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Exploitation of evolutionarily conserved amoeba and mammalian processes by *Legionella*

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Abstract

Legionella pneumophila proliferates within various protists and metazoan cells, where a cadre of ~300 effectors is injected into the host cell by the defect in organelle trafficking/intracellular multiplication (Dot/Icm) type IVB translocation system. Interkingdom horizontal gene transfer of genes of protists and their subsequent convergent evolution to become translocated effectors has probably enabled *L. pneumophila* to adapt to the intracellular life within various protists and metazoan cells through exploitation of evolutionarily eukaryotic processes, such as endoplasmic reticulum-to-Golgi vesicle traffic, phosphoinositol metabolism, AMPylation, deAMPylation, prenylation, polyubiquitination, proteasomal degradation and cytosolic amino- and oligo-peptidases. This is highlighted by the ankyrin B (AnkB) F-box effector that exploits multiple conserved eukaryotic machineries to generate high levels of free amino acids as sources of carbon and energy essential for intracellular proliferation in protists and metazoan cells and for manifestation of pulmonary disease in mammals.

Keywords

prenylation; farnesylation; polyubiquitination; phosphatidylinositol; F-box; AnkB; proteasome

Intracellular life cycle of *L. pneumophila* within mammalian and protozoan cells

L. pneumophila is ubiquitous in aquatic habitats where it survives and replicates within a wide range of protists, such as amoeba and ciliated protozoa [1]. Upon transmission to humans, *L. pneumophila* replicates in alveolar macrophages leading to Legionnaires' disease [1,2].

The molecular and cellular aspects of infection by *L. pneumophila* in both protozoa and mammalian phagocytes are similar [3,4]. When either protozoan hosts or macrophages engulf *L. pneumophila*, the bacterium evades targeting the *Legionella*-containing vacuole (LCV) to degradation by the lysosomes [1,5,6]. Instead, mitochondria and endoplasmic reticulum (ER)-derived vesicle are rapidly recruited to the LCV [4–7]. Within a few minutes, the LCV becomes remodeled into an ER-derived compartment [1,6–8]. Within the ER-remodeled LCV, *L. pneumophila* replicates to high numbers until it disrupts the phagosomal membrane and escapes into the host cell cytosol where the last one to two

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rounds of replication occurs [9]. This culminates in the expression of various bacterial virulence traits and bacterial escape to the extracellular environment [10,11].

The ability to evade phagocytic killing and to remodel the LCV within both hosts is mediated by the type IVB secretion system known as the Dot/Icm secretion system [6] that injects ~300 effectors into the host cell [12]. This exceptional number of translocated bacterial effectors includes many substrates harboring eukaryotic domains leading to interference or exploitation of various eukaryotic cellular processes and some of the cellular processes are highly conserved through evolution [7,13–16]. This may not be surprising because the molecular and cellular aspects of intracellular proliferation of *L. pneumophila* within protists and metazoan cells are similar. This review highlights the conserved eukaryotic machineries exploited by *L. pneumophila* and the translocated bacterial effectors involved.

Exploitation of the eukaryotic prenylation pathway by *L. pneumophila*

Many of the injected effectors of *L. pneumophila* are targeted into various host cell membranes and the LCV membrane [13,14,17–20], through exploitation of various cellular processes such as prenylation [21] or lipid phosphatidylinositol (PI) derivatives [22]. Prenylation is an evolutionarily conserved post-translational lipid modification of eukaryotic proteins that increases protein hydrophobicity to enable anchoring a hydrophilic protein to the lipid bilayer of membranes or to associate with other hydrophobic proteins [21]. Prenylation is initiated by covalent linkage of a farnesyl group (15C) or geranyl-geranyl group (20C) to a cysteine residue in a conserved C-terminal CaaX motif of eukaryotic proteins by farnesyl transferase (FTase) or two geranyl-geranyl tranferases (GGTases I and II), respectively [21]. The terminal aaX tripeptide is then cleaved by the endoprotease Ras-converting enzyme-1 (RCE1) followed by carboxyl methylation by the isoprenyl cysteine carboxyl methyl transferase (IcmT) and membrane association of the prenylated proteins (Figure 1a). The enzymatic machinery of prenylation is evolutionarily conserved in eukaryotes but is not present in prokaryotes. Prenylation plays a key role in functional activity of many eukaryotic proteins such as the Ras and Rab proteins, involved in signaling and vesicle fusions, respectively [21].

L. pneumophila is the first example of a pathogen that hijacks the eukaryotic prenylation machinery to anchor several bacterial effectors into the pathogen-containing vacuolar membrane and other host membranes [13,14,19–21]. Among these membrane-associated effectors is the well characterized ankyrin B (AnkB), which is found in all sequenced *L. pneumophila* strains (*lpg2144, lpa03071, lpl2072, lpp2082, lpc1593, legAU13)* and is present in 211 of 217 tested strains [23–25]. During macrophage and amoeba infection with *L. pneumophila*, the C-terminal CaaX (CLVC) motif of AnkB is modified by the host farnesylation machinery [13,14,20]. This host-mediated farnesylation is essential for anchoring AnkB to the cytosolic face of the LCV membrane (Figure 2) [13,14,21]. The CaaX (CLVC) motif of AnkB is essential for anchoring ectopically expressed AnkB to the plasma membrane of human cells and amoeba [13,14,21]. A substitution of the conserved cysteine residue in the CaaX motif with alanine ($AnkB^{169}C/A$) results in redistribution of ectopically expressed $AnkB^{169}C/A$ into the cytosol of human cells and amoeba. Expression of the $AnkB^{169}C/A$ variant by *Legionella* during infection results in failure to anchor AnkB to the LCV membrane, severe defects in intracellular proliferation in amoeba and macrophages, and attenuation of intrapulmonary proliferation in the mouse model, similar to ablation of *ankB* [14]. Disruption of the host farnesylation machinery by chemical inhibitors or by silencing FTase by RNAi results in loss of membrane anchoring and biological function of AnkB [14].

Covalent linkage of the 15C farnesyl moiety to cysteine within the CaaX motif occurs in the cytoplasm where FTase is localized. Then cleavage of the aaX terminal tripeptide and methylation of the farnesyl group takes place at the cytosolic face of the ER where RCE1 and IcmT are located (Figure 1a) [21]. However, host-mediated farnesylation of AnkB probably occurs locally at the ER-derived LCVs in both hosts, where the three host farnesylation enzymes FTase, RCE1 and IcmT are recruited to the LCV in a Dot/Icm-dependent manner. It is thought that there are specific Dot/Icm effectors involved in recruiting FTase, whereas IcmT and RCE1 are part of the ER [13,14].

Besides AnkB, the LCV is highly enriched with other prenylated proteins [13,14,20]. *In silico* analyses of four sequenced *L. pneumophila* genomes revealed the presence of at least 11 genes that encode CaaX motif-containing proteins and those proteins have been designated as prenylated effectors of *Legionella* (Pel)/CaaX motif protein (CMP) [19,20]. Most of the PelA-K effectors are translocated substrates of the Dot/Icm type IV secretion system, but their functions are unknown. During ectopic expression, Pels/CMPs are modified by the mammalian prenylation machinery and exhibit distinct membrane localization in HEK293 and COS-1 cells [19,20]. The Pels/CMPs exhibit a cytosolic shift in their cellular localization upon chemical inhibition of either FTase or GGTases [19,20]. Although none of the Pels/CMPs have been tested in amoeba, we speculate that some if not all of the Pels/CMPs are modified by the amoeba prenylation machinery because the LCV harboring the *ankB* null mutant is decorated with farnesylated proteins, which is mediated by the Dot/Icm secretion system [13,14]. The strategy of exploitation of the evolutionarily conserved host prenylation machinery is a remarkable example of pathogenic evolution of *Legionella* and its host adaptation to infect phylogenetically diverse hosts.

Numerous other bacterial pathogens have the potential to exploit the prenylation machinery to anchor their injected effectors into the pathogen-containing vacuolar membrane or other host membranes [20,21]. The *Salmonella* effector SifA was the first reported prenylation-dependent membrane-anchored effector that is also modified by host *S*-palmitoylation [21]. It is possible that prenylation inhibitors might be used as a target for therapeutic agents against *Legionella* and other pathogens that require the host prenylation machinery to cause disease.

Exploitation of host phosphoinositides to anchor *Legionella* effectors to the LCV

Many pathogens including *Listeria, Shigella, Salmonella, Brucella* and *Mycobacterium* spp. exploit host cell lipid PI derivatives to anchor their effector proteins to various host membranes to promote host cell invasion or establish a suitable replicative niche [26–28]. PI can be phosphorylated/dephosphorylated at the 3, 4 and/or 5 positions on the inositol ring by PI kinases or phosphatases, respectively [22]. The PI derivatives are highly conserved through evolution and play roles in various eukaryotic cellular processes, including cell signaling, actin remodeling and membrane trafficking in amoebae and mammalian cells [29].

The presence of PI derivatives on the cytosolic side of the LCV membrane allows *L. pneumophila* to employ them for anchoring some of the translocated effectors on the LCV [22]. The main PI derivative present on the LCVs is phosphatidylinositol (4) phosphate [PI(4)P], which is a Golgi marker that acts as a second messenger to mediate export of early secretory vesicles from ER exit sites [22]. A major PI(4)P-binding effector on the LCVs is SidC and its paralog SdcA [30]. Anchoring those effectors to the LCV membrane through PI(4)P promotes recruitment of ER vesicles to the LCV within amoeba and macrophages [31–33]. The SidM/DrrA effector is a competitor of SidC for free PI(4)P-binding sites on the

LCV (Figure 3). Anchoring SidM/DrrA to the LCV via PI(4)P recruits Rab1 to the LCVs. The SidM/DrrA effector modulates the activity of Rab1 through its guanine nucleotide exchange factor (GEF) activity [18], its GDI displacement factor (GDF) activity [34], and its AMPylation and deAMPylation of Rab1 [46–39] to promote recruitment of early secretory vesicles to LCV [5,7]. The GEF activity of SidM is promoted by the LidA effector, which is localized to the LCV. Anchoring LidA to the LCV membrane is achieved by interacting with PI(4)P and with lower affinity to PI(3)P (Figure 3) [38,39]. Interestingly, the Rab1-modulating SidM/DrrA is sufficient to stimulate non-canonical membrane vesicle fusion [31].

Another effector of *L. pneumophila* that interacts with PI(3)P is LpnE. The LpnE effector is a Sel1 repeat-containing protein, which is exported independent of both type II and the Dot/Icm type IV secretion systems, and is involved in the entry of *L. pneumophila* into macrophages and epithelial cells and for virulence in mice [40]. This effector binds exclusively to PI(3)P (Figure 3) [28], and to PI-metabolizing enzymes; the mammalian oculocerebrorenal syndrome of Lowe 1 (OCRL1) and its *Dictyostelium* homolog *Dictyostelium discoideum* 5-phosphatase 4 (Dd5P4), which hydrolyze PI(4,5)P_2 to yield PI(4)P [22].

Although SidC and its paralog SdcA are the only effectors known to bind PI(4)P within amoeba and macrophages [32,33], it is likely that other *L. pneumophila* effectors exploit host PI derivatives in a similar strategy within both hosts because the LCVs in both hosts is decorated with PI(4)P that interferes with signal transduction and promotes host vesicle trafficking [22,33]. Therefore, *L. pneumophila* exploits at least two evolutionary conserved eukaryotic processes, PI metabolism and prenylation, to anchor translocated effectors to the LCV membrane within phylogenetically diverse eukaryotic hosts.

Exploitation of eukaryotic ubiquitination by F-box effectors of *Legionella*

Ubiquitination is another evolutionarily conserved post-translational modification process that is essential for various eukaryotic processes but it is absent in prokaryotes [41]. Polyubiquitination involves covalent polymerization of the 76-amino acid ubiquitin monomers through one of the seven lysine residues in ubiquitin (K6, K11, K27, K29, K33, K48 or K63) [42]. Polyubiquitination through K48 linkages is a hallmark for degradation by the 26S proteasomes. However, K63 linkages in polyubiquitin modulate the biological activity or location of the polyubiquitinated proteins.

Ubiquitination is mediated by sequential action of three classes of enzymes (E1–E3) [42]. The ubiquitin activating enzyme (E1) transfers ubiquitin to a conjugating enzyme (E2), followed by a substrate-specific E3 ubiquitin ligase that links ubiquitin to the target protein (Figure 1b) [42]. Several classes of E3 ubiquitin ligases have been described based on the presence of U-box or F-box domains [43]. One well-characterized E3 ubiquitin ligase family is the multi-protein SCF1 complex that is composed of SKP1, Cul1 and RBX1 [44]. It is not surprising that many pathogens exploit host ubiquitination [45,46], which is achieved via pathogen-injected effectors that mimic E3 ubiquitin ligases or by *bona fide* U/F-box effectors [16,45–48].

In silico genomic analyses of *Legionella* reveals the presence of F- or U-box containing effector encoding genes. The AnkB F-box effector is one of very few Dot/Icm-translocated effectors that plays a major role in intracellular replication within both macrophages and protozoa, and is the only effector known to be required for intrapulmonary proliferation in mice [11,16,23,24,48]. The AnkB effector harbors an F-box domain, two ankyrin (ANK) protein–protein interaction domains in addition to the c-terminal farnesylation CaaX (CLVC) motif [11,16,23,24,49]. In both macrophages and protozoa, AnkB functions as a

bona fide F-box protein where it interacts with the host SCF1 complex and functions as a platform for the docking of polyubiquitinated proteins to the LCV membrane in macrophages and amoeba (Figure 2) [13,16,48,49].

In addition to AnkB, the genome sequence of *L. pneumophila* reveals the presence of at least another four F-box-containing proteins [47]. These include Lpg2224 (PpgA), Lpg2525 and LicA [50]. LegU1 is another F-box effector that interacts with the SCF1 complex, and targets a host protein for ubiquitination. However, in the Philadelphia strain, the mutant that harbors in-frame deletion of the F-box domain of *legU1* does not display any defects in either the accumulation of polyubiquitinated proteins on the LCV or intracellular proliferation as compared to the wild type strain in either primary bone marrow-derived mouse macrophages or amoeba [50]. Thus, multiple *L. pneumophila* F-box proteins hijack the highly conserved SCF1 ubiquitin ligase of amoeba and mammalian cells.

Exploitation of eukaryotic ubiquitination by U-box effectors of *Legionella*

The *Legionella* U-box protein (LubX) effector mimics the host E3 ubiquitin ligase activity during mammalian infection [51]. A U-box is a class of eukaryotic E3 ubiquitin ligases that serves as a docking site for E2 ubiquitin conjugating enzymes [52]. LubX harbors two U-box domains where both U-box domains are essential for the LubX biological activity [51]. LubX acts as a negative regulator of the SidH effector by targeting it for proteasomal degradation at later stages of the infection [51]. Interestingly, although LubX triggers ubiquitination of mammalian Cdc2-like kinase 1 (Clk1), its role in the protozoan host has not been reported. It is likely that LubX mimics the host E3 ubiquitin ligase activity in similar fashion during the infection within both evolutionarily distant hosts. There is no doubt that identifying the host targets of U-box and F-box effectors will provide an insight into how *Legionella* exploits evolutionarily conserved E3 ubiquitin ligases of phylogenetically diverse hosts.

AnkB promotes proteasome-mediated generation of free amino acids essential as energy and carbon sources

Recent studies have shown that the polyubiquitinated proteins assembled by AnkB on the LCV are preferentially enriched for Lys48-linked polyubiquitinated proteins, which is a hallmark for proteasomal degradation, that generate 2–24 amino acid peptides [53]. Substitution of Lys48 to Arg abolishes the decoration of the LCV with polyubiquitinated proteins and blocks intracellular proliferation [53]. This indicates that turnover of Lys48-linked polyubiquitinated proteins is needed for continuous and dynamic remodeling of the LCV or that proteasomal degradation is needed to generate a surplus of amino acids for bacterial proliferation of *L. pneumophila*. Inhibition of the proteasome, or the host amino- and oligo-peptidases that degrade the short peptides generated by proteasomal degradation [54,55], blocks intracellular proliferation. However, both inhibitions are bypassed by excess amino acid supplementation [53]. Interestingly, failure of wild type *L. pneumophila* to proliferate in human cells overexpressing ubiquitin variants with Lys48-Arg substitution is accompanied with a strong bacterial starvation response for amino acids, and this response is completely suppressed upon supplementation of the infected cells with excess amino acids, which also rescue intracellular bacterial proliferation [53]. Cells infected by the wild type strain have high levels of free amino acids compared to the *ankB* mutant-infected cells, which are similar to uninfected cells [53]. Importantly, supplementation of *ankB* mutant-infected amoeba or macrophages with excess amino acids rescue the mutant for intracellular proliferation and suppress its amino acids starvation response, similar to genetic complementation by the native *ankB* allele [53]. Screening the 20 amino acids individually for their ability to rescue the *ankB* mutant for intracellular proliferation in macrophages and

amoeba has shown that cysteine or serine supplementation rescue the *ankB* mutant as efficiently as the mixture of amino acids [53].

Cysteine and serine are converted by *Legionella* into pyruvate that feeds the tricarboxylic acid (TCA) cycle, and *L. pneumophila* relies on amino acids as the major sources of carbon and energy production through the TCA cycle. Interestingly, supplementation of amoeba or macrophages with pyruvate or citrate rescues the *ankB* mutant for intracellular proliferation within amoeba and macrophages and for its starvation response, similar to the supplementation by the mixture of amino acids. Remarkably, injection of mice with the mixture of amino acids or with cysteine specifically rescues the *ankB* mutant for intrapulmonary proliferation, similar to genetic complementation [53]. These findings indicate that the ultimate goal of exploitation of multiple evolutionarily conserved eukaryotic processes by AnkB is to generate amino acids by promoting host proteasomal degradation of the K48-linked polyubiquitinated proteins. The generated amino acids are required as sources of carbon and energy for intracellular growth of *Legionella* (Figure 2). Because either cysteine or serine are sufficient to rescue the *ankB* mutant, it is most likely that *L. pneumophila* has sufficient levels of cellular amino acids with the exception of high levels of a major source of carbon and energy to feed the TCA cycle. Interestingly, cysteine and serine are non-essential amino acids for *Acanthamoeba* or human cells. In addition, *Legionella* is auxotrophic for Arg, Cys, Ile, Leu, Met, Val and Thr, which include all the amino acids that *Acanthamoeba* is auxotrophic for (Arg, Ile, Leu, Met and Val) [56]. With the exception of Cys, human cells are auxotrophic for all the amino acids that *Legionella* is auxotrophic for, but Cys is relatively scarce in human cells. This indicates a tremendous patho-adaptation of *Legionella* to the intracellular life within *Acanthamoeba* and human cells and amoeba. The mechanism of generation of a surplus of amino acids through promoting host proteasomal degradation by an F-box effector *Legionella* is the first example of a microbial strategy dedicated to generate sources of carbon and energy needed for microbial proliferation *in vivo*. Remarkably, the amino acids essential for *Legionella*, due to deficiency of *de novo* synthesis, are also essential for the two evolutionarily distant hosts, with the exception of Cys, which is scarce in human cells. Whether other organisms exploit similar, or idiosyncratic, strategies to access host sources of carbon and energy *in vivo* remains to be explored [57].

Evolution of *Legionella* translocated effectors

The high number of *L. pneumophila* eukaryotic-like proteins that exhibit structural and functional mimicry of eukaryotic proteins may reflect the diversity of eukaryotic pathways that are exploited by *L. pneumophila* during infection of phylogenetically diverse eukaryotic hosts [13,16,23,58]. Even though ~10% of the *Legionella* genome is dedicated to injecting effectors into host cells, most of these effectors do not play critical roles in intracellular proliferation in macrophages, suggesting potential host tropism for redundant effectors that are tailored to exploit a certain host in the environment. *In silico* analysis of the *L. pneumophila* genome identified several genomic islands that were indispensable for bacterial growth within protists but not in macrophages or nutrient-rich media [59]. Interestingly, the necessity of individual genomic islands for intracellular proliferation varies among the amoeba species. This indicates that *Legionella* is a generalist pathogen, which distinguishes this bacterial species from most other specialist pathogens [59].

It is likely that horizontal gene transfer (HGT) and convergent evolution are two key mechanisms for the evolution of eukaryotic-like effectors during long term co-evolution of *L. pneumophila* with diverse primitive eukaryotic hosts (Box 1). The HGT theory is supported by several lines of evidences. First, *L. pneumophila* is naturally competent for DNA uptake and the Dot/Icm translocation system can exchange DNA between bacteria

through conjugation [60]. Second, the difference of G + C content of the eukaryotic-domain encoding genes compared to the rest of the genome suggests more recent acquisition of those genes [61]. Subsequent multiple convergent evolutions of the host gene acquired through HGT is essential for the encoded protein to become a translocated effector functional in the host (Box 1).

Box 1

Obstacles for a eukaryotic gene to encode a translocated functional bacterial effector

The long close association between *Legionella* and protozoa in the environment provides the opportunity for HGT to occur. The acquired gene must undergo a number of changes, probably through convergent evolution, to become a translocated functional effector. This involves multiple processes such as removal of the introns, acquisition of a prokaryotic promoter along with regulatory elements and a ribosomal binding site. To become an effector recognized and translocated by the Icm/Dot secretion system, a translocation signal must be acquired along with domains that interact with chaperones needed for effector stability and recognition by the translocation machinery [71]. To become biologically functional, some effectors must also acquire eukaryotic post-translational modification motifs such as prenylation or the *S*-palmitoylation motif. Convergent evolution of the genes acquired through interkingdom HGT is probably the main mechanism of long term evolution to result in translocated effectors that exhibit biological function in the host cytosol.

It seems more likely that AnkB has been acquired by *L. pneumophila* through interkingdom HGT as a whole eukaryotic gene or even as fragments during the long intimate association with primitive unicellular eukaryotes such as amoeba or even indirectly from another intracellular bacterium or endosymbiont of unicellular eukaryotes [47,72]. The domain architecture of AnkB of *L. pneumophila* more closely resembles the F-box proteins of unicellular eukaryotes, where the protein–protein interaction domains of F-box proteins are ankyrin domains (ANK) [47]. By contrast, ANK domains are absent from F-box proteins of metazoans such as mammals. Instead, mammalian F-box proteins have WD repeats or leucine-rich repeats (LRR) as protein–protein interaction domains [73]. Interestingly, all eukaryotic F-box proteins are cytosolic, whereas AnkB is a membrane-anchored protein. The CaaX motif of AnkB could have gradually evolved through convergent evolution to be anchored to the LCV membrane. Nevertheless, acquisition of the CaaX motif through horizontal gene transfer cannot be excluded [13,14]. There is a growing list of *L. pneumophila* effectors that have probably been acquired by HGT from protozoan host, such as the LegK1 and LegK3, which exhibit similarity to amoeba protein kinases [72]. Moreover, the *L. pneumophila* LegS2 effector is highly homologous to the protozoan sphingosine-1-phosphate lyase (SPL) [71]. Evidence shows that many genes of *Legionella* have been acquired from other organisms that reside within amoeba such as Lpg2416, which is an ankyrin-containing protein that has homologs only in *Acanthamoeba polyphaga* mimivirus [74].

Patho-adaptation of *L. pneumophila* to metazoan hosts

The accumulation of acquired genetic materials through HGT helps *L. pneumophila* to increase its fitness and adaption to diverse hosts, which provides selective pressure for maintaining the newly acquired genes and expanding the host range [59]. With the presence of high numbers of translocated bacterial effectors, functional redundancy is observed, where mutation in a gene does not have any phenotype in either macrophages or protozoa. It is likely that the eukaryotic-like proteins that exploit conserved eukaryotic processes initially

evolved to manipulate diverse protozoan hosts. However, the presence of conserved targets in mammalian cells enables *L. pneumophila* to exploit conserved pathways in mammalian cells via the same effector such as AnkB [13,14,16,48]. However, *Legionella* modulates mammalian-specific processes that are absent from unicellular eukaryotes [62]. One example is modulation of programmed cell death by *L. pneumophila*, where it is thought that *L. pneumophila* generates a delicate balance between triggering both pro-apoptotic and anti-apoptotic pathways to render mammalian cells permissive for intracellular proliferation [12,62]. Even though caspase-3 is activated in *L. pneumophila*-infected human macrophages, *L. pneumophila* inhibits apoptosis by triggering anti-apoptotic pathways via SdhA and SidF effectors [12] until later stages of infection, giving enough time for *L. pneumophila* to replicate [4,63–65]. However, *L. pneumophila* is incapable of inducing apoptosis in amoebae [66] because amoeba has a primitive program of cell death that lacks apoptosis regulatory proteins such as the Bcl2 and caspase families of proteins [67]. In addition, modulation of activation of the inflammasomes and nuclear factor-kappaB (NF-κB) by *Legionella* is also specific to mammals and is absent from unicellular eukaryotes [62,68]. The Dot/Icm-mediated translocation of at least four kinases (LegK1–LegK4) to modulate host phosphorylation activities by *L. pneumophila* is a clear indication of interference with host signaling by *L. pneumophila*. Some of this interference with host signaling may be limited to the mammalian host, such as phosphorylation and activation of NF-κB by LegK1 [10,68–70]. It may also be limited to the protozoan host, such as the kinase activity of LegK2 is essential for remodeling the LCV by the ER, and for intracellular proliferation in *Acanthamoeba* [68]. Whether LegK2 is functionally active and is required for intracellular proliferation in mammalian cells is still to be determined. Therefore, it is most likely that *Legionella* required a lengthy period of interaction with metazoan hosts, such as mammals, before pathogenesis of disease manifestation was exhibited.

Concluding remarks

Approximately 10% of the coding capacity of the *L. pneumophila* genome is dedicated to translocation of ~300 effectors, which is the largest cadre of effectors relative to any other known pathogen. This exceptional number of effectors is likely to have equipped *L. pneumophila* with the capacity to exploit multiple protist hosts with effectors tailored to certain hosts. Many host pathways are exploited, and the few discovered so far are undoubtedly the tip of the iceberg of this bacteria–host interaction. Much of the host exploitation is achieved by eukaryotic-like effectors that mimic functions of eukaryotic proteins. Genes encoding effectors have probably been acquired by HGT through long adaptation and co-evolution with unicellular eukaryotes in the aquatic environment and by subsequent convergent evolution such as the AnkB, LegK3 and LegS2 effectors that exhibit high similarity to amoeba proteins. There is no doubt that protozoa have been a productive ‘training’ ground for intracellular proliferation of *L. pneumophila*, which has a remarkable capacity to exploit the structural and molecular conservation of cellular processes in unicellular eukaryotes to emerge and infect more evolved mammalian cells. The AnkB effector is a fascinating example of exploitation of multiple evolutionarily conserved processes, and its biological function is ultimately dedicated to generate a surplus of host amino acids as sources of carbon and energy needed for intracellular proliferation within protists and metazoan hosts. Many new exciting studies on the role of SidM/DrrA in modulating Rab1 activity in vesicle traffic in metazoan cells have not examined the role in protists [5,7,18,34–39]. However, considering the high conservation of vesicle traffic through evolution, we speculate that the role of SidM/DrrA in modulating Rab1 activity to intercept vesicle traffic from the ER [5,7] is also exhibited in amoeba [18,34–39].

However, the ability of *Legionella* to exploit many highly evolved eukaryotic processes, such as the inflammasomes, pro- and anti-apoptotic pathways that are absent in protozoa

suggests long term co-evolution of *Legionella* with more evolved eukaryotic hosts. Regardless of the origin of the acquired gene by HGT, the gene must undergo long term evolution before the encoded proteins becomes an injected effector that modulates cellular processes. It is possible that many *Legionella* eukaryotic-like proteins that are not translocated are still undergoing evolution of acquisition of various motifs needed for translocation. Further understanding of the molecular aspects of *Legionella*–protist interaction will continue to fascinate us and educate us about a remarkable evolution of a bacterial pathogen from interacting with protists to its patho-adaptation to metazoans.

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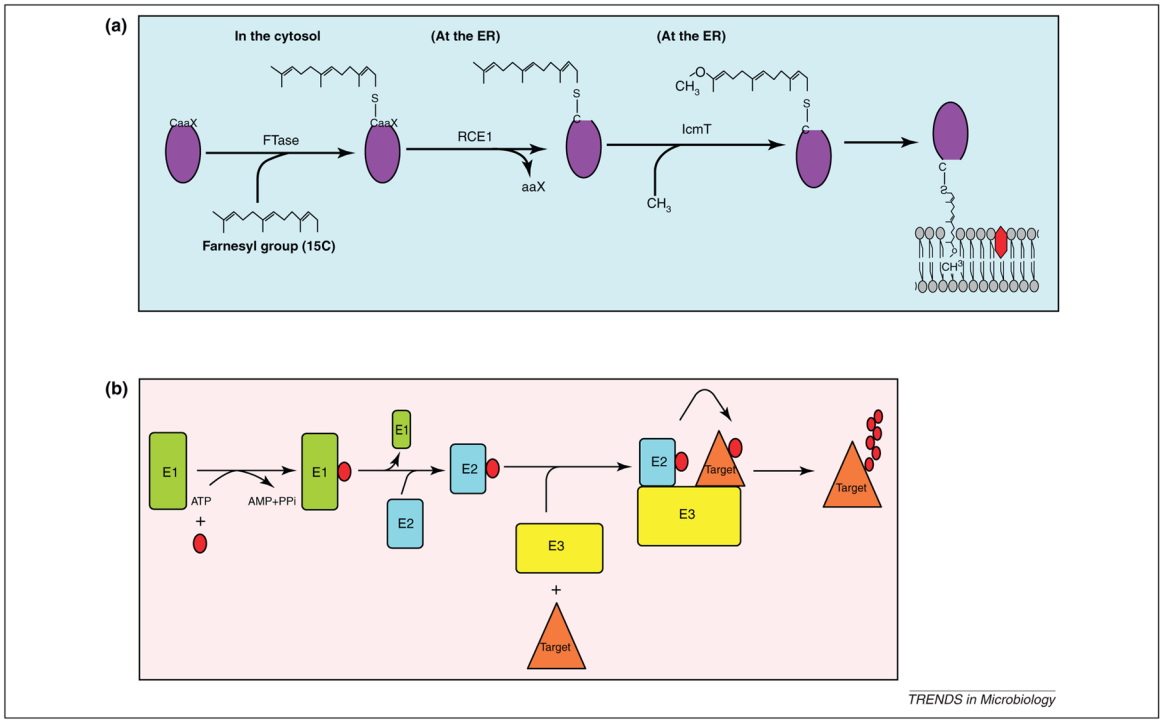

Figure 1.

Evolutionarily conserved eukaryotic pathways exploited by *Legionella* in amoeba and mammals. **(a)** Farnesylation modification of eukaryotic proteins. Proteins that contain CaaX motif are recognized by the cytosolic farnesyl transferase (FTase), which modifies the protein by the addition of a 15-carbon farnesyl group to the conserved cysteine residue within the C-terminal CaaX motif. The farnesylated proteins are trafficked to the endoplasmic reticulum (ER) where the aaX tripeptide is cleaved by the Ras-converting enzyme-1 (RCE1) protease, followed by methylation of the farnesyl group by the isoprenyl cysteine carboxyl methyl transferase (IcmT) enzyme. The modified protein is subsequently targeted to specific membranes. **(b)** The eukaryotic ubiquitination pathway. Ubiquitin modification is an ATP-dependent process involving the sequential action of three enzymes (E1–E3). Ubiquitin activating enzyme (E1) activates ubiquitin (red circle) via the generation of a high energy thioester bond between ubiquitin and an E1 cysteine residue. The activated ubiquitin is then transferred to ubiquitin conjugating enzyme (E2). The final step of the ubiquitination is where substrate-specific ligase (E3) binds simultaneously to E2 and the substrate, resulting in transfer of ubiquitin monomer from the E2 enzyme to a target protein. The fate of the modified protein is determined by the lysine linkages utilized in polyubiquitination.

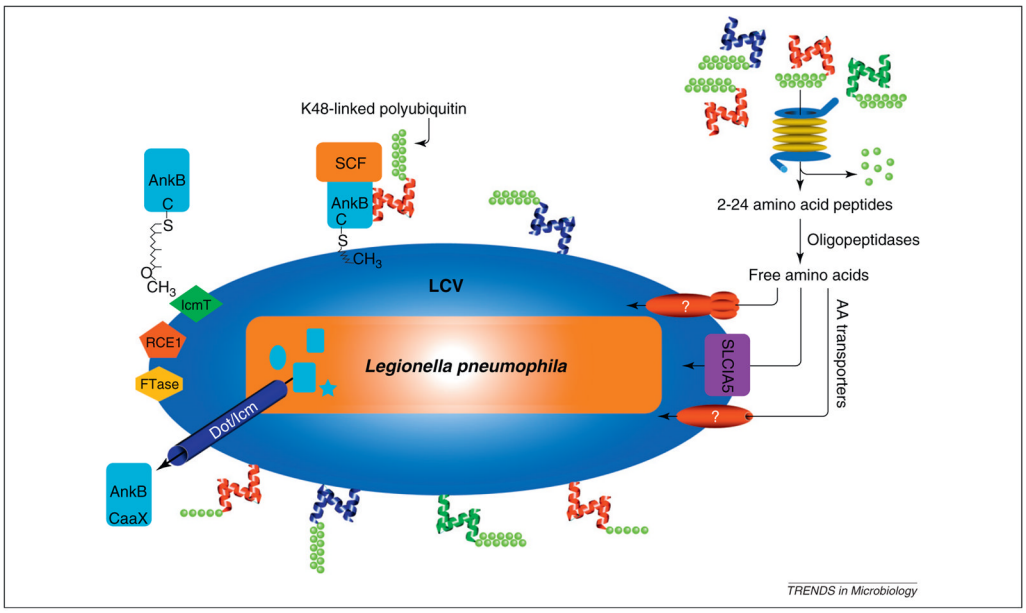

Figure 2.

Ankyrin B (AnkB)-mediated generation of amino acids through promoting proteasomal degradation of amoeba and mammalian hosts. Upon translocation of AnkB by the defect in organelle trafficking/intracellular multiplication (Dot/Icm) type IV secretion system, AnkB is farnesylated by the three host enzymes farnesyl transferase (FTase), Ras-converting enzyme-1 (RCE1) and isoprenyl cysteine carboxyl methyl transferase (IcmT) that are recruited to the *Legionella*-containing vacuole (LCV) by the Dot/Icm system. Upon farnesylation, AnkB is anchored into the cytosolic face of the LCV membrane, where it interacts with the SCF1 E3 ubiquitin ligase complex and acts as a platform for the docking of K48-linked polyubiquitinated proteins to the LCV. The K48-linked polyubiquitinated proteins are targeted for proteasomal degradation that generates short peptides that are rapidly degraded by cytosolic oligo- and amino-peptidases. This results in generation of high levels of free amino acids that are imported to the LCV by various amino acid (AA) transporters, such as SLC1A5. The whole process is highly conserved and is essential for intracellular proliferation of *Legionella* in amoeba and human cells and for intrapulmonary proliferation in mice. Adapted from [53].

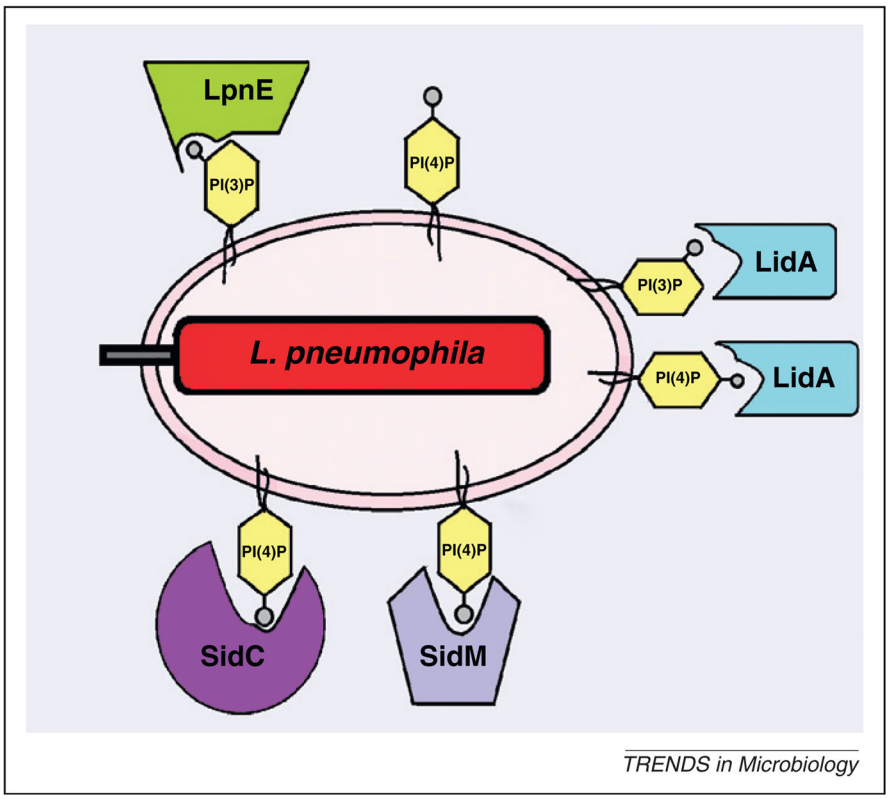

Figure 3.
Exploitation of host cell lipid phosphatidylinositol (PI) derivatives to anchor *Legionella pneumophila* effectors. Many defects in organelle trafficking/intracellular multiplication (Dot/Icm)-translocated effectors such as SidC, SidM, LidA and LpnE are anchored to the *Legionella*-containing vacuole (LCV) membrane through PI(4)P or PI(3)P to interfere with signal transduction and promote host vesicle trafficking.