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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

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Summary

***Anaplasma phagocytophilum*, an obligate intracellular bacterium, is the aetiologic 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.**

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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.

Results

Intracellular upregulation of virB6 *and* virB9 *transcription in human neutrophils*

In the *A. phagocytophilum* genome, *virB/D* genes cluster mainly in two separate operons, and both genomic loci are polycistronically transcribed in HL-60 cells (Ohashi *et al*., 2002). To investigate *virB* transcription during *A. phagocytophilum* infection cycle in human neutrophils, *virB9* and *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 *virB9* and *virB6* per bacterium in human neutrophils were upregulated greater than 100-fold at 35 h post infection (PI) compared at 3 h PI (Fig. 1). Transcription of neither *virB6* nor *virB9* was detected at 3 h PI under this condition. This result showed the intracellular upregulation of *virB9* and *virB6* transcription in two separate *virB/D* loci in *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. 2A). When the protein

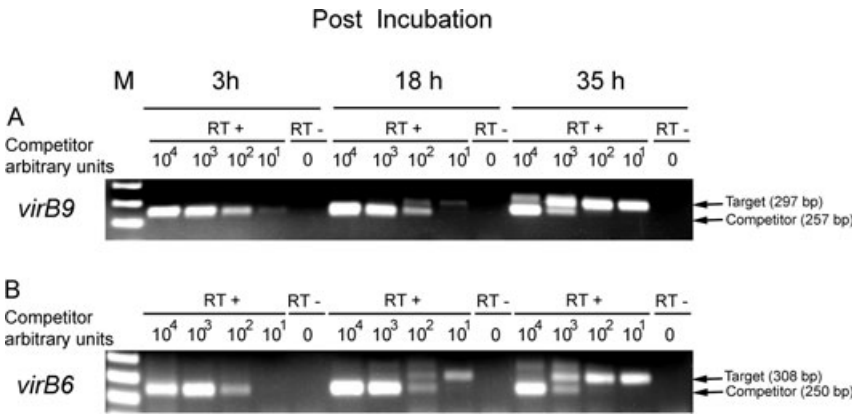

Fig. 1. Temporal expression of *virB9* and *virB6* by *A. phagocytophilum* in neutrophils. cDNA was synthesized from total RNA extracted from infected human neutrophils at three different time points (3, 18 and 35 h PI). cDNA levels were normalized by the number of bacteria as determined by 16S rRNA 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 1.

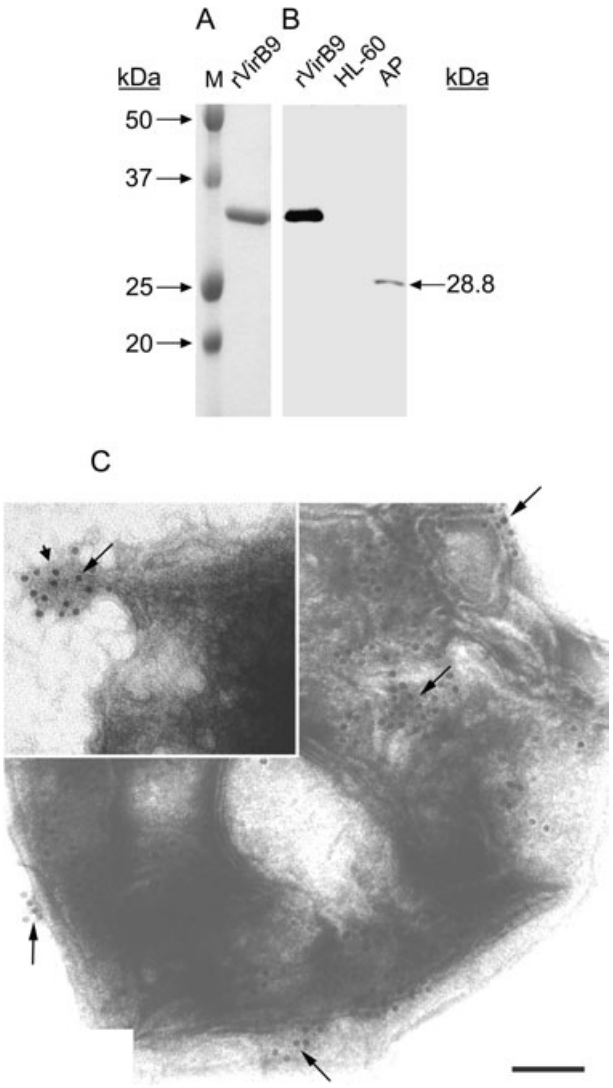

Fig. 2. Immunogold labelling of *A. phagocytophilum* using the antibody against *A. phagocytophilum* rVirB9.
A. rVirB9 expressed in *E. coli* was purified by immobilized Ni^{2+} affinity chromatography, separated by SDS-PAGE, and stained with Coomassie blue. Lanes: M, protein molecular size marker; rVirB9, 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.

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. 2B). The antibody did not react with uninfected HL-60 cells (Fig. 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. 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. 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. 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-VirB9 antibodies was specific (Fig. 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. 3A). This result suggests that VirB9 protein expression was downregulated prior to spontaneous release.

To further analyse 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%

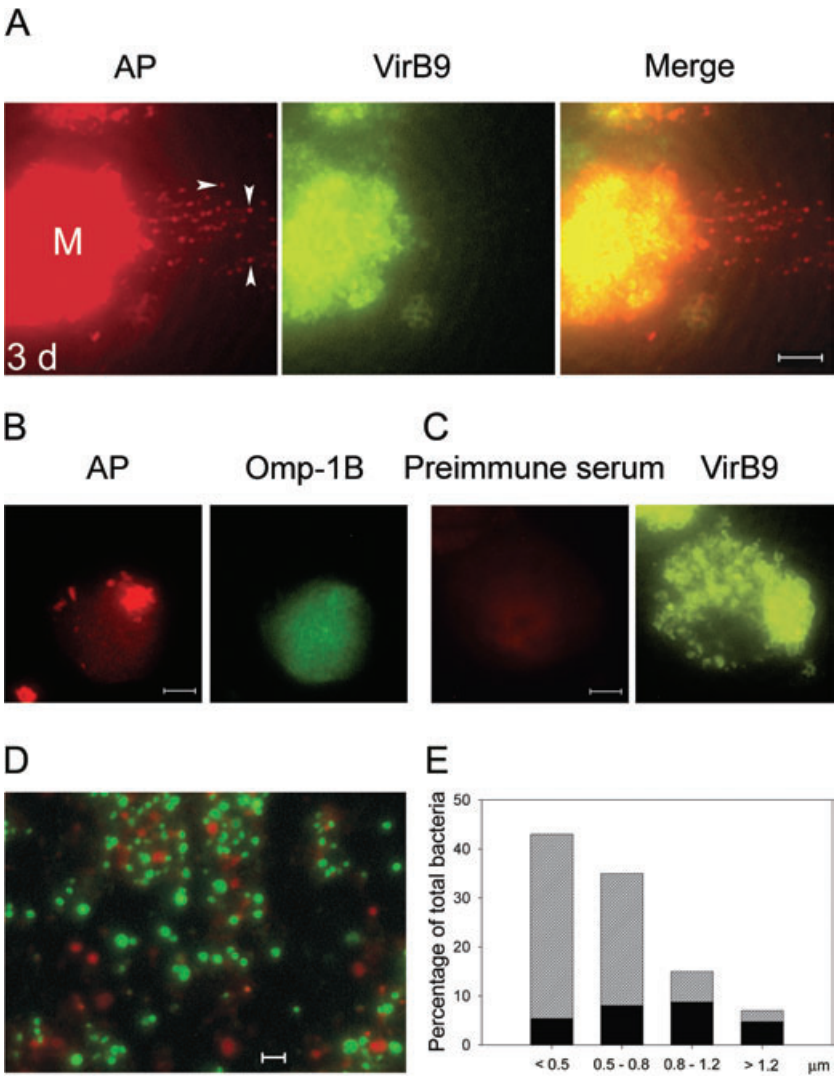

Fig. 3. VirB9 expression by *A. phagocytophilum* in morulae and in spontaneous release.
A. *A. phagocytophilum*-infected HL-60 cells harvested on day 3 PI were immunofluorescence-labelled with two antibodies, horse anti-*A. phagocytophilum* (AP) (Cy3, red) and rabbit anti-rVirB9 (VirB9) (Alexa Fluor 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.

($n = 3$ 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. 3D and E). Bacteria spontaneously released from HL-60 cells showed high (> 75%) viability as determined by LIVE/DEAD BacLight Bacterial Viability Kit (Fig. 3D). Generally viabilities of bacteria of smaller sizes were greater than those of larger sizes (Fig. 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. 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. 4, panels B and E). At approximately 40 h PI, 80% of morulae were strongly labelled with anti-rVirB9 antibody (Fig. 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. 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. 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. 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. 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*

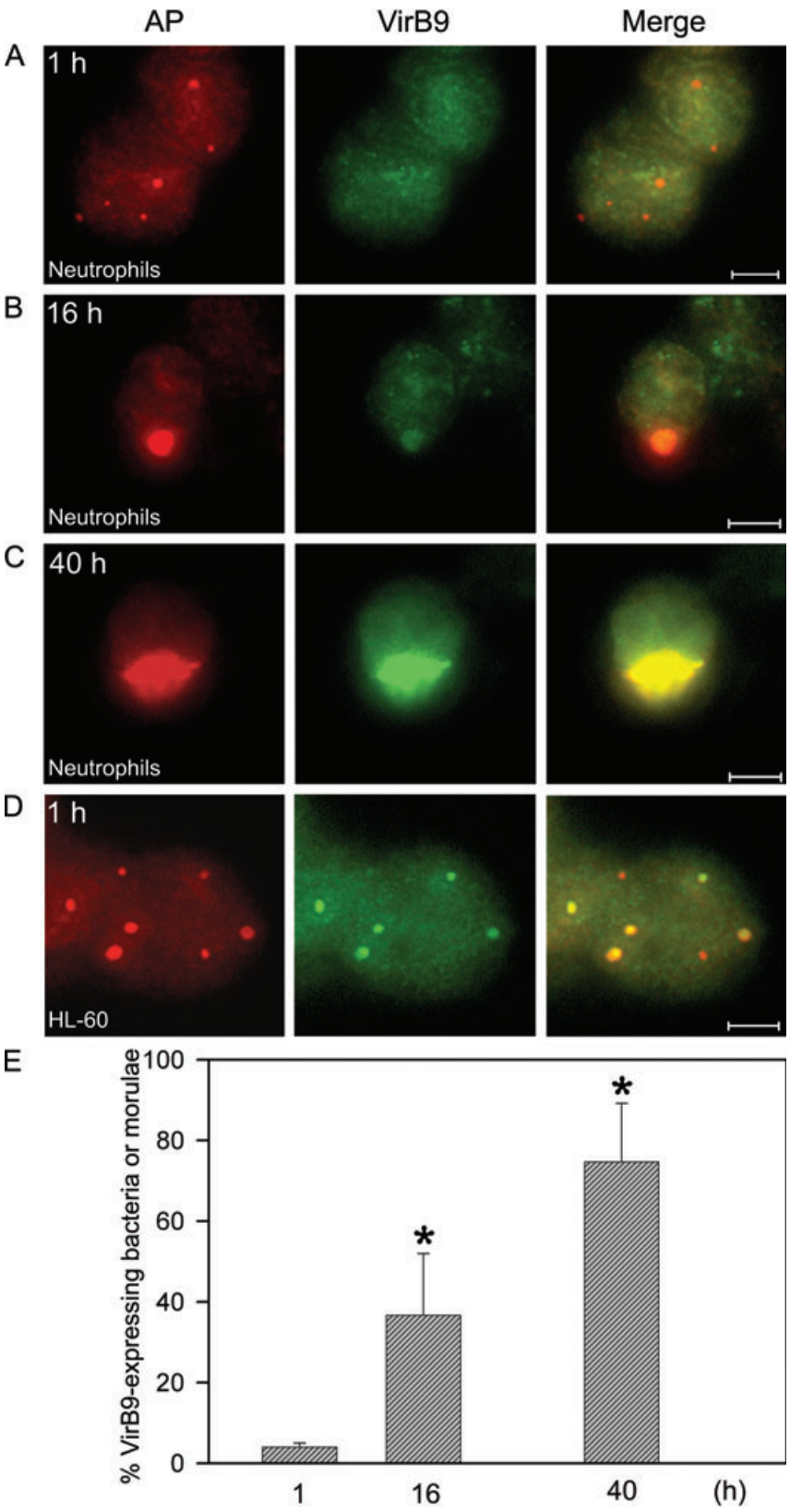

Fig. 4. Binding and internalization of VirB9-expressing and -undetectable *A. phagocytophilum* into human neutrophils and HL-60 cells. *A. phagocytophilum* organisms mechanically released from infected HL-60 cells were used to infect human neutrophils or HL-60 cells. Double immunofluorescence labelling 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 ($P < 0.01$).

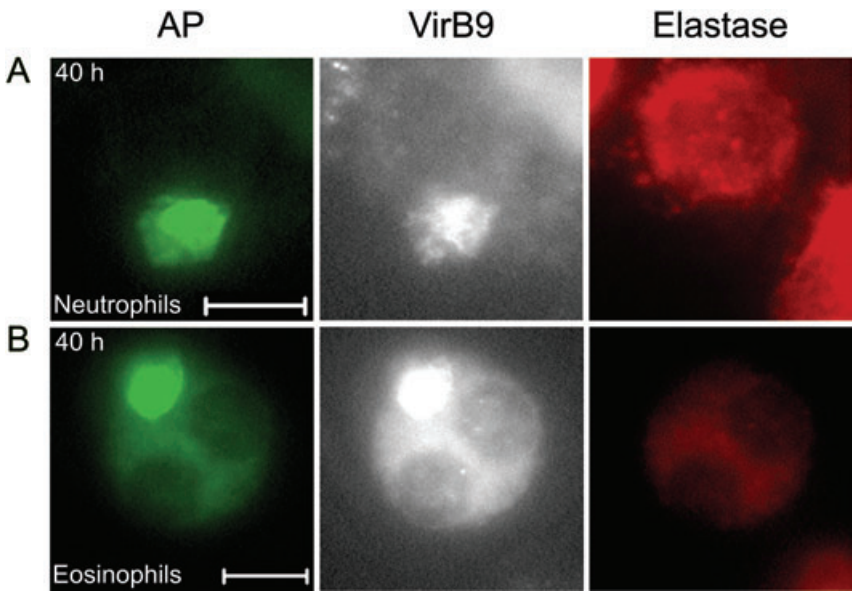

Fig. 5. VirB9 expression in *A. phagocytophilum*-infected human neutrophils and eosinophils. Triple immunofluorescence labelling was performed to investigate VirB9 expression in human neutrophils (A) and eosinophils (B) 40 h PI using three antibodies, horse anti-*A. phagocytophilum* (AP) (FITC, green), 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.

enter HL-60 cells through different receptors and internalization pathways.

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://

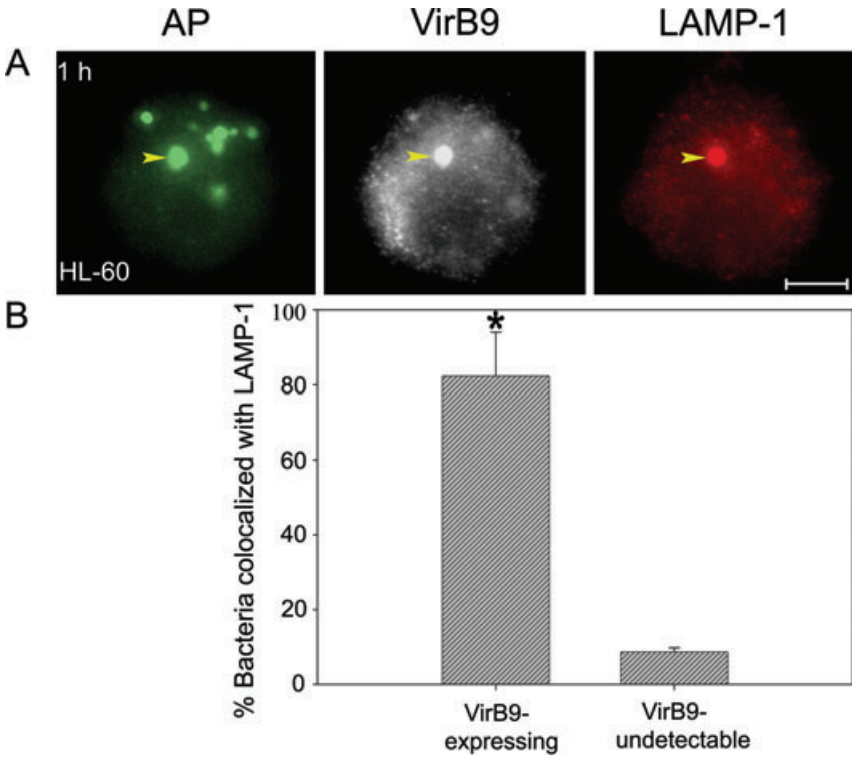

Fig. 6. Intracellular localization of VirB9-expressing and -undetectable *A. phagocytophilum* within HL-60 cells.
A. Triple immunofluorescence labelling was performed to examine the colocalization of *A. phagocytophilum* with LAMP-1 at 1 h PI using three antibodies, horse anti-*A. phagocytophilum* (FITC, 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 ($P < 0.01$).

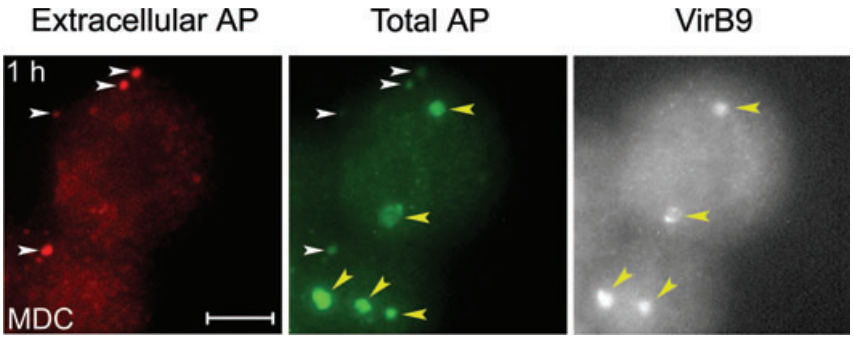

Fig. 7. Internalization of VirB9-expressing *A. phagocytophilum* into HL-60 cells by phagocytosis. Triple immunofluorescence labelling was performed to examine VirB9 expression by *A. phagocytophilum* internalized into HL-60 cells in the presence of monodansylcadevaline using two primary antibodies (horse 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.

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/D*s 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.

Experimental procedures

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% CO_2–95% 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 were infected, cells were lysed by nitrogen cavitation at 4×10^6 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 Na_2HPO_4, and 2 mM KH_2PO_4 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 3×10^6 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 (1.5×10^7) were incubated with *A. phagocytophilum* derived from 2×10^7 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 v*irB6* 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 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 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* INVaF′ 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 analysed 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–

Table 1. Oligonucleotide primers used in PCR and construction of competitors.

Gene name	P1 (used for construction of competitor and competitive PCR)	P2 (used for construction of competitor)	P3 (used for construction of competitor)	P4 (used for construction of competitor and competitive PCR)	Size (bp) (target/competitor)
virB9	5′-gctttcgcgctgttctctatg-3′	5′-gtctcacttaaccggaaaaacctcattct-3′	5′-tttccggttaaggtgagacggtgcgagtt-3′	5′-ccgcccctactcgtttcta-3′	297/257
virB6	5′-gcggcgggcgatgctact-3′	5′-atccaacaatatgctacctatctttgcaag-3′	5′-taggtagcatattgttggatttaggcctct-3′	5′-aacacgggatgacggacaaaactc-3′	308/250
16S *rRNA*	5′-cgggggaaagatttatcgctatta-3′	5′-ggatcaggcttaggagtctggaccgtatct-3′	5′-cagactcctaagcctgatccagctatgccg-3′	5′-cgcttgcccctccgtatta-3′	323/285

The complementary sequences in primer P2 and P3 are underlined.

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

Purified rVirB9 (50 μg) and 2×10^6 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 semidry 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} diaminobenzidine (Nakarai, Japan) in 50 mM Tris–HCl buffer (pH 7.2) containing 0.1% H_2O_2 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-*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 *t*-test ($P < 0.05$ was considered significant).

Negative-staining of immunogold-labelled A. phagocytophilum

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 $\mu g\ ml^{-1}$) affinity-purified with membrane-bound rVirB9 (Sambrook *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.

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