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Breaking In and Grabbing A Meal: *Anaplasma phagocytophilum* Cellular Invasion, Nutrient Acquisition, and Promising Tools for Their Study

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Abstract

Anaplasma phagocytophilum invades neutrophils to cause the emerging infection, human granulocytic anaplasmosis. Here, we provide a focused review of the *A. phagocytophilum* invasin-host cell receptor interactions that promote bacterial entry and the degradative and membrane traffic pathways that the organism exploits to route nutrients to the organelle in which it resides. Because its obligatory intracellular nature hinders knock out-complementation approaches, we also discuss current methods used to study *A. phagocytophilum* gene function and the potential benefit of applying novel tools that have advanced studies of other obligate intracellular bacterial pathogens.

INTRODUCTION

Anaplasma phagocytophilum is a tick-borne, obligate intracellular bacterium of the family *Anaplasmataceae* that infects granulocytes, bone marrow progenitor cells, and endothelial cells of various mammalian species, including humans [1, 2]. *A. phagocytophilum* is unusual in its ability to flourish within neutrophils, which are important effector cells of microbial killing. Infection of humans by *A. phagocytophilum* causes human granulocytic anaplasmosis (HGA), an emerging infectious disease first detected in 1994 [3]. Cases of HGA have also been documented in Europe and Asia. Though incidence is on the rise, HGA remains an underreported disease. HGA is an acute febrile infection accompanied by many non-specific symptoms including chills, headache, malaise, and myalgia. Clinical manifestations include leukopenia, thrombocytopenia, and elevations in serum hepatic aminotransferases. The disease is generally self-limiting in healthy individuals, though up to 50% of symptomatic patients require hospitalization [1]. Potential complications include rhabdomyolysis, pneumonitis, shock, seizures, hemorrhage, and increased susceptibility to potentially fatal secondary infections. Risk for complications and death is greater for individuals having pre-existing immunocompromise, the elderly, and when antibiotic therapy is delayed [1, 4].

A. phagocytophilum's obligatory intracellular nature is predicated on its need to parasitize host cell nutrients. Following invasion, the bacterium resides in a host cell-derived vacuole

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that it remodels into a protective niche [1]. Membrane traffic is rerouted to the *A. phagocytophilum*-occupied vacuole (ApV) to deliver vital nutrients and host cell membrane material. This review describes our current understanding of the molecular events involved in *A. phagocytophilum* cellular invasion and molecular parasitism. We also discuss the challenges of studying the organism and conclude with a survey of recent advances in the genetic manipulation of other obligate intracellular bacteria and their potential applications to studying *A. phagocytophilum.*

UNLOCKING THE DOORS: *A. phagocytophilum* cellular invasion

A. phagocytophilum must attach to and enter host cells in order to survive (Figure 1). This process is facilitated by multiple bacterial adhesins/invasins that cooperatively recognize host cell receptors and initiate signaling cascades to promote pathogen internalization.

Host cell receptors

The most well characterized *A. phagocytophilum* receptor is P-selectin glycoprotein ligand-1 (PSGL-1). Engaging PSGL-1 is critical for infection of human neutrophils, bone marrow progenitors, and promyelocytic HL-60 cells [5, 6] and initiates a Syk- and ROCK1-dependent signaling cascade that promotes bacterial uptake [7]. PSGL-1 is capped by an O-glycan that is terminally decorated with sialyl Lewis x, a tetrasaccharide that includes α2,3-sialic acid and α1,3-fucose [8]. The bacterium cooperatively binds three structural determinants of human PSGL-1: (i) PSGL-1 N-terminal peptide, (ii) α1,3-fucose of sLe^x, and (iii) α2,3-sialic acid of sLe^x [9]. Binding to α2,3-sialic acid promotes cellular entry, but is dispensable for adhesion whereas recognition of PSGL-1 and α1,3-fucose are important for binding and invasion [5, 6, 10]. *A. phagocytophilum* infection of murine neutrophils does not involve PSGL-1 because murine PSGL-1 lacks the DFLPE peptide that is found in the human PSGL-1 N-terminus and is critical for *A. phagocytophilum* binding [9, 10]. α2,3-sialic acid and α1,3-fucose are important and necessary, respectively, for *A. phagocytophilum* infection of murine neutrophils [10]. α1,3-fucose but not sialic acid is essential for *A. phagocytophilum* to colonize ixodid ticks [11]. Therefore, α1,3-fucose is a unifying determinant that *A. phagocytophilum* targets to infect its natural murine and arthropod reservoirs and accidental human hosts.

A. phagocytophilum adhesion and invasion also occur through PSGL-1-independent routes that involve β2 integrin and lipid rafts. Differences in receptor binding occur when *A. phagocytophilum*-neutrophil interactions are examined under shear flow conditions similar to those in a blood vessel [12]. Whereas the pathogen solely engages PSGL-1 under static conditions [5, 6, 13], it binds both PSGL-1 and β2 integrin under flow [12]. Lipid rafts are important signaling platforms and contain enriched amounts of glycophosphatidylinositol (GPI)-anchored proteins and caveolin-1. GPI-anchored proteins are required for infection, and caveolin-1 colocalizes with early *A. phagocytophilum* vacuoles, suggesting that the bacterium enters host cells at lipid rafts [14]. PSGL-1 is found in lipid rafts, and β2 integrin mobilization into lipid rafts has been linked to bacterial pathogenesis [15, 16]. Prolonged cultivation in α1,3-fucosyltransferase- and sialyltransferase-defective cell lines selects for *A. phagocytophilum* organisms that no longer rely on sLe^x, PSGL-1, or Syk for entry [13, 17, 18]. Whether this enriched subpopulation consists of phenotypic or genotypic variants that target β2 integrin, lipid rafts, or other receptors is unknown.

A. phagocytophilum surface proteins implicated in infection

Several *A. phagocytophilum* outer membrane proteins (OMPs) have been indicted in mediating attachment to and invasion of mammalian host cells. The most thoroughly dissected are OmpA and Asp14 (14-kDa *A. phagocytophilum* surface protein). Both are

transcriptionally induced during the tick transmission bloodmeal and are upregulated when the bacterium engages sLe^x-capped PSGL-1, suggesting their importance in establishing infection [19, 20]. The OmpA predicted N-terminal extracellular domain, specifically amino acids 19–74, binds α2,3-sialic acid of sLe^x. Bacterial entry (but not attachment to host cells) is significantly impaired by OmpA antibody and is antagonized by recombinant forms of OmpA or its extracellular domain. Competitive inhibition of *A. phagocytophilum* binding to sLe^x-capped PSGL-1 by recombinant OmpA mimics the inhibition afforded by a monoclonal antibody specific for α2,3-sialic acid of sLe^x [20]. Asp14 is also critical for cellular invasion but not adhesion. The Asp14 invasion domain maps to the protein's C-terminal 12 to 24 amino acids. While its receptor is unknown, it is clear that Asp14 functions in cooperation with OmpA to promote optimal *A. phagocytophilum* entry. Treating host cells with recombinant OmpA or Asp14 prior to bacterial addition results in a 57 to 65% reduction in infection, whereas pretreatment with both proteins results in a 90% reduction [19]. This result also implies that OmpA and Asp14 promote infection through complementary signaling pathways.

Asp55, Asp62, and APH1235 are other *A. phagocytophilum* proteins implicated in adhesion and invasion. Antibodies specific for Asp55 and Asp62 significantly inhibit *A. phagocytophilum* infection of host cells [21]. Since the Asp55 and Asp62 receptors are undefined, it is difficult to interpret if this inhibition is specific. APH1235 is induced during transmission feeding of infected ticks and when non-infectious RC bacteria convert to the infectious DC form. Reports are conflicting as to whether APH1235 is exposed on the bacterial surface [22, 23]. However, propagating *A. phagocytophilum* in HL-60 cell culture in the presence of APH1235 antibody significantly reduces bacterial load [22].

Msp2 (P44) is the bacterium's major surface protein and consists of conserved N- and C-terminal domains and a central hypervariable region. The *A. phagocytophilum* genome carries a repertoire of 113 *p44 (msp2)* paralogs and a single expression site, which enables the organism to utilize gene conversion to vary the P44 (Msp2) protein that it expresses. This phenomenon and its role in antigenic variation has been reported and reviewed elsewhere [1, 4, 24–26]. Evidence suggests that P44 (Msp2) is also involved in cellular invasion. Recombinant Msp2 (P44), the specific paralog of which was not clarified in the referenced study, binds myeloid cells and thereby inhibits *A. phagocytophilum* attachment and internalization [27]. It also antagonizes bacterial binding to α1,3-fucosylated PSGL-1 [27], indicating that a specific paralog or perhaps a group of structurally related Msp2 (P44) proteins may recognize α1,3-fucose and/or PSGL-1 N-terminal peptide. The latter possibility is more likely since *A. phagocytophilum* more frequently expresses individual Msp2 (P44) paralogs during cultivation in human myeloid cells and more frequently expresses certain clades of Msp2 (P44) paralogs during infection of specific mammalian hosts [24, 28–30]. If Msp2 (P44) is involved in cellular invasion, then its receptor binding domain is presumably located in a conserved region. Alternatively, it could exist in the hypervariable region if it is a conformational binding determinant and is structurally conserved. Pretreatment of *A. phagocytophilum* with monoclonal antibodies specific for epitopes in the Msp2 (P44) N-terminal conserved region or the hypervariable region of a specific paralog, Msp2 (P44)-18, block bacterial binding to or replication in human myeloid cells, respectively [31].

ORDERING ROOM SERVICE: *A. phagocytophilum* selectively routes degradative and membrane trafficking pathways to its vacuole

Nutritional virulence, as coined by Abu Kwaik and Bumann means that, "without proper nutritional resources for survival/proliferation in the host, bacterial pathogens do not cause disease [32]." *A. phagocytophilum* is auxotrophic for 16 amino acids and requires

cholesterol for intracellular survival [33–36]. It must not only parasitize these essential nutrients but also must route them to its organelle. A series of recent key findings have begun to illuminate *A. phagocytophilum* nutritional virulence strategies.

Exploiting autophagy

Autophagy is a eukaryotic cellular homeostasis process that digests unwanted intracellular objects, including damaged organelles. It also targets intracellular pathogens and is therefore an important arm of the innate immune response. Autophagosome formation is controlled by the sequential assembly of autophagy-related (ATG) proteins and is initiated by the formation of a complex that includes ATG14 and BECN1 (Beclin 1) [37]. The complex promotes formation of a structure called the omegasome, from which the phagophore forms to elongate and enclose cytoplasmic contents with the help of a pair of ubiquitin-like conjugation systems, one of which includes the protein, LC3 [37, 38].

The ApV resembles the early autophagosome based on association of double-lipid bilayer membranes, Beclin 1, and LC3. Early in infection, clumps of Beclin1 and LC3 form, signifying the induction of autophagy, and these proteins localize to the bacterial inclusion. However, rather than clear the infection, autophagy is exploited by *A. phagocytophilum* as a clever way to pirate amino acids. The T4SS effector, Ats-1 (*A. phagocytophilum* translocated substrate 1) is secreted from the organism across the inclusion membrane into the cytoplasm where it binds both Beclin 1 and ATG14 to induce omegasome formation. The isolation membrane elongates to envelope cytoplasmic content in a double-membraned, LC3-decorated autophagosome. Autophagosomes are targeted to the ApV, where they subsequently fuse to deliver autophagic body-like vesicles into the vacuole's lumen. Supplementation of excessive amino acids partially overrides the growth inhibition of *A. phagocytophilum* that occurs in the presence of the autophagic pathway inhibitor, 3-methyl-adenine [39]. How the autophagic-like bodies are broken down to yield free amino acids within the ApV lumen is unclear, but it has been speculated that bacterial surface-localized proteases may be responsible. Support for this hypothesis is provided by the observation that treatment of *A. phagocytophilum* infected host cells with a membrane-permeable serine-protease inhibitor retards infection [39]. Interestingly, Ats-1 is a dual-function effector, as it is also imported into the mitochondria where it interferes with apoptosis induction [40].

Recent evidence suggests the involvement of mono- and polyubiquitinated proteins in autophagy [41, 42]. Inclusion bodies that are too large for degradation in the 26S proteasome are targets for selective autophagy and contain polyubiquitinated proteins and LC3 [42]. Additionally, labeling of intravacuolar bacteria with monoubiquitin has been shown to target bacteria for destruction in the autophagosome [41], though some bacteria such as *Salmonella enterica* subvert this process [43]. Interestingly, while the ApV is decorated with LC3, it is not decorated with polyubiquitinated proteins. Rather, ApVs stain positive for monoubiquitin in mammalian and, to a lesser extent, tick host cells. *De novo* bacterial protein synthesis is important for continued association of monoubiquitin with the ApV suggesting this is a bacterial-mediated process [44]. Monoubiquitinated proteins play various other roles in the cell including directing endocytic traffic [45]. Thus, whether Ap uses monoubiquitinated proteins specifically to aid in autophagosome fusion remains unknown.

Cholesterol acquisition

The *A. phagocytophilum* genome does not contain any genes for synthesis of lipid A or the full complement required for peptidoglycan synthesis [33, 34]. As such, the bacterium stabilizes its outer membrane by incorporating cholesterol. A lack of genes related to cholesterol synthesis or modification obligates it to hijack cholesterol from its mammalian

host [34]. Indeed, exogenous cholesterol is taken up by the bacterium [34], endogenous cholesterol synthesis is upregulated in infected cells, and free (unesterified) cholesterol is enriched in the *A. phagocytophilum*-occupied vacuole (ApV) [35]. Both extraction of cholesterol from the membranes of host cell-free bacteria with methyl-β-cyclodextrin (MβCD) and addition of the structural but poorly functional cholesterol derivative, NBD-cholesterol, significantly reduce infectivity [34], suggesting that cholesterol is also important for *A. phagocytophilum* virulence.

Host leukocyte cholesterol is either synthesized in the smooth endoplasmic reticulum (ER) or acquired mostly through low-density lipoprotein receptor (LDLR)-mediated endocytosis. Culture of infected cells in lipoprotein-deficient serum, treatment of host cells with an LDLR antibody and pharmacological inhibitors of the LDLR pathway, but not inhibitors of cholesterol synthesis, significantly reduce *A. phagocytophilum* infection and replication, implicating LDLR-mediated endocytosis in cholesterol acquisition by the bacterium. *A. phagocytophilum* infection increases LDLR mRNA stability, resulting in a marked increase in LDLR protein compared to uninfected cells [35], providing a functional explanation for the increased cholesterol levels associated with infection. In LDLR-mediated endocytosis, cholesterol esters are hydrolyzed in acidified endosomes while free cholesterol is trafficked in a vesicular compartment containing the cholesterol-binding protein Niemann-Pick type C1 (NPC1) to the ER, sometimes by way of the *trans*-Golgi network (TGN). In the ER, excess free cholesterol is esterified and stored or trafficked back to the plasma membrane [46]. NPC1 and NPC2 are upregulated in *A. phagocytophilum*-infected cells and localize to and in the ApV, respectively. siRNA knockdown of NPC1 significantly reduces infection and cholesterol levels in purified bacteria, indicating the importance of this pathway in cholesterol acquisition. Treatment of host cells with U18666A, a compound that blocks free cholesterol egress from the endosomal pathway to the ER or TGN to produce an NPC1-deficient phenotype, impedes infection. During infection and subsequent increase in intracellular cholesterol, ER-resident sterol regulatory element-binding proteins (SREBPs), key transcriptional regulators maintaining cholesterol homeostasis in the cell, are not activated, suggesting that the ApV intercepts NPC1 vesicles downstream of the U18666A target site and upstream of their delivery to the ER [36]. Furthermore, siRNA knockdown of VAMP4, a protein that is important for delivery of NPC1 vesicles to the TGN and localizes to the ApV [46], only marginally reduces infection and does not alter *A. phagocytophilum* cholesterol uptake [36]. These studies indicate a novel mechanism whereby the pathogen hijacks free cholesterol from NPC1 vesicles en route to the TGN and/or ER. Lipid droplets may be an additional cholesterol source for *A. phagocytophilum* [35]. Perlipin, a lipid droplet-associated phosphoprotein that is important for lipolysis and cholesterol synthesis, is transcriptionally upregulated in *A. phagocytophilum* infected cells and siRNA knockdown of perilipin inhibits bacterial infection and growth [47].

A. phagocytophilum's dependence on cholesterol indicates that patients with hypercholesterolemia may experience more severe infection [48]. Indeed, a high cholesterol diet facilitates increased infection of apolipoprotein E-deficient (apoE$^{-/-}$) mice [49]. HGA is more commonly associated with older individuals, contrary to the median age of persons inflicted with other tick-borne pathogens. Older individuals generally have weakened immune systems and elevated levels of blood cholesterol. Thus, *A. phagocytophilum*'s cholesterol dependence may at least partially explain the increase in median age. It has been speculated that therapeutics aimed at lowering plasma cholesterol levels could aid control of HGA in this population [35, 48, 49].

Selective targeting of Rab GTPases

Rab GTPases are master regulators of membrane dynamics on organelles. The Rab family consists of nearly 70 members, each of which is involved in controlling a defined vesicular transport step [50, 51]. *A. phagocytophilum* selectively targets a subset of Rab GTPases to its vacuole [52]. Doing so provides a means of molecular camouflage and likely contributes to the bacterium satisfying its obligatory intracellular nutritional requirements for amino acids and cholesterol. It also conceivably provides a continuous supply of host membrane material to enable of expansion of the vacuolar membrane such that it can accommodate the growing intravacuolar bacterial population. Rab1, Rab4A, Rab10, Rab11A, Rab14, Rab22A, and Rab35 localize to the ApV [52] (Table 1). These Rabs direct vesicular traffic associated with clathrin-dependent endocytic recycling (Rab4A, Rab35) [53], clathrin-independent endocytic recycling (Rab10, Rab11A, Rab14, Rab22A) [51, 53], the endoplasmic reticulum (ER; Rab1, Rab10) [54], and the *trans*-Golgi network (TGN; Rab10, Rab11A, Rab14, Rab22A) [50, 51, 53]. This phenomenon is driven by nascent bacterial protein synthesis and is critical for the ApV to evade lysosomal fusion [52].

From the exclusivity of the pathways that Rab1 and Rab4A regulate [53], it is clear that the ApV intercepts ER traffic and clathrin-dependent recycling endosomes. Rab35 localizes with Rab4 on clathrin-dependent recycling endosomes and is found along with Rab10 and Rab22A on tubular recycling endosomes of the clathrin-independent recycling pathway [53]. Since the frequency of Rab35 localization to the ApV both pronouncedly exceeds that of Rab4 and is comparable to the frequencies by which Rab10 and Rab22A associate with the ApV [52], it can be inferred that the ApV also co-opts clathrin-independent endocytic recycling. Proteins involved in amino acid uptake and cholesterol both traffic through recycling endosomes [53].

Rab10, Rab11A, Rab14, and Rab22A regulate endocytic recycling and also mediate transport of vesicles from (Rab10, Rab11A, Rab14) and to (Rab22A) the TGN [53]. VAMP4 and syntaxin-16, two SNARE proteins that are important for cargo transport between recycling endosomes and the TGN [46], also localize to the ApV membrane [36]. Thus, the ApV may intercept RE-to-TGN traffic and/or may hijack TGN-derived vesicles. Neither Rab10 and Rab14 recruitment to the ApV nor *A. phagocytophilum* growth is impaired by the Golgi apparatus destabilizing agent, Brefeldin A [52], which implies that an intact Golgi apparatus is not required for either phenomena. Indeed, *cis*-Golgi fragmentation has been observed in cells that are heavily infected with *A. phagocytophilum* [36], suggesting that the pathogen itself promotes Golgi destabilization, at least under conditions of high bacterial load. Golgi fragments, particularly those derived from the TGN, would be rich in cholesterol. *Chlamydia trachomatis* induces Golgi fragmentation and routes lipid-rich TGN-derived vesicles to its inclusion, a process that is important for chlamydial survival and generating infectious progeny [55]. Thus, thorough examination of whether *A. phagocytophilum* targets the TGN is warranted.

Rab proteins cycle between an inactive cytosolic GDP-bound form and an active GTP-bound form that associates with organelle membranes to regulate membrane fusion events [51]. In line with normal Rab membrane cycling, GTP- but not GDP-bound forms of Rab1, Rab4A, and Rab11A [52], as well as Rab14, Rab22A, and Rab35 (Huang and Carlyon, unpublished observations) associate with the ApV. Strikingly, GTP-bound, GDP-bound, and guanine nucleotide-free forms of Rab10 localize to the ApV with comparable efficiencies, an observation that suggests an *A. phagocytophilum* protein may target Rab10 to the ApV [52].

HELP IS ON THE WAY: Promising tools for elucidating *A. phagocytophilum* gene function

A major hindrance to fully characterizing *A. phagocytophilum* virulence mechanisms is the bacterium's intractability to traditional genetic manipulation methods. For instance, due to its obligate intracellular nature knocking out a gene that is essential for cellular invasion or intracellular survival would prohibit the mutant's selection. In addition, its reduced genome size could affect both the stability and length of clonal passages of genomic insertions. However, in line with the proverbial saying, "necessity is the mother of invention," scientists have developed methods that circumvent and even nullify the challenges that obligate intracellular bacteria present to genetic manipulation. Here we summarize what has been used to study *A. phagocytophilum* and discuss additional genetic tools developed through the study of other obligate intracellular bacteria that would potentially benefit *A. phagocytophilum* research. For a more detailed review of these methods, see Beare *et al* [56].

Tools used to study *A. phagocytophilum* gene function

Nearly a decade ago, it was demonstrated that *A. phagocytophilum* could be transformed using the *Himar1* transposon system when cassettes encoding fluorescent proteins and antibiotic resistance were stably inserted into the bacterium's chromosome [57, 58]. Since then, the Himar1 system has been applied to study other obligate intracellular bacteria including *A. marginale*, *Ehrlichia chaffeensis*, *Rickettsia prowazekii*, and *Coxiella burnetii* [57, 59–61]. *Himar1* cassette-mediated disruption of the dihydrolipoamide dehydrogenase 1 (LPDA1) gene (*aph0065*) enabled researchers to identify LPDA1 as an important *A. phagocytophilum* immunopathological molecule [62]. Transcriptional profiling of an *A. marginale Himar1* transformant that exhibited a slow growth phenotype revealed a gene network that was transcriptionally altered *in trans* by the *Himar1* insertion [63]. While both examples highlight the *Himar1* transposon system as an important tool for the field and a first step toward the development of a system for studying *Anaplasma* spp. gene function, the latter example also denotes that, because of the random nature of *Himar1* transposition, unwanted off target effects can result. Moreover, because *Himar1* cannot be used for site-specific gene disruption, libraries of *Himar1* mutants must be laboriously screened to first identify a discernable phenotype, next the mutant must be sequenced to identify the disrupted gene, and only then can testable hypotheses in regards to the disrupted gene's function be formed. Furthermore, random insertion into genomic regions that are critical for infection of host cells, replication, or genome maintenance would be missed because the resulting transgenic populations would be unable to invade or survive in host cells and would consequently die or would be outgrown by host cells and other more fit *A. phagocytophilum* organisms.

Tools developed through study of other obligate intracellular bacteria

Systems that enable stable, site-specific insertions into the chromosome or that effectively deliver antisense oligonucleotides to modulate bacterial gene expression are needed to efficiently elucidate *A. phagocytophilum* gene function. Several promising tools developed for studying other obligate intracellular bacteria satisfy these criteria. Homologous recombination by the modified mobile group II intron (TargeTron) method was used to insert cassettes into constitutive and differentially expressed genes and intergenic regions of the *Ehrlichia chaffeensis* chromosome [60]. Though the mutants survived for only eight days, this study proved that site-specific mutagenesis of *Anaplasmataceae* bacteria is achievable. Self-replicating plasmids for transforming *A. phagocytophilum* would enable genetic manipulation of the bacterium without targeting or disrupting the bacterial genome

itself and would potentially generate more stable transformants. Like *A. phagocytophilum*, plasmids have not been found in *R. prowazekii*. Yet, researchers were able to transform *R. prowazekii* with a replicating plasmid from the less pathogenic species, *R. amblyomii* [64], marking the first plasmid to be stably maintained in *R. prowazekii*. A shuttle plasmid for transforming *C. trachomatis* has also been developed [65].

The formulation of an axenic growth medium for *C. burnetii* has greatly accelerated the development of genetic techniques for this organism [66]. Because *C. burnetii* colonies can be grown on semi-solid media, isogenic clones can be selected, enabling recovery of mutants carrying site-directed insertions, inducible expression systems, and targeted gene deletions [59, 67]. Host cell-free growth has also recently been achieved for *C. trachomatis* [68], which is a harbinger that, once the nutritional requirements of an obligate intracellular bacterium are understood, a medium that supports axenic growth can be developed.

A very promising method for rapidly modulating obligate intracellular bacterial gene expression is to use polyamidoamine (PAMAM) dendrimers to deliver DNA into bacteria that are actively growing inside eukaryotic host cells [69, 70]. Indeed, highly efficient knockdown of gene expression was achieved within six hours of incubation of PAMAM dendrimer-antisense oligonucleotide complexes with *C. trachomatis* infected cells [70]. This approach is especially appealing for studying genes that are critical for cellular invasion or intracellular survival in *A. phagocytophilum* and other obligate intracellular bacteria because it circumvents having to generate a mutation that is potentially fatal or would result in less fit organisms that could not be recovered. Dendrimers were also used to efficiently deliver a 7.5-kb plasmid into *C. pneumoniae* inclusions inside host cells. The chlamydiae took up the plasmid and maintained the plasmid for five passages, resulting in heterologous GFP-expressing organisms that expressed all the plasmid's genes [69]. It has yet to be demonstrated whether an isogenic population can be obtained following dendrimer-mediated transformation, but given its high efficiency, it is certainly plausible that clonal organisms could be isolated from a transformant population via limiting dilution.

CONCLUSIONS

The invasion and nutrient parasitism mechanisms described here highlight many of *A. phagocytophilum*'s clever virulence tactics. Though these strategies have been thoroughly researched, knockout-complementation and other genetic approaches will provide proof of principle, examine non-redundancy of function, and further fine-tune understanding of these and other virulence mechanisms. To accomplish this, it will be critical to explore new tools, such as those that have been effectively used to study other obligate intracellular bacterial pathogens. Both the development of an axenic media for cell-free cultivation of *A. phagocytophilum* and using dendrimers to knockdown target gene expression in actively growing organisms are particularly promising.

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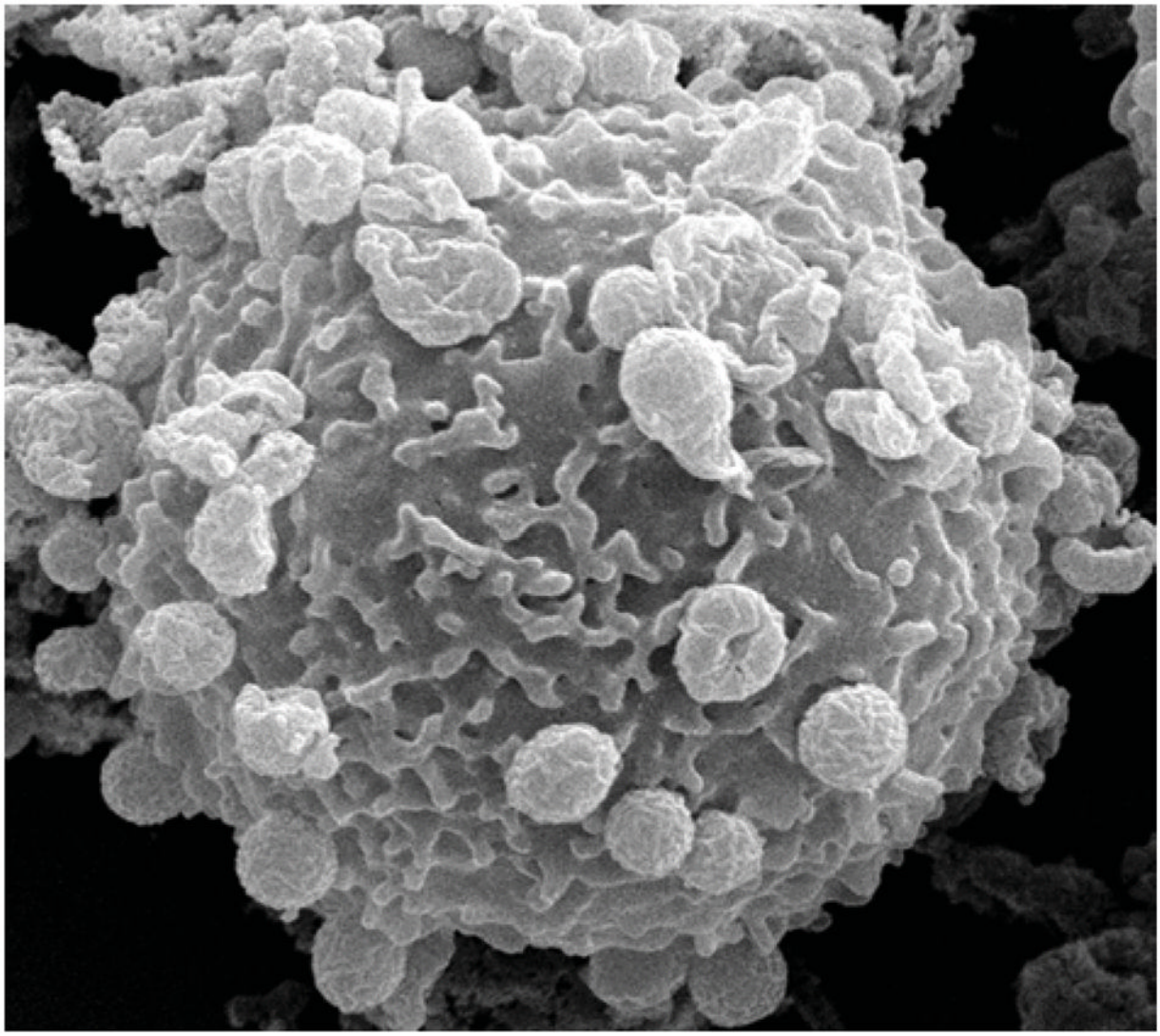

Figure 1.
Scanning electron micrograph of a human neutrophil with numerous *A. phagocytophilum* bacteria attached to the host cell surface.

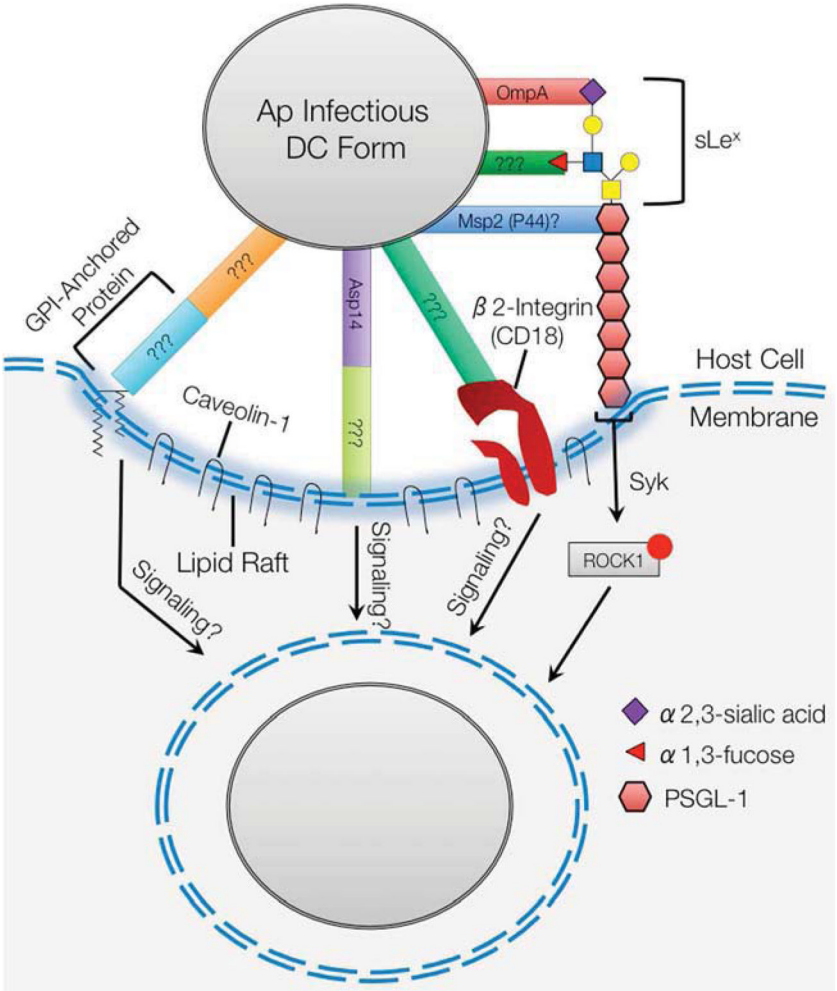

Figure 2.

A. phagocytophilum cellular invasion. The infectious dense-cored form of the bacterium utilizes multiple surface proteins to cooperatively bind PSGL-1 at an N- terminal amino acid sequence and α2,3-sialic acid and α1,3-fucose of the sLe^x tetrasaccharide that caps PSGL-1. Binding to PSGL-1 initiates a signaling cascade that involves Syk and phosphorylation of ROCK1 and promotes bacterial internalization. *A. phagocytophilum* also binds β2-integrin, but the relevance of this interaction is only detectable under conditions of shear flow. The pathogen binds at lipid rafts enriched for caveolin-1. Bacterial adherence to and infection of host cells from which GPI-anchored proteins have been cleaved is significantly impaired. OmpA, specifically a domain contained with amino acids 19–74, binds to α2,3-sialic acid and is the only *A. phagocytophilum* invasin for which a receptor is known. Asp14, specifically a domain contained with amino acids 100–124, is also important for invasion. Msp2 (P44), or a least a paralog thereof, may be involved in binding to PSGL-1. ???, unidentified *A. phagocytophilum* adhesins/invasins or host cell receptors.

Table 1

Rab GTPases that are recruited to the *A. phagocytophilum*-occupied vacuole

Rab GTPase[a]	Site(s) of Action[b]	Transport Function(s)
Rab1	ER[c]	ER to Golgi, Golgi to ER, IC to PM
Rab4A	Clathrin-dependent recycling endosomes	Rapid endocytic recycling
Rab10	ER Clathrin-independent recycling endosomes TGN	Dynamic ER tubules Endocytic recycling TGN to PM TGN to RE
Rab11A	Clathrin-independent recycling endosomes TGN	Endocytic recycling TGN to PM
Rab14	Clathrin-independent recycling endosomes TGN	Endocytic recycling TGN to EE
Rab22A	Clathrin-independent recycling endosomes TGN	Endocytic recycling EE to TGN
Rab35	Clathrin-dependent recycling endosomes	Endocytic recycling

[a] Rab GTPase localization data from Huang et al (2010)

[b] Site of Action and Transport Function data summarized from references by Grant & Donaldson (2009), Stenmark (2009), Liu & Storrie (2012), and English & Voeltz (2013)

[c] ER, endoplasmic reticulum; IC, pre-Golgi intermediate compartment; PM, plasma membrane; RE, recycling endosomes; TGN, *trans*-Golgi network; EE, early endosome