Amoebae and Mammals Deliver Protein-Rich Atkins Diet Meals to Legionella Cells

Legionella pneumophila promotes proteasome-mediated protein degradation of its diverse hosts to meet its specific carbon and energy needs

Christopher T. D. Price and Yousef Abu Kwaik

Key to survival of any organism is its harnessing of nutrients. For intracellular bacterial pathogens, gaining access to sufficient nutrients poses a particular challenge. The host sequesters some necessary nutrients while others are present in insufficient quantities. Although microbial acquisition of such nutrients and in vivo bacterial metabolism are fundamental to pathogen-host interactions, our understanding of these processes is limited.

Numerous bacterial pathogens inject their host cells with a diverse array of effectors that target various cellular processes such as signaling, biosynthetic and degradation pathways, innate immunity, pro- and anti-apoptosis, autophagy, and vesicular traffic. Potential sources of microbial nutrition in vivo include exploiting host biosynthetic and degradation pathways. Translocated effectors of microbial pathogens promote host degradation pathways, but little is known about how effective this is in providing carbon and energy needs.

Legionella pneumophila, which causes Legionnaires’ disease, is abundant in the aquatic environment. It shares an intimate intracellular relationship with diverse amoeba and ciliate species, which normally graze on bacteria. When L. pneumophila infects amoeba and humans, they use the AnkB translocated effector to promote host proteasomal degradation of proteins to generate higher levels of amino acids, allowing the pathogen to grow within the host cell. We suspect that there are other similarly intriguing strategies by which intracellular pathogens outsmart their eukaryotic hosts in the quest for limited food sources.

Two Distinct Hosts but Similar Intracellular Lifestyles for L. pneumophila

Upon invading amoeba and human cells, L. pneumophila evades the endosomal-lysosomal degradation pathway by entering a vacuole, designated the Legionella-containing vacuole (LCV) (Fig. 1). The LCV intercepts endoplasmic reticulum (ER)-to-Golgi vesicular traffic, and becomes quickly remodeled into an ER-derived vacuole (Fig. 1). L. pneumophila rapidly replicates in the LCV to a high density but, prior to egress, the bacteria escape into the cytosol for another 1–2 rounds of proliferation (Fig. 1).

People who inhale Legionella-contaminated aerosols can become infected when the bacteria invade and replicate within alveolar macrophages, using mechanisms that also operate...
within the environmental host amoeba. The Dot/Icm type IV secretion apparatus injects into host cells about 300 different effector proteins, close to 10% of the coding capacity of the *L. pneumophila* genome, which is a record number among bacterial pathogens. However, only a few of these effectors prove essential for intracellular replication. Some effectors are structurally and functionally similar to eukaryotic proteins and interact with various host processes, including signaling, vesicular trafficking, protein synthesis, apoptosis, posttranslational modifications, ubiquitination, and proteasomal degradation.

Once in the host cell cytosol during late stages of the intracellular life cycle, postexponential (PE) *L. pneumophila* cells develop motility and
increased infectivity. This dramatic phenotypic switch occurs after amino acid and fatty acids supplies are exhausted, triggering RelA and SpoT to synthesize the bacterial alarmone ppGpp, which is a master regulator of numerous genes required for Legionella pathogenesis.

Before that switch, L. pneumophila cells replicate with a generation time of about 40 minutes, devouring tremendous amounts of carbon and nitrogen for energy and biomass. Early efforts to develop a suitable culture medium for L. pneumophila recognized that this organism relies on amino acids, particularly serine, for energy, with little to no use for carbohydrates. Thus, L. pneumophila is routinely cultured on buffered charcoal yeast extract (BCYE) agar with a supplement of 3 mM cysteine, whose basis was unraveled recently. This organism is auxotrophic not only for cysteine but also arginine, isoleucine, leucine, methionine, valine, and threonine. Moreover, its glycolytic pathway is incomplete but the tricarboxylic acid (TCA) cycle is intact. Serine is directly metabolized to pyruvate to feed the TCA cycle, and genetic evidence suggests cysteine and other amino acids also feed the TCA cycle.

**Elaborate Legionella Strategy for Meeting Nutrient Needs**

How does L. pneumophila meet its nutritional needs for amino acids in amoeba and mammalian host cells? Within few minutes of forming the LCV in amoeba and human cells, it becomes decorated with polyubiquitinated proteins (Fig. 2C),

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**FIGURE 2**

The eukaryotic-like domain architecture of the L. pneumophila AnkB translocated effector. (A) Representation of the AnkB protein with its eukaryotic F-box and two ankyrin domains and a eukaryotic CaaX farnesylation motif at its C-terminus. The F-box domain interacts with the eukaryotic protein SKP1, part of the E3 SCF ubiquitin ligase. The two ankyrin domains are involved in diverse protein-protein interactions. The CaaX motif ("C" for cysteine, “a” for any aliphatic amino acid, and “X” for any amino acid) allows this effector protein to be farnesylated by host enzymes resulting in increased hydrophobicity and membrane anchoring of AnkB. (B) Confocal microscopy images of infected cells at 2 h postinfection for colocalization of the LCVs (green) with polyubiquitinated (PolyUb) proteins (red). The arrow indicates heavy colocalization of polyubiquitin with the WT strain.
Eukaryotic farnesylation and polyubiquitination. (A) Eukaryotic proteins harboring a C-terminal CaaX motif are targeted for farnesylation by the cytosolic enzyme farnesyltransferase (FTase). Farnesylated proteins are further processed by two ER membrane proteins, RCE1 and ICMT. (B) Ubiquitination is mediated by three classes of enzymes (E1-E3). The ubiquitin activating enzyme (E1) transfers ubiquitin to a conjugating enzyme (E2), followed by E3 ubiquitin ligase linking ubiquitin to the target protein. If polyubiquitination involves K48 linkages, the modified protein is targeted to the multi-subunit proteasome where the protein is degraded into short peptides that are then converted into amino acids by cytosolic peptidases.
a process that depends on the AnkB Dot/Icm-translocated effector being anchored into the LCV membrane. The AnkB protein is found in 211 of 217 tested strains. It is composed of several eukaryotic-like domains, including an F-box, two ankyrin domains, and a C-terminal CaaX prenylation motif (Fig. 2).

Prenylation is a highly conserved eukaryotic posttranslational modification that covalently links a 15-carbon farnesyl or 20-carbon geranylgeranyl lipid moiety to a cysteine residue at the -4 position from the C-terminus of a protein, increasing hydrophobicity and enabling the lipiddated protein to be anchored into membranes (Fig. 3A). When farnesylated, AnkB attaches to the outer leaflets of LCV membranes within amoeba and human cells. The LCV is the only pathogen-containing vacuole known to hijack the highly conserved host farnesylation enzymatic machinery, which is recruited by Legionella to the LCV membrane.

Another highly conserved eukaryotic post-translational process hijacked by AnkB is ubiquitination. This process covalently tags proteins with repeating units of the 76-amino acid polypeptide called ubiquitin, and it involves three enzymes, E1, E2, and E3 (Fig. 3B). Eukaryotic F-box proteins interact with the E3 SCF1 (SKP1/CUL1/F-box) ubiquitin ligase complex to modify protein targets with a ubiquitin monomer or polymer (Fig. 3B). The F-box domain binds the E3 ubiquitin ligase, while the ubiquitination target protein binds the F-box protein through a protein-protein interaction repeat domain, such as LRR or WD 40 in higher eukaryotes, or Ankyrin repeats in unicellular eukaryotes such as amoebae.

Findings from Ralph Isberg’s lab at Tufts University in Boston, Mass., have shown the LCV to be decorated with polyubiquitinated proteins, but the molecular bases of this were not known. Simultaneous and independent work from Carmen Buchrieser’s lab at the Institut Pasteur in Paris and by our lab have shown the AnkB F-box effector of two distinct clinical isolates to be directly responsible for this decoration of the LCV. Through molecular and biochemical mimicry, AnkB functions on the LCV membrane within amoeba and mammalian cells as a bona fide eukaryotic F-box protein that interacts with the host SCF1 ubiquitin ligase complex (Fig. 4A).

Polyubiquitination of proteins plays a key role in their fate, including degradation, cellular localization, and functional modification, and this fate depends on the linkage pattern between the ubiquitin monomers in the polyubiquitin polymer. Ubiquitin monomers are covalently linked through any of the seven lysine (K) residues of ubiquitin. Proteins tagged with a K48-linked polyubiquitin chain are destined for degradation via the proteasome, a large, multisubunit proteolytic complex that yields short peptides (~8 amino acids), which cytosolic peptidases hydrolyze to amino acids (Fig 3B).

**Intricacies of L. pneumophila Revealed by the ankB Mutant**

Although the ankB mutant of L. pneumophila is severely defective in intracellular proliferation in amoeba and human cells, it evades lysosomal fusion and forms the ER-remodeled LCVs similar to the wild-type strain (Fig. 1). However, while the LCV harboring the wild-type strain is preferentially decorated with K48-linked polyubiquitinated proteins, a process essential for intracellular proliferation, polyubiquitinated proteins do not decorate LCVs containing the ankB mutant within amoeba and human cells.

These results led us to ask why decorating the LCV with K48-linked polyubiquitinated proteins enables L. pneumophila to proliferate. Perhaps AnkB targets particular host proteins or L. pneumophila effectors for proteasome-mediated degradation. Alternatively, the higher levels of cellular amino acids generated through proteasome-mediated degradation of polyubiquitinated proteins is needed to power intracellular replication of L. pneumophila.

Several findings lend support to this second possibility, including: (i) the ankB mutant exhibits an amino acid starvation response; (ii) substituting arginine for K48 of ubiquitin abolishes LCV decoration, induces an amino acid starvation response, and blocks intracellular proliferation of the wild-type strain; (iii) host proteasome and cytosolic peptidase activities are required for replication of the wild-type strain; and (iv) after L. pneumophila infects cells, levels of cellular amino acids rise dramatically, and this rise is totally dependent on AnkB.

Therefore, our simple approach was to examine if we can rescue the ankB mutant by providing amoeba and human cells with excess amino acids to make up for the defect of AnkB-dependent rise in cellular amino acids. Intriguingly, adding
The AnkB effector helps *L. pneumophila* to adapt to host cells where the bacteria exploit highly conserved processes. (A) The AnkB effector is translocated into host cells by the Dot/Icm type IV secretion system and is immediately farnesylated by the three host enzymes FTase, RCE1, and ICMT. The farnesylated AnkB anchors into the cytosolic face of the LCV membrane where it interacts with the eukaryotic ubiquitin ligase complex and serves as a platform for the docking of K48-linked polyubiquitinated proteins. Proteasomal degradation of the polyubiquitinated protein generates peptides and then amino acids that are imported into the LCV. (B) Cysteine and serine are metabolized to pyruvate, which is converted into acetyl CoA to feed the TCA cycle, the main source of carbon and energy for *L. pneumophila*. (C) Synchronizing its amino acid needs with those of its evolutionarily distant hosts appears to be essential for *L. pneumophila* pathogenesis in humans.
a amino acids reverses the intracellular growth defect of the ankB mutant and also restores growth of wild-type L. pneumophila in human cells over-expressing the K48-R ubiquitin variant or in cells in which proteasomal degradation or cytosolic peptidases are inhibited. Moreover, adding certain single amino acids, such as cysteine or serine, also can restore intracellular growth of the ankB mutant in amoeba and human cells. This result reflects the fact that L. pneumophila metabolizes these amino acids to feed the TCA cycle, where they are converted into pyruvate and then acetyl CoA (Fig. 4). Recent studies from Anne Bertolotti’s lab at the MRC labs of Molecular Biology in Cambridge, UK, have shown that inhibition of proteasomal protein degradation in yeast, mammals, and flies leads to cell death due to depletion of cellular amino acids. The lethality of amino acid depletion upon inhibition of the proteasomes is reversed by supplementation of cysteine, which is the least abundant cellular amino acid. Thus, Legionella hijacks a major cellular protein degradation pathway essential for generating amino acids, and cysteine in particular, in all eukaryotes.

This case is the first of its kind in which a translocated effector manipulates several host pathways, but the ultimate goal of these manipulations is to generate carbon and energy needed for intracellular bacterial growth. AnkB not only links cellular microbiology and virulence of L. pneumophila to its metabolism, it also exploits eukaryotic farnesylation, polyubiquitination, and proteasomal degradation machineries that are conserved throughout the eukaryotic kingdom.

Adventages of Hijacking Host Metabolic Processes

What is the advantage of hijacking host proteasome machinery to generate food sources—particularly for an accidental pathogen? To survive in waterways, L. pneumophila invades amoeba, ciliates, and possibly other eukaryotes. Unable to make cysteine, L. pneumophila targets this highly conserved proteolytic complex to generate higher levels of cellular amino acids and cysteine in particular, improving survival chances in whatever eukaryote it invades, ranging from unicellular amoebae to humans.

Acanthamoebae, a primary aquatic host of L. pneumophila, are auxotrophic for several amino acids, including cysteine (Fig. 4C). Another host, the social amoeba Dictostelium discoideum, is auxotrophic for 11 amino acids, including cysteine, while cysteine is semi-essential in humans. Why did Legionella evolve to be so dependent on cysteine, which is essential for the amoeba host? Long-term evolution of L. pneumophila with its amoeba host has resulted in L. pneumophila synchronizing amino acid auxotrophy with the host, a remarkable nutritional adaptation (Fig. 4C).

We speculate that during times of nutrient abundance for the amoeba host, protein production in the host would be unhindered, and intracellular L. pneumophila can promote host degradation of polyubiquitinated proteins to generate higher levels of cellular amino acids and proliferate. However if key amino acids, such as cysteine, are scarce, amoebae will differentiate into metabolically inactive cysts to survive the starvation conditions, while L. pneumophila would also be starved and no longer replicate within amoebae. In support of this, the ankB mutant bacteria merely wait within the LCV until amino acids become available again.

Thus, by synchronizing amino acid auxotrophy with its host, the bacteria are likely to be protected within an amoeba cyst until nutrients are again abundant. The strict reliance of L. pneumophila on eukaryotic proteasomes for amino acids points to potential water treatment strategies that could reduce the L. pneumophila burden there through inhibiting the amoeba proteasomes.

Since all the functional domains and motifs of AnkB are eukaryotic-like, one wonders where L. pneumophila acquired the ankB gene. It seems likely that L. pneumophila acquired AnkB through interkingdom horizontal gene transfer while intimately associated with primitive unicellular eukaryotes such as amoebae. Indeed, the domain architecture of AnkB of L. pneumophila more closely resembles F-box proteins of unicellular eukaryotes than it does those from mammals. This is evident from the absence of Ankyrin domains in mammalian F-box proteins and their presence in amoeba F-box proteins. It is most likely that adaptation of L. pneumophila to the amoeba host has enabled this bacterium to exploit highly conserved eukaryotic pathways to infect humans. However, the ability of L. pneumophila to modulate other mammalian processes not present in amoeba, such as the inflammasome and pro-and anti-apoptotic pathways, suggests additional long-term adaptation to and
interactions of *L. pneumophila* with other, more complex multicellular eukaryotic hosts.

Christopher T. D. Price is a Research Associate and Yousef Abu Kwaik is the Bumgardner Professor of Molecular Pathogenesis in the Department of Microbiology and Immunology, College of Medicine, University of Louisville, Ky.

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**Suggested Reading**


