Legionella pneumophila is an aquatic organism that interacts with amoebae and ciliated protozoa as the natural hosts, and this interaction plays a central role in bacterial ecology and infectivity. Upon transmission to humans, L. pneumophila infect and replicate within alveolar macrophages causing pneumonia. Intracellular proliferation of L. pneumophila within the two evolutionarily distant hosts is facilitated by bacterial exploitation of evolutionarily conserved host processes that are targeted by bacterial protein effectors injected into the host cell by the Dot/Icm type VIb translocation system. Although cysteine is semi-essential for humans and essential for amoebae, it is a metabolically favorable source of carbon and energy generation by L. pneumophila. To counteract host limitation of cysteine, L. pneumophila utilizes the AnkB Dot/Icm-translocated F-box effector to promote host proteasomal degradation of polyubiquitinated proteins within amoeba and human cells. Evidence indicates ankB and other Dot/Icm-translocated effector genes have been acquired through inter-kingdom horizontal gene transfer.

**Legionnaire Disease**

The first recognized outbreak of Legionnaire disease occurred in 1976 during the 56th annual American Legion Convention in Philadelphia. There were 180 individuals who were diagnosed with severe pneumonia and of these individuals, 29 died. Less than one year after the outbreak the causative agent of the disease was isolated. The bacterium, which was designated Legionella pneumophila, is a gram-negative facultative intracellular bacterium that proliferates within alveolar macrophages, causing Legionnaire disease. There are more than 50 species of Legionellae, but L. pneumophila continues to be responsible for more than 80% of cases of Legionnaire disease in most of the world. The exception is Western Australia, where L. longbeachae is the most predominant species in causing disease, but its pathogenesis is distinct from L. pneumophila.

Legionnaire disease and Pontiac fever, which is a flu-like illness caused by Legionella, have only emerged within the past few decades due to human alterations to the environment. These alterations include the use of air conditioning systems, whirlpools, and water cooling towers that generate aerosols as a vehicle to transmit Legionella from aquatic sources (Fig. 1). To date there has been no documented cases of L. pneumophila transmission between individuals, and transmission from the environment to the human host is considered to be the main mode of transmission of L. pneumophila. Once L. pneumophila infects the human host, its intracellular lifecycle has a striking similarity to that within the evolutionarily distant natural host, amoebae (Fig. 1).

**Legionella–Amoebae Interaction**

L. pneumophila is ubiquitous in freshwater environments as well as many man-made water systems worldwide, often in close association with freshwater protozoa. L. pneumophila replicate at temperatures of 25–42 °C with an optimal growth temperature of 35 °C. Consistent with what L. pneumophila would encounter in the environment, motility and adherence to host cells are optimal at temperatures below 37 °C. When the temperature in the aquatic environment is increased, the balance between bacteria and amoebae can shift, which results in rapid multiplication of L. pneumophila. Encysted amoeba is a highly resistant developmental stage that contributes to the resistance of intracellular L. pneumophila to different chemical and physical agents. Therefore, the relationship between L. pneumophila and amoebae plays an important role in ecology and pathogenicity of the bacterium.

One mechanism by which the bacteria are released from amoebae is within excreted vesicles similar to exocytosis of food vacuoles. L. pneumophila can still be cultured after 6 mo of residence within excreted vesicles. The number of bacteria isolated from water sources of transmission to humans during Legionnaire disease outbreaks is usually low or undetectable. It is thought that the enhanced infectivity of L. pneumophila as
A result of growth within amoebae could compensate for the low infectious dose in the water sources.\textsuperscript{17-20} The ability of \textit{L. pneumophila} to parasitize human macrophages and to cause human disease is thought to be a consequence of its prior adaptation to intracellular growth within various protozoan hosts.\textsuperscript{7,8} This is most likely due to bacterial acquisition of eukaryotic genes during its co-evolution with amoebae and adaptation to the intracellular life within primitive eukaryotic hosts.\textsuperscript{7,21-24}

\textit{L. pneumophila} and amoebae have been isolated from the same source of infection during outbreaks of Legionnaire disease.\textsuperscript{8} The isolated amoebae have also been shown to support intracellular replication of \textit{L. pneumophila}.\textsuperscript{25} It has been shown that \textit{L. pneumophila} that cannot be cultured in vitro using classical methods can be resuscitated and proliferate if they are co-cultured with amoebae.\textsuperscript{8,26-27} \textit{Dictyostelium discoideum} has been adapted as a genetically amenable amoeba model system to decipher molecular and cellular bases of \textit{L. pneumophila}-amoeba interaction.\textsuperscript{28} Therefore, amoebae are not only the environmental host for this human pathogen, but constitute a genetically amenable model system to study pathogenesis of \textit{L. pneumophila}. These findings demonstrate that studies on the \textit{L. pneumophila}-amoeba interaction will continue to contribute to our knowledge of the central role of the amoeba host in the pathogenesis of these bacteria.

\textbf{Amoebae Aid in the Persistence of \textit{Legionella pneumophila} in the Environment}

Amoebae not only enhance the pathogenicity of \textit{L. pneumophila}, but they also enable the bacteria to persist in the environment. Fourteen species of amoebae, with \textit{Harmonamoeba} and \textit{Acanthamoeba} being the most prominent, and two species of ciliated protozoa have been shown to support intracellular replication of \textit{L. pneumophila}.\textsuperscript{8} \textit{L. pneumophila} infects the trophozoite form of amoeba and the presence of the bacteria within amoebae serves to protect the bacteria from harsh environments.\textsuperscript{22} \textit{L. pneumophila} does not proliferate within encysted amoeba but when conditions become unfavorable, protozoa can differentiate from their trophozoite form into a cyst form that protects the organisms and ensures their survival (Fig. 1). \textit{L. pneumophila} released from free-living amoebae also show an increased resistance to harsh conditions compared with those grown in vitro.\textsuperscript{29-32} When compared with bacteria grown in vitro, bacteria grown in amoebae have changes in biochemistry, physiology, and virulence potential.\textsuperscript{32} These changes include an enhanced resistance to chemicals and antibiotics, an altered fatty acid profile and protein profile, shorter size, motility, an increased ability to infect amoebae and mammalian cells, an increase in environmental fitness, and an increase in uptake.\textsuperscript{8,19,20,26,27} In addition, the bacteria found within vesicles excreted from amoebae are highly resistant to biocides while the vesicle is resistant to freezing and sonication.\textsuperscript{37} After prolonged starvation of \textit{L. pneumophila} or treatment with chlorine that renders \textit{L. pneumophila} non-culturable in aquatic environments, the bacteria can’t be cultured on rich media but they can be resuscitated by infection of amoebae, clearly indicating the remarkable protection of \textit{L. pneumophila} through its intracellular niche within amoebae.\textsuperscript{26,27}

Numerous methods have been employed to attempt to eradicate \textit{L. pneumophila} from aquatic environments, with little success. These attempts, which include chemical biocides, overheating water, and UV irradiation, have been successful for short periods after which the bacteria can be again detected. It has been suggested that in order to eradicate \textit{L. pneumophila} from aquatic environments continuous treatments effective against both the bacteria and the protozoan host should be employed.\textsuperscript{8,12,27-33}

\textbf{Legionella-Like Amoebal Pathogens}

There are \textit{Legionella}-like species that cannot be grown on bacteriologic media but must be co-cultured with protozoa and are referred to as \textit{Legionella}-like amoebal pathogens (LLAP).\textsuperscript{14} The LLAPs are closely related to \textit{Legionella} phylogenetically and acquired their name because of their ability to infect and multiply within amoebae.\textsuperscript{14} It is thought the LLAPs play a role in community-acquired pneumonia and usually act as a co-pathogen but not as the sole pathogen.\textsuperscript{34} Little is known about LLAPs and future studies are needed to gain a better understanding of the significance of these organisms in pulmonary infections.

\textbf{Entry and Intracellular Trafficking of \textit{L. pneumophila}}

\textit{L. pneumophila} infection of human alveolar macrophages is an accidental infection and is thought to be a diversion from its natural life cycle within amoebae. In the aquatic environment, \textit{L. pneumophila} resides in protozoa or in biofilms.\textsuperscript{35,36} Amoebae play a central role in the life cycle of \textit{L. pneumophila}, and this was first described in 1980.\textsuperscript{37} Upon initial interaction between \textit{L. pneumophila} and amoebae, \textit{L. pneumophila} is often engulfed by coiling phagocytosis but other forms of internalization also occur through a Gal/Gal-NAC specific receptor.\textsuperscript{38,39} After internalization of \textit{L. pneumophila} into the trophozoite of amoeba, proliferation occurs within the \textit{Legionella}-containing vacuole (LCV) followed by bacterial release from the cell to seek a new host (Fig. 1).\textsuperscript{40-42} Once amoebae or mammalian cells engulf \textit{L. pneumophila}, the bacteria evade the default trafficking pathways into the lysosomal network (Fig. 1). The LCV recruits host cell organelles, like mitochondria, ribosomes, and small vesicles to its surface.\textsuperscript{43} This accumulation begins during uptake into the cell and is completed within a few minutes.\textsuperscript{39} The ER-to-Golgi vesicle traffic is intercepted by the LCV and the LCV membrane becomes derived from the ER (Fig. 1).\textsuperscript{13,43} The LCV becomes rapidly decorated with polyubiquitinated proteins within amoebae and human cells.\textsuperscript{44-46} Following maturation of the ER-remodeled LCV and its decoration with polyubiquitinated proteins, rapid replication of \textit{L. pneumophila} commences (Fig. 1). During late stages of intracellular proliferation, \textit{L. pneumophila} escape from the LCV to the cytosol where the bacteria finish the last 1–2 rounds of proliferation along with phenotypic modulations in response to nutrient depletion in the host (Fig. 1).\textsuperscript{8,40,47}

The strategy used by \textit{L. pneumophila} to avoid lysosomes and to modulate cellular processes is dependent on the Dot/Icm type IVB secretion system.\textsuperscript{43,48,49} This secretion system injects ~300 effector proteins into the host cell, which accounts for ~10%
exponential phase of intracellular growth within macrophages and Acanthamoeba, but the function of most of these proteins has yet to be determined.

Figure 1. The environmental life cycle of L. pneumophila within protozoa. (1) Flagellated L. pneumophila infect protozoa in the aquatic environment. (2) The LCV evades the default endosomal–lysosomal degradation pathway and becomes rapidly remodeled by the ER through intercepting ER-to-Golgi vesicle traffic and becomes rapidly decorated with polyubiquitinated proteins in an AnkB-dependent manner. (3) Under unfavorable stress conditions, such as nutrient deprivation, amoebae encyst, and bacterial proliferation will not occur due to nutrient limitation. Under growth-permissive conditions for the amoeba, the LCV is decorated with polyubiquitinated proteins, which are targeted for proteasomal degradation leading to elevated cellular levels of amino acids (AA) that power bacterial proliferation of the wild-type strain, while the ankB mutant is defective in this process and is unable to grow despite formation of ER-remodeled replicative LCV. (4) During late stages of infection, the LCV becomes disrupted leading to bacterial egress into the cytosol where the last 1–2 rounds of proliferations are completed. Upon nutrient depletion (see magnified box), RelA and SpoT are triggered leading to increased level of ppGpp, which triggers phenotypic transition into a flagellated virulent phenotype followed by lysis of the amoeba and bacterial escape from the host cell. Excreted vesicles filled with bacteria are also released. The infectious particle is not known but may include excreted Legionella-filled vesicles, intact Legionella-filled amoebae, or free Legionella that have been released from host cell. (5) Transmission to humans occurs via aerosols generated from man-made devices and installations, such as cooling towers, whirlpools, and showerheads.

of the coding capacity of the genome of L. pneumophila. Although a large number of effectors are injected into the host cell, but with only few exceptions, deletion of individual effectors does not result in reduced intracellular proliferation, suggesting potential functional redundancy. Strikingly, many L. pneumophila effector proteins harbor eukaryotic protein domains, which include ankyrin repeats, leucine-rich repeats, Sel-1, U-box, F-box, and a C-terminal CaaX prenylation motif. Expression of a large number of effectors is induced during the exponential phase of intracellular growth within macrophages and Acanthamoeba, but the function of most of these proteins has yet to be determined.

Growth phase-dependent regulation of bacterial virulence. The intracellular lifecycle of L. pneumophila consists of a replicative phase within the LCV, and a transmissive phase, exhibited upon escape into the cytosol. This biphasic lifestyle is characterized by dramatic changes in the transcriptome, that result in phenotypic modulations. During the replicative
phase, the bacterium is undergoing exponential (E) growth; it is non-motile and represses transmissive traits, such as lysosomal evasion (Fig. 1).64 The stringent-like response is triggered upon transition of L. pneumophila into post-exponential (PE) growth, when the bacteria become cytotoxic, motile, sodium-sensitive, osmotic-resistant, and capable of lysosomal evasion.65,66 These phenotypic modulations are necessary to escape from the wasted host and invade a new host to start a second cycle of intracellular proliferation.41,42,47,59,60,63-65

The transition between replicative and transmissive phenotypes is highly orchestrated, and is governed by many factors that are influenced by intracellular nutrient levels.67 Upon amino acid depletion, uncharged bacterial tRNAs activate RelA to synthesize the bacterial alarmone 3′,5′-bispyrophosphate (ppGpp), a master regulator of numerous genes of L. pneumophila, which triggers phenotypic modulations upon transition into the PE phase.68 SpoT, a bifunctional synthetase/hydrolase that responds to a variety of stimuli, such as fatty acid starvation, also synthesizes ppGpp leading to increased levels of the alarmone (Fig. 1).67 RpoS and several global response two-component regulators, such as LetA/S and PmrA/B, function as downstream cascades of regulatory networks that govern phenotypic modulations at the PE phase.69-72 Small non-coding RNAs, such as RsmY and RsmZ, are induced at the PE phase by the regulatory cascade of networks triggered by elevated ppGpp levels.66,68,71 The RNA polymerase interacting protein, DskA, also responds to increased levels of ppGpp and other stress signals to coordinate phenotypic modulations of L. pneumophila at the PE phase and its transmission to a new host.67

In addition to triggering flagellation and various virulence-related traits, elevated ppGpp levels result in upregulation of the type IV secretion components and many of its exported effectors.59-61 One of the Dot-lcm-translocated effectors important in the intracellular infection of amoebae and human cells is the eukaryotic-like AnkB,33,44,45 which is temporally and differentially regulated at the PE phase.33,35,61,72 Therefore, complex cascades of regulatory networks govern phenotypic transition at the PE phase and most or all of these networks are under the direct or indirect control of ppGpp.

In addition to phenotypic modulations at the PE phase, L. pneumophila undergoes a differentiation cycle that is dimorphic, cycling between a replicating form and a planktonic spore-like cyst form, designated as mature intracellular form (MIF).73-75 The MIF is near dormant metabolically, resistant to detergents and antibiotics, and is more invasive.76 The MIFs are formed later in HeLa cells.73 The MIFs of L. pneumophila do not form in macrophages, which is likely due to early apoptotic lysis within 1–3 d of the infection, while the MIFs are formed later in HeLa cells.73 The MIFs of L. pneumophila germinate following entry into a susceptible eukaryotic host cell or in rich media in vitro. It is possible that the MIFs contribute to the ecology of L. pneumophila during starvation in the water system when nutrients are depleted and the amoebal hosts are encysted and not susceptible to infection.

Exploitation of conserved eukaryotic processes by the eukaryotic-like AnkB effector of L. pneumophila. L. pneumophila harbors a plethora of eukaryotic-like effectors that interfere with host processes by mimicking eukaryotic functions.21,23,33,54 Many translocated effectors of L. pneumophila are functionally and structurally similar to eukaryotic proteins and interact with and disrupt various eukaryotic processes such as signaling, protein synthesis, apoptosis, posttranslational modification, vesicular trafficking, ubiquitination, and proteasomal degradation.43 Among the ~300 effectors of L. pneumophila, AnkB is the only effector known to be indispensable for the intracellular infection of both human cells and amoebae, and the biological function of this effector has been deciphered. It is not surprising that recent studies on the AnkB effector and its exploitation of multiple highly conserved eukaryotic processes may just be the tip of the iceberg of our continued unraveling of L. pneumophila-host interaction and its evolution from invading amoebae to invading human cells and causing pneumonia.

The AnkB effector harbors multiple eukaryotic domains that enable this protein to hijack a number of evolutionarily conserved eukaryotic processes, and is essential for intracellular proliferation of L. pneumophila in amoebae and human cells and for virulence in the mouse model.33,44,45,76 The AnkB effector harbors two Ankyrin domains (ANK), 33-residue repeats involved in protein-protein interactions, and is the most common domain in eukaryotic proteins.43 AnkB also contains a C-terminal eukaryotic CaaX motif (C, cysteine; a, aliphatic amino acid; X, I any amino acid) that allows the protein to be lipidated through farnesylation by the host farnesyltransferase (FTase), which anchors AnkB into the LCV membrane (Fig. 2).58,77-79 Farnesylation is a type of prenylation that covalently links a 15-carbon lipid moiety to a conserved cysteine residue within the C-terminus “CaaX” motif, which confers hydrophobicity enabling the lipidated protein to be anchored into the lipid bi-layer of eukaryotic membranes.58 However, there is variation in the C-terminus CaaX motif of AnkB, as some isolates, such as the Paris strain of L. pneumophila, have a truncated C-terminus without a CaaX motif.45 The biological relevance of this variation is still to be determined.

Ubiquitination of proteins is a highly conserved eukaryotic post-translation modification that is mediated by three enzymes (E1–E3); E1 is the activating enzyme which transfers a 76-amino acid ubiquitin polypeptide to the conjugating enzyme (E2) while the E3 ubiquitin ligase links ubiquitin to the target protein.76 Polyubiquitin is formed by linking ubiquitin monomers through one of the 7 lysine (K) residues of ubiquitin. Polyubiquitination through K48 linkages targets the modified protein for proteolytic degradation by the proteasomes.43 The AnkB effector is a bona fide F-box protein that binds the E3 eukaryotic ubiquitin ligase and functions as a platform for the assembly of K48-linked polyubiquitinated proteins on the LCV (Fig. 2),44,45 which occurs within a few minutes of bacterial entry.49,50 Proteasomal degradation of K48-linked polyubiquitinated proteins results in increased cellular levels of amino acids (Fig. 2),76 which are essential for intracellular proliferation of L. pneumophila that is dependent on amino acids as the major source of carbon and energy to feed the tri-carboxylic acid (TCA) cycle.41

The ankB mutant of L. pneumophila is severely defective in intracellular proliferation in amoebae and human macrophages due to the defect in assembly of K48-linked polyubiquitinated
proteins decorating the LCV (Fig. 1). Due to lack of proteasomal degradation of K48-linked polyubiquitin during infection by the ankB mutant, cellular levels of amino acid do not increase. This triggers a bacterial starvation response, mediated by the induced expression of RelA and SpoT, and results in elevated ppGpp levels. Intracellular growth can be restored to the ankB mutant within amoebae and human cells by supplementing excess amino acids. Thus, higher levels of cellular amino acids are needed for intracellular replication of L. pneumophila.

Remarkably, supplementation of infected cells with certain single amino acids, such as cysteine, reverses the growth defect of the ankB mutant in amoebae and human cells by supplementing excess amino acids. Thus, higher levels of cellular amino acids are needed for intracellular replication of L. pneumophila. Remarkably, supplementation of infected cells with certain single amino acids, such as cysteine, reverses the growth defect of the ankB mutant in amoebae and human cells. Interestingly, in human cells cysteine is semi-essential and is the least abundant amino acid, but in amoebae cysteine is essential. Similar to cysteine, supplementation of infected cells with pyruvate or citrate to feed the TCA cycle, rescues the ankB mutant for intracellular proliferation. Interestingly, in vitro growth of L. pneumophila in rich medium requires supplementation with 3.3 mM cysteine. Therefore, AnkB is a remarkable example of an effector involved in exploitation of multiple host processes that are highly conserved in unicellular eukaryotes and mammals.

By promoting proteasomal degradation in amoebae and human cells though the AnkB F-box effector, L. pneumophila generates a gratuitous supply of cellular amino acids within the cytosol of L. pneumophila-infected amoebae and human cells. The amino acids are imported into the LCV through various host amino acid transporters present in the LCV membrane, including the neutral amino acid transporter SLC1A5, which imports Cys, and subsequently into L. pneumophila through numerous ABC transporters and amino acid permeases such as the threonine transporter PhtA.24

**Figure 2.** Nutritional and metabolic adaptation of L. pneumophila to the intracellular life within amoebae and human cells is facilitated by the AnkB effector and its exploitation of multiple highly conserved eukaryotic processes. The AnkB effector is translocated into host cells by the Dot/Icm type IV secretion system of L. pneumophila, and it is immediately farnesylated by the three host enzymes FTase, RCE1, and ICMT, that are recruited to the LCV by the Dot/Icm system. Farnesylation of AnkB results in its anchoring into the cytosolic face of the LCV membrane where it interacts with the eukaryotic SCF1 ubiquitin ligase complex. The AnkB effector functions as a platform for the docking of K48-linked polyubiquitinated proteins to the LCV. Proteasomal degradation of the K48-linked polyubiquitinated protein generates 2–24 amino acid (AA) peptides that are rapidly degraded by oligo- and amino-peptidases. This generates a surplus of cellular amino acids within the cytosol of L. pneumophila-infected amoebae and human cells. The amino acids are imported into the LCV through various host amino acid transporters present in the LCV membrane, including the neutral amino acid transporter SLC1A5, which imports Cys, and subsequently into L. pneumophila through numerous ABC transporters and amino acid permeases such as the threonine transporter PhtA.24
through high nutritional dependence of *L. pneumophila* on host limiting amino acids, such as Cys, and synchronization of amino acid auxotrophy with its host, *L. pneumophila* synchronizes its nutritional needs for growth with the availability of nutrients for the host. This remarkable nutritional adaptation could be what allows the bacteria to be protected during amoebal encystation upon nutrient depletion, leading to cessation of intracellular bacterial growth (Fig. 1). Encysted amoebae are thought to be protected against invasion by *L. pneumophila*. Therefore, potential growth and release of *L. pneumophila* from amoeba in an aquatic environment in which amoebae have encysted in response to nutrient depletion is unlikely to happen, since it would result in free-living bacteria without a susceptible host, which may result is eventual loss of bacterial viability. However, potential differentiation into the MIF under starvation conditions may facilitate continued presence of *L. pneumophila* in the aquatic environment under these conditions.

**Acquisition of eukaryotic genes through inter-kingdom horizontal gene transfer.** The long-term co-evolution of *L. pneumophila* with various protists and metazoa has influenced the genomic structure of this organism through inter-kingdom horizontal gene transfer (HGT).

This long-term co-evolution is likely what gave rise to the acquisition of eukaryotic host genes encoding proteins with eukaryotic-like functions and structures. Amoeba may act as a gene melting pot, allowing diverse microorganisms to evolve by gene acquisition and loss, and then either adapt to the intra-amoebal lifestyle or evolve into new pathogens. Interestingly, mammalian F-box proteins do not have the ANK domain, while F-box proteins from amoeba do.

Therefore, it is more likely that ankB had been acquired through inter-kingdom HGT from a primitive eukaryotic host. *L. pneumophila* is a naturally competent organism that takes up DNA and can exchange DNA between bacteria through conjugation. Long-term convergent evolution and modification of the genes acquired through HGT, splitting of introns, acquisition of prokaryotic promoters and regulators, and translocation motifs is likely what allowed eukaryotic-like proteins to become translocated effectors with functional activities in the host cell. It is to be expected that many of the eukaryotic-like proteins in *L. pneumophila* are still undergoing convergent evolution through modifications that might enable them to become translocated and functionally active effectors.

Long-term co-evolution with its protozoan hosts has likely contributed to the ability of *L. pneumophila* to cause disease in humans, perpetuated by changes in human lifestyle. Understanding its association with amoebae will give us a better understanding of how *L. pneumophila* causes human disease though exploitation of evolutionary conserved eukaryotic processes. Since *L. pneumophila* also exploits mammalian-specific processes such as the inflammasomes and pro- and anti-apoptosis, it is likely that additional virulence properties have been acquired by *L. pneumophila* to enhance its capacity to infect humans. Since many other pathogens are detected within amoebae, this primitive eukaryotic host may represent a reservoir for many human pathogens.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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