophagosomes with lysosomes. Inclusions containing DAPI-stained *A. phagocytophilum* were surrounded with GFP-LC3 in punctate pattern at 48 h p.i. Lysosomes were labeled with PE-conjugated anti-LAMP-3 antibody. The nucleus of RF/6A cell was marked ‘N’ and with dashed lines. Scale bar = 10 µm.
Figure 2.2

A. LC3  Anaplasma  Merge

B. LC3  Anaplasma  Merge

C. 14 h.p.i.

D. LC3ΔC22  Anaplasma  Merge

E. 14 h.p.i.

F. 48 h.p.i.

G. 72 h.p.i.

H. % Colocalization of GFP and bacteria

I. Uninfected 2 d  Anaplasma 2 d  Uninfected 3 d  Anaplasma 3 d

J. Bacterium number (x 10^7)

K. DAPI  DAPI (gray)  GFP-LC3  LAMP-3  Merge

14  48  72 h.p.i.
Figure 2.3. Colocalization of *A. phagocytophilum* inclusions with Beclin 1 by confocal double immunofluorescence microscopy. *A. phagocytophilum*-infected HL-60 cells harvested at 3 h (A), 20 h (B), 32 h (C), and 50 h (D) p.i. were double immunofluorescence-labeled with horse anti-*A. phagocytophilum* (Anaplasma) (FITC, green) and mouse monoclonal anti-Beclin 1 (Cy3, red). Scale bar = 5 µm.

E. The percentage of *A. phagocytophilum* inclusions that colocalized with Beclin 1. The percentage of colocalization was scored in 100 inclusions. Data are presented as the means and standard deviations of triplicate samples. *, and ** indicate a significant difference compared to the sample at 3 h p.i. by analysis of variance and Tukey honestly significant differences (HSD) (*P* < 0.05).
Figure 2.3

A

Anaplasma  Beclin 1  Merge

3 h p.i.

B

20 h p.i.

C

32 h p.i.

D

50 h p.i.

E

% Colocalization of Beclin 1 and bacteria

**

*
Figure 2.4. 3-MA inhibits *A. phagocytophilum* replication.

**A.** Dose response of 3-MA on *A. phagocytophilum* infection. HL-60 cells pretreated with the indicated concentrations of 3-MA were infected with *A. phagocytophilum* and the bacteria were allowed to replicate in the presence of 3-MA until 2 d p.i. The percentage of infected HL-60 cells was determined based on the presence of inclusions in 100 HL-60 cells. Data are presented as the means and standard deviations of triplicate samples.* indicates a significant difference compared with no 3-MA control by the Tukey HSD test (*P* < 0.01).

**B.** Temporal effect of 3-MA addition on *A. phagocytophilum* infection. HL-60 cells were infected with *A. phagocytophilum* for 5, 10, and 20 h. 3-MA was added to the culture at a final concentration of 2 mM. The cultures were allowed to further incubate until 2 d p.i. Data are presented as the means and standard deviations of triplicate samples. * indicates a significant difference compared with the RPMI control by the Tukey HSD test (*P* < 0.01).
Figure 2.5. 3-MA neither alters internalization of *A. phagocytophilum* nor induces the colocalization of *A. phagocytophilum* with LAMP-1, but reversibly inhibits *A. phagocytophilum* growth.

Immunofluorescence labeling was performed to examine the internalization of *A. phagocytophilum* into HL-60 cells in the control medium (RPMI) (**A**) or in the presence of 3-MA (**B**) at 2 h p.i. Extracellular bacteria were stained prior to permeabilization, with horse anti-*A. phagocytophilum* and Cy3 (red)-conjugated goat anti-horse IgG. Total bacteria were stained after permeabilization with saponin, with horse anti-*A. phagocytophilum* and FITC (green)-conjugated goat anti-horse IgG. Intracellular bacteria were indicated with white arrows. Scale bar = 5 µm.

Lack of colocalization of *A. phagocytophilum* with LAMP-1 after treatment with 3-MA as determined by confocal double immunofluorescence microscopy. *A. phagocytophilum*-infected HL-60 cells treated with RPMI medium (**C**) or with 3-MA (**D**) were labeled with two primary antibodies (horse anti-*A. phagocytophilum*, and mouse monoclonal anti-LAMP-1) and two secondary antibodies (FITC-conjugated goat anti-horse IgG, and Cy3-conjugated goat anti-mouse IgG) and observed under confocal immunofluorescence microscopy. Scale bar = 5 µm.

**E.** The percentage of intracellular *A. phagocytophilum* at 2 h p.i. One hundred *A. phagocytophilum* in each group were scored and the percentage of intracellular *A. phagocytophilum* was determined. Data are presented as the means and standard deviations of triplicate samples. There is no significant difference between groups treated with 3-MA or RPMI medium control by Student’s *t*-test (*P > 0.05*).
F. The percentage of *A. phagocytophilum* inclusions that did not colocalize with LAMP-1. One hundred *A. phagocytophilum* inclusions in each group were scored and the percentage of *A. phagocytophilum* inclusions which did not colocalize with LAMP-1 was determined. Data are presented as the means and standard deviations of triplicate samples. There is no significant difference between groups treated with 3-MA or RPMI medium control by Student’s *t*-test (*P* > 0.05).

G-L. The inhibition of the replication of *A. phagocytophilum* by 3-MA is reversible.

G. *A. phagocytophilum* in HL-60 cells at 20 h p.i., without 3-MA treatment.

H. *A. phagocytophilum* in HL-60 cells at 25 h p.i., without 3-MA treatment.

I. *A. phagocytophilum* in HL-60 cells at 44 h p.i., without 3-MA treatment.

J. *A. phagocytophilum* in HL-60 cells was incubated for 20 h without 3-MA, then incubated for 5 h in the presence of 10 mM 3-MA.

K. *A. phagocytophilum* in HL-60 cells was incubated for 20 h without 3-MA, then incubated for 5 h in the presence of 10 mM 3-MA, and finally 3-MA was removed and continuously incubated for additional 19 h.

L. *A. phagocytophilum* in HL-60 cells was incubated for 20 h without 3-MA, then incubated for 24 h in the presence of 10 mM 3-MA.

Arrows indicate *A. phagocytophilum* inclusions. Scale bar = 5 µm
Figure 2.5
Figure 2.6. Rapamycin enhances *A. phagocytophilum* infection.

A. Rapamycin enhanced *A. phagocytophilum* infection in HL-60 cells. HL-60 cells pretreated with rapamycin at 50 ng ml\(^{-1}\) for 3 h were infected with *A. phagocytophilum* in the continued presence of rapamycin until 2 d p.i. Data are presented as the means and standard deviations of triplicate samples. * indicates a significant difference compared with the DMSO solvent alone control by Student’s \(t\)-test (\(P < 0.01\)).

B. Western blot analysis of *A. phagocytophilum* infection in rapamycin-treated cells. HL-60 cells pretreated with rapamycin at 50 ng ml\(^{-1}\) for 3 h were infected with *A. phagocytophilum* in the continued presence of rapamycin until 2 d p.i. The infected cells (4.5 \(\times\) 10\(^5\)) were used for Western blot analysis with mouse monoclonal anti-P44. The loading amount was normalized using \(\alpha\)-tubulin. Results shown are representative of three independent experiments.
Figure 2.6

A

B
CHAPTER 3

DIFFERENTIAL EXPRESSION OF VIRB9 AND VIRB6 DURING THE LIFE CYCLE OF ANAPLASMA PHAGOCYTOPHILUM IN HUMAN LEUCOCYTES IS ASSOCIATED WITH DIFFERENTIAL BINDING AND AVOIDANCE OF LYSOSOME PATHWAY

3.1 Abstract

Anaplasma phagocytophilum, an obligate intracellular bacterium, is the aetiological agent of human granulocytic anaplasmosis (HGA). A. phagocytophilum virB/D operons encoding type IV secretion system are expressed in cell culture and in the blood of HGA patients. In the present study, their expression across the A. phagocytophilum intracellular developmental cycle was investigated. We found that mRNA levels of both virB9 and virB6 were upregulated during infection of human neutrophils in vitro. The antibody against the recombinant VirB9 protein was prepared and immunogold and immunofluorescence labelling were used to determine the VirB9 protein expression by individual organisms. Majority of A. phagocytophilum spontaneously released from the infected host cells poorly expressed VirB9. At 1 h post infection, VirB9 was not detectable on most bacteria associated with neutrophils. However, VirB9 was strongly expressed by A. phagocytophilum during proliferation in neutrophils. In contrast, with
HL-60 cells, approximately 80% of *A. phagocytophilum* organisms associated at 1 h post infection expressed VirB9 protein and were colocalized with lysosome-associated membrane protein-1 (LAMP-1), whereas, VirB9-undetectable bacteria were not colocalized with LAMP-1. These results indicate developmental regulation of expression of components of type IV secretion system during *A. phagocytophilum* intracellular life cycle and suggest that bacterial developmental stages influence the nature of binding to the hosts and early avoidance of late endosome-lysosome pathway.

### 3.2 Introduction

*Anaplasma phagocytophilum* is the agent of human granulocytic anaplasmosis (HGA, formerly called human granulocytic ehrlichiosis [HGE]), an emerging infectious disease in the United States and other countries (Bakken *et al.*, 1994; Brouqui *et al.*, 1995). HGA is an acute febrile illness often accompanied with prominent haematological abnormalities (such as leucopenia, relative lymphocytosis and thrombocytopenia), and elevation of hepatic aminotransferase activity (Dumler and Bakken, 1998). HGA became nationally reportable in 1998 (McQuiston *et al.*, 1999). *A. phagocytophilum* has the remarkable ability to parasitize first-line immune defensive cells, neutrophils, as its primary survival and replication sites (Rikihisa, 2003). After being liberated from infected host cells, *A. phagocytophilum* enters new host cells via caveola-mediated endocytosis and replicates in membrane-bound inclusions called morulae in the cytoplasm of host cells, secluded from host immune surveillance and destruction by lysosomes and reactive oxygen intermediates (Webster *et al.*, 1998; Mott and Rikihisa, 2000; Lin and Rikihisa, 2003; Rikihisa, 2000; 2003). Although several host cell factors
required for *A. phagocytophilum* infection have been characterized (Lin *et al.*, 2002; Mott *et al.*, 2002; Lin and Rikihisa, 2003), bacterial factors that promote obligatory intracellular life cycle are largely unknown.

The type IV secretion system (TFSS) transports macromolecules across the membrane in an ATP-dependent manner and is ancestrally related to the conjugation system of Gram negative bacteria. The TFSS is increasingly recognized as a virulence factor delivery mechanism to modulate eukaryotic cell functions by pathogens (Cascales and Christie, 2003). In the most extensively studied *Agrobacterium tumefaciens* TFSS, the single virB operon along with virD4, encodes 12 membrane-associated proteins that form a transmembrane channel complex (Christie, 1997). The split virB/D operons encoding the TFSS machinery have been found in the obligate intracellular parasites *Ehrlichia chaffeensis* and *A. phagocytophilum* (Ohashi *et al.*, 2002), and analysis of recent whole-genome sequence databases indicates conservation of this split operon structure in other members of the order *Rickettsiales* (Andersson *et al.*, 1998; Malek *et al.*, 2004; Brayton *et al.*, 2005; Collins *et al.*, 2005). Analysis of molecular interaction of several VirB/D proteins of *Rickettsia sibirica* by using bacterial two-hybrid system suggests that rickettsial virB/D gene products may be assembled to form TFSS as those of other bacteria with some modification (Malek *et al.*, 2004).

*Anaplasma phagocytophilum* TFSS is expected to function during granulocyte infection *in vitro* and *in vivo*, because (i) both virB8–virD4 and sodB–virB6 operons are polycistronically transcribed in *A. phagocytophilum* growing in HL-60 promyelocytic leukaemia cells, and (ii) *A. phagocytophilum* virB9 gene is transcribed in peripheral blood leucocytes from HGA patients and from experimentally infected animals (Ohashi *et al.*,
2002). In a closely related monocyte-tropic obligatory intracellular bacterium *Ehrlichia canis*, *virB9* is expressed in the blood from infected dogs, in the infected tick tissues, and infected canine monocyte cell culture (Felek *et al.*, 2003). Although there is no report for obligatory intracellular bacteria, for facultative intracellular bacteria including *Legionella, Bartonella* and *Brucella* species, TFSS is essential for their intracellular survival (Roy *et al.*, 1998; Schulein and Dehio, 2002; Celli *et al.*, 2003). *Brucella suis* *virB/D* operon is not expressed in free-living bacteria, but expressed 3 h after internalization into macrophages (Boschiroli *et al.*, 2002). Therefore, TFSS is required for *Brucella* proliferation and survival, but not for internalization. In contrast, in *Legionella*, DotA, one component of TFSS, is expressed prior to, or at the beginning of infection of the host cells, and the DotA mutant cannot evade the fusion with lysosomes (Roy *et al.*, 1998). It is possible that expression of TFSS may be also regulated and associated with intracellular survival in obligatory intracellular bacteria. In the present study, we examined expression of components of TFSS by *A. phagocytophilum* during obligatory intracellular life cycle, and early cellular trafficking of *VirB9*-expressing and -undetectable *A. phagocytophilum* in human neutrophils and HL-60 cells.

3.3 Materials and Methods

**Anaplasma phagocytophilum**

The *A. phagocytophilum* HZ strain was propagated in HL-60 cells (American Type Culture Collection, Manassas, VA) in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (US Biotechnologies, Parkerford, PA) and 2 mM L-glutamine (Invitrogen). Cultures were incubated at 37°C in a humidified 5%
CO₂–95% air atmosphere. No antibiotic was used throughout the study. *A. phagocytophilum* infection was examined with Diff-Quik staining (Baxter Scientific Products, Obetz, OH) after centrifugation of cells onto microscope slides in a cytocentrifuge (Thermoshandon, Pittsburgh, PA). When >95% of HL-60 cells was infected, cells were lysed by nitrogen cavitation at 4 × 10⁶ cells per millilitre under 800 psi in a nitrogen bomb (model 4639, Parr Instruments, Moline, IL). After centrifugation at 2000 g for 5 min to remove HL-60 cell debris, the supernatant was used to infect new host cells. Determination of viability of organisms after spontaneous release from infected HL-60 cells was performed with the LIVE/DEAD BacLight Bacterial Viability Kit (Molecular probes, Eugene, OR) as described (Lin and Rikihisa, 2005).

**Isolation of human neutrophils**

Human neutrophils were purified as described elsewhere (Le Cabec and Maridonneau-Parini, 1995) with minor modifications. Briefly, 4.5% Dextran T500 (Pharmacia Biotech AB, Uppsala, Sweden) suspended in 1× PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 2 mM KH₂PO₄ at pH 7.4) was added to 20 ml blood samples drawn from healthy volunteers to a final concentration of 1.5%, then allowed to settle at 1 g for 20 min at RT. The leucocyte-rich plasma above the sedimented erythrocytes was removed and centrifuged at 500 g for 10 min. Each pellet was resuspended in 3 ml of PBS, and the remaining red blood cells were lysed by adding 38 ml of water. Haemolysis was stopped 22 s later by adding 2 ml of 20× PBS. The pellet was resuspended in 10 ml of 1× PBS and gently overlaid onto 10 ml Histopaque 1077 (Sigma Diagnostics, St. Louis, MO) and centrifuged at 750 g for 20 min. After washing
the pellet once with 4 ml of PBS, neutrophils were resuspended in RPMI medium to a concentration of $3 \times 10^6$ cells per millilitre. Cells were 99% polymorphonuclear (3–5% eosinophils) and 1% mononuclear (lymphocytes and monocytes) as determined by Diff-Quik staining.

Infection of human neutrophils and HL-60 cells with host cell-free *A. phagocytophilum*: neutrophils or HL-60 cells ($1.5 \times 10^7$) were incubated with *A. phagocytophilum* derived from $2 \times 10^7$ infected HL-60 cells in 15 ml of complete RPMI medium (approximate multiplicity of infection: 1:100). After 20 min of shaking at RT and 30 min of incubation at 37°C, infected cells were washed twice with PBS to remove unbound bacteria, and then incubated at 37°C to allow *A. phagocytophilum* to replicate for total 16 and 40 h. To study intracellular trafficking of *A. phagocytophilum* in HL-60 cells in the early stage of infection, *A. phagocytophilum*-infected HL-60 cells were washed twice with ice-cold PBS after incubation for 20 min at RT and resuspended with prewarmed RPMI medium. Internalization was allowed to occur for 30 min at 37°C prior to fixation with paraformaldehyde.

**Competitive PCR and competitive RT-PCR**

Three PCR competitors for 16S rRNA gene, *virB9*, or *virB6* with an internal deletion of approximately 20% of target DNAs were constructed by separately amplifying two DNA fragments flanking the internal deletion with two pairs of primers (P1 and P2, and P3 and P4) (Table 3.1). In order to ligate the 5'- and 3' fragments, the 5' end of reverse primer P2 for the 5' fragment was complementary to the 5' end of forward primer in P3 for the 3' fragment (Table 3.1). Two fragments were mixed, denatured,
annealed and extended by Taq DNA polymerase using P1 and P4 primer pair. The PCR products were cloned into a pCRII vector (Invitrogen). The plasmid was amplified in *E. coli* INVαF' strain (Invitrogen), purified, and used as competitors. To determine the number of ehrlichial organisms, genomic DNA was extracted from one-half of infected cells using a QIAamp blood kit (Qiagen, Valencia, CA). The serially diluted 16S rRNA gene competitor was added into the PCR mixture which contained 0.2–0.3 µg of the genomic DNA. As there is a single gene of 16S rRNA in the genome (Massung *et al*., 2002), the number of 16S rRNA gene determined by competitive PCR corresponds to that of bacterium in the respective samples. Total RNA was extracted from another half of infected cells using RNeasy Mini RNA extraction kit (Qiagen). The extracted RNA (1.0 µg) was treated with 1 unit of DNase I (Invitrogen) at RT for 15 min, inactivated by adding EDTA to final concentration 2.5 mM and heating for 10 min at 65°C and subjected to reverse transcription using Superscript II (Invitrogen) with random hexamer at 42°C for 50 min. The cDNAs derived from the same number of bacterium in respective samples were used for PCR reactions with serially diluted competitor plasmids to compare the copy numbers of *virB9* cDNA and *virB6* cDNA across specimens. The PCR conditions were 45 cycles consisting of 1 min of denaturation at 94°C, 1 min of annealing at 60°C, and 1 min of extension at 72°C. The PCR products were electrophoresed and visualized after ethidium bromide staining. The gel images were digitally captured and analyzed by using a gel video system (FujiFilm, Stamford, CT).
Cloning, expression of recombinant VirB9, and antibody production

*Anaplasma phagocytophilum* genomic DNA was isolated from organisms purified by Percoll density gradient centrifugation as described elsewhere (Ohashi *et al.*, 2002). A 798 bp DNA fragment encoding the putative mature VirB9 of *A. phagocytophilum* was amplified with the following pair of primers: 5′-CGG GAT CCG AAA GCA TGC TTT GCC AGC-3′ and 5′-TGC GGC CGC CTA ACT AAG AGC CTG ATT C-3′; BamHI and NotI sites, respectively, are underlined. The amplified fragment was digested with BamHI and NotI and ligated into BamHI–NotI-digested pET33b (+) (Novagen, Madison, WI). The sequence of the insert and junctions was verified using a dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) and run on an ABI Prism 377 DNA sequencer (Applied Biosystems). *E. coli* BL21(DE3)pLysS (Novagen) was transformed with this recombinant expression vector. Cultures were grown in Luria–Bertani (LB) medium containing 34 mg ml\(^{-1}\) kanamycin at 37°C with shaking at 250 rpm. When cultures were in the mid-logarithmic phase of growth (OD\(_{600}\) value of 0.4), expression of recombinant VirB9 (rVirB9) was induced at 25°C for 5 h by adding IPTG (isopropyl-beta-D-thiogalactopyranoside) to a final concentration of 1 mM. Bacteria were harvested by centrifugation at 10 000 g for 10 min at 4°C and suspended in 1 × His binding buffer (0.5 M NaCl, 20 mM Tris–HCl and 5 mM imidazole at pH 7.9), then sonicated on ice. The lysates were centrifuged at 14 000 g for 20 min at 4°C. The supernatant containing the His-tagged rVirB9 was affinity-purified on a HisBind Quick Column under native conditions as recommended by the manufacturer (Novagen). The concentration of purified rVirB9 was measured by the BCA (bicinchoninic acid) method with a protein assay kit (Pierce, Rockford, IL). After
separation of rVirB9 by SDS-PAGE, the rVirB9 band was excised and used to prepare antiserum in rabbits (Prosci Incorporated, Poway, CA).

**SDS-PAGE and Western blotting analysis**

Purified rVirB9 (50 µg) and 2 × 10⁶ HL-60 cells or *A. phagocytophilum*-infected HL-60 cells (infectivity > 95%) were dissolved in 100 µl of 2× SDS-PAGE loading buffer (4% SDS, 135 mM Tris–HCl [pH 6.8], 10% glycerol, and 10%β-mercaptoethanol). Samples were separated by SDS-PAGE with 12% polyacrylamide resolving gels, then stained with Coomassie blue or transferred to a nitrocellulose membrane using a semidyry blotter (WEP, Seattle, WA). The membrane was blocked using 5% (wt/vol) skim milk (Kroger, Cincinnati, OH) in Tris-buffered saline (150 mM NaCl and 50 mM Tris at pH 7.5), then incubated with rabbit anti-rVirB9 antibody (1:1000 dilution) at 4°C for 12 h, and subsequently with peroxidase-conjugated goat anti-rabbit IgG (KPL, Gaithersburg, MD) in 1:1000 dilution at RT for 3 h. The bound antibody was detected by incubation with 0.2 mg ml⁻¹ dianinobenzidine (Nakarai, Japan) in 50 mM Tris–HCl buffer (pH 7.2) containing 0.1% H₂O₂ or by chemiluminescence (Amersham Pharmacia, Piscataway, NJ).

**Immunofluorescence microscopy**

Infected cells or host cell-free *A. phagocytophilum* organisms were fixed with 1% paraformaldehyde in PBS at RT for 30 min and then washed three times in PBS. Permeabilization and blocking were performed with PGS solution (PBS containing 0.4% BSA, 0.2% gelatin and 0.3% saponin) at RT for 1 h. For double immunofluorescence labelling, the permeabilized cells were incubated with a mixture of horse antibody against
whole *A. phagocytophilum* (diluted 1:150) and rabbit antibody against rVirB9 (diluted 1:80) in PGS solution for 1 h. Horse and rabbit antibodies were pre-adsorbed with acetone-fixed HL-60 cells to decrease background as described elsewhere (Sambrook *et al.*, 1989). The cells were washed with PBS three times to remove unbound antibody; the bound antibodies were detected with a mixture of Cy3-conjugated goat anti-horse IgG (diluted 1:100, Jackson ImmunoResearch Laboratories, West Grove, PA) and Alexa Fluor 488-conjugated goat anti-rabbit IgG (diluted 1:300, Molecular Probes).

Triple immunofluorescence labelling was used to study the intracellular trafficking of *A. phagocytophilum* in HL-60 cells or to identify the presence of *A. phagocytophilum* in human neutrophils. In addition to primary antibodies against VirB9 or whole *A. phagocytophilum*, other primary antibodies were included: mouse monoclonal antibodies against LAMP-1 (Clone 25, BD Biosciences Pharmingen, diluted 1:50) or against human neutrophil elastase (Clone NP57, DakoCytomation, Denmark, diluted 1:20); secondary antibodies were FITC-conjugated goat anti-horse IgG (diluted 1:50, Jackson ImmunoResearch Laboratories), Alexa Fluor 350-conjugated goat anti-rabbit IgG (diluted 1:300, Molecular Probes), and Alexa Fluor 555-conjugated goat anti-mouse IgG (diluted 1:300, Molecular Probes). To study the effects of cytochalasin D and monodansylcadaverine on the internalization of VirB9-expressing and -undetectable *A. phagocytophilum* into HL-60 cells, extracellular bacteria were first stained with horse anti-*A. phagocytophilum* and Cy3-conjugated goat anti-horse IgG in the absence of saponin after fixation with 1% paraformaldehyde. Then extracellular and intracellular bacteria were stained with horse anti-*A. phagocytophilum* and FITC-conjugated goat anti-horse IgG, and VirB9 was stained with rabbit anti-rVirB9 and Alexa Fluor 350-
conjugated goat anti-rabbit IgG in the presence of saponin, a permeabilizing reagent. All cells were washed three times with PBS to remove unbound secondary antibodies before observation with a Nikon Eclipse E400 fluorescence microscope with xenon–mercury light source (Nikon Instruments, Melville, NY). Images were taken with a SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI). The integrated SPOT RT image software (version 3.5) was used to measure the sizes of individual fluorescence-labelled bacteria and add a calibration bar to images. The original colour emitted by excited Alexa Fluor 350 (blue) was transformed to gray pseudocolour for clear viewing with Photoshop 7.0 software (Adobe, San Jose, CA). One hundred anti-\textit{A. phagocytophilum}-positive bacteria or inclusions were scored for VirB9 expression or LAMP-1 staining in three independent experiments. The data were compared by Tukey HSD test or Student's \textit{t}-test (\(P < 0.05\) was considered significant).

\textbf{Negative-staining of immunogold-labelled \textit{A. phagocytophilum}}

\textit{Anaplasma phagocytophilum} organisms released from host cells by nitrogen cavitation were fixed with 1% paraformaldehyde in PBS at RT for 30 min and then washed three times in PBS. Samples were then incubated with the rabbit anti-rVirB9 antibody (32 \(\mu\text{g}\text{ ml}^{-1}\)) affinity-purified with membrane-bound rVirB9 (Sambrook \textit{et al.}, 1989) for 1 h at 37°C. After washing several times with PBS, the bound antibodies were further incubated with gold-conjugated goat anti-rabbit IgG (1:20 dilution; 12 nm in diameter, Jackson ImmunoResearch Laboratories) for 1 h at RT. After washing with PBS, samples were adsorbed onto Formvar-carbon-coated copper grids (200 mesh; Electron Microscopy Sciences, Hatfield, PA), washed in water and stained with 1.0%
aqueous uranyl acetate. After air-drying, samples were examined in a Philips 300 transmission electron microscope at 60 kV.

3. 4 Results

Intracellular upregulation of \textit{virB6} and \textit{virB9} transcription in human neutrophils

In the \textit{A. phagocytophilum} genome, \textit{virB/D} genes cluster mainly in two separate operons, and both genomic loci are polycistrionically transcribed in HL-60 cells (Ohashi \textit{et al.}, 2002). To investigate \textit{virB} transcription during \textit{A. phagocytophilum} infection cycle in human neutrophils, \textit{virB9} and \textit{virB6} were used as representative for each operon. Competitive reverse transcription polymerase chain reaction (RT-PCR) assay was developed to detect a very low level of transcription at initial stage of infection. We chose the competitive RT-PCR over other polymerase chain reaction (PCR) methods, because in this method the competitor coexists with the target cDNA in the same reaction tube, also serves as internal control for variation of each PCR. Transcription levels were normalized based on bacterium genome equivalent as determined by the 16S rRNA gene-based competitive DNA PCR. Relative cDNA levels of \textit{virB9} and \textit{virB6} per bacterium in human neutrophils were upregulated greater than 100-fold at 35 h post infection (PI) compared at 3 h PI (Fig. 3.1). Transcription of neither \textit{virB6} nor \textit{virB9} was detected at 3 h PI under this condition. This result showed the intracellular upregulation of \textit{virB9} and \textit{virB6} transcription in two separate \textit{virB/D} loci in \textit{A. phagocytophilum} genome.
Cloning, expression of recombinant *A. phagocytophilum* VirB9, and antibody production

The virB9 coding region without the sequence encoding the putative 22-amino-acid N-terminal signal peptide (28.8 kDa) was cloned and expressed in *Escherichia coli* as an N-terminal histidine-tagged fusion protein (designated as rVirB9). After Ni\(^{2+}\) affinity chromatography, the purified 298-amino-acid expression product (which included 39 vector-encoded amino acids) appeared as a 33.0 kDa band on SDS-PAGE (Fig. 3.2A). When the protein from this band was used to immunize rabbits, the resulting monospecific antibody specifically bound to the 33.0 kDa rVirB9 and to the 28.8 kDa mature native VirB9 of *A. phagocytophilum* growing in cultured HL-60 cells by Western blot analysis (Fig. 3.2B). The antibody did not react with uninfected HL-60 cells (Fig. 3.2B). The pre-immune rabbit serum did not react with rVirB9 or native VirB9 (data not shown). Immunogold labelling of paraformaldehyde-fixed *A. phagocytophilum* liberated from HL-60 cells by nitrogen cavitation showed the highly wrinkled outer membrane as previously noted (Rikihisa *et al*., 1997; Popov *et al*., 1998) and patchy surface labelling of VirB9 protein as well as occasional labelling of short surface protrusions (Fig. 3.2C). Thus, the rabbit antibody was specific and recognized the native *A. phagocytophilum* VirB9 protein. These data also indicate that *A. phagocytophilum* growing in HL-60 cell cultures expressed the VirB9 protein.
VirB9 was strongly expressed by replicating *A. phagocytophilum*, whereas undetectable in majority of *A. phagocytophilum* spontaneously liberated from host cells

As mRNA of *virB9* was not detected at 3 h PI in neutrophils infected with *A. phagocytophilum* liberated from heavily infected HL-60 cells (Fig. 3.1), VirB9 protein expression by *A. phagocytophilum* in HL-60 cells was examined by double immunofluorescence labelling. Morulae (mulberry-like intracellular microcolonies characteristic to this group of bacteria) in HL-60 cells were strongly labelled with both anti-VirB9 antibody and anti-*A. phagocytophilum* antibody, although labelling of individual bacteria was difficult to discern due to tight packing of *A. phagocytophilum* organisms in the cytoplasmic inclusion (Fig. 3.3A). As negative controls for immunofluorescence labelling, *A. phagocytophilum*-infected HL-60 cells were incubated with the rabbit antibody against an unrelated recombinant protein (OMP-1B protein of *Ehrlichia chaffeensis*, H. Huang and Y. Rikihisa, unpublished) and secondary conjugated antibodies, or with pre-immune horse serum and secondary conjugated antibodies. There was no detectable labelling, indicating that labelling with both anti-*A. phagocytophilum* and anti-rVirB9 antibodies was specific (Fig. 3.3, panels B and C). In contrast, majority of the bacteria being spontaneously released from host cells were individually dispersed and not labelled with anti-VirB9 antibody, but they were strongly labelled with anti-*A. phagocytophilum* antibody (Fig. 3.3A). This result suggests that VirB9 protein expression was downregulated prior to spontaneous release.

To further analyze VirB9 protein expression by individual bacteria, host cell-free *A. phagocytophilum* spontaneously released and mechanically released from infected HL-
60 cells were examined by double immunofluorescence labelling. Images were taken with a SPOT digital camera with the integrated SPOT RT image software to estimate the proportion of anti-rVirB9 positively labelled bacteria and the size of individual bacteria. When bacteria spontaneously released at 3 days PI were examined, expression of VirB9 protein was detected in only 20 ± 12% \((n = 3\) independent experiments) of *A. phagocytophilum*. In comparison, 50 ± 10% \((n = 3\) independent experiments) of *A. phagocytophilum* liberated by nitrogen cavitation from infected HL-60 cells at 3 days PI were VirB9-detectable, suggesting preferential spontaneous release of VirB9 undetectable bacteria. *A. phagocytophilum* is a pleomorphic bacterium that varies in diameter from 0.2 to 2.0 µm by electron microscopy (Woldehiwet and Scott, 1982; Rikihisa, 1991; Popov *et al.*, 1998; Webster *et al.*, 1998; Munderloh *et al.*, 1999). Similar size distribution was found among host cell-free bacteria by fluorescence microscopy as determined by SPOT RT image software (Fig. 3.3D and E). Bacteria spontaneously released from HL-60 cells showed high (>75%) viability as determined by LIVE/DEAD BacLight Bacterial Viability Kit (Fig. 3.3D). Generally viabilities of bacteria of smaller sizes were greater than those of larger sizes (Fig. 3.3E). To determine whether *A. phagocytophilum* organisms of different sizes differ in VirB9 protein expression levels, we scored the percentages of bacteria expressing VirB9 protein in two arbitrary size categories: small (<1 µm) and large (>1 µm). Only 25 ± 12% \((n = 3\) independent experiments) of 100 small bacteria expressed VirB9 protein, whereas 75 ± 12% \((n = 3\) independent experiments) of 100 large bacteria expressed VirB9. Thus this result implies that there were populations of *A. phagocytophilum*, differing in size as well as VirB9 protein expression level.
Preferential binding and internalization of VirB9-undetectable *A. phagocytophilum* to human neutrophils

As VirB9-expressing and -undetectable *A. phagocytophilum* coexist after being liberated from infected host cells, and mRNA of *virB9* was not detected at 3 h PI in neutrophils infected with host cell-free *A. phagocytophilum*, VirB9 and the total *A. phagocytophilum* protein expression by *A. phagocytophilum* were examined by double immunofluorescence labelling. Neutrophils were incubated with *A. phagocytophilum* liberated by nitrogen cavitation from infected HL-60 cells, to allow binding and internalization for approximately 1 h (20 min at room temperature [RT] and 30 min at 37°C), then washed and examined by double immunofluorescence labelling. The bacteria associated with neutrophils were strongly labelled with the horse anti-*A. phagocytophilum* antibody; however, more than 95% of the bacteria associated with neutrophils were not labelled with anti-VirB9 antibody and were smaller than 1 µm (Fig. 3.4, panels A and E). Larger VirB9-positive *A. phagocytophilum* rarely associated with human neutrophils. This result suggests that the VirB9-undetectable, small form of *A. phagocytophilum*, binds and infects human neutrophils *in vitro*. This is likely the reason why the *virB9* transcript could not be detected at the early stage of infection in neutrophils.

Intracellular induction of VirB9 protein expression in human neutrophils and eosinophils

As *virB9* transcription was upregulated greater than 100-fold per bacterium at 35 h PI compared at 3 h PI in human neutrophils, temporal VirB9 protein expression by *A. phagocytophilum* in neutrophils was examined. At approximately 16 h PI, most Diff-
Quik-stained intracellular *A. phagocytophilum* inclusions were larger than 2 µm. Approximately 40% of morulae were immunostained positive for VirB9 protein (Fig. 3.4, panels B and E). At approximately 40 h PI, 80% of morulae were strongly labelled with anti-rVirB9 antibody (Fig. 3.4, panels C and E). Thus VirB9 protein as well as mRNA expression was upregulated during infection of neutrophils. *A. phagocytophilum* infection inhibits spontaneous apoptosis of human neutrophils (Yoshiie *et al*., 2000), thus ~50% of neutrophils remained viable at 40 h PI *in vitro*.

The human peripheral blood granulocyte preparation contains eosinophils at various levels. *A. phagocytophilum* also infects human eosinophils and infected eosinophils survive longer than neutrophils in culture (Yoshiie *et al*., 2000). To determine whether VirB9 protein was also expressed by *A. phagocytophilum* in human eosinophils, we performed triple immunofluorescence labelling with a monoclonal anti-human neutrophil elastase antibody (which specifically labels neutrophils), anti-rVirB9 antibody, and anti-*A. phagocytophilum* antibody. Under the fluorescence microscope, eosinophils were identified by the absence of neutrophil elastase and the presence of autofluorescence, due to eosinophil granule-associated flavin adenine dinucleotide (Mayeno *et al*., 1992) (Fig. 3.5). VirB9 protein expression by *A. phagocytophilum* was found in eosinophils. Thus VirB9 upregulation was not limited to neutrophils, but perhaps associated with *A. phagocytophilum* infection cycle in the natural host cells.
Binding and internalization of VirB9-expressing and -undetectable *A. phagocytophilum* into HL-60 cells

HL-60 cells, a human promyelocytic leukaemia cell line (Collins *et al.*, 1977) has been used extensively for *A. phagocytophilum* cultivation and cell biology research. To extend our observation using human neutrophils, we studied the binding and internalization of VirB9-expressing and -undetectable *A. phagocytophilum* into HL-60 cells. After HL-60 cells were incubated for 20 min at RT and for 30 min at 37°C with *A. phagocytophilum* which was mechanically released from infected HL-60 cells, up to 80% of the *A. phagocytophilum* organisms bound to HL-60 cells expressed VirB9 protein (Fig. 3.4, panel D). This result suggests that HL-60 cells are different from neutrophils by having a binding site for VirB9-expressing *A. phagocytophilum* in addition to the binding site for VirB9-undetectable *A. phagocytophilum*.

VirB9-undetectable *A. phagocytophilum* evaded fusion with late endosomes and lysosomes

*Anaplasma phagocytophilum* replicative inclusions within HL-60 cells lack markers for late endosomes or lysosomes (Mott *et al.*, 1999). Because both VirB9-expressing and -undetectable *A. phagocytophilum* bound to and were internalized by HL-60 cells, their intracellular compartment at 1 h PI was examined using lysosome-associated membrane protein-1 (LAMP-1) as a marker for late endosomes and lysosomes. Triple immunofluorescence labelling revealed that approximately 80% of VirB9-expressing *A. phagocytophilum* in HL-60 cells colocalized with LAMP-1, whereas less than 20% of VirB9-undetectable *A. phagocytophilum* colocalized with LAMP-1 in HL-60 cells after 1 h of co-incubation (Fig. 3.6). These data indicate that majority of
VirB9-undetectable small *A. phagocytophilum* organisms evaded fusion with late endosomes and lysosomes in HL-60 cells at 1 h PI, in contrast, majority of VirB9-expressing *A. phagocytophilum* organisms were routed to lysosomes. Although pretreatment of human neutrophils with monodansylcadaverine, an inhibitor of transglutaminase (Levitzki *et al.*, 1980), blocks *A. phagocytophilum* infection of neutrophils (Yoshiie *et al.*, 2000), it did not inhibit the entry of VirB9-expressing *A. phagocytophilum* into HL-60 cells (Fig. 3.7). Entry of VirB9-expressing *A. phagocytophilum* was completely blocked by pretreatment of HL-60 cells with 4 µM cytochalasin D for 30 min (data not shown). This result, as well as the one above, supported the hypothesis that VirB9-expressing and -undetectable *A. phagocytophilum* enter HL-60 cells through different receptors and internalization pathways.

### 3.5 Discussion

This study is, to our knowledge, the first report on investigation of TFSS protein expression by obligate intracellular bacteria during intracellular life cycle. Immunogold labelling showed surface exposure of VirB9 protein in *A. phagocytophilum*. VirB9 in *Agrobacterium tumefaciens* is an outer membrane-associated protein (Jakubowski *et al.*, 2005). In *Helicobacter pylori*, VirB9-homologue was associated with a filamentous macromolecular structure protruding from the bacterial envelope localized by immunogold electron microscopy along the length of the pilus (Tanaka *et al.*, 2003). We could not find homologues of *virB2, virB5* and *virB7* that encode proteins making the external pilus projection in *A. tumefaciens* (Sagulenko *et al.*, 2001) in the *A. phagocytophilum* genome (http://www.tigr.org). Patchy distribution of the VirB9
protein and occasional short surface protrusions (∼80 nm in diameter) containing the VirB9 protein suggest these protein clusters as a part of the type IV secretion apparatus. Relating to this observation, molecular interactions between VirB9 protein with VirB9 as well as with other VirB proteins encoded by Rickettsia sibirica were reported (Malek et al., 2004). However, we cannot deny the possibility that the method employed to isolate A. phagocytophilum from the host cells and to carry out immunoelectron microscopy may have changed the distribution and the structure of the type IV secretion apparatus. Further studies are necessary to define the structure of the type IV secretion apparatus in A. phagocytophilum.

Our study revealed developmental regulation of TFSS component expression in A. phagocytophilum and biological differences between different stages or sizes of A. phagocytophilum. Generally at the initiation of infection and prior to the spontaneous release from the host cells, bacteria were smaller in size than bacteria replicating in the inclusion. It is possible that virB/Ds represent many other genes regulated during A. phagocytophilum developmental cycle. virB6 and virB9 expression was transcriptionally upregulated in A. phagocytophilum replicating in human neutrophils. Expression of the virB operon is tightly regulated in some bacteria, such as Brucella suis, in which the virB promoter is induced within 3 h inside macrophages by sensing the acidic environment of the phagosome (Boschiroli et al., 2002). The signal upregulating the virB6 and virB9 expression in A. phagocytophilum is unknown, but it is unlikely low pH, because the A. phagocytophilum replicative inclusion compartment in HL-60 cells is not acidic (Webster et al., 1998; Mott et al., 1999). Majority of spontaneously released A. phagocytophilum did not express VirB9. The signal which downregulates the VirB9
expression in *A. phagocytophilum* remains to be determined. For other bacteria such as *B. suis* and *B. melitensis*, virB9 transcription is downregulated when the growth of free-living bacteria reaches stationary phase by a quorum-sensing mechanism (Taminiau *et al.*, 2002).

Our data showed that in the early stage of infection, VirB9-undetectable *A. phagocytophilum* could evade fusion with late endosomes and lysosomes in HL-60 cells, whereas VirB9-expressing *A. phagocytophilum* could not. This is somewhat similar to *Legionella*. *Legionella* at stationary stage culture can evade the endosome-lysosome pathway in macrophages, but *Legionella* at the exponential stage cannot (Joshi *et al.*, 2001). A recent study showed that *Legionella dotA* or *dotB* mutant exposed to pH 6.5 enters amoeba and macrophage hosts independently of TFSS, suggesting that TFSS is not essential, but a mild acid-inducible factor is required for entry into two types of host cells (Bandyopadhyay *et al.*, 2004). Similarly, our studies suggest that VirB9 protein is not essential for entry of *A. phagocytophilum* to establish infection in both human neutrophils and eosinophils *in vitro*.

Present findings show that in addition to a receptor for VirB9-undetectable small *A. phagocytophilum*, promyelocytic leukaemia HL-60 cells have a receptor for the internalization of VirB9-expressing large *A. phagocytophilum*. The receptor seems to be coupled with phagocytosis and does not seem to be conducive to infection, because it directs *A. phagocytophilum* to late endosomes or lysosomes. Our finding also implies that different developmental stages of *A. phagocytophilum* have distinct surface or biological properties that allow differential binding to neutrophils and HL-60 cells. Thus it might help the analysis if this difference is taken into consideration in the future studies of
*A. phagocytophilum* ligand-receptor, especially when HL-60 cells are used as a model host cells. As currently separation of different developmental stages of *A. phagocytophilum* is not possible and size differences are difficult to discern, especially in the host cells, VirB9 protein may serve as a biological marker to distinguish bacterial developmental stages. Having identified the stage of TFSS component expression during the intracellular life cycle of *A. phagocytophilum* will facilitate our future investigation of the role of TFSS in obligatory intracellular bacterial infection.
<table>
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<tr>
<th>Gene name</th>
<th>P1 (used for construction of competitor and competitive PCR)</th>
<th>P2 (used for construction of competitor)</th>
<th>P3 (used for construction of competitor)</th>
<th>P4 (used for construction of competitor and competitive PCR)</th>
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Table 3.1. Oligonucleotide primers used in PCR and construction of competitors.

Note: The complementary sequences in primer P2 and P3 are underlined.
Figure 3.1. Temporal expression of *virB9* and *virB6* by *A. phagocytophilum* in neutrophils. cDNA was synthesized from total RNA extracted from infected human neutrophils at three different time points (3, 18 and 35 h PI). cDNA levels were normalized by the number of bacteria as determined by 16S rRNA gene-based competitive PCR. The relative amount of competitor plasmids used in each reaction is indicated at the top of each panel. The result is a representative from three independent experiments which showed similar results. RT+: with reverse transcription; RT−: without reverse transcription. Primers for PCRs and construction of competitors are shown in Table 3.1.
Figure 3.1

<table>
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</table>

**A**
- virB9 Target (297 bp)
- Competitor (257 bp)

**B**
- virB6 Target (308 bp)
- Competitor (250 bp)
Figure 3.2. Immunogold labelling of *A. phagocytophilum* using the antibody against *A. phagocytophilum* rVirB9.

A. rVirB9 expressed in *E. coli* was purified by immobilized Ni$^{2+}$ affinity chromatography, separated by SDS-PAGE, and stained with Coomassie blue. Lanes: M, protein molecular size marker; rVirB9, recombinant *A. phagocytophilum* VirB9.

B. Western blot analysis was performed using rabbit antibody against *A. phagocytophilum* rVirB9. Lanes: rVirB9, recombinant VirB9 of *A. phagocytophilum*; HL-60, HL-60 cells; AP: *A. phagocytophilum*-infected HL-60 cells.

C. Immunogold labelling of paraformaldehyde-fixed *A. phagocytophilum* liberated from HL-60 cells by nitrogen cavitation showed highly wrinkled membrane and patchy surface labelling of VirB9 protein (arrows) as well as occasional labelling of stubby surface protrusion (arrowhead, Insert). Scale bar = 0.1 μm.
Figure 3.2
Figure 3.3. VirB9 expression by *A. phagocytophilum* in morulae and in spontaneous release.

A. *A. phagocytophilum*-infected HL-60 cells harvested on day 3 PI were immunofluorescence-labelled with two antibodies, horse anti-*A. phagocytophilum* (AP) (Cy3, red) and rabbit anti-rVirB9 (VirB9) (Alexa Fluor 488, green) of *A. phagocytophilum*. M: morula; white arrowheads point to bacteria being released from the host cells. Note strong anti-VirB9 antibody labelling of the morula and absence of labelling of numerous individual bacteria at the moment of spontaneous release. Scale bar = 5 µm.

B. *A. phagocytophilum* in HL-60 cells were labelled with horse anti-*A. phagocytophilum* (AP) (Cy3, red), but not with rabbit anti-OMP-1B of *E. chaffeensis* (Alexa Fluor 488, green) (negative control). Scale bar = 5 µm.

C. *A. phagocytophilum* in HL-60 cells were labelled with rabbit anti-rVirB9 (VirB9) (Alexa Fluor 488, green), but not with pre-immune horse serum (Cy3, red) (negative control). Scale bar = 5 µm.

D. Spontaneously released *A. phagocytophilum* stained with the LIVE/DEAD BacLight Bacterial Viability Kit. Majority of bacteria were viable as shown by intense green staining with SYTO 9. A minority of bacteria with damaged membranes were stained red with propidium iodide. Scale bar = 2 µm.

E. Size distribution and proportions of viable bacteria among spontaneously released *A. phagocytophilum* as determined by Bacterial Viability Kit and SPOT RT image software (version 3.5). Gray bar with fine texture: viable bacteria; Black bar: dead bacteria.
Figure 3.3

A

AP  VirB9  Merge

3 d

B

AP  Omp-1B  Preimmune serum  VirB9

C

D

E

Percentage of total bacteria

< 0.5  0.5 - 0.8  0.8 - 1.2  > 1.2 µm
Figure 3.4. Binding and internalization of VirB9-expressing and undetectable A. phagocytophilum into human neutrophils and HL-60 cells. A. phagocytophilum organisms mechanically released from infected HL-60 cells were used to infect human neutrophils or HL-60 cells. Double immunofluorescence labelling was performed using horse anti-A. phagocytophilum (Cy3, red) and rabbit anti-rVirB9 of A. phagocytophilum (Alexa Fluor 488, green) for samples harvested at approximately 1 h (20 min at RT and 30 min at 37°C; A, human neutrophils; D, HL-60 cells), 16 h (B, human neutrophils) or 40 h (C, human neutrophils) PI. Scale bar = 5 μm. (E) The percentage of VirB9-expressing A. phagocytophilum in human neutrophils was calculated at three time points, based on examination of 100 individual bacteria (1 h PI) or 100 morulae (16 and 40 h PI). Data are presented as means and standard deviations of three independent experiments. *Significantly different from 1 h by Tukey HSD test (P < 0.01).
Figure 3.4
Figure 3.5. VirB9 expression in *A. phagocytophilum*-infected human neutrophils and eosinophils. Triple immunofluorescence labelling was performed to investigate VirB9 expression in human neutrophils (A) and eosinophils (B) 40 h PI using three antibodies, horse anti-*A. phagocytophilum* (AP) (FITC, green), rabbit anti-rVirB9 (VirB9) (Alexa Fluor 350, blue, pseudocoloured gray) and mouse anti-human neutrophil elastase monoclonal antibody (Alexa Fluor 555, red). Scale bar = 5 µm.
Figure 3.5
Figure 3.6. Intracellular localization of VirB9-expressing and -undetectable *A. phagocytophilum* within HL-60 cells.

A. Triple immunofluorescence labelling was performed to examine the colocalization of *A. phagocytophilum* with LAMP-1 at 1 h PI using three antibodies, horse anti-*A. phagocytophilum* (FITC, green), rabbit anti-rVirB9 (Alexa Fluor 350, blue, pseudocoloured gray) and mouse monoclonal antibody against LAMP-1 (Alexa Fluor 555, red). Yellow arrowheads point to VirB9-expressing bacteria colocalized with LAMP-1. Scale bar = 5 µm.

B. Percentages of LAMP-1 positive labelling of vacuoles containing VirB9-expressing and -undetectable *A. phagocytophilum* were calculated based on 100 bacteria in each category. Data are presented as means and standard deviations of three independent assays. *Significantly different between two categories by Student's t-test (P < 0.01).*
Figure 3.6
Figure 3.7. Internalization of VirB9-expressing *A. phagocytophilum* into HL-60 cells by phagocytosis. Triple immunofluorescence labelling was performed to examine VirB9 expression by *A. phagocytophilum* internalized into HL-60 cells in the presence of monodansylcadaverine using two primary antibodies (horse anti-*A. phagocytophilum* and rabbit anti-rVirB9) and three secondary antibodies (Cy3-conjugated goat anti-horse IgG, FITC-conjugated goat anti-horse IgG, and Alexa Fluor 350-conjugated goat anti-rabbit IgG). Extracellular bacteria (Cy3, red, white arrowhead) were stained prior to permeabilization with saponin and total bacteria (extracellular and intracellular; FITC, green) were stained after permeabilization. VirB9 was shown in pseudocoloured gray (Alexa Fluor 350, blue). Most of intracellular bacteria (yellow arrowhead) were > 1 µm and positively stained with anti-rVirB9 antibody. Scale bar = 5 µm.
Figure 3.7
CHAPTER 4

ANAPLASMA PHAGOCYTOPHILUM ATS-1 TARGETS MITOCHONDRIA AND INHIBITS HOST CELL APOPTOSIS

4.1 Abstract

*Anaplasma phagocytophilum* is an obligatory intracellular bacterium that causes human granulocytic anaplasmosis. To replicate inside short-lived neutrophils *A. phagocytophilum* inhibits apoptosis of infected neutrophils. Here, using *A. phagocytophilum* VirD4, a component of type IV secretion apparatus as bait, and the *A. phagocytophilum* genomic DNA library as prey in bacterial two-hybrid system, we identified a hypothetical protein, named *Anaplasma* translocation substrate 1 (Ats-1) that binds to VirD4. We demonstrated that Ats-1 was expressed by *A. phagocytophilum* in the vacuole, translocated across the vacuolar membrane, and localized in the mitochondria of infected-human neutrophils and HL-60 cells. Ats-1 had the intrinsic activity to target mitochondria when ectopically expressed in the absence of bacteria. The N-terminal region of Ats-1 was required for mitochondrial localization and the cleavage of presequence. Substitution of FYH of Ats-1 (amino acid 55-57) to AAA did not prevent translocation of Ats-1 to mitochondria, but prevented presequence cleavage. Ectopic expression of Ats-1 inhibited etoposide-induced apoptosis and cytochrome c release from
mitochondria in RF/6A cells. Taken together, *A. phagocytophilum* inhibits apoptosis of the host cells by secreting Ats-1, a novel mitochondria-targeting protein that maintains mitochondrial membrane integrity.

### 4.2 Introduction

*Anaplasma phagocytophilum*, is an obligatory intracellular Gram-negative bacterium, which replicates in the membrane-bound inclusion of granulocytes. Bacteria are transmitted among wild animals by the tick bite. When humans are bitten by the infected ticks, they come down with potentially fatal influenza-like illness called human granulocytic anaplasmosis (HGA, formerly human granulocytic ehrlichiosis) (Bakken *et al.*, 1994; Goodman *et al.*, 1996). *A. phagocytophilum* has the remarkable ability to parasitize first-line immune defensive cells, neutrophils and replicate in membrane-bound inclusions in the cytoplasm of the host cells by blocking the production of reactive oxygen intermediates, and avoiding the fusion with lysosomes (Webster *et al.*, 1998; Mott and Rikihisa, 2000; Rikihisa, 2000; 2003). Neutrophils typically undergo apoptosis 6 – 12 h after their release from the bone marrow (Akgul *et al.*, 2001). Yoshiie *et al.* (Yoshiie *et al.*, 2000) found that the spontaneous apoptosis of human neutrophils was delayed by *A. phagocytophilum* infection *in vitro*, allowing bacteria to complete the replication cycle in life-extended neutrophils. This anti-apoptotic phenomenon has been confirmed in the study of ovine neutrophils infected *in vivo* with a sheep *A. phagocytophilum* isolate (Scaife *et al.*, 2003). The mechanisms by which *A. phagocytophilum* inhibits the spontaneous apoptosis of human neutrophils have been partially elucidated. Ge *et al.* (Ge *et al.*, 2006) found that *A. phagocytophilum* infection
inhibits human neutrophil apoptosis by blocking both extrinsic and intrinsic apoptosis pathways: cell surface Fas clustering, cleavage of pro-caspase 8 and Bid, and translocation of Bax into mitochondria were found to be blocked. Additionally it was shown that the transcription of the gene encoding Bfl-1, an anti-apoptotic Bcl-2 family member is upregulated, mitochondrial membrane potential is maintained, and caspase 3 activation is prevented in \textit{A. phagocytophilum}-infected neutrophils (Ge et al., 2005). However, bacterial factors that contribute to the inhibition of neutrophil apoptosis have been unknown.

Ancestrally related to the conjugation system of Gram-negative bacteria, type IV secretion system (TFSS) transports macromolecules across the bacterial membrane in an ATP-dependent manner to a diverse range of bacterial and eukaryotic cells (Cascales and Christie, 2003). The TFSS is increasingly recognized as virulence factor delivery machinery by pathogens. The delivered bacterial macromolecules referred as TFSS substrates or effectors can dysregulate or modulate diverse eukaryotic target cell functions, resulting in disease development (Cascales and Christie, 2003). For example, facultative intracellular bacteria such as \textit{Legionella pneumophila}, and \textit{Bartonella henselae} secrete TFSS substrates/effectors that modulate host cell vesicular trafficking and apoptosis for their advantage (Schmid et al., 2006; Shin and Roy, 2008). As for an obligatory intracellular bacterium \textit{A. phagocytophilum}, genes encoding TFSS apparatus were identified (Ohashi et al., 2002; Hotopp et al., 2006). During the infection of human neutrophils \textit{in vitro}, both \textit{virB9} and \textit{virB6} of \textit{A. phagocytophilum} at the mRNA level, and VirB9 at the protein level are up-regulated (Niu et al., 2006). The TFSS is functional in
*A. phagocytophilum* during infection of host cells, as indicated by translocation of AnkA, a TFSS substrate of *A. phagocytophilum* (Lin et al., 2007).

*Agrobacterium tumefaciens* VirD4, a component of TFSS apparatus is called a coupling protein, as it recognizes TFSS substrate proteins at their C-terminus prior to delivery in the VirB transmembrane channel (Cascales and Christie, 2003). In the present study, we screened the *A. phagocytophilum* genomic DNA library for TFSS substrates by bacterial two-hybrid system using *A. phagocytophilum* VirD4 as bait. A hypothetical protein was identified as a putative substrate, hereby named *Anaplasma* translocation substrate 1 (Ats-1). This study showed that Ats-1, a novel mitochondria-targeting protein, directly inhibits host cell apoptosis.

**4.3 Materials and Methods**

*Anaplasma phagocytophilum and cell cultures*

*A. phagocytophilum* HZ strain was propagated in HL-60 cells (American Type Culture Collection, Manassas, VA) in complete RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (US Biotechnologies, Parkerford, PA) and 2 mM l-glutamine (Invitrogen). *A. phagocytophilum* HZ strain was also maintained in monkey endothelial cell line RF/6A (ATCC) in advanced MEM (Invitrogen), supplemented with 10% fetal bovine serum and 2 mM l-glutamine. Cultures were incubated at 37°C in a humidified 5% CO₂–95% air atmosphere. Human neutrophils were isolated by Dextran sedimentation, followed by Histopaque gradient centrifugation, and subjected to the infection with *A. phagocytophilum*, as described previously (Niu et al., 2006). *A. phagocytophilum* infection was examined with Diff-Quik
staining (Baxter Scientific Products, Obetz, OH) after centrifugation of cells onto microscope slides in a cytocentrifuge (Thermoshandon, Pittsburgh, PA). Host cell-free
*A. phagocytophilum* was prepared by sonication or nitrogen cavitation, as described elsewhere (Mott *et al.*, 2002; Niu, *et al.*, 2006). For cellular fractionation, 1.5 x 10^8 *A. phagocytophilum*-infected HL-60 cells were resuspended in 25 ml RPMI medium and subjected to nitrogen cavitation. Bacterial pellet and cytosol of infected HL-60 cells were separated after centrifugation at 10,000 x g for 10 min at 4°C. *A. phagocytophilum* was further purified from bacterial pellet by Percoll density-gradient centrifugation, as described elsewhere (Lin and Rikihisa, 2003).

**Bacterial two-hybrid system**

To make a bait, the genomic DNA was isolated from *A. phagocytophilum* purified by Percoll density-gradient centrifugation. The DNA fragment encoding *A. phagocytophilum* VirD4 (amino acid 2-740) was amplified using a pair of primer (forward primer: 5’-AGT GCG GCC GCA CAT AGT TCC AAT CAT ATA CGA AA -3’, Not I site is underlined; reverse primer: 5’-TTA CTC GAG CTA CTT TAG TCT TCC GTT ACT -3’, Xho I site is underlined) from isolated genomic DNA. High fidelity Platinum *Pfx* DNA polymerase (Invitrogen) was used for this and the following amplifications. PCR product was digested with Not I and Xho I, and ligated with Not I and Xho I-digested pBT plasmid of BacterioMatch II two-hybrid system (Stratagene, La Jolla, CA) using T4 DNA ligase (Invitrogen). Ligated products were transformed into XL1-Blue MRF’ Kan strain (Stratagene). The sequence of the insert and junctions was verified using a dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) in an ABI Prism 377 DNA sequencer (Applied Biosystems). The recombinant bait
plasmid named as pBT-VirD4 was purified using QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA).

To construct random genomic DNA library of *A. phagocytophilum*, pTRG plasmid from BacterioMatch II two-hybrid system was first modified by introducing two BstXI sites into its multiple cloning sites. Two pairs of primers were designed for introducing two BstXI sites into multiple cloning sites sequentially. After running PCR using the first pair of primers (pTRG BstXI Forward I: 5′ - CGA GGA TCC AGT GTG GTG GCG GCC GCA AGA ATT CAG TCT - 3′, BamH I and Not I sites are underlined, BstXI site is italicized; pTRG BstXI Reverse I: 5′ - CGC GGA TCC GGC CGC CTC TGG TTT CTC TT - 3′, BamH I site is underlined), PCR product was digested with BamH I and ligated itself by T4 DNA ligase, resulting in the insertion of first BstXI site between BamH I and Not I sites in pTRG vector. For the insertion of second BstXI site, PCR was performed using the second pair of primers (pTRG BstXI Forward II: 5′ - CGA CTC GAG CCA GCA CAG TGG TAA TTA ATT AAT TAA TGA ACT AGT GAG ATC - 3′, Xho I site is underlined and BstXI site is italicized; pTRG BstXI Reverse II: 5′ - CGC CTC GAG CGC CAG CTC AGA CTG AAT TC - 3′, Xho I site is underlined) and previously partially-modified pTRG plasmid as template, PCR product was digested with Xho I and ligated by T4 DNA ligase, resulting in the insertion of second BstXI site downstream of Xho I site. The two BstXI sites and their junction sequence were verified by DNA sequencing.

*A. phagocytophilum* genomic DNA of 10 µg was fragmented by nebulization (Invitrogen) according to the manufacturer’s instruction. Fragmented DNA (0.5 to 3.0 kb) was ligated with BstXI adapter (Invitrogen) after its ends were repaired by T4 DNA
polymerase (Invitrogen), and phosphorylated by T4 polynucleotide kinase (Invitrogen). BstXI-adapted DNA was ligated with BstXI-digested modified pTRG vector. Ligation product was transformed into XL1-Blue MRF’ Kan cells by electroporation, according to manufacturer’s instruction (MicroPulser Electroporator, Bio-Rad, Hercules, CA). After incubation at 30 °C for 24 h, these transformants were harvested, and recombinant plasmids were purified using Qiagen plasmid Maxi kit.

**Screening of random genomic DNA library for VirD4-interacting protein**

The mixture of 50 ng pBT-VirD4 vector and 50 ng pTRG library was electroporated into BacterioMatch II electrocompetent reporter cells (Stratagene). Transformants were screened according to the manufacturer’s instruction. Interactions were determined to be positive as measured by growth on selective screening medium consisting of minimal medium plus 5 mM 3-amino-1,2,4-triazole (3-AT), 25 ng ml⁻¹ chloramphenicol, and 12.5 ng ml⁻¹ tetracycline for 40 h (24 h at 37°C, and then 16 h at RT) and validated by growth on dual selective screening medium consisting of minimal medium plus 5 mM 3-AT and 12.5 ng ml⁻¹ streptomycin, 25 ng ml⁻¹ chloramphenicol, and 12.5 ng ml⁻¹ tetracycline, with all media prepared as outlined by the instruction manual of BacterioMatch II two-hybrid system. Self-activation controls for bait and prey were performed using pBT-VirD4 and empty prey vector, or empty bait vector and pTRG-prey.

**Expression of recombinant APH0859, antibody production, and affinity purification**

A 765 bp DNA fragment encoding the APH0859 of *A. phagocytophilum* was amplified with the following pair of primers: 5’-GTG CGC CGG CCA TGG ATA TTG GCG CCA GAA TG-3’ and 5’-TTA CTC GAG TTA CCT CGT ACC TTT ACC ATG
TG-3’; NotI and XhoI sites, respectively, are underlined. The amplified fragment was digested with NotI and XhoI and ligated into BamHI–NotI-digested pET33b (+) (Novagen, Madison, WI). The sequence of the insert and junctions was verified by DNA sequencing. *E. coli* BL21(DE3) (Novagen) was transformed with this recombinant expression vector. Cultures were grown in Luria–Bertani (LB) medium containing 34 mg ml⁻¹ kanamycin at 37°C with shaking at 250 rpm. When cultures were in the mid-logarithmic phase of growth (OD₆₀₀ value of 0.4), expression of recombinant APH0859 (rAPH0859) was induced at 25°C for 5 h by adding IPTG (isopropyl-beta-D-thiogalactopyranoside) to a final concentration of 1 mM. Bacteria were harvested by centrifugation at 10,000 x g for 10 min at 4°C and suspended in 1× His binding buffer (0.5 M NaCl, 20 mM Tris–HCl and 5 mM imidazole at pH 7.9), then sonicated on ice. The lysates were centrifuged at 14,000 x g for 20 min at 4°C. The supernatant containing the His-tagged rAPH0859 was affinity-purified on a HisBind Quick Column under non-denatured conditions as recommended by the manufacturer (Novagen). The concentration of purified rAPH0859 was measured by the BCA (bicinchoninic acid) method with a protein assay kit (Pierce, Rockford, IL). After separation of rAPH0859 by SDS-PAGE, the rAPH0859 band was excised and used to prepare antiserum in rabbits (Prosci Incorporated, Poway, CA). For affinity purification of the anti-rAPH0859 serum, 3 ml of purified rAPH0859 (4 mg ml⁻¹) dialyzed against 0.1 M MOPS, pH 7.5, was coupled to 1 ml Affi-Gel 10 (Bio-Rad) at 4°C for 4 h with gentle shaking. After removing the supernatant from Affi-gel 10, the remaining reactive esters were blocked by addition of 0.1 ml 1 M ethanolamine HCl, pH 8.0. It was then washed twice with 0.1 M MOPS, pH 7.5, and twice with elution buffer (0.1 M glycine, pH 2.5). The washed slurry was
neutralized by successive washing with 50 mM Tris-HCl, pH 7.4 until neutral pH was reached. Heat-inactivated anti-rAPH0859 serum of 2ml was diluted with 18 ml of 1 x TBS (50 Tris-HCl, pH 7.4, 150 mM NaCl), and loaded to an Econo-Column chromatography column (Bio-Rad) containing the resin. The column was washed four times with 1 x TBS, 5 ml each time. The antibody was eluted with 0.1 M glycine-HCl, pH 2.5, and neutralized by addition of 0.1 volume of 1.0 M Tris-HCl, pH 8.8. The affinity-purified rabbit polyclonal anti-rAPH0859 antibody was used for Western analysis at a dilution of 1: 1,000, and immunofluorescence-labeling at a dilution of 1: 100.

**Protein identification by mass spectrometry**

*A. phagocytophilum*-infected HL-60 cells of 1. 0 x 10$^8$ were centrifuged, washed twice with cold 1 x PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, and 2 mM KH$_2$PO$_4$ at pH 7.4), and lysed in 2.0 ml cold lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA (pH 8.0), and 1% Triton X-100, supplemented with 2% volume of protease inhibitors cocktail set III [EMD Chemicals, San Diego, CA]). The lysate was pre-cleared with 100 µl protein A-agarose (Santa Cruze Biotechnology, Santa Cruz, CA), and incubated with 50 µg affinity-purified rabbit anti-rAPH0859 antibody or rabbit pre-immune IgG for 2 h at 4 °C. The immune complex was precipitated by adding 300 µl protein A-agarose for overnight incubation. The agarose beads were washed four times with 1 x PBS, and protein was eluted by adding 100 µl 2 x SDS-PAGE loading buffer (4% SDS, 135 mM Tris–HCl [pH 6.8], 10% glycerol, and 10% β-mercaptoethanol) and boiling for 5 min. Protein was separated in SDS-PAGE, and the band with molecular mass of 48 kDa, which was specifically immunoprecipitated by anti-
rAPH0859 antibody, was excised and digested with chymotrypsin, and subjected to protein identification by mass spectrometry (Mass Spectrometry & Proteomics Facility at The Ohio State University) after fixation and staining with GelCode Blue (Pierce).

**Construction of recombinant ats-1 in mammalian expression plasmid and transfection**

All the primers used for construction of ats-1 in mammalian expression plasmid are listed in Table 4.2. For expression full-length Ats-1, PCR was performed using a pair of primers (Ats-1 Forward, and Ats-1 Reverse). To make mitochondria-targeting sequence deletion mutant, the DNA fragment was amplified using *A. phagocytophilum* genomic DNA as template and a pair of primers (Ats-1 ∆N17 Forward, and Ats-1 Reverse). To insert HA tag (YPYDVPDYA) into different sites of Ats-1 to study the cleavage site of Ats-1, several PCRs were performed sequentially (Fig. 4.2). First, Ats-1 gene was amplified as two fragments, each of which encodes N-terminus and C-terminus of Ats-1, respectively. The DNA fragment encoding N-terminal Ats-1 was amplified by a pair of primers (Ats-1 Forward and HA Reverse N), and the DNA fragment encoding C-terminal Ats-1 was amplified by another pair of primers (HA Forward C1 and Ats-1 Reverse). Both the primers, HA Reverse N and HA Forward C1, contain the sequence encoding a part of HA tag at their 5’-end to facilitate the insertion of HA tag into Ats-1. The PCR products were designated as HA N for the DNA fragment encoding N-terminal Ats-1, and HA C1 for the DNA fragment encoding C-terminal Ats-1, respectively. Second, 5’-end extended-HA C1 named as HA C2 was amplified by PCR using HA C1 as template, and a pair of primers (HA Forward C2 and Ats-1 Reverse). Third, the 5’-end of HA C2 was extended further by PCR using HA C2 as template, and a pair of primer
(HA Forward C3 and Ats-1 Reverse). This PCR product was designated as HA C3. Finally, based on the DNA sequence complementation of 3’-end of DNA fragment HA N to the 5’-end of HA C3, the two DNA fragments of HA N, and HA C3 were mixed, denatured, annealed, and extended, resulting in the DNA fragment encoding Ats-1 with a HA insertion. To substitute the amino acids between 45th – 61st of Ats-1, site-direct mutagenesis was performed by PCR. Similar to the method used above for the insertion of HA into Ats-1, two PCRs were performed using A. phagocytophilum genomic DNA as template and two pairs of primers (Ats-1 forward, and AAA (or AGA) Reverse, AAA (or AGA) Forward, and Ats-1 Reverse), resulting in the two DNA fragments encoding N- and C-termini of mutated Ats-1, respectively. Then, these two DNA fragments were mixed, denatured, annealed, and extended, resulting in the DNA fragment encoding Ats-1 with amino acid substitution. These resulting PCR products were digested with SalI and NotI, and cloned into SalI-NotI-digested pEGFP-N1 vector (Clontech), in which the DNA sequence encoding enhanced green fluorescent protein (EGFP) was removed. These resulting recombinant plasmids were named \( \text{pats-1} \) for full-length Ats-1, \( \text{pats-1-HA30} \) for HA insertion between 30th – 31st of Ats-1, \( \text{pats-1-HA45} \) for HA insertion between 45th – 46th of Ats-1, \( \text{pats-1-HA60} \) for HA insertion between 60th – 61st of Ats-1, \( \text{pats-1-HA72} \) for HA insertion between 72nd – 73rd of Ats-1, \( \text{pats-1} \) (NGL46 - 48AAA) for amino acid substitution at 46th to 48th, \( \text{pats-1} \) (MGK49 - 51AAA) for amino acid substitution at 49th to 51st, \( \text{pats-1} \) (GKP52 - 54AAA) for amino acid substitution at 52nd to 54th, \( \text{pats-1} \) (FYH55 - 57AAA) for amino acid substitution at 55th to 57th, \( \text{pats-1} \) (RAS58 - 60AGA) for amino acid substitution at 58th to 60th, and \( \text{pats-1} \) ΔN17 for mitochondria-targeting sequence deletion.
Recombinant plasmids were purified using EndoFree Plasmid Maxi Kit (Qiagen, Valencia, CA) according to the manufacturer’s instruction. Transfection of RF/6A endothelial cells with these plasmids was performed using electroporation. Briefly, RF/6A cells were washed once using 1 x PBS, and resuspended in RPMI-1640 without serum at a final cell density of $2 \times 10^7 \text{ ml}^{-1}$. RF/6A cells (80 µl) was mixed with 5 µg plasmid, and subjected to a pulse from Gene Pulser Xcell System (Bio-Rad, Hercules, CA) in 0.2-cm cuvette. The setting was 100 V, 1000 µF, which resulted in a pulse time of 50 ms.

**Time-course infection by *A. phagocytophilum***

Infection of HL-60 cells or human neutrophils with host cell-free *A. phagocytophilum* was performed, as described previously with minor modification (Niu *et al.*, 2006). Briefly, human neutrophils or HL-60 cells ($1.5 \times 10^7$) were incubated with *A. phagocytophilum* derived from $2 \times 10^7$ infected HL-60 cells in 15 ml of complete RPMI medium (approximate bacteria and host cell ratio of 100:1). After 2 h of incubation at 37°C, infected cells were washed twice with PBS to remove unbound bacteria, and then incubated at 37°C for designated time period. *A. phagocytophilum*-infected neutrophils or HL-60 cells were fixed with paraformaldehyde and were subjected to immunofluorescence labeling.

**Induction of apoptosis**

$1.6 \times 10^5$ transfected RF/6A cells growing in glass coverslip in 2 ml advanced MEM medium were treated with 100 µM etoposide (Sigma) 20 h post transfection for designated time. Etoposide-treated transfected RF/6A cells were fixed with 2%
paraformaldehyde in PBS for fluorescence microscopy to determine the cell morphology, nucleus condensation and fragmentation, and release of cytochrome $c$ into cytosol.

**Immunofluorescence microscopy**

Infected cells or transfected RF/6A cells were fixed with 2% paraformaldehyde in PBS at RT for 25 min and then washed three times in PBS. Permeabilization and blocking were performed with PGS solution (PBS containing 0.4% BSA, 0.2% gelatin and 0.3% saponin) at RT for 1 h. For the determination of Ats-1 translocation in *A. phagocytophilum*-infected HL-60 cells, double immunofluorescence labeling was performed using affinity-purified rabbit anti-rAts-1, and mAb 5C11 (anti-P44), and Alexa Fluor 555-conjugated goat anti-rabbit IgG, and Alexa Fluor-488 conjugated goat anti-mouse IgG as secondary antibody (Molecular Probes) (Kim and Rikihisa, 1998). To investigate the targeting of translocated Ats-1 to mitochondria in *A. phagocytophilum*-infected HL-60 cells, or human neutrophils, triple immunofluorescence labeling was performed using horse anti-*A. phagocytophilum* serum, affinity-purified rabbit anti-rAts-1, and mouse mAb against Mn-Sod (clone MnS-1; Alexis, San Diego, CA), and Affipure Cy3-conjugated goat anti-horse IgG (Jackson ImmunoResearch Laboratories), Alexa Fluor 488-conjugated goat anti-rabbit IgG, and Alexa Fluor 350-conjugated goat anti-mouse IgG as secondary antibody (Molecular Probes). To stain Mn-Sod or cytochrome $c$ in *ats-l*- or *gfp*-transfected RF/6A cells, cells were labeled with affinity-purified rabbit anti-rAts-1, and mouse mAb against Mn-Sod or cytochrome $c$ (clone 2G8, Santa Cluz biotechnology), and Alexa Fluor 488-conjugated goat anti-rabbit IgG, and Alexa Fluor 555-conjugated goat anti-mouse IgG as secondary antibody (Molecular Probes). For the observation of apoptosis induction in transfected RF/6A cells, nuclei of RF/6A cells were
stained 300 nM 4',6-diamidino-2-phenylindole, dilactate (DAPI, Molecular Probes) for 5 min prior to viewing under a fluorescence microscope. The permeabilized cells were incubated with primary antibodies in PGS solution for 1 h. The cells were washed with PBS three times to remove unbound antibody; the bound antibodies were detected with dye-conjugated secondary antibodies. Fluorescence images were analyzed by a Nikon Eclipse E400 fluorescence microscope with a xenon-mercury light source (Nikon Instruments, Melville, NY) or LSM 510 laser-scanning confocal microscope (Carl Zeiss, Thornwood, NY). The original colour emitted by excited Alexa Fluor 350 (blue) was transformed to gray pseudocolour for clear viewing with Photoshop 7.0 software (Adobe, San Jose, CA).

**Western blot analysis**

$2 \times 10^6$ HL-60 cells, *A. phagocytophilum*-infected HL-60 cells (infectivity > 95%) or $1.6 \times 10^6$ transfected RF/6A cells were dissolved in 100 µl of 2× SDS-PAGE loading buffer (4% SDS, 135 mM Tris–HCl [pH 6.8], 10% glycerol, and 10% β-mercaptoethanol). Samples were separated by SDS-PAGE with 10% polyacrylamide resolving gels, then transferred to a nitrocellulose membrane using a semidry blotter (WEP, Seattle, WA). The membrane was blocked using 5% (wt/vol) skim milk in Tris-buffered saline (150 mM NaCl and 50 mM Tris at pH 7.5), then incubated with affinity-purified rabbit anti-rAts-1 antibody (1:1,000 dilution), or mouse mAb anti-HA (HA.11, Clone 16B12, Covance, 1:1,000 dilution) at 4°C for 12 h, and subsequently with peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse (KPL, Gaithersburg, MD) in 1:2,000 dilution at RT for 3 h. The bound antibody was detected by
chemiluminescence (Pierce Biotechnology, Rockford, IL). The images were captured by a CCD camera (Fuji LAS-3000 imaging system).

4.4 Results

Identification of VirD4-interacting proteins

To identify potential TFSS effectors/substrates, we performed a screening of the *A. phagocytophilum* genomic library by a bacterial two-hybrid system using full-length *A. phagocytophilum* VirD4 as bait. Of 100 positive colonies sequenced, majority (65%) encoded a hypothetical protein APH0859 in *A. phagocytophilum* genome. Eight colonies encoded VirB11 (Table 4.1). The remaining colonies encoded various *A. phagocytophilum* proteins at a low frequency. No colonies grew in the selective media when empty bait vector pBT and prey, pTRG-aph0859 were used to co-transform the *E. coli* reporter strain, suggesting that the interaction between VirD4 and APH0859 in bacterial two-hybrid system was specific (Fig. 4.2). This result suggested that APH0859 is a potential substrate for TFSS of *A. phagocytophilum*. APH0859 is a 253-amino acid protein with predicted molecular mass of 27 kDa and pI 6.64 (Hotopp *et al.*, 2006). The C-terminal 20-residue of APH0859 (VTPLVSAQNRPETHGKGTR) contains a higher incidence of basic amino acids (Arg/Lys) than the remaining section of APH0859, and has a net positive charge of +2.077 at pH 7.0 (pI 10.89), a signal reported to be characteristic for substrates of *A. tumefaciens* TFSS substrates (Vergunst *et al.*, 2005) and found in the proven *A. phagocytophilum* TFSS substrate, AnkA (Lin *et al.*, 2007). The interaction between *A. phagocytophilum* VirD4 and *A. phagocytophilum* VirB11 suggested that *A. phagocytophilum* VirD4 may usher TFSS substrates to *A. phagocytophilum*. 

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*phagocytophilum* VirB11 prior to delivery to the *A. phagocytophilum* core TFSS apparatus as demonstrated in *A. tumefaciens* for delivery of T-DNA (Cascales *et al.*, 2004).

**Translocation of APH0859 into the cytoplasm of HL-60 cells by *Anaplasma phagocytophilum***

The DNA fragment encoding full-length APH0859 of 253 amino acids (GenBank Accession No, YP_505436) was cloned into pET-33b (+) vector and expressed as a recombinant protein in *E. coli*. Rabbits were immunized with the purified recombinant protein (Fig. 4.3A). Monospecific antibody against recombinant APH0859 (rAPH0859) was affinity-purified by using rAPH0859-conjugated Affil-gel 10 agarose. Western blot analysis showed one major band of approximately 48 kDa, and one minor band of approximately 35 kDa in *A. phagocytophilum*-infected HL-60 cells by using anti-rAPH0859 antibody, whereas no bands were detected in uninfected HL-60 cells (Fig. 4.3B). Since APH0859 had a larger mass in Western blot analysis than expected molecular mass (27.0 kDa), the open read frame (ORF) of *aph0859* was reanalyzed. The newly defined ORF of *aph0859* encodes a protein of 376 amino acids (expected molecular mass 40.3 kDa), and the most upstream ATG was designated as the translational start site. The new ORF was supported by several evidences. First, when the protein with molecular mass of 48 kDa in SDS-PAGE was immunoprecipitated from the lysate of *A. phagocytophilum*-infected HL-60 cells with anti-rAPH0859 antibody, and subjected to protein identification by mass spectrometry, a peptide which only matched the new ORF of APH0859 was detected, in addition to several peptides which belong to previously annotated APH0859 (Fig. 4.3C). Second, when the new ORF was expressed
in *E. coli* as a recombinant protein without plasmid-derived peptide, it showed the same molecular mass as native APH0859 in SDS-PAGE (data, not shown). Third, when the new ORF sequence was aligned with its ortholog, AM410 (predicted molecular mass of 43 kDa) in the genome of *Anaplasma marginale* (GenBank Accession No. YP_153722), the N-terminal region of newly defined APH0859 (1-123) shares a high similarity to that of AM410 (E value is 1.2e-09). The 48-kDa protein encoded by this newly defined *aph0859* ORF was named as *Anaplasma* translocation substrate 1 (Ats-1). It is currently unknown why Ats-1 with expected molecular mass of 40.3 kDa showed as the band of approximately 48 kDa in SDS-PAGE.

To determine the translocation of Ats-1 from *A. phagocytophilum* into host cell cytoplasm across the inclusion and bacterial membranes, immunofluorescence-labeling was performed. P44 is the major outer membrane protein of *A. phagocytophilum* (Zhi *et al.*, 1998). Monoclonal anti-P44 antibody 5C11 (Kim and Rikihisa, 1998; Wang *et al.*, 2006) was used to localize the *A. phagocytophilum*, and rabbit anti-rAts-1 antibody was used to localize the substrate. In the time-course infection experiment, Ats-1 translocation was not detected by double immunofluorescence-labeling by 22 h post-infection (p.i.), but the translocation was clearly observed at 32 h p.i. in HL-60 cells (Fig. 4.3D).

**Localization of Ats-1 with mitochondria**

We found that Ats-1 has a mitochondrial localization signal at its N-terminus based on two prediction programs: MitoProt (http://ihg.gsf.de/ihg/mitoprot.html) and Predotar (http://urgi.versailles.inra.fr/predotar/predotar.html) (Fig. 4.3C) (Claros and Vincens, 1996; Small *et al.*, 2004). Therefore, we examined whether translocated Ats-1 localizes with mitochondria in *A. phagocytophilum*-infected HL-60 cells and human
neutrophils by triple immunofluorescence labeling. Horse anti-*A. phagocytophilum* serum and Cy3-conjugated goat anti-horse IgG were used to label *A. phagocytophilum*; rabbit anti-rAts-1 antibody and Alexa Fluor 488-conjugated goat anti-rabbit IgG were used for Ats-1 labeling; and mouse monoclonal anti-manganese superoxide dismutase (Mn-Sod), a mitochondrial marker (Ge *et al.*, 2006), and Alexa Fluor 350-conjugated goat anti-mouse IgG were used for labeling mitochondria as reported previously. The translocated Ats-1, which did not colocalize with *A. phagocytophilum*, was found to colocalize with mitochondria (Fig. 4.4A and B). This result suggested that Ats-1 is targeted to mitochondria after translocation into host cell cytoplasm.

To determine whether translocation to the mitochondria is the intrinsic activity of Ats-1, *ats-1* was cloned into a mammalian expression vector, and used to transfect monkey endothelial cell RF/6A. Endothelial cells can be infected with *A. phagocytophilum* in vitro and in vivo (Munderloh *et al.*, 2004; Herron *et al.*, 2005). By double immuno-fluorescence labeling, Ats-1 was found to be expressed and colocalize with Mn-Sod in *ats-1*-transfected RF/6A cells, indicating Ats-1, in the absence of bacteria, can target to the mitochondria (Fig. 4.4C).

**Cleavage of Ats-1 in mitochondria**

Because the smaller band of approximately 35 kDa was detected in *A. phagocytophilum*-infected HL-60 cells (Fig. 4.3B), and the presequence containing N-terminal mitochondria-targeting sequence normally is cleaved from the precursor protein after translocation into mitochondrial matrix (Omura, 1998), we examined whether Ats-1 expressed by *A. phagocytophilum* as a full-length precursor protein that was subsequently cleaved in the mitochondria. Only a single band of 48 kDa was detected in *A.
phagocytophilum purified by Percoll density-gradient centrifugation. However, one additional band of 35 kDa was detected in the cellular fraction containing A. phagocytophilum and mitochondria (Fig. 4.5A). This result suggests that Ats-1 is translated in full-length (48 kDa) in A. phagocytophilum, and is cleaved by a protease to 35 kDa after translocation. To determine whether Ats-1 was cleaved by the host cell protease in the absence of A. phagocytophilum in transfected endothelial cells, RF/6A cells transfected with ats-1 were subjected to Western blot analysis. Majority of expressed Ats-1 was cleaved in transfected RF/6A cells (Fig. 4.5B). One extra band of larger molecular mass (65 kDa) was detected with anti-rAts-1 in Western blot analysis, suggesting Ats-1 formed a complex in transfected RF/6A cells. To determine whether the N-terminal sequence of Ats-1 is essential for mitochondrial localization and cleavage, N-terminal amino acid sequence of Ats-1 was deleted. Deletion of the N-terminal 17 amino acids abrogated mitochondrial localization as well as the Ats-1 cleavage (Fig. 4.5C and D). These results indicate that the N-terminal sequence is required for Ats-1 mitochondria targeting and cleavage.

The cleavage site of Ats-1

To determine the cleavage site in Ats-1, a haemoagglutinin (HA) tag was inserted into Ats-1 at serial locations (Fig. 4.6A). Since the molecular mass of the cleaved Ats-1 was approximately 35 kDa, the HA tag was inserted into Ats-1 at one of four sites (between 30\textsuperscript{th} - 31\textsuperscript{st}, 45\textsuperscript{th} – 46\textsuperscript{th}, 60\textsuperscript{th} – 61\textsuperscript{st}, or 72\textsuperscript{nd} – 73\textsuperscript{rd}), and Western blot analysis was performed on transfected RF/6A cells using anti-Ats-1 and anti-HA. The C-terminal cleaved product of two mutants (Ats-1-HA30, Ats-1-HA45) lost HA tag, but the C-terminal cleaved product from the other two mutants (Ats-1-HA60, Ats-1-HA72) retained...
HA tag (Fig. 4.6B), indicating that the cleavage site of Ats-1 was between 45\textsuperscript{th} – 60\textsuperscript{th} amino acid of Ats-1. Of note, the larger band (66 kDa) from Ats-1-HA30 and Ats-1-HA45 was not reacted with anti-HA antibody, but it was detected in Ats-1-HA60 and Ats-1-HA72 with anti-HA antibody, suggesting that the 66 kDa complex was formed by the cleaved C-terminal peptide, but not by the precursor protein.

**FYH at 55\textsuperscript{th}-57\textsuperscript{th} amino acid of Ats-1 is important for cleavage, but not essential for targeting**

To determine which amino acids between 45\textsuperscript{th} – 60\textsuperscript{th} are important for cleavage, this region of Ats-1 were sequentially substituted with AAA or AGA by the order of 3 amino acids (Fig. 4.6C). RF/6A cells were transfected with these *ats-1* mutants encoding different substitutions, and subjected to Western blot analysis using affinity-purified anti-rAts-1 (Fig. 4.6D). Among five mutants, *ats-1* mutation (FYH55-57AAA) greatly inhibited the cleavage. To determine whether this substitution is essential for Ats-1 targeting to mitochondria, *ats-1* (FYH55-57AAA)-transfected RF/6A cells were subjected to immunofluorescence labeling using affinity-purified anti-rAts-1. Ats-1 staining still showed the mitochondrial pattern (Fig. 4.6E). This result suggests that the FYH at 55\textsuperscript{th} – 57\textsuperscript{th} of Ats-1 is important for cleavage, but not essential for targeting. The diagram of mitochondrial targeting sequence (MTS) location and the possible cleavage site for presequence in Ats-1 is summarized in Fig. 4.6A.

**Ats-1 blocked induced apoptosis**

Mitochondria play a critical role in regulation of apoptosis, and loss of mitochondrial membrane potential is delayed by *A. phagocytophilum* infection of human neutrophils (Ge *et al.*, 2005). As Ats-1 targeted to mitochondria, the effect of Ats-1 on
host cell apoptosis was determined. When apoptosis was induced in RF/6A cells by etoposide, a topoisomerase II inhibitor (Robertson et al., 2002), ats-1-transfected cells inhibited this induced apoptosis. 50% and 40% GFP-expressing RF/6A cells went to apoptosis at 1 day and 3 day post treatment (p.t.) by etoposide treatment, respectively (Fig. 4.7B and C), as determined by rounded cell morphology, condensed nuclei, and release of cytochrome c into the cytosol. In contrast, less than 25% of Ats-1-expressing RF/6A cells were apoptotic at 1 day and 3 day p.t. (Fig. 4.7A and C).

4.5 Discussion

The present data identified a bacterial secreted protein that has a eukaryotic mitochondria targeting structure, translocates to mitochondria, and inhibits induced-apoptosis of the host cells by stabilizing the mitochondrial membrane integrity. Apoptosis can be viewed as a three-stage process: initiation, decision/commitment and degradation/execution (Boya et al., 2001). During the initiation phase, different pro-apoptotic signal transduction pathways are activated and finally act on mitochondria to increase membrane permeability. During the decision or commitment phase, mitochondrial membrane permeabilization (MMP) occurs, leading to release of death-promoting molecules, such as cytochrome c from intermembrane space, and dissipation of the mitochondrial transmembrane potential ($\Delta\psi_m$) across inner membrane. The degradation or execution phase is triggered by the formation of apoptosome with cytochrome c, subsequently activating caspases (apoptosis-specific cysteine proteases) and nucleases. The present study suggests that Ats-1 inhibited MMP in the decision or commitment phase as suggested by the inhibition of cytochrome c release from
mitochondria in apoptosis-induced RF/6A cells. This is in agreement with the report by Ge et al. (2005) showing the inhibition of loss of $\Delta \psi_m$ in spontaneous neutrophil apoptosis by *A. phagocytophilum* infection.

*A. phagocytophilum* infection inhibits human neutrophil apoptosis by blocking extrinsic and intrinsic pathways. In addition to the maintenance of mitochondrial membrane potential, Fas clustering is also blocked, which is initial step for apoptosis in extrinsic pathways. Because Ats-1 targets mitochondria, the organelle acting downstream in both extrinsic and intrinsic pathways, there may be other anti-apoptotic factors produced by *A. phagocytophilum*. Having redundant ability to inhibit apoptosis is important for some pathogens to prevent the premature death of host cells, as revealed by many viruses which encode multiple proteins to inhibit apoptosis induced by different stimulators (Dobbelstein and Shenk, 1996; Guo et al., 2005; Stewart et al., 2005). One common way used by virus to inhibit host cell apoptosis is to encode a viral mimic of host cell pro-apoptotic protein Bcl-2, as revealed in Murine $\gamma$-herpesvirus 68 ($\gamma$HV-68) (Cuconati and White, 2002). In addition to the viral Bcl-2, which contributes to latency establishment, $\gamma$HV-68 also encodes another anti-apoptotic protein, vMAP. vMAP localizes to host cell mitochondria through its N-terminal mitochondria-targeting sequence, and exerts its anti-apoptotic activity by interacting with Bcl-2, and voltage-dependent anion channel 1 (VDAC1) (Feng et al., 2007). VDAC was conceived to form the mitochondrial permeability transition pore (mPTP) with inner membrane protein, adenine nucleotide translocase (ANT), matrix protein, cyclophilin D, and other proteins (Crompton, 1999). Ats-1 was also targeted to mitochondria and inhibited apoptosis, and whether it also interacts with mPTP is under investigation.
Most mitochondrial proteins are encoded in the nucleus and synthesized by ribosomes in cytoplasm as precursor proteins with N-terminal presequences that are essential for targeting to mitochondria (Omura, 1998). The presequence is cleaved off from proproteins by matrix-located mitochondrial processing peptidase (MPP) after they are imported into the mitochondrial matrix. Presequence varies in length, and shares little similarity in primary sequence. However it has a common characteristic feature, the high content of positively charged residues and the capacity to form an amphiphilic α-helix (Omura, 1998). One common feature for cleavage site is a proximal basic amino acid residue, usually arginine, at the -2 position (Hendrick et al., 1989). The cleavage site of Ats-1 is between 46th to 60th of Ats-1. After carefully reviewing the sequence in this region (NGLMGKGPYHRAS) and its flanking sequence, we speculate that the cleavage site is between 59th and 60th, as arginine is present at 58th (-2 position).

Of note, a protein complex composed of cleaved Ats-1 was detected in _ats-1_-transfected RF/6A cells, but not detected in _A. phagocytophilum_-infected HL-60 cells. The failure to detect this protein complex in infected HL-60 cells may be due to limited amount of protein complex or lack of formation of protein complex by cleaved Ats-1. Several mitochondrial inner membrane proteins form homodimers, such as mitochondrial ATP synthase (Minauro-Sanmiguel et al., 2005). However, biological significance of the Ats-1 complex is currently unknown.

To show that Ats-1 can be secreted in VirB/D-dependent manner into eukaryotic host cells, Cre recombinase reporter assay for translocation (CRAfT) was performed (Hua Niu, Amke den Dulk-Ras, Paul J. J. Hooykaas, and Yasuko Rikihisa, unpublished data). Although CRAfT has been used successfully to demonstrate _A. tumefaciens_ VirD4-
dependent translocation of AnkA from *A. tumefaciens* to root explants of *Arabidopsis thaliana* (Lin *et al.*, 2007), we are not so far able to demonstrate *A. tumefaciens* VirD4-dependent Ats-1 translocation. As CRAfT requires translocation of Cre-Ats-1 fusion protein to the plant cell nucleus, it is possible some signals present within Ats-1 molecule prevent it from nuclear translocation as indicated in the recent study that was unable to demonstrate the translocation of *B. henselae* BepA by CRAfT (Schmid *et al.*, 2006).

Nonetheless, the Ats-1 was identified to bind to *A. phagocytophilum* VirD4, have the mitochondria-targeting sequence at N-terminus, and demonstrated to localize in the mitochondria of host cells after translocation. Ats-1 alone without any other *A. phagocytophilum* components can inhibit etoposide-induced apoptosis in RF/6A cells by maintaining mitochondrial membrane integrity. There are a number of bacteria that inhibit apoptosis of eukaryotic cells by different mechanisms (Faherty and Maurelli, 2008). *Neisseria gonorrhoea* and *Neisseria meningitides* porins are first bacterial proteins known to target to the host cell mitochondria and induce or inhibit the host cell apoptosis, although how porins are translocated across the host membrane to the mitochondria remains unknown (Massari *et al.*, 2000; Muller *et al.*, 2000). *Chlamydia* secretes chlamydial proteasome-like activity factor (CPAF), a protease to degrade pro-apoptotic proteins with a BH3 domain (Fischer *et al.*, 2004).

To our knowledge, Ats-1 is the first bacterial mitochondria-targeting protein related to TFSS. Discovery of the apoptosis inhibition by Ats-1 may help us understand the unique anti-apoptotic mechanism in *A. phagocytophilum* pathogenesis. In the future, vaccines or drugs designed to target *A. phagocytophilum* anti-apoptotic signaling in
neutrophils may prevent this organism from proliferating in its host cells and eventually control the disease of HGA.
<table>
<thead>
<tr>
<th>Protein name</th>
<th>GenBank accession number</th>
<th>Total amino acid number</th>
<th>Colony number</th>
<th>Interacting region</th>
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<tr>
<td>Hypothetical protein</td>
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<td>253</td>
<td>65</td>
<td>From 9\textsuperscript{th} to 253\textsuperscript{rd}</td>
</tr>
<tr>
<td>(APH0859)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VirB11</td>
<td>YP_505895</td>
<td>332</td>
<td>8</td>
<td>From 210\textsuperscript{th} to 332\textsuperscript{nd}</td>
</tr>
</tbody>
</table>

Table 4.1. Proteins identified by bacterial two-hybrid system using *A. phagocytophilum* VirD4 as bait

100 positive colonies in bacterial two-hybrid screening were subjected to sequencing. Two of identified proteins are listed in table 4.1, including protein name, GenBank accession number, total amino acid number, colony number in 100 colonies, and interacting region.
<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ats-1 Forward</td>
<td>5’-GCAGTCGACGCCACCATGGTGCTAATAAGAAGAATTCTGAC-3’</td>
<td>SalI site is underlined</td>
</tr>
<tr>
<td>Ats-1 Reverse</td>
<td>5’-AGTGGGCGCCGCTTACCTCGTACCTTTACCATGTG-3’</td>
<td>NotI site is underlined</td>
</tr>
<tr>
<td>30 – 31 HA</td>
<td>5’-CATCGTATGGGTAGCTAGTATTTTCAGTGCTGG-3’</td>
<td>Sequence encoding HA tag is italicized</td>
</tr>
<tr>
<td>Reverse N</td>
<td>5’-CATCGTATGGGTAGCTAGTATTTTCAGTGCTGG-3’</td>
<td>Sequence encoding HA tag is italicized</td>
</tr>
<tr>
<td>30 – 31 HA</td>
<td>5’-CCAGATTACGCTGCGGAGAACCTCAAGAAATCTC-3’</td>
<td>Sequence encoding HA tag is italicized</td>
</tr>
<tr>
<td>Forward C1</td>
<td>5’-CATCGTATGGGTAGCTAGTATTTTCAGTGCTGG-3’</td>
<td>Sequence encoding HA tag is italicized</td>
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<tr>
<td>30 – 31 HA</td>
<td>5’-TACCCATAAGCTTCCAGATTACGCTGCGGAGAACCTCAAGAAATCTC-3’</td>
<td>Sequence encoding HA tag is italicized</td>
</tr>
<tr>
<td>Forward C2</td>
<td>5’-CATCGTATGGGTAGCTAGTATTTTCAGTGCTGG-3’</td>
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<tr>
<td>30 – 31 HA</td>
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<tr>
<td>Forward C3</td>
<td>5’-CATCGTATGGGTAAAGAATGGCATGGGTAAAGGA-3’</td>
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<td>45 – 46 HA</td>
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<td>45 – 46 HA</td>
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Table 4.2. Oligonucleotide primers used for amplification of different Ats-1 gene fragments by PCR (Continued)
Table 4.2: Continued

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<thead>
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<tr>
<td>45 – 46 HA Forward C3</td>
<td>5’- ( \text{CAGAAATTCTTTTACCCATACGATTTCCAGAT} ) -3’</td>
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<tr>
<td>60 – 61 HA Reverse N</td>
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<td>60 - 61 HA Forward C1</td>
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<td>60 - 61 HA Forward C2</td>
<td>5’- ( \text{TCACACGATTTTCCAGATTACCAGCTGAGATGCAG} ) -3’</td>
<td>Sequence encoding HA tag is italicized</td>
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<tr>
<td>60 - 61 HA Forward C3</td>
<td>5’- ( \text{CCATCGCGCTTCTTTACCCATACGATTTCCAGAT} ) -3’</td>
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<tr>
<td>72 – 73 HA Reverse N</td>
<td>5’- ( \text{CGTATGGGTAGCCCCCTCTTTATCCCAGG} ) -3’</td>
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<td>72 - 73 HA Forward C1</td>
<td>5’- ( \text{CCAGATTACGCTACAAAAATAAGTTTCGCCACTATGC} ) -3’</td>
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(Continued)
Table 4.2: Continued

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<th>Sequence</th>
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<td>5' -</td>
<td>encoding HA tag is italicized</td>
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<tr>
<td>72 - 73 HA Forward C3</td>
<td>5'-AAGAGAGGGGCTACCCATACGATGGTCCAGA-3'</td>
<td>3'</td>
<td>encoding HA tag is italicized</td>
</tr>
<tr>
<td>46 – 48 AAA Forward</td>
<td>5' -</td>
<td>TTCTTTGCTGCTGCTATGGTGAAAGGAAAGCCTTTTA-3'</td>
<td>5' -</td>
</tr>
<tr>
<td>46 – 48 AAA Reverse</td>
<td>5' -</td>
<td>ACCCATAGCAGCAGCAAAGAAATTTCCCTGTGTAC</td>
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<tr>
<td>49 – 51 AAA Forward</td>
<td>5' -</td>
<td>GGCCTCGCTGCTGCTGGAAGGCCTTTTACCATCG</td>
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<tr>
<td>49 – 51 AAA Reverse</td>
<td>5' -</td>
<td>CTTCCAGCAGCAGCAGGCCATTAAAGAAATTTCC</td>
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(Continued)

115
<table>
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<tr>
<th>Region</th>
<th>5’- Sequence 3’</th>
<th>Underlined Sequence for Amino Acid Substitution</th>
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<td>52 – 54 AAA Forward</td>
<td>GGTAAGCTGCTGCTTTTTACCATCGCGCTTCTGA</td>
<td>5’- G-3’</td>
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<tr>
<td>52 – 54 AAA Reverse</td>
<td>GCTGCTGCTTTTTACCATCGCGCTTCTGA</td>
<td>5’- AG-3’</td>
</tr>
<tr>
<td>55 – 57 AAA Forward</td>
<td>AAGCCTGCTGCTGCTCGCGTTCTGAGATGCAGAA</td>
<td>5’- T-3’</td>
</tr>
<tr>
<td>55 – 57 AAA Reverse</td>
<td>AGCGCGAGCAGCAGCAGCGAGCGCTGCTGCTTTTACCATGAGGCATTAA</td>
<td>5’- AG-3’</td>
</tr>
<tr>
<td>58 – 60 AGA Forward</td>
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(Continued)
Table 4.2: Continued

| Ats-1 ΔN17 Forward | 5’- GCAGTCGACGCCACCATGGCGCATTGTTTCTGG ATTC-3' | SalI site is underlined |
Figure 4.1. The diagram shows the procedure how to insert HA tag into Ats-1.

First step, Ats-1 gene was amplified as two fragments, each of which encodes N-terminus and C-terminus of Ats-1. The DNA fragment encoding N-terminal Ats-1 was amplified by a pair of primers (Ats-1 Forward N and HA Reverse N), and the DNA fragment encoding C-terminal Ats-1 was amplified by another pair of primers (HA Forward C1 and Ats-1 Reverse). Both the primers, HA Reverse N and HA Forward C1, contain the sequence encoding a part of HA tag at their 5’-end. The PCR products were designated as HA N for the DNA fragment encoding N-terminal Ats-1, and HA C1 for the DNA fragment encoding C-terminal Ats-1, respectively.

Second step, HA C2 was amplified by PCR using HA C1 as template, and a pair of primers (HA Forward C2 and Ats-1 Reverse).

Third step, the 5’-end of HA C2 was extended further by PCR using HA C2 as template, and a pair of primer (HA Forward C3 and Ats-1 Reverse). This PCR product was designated as HA C3.

Fourth step, based on the DNA sequence complementation of 3’-end of DNA fragment HA N to the 5’-end of HA C3, the two DNA fragments of HA N, and HA C3 were mixed, denatured, annealed, and extended, resulting in the DNA fragment encoding Ats-1 with a HA insertion.

The black bars show the DNA sequence encoding Ats-1, Red, yellow and purple bars show the DNA sequence encoding HA tag.
Figure 4.1

[Diagram of molecular biology process showing PCR steps for Ats-1 and HA vectors.]
Figure 4.2. *A. phagocytophilum* proteins that bind to *A. phagocytophilum* VirD4.

Confirmation of specific interaction between *A. phagocytophilum* VirD4 and APH0859 identified by bacterial two-hybrid system using *A. phagocytophilum* VirD4 as bait. The *E. coli* reporter strain was co-transformed with empty bait vector, or pBT-*virD4* plasmid with *aph0859* plasmid, and incubated in selective media.
Figure 4.2

Empty bait pBT + aph0859

pBT-virD4 + aph0859
Figure 4.3. Expression of recombinant APH0859, new definition of *aph0859* ORF, and translocation of Ats-1 from *A. phagocytophilum* into cytoplasm of HL-60 cells.

**A.** Truncated rAts-1 (old APH0859 ORF) expressed in *E. coli* was purified by immobilized Ni\(^{2+}\) affinity chromatography, separated by SDS-PAGE, and stained with Coomassie Brilliant blue. Lanes: M, protein molecular size marker; rAts-1, recombinant truncated *A. phagocytophilum* Ats-1. Arrow indicates rAts-1.

**B.** Western blot analysis was performed using affinity-purified rabbit antibody against *A. phagocytophilum* rAts-1. Lanes: HL-60, HL-60 cells; Anaplasma: *A. phagocytophilum*-infected HL-60 cells. Arrows 1 and 2 indicate full-length native Ats-1 and cleaved Ats-1, respectively.

**C.** The amino acid sequence deduced from newly defined *aph0859* ORF (376 amino acids). The amino acid sequences identified in mass spectrometry are highlighted with bold letters. Mitochondria-targeting sequence (MTS) of N-terminal 17 aa predicted by Mitoprot program (Claros and Vincens, 1996), is indicated with shaded area in bold letters. Bent arrows 1 and 2 indicate, newly defined and previously predicted translational start site, respectively.

**D.** Double immunofluorescence labeling of Ats-1 in *A. phagocytophilum*-infected HL-60 cells. Uninfected HL-60 cells, *A. phagocytophilum*-infected HL-60 cells at 22 or 32 h post infection were double immunofluorescence-labeled with mouse mAb anti-P44 (P44; Alexa Fluor 488, green) and affinity-purified rabbit anti-rAts-1 (Ats-1; Alexa Fluor 555, red). Merged image with phase contrast micrograph (Merge). N: nucleus. Arrowhead indicates Ats-1 translocated to the cytoplasm of host cells. Bar: 5 µm.
Figure 4.3

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C

Newly defined translational start site

MLIRRLTTSRNVAARIVSFGTAPENTSARTSRNLGLTTCGNFFNG
LMGKIGKPYHRASEMQLNPWTDKERGTKISSHYAQTGQLVLQIGD

Previously predicted translational start site

GRVSEGALQMLEGALDDSVGELDPSSKGLNPGMDIGARMHDHNRAKNECGALLDLRKKLEETGGKISVERTGDGFRMLVIKIDTKNKESEEEVEKEVQLVLGLTGVSGLKILAKSAKIELMHQAKTDMNALAPVSHTPAQEKPDSDIQENSEKSAADAKNSQADQVQNEENSREDTTRRNSTTNGEERIFLSGDASPSRPSSGAGTDQAVQQAHAFLRDSEDRVHGSGITNOQGAMMQAVLSAARGLSVDSHDSSAQTQGPNPTVTPLVSAQRGPRPETHGKGTTR*

D

P44

Uninfected HL-60

22 h post infection

32 h post infection

AlS-1

Merge

N

N

N

N
Figure 4.4. Ats-1 localizes in mitochondria of host cells.

**A and B.** Triple immunofluorescence labeling of *A. phagocytophilum*-infected HL-60 cells (A) and human neutrophils (B) using horse anti-*A. phagocytophilum* (Anaplasma; Cy3, red), rabbit anti-rAts-1 (Ats-1; Alexa Fluor 488, green), mAb anti-Mn-Sod (Mn-Sod; Alexa Fluor 350, white pseudocolor). Arrowheads indicate Ats-1 colocalized with mitochondria. Bar: 5 µm.

**C.** Confocal double immunofluorescence labeling of *ats*-1-transfected RF/6A cells using rabbit anti-Ats-1 (Ats-1; Alexa Fluor 488, green), mAb anti-Mn-Sod (Mn-SOD; Alexa Fluor 555, red). Bar: 10 µm.
Figure 4.4

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Figure 4.5. Requirement of N-terminal sequence of Ats-1 in targeting mitochondria and cleavage in host cells.

A. Cellular fractionation was performed for *A. phagocytophilum*-infected HL-60 cells. *A. phagocytophilum*-infected HL-60 cells were disrupted by nitrogen cavitation. *A. phagocytophilum* and mitochondria-containing pellet (Pellet) was obtained by centrifugation at 10,000 x g. *A. phagocytophilum* [AP (Percoll)] was further purified by Percoll density-gradient centrifugation from Pellet. Western blot analysis was performed for Pellet and AP (Percoll) using affinity-purified rabbit anti-Ats-1. Arrows 1 and 2 indicate full-length native Ats-1 and cleaved Ats-1, respectively.

B. Cleavage of Ats-1 in *ats-1*-transfected cells.

RF/6A cells were transfected with the *ats-1* plasmid, and subjected to Western blot analysis using affinity-purified rabbit anti-rAts-1.

Anaplasma, *A. phagocytophilum*-infected HL-60 cells. Ats-1, *ats-1*-transfected RF/6A cells. Arrows 1 and 2 indicate full-length Ats-1 and cleaved Ats-1, respectively.

A band with larger molecular mass (65 kDa) in the lane of *ats-1*-transfected RF/6A cells is highlighted with an arrowhead.

C. No cleavage of Ats-1 in *ats-1ΔN17*-transfected RF/6A cells. RF/6A cells were transfected with the *ats-1ΔN17* plasmid, and subjected to Western blot analysis using affinity-purified rabbit anti-rAts-1. Arrow 1 indicates full-length Ats-1. No smaller band was detected.

D. Immunofluorescence-labeling of RF/6A cells transfected with *ats-1ΔN17*, using affinity-purified rabbit anti-rAts-1. Note diffuse distribution of Ats-1. Bar: 10 µm
Figure 4.5

A

Pellet AP (Percoll)

kDa

50.0
37.0

1
2

B

Anaplasma Ats-1

kDa

75.0
50.0
37.0

1
2
3

C

Ats-1ΔN17

kDa

50.0
37.0

1

D

Ats-1ΔN17

N
Figure 4.6. Cleavage site of Ats-1 and determination of amino acids important for cleavage.

A. The diagram shows the site of HA insertion in Ats-1, and cleavage products containing HA tag. The HA tag was inserted between 30\textsuperscript{th}-31\textsuperscript{st}, 45\textsuperscript{th}-46\textsuperscript{th}, 60\textsuperscript{th}-61\textsuperscript{st}, and 72\textsuperscript{nd}-73\textsuperscript{rd} amino acid of Ats-1. MTS sequence is shown as white bar. The cleavage site is indicated at the end of solid gray bar.

The result of Western blot analysis in Fig. 4.5\textbf{B} is also summarized.

B. RF/6A cells were transfected with recombinant plasmids encoding Ats-1 with HA insertion at different sites. Western blot analysis was performed for these transfectants using affinity-purified rabbit anti-rAts-1 or mAb anti-HA.

C. The amino acid sequence between 46\textsuperscript{th}-60\textsuperscript{th} of Ats-1 was sequentially substituted with AAA or AGA.

D. RF/6A cells were transfected with recombinant plasmids encoding Ats-1 with different amino acid substitution as shown in C, and subjected to Western blot analysis using affinity-purified rabbit anti-rAts-1. Cleavage was greatly inhibited only with Ats-1 (FYH55-57AAA).

E. Immunofluorescence-labeling of RF/6A cells transfected with \textit{ats-1} (FYH55-57AAA), using affinity-purified rabbit anti-rAts-1, and mose anti-Mn-Sod. Note mitochondrial localization of Ats-1. Bar: 10 \(\mu\text{m}\)
Figure 4.7. Ats-1 inhibits etoposide-induced apoptosis in *ats-1*-transfected RF/6A cells.

**A.** Triple fluorescence labeling of *ats-1* (Ats-1)-, or *gfp* (GFP)-transfected RF/6A cells at 3 days post treatment with etoposide with affinity-purified rabbit anti-rAts-1, mAb anti-cytochrome *c*, and DAPI. Bar: 10 µm.

**B.** Percentage of apoptotic cells in Ats-1- or GFP-expressing RF/6A cells at 1 or 3 days post treatment with etoposide. Data are presented as means and standard deviations of triplicate samples. The asterisk indicates a significant difference compared with GFP-expressing RF/6A cells by Student’s *t*-test (*P* < 0.01) within each group at each time point.
Figure 4.7
CHAPTER 5

DEVELOPMENT OF A REPORTER SYSTEM FOR DETECTING TYPE IV SECRETION SUBSTRATE TRANSLOCATION

5.1 Introduction

Anaplasma phagocytophilum, is an obligatory intracellular Gram-negative bacterium, which replicates in the membrane-bound inclusion of granulocytes. Bacteria are transmitted among wild animals by the tick bite. When humans are bitten by the infected ticks, they come down with severe influenza-like illness called human granulocytic anaplasmosis (HGA, formerly human granulocytic ehrlichiosis) (Bakken et al., 1994; Goodman et al., 1996). A. phagocytophilum has the remarkable ability to parasitize first-line immune defensive cells, neutrophils and replicate in membrane-bound inclusions in the cytoplasm of the host cells by blocking the production of reactive oxygen intermediates, inhibiting neutrophil apoptosis, recruiting cholesterol to A. phagocytophilum inclusions, and avoiding the fusion with lysosomes (Webster et al., 1998; Mott et al., 1999; Mott and Rikihisa, 2000; Lin and Rikihisa, 2003; Rikihisa, 2000; 2003). It is expected that the proteins secreted from A. phagocytophilum into host cells are responsible for some of these phenomena. Genes encoding several kinds of export or secretion systems were identified in the A. phagocytophilum genome, including Sec-dependent pathway, twin-arginine translocation (TAT) pathway, type I secretion system,
and type IV secretion system (Hotopp et al., 2006). More than five protein secretion systems were found in the gram-negative bacteria. Base on the dependence on the Sec apparatus in the inner membrane (IM), they are grouped into Sec-dependent, or independent pathway. Sec-dependent pathway exports unfolded substrates to periplasm or IM by Sec apparatus in ATP-dependent manner as the first step (Desvaux et al., 2004). Signal peptide is required to be present in the substrate in Sec-dependent pathway. TAT pathway exports folded proteins from the cytoplasm across IM in Sec-independent manner (Lee et al., 2006). Three integral membrane proteins, one from each of the TatA, TatB, and TatC families are the minimal set of components required for Tat translocation in Escherichia coli (Lee et al., 2006). All the three components were found to be encoded in the A. phagocytophilum genome, suggesting that A. phagocytophilum may have a functional TAT secretion system (Hotopp et al., 2006). Type I secretion system transports the substrates directly from the cytoplasm to the extracellular medium, without periplasmic intermediate. The secretion apparatus spans IM and outer membrane (OM) through a complex composed of ABC transporter, a membrane fusion protein (MFP), and an outer membrane protein (OMP). The signal sequences for type I secretion system are found in the C terminus and remain uncleaved upon export (Delepeleaire 2004). Without periplasmic intermediate in the most cases, type IV secretion system transports substrates directly from cytoplasm to extracellular medium. The signal sequences also lie in the C-termini, and remain uncleaved after translocation. The type IV secretion apparatus is composed of multiple components (Christie et al., 2005). However, only type IV secretion system in the secretion system encoding in A. phagocytophilum genome is able to transport substrate across host cell membrane, as shown by the translocation of AnkA.
into the cytoplasm of HL-60 cells from *A. phagocytophilum* by type IV secretion system (Lin *et al*., 2007).

Several methods have been developed to detect the secreted substrates from bacteria into the cytoplasm of eukaryotic cells. Substrates fused with tags make them visible or detectable only after translocation into host cells. These tags used in previous studies are calmodulin-dependent adenylate cyclase (Cya) of *Bordetella pertussis*, Cre recombinase, and phosphorylable glycogen synthase kinase tag (Sory and Cornelis, 1994; Nagai *et al*., 2005; Schulein *et al*., 2005; Vergunst *et al*., 2005; Garcia *et al*., 2006; Hohlfeld *et al*., 2006). Cya produces cAMP in a calmodulin-dependent manner. As calmodulin is only present cytoplasm of host cells, but not in the bacteria, increase in cAMP concentration shows the translocation of substrates. Cre recombinase, a site-specific recombinase, can specifically cleave the DNA sequence flanked with *loxP* sites. In reporter host cells, the expression of reporter genes is inhibited by a DNA sequence flanked with *loxP* sites. When Cre-substrates tagged with a nuclear localization signal are translocated into reporter host cell nucleus from bacteria, the expression of reporter genes are activated by removing the DNA sequence flanked with *loxP*. A peptide of 13 amino acids (GSK tag) derived from glycogen synthase 3-β kinase was used as a reporter tag because it could be phosphorylated by host cell phosphate kinase. When GSK-substrates are translocated into host cells, they are phosphorylated and can be detected with phosphospecific GSK-3β antibodies. Compared to other tags, GSK tag has some advantage, such as its small size, (13 amino acids) which less interferes with the secretion and function of substrates (Garcia *et al*., 2006).
Recently, *A. phagocytophilum* became genetically amenable. Felsheim *et al.* (Felsheim *et al.*, 2006) successfully transformed *A. phagocytophilum* with gfp based on Himar1 mariner transposon system. In the present study, we made efforts to develop a reporter system in *A. phagocytophilum* to detect the translocated protein from the bacteria to host cells using Ats-1 as a model protein. As shown in the chapter 4 in this dissertation that Ats-1 is secreted from *A. phagocytophilum* to the host cell cytoplasm.

### 5.2 Materials and Methods

#### Construction of recombinant plasmid

To produce recombinant Ats-1 which contains GSK tag (MSGPRPTTSFAES) at 110th-111th, and HA tag (YPYDVPDYA) at 60th-61st, of Ats-1, the recombinant plasmid was constructed by a serial PCRs. All of primers used in this chapter are listed in Table 5.1. To insert the DNA sequence encoding GSK tag into *ats-1*, several PCRs were performed sequentially. First, Ats-1 gene was amplified as two fragments, each of which encodes N-terminus and C-terminus of Ats-1, respectively using the recombinant plasmid, *pats-1* (60HA61) constructed in chapter 4 in this dissertation as template, as DNA sequence encoding HA tag was already inserted into *ats-1* at the desired site. The DNA fragment encoding N-terminal Ats-1 was amplified by a pair of primers (Ats-1 GSK Forward N and GSK Reverse N), and the DNA fragment encoding C-terminal Ats-1 was amplified by another pair of primers (Ats-1 GSK Forward C1 and Ats-1 Reverse for C-terminus). Both the primers, GSK Reverse N and Ats-1 GSK Forward C1, contain the sequence encoding a part of GSK tag at their 5’-end to facilitate the insertion of GSK tag DNA sequence into *ats-1*. The PCR products were designated as GSK N for the DNA
fragment encoding N-terminal Ats-1, and GSK C1 for the DNA fragment encoding C-terminal Ats-1, respectively. Second, 5’-end extended-GSK C1 named as GSK C2 was amplified by PCR using GSK C1 as template, and a pair of primers (Ats-1 GSK Forward C2 and Ats-1 Reverse). Third, the 5’-end of GSK C2 was extended further by PCR using GSK C2 as template, and a pair of primer (Ats-1 GSK Forward C3 and Ats-1 Reverse). This PCR product was designated as GSK C3. Finally, based on the DNA sequence complementation of 3’-end of DNA fragment GSK N to the 5’-end of GSK C3, the two DNA fragments of GSK N, and GSK C3 were mixed, denatured, annealed, and extended, resulting in the DNA fragment encoding Ats-1 with a GSK and HA insertions (Fig. 5.1). This PCR product was named as ats-1 (HA-GSK) and digested with BamHI and NotI.

To modify the pHimar1 UV-SS plasmid (gift of Dr. Roderick Felsheim at Department of Entomology, University of Minnesota) by insertion of BamHI and NotI sites, and removing DNA sequence encoding GFPuv, a PCR was performed using pHimar1 UV-SS plasmid as template and a pair of primers (Himar1 Forward and Himar1 Reverse), resulting in a DNA fragment in which DNA sequence encoding GFPuv was removed, and a BamHI site [(just downstream of translational start condon of gfp (5’-ATG GGA TCC GG-3’, the underlined is BamHI site)] and a NotI site [(upstream of translation start site of spectinomycin resistance gene, aadA (5’-GTGCGGCGCGGATTAATTATG-3’, NotI site and translation start codon for aadA are underlined)] were inserted. This PCR product digested with NotI and BamHI, was ligated with BamHI-NotI-digested ats-1 (HA-GSK) and transformed into the competent E. coli DH5α strain. The resulting recombinant plasmid was named as pHimar1 Ats-1(HA-GSK)-SS. pHimar1 Ats-1(HA-GSK)-SS was transformed into the dam and dcm-deficient E. coli ER2925 strain (New
England BioLabs, Ipswich, MA) to prepare modification-less plasmid for *A. phagocytophilum* transformation.

To determine GSK tag still is phosphorylatable by mammalian cells when it was inserted into Ats-1. The PCR product amplified using pHimar1 Ats-1(HA-GSK)-SS plasmid as template, and a pair of primers (Ats-1 Forward and Ats-1 Reverse), was digested with SalI and NotI, and ligated with SalI-NotI-digested pEGFP-N1 vector (Clontech, Mountain View, CA). The DNA fragment encoding GFP was removed from pEGFP-N1 vector after digestion with SalI and NotI. The ligated product was transformed into *E. coli* DH5α. This recombinant plasmid named as *pats-1* (HA-GSK) was purified using EndoFree Plasmid Maxi Kit (Qiagen, Valencia, CA) according to the manufacturer’s instruction. Transfection of RF/6A endothelial cells with *pats-1* (HA-GSK), or *pats-1* constructs was performed using electroporation as previously described (Niu *et al.*, 2008).

**SDS-PAGE and Western blotting analysis**

For determination of expression of Ats-1 (HA-GSK) in *E. coli*, 1 ml overnight culture of *E. coli* DH5α harboring pHimar1 Ats-1(HA-GSK)-SS was centrifuged at 10,000 *x* g for 1 min, and resuspended in 100 µl of 2 × SDS-PAGE loading buffer (4% SDS, 135 mM Tris–HCl [pH 6.8], 10% glycerol, and 10% β-mercaptoethanol). For investigation of phosphorylation of Ats-1(HA-GSK) in RF/6A cells, 1.6 x 10⁵ transfected RF/6A cells in one well of a 6-well plate were dissolved in 2 × SDS-PAGE, supplemented with 2% phosphatase inhibitor cocktail 1 (Sigma). Samples were separated by SDS-PAGE with 10% polyacrylamide resolving gels, and transferred to a nitrocellulose membrane using a semidy blotter (WEP, Seattle, WA). The membrane
was blocked using 5% (wt/vol) skim milk (Difco, Sparks, MD) in Tris-buffered saline (150 mM NaCl and 50 mM Tris-HCl at pH 7.5), then incubated with rabbit anti-rAts-1 (1:1,000 dilution), rabbit anti-GSK 3β tag (Cell Signaling Technology, Danvers, MA, 1:1000 dilution), rabbit anti-phospho-GSK-3β (Cell Signaling Technology, 1:1,000 dilution), or mouse monoclonal anti-HA (HA.11, Clone 16B12, Covance, 1:1,000 dilution) at 4°C for 12 h, and subsequently with peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (KPL, Gaithersburg, MD) in 1:2,000 dilution at RT for 3 h. The bound antibodies were detected by chemiluminescence (Pierce Biotechnology, Rockford, IL). The images were captured by a CCD camera (Fuji LAS-3000 imaging system).

5.3 Results

Expression of Ats-1(HA-GSK) in Escherichia coli

Since the rabbit anti-GSK tag commercially acquired can not recognize this tag when it was expressed at the N-terminus of protein (data, not shown), GSK tag was inserted between 110th and 111th of Ats-1. As spontaneous spectinomycin-resistant A. phagocytophilum mutants may arise after transformation, leading to the dilution of Ats-1(HA-GSK)-expressing A. phagocytophilum, HA tag was inserted into Ats-1 for enrichment of Ats-1(HA-GSK) from the cytosol of host cells infected with Ats-1(HA-GSK)-expressing A. phagocytophilum. Since the transcription of ats-1(HA-GSK) is under the control of Anaplasma marginale tr promoter, which is functional in E. coli, we determined the expression of Ats-1(HA-GSK) in E. coli transformed with pHimar1 Ats-1(HA-GSK)-SS. Ats-1(HA-GSK) was detected as approximately 50-kDa bands by
Western blot analysis using anti-rAts-1, anti-GSK-3β tag, and anti-HA, whereas no band was detected in *E. coli* transformed with pHimar1 UV-SS (Fig. 5.2A). Several bands of smaller than 50 kDa were detected, suggesting Ats-1 (HA-GSK) was unstable when expressed in *E. coli* (Fig. 5.2A). The expected molecular mass of Ats-1 (HA-GSK) is 43 kDa. It is currently unknown why Ats-1 ran more slowly in SDS-PAGE than expected.

**Ats-1 (HA-GSK) is phosphorylatable when expressed in mammalian cells**

To determine whether GSK tag still have potential to be phosphorylated by mammalian cells when it is inserted into Ats-1, RF/6A cells were transfected with *ats-1* (HA-GSK) cloned into a mammalian expression vector, and subjected to Western blot analysis, using anti-rAts-1, anti-GSK-3β tag, or anti-phospho-GSK-3β. Ats-1 (HA-GSK) was expressed and phosphorylated in RF/6A cells (Fig. 5.2B).

**5.4 Discussion**

The study in the field of *Rickettsia*, including *A. phagocytophilum* is greatly hampered by the inability of genetic manipulation. The recent work by Felsheim *et al.* opened the possibility to study *A. phagocytophilum* by genetic modification (Felsheim *et al.*, 2006). In this study we did preliminary work for the development of a reporter system to study the secreted substrates of *A. phagocytophilum*. The plasmid encoding Ats-1 (HA-GSK) was constructed, and it was showed that Ats-1 (HA-GSK) was phosphorylatable when expressed in mammalian cells. In the near future, HL-60 cells will be infected with Ats-1(HA-GSK)-transformed *A. phagocytophilum*. Ats-1(HA-GAK) will be immunoprecipitated with anti-HA from the cytosol of infected HL-60 cells for enrichment, and subjected to phosphorylation assay. After this method is firmly
established, we will apply it to screen the hypothetical proteins encoded by \textit{A. phagocytophilum} for substrate candidate of type IV secretion system.
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Table 5.1. Oligonucleotide primers used in PCR
Figure 5.1. The diagram shows the procedure how to insert GSK tag into Ats-1.

First step, Ats-1 gene was amplified as two fragments, each of which encodes N-terminus and C-terminus of Ats-1. The DNA fragment encoding N-terminal Ats-1 was amplified by a pair of primers [Ats-1 GSK Forward N (Forward N) and GSK Reverse N(Reverse N)], and the DNA fragment encoding C-terminal Ats-1 was amplified by another pair of primers [Ats-1 GSK Forward C1 (Forward C1) and Ats-1 Reverse for C-terminus(Reverse C)]. Both the primers, GSK Reverse N and Ats-1 GSK Forward C1, contain the sequence encoding a part of GSK tag at their 5’-end. The PCR products were designated as GSK N for the DNA fragment encoding N-terminal Ats-1, and GSK C1 for the DNA fragment encoding C-terminal Ats-1, respectively.

Second step, GSK C2 was amplified by PCR using GSK C1 as template, and a pair of primers (Ats-1 GSK Forward C2 and Reverse C).

Third step, the 5’-end of GSK C2 was extended further by PCR using GSK C2 as template, and a pair of primer (Ats-1 GSK Forward C3 and Reverse C). This PCR product was designated as GSK C3.

Fourth step, based on the DNA sequence complementation of 3’-end of DNA fragment GSK N to the 5’-end of GSK C3, the two DNA fragments of GSK N, and GSK C3 were mixed, denatured, annealed, and extended, resulting in the DNA fragment encoding Ats-1 with a GSK and HA insertion.

The black bars show the DNA sequence encoding Ats-1, Red, yellow and purple bars show the DNA sequence encoding GSK tag.
Figure 5.1

[Diagram showing a PCR process with steps for amplifying DNA sequences.]

1. **1st step**: PCR reaction with Forward N and Reverse N primers.
2. **2nd step**: PCR reaction with Forward C and Reverse C primers.
3. **3rd step**: PCR reaction with Forward C and Reverse C primers.
4. **4th step**: Mixture of PCR products.
5. **Extension**: Final step to form the desired DNA sequence.
Figure 5.2. Expression of Ats-1 (HA-GSK) in *E. coli*, and phosphorylation in transfected RF/6A cells.

A. *E. coli* transformed with pHimar1 UV-SS, or pHimar1 Ats-1(HA-GSK)-SS, was subjected to Western blot analysis, using anti-rAts-1, anti-GSK 3β tag, and anti-HA. UV-SS: pHimar1 UV-SS plasmid; HA-GSK: pHimar1 Ats-1(HA-GSK)-SS plasmid.

B. RF/6A cells transfected with pats-1, or pats-1(HA-GSK), were subjected to Western blot analysis, using anti-rAts-1, anti-GSK 3β tag, and anti-phospho-GSK-3β. Ats-1: pats-1 plasmid; HA-GSK: pats-1(HA-GSK) plasmid.
Figure 5.2

A

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BIBLIOGRAPHY


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