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Nutritional virulence of Legionella pneumophila.

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NUTRITIONAL VIRULENCE OF *LEGIONELLA PNEUMOPHILA*

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A Dissertation Approved on

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DEDICATION

This dissertation is dedicated to my parents, who are embarrassingly proud of me and my accomplishments, and to Andrew Best, for being the best husband.

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ABSTRACT

NUTRITIONAL VIRULENCE OF *LEGIONELLA PNEUMOPHILA*

Ashley Best

April 18, 2018

Legionella pneumophila is an environment organism that parasitizes a wide range of protozoa. Growth within the environmental host primes *L. pneumophila* for infection of human alveolar macrophages when contaminated aerosols are inhaled. Intracellular replication within either host requires the establishment a replicative niche, known as the *Legionella*-containing vacuole (LCV). Biogenesis of the LCV depends on the type IVb translocation system, the Dot/Icm, to translocation >320 effectors into the host cytosol. Effectors are responsible for preventing lysosome fusion to the LCV, recruitment of ER-derived vesicles to the LCV, and modulation of a plethora of host processes to promote the intracellular survival and replication of *L. pneumophila*. Nutrient requirements of the pathogen are reflective of its intracellular lifecycle, consuming host amino acids for carbon and energy. Amino acids, particularly serine and cysteine, are used to generate pyruvate to feed into the TCA cycle, which is the main metabolic pathway for generation of energy. Endogenous levels of host amino acids are insufficient to support robust intracellular replication. Excess host amino acids are generated by the AnkB effector through ubiquitination and proteasomal degradation of host proteins in the cytosol. Host amino acids must be transported across the LCV membrane to be utilized by *L. pneumophila*. Host solute

carrier (SLC) transporters are the most likely candidate to import amino acids into the LCV lumen, as they have been detected in the LCV proteome of multiple mass-spectrometry studies. We sought to confirm the role of human SLCs in nutrient acquisition during intracellular growth of *L. pneumophila*. No amino acid-transporting SLCs were confirmed to colocalize to the LCV by confocal microscopy. However, a glucose transporter, SLC2a1/Glut1 was shown to be recruited the LCV in a Dot/Icm-dependent manner. The role of glucose in intracellular replication of *L. pneumophila* is poorly understood. Glucose minimally used through glycolysis, but metabolized through the Enter-Doudoroff pathway. Glucose does not support the replication of *L. pneumophila* during *in vitro growth.* We identified 10 SLC-like transporters in *L. pneumophila* based on their structural similarity to human SLCs. We characterized the role of two putative SLC-like glucose transporters, LstA and LstB of *L. pneumophila*, in import of glucose and in intracellular replication within human macrophages and amoebae. Single transporter mutants decrease *L. pneumophila*'s ability to import glucose but do not affect the ability to replicate within the host. Interestingly, the double mutant, *lstA/lstB*, is severely defective for import of glucose and for intracellular replication within human macrophages and *Acanthamoeba polyphaga.* These data show that glucose uptake by the redundant transporters, LstA and LstB, is required for *in vivo* growth. *L. pneumophila* encodes putative amylases effectors that may be degrading host glycogen as a means to generate glucose that is imported by LstA and LstB. We characterized the *L. pneumophila* amylase, LamB, because of its uniqueness amongst *Legionella* species. Here we describe LamB as a functional amylase that is required for intracellular replication of *L. pneumophila* in human macrophages and *A. polyphaga*. Additionally, the *lamB* mutant is completely attenuated in intra-pulmonary proliferation in

the A/J mouse model. Taken together, these data further characterize nutritional virulence of *L. pneumophila*.

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INTRODUCTION

Eponymous outbreak of Legionnaires' disease

Legionella pneumophila has intrigued scientists since it first appeared on the world stage and continues to do so today. The first recognized outbreak of Legionnaires' disease occurred in 1976 during the 56th annual American Legion Convention in Philadelphia at the Bellevue-Stratford Hotel corresponding with the nation's bicentennial celebrations [1]. There were 180 individuals who were diagnosed with severe pneumonia, 34 of which resulted in death [1, 2]. After six months of microbiological and epidemiological studies, preceded by viral and toxic etiologies, it was determined by CDC scientists, that the causative agent of the pneumonia in Philadelphia was a previously unidentified bacterium, *Legionella pneumophila* [3, 4]. It was designated as such after the American Legion, to which a large number of infected individuals belonged, and Philadelphia, for the city of the outbreak.

What scientists did not know then was that the bacterium was unique in its nutrient requirements and therefore needed special agar plates to be cultured, adding to its difficulties in being isolated. Additionally, the bacteria could only replicate in alveolar macrophages, making identification from lung tissue samples difficult. The investigation ended up lasting over six months and involved teams of toxicologists, parasitologists, virologists, bacteriologists, and epidemiologists. Interestingly, had the CDC not used guinea pigs to confirm the bacterium identified in a tissue sample was the causative

agent, and instead used mice – the CDC would have been unable to identify the pathogen, as mice are not susceptible to Legionnaire's disease [3].

Immune compromised individuals and smokers are more susceptible to Legionnaire's disease but healthy individuals acquire the disease [5, 6]. In the Philadelphia outbreak, many of the victims were elderly, male smokers, who were staying at the Bellevue-Stratford Hotel. It was suspected later that a contaminated cooling tower of the hotel was the specific source for the 1976 outbreak, based on epidemiological studies of other *Legionella* outbreaks; however, the proper environmental tests for *Legionella* were not feasible in 1976, so the exact source remains unknown [7].

The prevailing dogma, for nearly four decades, was that transmission from the environment to the human host was the only mechanism of transmission [8-10]. There were no documented cases of *L. pneumophila* transmission between individuals. However, an unusual case report from Portugal outlines the transmission of *L. pneumophila* from a maintenance worker at an industrial cooling tower to his elderly mother he visited, 180+ miles from the outbreak [11, 12]. Nearly forty years passed between the first described outbreak and an incident of person-to-person transmission was documented. This was not due to changes in the bacterium to allow it to be more transmissive but rather the difficulty in isolating a single case that can be directly attributed to person-to-person contact. Multiple factors must be considered for this to be possible; the second individual must have had absolutely no contact with the infected site, the second individual must also be immunocompromised, and the two need to have prolong contact. All of these factors were present in the this unusual case report from

Portugal where the first infected individual had very close, repeated contact with his sick mother for a few days [12].

Outbreaks dating back to 1959 were retroactively attributed to *Legionella* [13]. In addition, many other previously identified, or left unidentified, bacteria would also be reclassified under this genus [14]. Outbreaks of *Legionella* infections have only emerged within the past few decades due to human alterations to the environment that generate water aerosols as a vehicle to transmit *Legionella* from aquatic sources. Some examples of these modifications are the use of cooling towers, air conditioning systems, whirlpools/ hot tubs, grocery store misters, shower heads, humidifiers, etc [15]. The general method of infection follows a similar process. First, *Legionella* within a protozoan host or free *Legionella* enter water holding units. Then, water within these units is, in some way, aerosolized. These aerosolized droplets contain the bacterium, or protozoan containing *Legionella*, which are inhaled to cause disease [7, 16-18].

Epidemiology of *L. pneumophila* infections

L. pneumophila is a Gram-negative facultative intracellular bacterium that proliferates within alveolar macrophages, causing Legionellosis [15]. There are more than 60 species of *Legionellae*, 30 of which are known to cause disease , but *L. pneumophila* continues to be responsible for more than 85% of cases of Legionellosis in most of the world with the exception of Western Australia, where *Legionella longbeachae* is the most predominant species, whose pathogenesis is distinct from *L. pneumophila* [15, 19-24].

Legionellosis manifests in two clinically distinct forms; Legionnaire's disease and Pontiac Fever [15]. Legionnaire's disease is a multisystem disease with pneumonia;

whereas, Pontiac fever is a self-limiting flu-like illness [25, 26]. The incubation period of both varies from 2-14 days. Symptoms of Legionnaires' disease include; cough, fever, muscle pains, shortness of breath, and headaches. Some patients also experience ataxia, diarrhea, vomiting, bloody sputum, abnormal electrolyte levels, and/or loss of appetite. Infection usually results in hospitalization with a fatality rate of 9% [27]. Cause of death is usually attributed to respiratory shock and/or multi-organ failure [22]. No vaccine exists for the prevention of infection. Disease can be treated with antibiotics: macrolides, tetracyclines, ketolides, and quinolones being the most effective [28-33].

The CDC reported that 6,100 cases of Legionnaire's disease were diagnosed in 2016 in the United States but acknowledge this number may be higher because it is an underdiagnosed disease [34]. Between 2001 and 2016, reported cases have grew more than four and a half times [35]. Patients that come into the clinic with pneumonia are usually just treated with antibiotics without further tests to prove the etiological agent. Confirmation of *Legionella* infection is done by urine-ELISA, which only detects *L. pneumophila* serogroup 1 [36-38]. Therefore, an exact case number is difficult to determine.

The best method of control is proper maintenance of water handling systems. However, many water-holding units are not properly cared for in order to prevent *Legionella* growth. This problem is wide-spread, since a CDC study found *Legionella* DNA in 84% of cooling towers tested across the United States [39].

Continual treatment of the contaminated water source is needed. For total eradication, methods such as continuous treatment with monochloramine or copper-silver ions and sustained temperatures above 55°C is recommended [40-42]. The usual course

of action is a single treatment by UV irradiation, biocides, or overheating of the water which is done following identification of *Legionellae* in the water [40, 42, 43]. This is successful as a short-term intervention but the organism can reappear after some time [42, 44].

Legionellae are ubiquitous in natural aquatic environments, usually in low numbers, but within human-made aquatic habitats, at temperatures between 25°C and 43°C, they are found in high quantities [45-47]. Additionally, *Legionellae* from infected amoebae, rather than free-living, are more resistant to chemical disinfectants and biocides, making eradication more difficult [40, 48, 49]. Given the prevalence in the environment, and lack of proper maintenance, infections with *L. pneumophila* will likely continue to be a public health risk.

L. pneumophila is an environmental, accidental human pathogen

It was first suggested by Rowbotham, in 1980, that *Legionella* could live intracellularly within amoebae, specifically *Acanthamoeba & Naegeria* [50]. The number of protozoa known to be infected by Legionella has grown to 19 species of amoebae and 9 species of non-amoebal protozoa, a number that is likely to rise as more continue to be identified (Table 1-1). Amoebae in the environment serve as the bacterium's source of carbon and energy, as *Legionella* consumes its host's amino acids [51]. *Legionella*'s unique nutrient requirements are representative of an intracellular lifestyle and thus are not commonly found growing free in the environment [52]. The bacterial nutrient requirements will be discussed in further detail in the section on nutrient acquisition.

Amoebae obtain their nutrients from consuming bacteria, yet *Legionellae* have evolved to evade the host's attempts at consuming them, a trait that isn't unique to

Legionellae. *Mycobacterium* sp., *Francisella tularensis*, *Escherchia coli* O157, and others, have transient associations with amoebae [53-57].

Table 1-1. Protozoan species that can support intracellular growth of *Legionella pneumophila*. *†Indicate non-amoebal species.*

Protozoan species	Reference
Acanthamoeba castellannii , *A. culbertsoni, A.* hatchetti, A. *polyphaga, A. royreba,*	[50, 58-60]
Balamuthia mandrillaris	[61]
Hartmanella (Vermamoeba) vermiformis, H. cantabrigiensis	[55, 59, 60]
Naegleria lovaniensis, *N. fowleri, N. gruberi, N. jadini*	[50, 56, 58]
Dictyostelium discoideum†	[62, 63]
Tetrahymena spp. (tropicalis, pyriformis, thermophila, vorax) †	[64-66]
Willaertia magna	[67]
Echinamoeba exundans	[59, 68]
Vahlkampfia jugosa, V. ustiana	[55, 60, 69]
Oxytricha bifaria†	[70]
Stylonychia mytilus†	[70]
Ciliophrya sp. †	[70]
Paramecium caudatum†	[71]
Comandonia operculata	[60]
Cochliopodium minus	[72]
Filamoeba nolandi	[60]

Some species of *Legionella* have evolved to be so in-tune with the amoebal host that they cannot be cultured by any means, except with co-cultivation with amoebae. These organisms are called *Legionella*-like amoebal pathogens (LLAP) [73]. One LLAP was isolated from a pneumonia patient's sputum, indicating that they are capable of causing disease in humans [73].

Legionellae can also survive outside of the amoebae in the environment within biofilms [74]. These biofilms usually exist with other microbial communities, which could provide the *Legionellae* with the nutrients they require to support growth. However, the relationship between *Legionellae* and other members of the biofilm communities is unknown.

Because amino acids, particularly serine, are the preferred carbon source, life within the amoebae is much more preferable due to the ease of access to amino acids [75, 76]. *L. pneumophila* has evolved to synchronize amino acid auxotrophies with that of the amoebae (for more information see "Nutrient acquisition by *L. pneumophila*") [52]. This allows the bacteria to better prepare for any nutrient stresses they may encounter [52].

In response to unfavorable conditions, the bacterium can enter a dormant state and when conditions become more favorable, become metabolically active again with the amoebae. This dormant state is characterized as "viable but non-culturable" (VNBC), in which the only known way of recovering these bacteria is through co-culture with amoebae [77, 78].

The largest impact of their environmental reservoirs on human disease is the priming of *L. pneumophila* for subsequent infection. Amoebae have been referred to as the "trojan horses of the microbial world" [79, 80]. This is because as *Legionella* prepare to exit the amoebae, they enter a transmissive state, becoming more virulent [79]. *L. pneumophila* that have escaped the amoebae are more infectious and can cause a more robust disease (see "Phase variation and stress response") [81-83]. Protozoa are also capable of releasing vesicles that contain many *L. pneumophila* which are of respirable size, thus increasing the dose of bacteria to the individual [84].

The intracellular lifestyle within amoebae and macrophages

To proliferate, *L. pneumophila* depletes its host of nutrients leading to lysis of the cell to find a new source of nutrients [85]. Therefore, the bacteria are constantly cycling through host cells. Whether it is its natural host, a protozoan, or its accidental host, a human macrophage, the intracellular lifecycle is very similar.

In short, a flagellated bacterium will enter the host, avoid fusion with the lysosomes and reside within a vacuole called the *Legionella*-containing vacuole (LCV), replicate in high numbers in the LCV, break out into the cytosol to replicate more, become flagellated again, and finally breaking out of host cell to repeat the cycle again (Fig. 1-1). The whole cycle lasts, under *in vitro* conditions, around 18 hrs and is a highly orchestrated event [86, 87].

In step one of Figure 1-1, a flagellated bacterium enters the host cell, primarily by micropinocytosis, although a unique form of entry has been observed, called coiling phagocytosis [54, 88]. Immediately upon attachment, *L. pneumophila* begins to alter the host by translocating effectors into the cytosol via the type 4B translocation system, Dot/Icm (more on this will be discussed in the next section) [89, 90].

Within the host, as seen in step two of Figure 1-1, the bacterium resides within the *Legionella*-containing vacuole (LCV). To create this protective environment, the bacterium avoids acidification and endosomal-lysosomal degradation [91, 92]. The vacuole is rapidly remodeled by intercepting ER-to-Golgi vesicles [93, 94]. Modification of the vacuole occurs immediately upon uptake [95]. This modified vacuole is surrounded by endoplasmic reticulum (ER) [91, 96]. Additionally, polyubiquitinated proteins begin to cloud around the LCV (Fig. 1-1) [97-99].

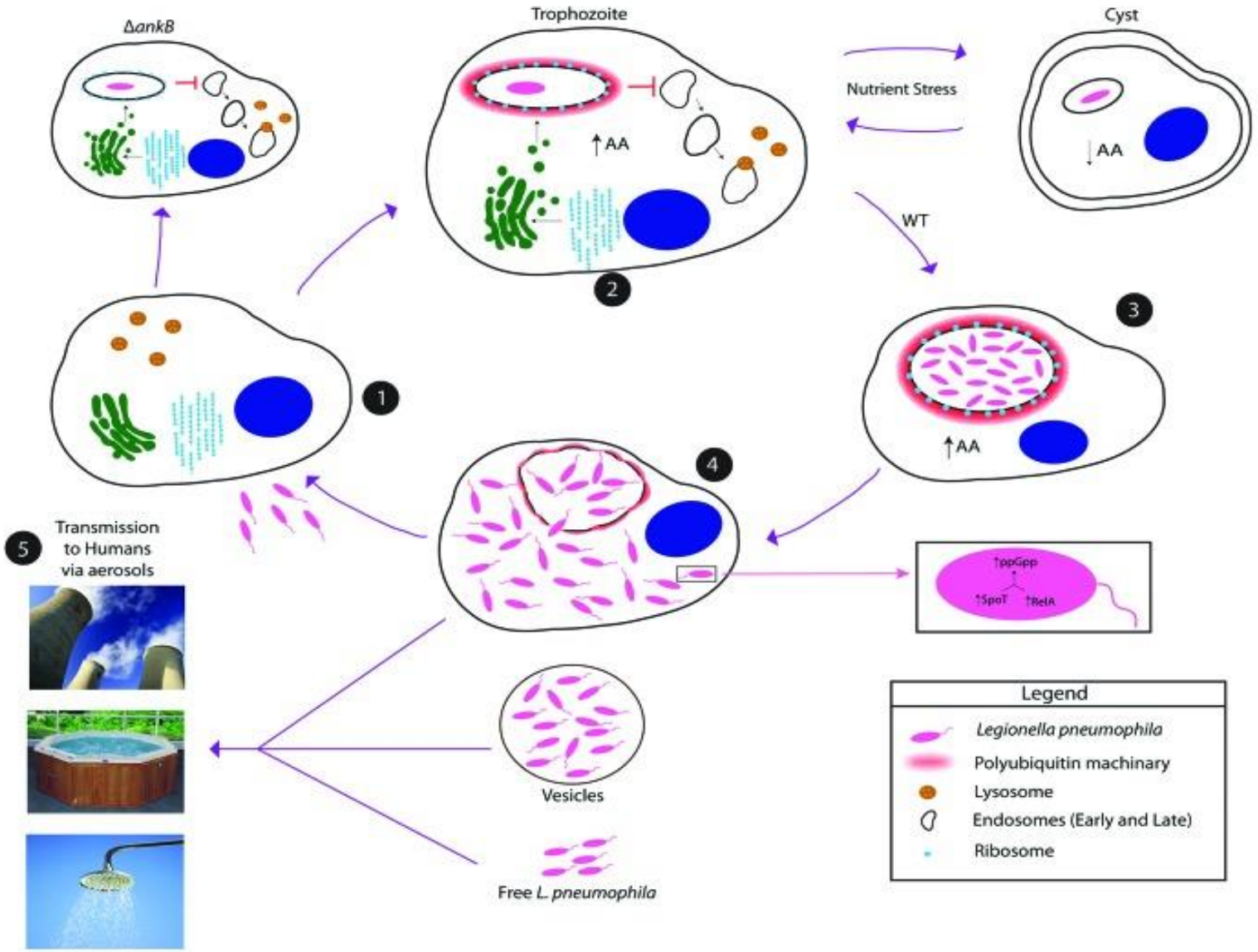
ΔankB
Trophozoite
Cyst
Nutrient Stress
↑AA
↓AA
WT
1
2
3
4
↑AA
5
Transmission
to Humans
via aerosols
↑ppGpp
↑SpoT
↑RelA
Vesicles
Free L. pneumophila
Legend
Legionella pneumophila
Polyubiquitin machinary
Lysosome
Endosomes (Early and Late)
Ribosome

Figure 1-1. The environmental life cycle of *L. pneumophila* within protozoa. 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 [100].

L. pneumophila is then able replicate to high numbers within the LCV, step three of Figure 1-1. Eventually, around 18hrs, the bacteria will outgrow the LCV and break-out into the host cytosol, step four of Figure 1-1 [79, 101, 102]. The bacteria will undergo a few more rounds of replication in the cytosol [101]. At this point, nutrient levels in the cytosol are very low, triggering the bacterial alarmone, ppGpp, and inducing a transition into the transmissive phase (see "Phase variation and stress response" for more detail) [85, 103-105].

One of the key changes in the transmissive phase is the production of the flagellum, which allows the bacteria to find a new host [105]. Free, flagellated bacteria can go on to repeat the cycle with a new host [106]. It is at this point that infection of humans can easily occur by aerosolization of infectious particles of either free bacteria, bacteria within vesicles, or even bacteria-filled amoebae, as seen in step five of Figure 1-1 [7]. Inhaled bacteria will enter the lungs and be taken up by resident alveolar macrophages and continue the cycle just as they would have in a protozoan host [91, 107]. As previously mentioned, growth within amoebae prepares the bacteria for subsequent infection, making them primed to cause disease in humans [108].

A type IVb translocation system, Dot/Icm, of *L. pneumophila* modulates host processes

Successful infection of any host cell by *L. pneumophila* depends on the presence of a functional translocation system, the Dot/Icm [109, 110]. Two loci involved in intracellular replication were identified in *L. pneumophila* by transposon mutagenesis; the *dot* (defect in organelle trafficking) locus and the *icm* (intracellular multiplication) locus [111, 112]. The *dot/icm* locus contains the genes necessary to produce a type IV

translocation apparatus and chaperone proteins needed for ushering some effectors into the apparatus [113, 114]. Defects in the Dot/Icm system lead to severe intracellular growth defects within every host cell tested. One such mutant, *dotA*, a common negative control for intracellular replication and translocation, is still able to enter a host cell but fails to generate a replicative LCV due to inability to translocate substrates, since DotA is an essential structural component of the Dot/Icm translocation apparatus [115].

The Dot/Icm is classified as a Type IV translocation system. Type IV translocation systems are divided into two subclasses; Type IVa are homologous to the *virB* operon of *Agrobacterium tumefaciens* and Type IVb are homologous to the conjugation machinery of IncI plasmids [116]. The Dot/Icm translocation system is classified as Type IVb and is capable of conjugation [116-118]. Interestingly, *L. pneumophila* also contains a Type IVa translocation system on the *lvh* locus but it is not required for intracellular replication [119].

The *dot/icm* locus is composed of 27 genes that make up both a translocation channel and a coupling protein/complex [116]. This machinery traverses the bacterial inner membrane and the cell envelope to deliver substrates into the host cytosol [114]. The coupling complex, DotL, DotM, and DotN, recruits substrates and delivers them to the translocation channel with the assistance of chaperone proteins, IcmW and IcmS [114, 120, 121]. IcmSW recruits substrates to DotL by binding to a recognition sequence on the C-terminus [122, 123]. However, some substrates can still bind to the DotMLN coupling complex without the help of IcmSW [90, 124]. These are considered to be IcmSW-independent substrates, whose binding appears to be mediated by a C-terminus that is glutamic acid-rich [90, 124].

Substrates of the Dot/Icm are collectively referred to as “effectors”. Large bioinformatics screens have led to the identification of over 300 effectors of the Dot/Icm [125-127]. The translocation of 300+ effectors, 10% of the genome, into the host cell by *L. pneumophila* is substantially greater than the next highest number of injected effectors by a pathogen, at >100 by *Coxiella burnetii [128]*.Delivery of a subset of these proteins occurs immediately upon attachment and occurs throughout intracellular growth [89, 90]. Intracellularly, Dot/Icm machinery is located at the poles of the bacterium [129]. Surprisingly, non-polar localization of the Dot/Icm structures fails to evade the lysosomes despite translocating effectors [129]. Regardless of translocating a large number of different effectors, on average, only 4 Dot/Icm structures are located at a pole [129]. It has been shown that only ~200 molecules of the effector VipD are delivered into the host cell, a relatively low number compared to the secretion of ~6,000 molecules of SipA by *Salmonella entrica* [130, 131]. However, host cell toxicity could play a role in the minimal translocation of individual effectors.

Few effector null mutants exhibit intracellular growth defects, which is likely due to the functional redundancy of many effectors [132-134]. Even minimalizing the *L. pneumophila* genome, eliminating 31% of the known effectors, barely caused any intracellular growth defect in mouse macrophages [134]. Redundancy among the *L. pneumophila* effectors occurs in different manners: molecular redundancy, target redundancy, pathway redundancy, cellular process redundancy, and system redundancy [132].

As an example of molecular redundancy, effectors have been shown to perform the same function on the same host cell target, like with the SidE family [133]. SidE,

SdeA, SdeB, and SdeC catalyze the ubiquitination of the host proteins Reticulon 4 and Rab33b [135, 136]. Deletion of all four of these effectors together, but not individually, impairs intracellular growth, which can be restored with complementation of just SdeA [136, 137]. Paralogs of VipD (VdpA, VdpB, and VdpC) may appear to be redundant but have actually do not bind the same proteins [130, 138]. The targeting of different components of the host endocytic pathway by VipD and SidK is an example of pathway redundancy [130, 139]. Target redundant effectors, like SidM and AnkX, both modulate the activity of host small GTPase Rab1 through different mechanisms [140-143]. Cellular process redundancy is the targeting of redundant or complementary host pathways that collectively govern a single process, like with SidF and SidP modulating host lipid metabolism and the abundance of phosphoinositides at the LCV [144-146]. System redundant effectors modulate more than one host cellular process to accomplish the same task; for example, inducing host cell survival mechanism by LegK1 (activates nuclear factor κB by degrading its inhibitor, IκB), Lgt1/Lgt2/Lgt3 (blocking host protein synthesis and thus replenishment of IκB), and SidF (inhibiting pro-apoptotic proteins) [145, 147, 148].

Redundancy in microbes is often lost over time, but *L. pneumophila* has retained a large number of seeming redundant effectors. Growth in a variety of environmental hosts and temporal regulation may explain why *L. pneumophila* have retained these effectors.

Effector redundancy is common among *Legionella* spp.; members of the genus do however, each contain their own set of effectors, which vary from 52 – 300+ putative effectors [149]. Of the 38 *Legionella* spp. analyzed, only seven of effectors were identified to be present across the genus [149]. These seven proteins were determined to

be “core effectors”, although the function of most is unknown: AnkH, MavN (iron acquisition), RavC, VipF (GNAT family N-aceytltransferase), cetLp1, Lpg3000, and Lpg2832 are present in all 41 *Legionella* spp. tested [149-152]. Another 30 effectors can be found in 31-40 species, while 78% of *Legionella* effectors are only shared by ten or fewer species [149]. *L. pneumophila* contains 30 species-specific effectors [149].

The GC content of these core effectors suggests they evolved as part of the *Legionella* genus over an extend period of time, as difference between the GC content core effectors (37.4%) and the genome (38.3%) is minimal [149]. However, the GC content of species-specific effectors is consistently lower than the GC content of the genome (~34%) for all tested *Legionella* species, indicating that these genes might have been recently acquired [149].

Protozoan genomes are typically characterized by a low GC content [153]. The long-term coevolution of *L. pneumophila* with various protozoa has likely influenced the genomic structure of this organism through inter-kingdom horizontal gene transfer (HGT) [154-157]. Even within strains of the same *Legionella* species, a high degree of plasticity is observed [158]. Between *L. pneumophila* strain Paris and *L. pneumophila* strain Lens, 2,664 genes are conserved but 428 and 280, respectively, are strain-specific genes [158]. Potential hot spots for genomic rearrangement have been identified that contribute to the plasticity of the organism [158, 159]. *L. pneumophila* strains contain independent plasmids and/or plasmids that have inserted into the genome [158]. Notably, the region containing the Type IVa secretion system locus, *lvh*, can be encoded in the chromosome or excised as a multicopy plasmid, and has a much higher GC content than the rest of the genome at 43% [158].

This *L. pneumophila* genomic plasticity and long-term coevolution with amoebae and intral-amoebal species likely has contributed to the large cadre of effectors in *L. pneumophila*, many of which contain eukaryotic-like domains and motifs [157, 158, 160,]. Amoebae may act as the 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.

L. pneumophila is a naturally competent organism that takes up DNA through conjugation as well as natural transformation [117, 118, 161]. Long-term convergent evolution and modification of the genes acquired through HGT by splicing of introns, acquiring prokaryotic promoters and regulators, and the addition of translocation motifs is likely what allowed eukaryotic proteins to become translocated bacterial effectors with functional activities in the host cell [156]. 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 [154].

Many *L. pneumophila* effectors contain eukaryotic proteins domains and motifs such as: F-box, U-box, ankyrin repeats, SEL-1 repeats, farnesylation motifs, and post-translational modification motifs [154, 155, 158, 162, 163]. These *L. pneumophila* effectors are involved in various host processes which include signaling, vesicular trafficking, apoptosis, protein synthesis, ubiquitination, etc; aiding in their ability to interfere in host processes using eukaryotic domains [155, 158, 160, 162].

Exploitation of conserved eukaryotic processes by the eukaryotic-like AnkB effector and its role in nutrient acquisition

Many effectors have been described for their function in LCV biogenesis. For example, SidM (DrrA) recruits, activates, and retains Rab1 at the LCV membrane causing vesicular trafficking between the ER and Golgi to be redirected to the LCV [164]. SidM works in conjunction with another effector, LidA, which enhances function of SidM [140]. Knockout of SidM has no effect on bacterial replication within host cells [140]. LidA, does however show a slight defect in replication [165]. As previously mentioned, this phenotype is common among knockouts in effector proteins due to the high number of redundant effectors that are translocated [140].

Among the ~300 effectors of *L. pneumophila*, AnkB is one of the few effectors known to be indispensable for the intracellular infection of both human macrophages and amoebae [97, 98, 127, 149, 166, 167]. Additionally, it is essential for virulence in the A/J mouse model [75, 168]. 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 and motifs that enable this protein to hijack a number of evolutionarily conserved eukaryotic processes [163, 167-170]. AnkB harbors three Ankyrin domains (ANK), 33-residue repeats involved in protein-protein interactions, which is the most common domain in eukaryotic proteins [163, 171]. These Ankyrin domains play a role in interaction of AnkB to the host SCF1 ubiquitin ligase complex [172]. This promotes the K^{48}-linked polyubiquitination of host proteins, targeting the proteins for degradation by the proteasome. AnkB also contains a

C-terminal eukaryotic CaaX motif (C, cysteine; a, aliphatic amino acid; X, any amino acid) which allows the protein to be lipidated through farnesylation by the host farnesyltransferase (FTase), thus anchoring AnkB into the LCV membrane [172, 173]. Because this process is localized to the LCV a "cloud" of polyubiquitinated proteins can be observed surrounding the LCV [75]. Increased host protein degradation provides an abundance of amino acids in the cytosol for metabolism by *L. pneumophila* (Figure 1-1) [75].

The *ankB* mutant of *L. pneumophila* is severely defective in intracellular proliferation in amoebae and human macrophages due to the defect in assembly of K^{48}-linked polyubiquitinated proteins decorating the LCV and subsequent lack of increased levels of amino acids (Figure 1-1) [75, 98, 154]. This triggers a bacterial starvation response, mediated by the induced expression of RelA and SpoT, and results in elevated ppGpp levels (more on this will be discussed in the next section) [75, 154].

Intracellular growth can be restored to the *ankB* mutant within amoebae and human cells by supplementing excess amino acids, similar to genetic complementation [75, 154, 174]. Thus, it is clear that higher levels of cellular amino acids are needed for intracellular replication of *L. pneumophila* than the endogenous amounts. Remarkably, supplementation of infected cells with certain single amino acids, such as serine or cysteine, reverses the growth defect of the *ankB* mutant in amoebae and human macrophages [75]. Interestingly, in human cells cysteine is semi-essential and is the least abundant amino acid, but in amoebae cysteine is essential [75, 174]. However, serine is not essential for either but is favored by *L. pneumophila* for use in the TCA cycle [175]. Similar to cysteine and serine, supplementation of infected cells with pyruvate or citrate

to feed the TCA cycle, rescues the *ankB* mutant for intracellular proliferation [75, 154, 174]. Additionally, *in vitro* growth of *L. pneumophila* in rich medium requires supplementation with 3.3 mM cysteine [75]. 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 [154]. By promoting proteasomal degradation of proteins in amoebae and human macrophages though the AnkB F-box effector, *L. pneumophila* generates a gratuitous supply of cellular amino acids, particularly the limiting and metabolically favorable ones such as Cys and Ser, which are the favorable source of carbon and energy for *L. pneumophila* to power intracellular growth within amoebae and human macrophages [174].

Nutritional based virulence is an emerging topic in microbial pathogenesis [176]. *Legionella*'s unique nutritional needs make it an interesting subject to study how virulence is tied to the drive for food.

Phase Variation and Stress Response

Nutrient availability governs the biphasic lifestyle of *L. pneumophila* [177-179]. When nutrient levels are high the organism exists in the replicative phase. When nutrient levels are low, the bacterium enters a transmissive phase, making it more capable of finding the next meal. The intracellular lifecycle of *L. pneumophila* within the LCV also rotates through replicative phase and a transmissive phase, exhibited upon escape into the cytosol [86, 101, 102, 180].

The biphasic lifestyle is characterized by dramatic changes in the transcriptome, which result in phenotypic modulations [177, 179, 181]. During the replicative phase, the bacterium is undergoing exponential growth (E); it is non-motile, and represses

transmissive traits, such as lysosomal evasion (Figure 1-1, step 2) [182]. The transmissive phase, during post-exponential (PE) growth prepares the bacteria for life outside of the protective environment of the LCV. Traits expressed during PE phase correspond with an increased virulence of the bacteria, which become cytotoxic, motile, sodium sensitive, and osmotically resistant [86, 182]. These changes are necessary to invade a new host and start a second cycle of intracellular proliferation [86, 102, 181-185].

The transition between replicative and transmissive phenotypes is highly orchestrated, and is governed by many factors that are influenced by intracellular nutrient levels [86, 87, 154, 186]. Upon amino acid depletion, uncharged bacterial tRNAs activate RelA to synthesize the bacterial alarmone guanosine-3'-5'-buspyrophosphate (ppGpp) (Fig. 1-2) [86]. 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. 1-2) [187]. DksA acts as a co-factor for ppGpp-dependent transcriptional regulation, but it is not required for replication in the macrophage, unlike the other factors of the regulatory cascade (Fig. 1-2) [104, 187, 188]. Accumulation of ppGGpp, activates RpoS, an alternative sigma factor [189-191]. RpoS regulates the two-component system CpxR/A and LqsR/S (Fig. 1-2) [192]. CpxR/A controls the expression of 27 Dot/Icm effectors and virulence substrates of the type-II secretion system [193, 194]. LqsR/S functions as the quorum sensing response regulator, resulting in production of *Legionella* autoinducer-1,3-hydroxypentadecane-4-one (LAI-1) [195].

Activation of the RNA binding protein and chaperone, Hfq, by RpoS and LetA/S, turns on transcription upon the onset of the late PE phase (Fig. 1-2) [196, 197]. Additionally, LetA/S activation by ppGpp accumulation activates the small, non-coding

RNAs, RsmX/Y/Z, which contain multiple binding sites for *csrA* [87, 198-200]. The sequestering action of RsmX/Y/Z on CsrA, a negative regulator of transmissive traits and activator of replication, allows for the translation of transmissive traits like motility, virulence, and stress resistance (Fig. 1-2) [201-203]. The PmrA/B two component system positive regulates CsrA and is responsible for post-translational repression of CsrA-regulated effectors (Fig. 1-2) [87, 179].

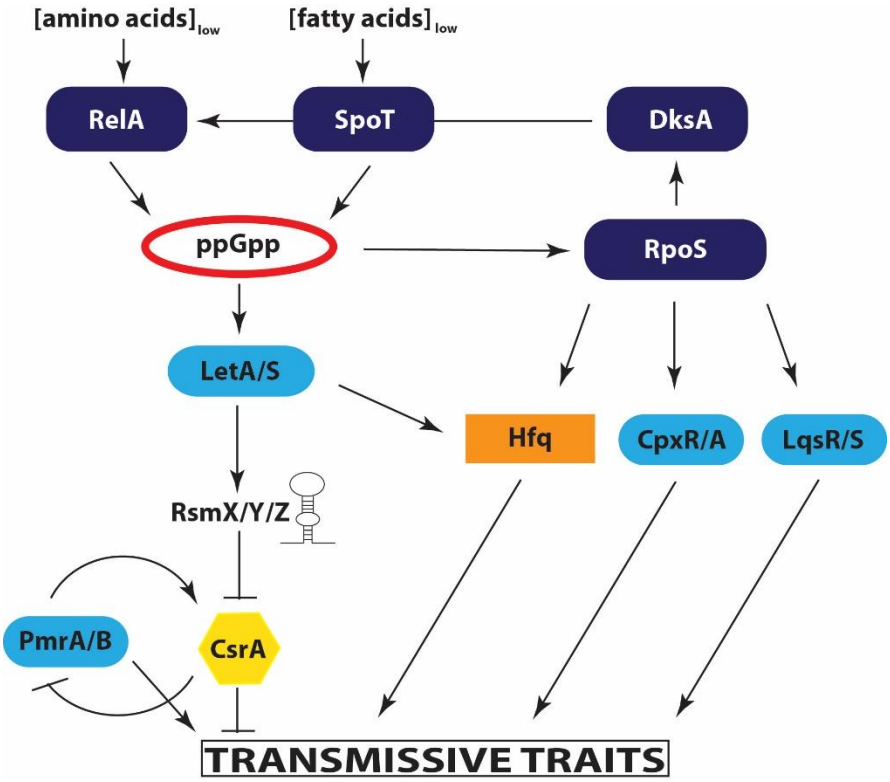

Figure 1-2. The starvation response in *L. pneumophila*. Starvation is triggered upon sensing depletion in the amino acids and fatty acids of the intracellular environment, which triggers RelA and SpoT, leading to an increase level of the alarmone, ppGpp. Accumulation of ppGpp is sensed by the two-component system LetA/S and the alternative sigma factor, RpoS. LetA/S induce the small non-coding RNAs, RsmX/Y/Z, which block the global repressor of transmissive traits, CsrA. RpoS regulates the Hfq, CpxR/A, and LqsR/S pathways, leading to an increase in transmissive traits. PmrA/B activates 43 effectors and positively regulates CsrA, acting as a switch upon entry to the transmissive phase.

In addition to triggering flagellation and various virulence-related traits, elevated ppGpp levels result in upregulation of the Dot/Icm effectors [177, 179, 181]. For example, during infection AnkB is temporally and differentially regulated at the PE phase [95, 97, 98, 166, 179, 204]. 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.

Nutrient Acquisition by *L. pneumophila*

It has been known since the discovery of *Legionella* that the bacteria have strict nutrient requirements, obtaining carbon and energy from amino acids [175, 205-208]. This is what contributed to the difficultly in isolating and growing *Legionella* [3]. Special agar plates, called Buffered Charcoal Yeast Extract (BCYE), are used for *Legionella* which are supplemented with cysteine and α-ketogluterate, to meet the high demand by the organism, and charcoal, to mitigate the effects of oxygen radicals generated during autoclaving of the agar [209].

Instead of using glycolysis to generate pyruvate from glucose, *L. pneumophila* uses amino acids [175, 205, 210]. Serine, cysteine, and alanine – in order of preference – are converted directly into pyruvate. Glutamate can be converted into the TCA cycle intermediate, α-ketogluterate, and aspartate into fumerate and oxaloacetate [174, 205, 208, 211] [174]. *L. pneumophila* can also take up host pyruvate to use directly in the TCA cycle [75, 154, 174]. Eylert *et al.* showed that the amino acids (alanine, glutamic acid, glycine, asparagine, leucine, threonine, arginine, isoleucine, valine, and aspartic

acid) are imported from *A. castellanii* into the LCV and are converted by *L. pneumophila*, suggesting their use in the TCA cycle [175].

While *L. pneumophila* relies on amino acids for carbon and energy it is also auxotrophic for seven amino acids (cysteine, leucine, methionine, valine, threonine, isoleucine, and arginine) [158, 162, 175]. These auxothropies are synced with their eukaryotic host, as a way of nutritional adaptation to the host (Fig. 1-3) [52]. Humans are auxotrophic for histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine [212]. Macrophages are additionally auxotrophic for glutamine and asparagine (Fig. 1-3) [212]. *Acanthamoeba*, one of the most prevalent environment hosts for *Legionella*, is auxotrophic for arginine, isoleucine, leucine, methionine, and valine [213].

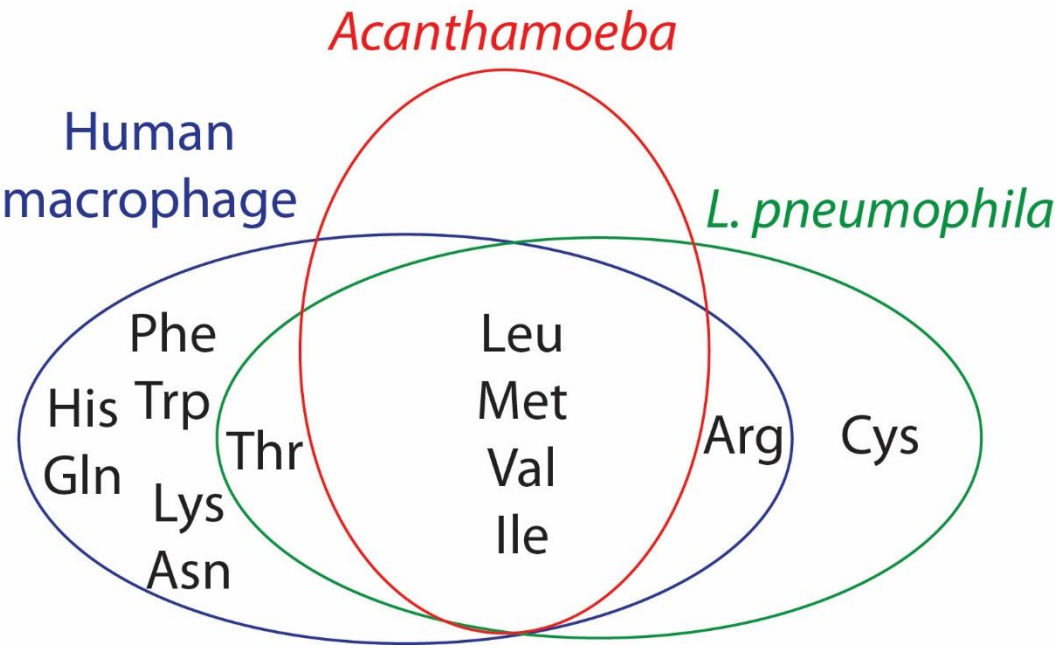

Figure 1-3. Amino acid auxotrophy in human macrophages, *Acanthamoeba,* and *L. pneumophila*. There is considerable overlap in auxotrophy between *L. pneumophila* and its most common environmental host, *Acanthamoeba*. Many of these auxothropies are also seen in human macrophages, the accidental host.

Why *L. pneumophila* prefers amino acids over glucose, like most other genera of bacteria studied, is unknown. The answer likely lies within its genome and through better understanding of its evolution with protozoa. Like most driving factors for evolution, use of amino acids could keep it reliant on its host, limiting growth only when within a host, thus giving *L. pneumophila* an evolutionary advantage. Nutrition of *L. pneumophila* within protozoa has been likely a major driving force in its evolution as an intracellular pathogen.

Differential glucose utilization by *L. pneumophila* and generation of a storage molecule

Historically, *L. pneumophila* was described as being defective in glycolysis. However, it does have a functional glycolytic pathway, also referred to as Embden-Meyeroff-Parnas (EMP) pathway, which is minimally utilized [175, 214]. Instead, it favors the Enter-Doudoroff (ED) pathway for glucose catabolism (Fig. 1-4) [175]. The Pentose Phosphate Pathway (PPP) functions only to generate mannose and histidine within *L. pneumophila* (Fig. 1-3) [214, 215]. During exponential (E) phase, isotopologue labelling demonstrates that serine is the preferred amino acid to generate pyruvate to feed into the TCA cycle (Fig. 1-4) [175, 214]. Some serine is diverted to the EMP and PPP to generate mannose and histidine, and to generate the storage molecule poly-3-hydroxybutyrate (PHB) (Fig. 1-4, 1-6) [175]. A shift into the post-exponential (PE) phase of growth occurs when amino acids (and fatty acids) are low, when glucose becomes the predominant molecule metabolized (Fig. 1-4) [175, 216-218]. Glucose is metabolized by ED pathway to generate pyruvate then acetyl-CoA, used for the synthesis of large stores of PHB (Fig. 1-3, 1-5).

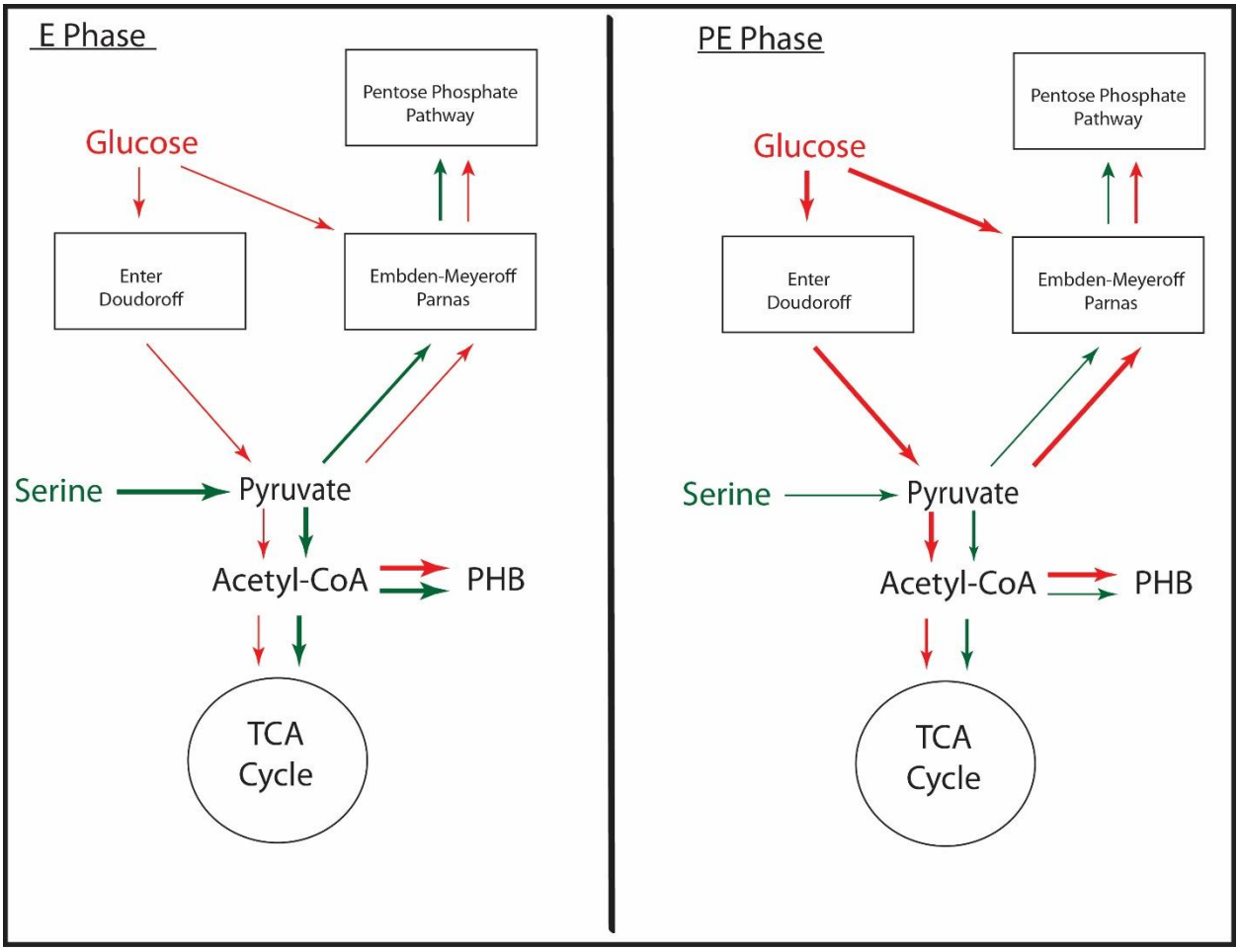

Figure 1-4. Utilization of serine and glucose is differentially regulated in exponential (E) phase and post-exponential (PE) phase. Isotopologue labeling experiments have demonstrated that in E phase of growth, serine is the preferentially used amino acid to generate pyruvate and feed into the TCA cycle. Also during this time, small amounts of serine (green) and glucose (red) are used to generate poly-3-hydroxybutyrate (PHB) from acetyl-CoA and are shuffled into the PPP to generate mannose and histidine. During PE phase growth, amino acids are low and glucose becomes the predominate molecule metabolize, which is primarily used to generate large stores of PHB.

This stage-specific metabolism is governed by the CsrA regulatory system [219]. The CsrA regulator binds directly to, and induces transcription of, the glucose utilization operon in *L. pneumophila,* which contains: a glucose-6-phosphate-1-dehydrogenase

(*lpg0416, zwf*), a 6-phosphogluconolactonase (*lpg0417, pgl*), a 6-phophogluconate dehydratase (*lpg0418, edd*), a glucokinase (*lpg0419, glk*), a KHG/KFPG aldose (*lpg0420, eda*), and a D-xylose proton symporter (*lpg0421, ywtG)* (Fig. 1-5)[175, 220]. Two separate reports on knockout of a glucose utilization gene cluster, in different strains showed conflicting requirement for glucose [175, 203, 220]. Harada *et al.* demonstrated a requirement for *edd, glk, eda,* and *ywtG* in a Philadelphia strain derivative, for intracellular growth in *A. culbertsoni*, the human epithelial cell line A549, and the A/J mouse model, [220]. In contrast, Eylert *et al.* demonstrated no requirement for any of the proteins in this glucose operon within the Paris strain of *L. pneumophila* and additionally showed that the glucose transporter, *ywtG*, was not part of the operon [175]. Therefore the need for glucose during infection is still unclear. While the need for glucose in long-term survival has yet to be investigated.

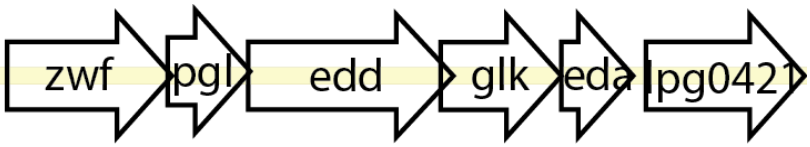

Figure 1-5. Glucose gene cluster in *L. pneumophila*. Zwf, Pgl, Glk, and Edd, of the glucose utilization cluster in *L. pneumophila* are responsible for glucose metabolism through the ED pathway. Eda converts an intermediate of the ED pathway into Glyceraldehyde-3-P, an intermediate of the EMP pathway. Lpg0421 (YwtG) is a glucose transporter.

Being completely dependent on the host can still cause its own set of problems for any pathogen; so it would be advantageous for the pathogen to evolve strategies to survive outside the host. For that, the bacteria use a carbon and energy storage molecule. Polyhydroxyalkanoates are a common family of carbon and energy reserve molecules generated by many bacterial species [221]. The most abundant member, poly-3-

hydroxybutyrate (PHB), is produced by many environmental microorganism such as, *Bradyrhizobium japonicum, Cupriavidus necator, Methylobacterium rhodesianum,* and *Bacilius megaterium* [221]. PHB is also produced by *Legionella* starting in the late exponential stage of growth [175, 214, 218]. PHB is a homopolymer of 3-hydroxybutyric acid which is generated from acetyl-CoA (Fig. 1-6) [221]. Large granules of PHB can be observed as empty spaces by electron microscopy, as PHB dissolves during the fixation process (Fig. 5) [222-225]. In early stages of growth, serine is converted into PHB until post-exponential growth, when glucose transport is greatly increased, and then glucose serves as the main precursor for PHB biosynthesis (Fig. 1-4, 1-6) [218, 220].

Generation of PHB helps to prepare the bacterium for life outside the cell, enabling it to survive nutrient-poor conditions [226]. Catabolism of PHB does occur during the VNBC state and in the stationary phase [218, 226]. PHB has been assumed as the major product of glucose utilization in *L. pneumophila* [175, 214]. Contribution of the glucose-utilization gene cluster to the generation of PHB has not been studied. Four PHB synthesis genes have been identified in *L. pneumophila* strain Paris [218]. A mutation in one gene, *lpp0650*, results in only 15% PHB compared to the WT strain but does not result in an intracellular growth defect in human macrophages nor *A. castellanii*, up to 72 hrs post-infection [218]. Glucose metabolism by the ED pathway is connected to PHB synthesis as a *zwf* mutant, which encodes the first enzyme of the ED pathway) has 68% less PHB than the WT strain [218].

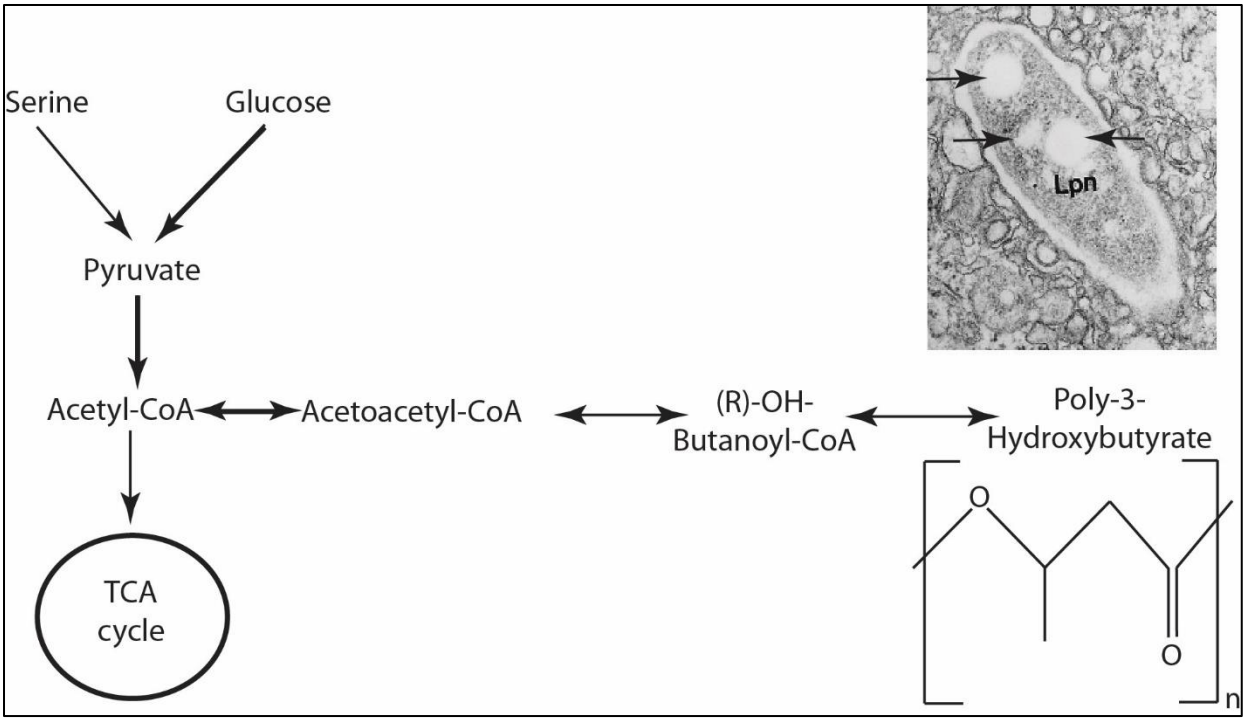

Figure 1-6. Serine and glucose are converted into poly-3-hydroxybutyrate. Glucose, the preferential molecule for PHB synthesis, is metabolized into pyruvate by the ED pathway, which is converted into acetyl-CoA. Acetyl-CoA can feed into the TCA cycle or converted into PHB through two intermediates, Acetoacetyl-CoA and (*R*)-OH-Butanoyl-CoA. PHB is a polymer that is used as a storage molecule. Granules of PHB can be seen during electron microscopy as large empty areas (insert), due to the fixation process [53].

SPECIFIC AIMS

It is clear that *L. pneumophila* relies on amino acids for optimal growth *in vivo* and that these can be acquired from the host cell [51, 75]. However, it is unclear by which mechanism *L. pneumophila* imports host amino acids from the cytosol into the LCV lumen. Human SLC transporters are a likely candidate of amino acid transporters, as they have been previously identified to be present on the LCV membrane by mass-spectrometry [227-229]. I sought to study the mechanism by which *L. pneumophila* acquires amino acids through human SLCs.

L. pneumophila has evolved with numerous eukaryotic-like genes acquired through inter-kingdom horizontal gene transfer. Therefore, I sought to identify eukaryotic SLC-like transporters in *L. pneumophila* that were structurally similar to human SLCs, due to the lack of characterized transporters in amoebae.

The role of glucose during intracellular infection has yet to be fully characterized. Glucose catabolism through the ED pathway is required for intracellular replication of *L. pneumophila* [220]. The mechanism of glucose import by *L. pneumophila* is not well understood. To better understand the role of glucose acquisition in *L. pneumophila*, I sought to characterize two putative eukaryotic SLC-like glucose transporters, LstA and LstB of *L. pneumophila*.

I hypothesize that *L. pneumophila* has acquired host solute carriers (SLCs) to important metabolites across the LCV membrane. Additionally, SLC-like transporters in *L. pneumophila* are important for acquisition of intracellular metabolites.

In addition to the generation of surplus amino acids in the cytosol, used to feed the TCA cycle, *L. pneumophila* contains multiple amylases that may contribute to nutritional virulence by increasing the host levels of glucose in the host cytosol. I sought

to characterize the putative amylase, LamB, to understand its role in intracellular replication.

I will test the hypothesis that, *L. pneumophila* produces amylases to generate a surplus of host glucose.

To test these two hypotheses, my specific aims are:

Specific Aim 1: Determine Dot/Icm-dependent recruitment of host SLCs to the LCV membrane during intracellular infection of human macrophages.

Specific Aim 2: Identify and characterize eukaryotic SLC-like proteins in *L. pneumophila* virulence.

Specific Aim 3: Identify and characterize the role of the LamB amylase of *L. pneumophila* in intracellular growth.

CHAPTER 2:

DOT/ICM RECRUITMENT OF HOST AMINO ACID TRANSPORTERS DURING INTRACELLULAR *L. PNEUMOPHILA* INFECTION

SUMMARY

Within the host cytosol, the *L. pneumophila* effector AnkB triggers polyubiquitination of host proteins, targeting them for proteasomal degradation [75, 97, 98, 170]. In order to be able to utilize the amino acids generated by AnkB, *L. pneumophila* must import them into the LCV. The only mechanism for amino acid movement across intact membranes is through active transport. Given that the LCV is derived from host membranes and many host Solute Carrier (SLC) transporters have been identified by mass-spectrometry to be present on the LCV, in numerous studies [227-229], I hypothesize that SLCs are responsible for importing amino acids to the LCV lumen [230-232]. Here I describe the attempts to validate localization of host SLC transporters on the LCV by confocal microscopy. Through a variety of cell types and time points, I was unable to confirm the presence of host amino acid-transporting SLCs on the LCV, in a Dot/Icm-dependent manner. I did identify amino acid-transporting SLCs present during infection of both WT *L. pneumophila* and *dotA,* the translocation-deficient mutant [110, 115]. Therefore, I could not confirm that amino acid-transporting SLCs are directed to the LCV, since the *dotA* mutant is contained within a phagolysosomes [233]. Instead, likely due to general vesicle trafficking and recruitment during infection, amino acid-transporting SLCs are "accidently" retained on the phagosomal membrane. Alternatively, this may be a very transient process which is difficult to capture through fixed-time point microscopy. However, I did confirm the presence of a glucose-transporting SLC, SLC2a1/Glut1, on the LCV, in a Dot/Icm-dependent manner, indicating an important role for glucose utilization by *L. pneumophila* during intracellular infection. Additional studies suggest SLC2a1/Glut1 is recruited during late stages of intracellular growth, corresponding to when *L. pneumophila* is utilizing glucose [181,

220]. The exact mechanism of *L. pneumophila*-mediated SLC2a1/Glut1 recruitment to LCV remains unclear. However, these studies were not possible to be continued due to termination of commercial antibody production and the lack of detection of SLC2a1/Glut1 on the LCV using 3 antibodies from other independent commercial sources (Table 2-7).

INTRODUCTION

Amino acids are the primary source of carbon and energy for *Legionella*; it is reasonable to conclude these are acquired from the host cell, macrophages or amoebae [76, 192, 205, 234]. Indeed, studies have shown during infection of *Acanthamoeba castellanii*, pre-fed with labeled glucose, which the amoebae converted into amino acids, that *L. pneumophila* acquires these amino acids from the amoebae [76]. However, the mechanism by which the bacterium accesses those amino acids is still unknown. Generation of large amounts of amino acids by the AnkB effector is done in the host cytosol, inaccessible to the replicating bacteria, and requires a transporter for amino acids to cross the LCV membrane [75, 231].

One likely set of candidates to aid in the transport of amino acids across the LCV membrane in human macrophages are the Solute Carrier (SLC) family of transporters, of which homologs are present in amoebae [231, 235]. The SLC group of membrane transporter proteins within humans is large and diverse; consisting of ~386 members, divided into 55 families [231, 232]. Of these, twelve families are involved in the transport of amino acids and/or oligopeptides (SLC1, 3, 6, 7, 15, 17, 18, 25, 26, 32, 36, and 38) [231, 235]. Within a family, members share primary sequence identity of ≥20%, to at least one other family member [232]. SLCs show tissue-specific tropism, and are

separated into families based on substrate-specificity, number of transmembrane helices, and mechanism of transport [231, 235]. Importantly, homologs of these transporters can be found in protozoa and can be found by comparing the amino acid sequences of SLCs to the database of protozoan genomes in BLAST but none to-date have been functionally described. They are members of an evolutionarily conserved family of transporters known as the Major Facilitator Superfamily (MFS), which are present in all kingdoms of life [236, 237]. Within the environmental host is where *L. pneumophila* would have acquired the ability to exploit these host transporters [156, 238]. However, a dearth of information on general biology and lack of molecular tools for transporters in protozoa make them a difficult target to study. Thus, the human counterparts are more feasible to study.

The mammalian SLC1a5 amino acid transporter is a high-affinity importer of glutamate but also imports neutral amino acids: cysteine, serine, and alanine [239, 240]. This transporter works in a super-complex with other SLCs and scaffolding proteins: SLC7a5, SLC3a2, SLC16a1, CD147, and Ep-CAM [241-245]. Signaling through this complex to mTOR that appropriate levels of amino acids are available leads to translation initiation by eIF3 and suppression of autophagy [241, 242]. In competing interest to *L. pneumophila,* increasing the activity of any of these transporters would increase the amino acid uptake of the host, but would initiate translation, causing the cell to use the free amino acids. Within mouse macrophages, *L. pneumophila* has been shown to inhibit mTORC1 to prevent protein synthesis, thus liberating more amino acids to drive intracellular replication, which could off-set any changes leading to increased uptake by the SLCs in the super-complex [246]. SLC1a5 has been shown to be required for

replication of *L. pneumophila* in the MM6 monocytic cell line via RNA silencing of SLC1a5 [247]. Additionally, knockdown of the aforementioned super-complex with the inhibitor BCH, leads to decreased replication in human macrophages [247]. To date, this is the only SLC shown to have a role in *L. pneumophila* intracellular replication. Presence of SLC1a5 on the LCV or recruitment of other SLCs to the LCV, in a Dot/Icm-dependent manner, has not been shown.

Glucose uptake in cells is achieved through the SLC2 (Glut) family of transporters [248]. Within the cell, once SLC2a1/Glut1 is synthesized, it remains in vesicles in the cytoplasm until a signal is received to traffic to the plasma membrane [249]. The primary signals for SLC2a1/Glut1 trafficking are IL-3 and granulocyte/macrophage colony-stimulating factor (GM-CSF) [249]. IL-3 and GM-CSF are involved in the proliferation of myeloid cells and the maturation of macrophages [250, 251]. Elevated levels of glucose (hyperglycemia), such as seen in diabetic patients, promote inflammatory activation of macrophages and aid in classical M1 activation of macrophages [252]. SLC2a1/Glut1 is the rate limited step of a proinflammatory phenotype in macrophages [253]. Overexpression of SLC2a1/Glut1 causes an increase in reactive oxygen species along with a pro-inflammatory response [253]. Outside of being a part of the macrophage response to general bacterial infections, no role for SLC2a1/Glut1 has been shown during intracellular infection of human macrophages with *L. pneumophila* or other intracellular bacterial pathogens.

Many SLCs have been identified to be part of the LCV proteome by mass-spectrometry in multiple studies [227-229]. Table 2-1, represents all the SLCs contained in the LCV-proteome identified by mass-spectrometry by various studies [227-229]. Each

study determined the proteins on the LCV in different host cells and time-points. Hoffmann *et al.* used the mouse macrophage cell line, RAW264.7, with a short infection of 1 hr [229]. The caveats of this approach is the use of RAW264.7, as these cells are of mouse origin restrictive to *Legionella* growth; but the transformed cell line is permissive to *L. pneumophila* growth [254-256]. Bruckert *et al*. used a human macrophage cell line, U937, with an infection duration of 4 hrs [228]. The longer time of infection could also allow for a variation in the dynamics of SLC recruitment or retention on the LCV. Finally, Naujoks *et al.* used primary macrophages but from C57BL/6 mice; these restrictive bone marrow-derived macrophages (BMM) were infected for 2 hrs with the *flaA* mutant, to prevent pyroptosis and pathogen restriction [227].

The amount of protein, and thus cells, needed to do mass-spectrometry is very high, making the use of primary human cells nearly impossible. Being able to overcome this technical limitation would provide more reliable data. Additionally, proteomics on the LCV of a well-annotated protozoan host would allow for comparisons of transporters that may be evolutionarily conserved. However, these three experiments provide a step towards identifying SLCs involvement during *L. pneumophila* infection.

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Table 2-1. Identification of SLCs on the LCV by mass-spectrometry studies.

SLC	Common synonym	Substrate	Hoffmann [229]	Bruckert [228]	Naujoks [227]
1a5	ASCT2	glutamine, alanine, cysteine, serine	✓	✓	✓
2a1	GLUT1	glucose	✓	✓	
2a6	GLUT6	glucose	✓		✓
3a2	CD98	valine, leucine, isoleucine, tryptophan, tyrosine	✓	✓	✓
7a5	LAT1	cysteine, alanine, serine, pheylalanine, tyrosine, leucine, arginine, tryptophan,	✓	✓	✓
9a3r1	NHEFR-1	Sodium/Hydrogen Exchanger - scaffold		✓	✓
15a3	OCTP	Oligopeptide	✓		✓
16a1	MCT1	Monocarboxylates	✓	✓	✓
16a3	MCT4	Monocarboxylates	✓	✓	✓
25a3	PTP/PHC	Phosphate (mitochondrial)	✓	✓	✓
25a4	ANT1	ADP (mitochondrial)	✓	✓	✓
25a5	ANT2	ADP (mitochondrial)	✓	✓	✓
25a10	DIC	Dicarboxylate (mitochondrial)	✓		✓
25a11	OGCP	Oxoglutarate/malate exchange (mitochondrial)	✓		✓
25a12	AGC1	Aspartate/Glutamate (mitochondrial)	✓		✓
25a13	CTLN2	Aspartate/Glutamate (mitochondrial)	✓	✓	✓
25a22	GC-1	Glutamate (mitochondrial)	✓		✓
25a24	APC1	ATP-MG/ Phosphate exchange (mitochondrial)	✓		✓
30a1	ANT1	Zinc		✓	✓

The amount of protein, and thus cells, needed to do mass-spectrometry is very high, making the use of primary human cells nearly impossible. Being able to overcome this technical limitation would provide more reliable data. Additionally, proteomics on the LCV of a well-annotated protozoan host would allow for comparisons of transporters that may be evolutionarily conserved. However, these three experiments provide a step towards identifying SLCs involvement during *L. pneumophila* infection [227-229].

The SLCs represented in Table 2-1 is not a complete list of all of those found in each individual study but a selection based on their appearance in two or more studies [227-229]. The Naujoks study alone identified 60 SLCs to be present on the LCV, while Hoffmann and Bruckert identified 20 and 21, respectively [227-229]. Differences in the number and type of SLCs identified could be due to numerous reasons, such as: cell type, length of infection, and experimental preparation [227-229]. This also could suggest a dynamic and temporal presence of SLCs on the LCV. Data from infections at late time-points, such as 8 hrs, could provide better representation of SLCs, as this is when the bacterium is preparing for replication and the demand for nutrients rises. The SLC25 family of transporters function at the mitochondrial membrane, rather than the plasma membrane [257]. LCVs have been shown to be closely associated with mitochondria during infection [56, 91, 258, 259]; therefore, their appearance on the LCV could be due to this close association contaminating LCV purification, through fusion of mitochondrial membranes with the LCV, or targeting of these by effectors. Most of these transporters target multiple substrates which contributes to strong overlap, especially for amino acids which feed into the TCA cycle (Ala, Cys, Ser, Trp, Arg, Phe, Pro, Met, Val, Iso, Gly, Leu, Tyr, Thr) and covers the amino acids for which *L. pneumophila* is auxotrophic (Thr, Arg, Iso, Met, Leu, Cys, Val) (Table 2-1) [52, 175].

Total human transcriptome microarray of hMDMs revealed minor changes in transcription of SLCs during infection with WT *L. pneumophila*, after 1 hr, compared to uninfected cells [260]. Only minimal fold changes between -1.2- and 1.4-fold were observed for SLCs [260]. For example, transcripts of SLC7a5 and SLC7a2 were increased by 1.2- and 1.4 -fold, respectively; whereas, SLC1a5 and SLC2a1 showed no

change [89, 260]. All but two of the mitochondrial transporters listed in Table 1 (SLC25a5 and SLC25a13) were found to be minimally down-regulated the hMDM microarray [260]. Given how minor the changes were, these data may not account for normal variation between hosts. This data suggests that *L. pneumophila* may not alter SLCs at the level of transcription, as there were no significant changes in the transcriptome of SLCs during infection with *L. pneumophila* or that changes to the transcription profile occur at later time-points during the infection.

Utilizing the findings from the three mass-spectrometry studies of the LCV proteome [227-229], along with the role of SLC1a5 in intracellular growth of *L. pneumophila* as a foundation [247], we choose to validate the presence of these specific SLCs on the LCV based on their repetitive appearance in the mass-spectrometry studies and their biological importance (essential and favored amino acids). We also included SLCs that transport other small molecules that are of use to *L. pneumophila* such as, monocarboxylates (pyruvate), tricarboxylates (citrate), and glucose. We did not take into consideration tissue-tropism, as the organism could be causing aberrant gene translation of SLCs.

The role of these SLCs and their mechanism of potential acquisition by the LCV, is unclear. It is possible that the SLCs are acquired by the LCV without intervention by the bacterium, as a consequence of recruiting ER-derived vesicles. Therefore, energy does not have to be expended by the bacterium to acquire transporters. Along this line, the SLCs present on the plasma membrane, during uptake, and retained on the LCV membrane may be sufficient. Alternatively, effectors could be responsible for the altered trafficking and expression of SLCs, in a direct or indirect manner.

Given that SLCs transporters are the only source of amino acid and small molecule movement in and out of mammalian cells, due to their identification on the LCV by mass-spectrometry, and the requirement for the SLC1a5 transporter during infection, we determined the presence of SLCs directly on the LCV in a Dot/Icm-dependent manner. Using single cell analysis by confocal microscopy we determined subcellular localization of SLCs during infection with WT *L. pneumophila* and the *dotA* mutant. Additionally, we sought to confirm hMDM transcriptome data by targeting select SLCs and directly measuring transcriptional changes during intracellular infection [260].

RESULTS

Effect of SLC transcription during infection with L. pneumophila

Alteration of subcellular expression may be the underlying mechanism for manipulation of host SLCs. SLCs are known to play a role in signaling pathways to the host, like the previously mentioned signaling of mTOR by the SLC1a5/SLC3a2/SLC7a5/SLC16a1 complex [241, 242]. *L. pneumophila* is known to inhibit signaling of mTORC1 by the SidE family of effectors in mouse macrophages, preventing free amino acids from being utilized for host translation [246]. Additionally, *L. pneumophila* could be dampening SLC translation, thus indirectly inhibiting mTOR signalling. Alternatively, increasing SLCs on the plasma membrane would allow for the uptake of more amino acids that *L. pneumophila* could use for carbon and energy, while still being able to mitigate the effects of mTOR signaling with the SidE family effectors [246].

We chose to look at the expression of the four SLCs known to affect mTOR signaling (*SLC1a5, SLC3a2, SLC7a5*, and *SLC16a1*), which includes *SLC1a5* that is

known to be required for intracellular replication of *L. pneumophila* [247]. After a 2 hr infection of hMDMs, transcript levels of *SLC1a5*, *SLC3a2*, *SLC7a5* and *SLC15a1* were quantified in the WT strain, *dotA* translocation-deficient mutant, and uninfected conditions, by qPCR, using three unique human donors (Table 2-2).

Table 2-2. Fold change of SLCs in hMDMs. Expression SLC1a5, SLC3a2, SLC7a5, and SLC16a1 in hMDMS infected with WT *L. pneumophila*, the *dotA* mutant, or uninfected cells, normalized to *gapdh*, using three individual human donors ([1,2,3]).

	SLC1a5	*SLC3a2*	*SLC7a5*	*SLC16a1*
Uninfected vs WT	-9.52[1] -1.46[2] -2.22[3]	-5.65[1] 1.11[2] -1.56[3]	-1.57[1] -1.26[2] 1.00[3]	-39.48[1] 1.09[2] -4.22[3]
Uninfected vs *dotA*	1.99[1] 1.97[2] 4.94[3]	-8.29[1] 1.02[2] 7.17[3]	5.79[1] 1.23[2] 9.25[3]	30.11[1] 2.37[2] 1.79[3]
WT vs *dotA*	-18.89[1] -2.95[2] -5.47[3]	1.47[1] 1.09[2] -5.56[3]	-5.79[1] -1.55[2] -4.45[3]	-30.11[1] -2.16[2] -4.37[3]

When comparing these results to that of the transcriptome microarray data of hMDMs, two transcriptional variants for *SLC1a5* showed varying fold changes (1.0-fold increase and a -1.1-fold decrease) when comparing WT to uninfected cells, indicating little-to-no change in transcription during infection [261]. When targeting *SLC1a5* directly in this experiment, using probes that recognize all transcriptional variants, a consistent down-regulation of *SLC1a5* is observed when comparing WT to uninfected hMDMS and a slight increase in transcript levels when comparing *dotA* to uninfected cells (Table 2-2). This supports the idea that *L. pneumophila* may be actively dampening the signaling through *SLC1a5,* possibly to prevent mTOR activation, through Dot/Icm

effectors. Indeed when comparing the WT strain to the *dotA* mutant, an even more drastic, negative fold-change of *SLC1a5* can be seen (Table 2-2). However, this is surprising given that SLC1a5 is required for intracellular replication of *L. pneumophila* in human-derived MM6 cells [247]. *L. pneumophila* may be finely tuning the function of SLC1a5 during infection to optimize amino acid uptake and host cell signaling or could be due to host species variations.

When examining the other SLCs involved in this mTOR-signaling complex, down-regulation of these transcripts is usually seen; however, there is much more variation. With each SLC, there is one replicate where there was a positive fold-change when comparing uninfected cells to *L. pneumophila* infected cells, which was not of the same donor (Table 2-2). Again, with *SLC3a2, SLC7a5,* and *SLC16a1*, there is an increase in the transcription of these transporters when comparing uninfected cells to infection with the *dotA* mutant as seem with SLC1a5 (Table 2-2). With *SLC7a5* and *SLC16a1*, there is again a strong repression when comparing WT to the *dotA* mutant, indicating that the presence of Dot/Icm effectors are playing a role altering the transcriptome profile of these SLCs (Table 2-2).

These genes are expressed and transcribed at low levels in the macrophage, thus making detection threshold prone to variable results [262]. This is supported by the fact that exponential amplification of the SLC transcripts by qPCR occurred between cycles 20 – 25. Additionally, there could be donor variability in hMDMs that contributes to the lack of a strong consistent trend.

L. pneumophila does not appear to play a large role in altering the transcription of these SLCs during infection, as seen in the transcriptome of *L. pneumophila* infected

hMDMs and this study [260]. With dozens of SLCs to target, *L. pneumophila* could be altering transcription of other SLCs we have not examined. When examining the hMDM transcriptome, with no changes greater than 1.4-fold, there does not seem to be any obvious candidate [260]. Since *L. pneumophila* was not significantly altering gene expression in this pilot study, it may act solely at the protein level and altering subcellular localization.

Screen of SLC transporters present on LCV during infection of HEK293T cells

There is considerable substrate overlap when looking at the SLCs identified by mass-spectrometry on the LCV (Table 2-1). In order to cover as many SLC transporters as possible, FLAG-tagged fusion proteins of SLCs were generated for detection of LCV-colocalization by confocal microscopy. FLAG-tag constructs for 19 SLCs, including transcriptional variants, were generated using cDNA from human macrophages as a template for insertion into the p3xFLAG-CMV10 vector, allowing for overexpression in mammalian cells. Human embryonic kidney cells, HEK293T, were chosen as the host for their ability to take up and express plasmids. *L. pneumophila* are capable of invading and replicating normally within these cells [150]. Nineteen transporters were examined for LCV-colocalization during infection, 7 of which had extremely low transfection efficiencies and therefore LCV-colocalization was not determined (SLC1a2, SLC1a5v2, SLC3a2, SLC6a5, SLC13a2, SLC17a6, and SLC25a2) (Table 2-3). The SLCs examined expands beyond that listed in Table 2-1 to prevent bias and perform a more comprehensive screen. Additional amino acid transporters were added into this study (SLC1a1, SLC1a2, SLC1a4, SLC1a6, SLC7a2, SLC7a10, SLC13a2, SLC16a7, SLC17a6, and SLC25a2), while many non-TCA cycle substrate transporters from Table

2-1 were not included (SLC2a1, SLC2a6, SLC9a3r1, SLC25a3, SLC25a4, SLC25a5, SLC25a10, SLCa24, SLC30a1, and SLC37a2).

Table 2-3. SLCs examined by overexpression in HEK293T.

SLC	Common synonym	Substrate
1a1	EAAT3	glutamate, aspartate
1a2	EAAT2	glutamate, aspartate
1a4 (v1, v2)	ASCT1	alanine, serine, cysteine, threonine
1a5 (v2, v3)	ASCT2	neutral amino acids
1a6	EAAT4	glutamate, aspartate
3a2	CD98	valine, leucine, isoleucine, tryptophan, tyrosine
6a5	NET1	glycine
7a2	CAT2	arginine, lysine, ornithine
7a3	CAT3	arginine, lysine, ornithine
7a5	LAT1	cysteine, alanine, serine, pheylalanine, tyrosine, leucine, arginine, tryptophan, L-DOPA, T3, T4
7a10	ASC-1	serine, alanine, glycine, threonine, cysteine
13a2	NaDC1	succinate, citrate
13a5	NaCT	citrate
16a1	MCT1	monocarboxylates
16a7	MCT2	monocarboxylates
17a6	VGLUT2	glutamate
25a2	ORC2	ornithine (mitochondrial)

The experiment infection was performed for 8 hrs, which corresponds to the start of bacterial replication, when nutrient demand is high. Additionally, this time-point could allow for sufficient accumulation of SLCs to the LCV and aid in visualization by microscopy. Positive colocalization was determined if >50% of the LCV was stained with α-FLAG when visualizing 3-dimensional LCVs. The WT strain and the *dotA* mutant were used in this study to determine if SLCs on the LCV are present in an effector-driven manner (Fig. 2-1).

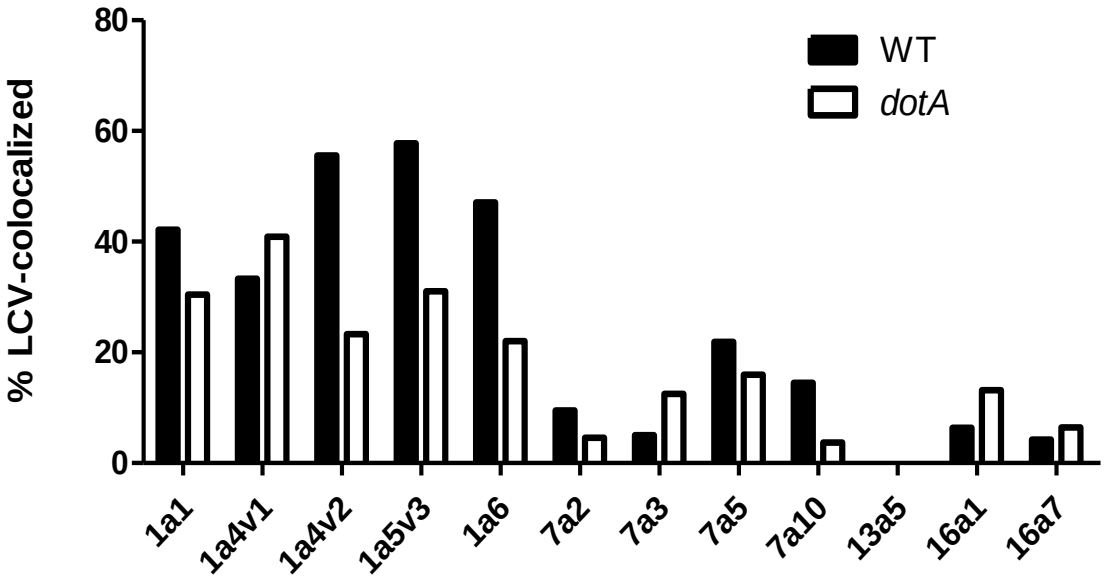


Figure 2-1. LCV-colocalized SLCs in HEK293Ts. Colocalization of FLAG-tagged host SLC transporters with the LCV was examined during infection of HEK293 cells with WT *L. pneumophila* and the *dotA* mutant, 8 hrs post-infection. LCV colocalization was evaluated in Z-stack confocal images and data points represent positive LCV-colocalization, indicated by >50% coverage of the vacuole, n>50 infected cells and are representative of one experiment.

The data suggest at least four SLCs could be detected on the LCV during infection of HEK293 cells: SLC1a1, SLC1a4 transcriptional variant 2 (SLC1a4v2), SLC1a5 transcriptional variant 3 (SLC1a5v3), and SLC1a6 (Fig. 2-1). The latter three appear to be dependent on the T4SS (Fig. 2-1). Our data are consistent with the requirement for SLC1a5, a neutral amino acid transporter, during infection with *L. pneumophila* [247]. SLC1a4 is responsible for the transport of alanine, serine, cysteine, and threonine, and SLC1a1 and SLC1a6 are transporters glutamate and aspartate, all of which can feed into the TCA cycle [263].

Functionally, this approach works to screen a large number of SLC transporters; however, it may not be a biologically relevant system and may produce false negatives.

Overexpressing just one of these transporters will not affect the expression of the other proteins within these complexes, like in the case of the previously described SLC1a5, which interacts with SLC3a2/SLC7a5/SLC16a1, potentially causing aberrant protein localization. Alternatively, overexpression can lead to false negatives because many of these SLCs function in heterodimers with other SLCs or other proteins. This setup gave us a starting point for narrowing down the list of SLCs involved in transport at the LCV membrane.

Screen of SLC transporters in the human macrophage cell line U937

We were interested in a more biologically relevant model to determine roles of SLCs transporters in the transport of nutrients across the LCV membrane. Therefore, we utilized the human monocytic cell line, U937, to determine native SLCs expression using protein-specific antibodies. SLCs are known to be poorly expressed in macrophages. A pool of SLCs were chosen to be studied based on data from HEK293 that show localization to the LCV: SLC1a4, SLC1a5, and SLC1a6 (Table 2-4). SLC3a2, SLC7a5, and SLC16a1 were included because they function in complex with SLC1a5 which was shown to be on the LCV (Table 2-4). For this study we chose to study SLC1a4, SLC1a5, SLC1a6, SLC3a2, SLC7a5, and SLC16a1.

Table 2-4. Selected SLCs for further study in U937.

SLC	Common synonym	Substrate
1a4	ASCT1	alanine, serine, cysteine, threonine
1a5	ASCT2	Neutral amino acids
1a6	EAAT4	aspartate, glutatmate
3a2	CD98	valine, leucine, isoleucine, tryptophan, tyrosine
7a5	LAT1	cysteine, alanine, serine, pheylalanine, tyrosine, leucine, arginine, tryptophan, L-DOPA, T3, T4
13a3	NaDC3	sodium-dicarboxylate cotransporter
16a1	MCT1	monocarboxylates

Detection of any of the selected native SLCs in U937 macrophages by confocal microscopy was not possible. The expression levels of these proteins were too low to detect even with high concentrations and extended incubations of primary and/or secondary antibody. Whole cells and isolated LCVs were both tested for SLC-colocalization to the LCV. With either method, SLCs could not be detected.

Screen of SLC transporters in human liver cell line HepG2

In order to continue on with our preliminary screens of SLCs, we utilized the liver cell line, HepG2. Due to the function of the liver, many SLCs are expressed in high quantities within these cells [264]. Therefore, this cell line may provide a starting point for identification of which native SLCs are recruited to the LCV and, allow for detection and testing of our antibodies where U937 cells did not. Like HEK293T cells, *L. pneumophila* also invades and replicates normally within this cell line. HepG2 cells were infected with WT *L. pneumophila* and the *dotA* mutant for 1, 4, and 8hrs.

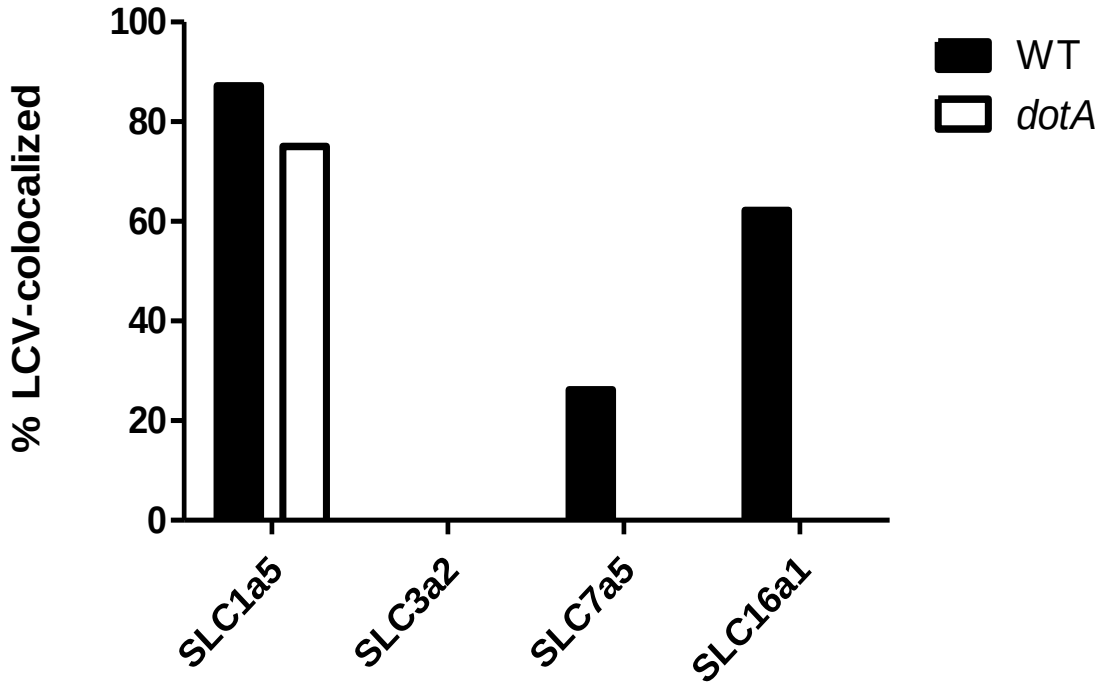


Figure 2-2. LCV-colocalization of native host SLC transporters in HepG2. Colocalization of SLCs to the LCV during infection of HepG2 cells with WT *L. pneumophila* and the *dotA* mutant, 8hrs post-infection. LCV colocalization was evaluated in Z-stack confocal images and data points represent positive LCV-colocalization, indicated by >50% coverage of the vacuole, n>50 infected cells and are representative of one experiment.

For this study we chose to examine LCV-localization of SLC1a4, SLC1a5, SLC1a6, SLC 2a1, SLC3a2, SLC7a5, and SLC16a1. Poor detection of SLC1a4, SLC1a6, and SLC2a1 in this cell line eliminated them from further examination. SLC1a4 should have been detectable as it is widespread throughout tissues, but is expressed the highest in brain tissues [263, 264]. SLC1a6 is primarily expressed in lung, skeletal muscle, intestine, kidney, and adipose tissue [263, 264]. SLC2a1 is also widespread but expression in the liver is lower than most organs [264]. Failure to detect these three transporters can be explained by their tissue-tropism. This also implies that expression in HepG2 cells is not altered by *L. pneumophila* infection.

No colocalization was observed for any transporter at 1 or 4 hrs post-infection. Colocalization was only observed at 8 hrs post-infection or this phenotype could be an artifact of the cell type. This supports the idea that SLCs may be accumulating on the LCV. The data supported, again, the role for SLC1a5 during infection, as previously seen in the study by Wieland *et al.* and with our preliminary HEK293T screen (Fig. 2-1, 2-2) [247]. However, in this cell type, there is no significant difference between the amounts of SLC1a5 on the LCV during infection with the WT strain or the *dotA* mutant, is localized in a phagolysosome . SLC1a5 either does not appear on the LCV during the early stages of infection or it does so in such low amounts that below the threshold of antibody detection. Interestingly, while there was staining of SLC3a2, which functions in complex with SLC1a5, there was no staining of the transporter on the LCVs of either the WT strain or the *dotA* mutant in HepG2 cells (Fig. 2-2). SLC7a5 and SLC16a1, also part of the same complex, were detected on the WT LCV but not the *dotA* LCV (Fig. 2-2).

The benefit of using cell lines is the ease of access and manipulation for studies. However, it was clear that the data generated in these studies was inconsistent, which may not necessarily be reflective of the true biological nature of SLCs during infection. However, this study allowed for confirmation of proper detection of some of our targeted SLCs, which was not possible in U937 macrophage.

Screen of SLCs during infection of human monocyte-derived macrophages

The previous cell types were used as a means to narrow the search for important SLCs. However, each system produced unique colocalization profile, along with a set of unique caveats. Therefore, we utilized human monocyte-derived macrophages (hMDMs). Infection with the WT strain or the *dotA* mutant proceeded for 8bhrs before being

visualized by confocal microscopy for LCV colocalization with SLCs. As previously, native expression of SLC1a4, SLC1a5, SLC1a6, SLC2a1, SLC3a2, SLC7a5, and SLC16a1 was tested. SLC2a2, a glucose transporter, and SLC16a3, a monocarboxylate transporter, were added to this study to broaden our scope outside of just amino acids. SLC16a3 can also function within the SLC1a5/SLC3a2/SLC7a5/SLC16a1 complex, which has been shown to be important for the intracellular replication of *L. pneumophila* when inhibited by BCH, by replacing SLC16a1 as they are functionally redundant [247, 265]. SLC2a2 is important in glucose sensing and glucose homeostasis [266]. Within these primary cells, detection of SLC1a4, SLC1a5, and SLC3a2 was not possible with antibodies; whereas in this study, SLC1a4 and SLC1a5 stained extracellular bacteria and therefore were not analyzed for LCV-colocalization.

Unlike what was observed during infection of HepG2 cells, neither SLC16a1 nor SLC7a5 were localized to the LCV during infection of hMDMs, nor the SLC16a1 replacement, SLC16a3 (Fig. 2-3). Interestingly, we did see strong colocalization, in a T4SS-dependent manner, with the glucose transporter SLC2a1/Glut1 (Fig. 2-3). This was specific to SLC2a1, as another glucose transporter, SLC2a2, did not colocalize to the LCV (Fig. 2-3). Tissue-tropism could explain this, as SLC2a2 is primarily expressed in the liver, intestine, and kidney, where SLC2a1 is widespread spread and main transporter for glucose in non-specialized cells [248]. These data were surprising given that *L. pneumophila* utilizes amino acids for carbon and energy, not glucose [175, 220].

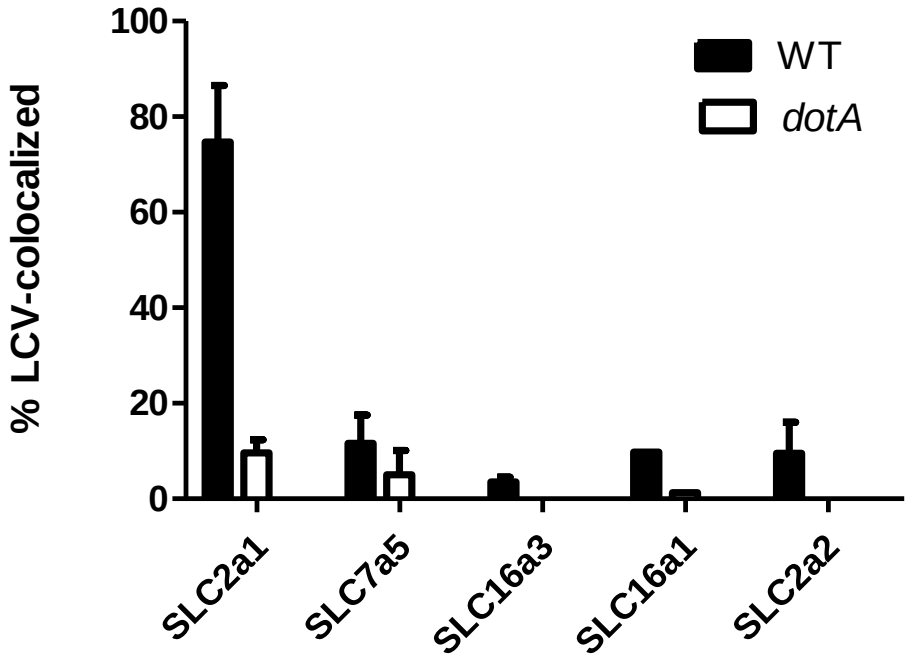


Figure 2-3. LCV-colocalization of native SLCs in hMDMs. Colocalization of SLCs with the LCV of hMDMs infected either WT *L. pneumophila* or the *dotA* mutant, 8hrs post-infection, was examined. LCV colocalization was evaluated in Z-stack confocal images and data points represent positive LCV-colocalization, indicated by >50% coverage of the vacuole, n>50 infected cells and are representative of three experiments.

Time-dependent LCV co-localization of SLC2a1/Glut1

Glucose is metabolized by *L. pneumophila* during late stages of growth [175, 220]. At which point, glucose utilization and uptake genes are upregulated [177, 181]. To determine if SLC2a1/Glut1 recruitment to the LCV followed this trend, hMDMs were infected with WT *L. pneumophila* and the *dotA* mutant for 2, 4, 8, and 10 hrs and visualized by confocal microscopy using antibody against Glut1 (Santa Cruz, H-43).

Indeed at 2 and 4 hrs, little colocalization of Glut1 to the LCV was observed, which was not affected by the Dot/Icm translocation system (Fig. 2-4). As observed in

our initial screen in hMDMs, colocalization of Glut1 to the WT LCV was near 80% at 8 hrs post-infection (Fig. 2-3 and 2-4). Surprisingly, like the early time points, little colocalization of Glut1 to LCVs was observed at 10 hrs post-infection (Fig. 2-4). This could have been due to experimental error, or change in LCV biogenesis after multiple rounds of replication in the vacuole.

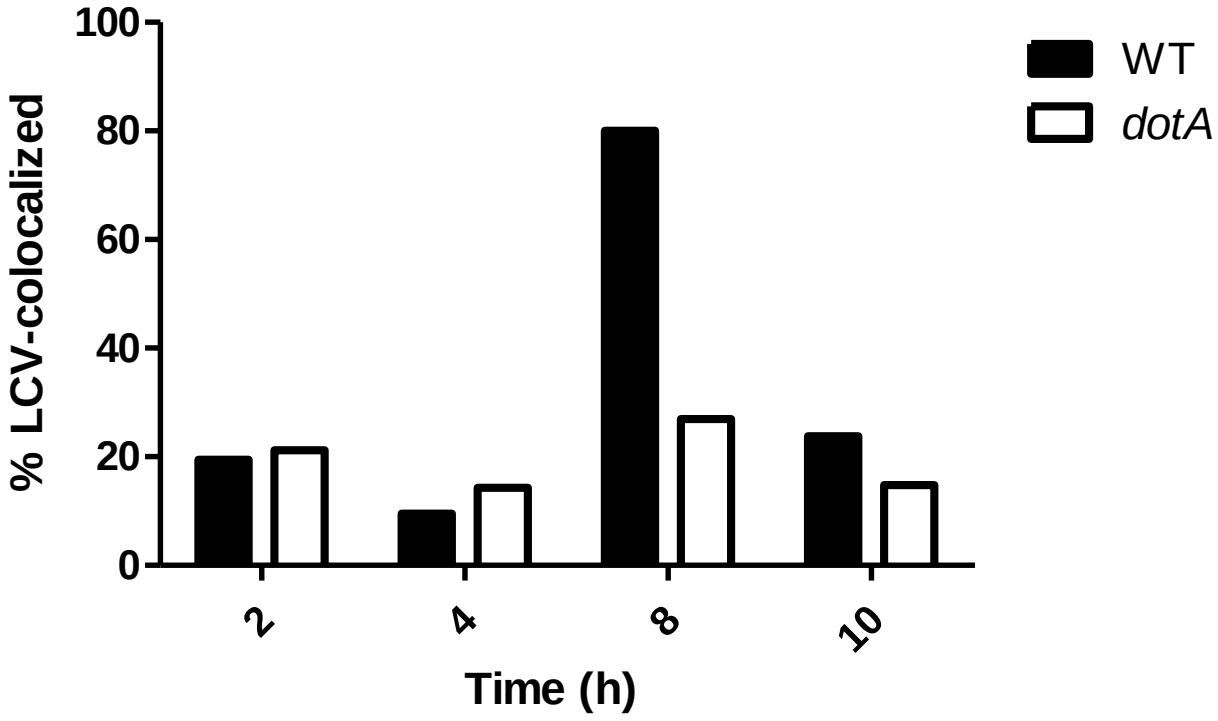


Figure 2-4. Time-dependent LCV-colocalization of native SLCs in hMDMs. Colocalization of SLCs with the LCV of hMDMs infected either WT L. pneumophila or the dotA mutant was examined at 2, 4, 8, and 10 hrs post-infection. LCV colocalization was evaluated in Z-stack confocal images and data points represent positive LCV-colocalization, indicated by >50% coverage of the vacuole, n>50 infected cells and are representative of one experiment.

Given that Glut1 is retained on ER-vesicles until appropriate signals are received that induce trafficking to the plasma membrane, like IL-3 or hyperglycemia, we sought to determine the subcellular localization of Glut1 during infection with *L. pneumophila* [249, 252]. Two distinct patterns of Glut1 localization can be observed, strong membrane

colocalization with cytosolic staining, and just cytosolic staining (Fig. 2-5B) [253]. Analyzing where Glut1 is localized, at 8 hrs post-infection from all previous studies, we see that when Glut1 is localized to the LCV it is predominately also membrane localized (Fig. 2-5AB).

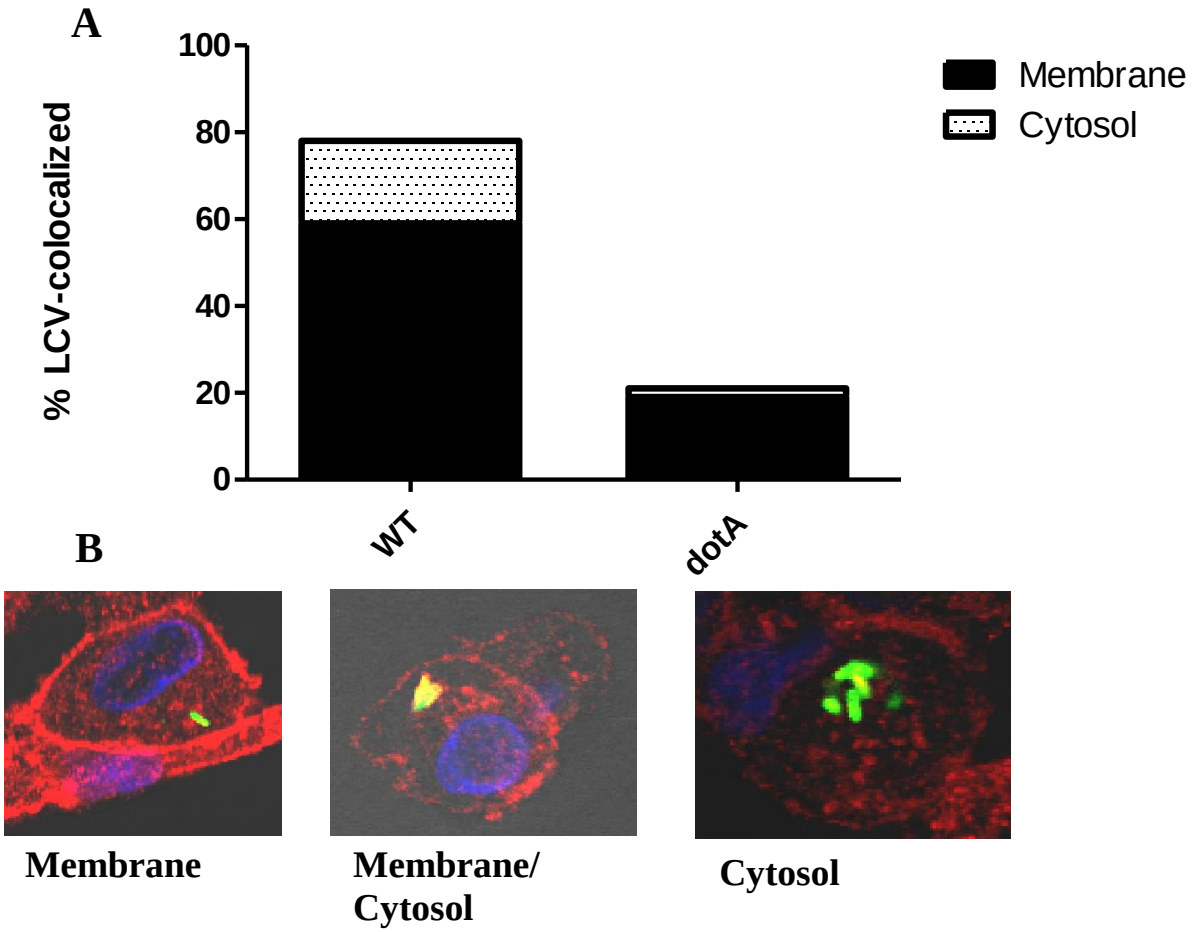


Figure 2-5. Subcellular localization of Glut1 during LCV-colocalization conditions. A) The trafficking patterns of Glut1 was analysed when positively colocalized to the LCV membrane during infection of hMDMs at 8 hrs post-infection with WT *L. pneumophila* or the *dotA* mutant. Glut1 subcellular localization and LCV colocalization was evaluated in Z-stack confocal images. Strong plasma membrane staining of Glut1 was used as a threshold for membrane localization despite appearance in the cytosol vs only cytosolic staining. Data points represent positive colocalization, indicated by >50% coverage of the vacuole, n>50 infected cells and data are representative of three experiments. B) Representative confocal images of membrane, membrane/cytosol, and cytosol staining of Glut1 (Red), during infection of *L. pneumophila* (green), with DAPI (blue).

Unfortunately, this data could not be reproduced to confirm time-dependent Glut1 colocalization. The Santa Cruz antibody (Glut1, H-43) used in this study and previous studies is no longer commercially produced (Table 2-5). Many antibodies from other companies were tested but none performed as well. Variations in staining patterns or failure to stain for confocal were the most common issues. One antibody failed to detected plasma membrane localized Glut1, even after triggering trafficking with IL-3 [249]. Various concentrations of antibody, staining length, and fixing methods were tested in order to continue studies with Glut1 but each were unsuccessful.

Table 2-5. SLC2a1/Glut1 antibodies tested in this study.

SLC2a1	Santa Cruz (H-43), rabbit
SLC2a1	Santa Cruz (A-4), mouse (poor staining)
SLC2a1	Proteintech 66290-1, mouse (poor staining)
SLC2a1	Genetex GTX15309, rabbit (no membrane staining)

These preliminarily data indicate that Glut1 may be recruited to the LCV in a time-dependent manner that coincides with increased glucose uptake and utilization by *L. pneumophila*, and recruited to the LCV in a Dot/Icm-dependent manner [177, 181, 220]. *L. pneumophila* may be intercepting Glut1 as it traffics to the membrane either through direct targeting of Glut1 or through recruitment of ER-derived vesicles for LCV maintenance. However, the lack of detection of Glut1 on the LCV by many other antibodies may indicate these data were an artifact of the Santa Cruz antibody.

Infection of L. pneumophila *increases expression of Glut1 that is independent of macrophage activation*

To determine if *L. pneumophila* infection is altering expression of Glut1, we determined the level of the Glut1 protein present in hMDMs at 2, 8, and 12 hrs post-infection. LPS/IFNγ-treated cells were used as a control for macrophage activation and untreated cells used for base-line expression of Glut1.

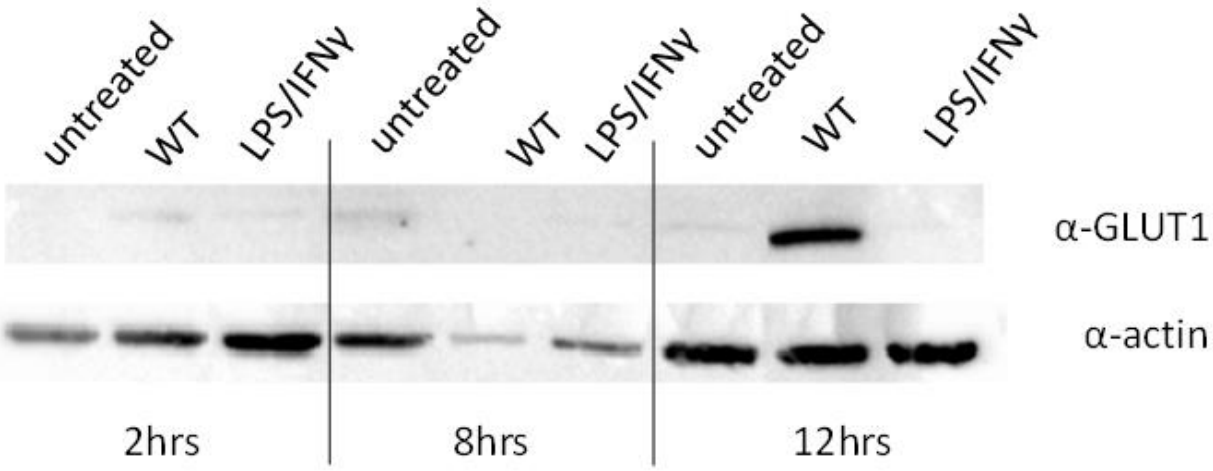


Figure 2-6. Expression of Glut1 in hMDMS. Untreated, *L. pneumophila* infected, or LPS/IFNγ treated hMDMs were examined for expression of Glut1 at 2, 8, and 12 hrs post-infection or treatment.

No significant changes in Glut1 expression were observed at 2 or 8 hrs post-infection (Fig. 2-6). At 12 hrs post-infection, a significant increase in the amount of Glut1 present was observed in cells infected with *L. pneumophila*, but not untreated or stimulated cells, indicating a direct effect on Glut1 by *L. pneumophila* that was not due to macrophage activation (Fig. 2-6).

This experiment was repeated using both IL-3 and excess glucose as positive controls and included the *dotA* mutant. At 2 hrs post-infection, an early increase in Glut1 was observed in hMDMs infected with the *dotA* mutant that diminished by 8 and 12 hrs

(Fig. 2-7). At 8 hrs, a minor increase in Glut1 was observed with the *lamA* mutant, but not under any other conditions (Fig. 2-7). By 12 h post-infection, hMDMs had responded to IL-3 and excess glucose treatment by increasing expression of Glut1, similar to that of the *lamA* mutant (Fig. 2-7). Infection with WT *L. pneumophila* also caused increased expression of Glut1 compared to uninfected cells, as previously seen (Fig. 2-6 and 2-7).

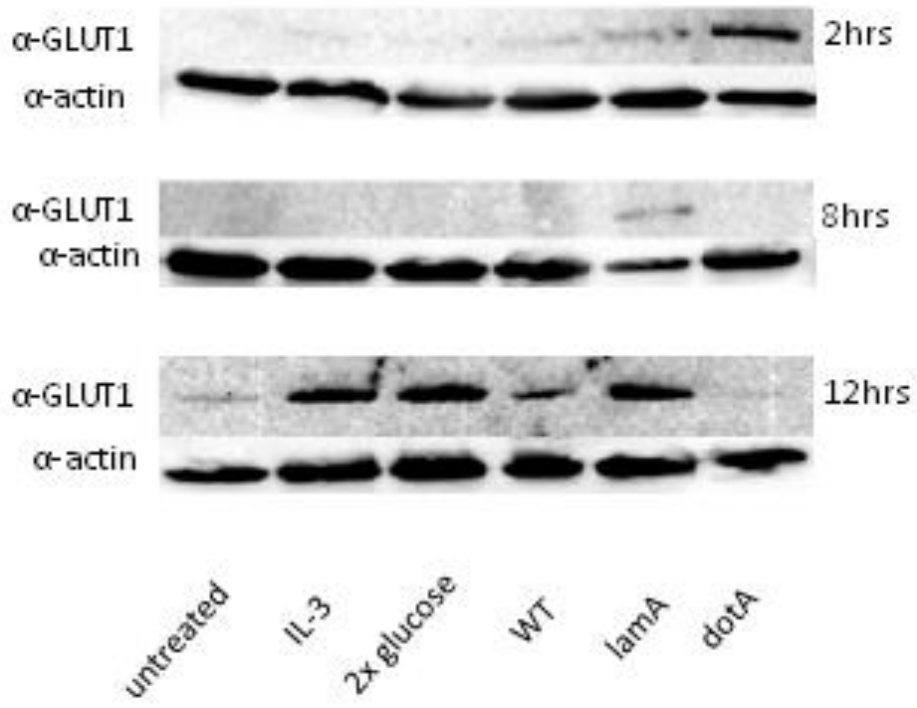


Figure 2-7. Glut1 expression in hMDMS. Untreated; *L. pneumophila, dotA,* or *lamA* infected; IL-3 treated, or excess glucose-treated hMDMs were examined for expression of Glut1 at 2, 8, and 12 hrs post-infection or treatment.

Again, confirmation of this data through repeat studies was not possible after the antibody being used was no longer being produced. As previously mentioned, other commercial antibodies did not perform as well and had non-specific binding that interfered with visualization of Glut1. However, these preliminary studies may indicate a

potential role for Glut1 during infection of macrophages with *L. pneumophila* that is Dot/Icm –dependent.

DISCUSSION

Transport of amino acids across the LCV has been an open question in *L. pneumophila* biology. It is apparent that the answer may not be as simple as identifying host SLCs under static conditions. The process of amino acid transport is likely very dynamic and thus difficult to determine by conventional methods. It could also be that individual host transporters present on the LCV are in low abundance, and below detection threshold by confocal microscopy. Many SLCs that transport similar substrates have been identified on LCVs [227, 228, 256]. Families of SLCs, rather than individual SLCs, may be employed, which would affect detection when limited to analyzing individual transporters at time. Alternatively, low numbers of transporters could be sufficient to transport the nutrients needed by *L. pneumophila* for intravacuolar replication. Any or multiple of these explanations may be what is occurring during infection. More in-depth experiments will need to be done to determine the role of host SLCs, if there are any.

We attempted to knockdown SLC expression by siRNA; however, strong knockdown was never achieved in primary macrophages for SLC1a5 and SLC2a1 (data not shown). Given that these transporters are part of essential homeostatic processes, it is understandable that knockdown could be detrimental and only those surviving cells that did not have high or complete knockdown are left, a problem that is confounded by the difficulty of knockdown in primary macrophages which are not altering their expression

profile. If this were achievable, metabolomics could be performed to determine the need of individual transporters for the intracellular replication and survival of *L. pneumophila.*

It remains unclear whether SLCs are responsible for transporting amino acids through the LCV membrane to the LCV lumen. Recently, it has been shown that the vacuole of *L. pneumophila* in hMDMs is semipermeable, as noted by galactin-3 staining of the LCV, which localizes to damaged host membranes [230]. A semipermeable LCV could be sufficient to allow amino acids into the LCV lumen. *L. pneumophila* may be providing its own transporters to serve this purpose on the LCV. Alternatively, it could be both host and bacterial transporters responsible for amino acid transport. With a variety of options for amino acid acquisition the bacteria are fit for survival. Having many types of SLCs, with overlapping substrates, would ensure the bacterium acquires the nutrients it needs to account for variations within protozoan hosts or in the event it encounters a host with an aberrant transporter profile, which would explain why numerous SLCs are found on the LCV by mass-spectrometry (Table 2-1). While many SLC homologs have been identified in the model protozoa, *Dictyostelium discodium*, *L. pneumophila* may not have the same ability to manipulate SLCs in the human host that it does in the environmental host.

Colocalization of SLC2a1/Glut1 to the LCV is a novel discovery if it can be reproduced using reliable antibodies. For macrophages, Glut1 is the most well studied glucose transporter, particularly for its involvement in macrophage polarization [267-272].

To facilitate rapid metabolic changes, under activation conditions macrophages prefer glycolysis, which is a faster method of generating ATP than through the TCA

cycle, even though it produces less ATP overall [253, 269, 272, 273]. In order to meet the increased demand of glucose, macrophages will increase the amount of SLC2a1/Glut1 present on the plasma membrane [249]. Using SLC2a1/Glut1 to increase cellular levels of glucose, allows for the macrophage to produce a stronger ROS response [253]. The presence of Glut1 on the LCV during infection of *L. pneumophila* could serve as a dual purpose, sequestering glucose from the macrophage would dampen the ROS attack, while allowing *L. pneumophila* to increase store of poly-3-hydroxybutyrate for survival outside of the host. Further studies to define the role of SLC2a1 during infection should be performed. Our attempts at characterizing SLC2a1 were hindered by the termination of the commercial production of the SLC2a1 antibody used in our studies. Antibodies against SLC2a1 from other companies were insufficient in the detection of the protein by confocal and western blot with hMDMs.

MATERIALS AND METHODS

Strains and cell lines

L. pneumophila strain AA100/130b (ATCC BAA-74) and the *dotA* T4SS-deficient mutant, were grown on Buffered Charcoal Yeast Extract (BCYE) agar, as we previously described [166]. For infection of cell monolayers, *L. pneumophila* strains were grown in BYE broth with appropriate antibiotic selection, at 37°C with shaking, to post-exponential phase (OD_{550nm} 2.1-2.2).

HEK293T cells were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum as previously described [97, 166]. U937 cells were cultured in RPMI (Corning) supplemented with 10% fetal bovine serum as previously described [168]. HepG2 cells were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum. Human monocyte-derived macrophages (hMDMs) were isolated from healthy donors and cultured in RPMI 1640, supplemented with 10% fetal bovine serum, as previously described [97, 166]. All methods were approved and carried out in accordance to the University of Louisville Institutional Review Board guidelines and blood donors gave informed consent as required by the University of Louisville Institutional Review Board (IRB # 04.0358).

Expression of SLCs during infection of hMDMs

To determine expression of SLCs during infection, 1 x 10^6 hMDMs were infected the WT strain or the *dotA* mutant, MOI of 10, for 2 hrs. RNA was isolated using *mir*VANA isolation kit (Invitrogen). RNA was converted into cDNA and used for Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) to quantitate SLC expression using the primers listed in Table 2-6. The expression of SLCs was assessed

quantitatively via the ΔΔCt method. Ct values corresponding to the SLCs were normalized to *gapdh*.

Analysis of SLC LCV-colocalization

RNA from hMDMs was isolated using the RNeasy PLUS mini kit (Qiagen), and used to generate cDNA. Human cDNA was used as a template for amplifying SLC genes using the primers listed in Table 2-6. SLC amplicons were cloned into the p3XFLAG-CMV10 expression vector.

Colocalization of SLC transporters to the LCV was determined using confocal microscopy. To achieve this, HEK293T, U937, HepG2, or hMDMs were plated into 24-well plates containing glass coverslips (2 x 10^5 cells per well). Coverslips for HEK293T cells were treated with poly-l-lysine. Monolayers were infected with either post-exponential phase WT or the *dotA* mutant, at an MOI of 25 for 1 h, and then treated for 1h with gentamicin to kill remaining extracellular bacteria as previously described [97, 166]. At various timepoints, the monolayers were fixed and permeabilized using methanol at -20°C for 5 min or 4% PFA for 15 mins. The monolayers were labeled with goat or rabbit anti-*Legionella* antiserum (1/500 dilution or 1/1000 dilution, respectively) and α-SLC antibody (Table 2-7), and counter-labelled with Alexa-Fluor 488 anti-rabbit or anti-goat IgG antibody and Alexa-Fluor 594 anti-mouse, anti-goat, or anti-rabit IgE (1/4000 dilution, Invitrogen) and DAPI to stain nuclei. The cells were examined by confocal microscopy using an Olympus FV1000 laser scanning confocal microscope (Olympus). Quantification of LCV-colocalization was performed manually by counting Z-stack images (8 μM depth with 0.2 μM slices) of infected cells. Over 50 infected cells were counted for each condition.

Table 2-6. Primers used in this study.

SLC1a1 F	AAGCTTATGGGGAAACCGGCG
SLC1a1 R	GAATTCCTAGAACTGTGAGGTCTGGGTGAAT
SLC1a2 F	GGGCAATCTCTGGACATCTTTATCT
SLC1a2 R	AGGCGAGACATGGAGAACACTTTA
SLC1a4 v1 F	AAGCTTATGGAGAAGAGCAACGAG
SLC1a4 v1 R	GAATTCTCACAGAACCGACTCCTT
SLC1a4 v2 F	AAGCTTATGAACATTTTAGGATTGGTC
SLC1a4 v2 R	GAATTCTCACAGAACCGACTCCTT
SLC1a5 v2 F	AAGCTTATGAACATCCTGGGCTTG
SLC1a5 v2 R	GAATTCTTACATGACTGATTCCTTCTCAGAG
SLC1a5 v3 F	AAGCTTATGTACTCTACCACCTATGAAGAGAG
SLC1a5 v3 R	GAATTCTTACATGACTGATTCCTTCTCAGAG
SLC1a6 F	GTGGCTTAGGGACTGGAAACATA
SLC1a6 R	GGGCACAGACCAGGACTCAC
SLC3a2 F	AAGCTTATGGAGCTACAGCCTCCTGAA
SLC3a2 R	GAATTCTCAGGCCGCGTAGGG
SLC6a5 F	CACCCTCCACCAGTTCAGTCT
SLC6a5 R	TGCGACACTATGCCTACTTTTCTA
SLC7a2 F	AGCTTATGAAGATAGAAACAAGTGGTTATAAC
SLC7a2 R	AAGCTTATGATTCCTTGCAGAGCC
SLC7a3 F	AAGCTTATGCCGTGGCAAGCAT
SLC7a3 R	AGATCTTCAAACTGAGTGGACATAGAGAGTG
SLC7a5 F	GGCCGGTGCGCAGAG
SLC7a5 R	GGCCCAAGGAGACCAAAAG
SLC7a10 F	GGCGGGACAGCGACATG
SLC7a10 R	TGCCAAAACACCTCCTCAATAAA
SLC13a2 F	AAGCTTATGGCCACCTGCTGGC
SLC13a2 R	GGTACCCTAGGGGCTTGGTGTGGT
SLC13a5 F	GCTGCCCCTCACTCGTCTC
SLC13a5 R	TGCCAGAAGGTTCGGTAGTC
SLC16a1 F	AAGCTTATGCCACCAGCAGTTG
SLC16a1 R	GGTACCTCAGACTGGACTTTCCTC
SLC16a7 F	AAGCTTATGCCACCAATGCCA
SLC16a7 R	GGTACCTTAAATGTTAGTTTCTCTTTCTGA
SLC17a6 F	GCCCGCAACTACTTTAAGAGAT
SLC17a6 R	TCCCCACCTAAAATTCTATGACTC
SLC25a2 F	AAGCTTATGAAGTCCGGTCCTG
SLC25a2 F	AGATCTTCAGTATGCTTCCAACTGTT
gapdh F (qPCR)	TGCACCACCAACTGCTTAGC
gapdh R (qPCR)	GGCATGGACTGTGGTCATGAG
SLC1a5 F (qPCR)	CCTCTTCCAGTTCCGCCACG
SLC1a5 R (qPCR)	GGCCGTGACCAGGATGGTGA
SLC3a2 F (qPCR)	TCCTGGACAGCCTATGGAGG
SLC3a2 R (qPCR)	CACTCTGGCCCTTCACAGTC

SLC7a5 F (qPCR)	TGGCCGTGGACTTCGG
SLC7a5 R (qPCR)	TCATCACACACGTGAACACG
SLC16a1 F (qPCR)	TATGGTGGAGGTCCTATCAGCA
SLC16a1 R (qPCR)	AGCCCAAGACCTCCAATGAC

Table 2-7. SLC antibodies used in this study and their source.

Protein	**Source**
SLC1a4	Santa Cruz (H-60), rabbit
SLC1a5	Santa Cruz (H-52), rabbit
SLC1a6	Santa Cruz (K-20), goat
SLC2a1	Santa Cruz (H-43), rabbit
SLC2a1	Santa Cruz (A-4), mouse
SLC2a1	Proteintech 66290-1, mouse
SLC2a1	Genetex GTX15309, rabbit
SLC2a2	Novus NBP2-2218, rabbit
SLC3a2	Abcam Ab193364, mouse
SLC3a2	R&D systems, MAB52920-SP, mouse
SLC7a5	Abcam Ab85226, rabbit
SLC13a5	Abnova, 152-206, rabbit
SLC16a1	Abcam ab90582, mouse
SLC16a3	Proteintech 22787-1-AP, rabbit

CHAPTER 3:

MAMMALIAN SOLUTE CARRIER (SLC)-LIKE TRANSPORTERS IN *LEGIONELLA PNEUMOPHILA**

* Best, A.M., Jones, S.C., Abu Kwaik, Y. Mammalian Solute Carrier (SLC)-like transporters in *Legionella pneumophila*. Sci Reports. *Manuscript in review*

Summary

Acquisition of nutrients during intra-vacuolar growth of *L. pneumophila* within macrophages or amoebae is poorly understood. Since many genes of *L. pneumophila* are acquired by inter-kingdom horizontal gene transfer from eukaryotic hosts, we examined the presence of human solute carrier (SLC)-like transporters in the *L. pneumophila* genome using I-TASSER to assess structural alignments. We identified 10 SLC-like putative transporters in *L. pneumophila* that are structurally similar to SLCs, seven of which are amino acid transporters, and one is a tricarboxylate transporter. The two other transporters, LstA and LstB, are structurally similar to the human glucose transporter, SLC2a1/Glut1. Single mutants of *lstA* or *lstB* have decreased ability to import, while the *lstA/lstB* double mutant is severely defective for uptake of glucose. While *lstA* or *lstB* single mutants are not defective in intracellular proliferation within *Acanthamoeba polyphaga* and human monocyte-derived macrophages, the *lstA*/*lstB* double mutant is severely defective in both host cells. The two phenotypic defects of the *lstA*/*lstB* double mutant in uptake of glucose and intracellular replication are both restored upon complementation of either *lstA* or *lstB*. Our data show that the two glucose transporters, LstA and LstB, are redundant and are required for intracellular replication within human macrophages and amoebae.

Introduction

Legionnaire's disease, an atypical pneumonia, is a result of inhalation of the bacteria *Legionella pneumophila* [1, 3, 274]. Within the human host, *L. pneumophila* primarily reside and replicate within alveolar macrophages [79, 100, 107]. Infection of humans is considered to be "accidental", as the natural hosts for *L. pneumophila* are protozoa in the aquatic environment [275]. Growth within either host occurs through

manipulation of evolutionarily conserved pathways, to avoid fusion to the lysosomes and to remodel the vacuole to become ER-derived, which is designated as the *Legionella*-containing vacuole (LCV) [91, 276, 277]. The Dot/Icm type IVb translocation system, which translocates >320 effector proteins into the host cytosol, is required for biogenesis of the LCV and for successful intracellular replication in macrophages and amoebae [126, 127, 278, 279]. A plethora of host cell processes are modulated by the translocation of effector proteins that allow *L. pneumophila* to evade innate immunity and acquire nutrients [75, 148, 280, 281].

L. pneumophila relies on host amino acids (such as, serine, cysteine, and alanine) to feed into TCA cycle as the main source of carbon and energy [76, 211]. The bacteria are in such high demand for amino acids that endogenous amounts within the host are below the threshold needed to support robust intracellular replication [75, 100]. To raise host cellular levels of amino acids, *L. pneumophila* translocates the AnkB effector, which hijacks the host ubiquitination-proteasome machinery to degrade proteins [75, 98, 170, 172].

Early studies pointed to a preference for amino acids as an energy source and identified auxotrophies for seven amino acids: threonine, arginine, isoleucine, methionine, leucine, cysteine, and valine [207, 211]. Many of these auxotrophies are shared with the amoeba host which may allow the bacterium to synchronize growth with that of the host, avoiding deleterious growth during times of environmental stress [52, 282]. *Legionella* can enter into a viable but non-culturable (VNBC) state when encountering nutritional stress, which has only been shown to be recovered by co-culturing with amoebae [77].

Nutritional virulence studies on *L. pneumophila* have focused on the generation and utilization of amino acids [75, 76, 89, 176]. Only recently has glucose metabolism been studied for its role during intracellular replication [175, 220]. However, supplementation of glucose *in vitro* does not enhance growth of *L. pneumophila* [220]. Glycolysis plays a minimal role in glucose catabolism, but is predominately metabolized through the ED pathway while the pentose phosphate pathway (PPP) functions only to generate mannose and histidine [175, 214, 220]. A gene cluster encoding enzymes for glucose catabolism, through the ED pathway of *L. pneumophila* has been shown to be required for growth in the A549 epithelial cell line, A/J mouse macrophages, and *Acanthamoeba culbertsoni,* indicating the importance of the ED pathway in intracellular replication of *L. pneumophila* [220]. Initial studies focused on poly-3-hydroxybuyterate (PHB), a 4-carbon storage molecule that is generated by metabolizing glucose, through the Enter-Doudoroff (ED) pathway into pyruvate, which gets converted acetyl-CoA, then PHB [226]. PHB is synthesized in late stages of growth and catabolized during stationary growth into acetyl-CoA to feed into the TCA cycle [218, 226]. The primary usage of glucose by *L. pneumophila* is considered to be conversion into PHB [175, 218].

How nutrients are imported by *L. pneumophila* is not well understood. To date, only one amino acid transporter, PhtA, of *L. pneumophila* has been shown to import threonine and is required for intracellular replication in macrophages [283]. Given that numerous genes in *L. pneumophila* have been acquired by inter-kingdom horizontal gene transfer from eukaryotic hosts, we sought to identify nutrient transporters in *L. pneumophila* based on their similarity to the human solute carrier (SLCs) transporters due to the lack of well annotated amoebal genomes [156, 284]. This superfamily of

transporters consists of over 55 families, grouped based on substrate specificity and tissue tropism [235]. They are considered to be part of a larger, evolutionarily conserved group of transporters known as the Major Facilitator Superfamily (MFS) [236, 237].

Ten putative amino acid SLC-like transporters were identified, including a citrate transporter, 7 amino acids transporters and 2 glucose transporters. We focused our studies on the two putative SLC-like glucose transporters, LstA and LstB, to further understand the import of glucose and its role in intracellular replication of *L. pneumophila*.

Results

Identification of human SLC-like transporters in L. pneumophila

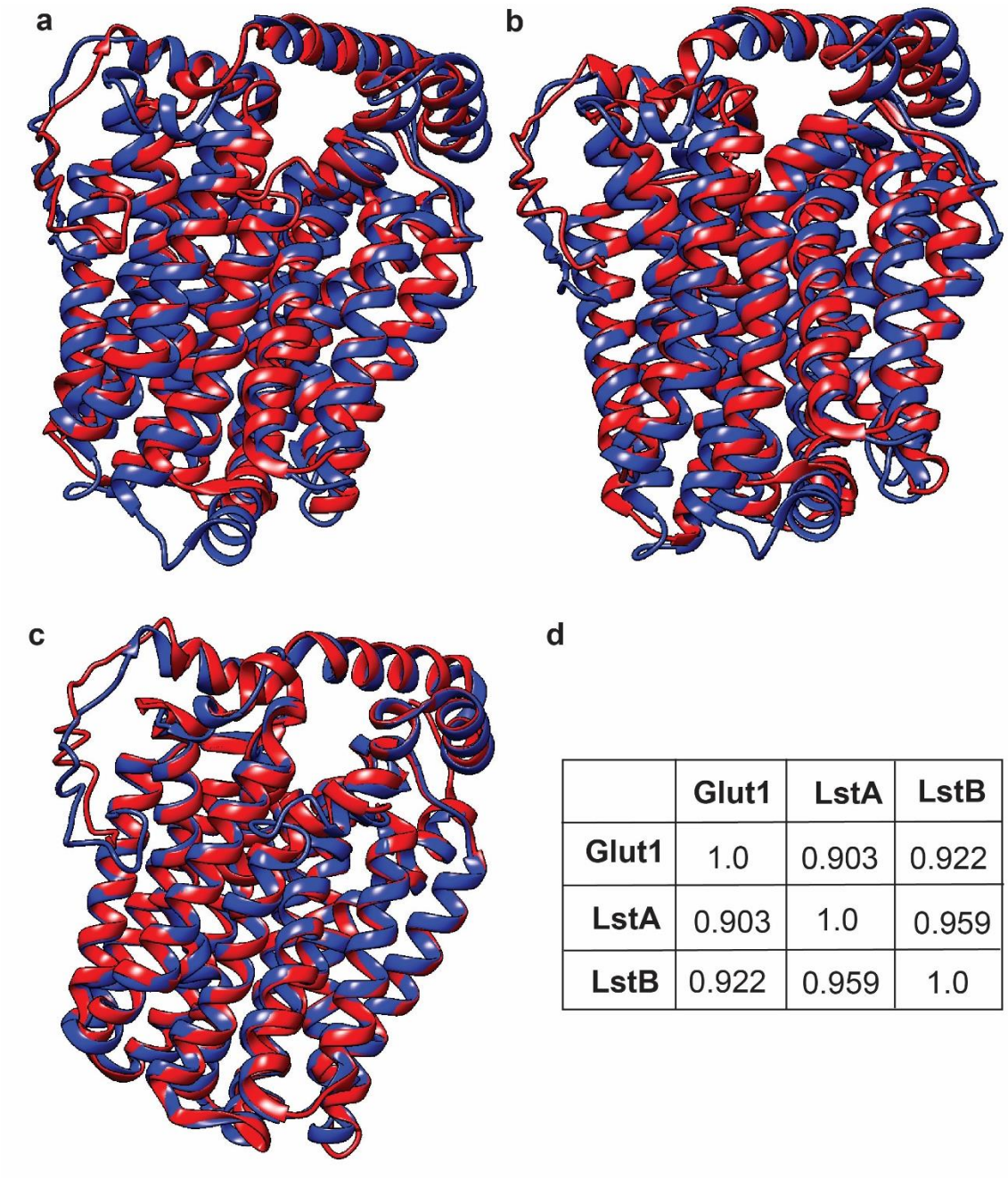
Using the primary amino acid sequences from the human cationic amino acid transporter family, SLC7, against the genome of *L. pneumophila* strain AA100/130b, seven putative SLC-like amino acid transporters in *L. pneumophila* were identified by BLAST with similarity of 56- 42% and identity of 25-37% (Table 3-1). In addition, using the primary amino acid sequence from the SLC13 family, one putative SLC-like transporter of tricarboxylates, *lpg2876* (24% / 51%), and using primary amino acid sequence from the SLC2 family, two putative SLC-like glucose transporters, *lpg0421* (33% / 50%) and *lpg1653* (30% / 48%), were also identified using BLAST (Table 3-1). Structural modeling of these proteins was done using the Iterative Threading Assembly Refinement (I-TASSER) server, which is a bioinformatics algorithm for predicting three-dimensional structure based on fold recognition [285-287]. Structural alignment was performed using TM-align, an algorithm that uses known or predicted protein structure to align proteins and determine structural similarity [285-287]. A TM-score is given for each alignment where, 1.0 indicates an exact copy, 0.0 - <0.3 indicates random structural similarity, and

0.5 - <1.0 indicating shared structural topology [287]. *L. pneumophila* proteins were compared to the human SLCs to determine structural homology, as measured by TM-scores (Table 3-1). Structural comparisons of these SLC-like transporters of *L. pneumophila* with human SLCs showed high structural similarity (TM-scores 0.78 – 0.977) (Table 3-1 and Fig. S3-1). Few homologs of mammalian SLCs have been identified within amoebae, which also can be used to identify *L. pneumophila* transporters; these are designated as CtrABC in *Dictyostelium discodium* (Fig. S3-2). We have designated these putative transporters as *Legionella* SLC-like transporters, LstA-J (Table 3-1).

Table 3-1. SLC-like putative proteins in *L. pneumophila* are homologous human SLCs. Ten transporters in *L. pneumophila* were identified by BLAST amino acid sequence homology with human SLC transporters. Predicated structures, generated by I-TASSER, were used to determine structural similarity with human SLCs by TM-score.

	Amino acid identity (BLAST)	Amino acid similarity (BLAST)	Putative substrates	Representative SLC, TM-score
LstA (Lpg0421)	37%	56%	Glucose and other monosaccharides	SLC2a1 (0.903)
LstB (Lpg1653)	25%	44%	Glucose and other monosaccharides	SLC2a1 (0.922)
LstC (Lpg0026)	25%	42%	Cationic amino acids (arginine, lysine, ornithine)	SLC7a1 (0.953)
LstD (Lpg0049)	25%	53%	Cationic amino acids (arginine, lysine, ornithine)	SLC7a5 (0.954)
LstE (Lpg0228)	33%	50%	Cationic amino acids (arginine, lysine, ornithine)	SLC7a1 (0.848)
LstF (Lpg0281)	25%	44%	Cationic amino acids (arginine, lysine, ornithine)	SLC7a4 (0.985)
LstG (Lpg0970)	30%	48%	Cationic amino acids (arginine, lysine, ornithine)	SLC7a4 (0.855)
LstH (Lpg1691)	27%	45%	Cationic amino acids (arginine, lysine, ornithine)	SLC7a2 (0.756)
LstI (Lpg2245)	25%	53%	Alanine, serine, cysteine, and threonine	SLC1a4 (0.961)
LstJ (Lpg2876)	24%	51%	Succinate, citrate, isocitrate, α-ketoglutarate	SLC13a3 (0.931

Since the uptake and the role of glucose in intracellular growth and metabolism of *L. pneumophila* is not well understood, we focused our studies on the two putative SLC-like transporters of *L. pneumophila* that shared strong structural similarity to SLC2a1/Glut1, LstA and LstB (Fig 3-1A, B). LstA of *L. pneumophila* and Glut1 of humans have a TM-score of 0.903, and LstB and Glut1 a TM-score of 0.922, indicating very strong structural similarity (Fig. 3-1A, B, D). Members within the Glut family do not share this degree of similarity (Glut1 and Glut3, TM-score of 0.88). When comparing alignment of LstA to LstB, the TM-score is 0.959, indicating potential redundancy (Fig. 3-1C, D). These two *L. pneumophila* proteins are smaller in size than their human counterpart proteins but the secondary structural alignment is conserved. LstA and LstB are likely members of the major facilitator superfamily (MFS), which are important transporters that have been maintained, in all domains of life, with little deviation through evolutionary history [237]. These two genes do not appear to be the result of gene duplication within *L. pneumophila* (Fig. S3-3), since the GC content of *lstA* is 39.7%, while *lstB* is 37.4%, indicating independent acquisition and divergent evolution.



	Glut1	LstA	LstB
Glut1	1.0	0.903	0.922
LstA	0.903	1.0	0.959
LstB	0.922	0.959	1.0

Figure 3-1. The predicted structure of two putative glucose transporters, LstA and LstB, of *L. pneumophila*. Structural alignment between A) Glut1 (blue) and LstA (red), B) Glut1 (blue) and LstB (red), and C) LstA (red) and LstB (blue), are shown using TM-align. D) TM-scores, indicating structural similiarity between Glut1, LstA, and LstB, as calculated by TM-align.

Glucose import by LstA and LstB

Predicted substrate binding, by I-TASSER, for LstA and LstB indicate glucose as a putative substrate [285, 286]. Given their high degree of structural similarity to Glut1, we hypothesized that both of these putative transporters were involved in the transport of glucose of *L. pneumophila*. To test if either LstA or LstB were required for the uptake of glucose, null mutants were generated, and uptake of glucose was analyzed by liquid scintillation using ^{14}C-glucose. *L. pneumophila* strains were grown to post-exponential phase in the presence of 0.1% uniformly labelled ^{14}C-glucose. Broth grown WT *L.* pneumophila was able to effectively take up ^{14}C-glucose *in vitro* (Fig. 3-2). As a control, excess, unlabeled glucose (10mM) was added, which abolished uptake of ^{14}C-glucose (Student *t*-test, $p < 0.001$) (Fig. 3-2). The *lstA* and *lstB* mutants had significantly reduced uptake of ^{14}C-glucose compared to the WT strain (Student *t*-test, $p < 0.001$), but glucose uptake was more reduced in the *lstA* mutant (Fig. 3-2). Complementation of the single mutants with the respective gene on a plasmid (*lstA*.C and *lstB*.C) restored uptake of glucose to that of the WT strain levels (Fig. 3-2).

To determine whether LstA and LstB were redundant, a *lstA/lstB* double mutant, was generated. Loss of both transporters abolished uptake of ^{14}C-glucose compared to the WT strain (Student *t*-test, $p < 0.001$) (Fig. 3-2). Upon supplementation of excess, unlabeled glucose (10mM) uptake of labeled glucose was inhibited in the complemented double mutants (Student *t*-test, $p < 0.001$) (Fig. 3-2). Interestingly, complementation with a single transporter, (*lstA*.C or *lstB*.C) restored uptake of ^{14}C-glucose to the double mutant similar to the WT strain levels (Fig. 3-2). These data show that LstA and LstB are glucose transporters.

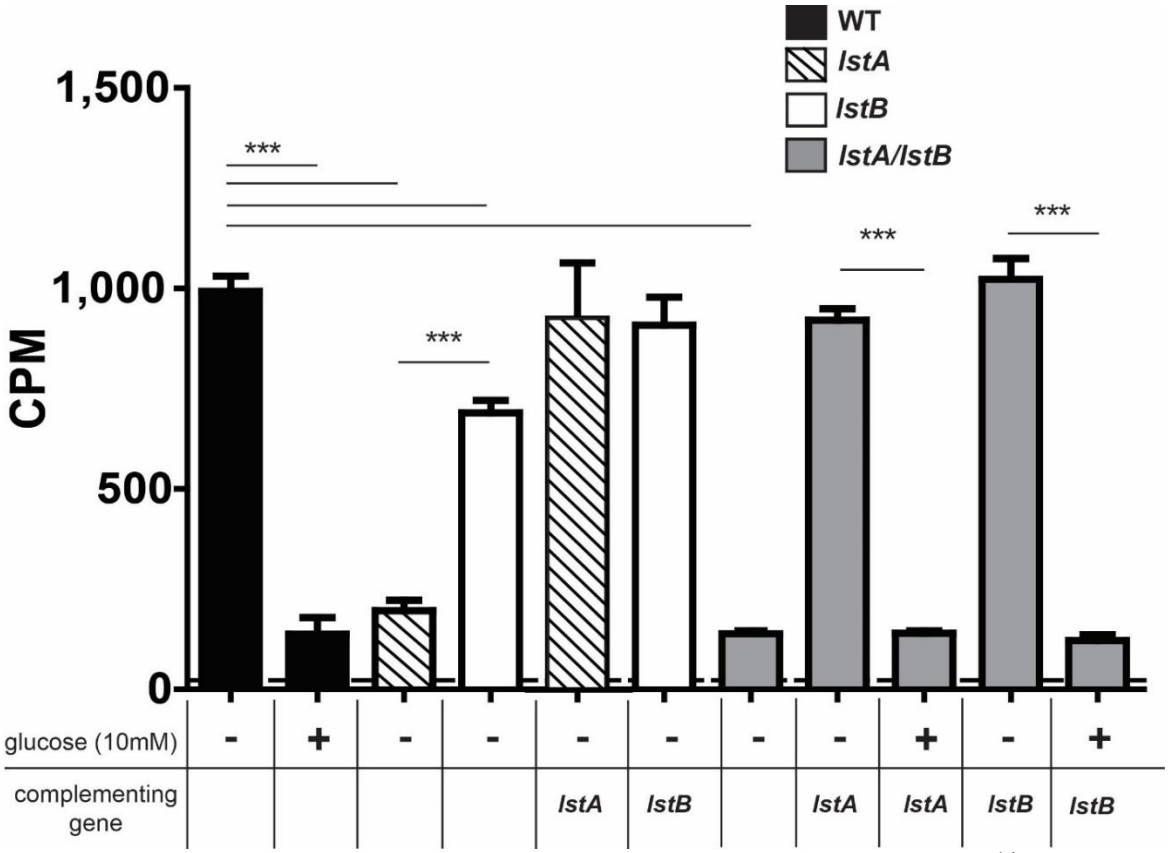


Figure 3-2. Glucose uptake in *L. pneumophila* by *lstA* and *lstB*. Uptake of ^{14}C-glucose measured in counts per minute (CPM), of WT *L. pneumophila* (black), single mutants (white), and double mutants (grey), was determined. Addition of unlabeled glucose (10mM) was used as a negative control (checkered). Data points represent mean CPM ± SD, n=4 and are representative of three independent experiments.

LstA and LstB CyaA-reporters are not translocated by Dot/Icm translocation system

While it is most likely that LstA and LstB are *L. pneumophila* membrane proteins, it is possible they are translocated into the host cell by the Dot/Icm. Translocation of these proteins could allow them to embed into the LCV membrane and access the pool of glucose in the host cytosol. Therefore, we sought to determine if LstA and LstB were translocated by the Dot/Icm translocation system [126, 127]. The adenylate cyclase (CyaA) reporter assay was used to assess translocation by measuring the level of cAMP generated by CyaA [137, 288]. Reporter plasmids were expressed in WT *L. pneumophila* and the *dotA* translocation-deficient mutant. The positive control, CyaA-RalF, was

effectively translocated in U937 human macrophages by the WT strain (Student *t*-test, $p < 0.001$) (Fig. 3-3). Neither CyaA-LstA nor CyaA-LstB reporters were translocated, as there was no significant difference in the level of cAMP between these reporters in WT *L. pneumophila* or the *dotA* mutant, compared to uninfected cells (Student *t*-test, $p > 0.05$) (Fig. 3-3).

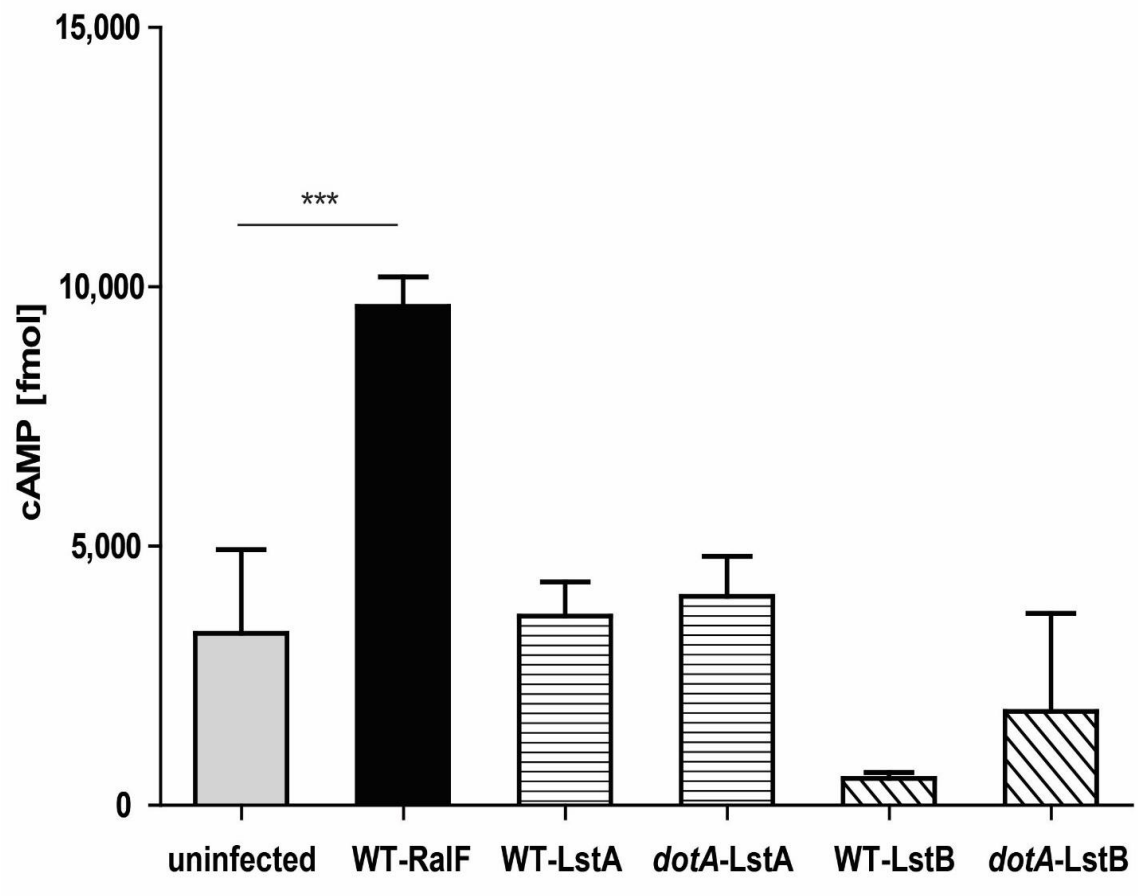


Figure 3-3. CyaA-LstA and CyaA-LstB reporters are not translocated by the Dot/Icm translocation system. Adenylate cyclase fusions of LstA and LstB were determined in triplicates at 1 h post-infection of U937 macrophages by the WT strain of *L. pneumophila* and the *dotA* mutant. A known T4SS effector (RalF) was used as a positive control. Data points represent mean CPM ± SD, n=4 and are representative of three independent experiments.

The LstA and LstB glucose transporters are required for growth in Acanthamoeba polyphaga *and human monocyte-derived macrophages*

We determined intracellular replication of glucose transporter single mutants, *lstA* and *lstB,* and the double mutant *lstA/lstB* in human monocyte-derived macrophages (hMDMs) and *A. polyphaga*. Single transporter null mutants, *lstA* and *lstB*, replicated similarly to WT *L. pneumophila* in *A. polyphaga* or hMDMs (Fig 3-4A, 3-5A), which is consistent with the idea that they are redundant transporters. Given that the *lstA/lstB* double mutant resulted in a severely diminished uptake of glucose, we determined the ability of the double mutant to replicate intracellularly. *In vitro* cultures of *lstA/lstB* grew similarly to the WT strain (Fig. S3-4). Preliminary studies indicate no defect in intracellular trafficking of the *lstA/lstB* double mutant determined by confocal microscopy to examine co-localization of the LCV with calnexin, LAMP1, and KDEL (data not shown).

Interestingly the *lstA/lstB* double mutant was severely defective for growth in *A. polyphaga* and hMDMs (Two-way ANOVA, $p < 0.001$) (Fig. 3-4B, 3-5B). Complementation of the double mutant, with individual single transporters, *lstA*.C or *lstB*.C, restored intracellular growth of the double mutant in *A. polyphaga* and hMDMs to almost that of WT *L. pneumophila* (Fig. 3-4B, 3-5B). These data show that the two glucose transporters, LstA and LstB, are required for intracellular growth of *L. pneumophila* within hMDMs and *A. polyphaga* and these two transporters are most likely to be redundant in their function to import glucose [220]. Our data show that uptake of glucose is required for intracellular replication of *L. pneumophila* within evolutionarily distant host cells.

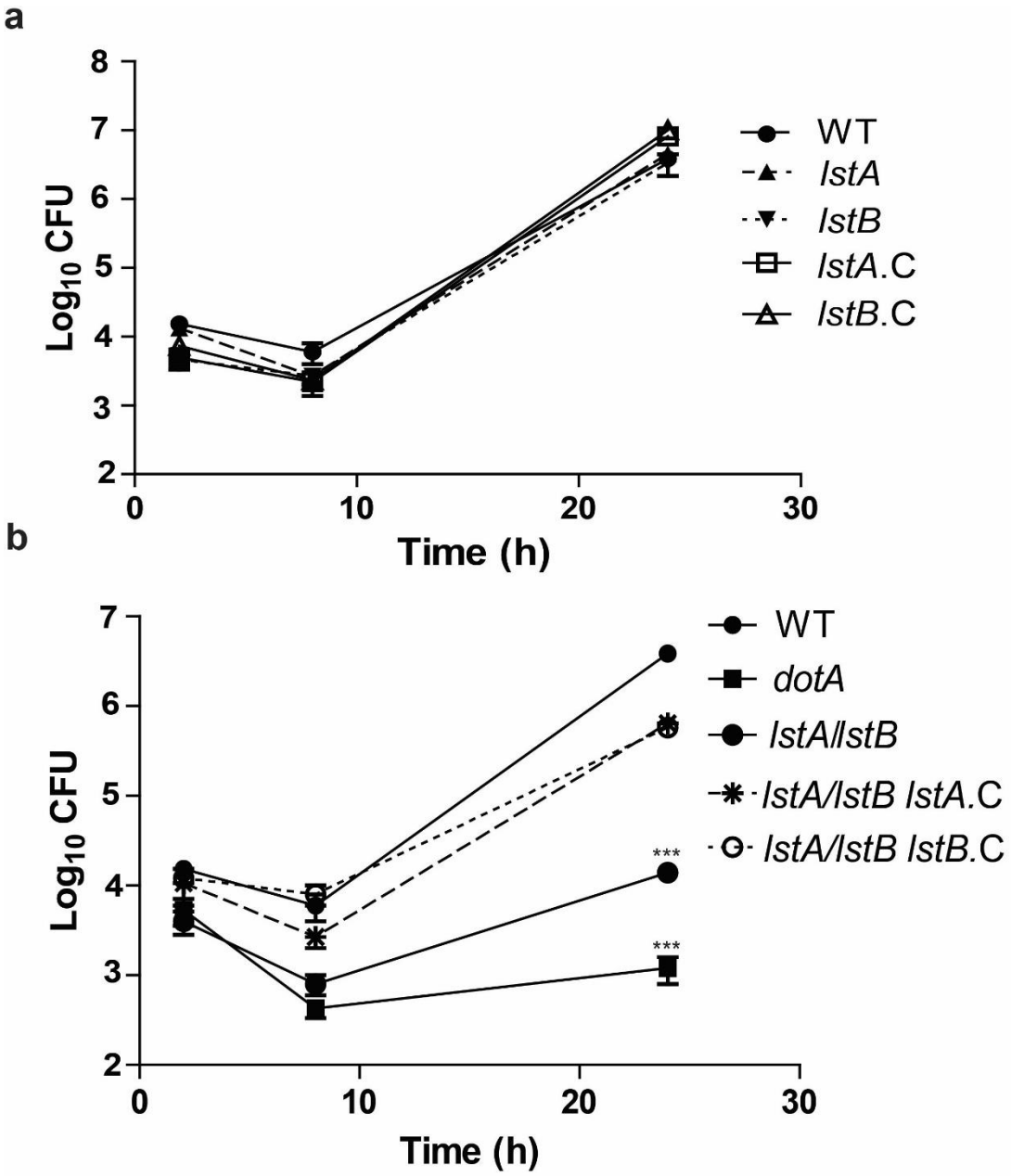


Figure 3-4. LstA and LstB are required for growth of *L. pneumophila* in amoebae. Intra-vacuolar replication of the A) the WT strain; the two single transporter mutants, *lstA* and *lstB*; and the complemented single mutant, *lstA*.C and *lstB*.C, was determined in *A. polyphaga*. B) The WT strain; the *dotA* mutant; the double mutant, *lstA/lstB*; and the complemented double mutant, *lstA/lstB lstA*.C and *lstA/lstB lstB*.C was also determined in *A. polyphaga*. The number of CFUs was determined at 2, 8, and 24 hrs post-infection. Data points represent mean CFUs ± SD, n=3 and are representative of three independent experiments.

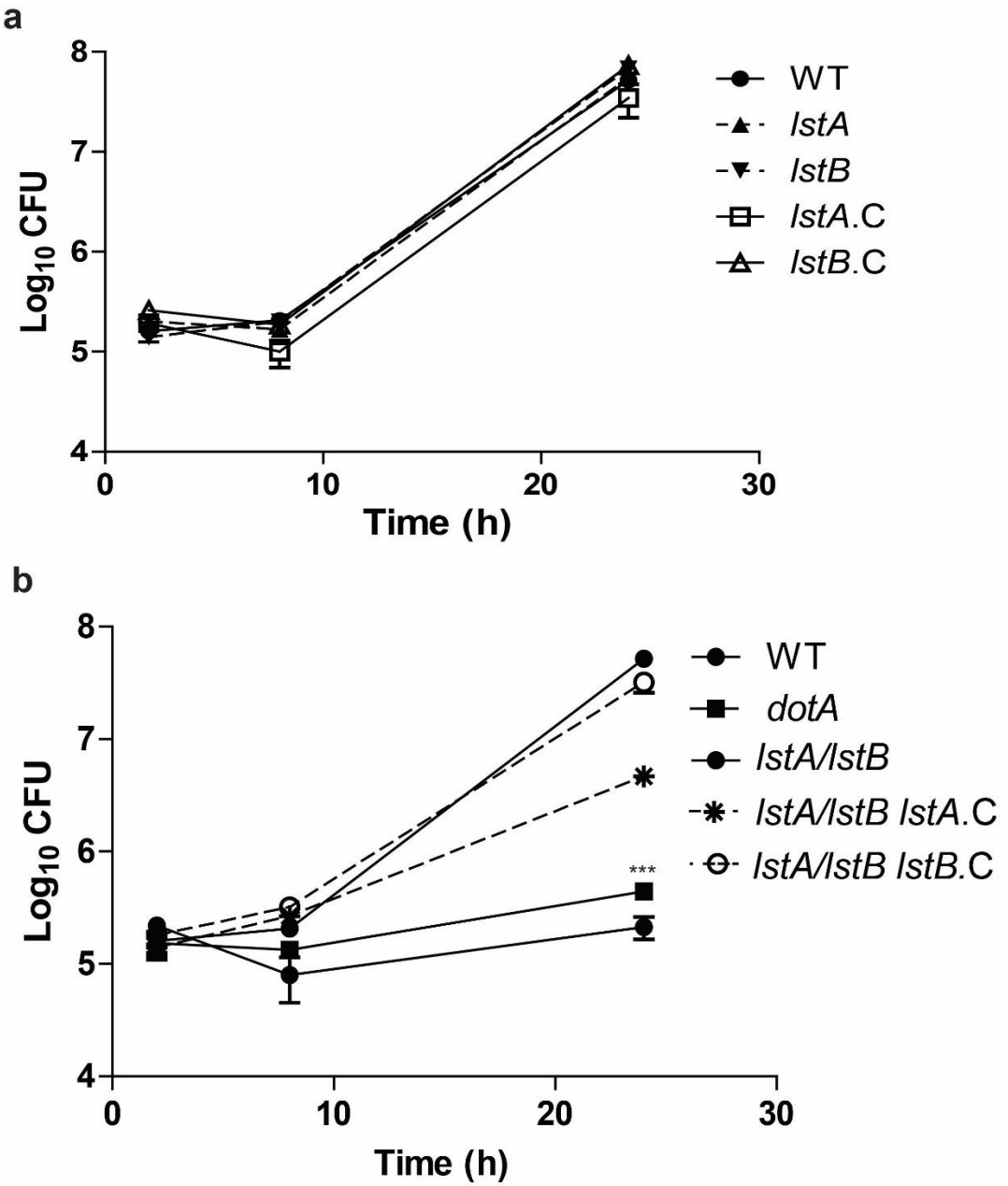


Figure 3-5. LstA and LstB are required for growth of *L. pneumophila* in hMDMs. Intra-vacuolar replication of the A) the WT strain; the two single transporter mutants, *lstA* and *lstB*; and the complemented single mutant, *lstA*.C and *lstB*.C, was determined in hMDMs. B) The WT strain; the *dotA* mutant; the double mutant, *lstA/lstB*; and the complemented double mutant, *lstA/lstB lstA*.C and *lstA/lstB lstB*.C was also determined hMDMs. The number of CFUs was determined at 2, 8, and 24 hrs post-infection. Data points represent mean CFUs ± SD, n=3 and are representative of three independent experiments.

Discussion

L. pneumophila generates copious amounts of host amino acids for carbon and energy but it is also reliant on host glucose [175, 220, 289]. Hauslein *et al.* described *L. pneumophila* metabolism as being "bipartite", where amino acids serve as the major energy supply in the exponential phase and carbohydrates at the post-exponential phase are used in anabolic processes [214]. The role of glucose during intracellular infection can be difficult to study; methods for altering the levels of glucose affect the host cells, which may have detrimental effects on intracellular growth of *L. pneumophila* independent of the glucose level. The glucose analog, 2-deoxy-D-glucose (2-DG) causes autophagy in macrophages [290]. Increasing the levels of glucose in macrophages increases the inflammatory response, while starving cells of glucose mimics treatment with 2-DG [253, 291]. Therefore, removing the *L. pneumophila*'s ability to access glucose by deletion of glucose transporters will best highlight the intracellular need for glucose without altering the host cell response to infection.

Our findings indicate that *L. pneumophila* utilizes two redundant glucose transporters, LstA and LstB, both of which transport glucose, and are required for growth within hMDMs and *A. polyphaga*. Surprisingly, the need for intracellular glucose is immediate, despite the fact that glucose is thought to be imported by *L. pneumophila* at the post-exponential phase and is thought to be primarily used in the late stages of growth for PHB synthesis [175, 218, 220, 226]. Consistent with this idea, *lstA* is highly upregulated in the post-exponential growth phase *in vitro*, when glucose is being utilized [181, 220]. However, *lstB* expression remains unchanged throughout the growth phases [181]. This could represent dual usages for glucose; LstB could transport low basal levels of glucose throughout intracellular growth while LstA transports large amounts of

glucose when the demand has increased during late stages of growth [175, 220]. Interestingly, LstA has been shown to be induced intracellularly during post-exponential growth of *L. pneumophila* in THP and *Acanthamoeba castellanii*, relative to growth *in vitro* [177, 181]. This supports the idea that glucose is required for intracellular replication but not for *in vitro* growth of *L. pneumophila* [220]. However, loss of either of the two transporters, *lstA* or *lstB*, is not sufficient to affect intracellular growth, which is most likely due to functional redundancy.

LstA is situated adjacent to a glucose utilization gene cluster, which is important for the catabolism of glucose via the ED pathway [175, 220]. Conflicting reports have shown that the gene cluster is required for growth in A549, A/J mouse macrophages, and *A. culbertsoni*, when mutated in *L. pneumophila* strain AM511 [220]. However, in the Paris strain of *L. pneumophila*, deletion of one of the genes, *zwf*, does not result in a growth defect in *Acanthamoeba castellannii* [175]. Additionally, the genetic organization of the gene cluster in the AM511 and the Paris strains is different [175, 220]. The *lstA* gene is 113bp downstream of the 3' end of *eda* of the glucose utilization gene cluster, which is sufficient for *lstA* to have its own promotor. This genetic organization is conserved among the Paris and AM511 strains, as well as our strain, AA100/130b.

The glucose transporter LstB/Lpg1653 is part of a myo-inositol catabolism gene cluster in *L. pneumophila* [292]. Although, LstB has been shown to transport inositol, inositol transporters are capable of transporting glucose, which is molecularly similar and acts as a competitive inhibitor of myo-inositol transport [292-294]. Therefore, it is possible that LstB has dual, or multi-, substrate specificity. Within *L. pneumophila*, myo-

inositol is also metabolized into acetyl-CoA, which could also support the generation of PHB [292].

Although CyaA-LstA and CyaA-LstB reporters are not translocated by the Dot/Icm system, CyaA reporter fusions are made at the N-terminus, since the C-terminus contains the recognition sequences for translocation by the Dot/Icm system [90]. This presents a caveat for trans-membrane proteins, such as for LstA and LstB, since the N-terminus tag may interfere with translocation or subcellular localization. Therefore, our data do not exclude the possibility that native LstA and LstB are translocated and embedded in the LCV membrane, providing *L. pneumophila* with access to the pool of glucose in the host cytosol. Alternatively, the LCV may acquire the host Glut1/SLC2a1 to import glucose into the LCV lumen while LstA and/or LstB are localized to the *L. pneumophila* outer membrane to import glucose from the LCV lumen.

Supplementation of glucose *in vitro* does not enhance the growth of *L. pneumophila* [220]. This may suggest the intracellular requirement for glucose does not support replication as a source of carbon and energy. Given that PHB is generated from glucose and is essential for survival outside of the host, lack of glucose uptake could be triggering stress response genes that prevent *L. pneumophila* from replicating [226]. If this were the case, starving host cells of glucose, would prevent the replication of intracellular *L. pneumophila*; however, starving host cells of glucose triggers cell death by autophagy [295]. Glucose is an important requirement for generating a reactive oxygen species (ROS) by amoebae and human macrophages in response to invading pathogens [253, 291]. Uptake of glucose by *L. pneumophila* could serve dual purposes of

sequestering glucose from the host, to dampen the immune response and to provide the precursor for PHB.

In summary, we have identified two redundant glucose transporters, LstA and LstB, which are required for intracellular replication of *L. pneumophila* in macrophages and amoebae. The requirement for glucose uptake by *L. pneumophila* is essential for intracellular growth in hMDMs and *A. polyphaga*, but not during growth *in vitro*. This presents an interesting question in *L. pneumophila* biology; why is glucose import required only during intracellular replication? This should be the focus of future studies.

Materials and Methods

Strains and cell lines

L. pneumophila strain AA100/130b (ATCC BAA-74) and the T4SS-deficient mutant, *dotA* were grown on Buffered Charcoal Yeast Extract (BCYE) agar, as we previously described [166]. To generate isogenic mutants in *lstA* and *lstB*, ~2 kb of flanking DNA on either side was amplified using primers listed in Table S3-2 and cloned into the shuttle vector, pBCSK+lstAKO and pBCSK+lstBKO (Table S3-1). The entire gene of either *lstA* or *lstB* was deleted via inverse PCR using the primers listed in Table S3-2, resulting in pBCSK+lstAKOi and pBCSK+lstBKOi (Table S3-1). The kanamycin cassette from the Ez-Tn5 transposon was amplified using primers listed in Table S2 and the resulting PCR product was subcloned into in pBCSK+lstAKOi and pBCSK+lstBKOi between the flanking regions of either *lstA* or *lstB*, using standard molecular procedures, resulting in in pBCSK+lstAKAN and pBCSK+lstBKAN (Table S3-1). Each resulting plasmid was independently introduced into *L. pneumophila* AA100/130b via natural transformation, as we previously described [161]. After three days, natural transformants were recovered by plated on BCYE supplemented with 50 μg/ml kanamycin, to generate *L. pneumophila lstA* and *L. pneumophila lstB* (Table S3-2). To confirm deletion of either *lstB* or *lstB*, the forward primer for sequencing and the reverse primer for generation of the knockout, listed in Table S3-2 were used. To generate double mutants, a gentamycin cassette was amplified using primers listed in Table S3-2 and the resulting PCR product was subcloned into in pBCSK+lstAKOi between the flanking regions of either *lstA*, using standard molecular procedures, resulting in in pBCSK+lstAGENT (Table S3-1). The resulting plasmid was introduced into *L. pneumophila lstB* via natural transformation, as previously described [161]. After three days, natural transformants were recovered by

plated on BCYE supplemented with 20 μg/ml kanamycin and 5 μg/ml gentamycin, to generate *L. pneumophila lstB/lstA* (Table S3-1). Deletions were confirmed using the same primers as described above.

To generate complement mutants of single deletions and double deletions, *lstA* or *lstB* with flanking upstream and downstream sequences were amplified by PCR using the primers listed in Table S3-2, and subcloned into pBCSK+, generating pBCSK+*lstA*.C and pBCSK-*lstB*.C (Table S3-1). The pBCSK+*lstA*.C plasmid was introduced into the *lstA* and *lstA/lstB* mutants and the pBCSK+*lstB*.C plasmid was introduced into the *lstB* and *lstA/lstB* mutants via electroporation as previously described (Table S3-1) [296]. All complement mutants were selected on BCYE plates supplemented with 5 μg/ml chloramphenicol, resulting in the following complement strains: *lstA*.C, *lstB*.C, *lstA/lstB lstA*.C , and *lstA/lstB lstB*.C (Table S3-2).

Human monocyte-derived macrophages (hMDMs) were isolated from healthy adult donors and cultured in RPMI 1640 (Corning) supplemented with 10% fetal bovine serum, as previously described [166]. All methods were carried out and approved in accordance to the University of Louisville Institutional Review Board guidelines and blood donors gave informed consent as required by the University of Louisville Institutional Review Board (IRB # 04.0358). U937 cells were cultured in RPMI 1640 (Corning) supplemented with 10% fetal bovine serum and *A. polyphaga* was cultured in PYG media, experiments were performed in PY media, as we previously described [166].

Structural comparison of glucose transporters

Predicted structures were generated via I-TASSER server from the Zhang Lab (https://zhanglab.ccmb.med.umich.edu/I-TASSER/) [285, 286, 297]. Structures generated

from I-TASSER were aligned using TM-align to determine structural alignment and TM-scores for similarity (https://zhanglab.ccmb.med.umich.edu/TM-align/) [287, 298].

Glucose uptake assay

Uptake of glucose was assayed by growing WT *L. pneumophila*, *lstA*, *lstB,* and complemented mutant strains individually in the presence of ^{14}C-label glucose (specific activity, 3.3MBq/µmol) (PerkinElmer) and followed the presence of glucose into the acid-insoluble fraction, as previously described [220]. One milliliter cultures were grown in Buffered Yeast Extract (BYE) broth supplemented with 0.1% D-[U-^{14}C] glucose (specific activity, 3.3MBq/µmol) at 37°C, shaking to post-exponential phase (>OD_{550} 2.0). For control, 10mM sterile glucose was added to broth cultures with 0.1% D-[U-^{14}C] glucose. Samples normalized to 10^9 bacteria in 5ml 1% Triton X-100 for 30 mins, and then incubated for 30mins with 5ml of chilled 10% (w/v) trichloroacetic acid, on ice. To capture radioactivity, samples were filtered through nitrocellulose filters (0.45-µm pore size; Milipore) and rinsed three times with chilled 5% trichloroacetic acid. Radioactivity of the whole sample was determined by liquid scintillation (Tri-Carb 2910 TR, PerkinElimer) with BetaBlend scintillation cocktail (MP Biochemical).

Intracellular replication

L. pneumophila; the isogenic single mutants, *dotA*, *lstA* and *lstB*; the double isogenic mutant *lstB/lstA*; and the complement mutants *lstA*.C, *lstB*.C, *lstB/lstA lstB*.C, and *lstB/lstA lstA*.C were grown to post-exponential on BCYE plates at 37°C prior to infection and used to infect hMDMs or *A. polyphaga*, as previously described [97, 166]. A total of 1 X 10^5 host cells per well were plated into 96 well plates and infected with *L. pneumophila* at an MOI of 10 for 1h then treated with gentamycin to kill remaining extracellular bacteria, as previously described [97, 166]. Host cells were lysed with sterile

water (hMDMs) or 0.02% Triton X-100 (*A. polyphaga*) at various timepoints over a 24h timecourse and *L. pneumophila* CFUs were determined by plating serial dilutions onto BCYE agar.

Translocation assay

To assay translocation of LstA and LstB by *L. pneumophila* T4SS, during infection of host cells, an adenylate cyclase fusion (CyaA) reporter was generated using standard biological techniques with primers listed in Table S3 [288]. A total of 1 x 10^6 U937 cells were infection with WT or *dotA* mutant *L. pneumophila* harboring plasmids expressing various adenylate cyclase fusions at an MOI of 20 for 1 h, as previously described (Table S2) [97, 288]. Following infection, cell monolayers were lysed in ddH_2O and processed to assess cAMP concentration by ELISA using the Direct cAMP ELISA kit (Enzo), according to the manufacturer's protocol and measured with a Syngergy H1 microplate reader (BioTek).

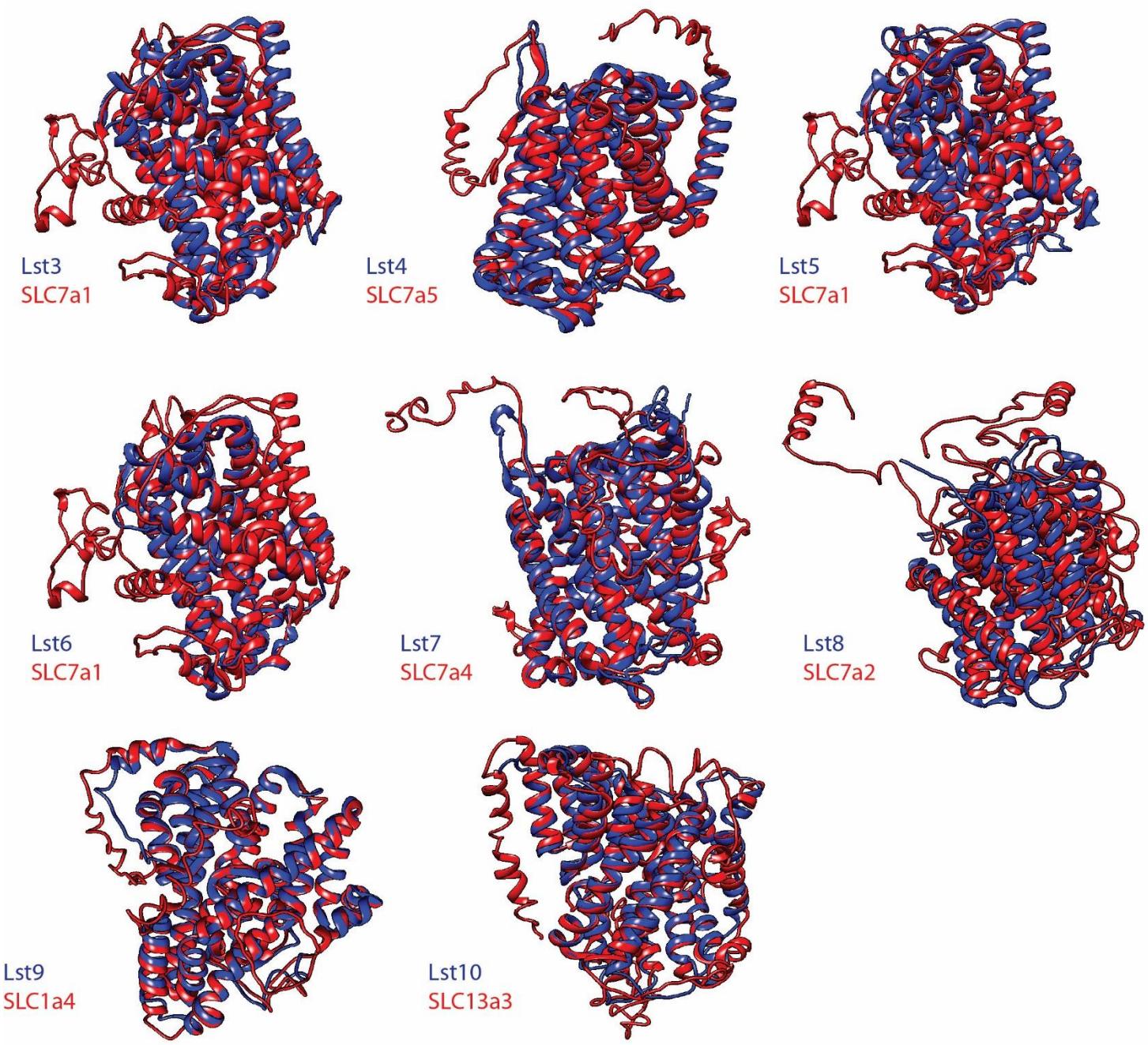


Figure S3-1. Structural alignment of human SLCs and SLC-like proteins in *L. pneumophila*. I-TASSER predicted structures of SLC-like proteins aligned, via TM-align, with human SLCs based on highest TM-score, indicate strong structural similarity.

	Amino acid identity (BLAST)	Amino acid similarity (BLAST)	Putative substrates	Protozoan transporter, TM-score
LstC	40%	58%	Cationic amino acids (arginine, lysine, ornithine)	CtrB (0.786)
LstE	26%	43%	Cationic amino acids (arginine, lysine, ornithine)	CtrA (0.977)
LstG	24%	42%	Cationic amino acids (arginine, lysine, ornithine)	CtrC (0.869)
SLC7a3	31%	50%	Cationic amino acids (arginine, lysine, ornithine)	CtrA (0.807)
SLC7a5	33%	48%	Cationic amino acids (arginine, lysine, ornithine)	CtrB (0.824)
SLC7a4	40%	58%	Cationic amino acids (arginine, lysine, ornithine)	CtrC (0.850)

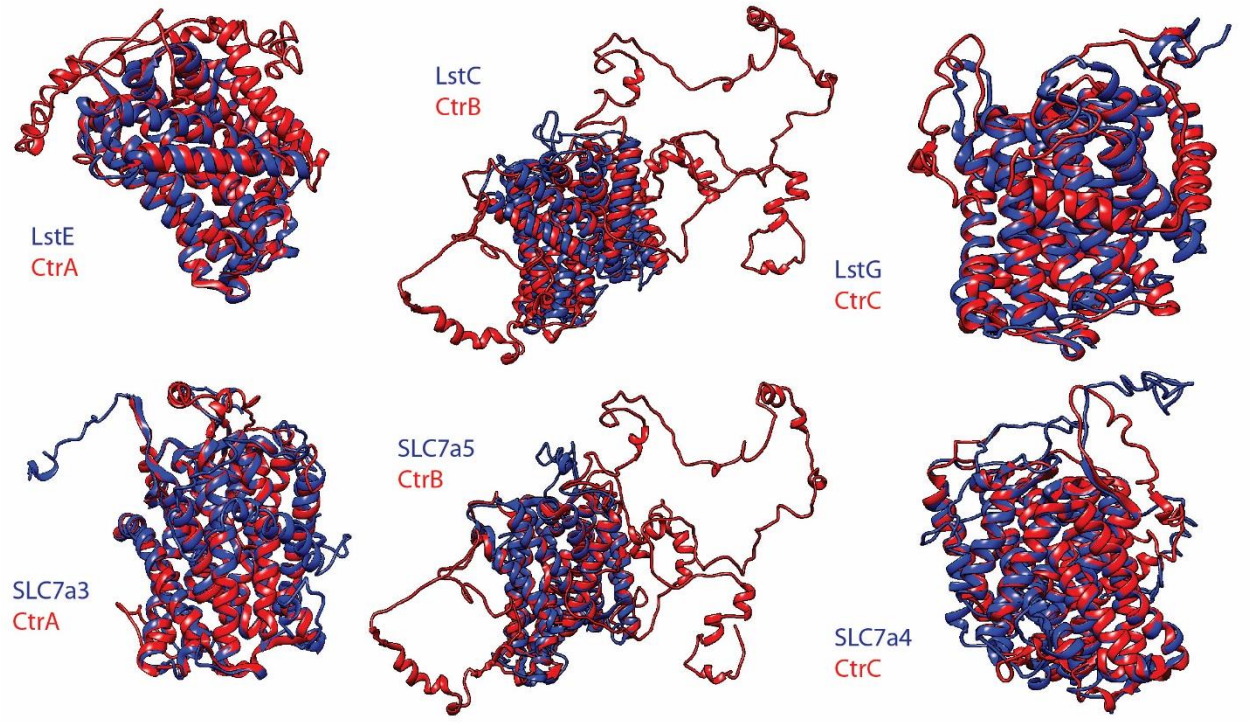


Figure S3-2. Similarity and identity of *L. pneumophila* SLC-like transporters and protozoan SLC-like transporters. SLC-like amino acid transporters are present in *Dictyostelium discodium*. These protozoan transporters share structural homology with the human SLC transporters and with *L. pneumophila* SLC-like transporters. A) Degrees of amino acid sequence identity and similarity between these transporters are shown in and B) structural alignment.

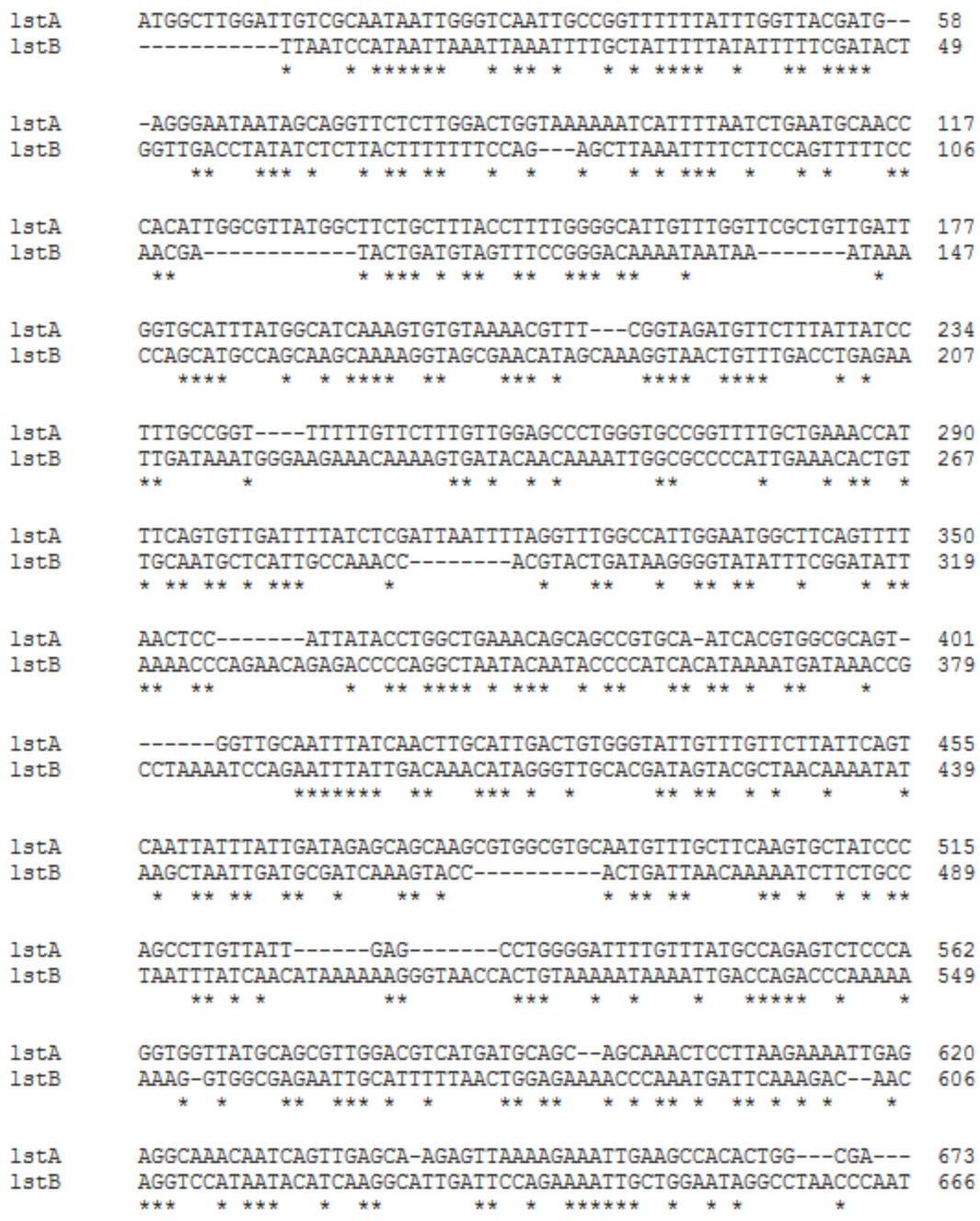


Figure S3-3. The *lstA* and *lstB* genes are likely not paralogs. Alignment of *lstA* within *lstB* indicates these two genes are likely not paralogs within *L. pneumophila*.

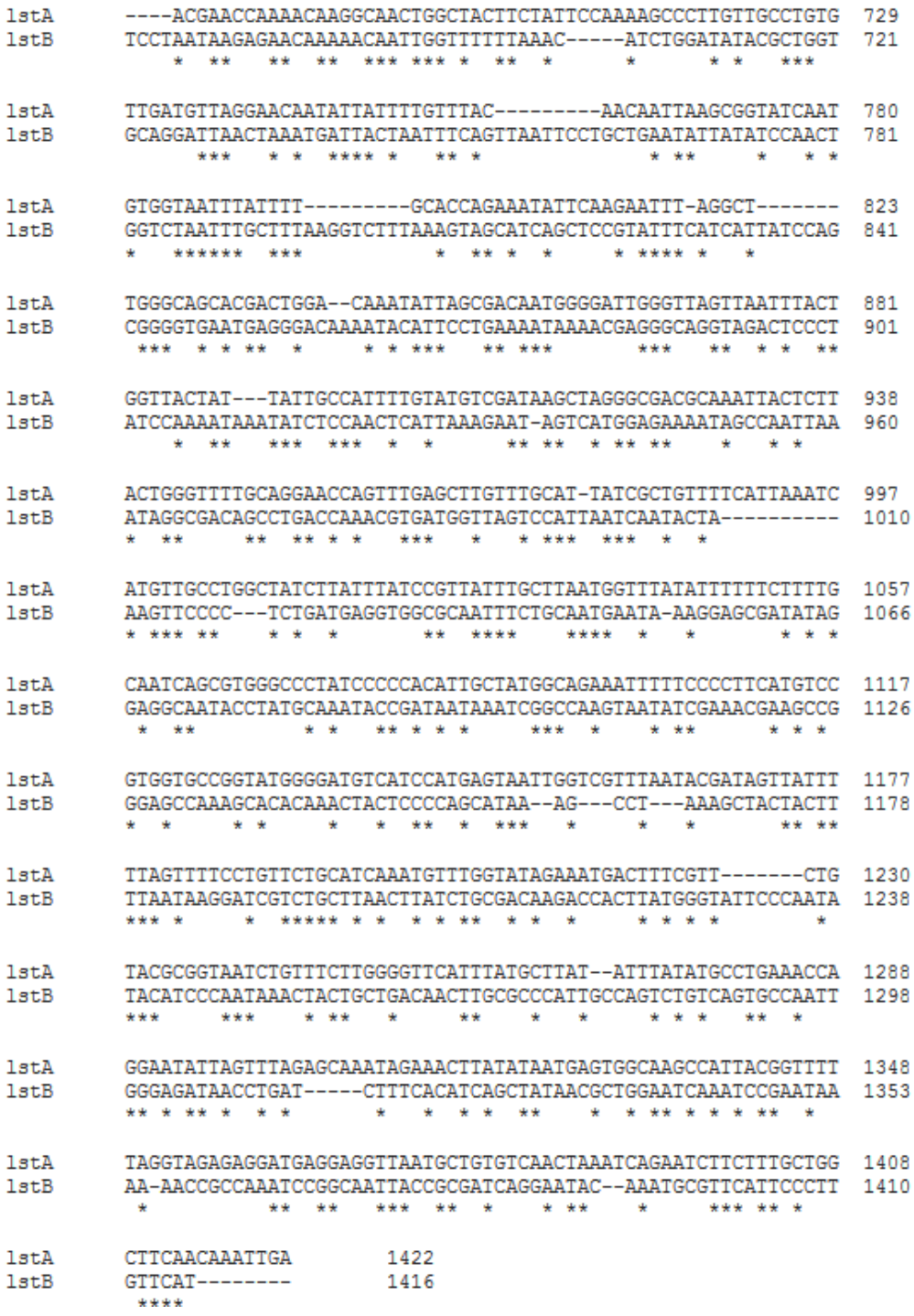


Figure S3-3. The *lstA* and *lstB* genes are likely not paralogs. Alignment of *lstA* within *lstB* indicates these two genes are likely not paralogs within *L. pneumophila*.

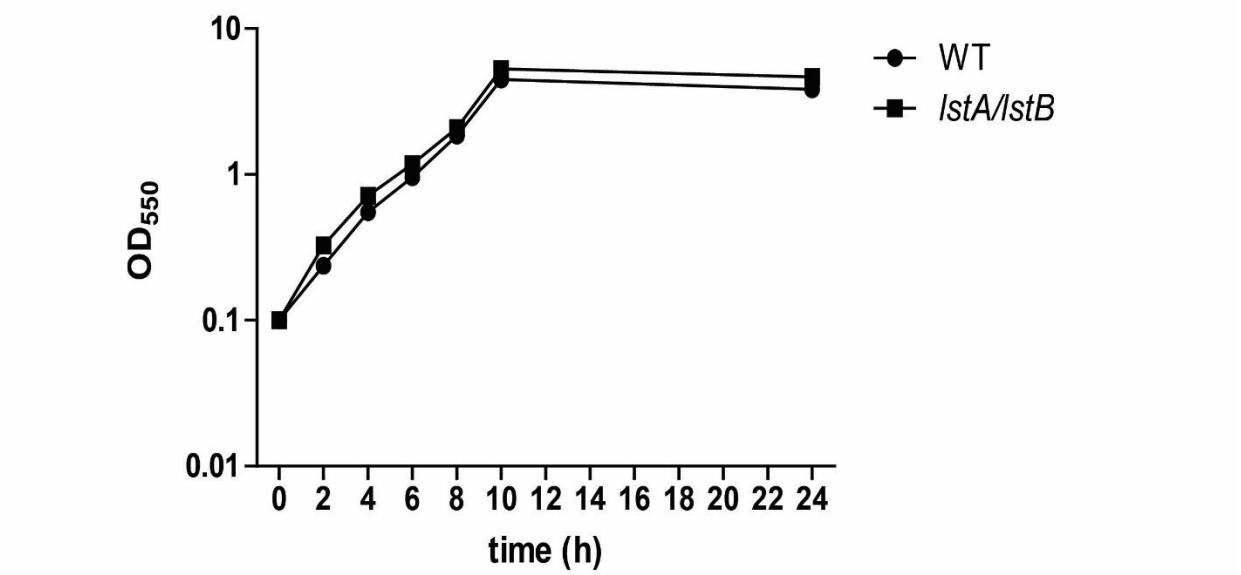


Figure S3-4. The *lstA* and *lstB* mutants grow similar to WT *in vitro*. Overnight cultures of WT and *lstA/lstB* in BYE broth diluted to OD_{550nm} of 0.05 and grown at 37°C for 24 hrs. Growth rates were determined by measuring OD_{550nm} every 2 hrs, for 12 hrs, then again at 24 hrs post-inoculation. Data are representative of three independent experiments.

Table S3-1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype
L. pneumophila	
AA100/130b	
dotA	
lstA	*lstA*, Kan^r substitution
lstB	*lstB*, Kan^r substitution
lstA/lstB	*lstB*, Kan^r substitution *lstA*, Gent^r substitution
lstA.C	*lstA* complemented with pBCsk+*lstA*.C
lstB.C	*lstB* complemented with pBCsk+*lstB*.C
lstB/lstA lstA.C	*lstB/lstA* complemented with pBCsk+*lstA*.C
lstB/lstA lstB.C	*lstB*/*lstA* complemented with pBCsk+*lstB*.C
CyaA-RalF	In WT
CyaA-LstA	In WT
CyaA-LstA	In *dotA*
CyaA-LstB	In WT
CyaA-LstB	In *dotA*
Plasmids	
pBCsk-	
pBCsk+*lstA*KO	2kB region upstream and downstream of *lstA*
pBCsk+*lstB*KO	2kB region upstream and downstream of *lstB*
pBCsk+*lstA*KOi	pBCSK+*lstA*KO with *lstA* removed
pBCsk+*lstB*KOi	pBCKSK+*lstB*KO with *lstB* removed
pBCsk+*lstA*Kan^r	pBCSK+*lstA*KO with *lstA* replaced with kanamycin resistance cassette
pBCsk+*lstB*Kan^r	pBCSK+*lstB*KO with *lstA* replaced with kanamycin resistance cassette
pBCsk+*lstA*Gent^r	pBCSK+*lstA*KO with *lstA* replaced with gentamycin resistance cassette
pBCsk+*lstA*.C	*lstA*, with 100bp promotor region
pBCsk+*lstB*.C	*lpg1649-lstB*, with 100bp promotor region
pCYA+*lstA*	CyaA-*lstA* fusion plasmid
pCYA+*lstB*	CyaA-*lstB* fusion plasmid

Table S3-2. Primers used in this study

Primer	Sequence
lstA-KO F	GGTACCATATGATTTTGATCGATTGT
lstA-KO R	GAGCTCAAGGAAGCACATAAATTAAAA
lstB-KOF	GGTACCAAAACATTCAATACTTCAACA
lstB-KO R	GAGCTCTCAGTGAATATAAATAAACGCT
lstA inverse F	TATAAATTTTCCTATGATGCCC
lstA inverse R	TTTTCACTCCTTGATTTTAAATTCT
lstB inverse F	GTAACTATCTGTCCCTAATGAAAATTC
lstB inverse R	TCATGAAAAAGAAAATATGTCGAAT
Kan F, R	/5Phos/CTGTCTCTTATACACATCTCAA
lstA sequence primer	TATCAAGAATCATAACTGGTCTTT
lstB sequence primer	TAATTTTTCTGCCCAATTCCTATCGA
Gent F	/5Phos/ATGTTACGCAGCAGCAACGAT
Gent R	/5Phos/TTAGGTGGCGGTACTTGGGTC
lstA-C F	GGTACCTTCGGGTTGAAAAAGCGT
lstA-C R	GAGCTCCGTGTCAGTCAAAGATAACA
lstB-C F	GGTACCGGATAATCTCCTTATTATATTG
lstB-C R	AGCTCATGCTTTGGTCAATTAAC
CyaA-*lstA* F	
CyaA-*lstA* R	
CyaA-*lstB* F	
CyaA-*lstB* R	

CHAPTER 4:

A *LEGIONELLA PNEUMOPHILA* AMYLASE IS ESSENTIAL FOR INTRACELLULAR REPLICATION IN HUMAN MACROPHAGES AND AMOEBAE*

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*Best, A.M. †, Price, C.T. †, Ozanic, M., Santic, M., Jones, S.C., Abu Kwaik, Y., A *Legionella pneumophila* amylase essential for intracellular replication in human macrophages and amoebae. Sci Rep, 2018. **8**(1): p. 6340 [299]

†These authors contributed equally

Summary

Legionella pneumophila invades protozoa with an "accidental" ability to cause pneumonia upon transmission to humans. To support its nutrition during intracellular residence, *L. pneumophila* relies on host amino acids as the main source of carbon and energy to feed the TCA cycle. Despite the apparent lack of a requirement for glucose for *L. pneumophila* growth *in vitro* and intracellularly, the organism contains multiple amylases, which hydrolyze polysaccharides into glucose monomers. Here we describe one predicted putative amylase, LamB, which is uniquely present only in *L. pneumophila* and *L. steigerwaltii* among the ~60 species of *Legionella*. Our data show that LamB has a strong amylase activity, which is abolished upon substitutions of amino acids that are conserved in the catalytic pocket of amylases. Loss of LamB or expression of catalytically-inactive variants of LamB results in a severe growth defect of *L. pneumophila* in *Acanthamoeba polyphaga* and human monocytes-derived macrophages. Importantly, the *lamB* null mutant is severely attenuated in intra-pulmonary proliferation in the mouse model and is defective in dissemination to the liver and spleen. Our data show an essential role for LamB in intracellular replication of *L. pneumophila* in amoeba and human macrophages and in virulence *in vivo*.

Introduction

The accidental human pathogen, *Legionella pneumophila*, causes an atypical pneumonia when water droplets, stemming from a contaminated water source such a cooling tower or humidifier, are inhaled by humans, which are considered as accidental host [3, 15, 300]. Over 20 protozoa species are known to harbor *Legionella* species, likely with more yet to be identified [100]. Growth within the natural protozoan host serves as a

"training grounds", priming for infection of human alveolar macrophages, as these bacteria are more infectious than their free-living counterparts [79, 301, 302]. Success in replicating in macrophages may have been facilitated by the exploitation of evolutionarily conserved host processes, which allow *L. pneumophila* to modulate conserved pathways in both macrophages and protozoa [77, 93, 100, 303]. Inhaled bacteria enter into the lungs where they primarily reside and proliferate within alveolar macrophages [107, 304, 305]. The intracellular lifecycle in the evolutionarily distant host cells is nearly identical [100]. Once the bacterium enters the host cell, it actively evades lysosomal fusion and intercepts ER-derived secretory vesicles to generate and ER-derived vacuole, known as the *Legionella*-containing vacuole (LCV) [53, 91, 96, 306], and modulate a plethora of cellular and innate immune processes [307-310].

Essential to intracellular replication is the Dot/Icm Type 4b secretion system (T4SS), which inject proteins, known as "effectors", from the bacterium to the host cytoplasm to modulate host processes [133, 278, 311]. Because of the broad host range, *L. pneumophila* has evolved over 320 effectors that are translocated by the Dot/Icm system and utilized as a "toolbox" to modulate cellular processes of various environmental hosts [127, 144, 149, 312, 313]. Many unique mechanisms of interfering with host processes, such as lysosomal-evasion and trafficking, have been identified and attributed to specific Dot/Icm effectors [20, 127, 143, 277-279]. The effector's ability to interfere with the function of eukaryotic host target proteins, comes from their evolutionary history; many effectors are derived from eukaryotic proteins acquired by inter-kingdom horizontal gene transfer (HGT) [149, 156, 284, 314].

The primary food source for *L. pneumophila* is amino acids which are used for carbon and energy through feeding the TCA cycle [175, 210, 211, 315].The generation of host amino acids by *L. pneumophila* is an effector-driven process [75]. Substantial generation of host amino acids is required in human macrophages and amoebae where the effector AnkB hijacks the host ubiquitin-proteasome protein degradation machinery, which is required for successful pathogen replication in the host [75, 98, 168, 316]. In contrast to human macrophages and amoebae, during infection of mouse macrophages with the *L. pneumophila* LP02 strain, the mammalian target of rapamycin complex 1 (mTORC1), a nutrient/energy sensor, is inhibited by multiple effectors to prevent protein synthesis, thus liberating amino acids for bacterial consumption [246]. Distinct pathogen mechanisms of generating host cell amino acids may be employed within diverse host cells and the pathogen mechanism may differ by various strains of *L. pneumophila* to acquire the high levels of host amino acids needed for replication.

Glucose is minimally metabolized by *L. pneumophila* through the Entner-Doudoroff (ED) pathway [175, 214, 220, 317]. Traditional glycolysis through the Embden-Meyeroff-Parnas (EMP) pathway is also minimal, despite all the necessary genes being present in the *L. pneumophila* genome [175, 220]. Glucose does not support growth of *L. pneumophila*, but it is predominantly imported by *L. pneumophila* upon termination of growth [175, 220], as the bacterium is preparing for cellular egress, and used mainly for the generation of the storage molecule, poly-3-hydroxybutyrate (PHB) through the ED pathway [218, 220]. During nutrition deprivation, PHB is converted to acetyl-CoA that feeds the TCA cycle [215, 222, 226]. Genes involved in glucose

metabolism and glucose uptake are up-regulated during growth in amoebae and may play a role in infection [181, 215].

Amylases are a conserved group of enzymes that catalyze hydrolysis of starch and glycogen into glucose [318]. They are members of a larger family, called glucosidases, and include other enzymes such as cellulase and lactase [319]. Interestingly, despite the minimal need of glucose by *L. pneumophila*, four putative amylases have been identified in the *L. pneumophila* genome, Lpg0422, Lpg1669, Lpg1671, and Lpg2528. The Lpg0422 (GamA) enzyme is the only characterized amylase [289]. It is secreted by the Type II secretion system (T2SS) and expressed during exponential growth but not required for intracellular growth [181, 289]. Lpg1669 is a putative amylase that lacks putative secretion signals for the T4SS or T2SS, based on bioinformatical analysis. Lpg1671 is predicted to be a T4SS substrate but its role in intracellular infection is not known [125, 126]. The gene for *gamA* is found in most *Legionella* species and *lpg1669* and *lpg1671* are found in three species of *Legionella*.

The predicted putative amylase, Lpg2528, has been designated as LamB. Among the 60 *Legionella* species, *L. pneumophila* and *L. steigerwaltii* are the only two *Legionella* species to harbor *lamB*. Since *L. pneumophila* is responsible for 85% of Legionnaire's disease cases, we decided to determine the role of LamB in the intracellular infection of amoebae and human macrophages[15, 24]. Here we show that despite the minimal role of glucose in *L. pneumophila* metabolism, the LamB amylase is surprisingly necessary for intracellular replication in amoebae and human macrophages, and is required for virulence *in vivo*, in the A/J mouse model.

Results

Identification of amylases in L. pneumophila

Based on domain sequence homology, three new putative amylases were identified in the *L. pneumophila* genome, in addition to the one described (GamA) amylase (Fig S4-1A, B, C) [289, 320]. The Lpg2528 putative amylase is designated as LamB, which is encoded by a monocistronic gene (Fig. 4-1D). Considering LamB is only present in *L. pneumophila* and *L. steigerwaltii*, of the 60 *Legionella* species, it is more likely that LamB has been acquired after the speciation event of *L. pneumophila* and suggests that *L. steigerwaltii* may have arisen recently from *L. pneumophila* (Fig. 4-1A). The evolution of this gene in *L. pneumophila* mirrors that of the strain evolution (Fig. 4-1A). *L. pneumophila* strain Lens is most related in genome sequence homology to strain 130b/AA100, which is seen with *lamB* (Branch length, 99) [321]. Similarly, *L. pneumophila* strain Alcoy is most homologous to strain Corby, as also seen with *lamB* (Branch length, 94) [321]. LamB shares amino acids sequence homology only with other soil and freshwater organisms such as, *Methylobacterium* and *Insolitispirillum* (see Supplementary Fig. S4-2 online). Thus, it is likely that *lamB* may have been acquired by HGT from other intra-amoebal or planktonic, environmental organisms. Because *L. pneumophila* is responsible for 85% of Legionnaire's disease cases, we characterized the role of this enzyme in the intracellular infections of human monocyte-derived macrophages (hMDMs) and *A. polyphaga* [15, 24].

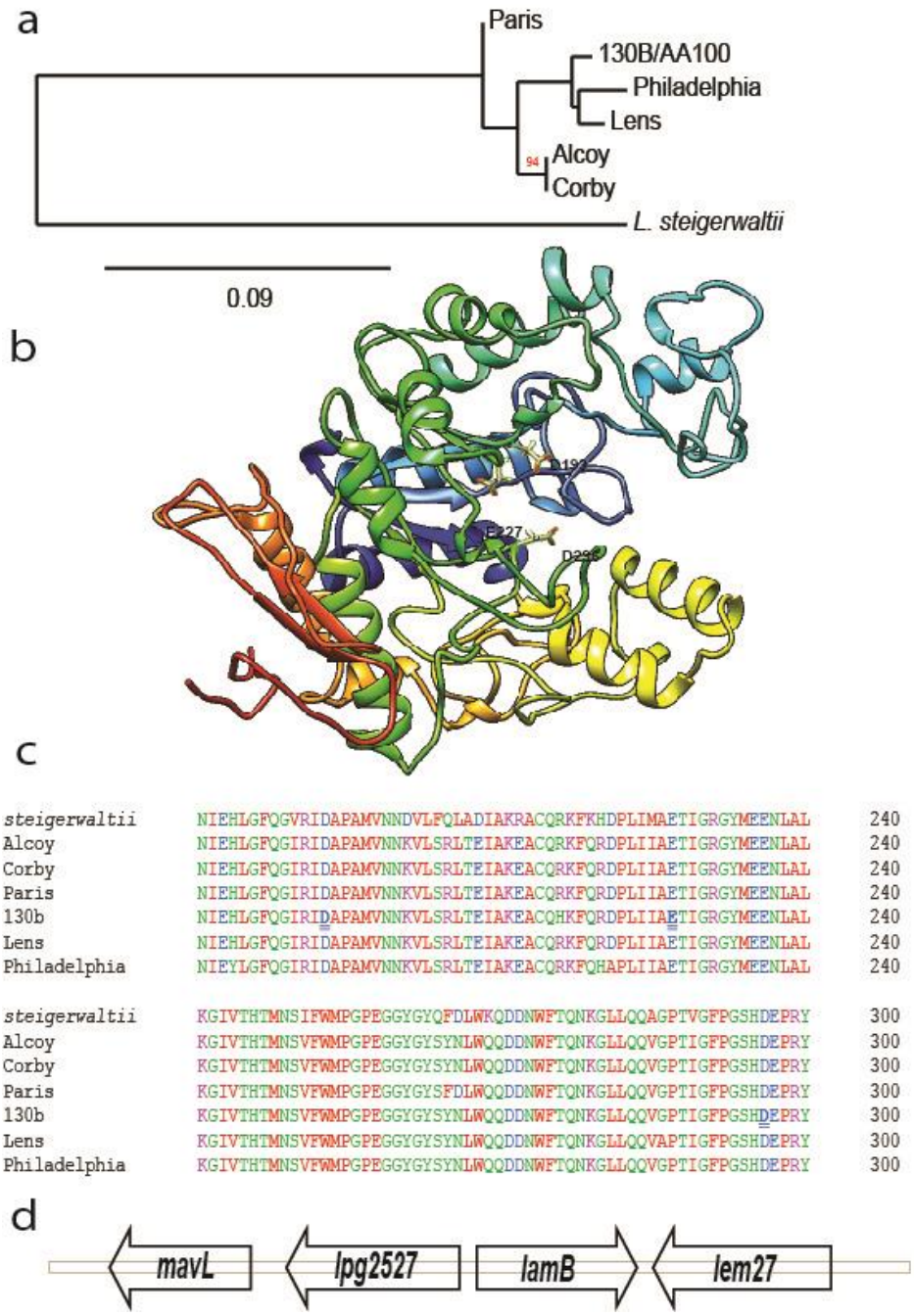

Figure 4-1. LamB is a putative amylase unique to *L. pneumophila*. A) Phylogram representation of LamB divergence in *L. pneumophila* strains and *L. steigerwaltii*. Measure of node support was determined by aLRT using Phylogeny.fr B) LamB is conserved among *L. pneumophila* strains and is only found in one other *Legionella* species, *L. steigerwaltii*. C) The structure of LamB, generated from I-TASSER server, which suggests it is an amylase [285]. Highlighted within the catalytic binding pocket of amylases are resides critical for catalytic activity, D193, E227, and D296. The *lamB* gene is found on a monocistronic operon within the *L. pneumophila* genome, this area of the chromosome is shown in D).

Structure of LamB and its potential secretion

LamB has an α-amylase domain (residues 18-376) that is structurally similar to the crystalized glucosidase of *Streptococcus mutants*, SmDG (Fig. S4-1D) [322, 323]. The putative catalytic site, which is located within the predicted catalytic pocket of the enzyme, is conversed amongst amylases and in LamB of *L. pneumophila* and *L. steigerwaltii* (Fig. 4-1B, C and Fig. S4-1D). Iterative Threading Assembly Refinement (I-TASSER) is a bioinformatics method of predicting the three-dimensional structure of proteins based on fold recognition [286, 297]. The server also predicts ligand binding sites, and gene ontology. Structural modeling of LamB using the I-TASSER database shows structural similarity with other crystalized glucosidases (Fig. 4-1B).

L. pneumophila proteins can access the host cytosol by two major routes, translocation via the T4SS or through secretion into the LCV lumen by the type II secretion system (T2SS) and into the cytosol through the semipermeable LCV membrane[133, 230]. *L. pneumophila* secretes many proteins via the T2SS, which is critical for intracellular growth and pulmonary disease [324-326]. Twenty proteins were identified to be secreted by the T2SS of *L. pneumophila,* while an additional 250+ proteins have been suggested to contain a putative T2SS signal [327]. LamB was not identified by any of these methods as potential type-II substrate [327]. In addition, LamB lacks the N-terminal secretion signal characteristic of T2SS substrates [326].

In order to be translocated by the Dot/Icm translocation system, effectors are recognized by a translocation signal on the C-terminus; alterations at the C-terminus of effectors causes a failure in translocation [90, 124, 126]. However, no translocation consensus sequence exists for all effectors of the Dot/Icm translocation system. Machine learning techniques have identified conserved bi- and tri-residues at the C-terminal end of

L. pneumophila effector proteins, 10% of known effectors do not contain at any of these motifs and some proteins harboring these motifs are not translocated effectors [126, 328]. LamB contains seventeen bi-residues identified to be heavily enriched in the last 100 amino acids of the C-terminus of T4SS effector proteins (see Supplementary Fig. S4-3 online) [328].

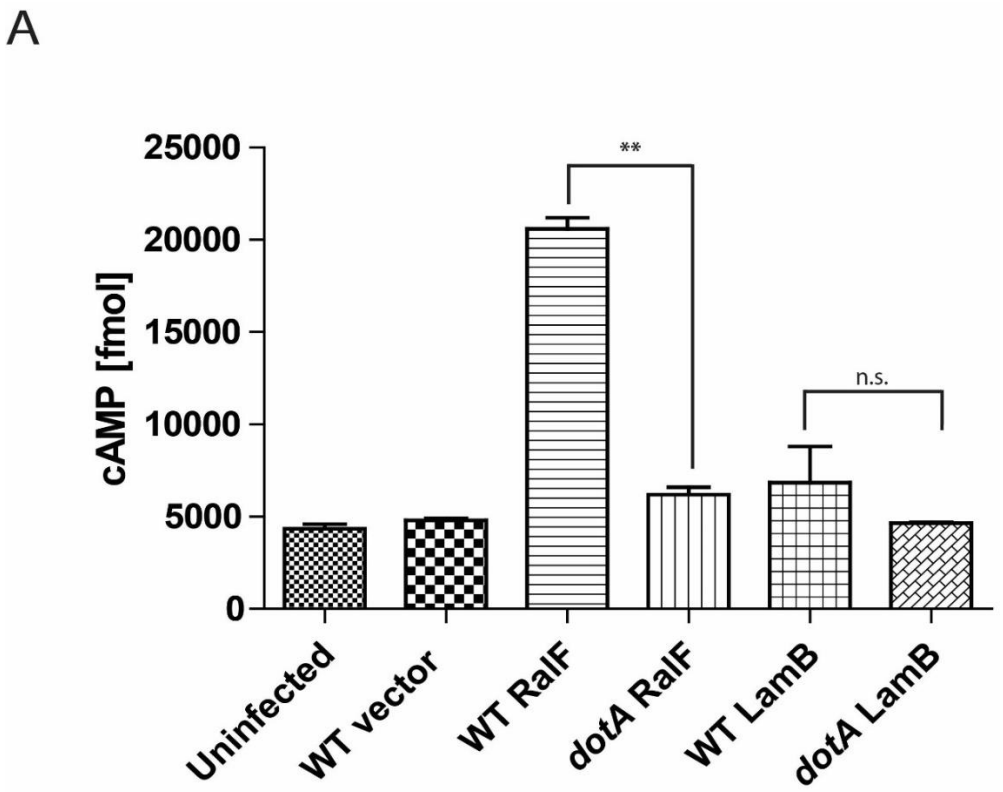

Figure 4-2. CyaA-LamB reporter is not translocated by the Dot/Icm T4SS. A) Adenylate cyclase fusions of LamB expressed in *L. pneumophila* and infected into hMDMs for 1hr, in triplicate, using known T4SS effector, RalF, as a positive control. Production of cAMP was assessed by ELISA. Data is shown as mean cAMP concentration ± SD, n=3 independent infections.

To determine whether LamB was Dot/Icm-translocated, the adenylate cyclase (CyaA) reporter assay was used [137, 288]. Transformation of plasmids expressing

reporters CyaA-LamB or the positive control, CyaA-RalF, as fusion proteins was performed in WT *L. pneumophila* and the translocation-deficient mutant, *dotA*. The data showed that the CyaA-LamB reporter was not translocated by the Dot/Icm translocation system, as there was no significant difference between the secretion of CyaA-LamB by WT *L. pneumophila* or the *dotA* mutant (Student *t*-test, $p > 0.2$); whereas, the control, CyaA-RalF, was readily translocated into the host cells during infection with WT *L. pneumophila* but not the *dotA* mutant (Student *t*-test, $p < 0.01$) (Fig. 4-2).

The amylase activity of LamB

To confirm the putative enzymatic activity of LamB as an amylase, *in vitro* biochemical activity was determined by using the standard amylase activity colorimetric assay, quantifying the cleavage of ethylidene-pNP-G7 to *p*-nitrophenol, which can be measured at 405nm [329, 330]. Three highly conserved residues, D193, E227, and D296 were identified in the catalytic pocket of LamB and confirmed with domain alignment to other amylases (Fig. 4-1B, C). Constructs harboring native LamB or three LamB variants of single amino acid substitutions in the catalytic pocket were expressed in *E. coli* as GST fusion proteins, controlled by an IPTG inducible promotor. Expression of these proteins was confirmed by western blot (Fig. S4-4). With IPTG induction, amylase activity was highest for the wild type protein compared to uninduced (Student *t*-test, $p < 0.001$) (Fig. 4-3). The three catalytic mutants showed no amylase activity after inducing with IPTG. These data confirmed that LamB is indeed an amylase and that the identified catalytic pocket is essential for enzymatic activity.

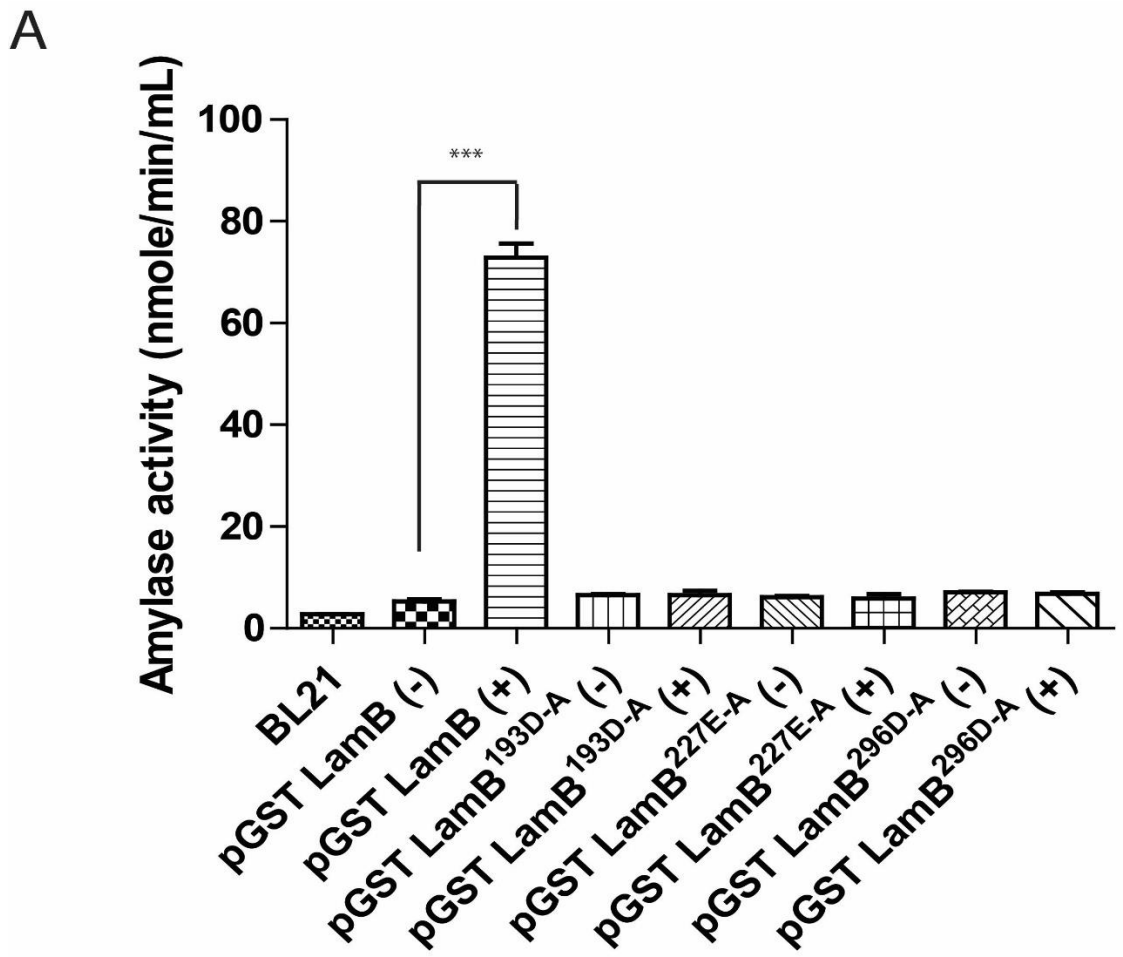

Figure 4-3. LamB is a functional amylase. A) Amylase activity of ITPG-inducible (+), GST-LamB fusions and catalytic site mutants, expressed in *E. coli,* was assessed by colormetric assay of cleavage of an artificial compound. Data are representative of three independent experiments represented by mean amylase activity, of triplicate sample, as measured by cleavage of ethylidene-pNP-G7 into *p*-nitrophenol, shown as mean amylase activity ± SD, n=3 independent cultures.

Requirement of LamB for growth in amoebae and hMDMs

In order to test the role of LamB in intracellular growth, a *lamB* null mutant was generated. Complementation was achieved by expression of *lamB* on a plasmid, *lamB*/C. The LamB variants with amino acid substitutions in the catalytic domain of LamB, D193A, E227A, and D296A were also introduced into the *lamB* null mutant (Fig. 4-1C). Infections of *Acanthamoeba polyphaga* or hMDMs were performed, as we previously described [166].

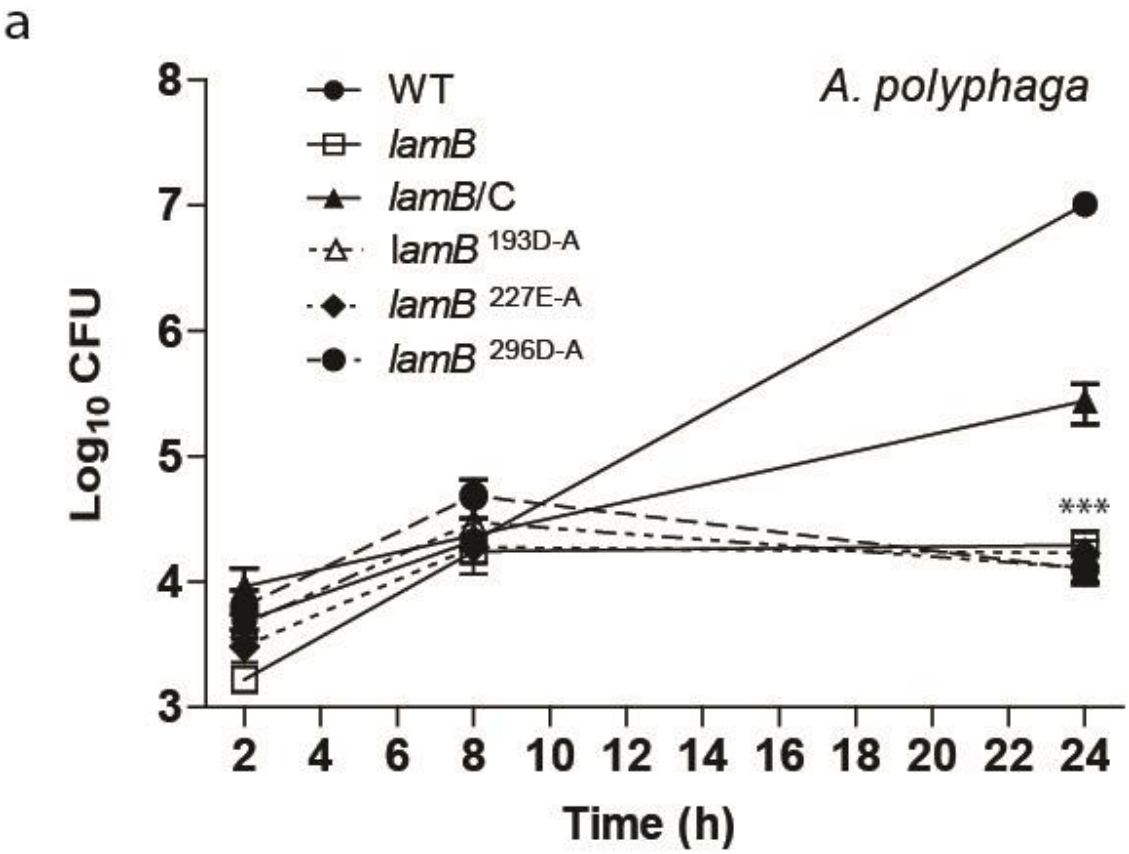

Figure 4-4. LamB is required for growth in amoebae. To determine intra-vacuolar replication of the WT strain, the *dotA* mutant, the *lamB* mutant, catalytic mutants (D193A, E227A, and D296A), and complemented *lamB* mutant (*lamB*/C), *A. polyphaga* were infected and number of CFUs were determined at 2, 8, and 24h post-infection. Data points represent (mean CFUs ± SD, n=3) and are representative of three independent experiments.

The *lamB* mutant was severely defective for intracellular growth in *A. polyphaga* and hMDMs (Fig 4-4, 4-5). At 24 h post-infection, there was a significant difference in the replication of the null mutant and the three catalytically inactive mutants compared to WT *L. pneumophila* (Two-way ANOVA, $p < 0.001$). The growth defect was partially restored to the mutant by in trans-complementation of the gene, which is likely due to loss of the plasmid. However, complementation of the null mutant with any of the three catalytic variants did not restore any growth to the *lamB* mutant in *A. polyphaga* or hMDMs (Fig. 4-4, 4-5). This defect is not attributed to a growth defect *in* vitro, as the *lamB* mutant grows just as well as the WT strain in broth (see Supplementary Fig. S4-5). These data show that LamB is necessary for intracellular replication of *L. pneumophila* in both hMDMs and *A. polyphaga*. Indeed, it is the amylase activity of LamB that contributes to its essential role in intracellular growth, indicating the requirement for degradation of polysaccharides by *L. pneumophila*. This is surprising, considering the minimal role of glucose in metabolism of *L. pneumophila*, and that it is mainly utilized during late stages of growth to synthesize the PHB storage compound.

To determine if generation of host glucose by LamB was necessary for intracellular replication, *A. polyphaga* was supplemented with exogenous glucose during infection (Fig. S4-6). Glucose supplementation did not rescue the *lamB* mutant for its defect in intracellularly replication nor did it alter the growth of the WT strain or the complemented mutant (Fig. S4-6).

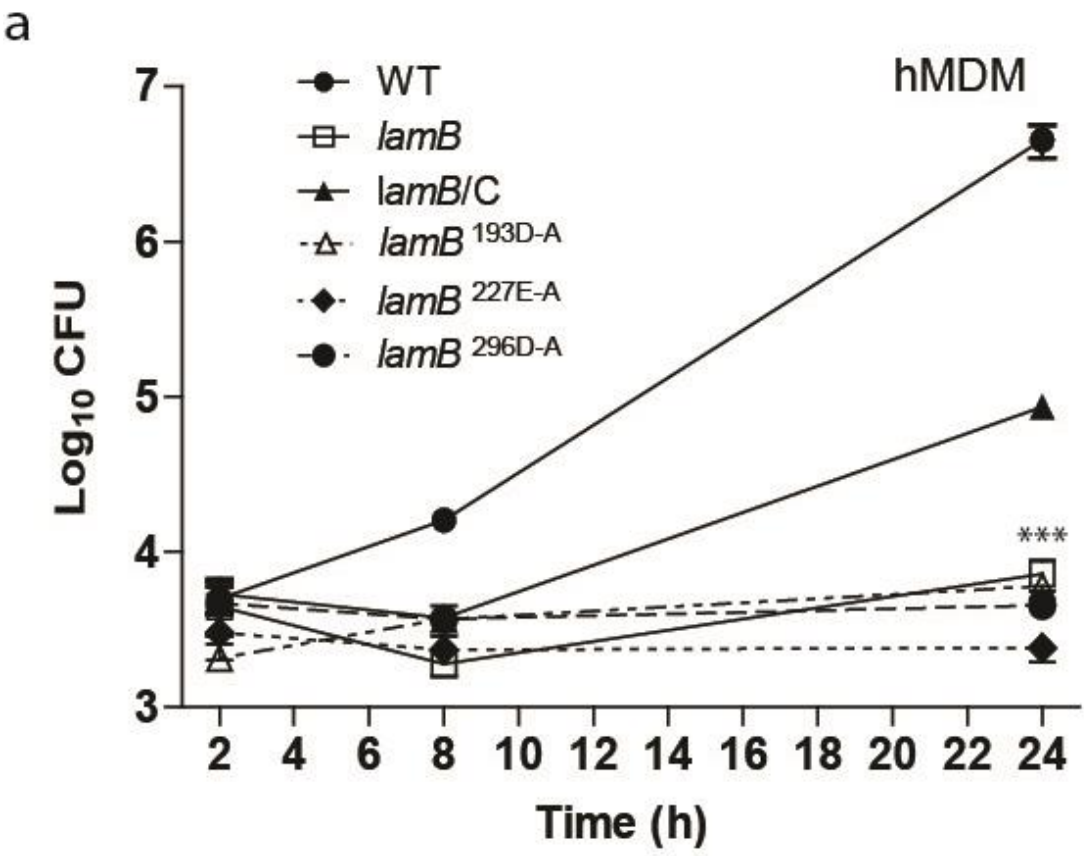

Figure 4-5. LamB is required for growth in hMDMs. To determine intra-vacuolar replication of the WT strain, the *dotA* mutant, the *lamB* mutant, catalytic mutants (D193A, E227A, and D296A), and complemented *lamB* mutant (*lamB/*C), hMDMs were infected and number of CFUs were determined at 2, 8, and 24h post-infection. Data points represent (mean CFUs ± SD, n=3) and are representative of three independent experiments.

Role of LamB in vivo

Given that *A. polyphaga* and hMDMs restrict the *lamB* mutant, we sought to determine the role of LamB in intrapulmonary growth in the mouse model. Intra-tracheal infection of A/J mice with WT *L. pneumophila,* the *lamB* mutant, or the complemented mutant (*lamB/C*) was performed, as we described previously [168]. Within 10 days, 50% of the mice infected with WT or the complemented mutant had died. However, 100% of mice infected with the *lamB* mutant survived for the 10 days of the study (Fig 4-6A).

Analysis of bacterial burden in the lungs of surviving mice, at 24, 48, and 72 hrs showed that the *lamB* mutant had significantly lower numbers of bacteria within the lungs, compared to the WT strain (Student *t*-test, $p < 0.05$) (Fig. 4-6B). The defective phenotype was completely recovered by complementation, indicating minimal loss of plasmid *in vivo* compared to *ex vivo* infection (Fig. 4-4, 4-5). Histopathology on pulmonary biopsies taken at 12 and 24 hrs post-infection, with wild type *L. pneumophila* showed severe inflammatory infiltrates of mononuclear cells (Fig. 4-6C, D). In contrast, following challenge with the *lamB* mutant, minimal inflammatory infiltration into the alveolar, bronchial, or peribronical spaces was observed (Fig. 4-6C, D).

The *lamB* mutant was less efficient in disseminating to the liver and spleen compared to the WT strain (Fig. 4-7A, B). At 48 hrs post-infection, there was a significantly lower amount of bacteria in the liver of mice infected with the *lamB* mutant compared to the WT strain (Student *t*-test, $p < 0.01$). Compared to the WT strain, fewer *lamB* mutants disseminated into the spleen at 48 hrs (Student *t*-test, $p < 0.05$) and 72 hrs (Student *t*-test, $p < 0.01$) post-infection compared to the WT strain. The reduced dissemination of the *lamB* mutant was completely restored upon complementation by *lamB*.

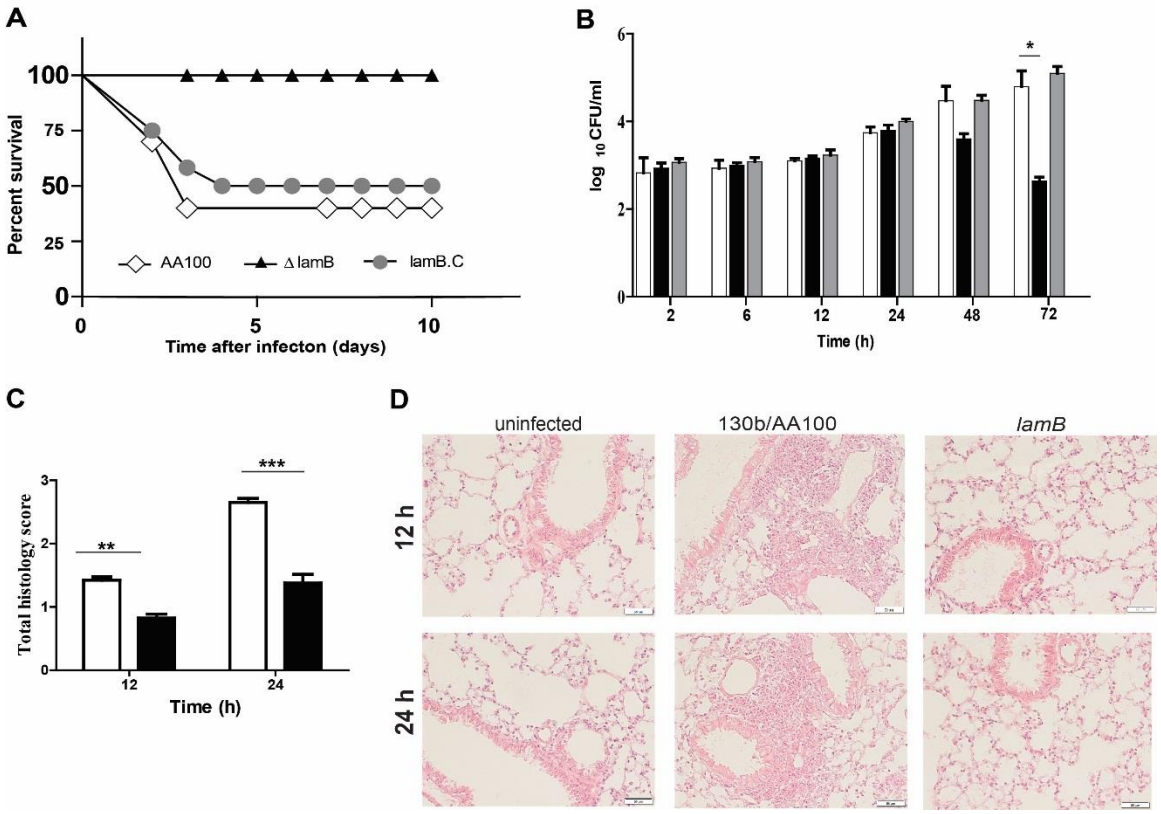

Figure 4-6. Role of LamB in virulence in A/J mice. Mice were infected intra-tracheally with 10^6 CFUs of WT (white), the *lamB* mutant (black), or the completed, *lamB*/C (grey). A) Survival of the mice over days and B) CFU organ burden in the lungs was assessed at the various time points. Pulmonary histopathology scores at 12 h (Student *t*-test, $p < 0.01$) and 24 h (Student *t*-test, $p < 0.005$) are shown in C) and representative images of uninfected, WT, and *lamB* are shown in D).

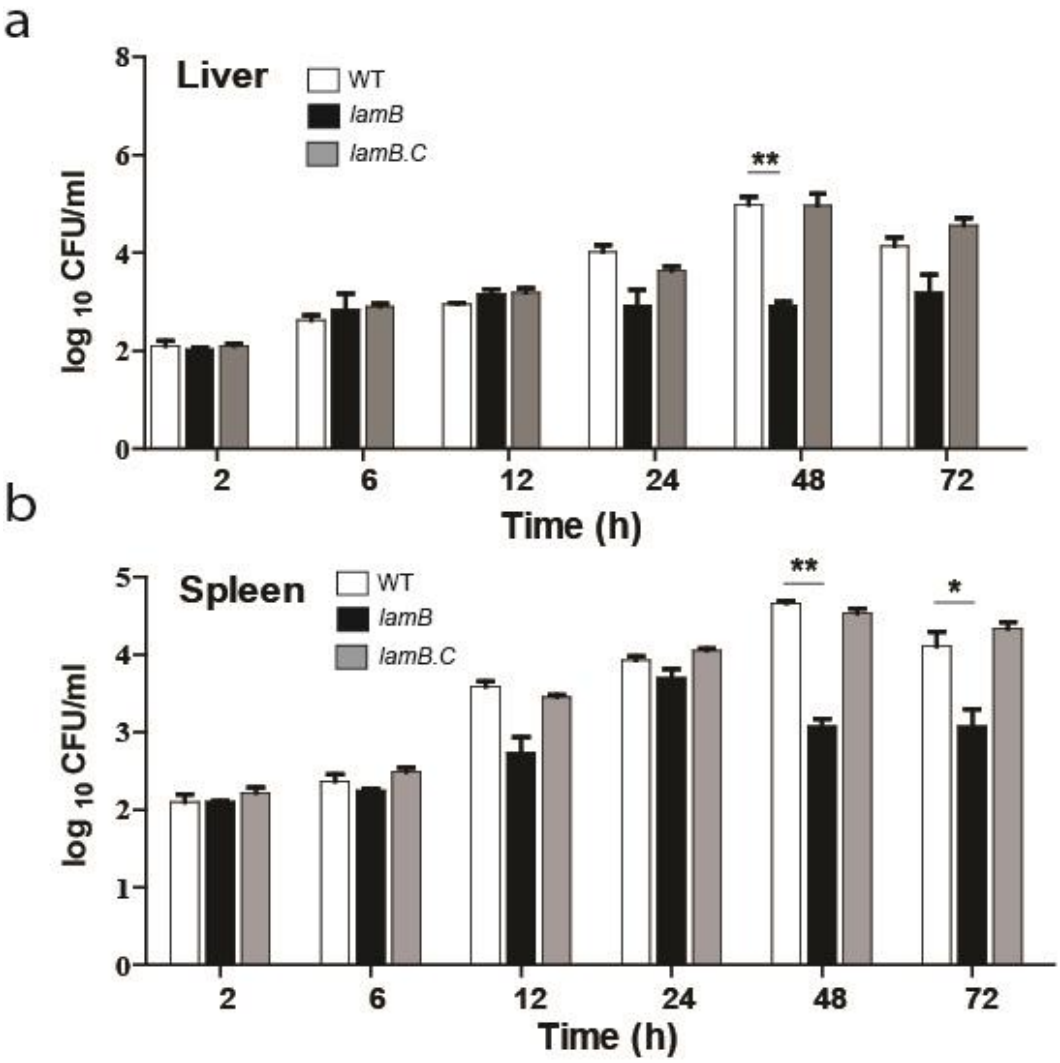

Figure 4-7. The role of LamB in dissemination *of L. pneumophila* in A/J mice. Mice were infected intra-tracheally with 10^6 CFUs of WT (white bars), the *lamB* mutant (black bars), or the completed, *lamB*/C (grey bars). Dissemination of the bacteria to the A) liver and B) spleen, as measured by CFU organ burden was assessed at the various time points.

DISCUSSION

A "bipartite" metabolism has been used to describe the nutritional needs and metabolic regulation of *L. pneumophila* [49, 100, 214, 222]. During early intracellular replication within human macrophages or amoebae, *L. pneumophila* relies on amino acids to generate carbon and energy from the TCA cycle [207, 211]. Once amino acid levels become low, the bacteria undergo growth phase transition, switching from the replicative phase to the transmissive phase [85, 187, 190, 331]. At this point, *L. pneumophila* increases uptake and utilization of glucose and converts it into the storage compound PHB [214, 220]. Experiments with ^{13}C-glucose demonstrated that glucose is used for *de novo* synthesis of amino acids and PHB during late stages of infection [175]. Additionally, labeling of glucose demonstrated a carbon flux from glucose to pyruvate via the Enter Doudoroff (ED) pathway but not the Pentose Phosphate Pathway (PPP) [175]. However, addition of excess exogenous glucose does not result in increased growth of the organism during any stage [220]. Therefore, generation of excess glucose in the host, as a source of carbon and energy, through degradation of polysaccharides by LamB is unlikely to support growth. Thus, it is surprising to identify a major role for LamB in intracellular growth, since *L. pneumophila* mainly utilizes amino acids for growth [332]. We speculate LamB is involved in processes other than nutritional virulence [176, 332].

Uptake of glucose is increased by *L. pneumophila* during post-exponential growth, most notably for the generation of the storage molecule, PHB [175, 218, 220], and nutrient importers are important for intracellular growth of *L. pneumophila* [333, 334]. Having large stores of PHB allows the organism to persist outside of the host for extended periods of time [226]. Transcriptomic studies in the human macrophage cell

line THP-1 have demonstrated that expression of *lamB* is highest early in infection (8 hrs) rather than later (14 hrs), opposite of when the organism starts increasing consumption of glucose[177, 220]. Faucher *et al*. also classified this gene as "highly induced in cells" [177]. LamB may be involved in increasing availability of glucose in the host ahead of when the organism prepares to utilize it during the late stages of infection. However, our data excludes that possibility. Increasing the availability of glucose with an amylase could allow *L. pneumophila* to generate more PHB, promoting long-term survival. It is possible that failure to store sufficient amounts of PHB by the *lamB* mutant results in an early defect in intracellular growth, due to the lack of a rapid influx of acetyl-CoA from reduced levels of PHB. Alternatively, since amylases are known to act on the glycosylation of proteins, LamB may be acting on the post-translational modification of host proteins to control processes important for replication, independent of nutrition or PHB storage [335]. This could allow the bacterium to evade some aspect of the innate host immune response necessary for successful intracellular replication. Targets of deglycosylation could be present in the host cytosol or within the LCV lumen from ER-to-Golgi-derived vesicles depositing glycosylated proteins in the lumen or on the luminal side of the LCV membrane. Future studies are aimed at identification of target(s) of LamB and how they contribute to infection. Considering LamB is unique to *L. pneumophila* and its loss causes complete defect in intracellular growth in macrophages and amoebae, and attenuation *in vivo*, it may contribute to the enhanced virulence of *L. pneumophila* and its prevalence as a disease-causing species compared to other *Legionella* species.

Bioinformatical analysis indicates that LamB does not contain a T2SS secretion signal, but it does however contain putative T4SS translocation signals [125, 126, 327]. However, through the CyaA reporter assay our data show that LamB is not translocated by the Dot/Icm. Previous reports have shown effectors that were not translocated as CyaA reporter assay, were actually translocated T4SS effectors [125, 127]. This reporter could interfere with the translocation of LamB, like seen with other effectors [127]. Predicted strength of the translocation signal is not a definitive answer to whether a protein is translocated, low-scoring predicted effectors have been shown to be translocated by the Dot/Icm System and high-scoring proteins have been shown to not be translocated [125]. Lifshitz *et al.* identified LamB to be a high-scoring putative effector and in the same study tested 10 new high-scoring putative effectors, of which three were confirmed to not be translocated by the Dot/Icm system using the CyaA reporter assay, but LamB was not tested [125]. Despite being a high-scoring putative effector, LamB may not be translocated, as observed in our CyaA reporter assay. Trans-rescue of the *lamB* mutant through expression of *lamB* in the host, could help determine if function of LamB in the host cytosol is what is required for intracellular replication of *L. pneumophila.* Loss of *lamB* does not affect *L. pneumophila*'s ability to grow *in vitro*, supporting the idea that LamB is likely secreted into the host cytosol or into the lumen of the LCV, but the mechanism remains to be determined.

In summary, we report an amylase essential for intracellular proliferation of *L. pneumophila* within the two evolutionarily distant hosts, human macrophages and amoebae. Given its uniqueness to *L. pneumophila*, LamB serves as an interesting enzyme

that may contribute to the prevalence and virulence of *L. pneumophila* compared to other *Legionella* species.

Materials and Methods

Strains and cell lines

L. pneumophila strain AA100/130b (ATCC BAA-74) and the *dotA* T4SS-deficient mutant, were grown on Buffered Charcoal Yeast Extract (BCYE) agar, as we previously described [166]. To generate the isogenic mutant *lamB* (*lpg2528*), 2 kb flanking DNA on either side of *lamB*, was amplified using PCR with primers listed in Table S4-1. The resulting amplicon was cloned into the shuttle vector, pBCSk+, to generate pBCSK+*lamB*KO. To delete the entire gene of *lamB*, inverse PCR was employed using the primers listed in Table S4-1, resulting in pBCSK+*lamB*KO2. The kanamycin resistance cassette from the Ez-Tn5 transposon was amplified using primers listed in Table S4-1. The resulting PCR product was subcloned in pBSCK+*lamB*KO2 between the *lamB* flanking regions using standard molecular procedures, resulting in pBCSK+*lamB*KO3. This plasmid was introduced into *L. pneumophila* AA100/130b via natural transformation, as we previously described [161]. Natural transformants were recovered by plating on BCYE agar supplemented with 50 μg/ml kanamycin. To complement the *lamB* mutant, PCR was used to amplify the *lamB* gene and its upstream promotor region, using primers listed in Table S4-1, and subcloned into pBCSK+, generating pBCSK+*lamB*/C. Complement mutants of *lamB* with mutations in the catalytic pocket were made by substituting the amino acid for alanine, to generate pBCSK+lamBD193A, pBCSK+lamBD296A, and pBCSK+lamBE227A, using primers listed in Table S4-1. These plasmid was introduced into the *lamA* mutant, via electroporation, as previously described [296]. Complemented *lamB* mutants were selected on BCYE plates supplemented with 5 μg/ml chloramphenicol, resulting in the complemented strains, *lamB*/C, *lamB*/D193A, *lamB*/D296A, and *lamB*/E227A.

Intracellular replication

For infection of cell monolayers, *L. pneumophila* strains were grown in BYE broth with appropriate antibiotic selection, at 37°C with shaking, to post-exponential phase (OD_{550nm} 2.1-2.2). *A. polyphaga* was cultured in PYG media at 22°C, experiments were performed in PY media at 35°C, as previously described [166]. Glucose supplementation experiments were done in presence of 100mM glucose in the media. Human monocyte-derived macrophages (hMDMs) were isolated from healthy donors and cultured in RPMI 1640, supplemented with 10% fetal bovine serum, as previously described [97, 166]. All methods were approved and carried out in accordance to the University of Louisville Institutional Review Board guidelines and blood donors gave informed consent as required by the University of Louisville Institutional Review Board (IRB # 04.0358).

The wild type strain; the isogenic mutants, *dotA* and *lamB*; and complements *lamB*/C, *lamB*/D193A, *lamB*/E227A, and *lamB*/D296A were grown to post-exponential phase in BYE broth at 37°C with shaking, prior to infection and used to infect hMDMs and *A. polyphaga,* as previously described [97, 166]. A total of 1 x 10^5 host cells were plated in 96 well plates and infected with *L. pneumophila* at an MOI of 10. Plates were centrifuged at 200 x g (5 mins), to synchronize infection. After 1 h, cells were treated for 1h with gentamicin to kill extracellular bacteria, as previously described [97, 166]. Over a 24h time course, host cells were lysed with sterile water (hMDMs) or 0.02% v/v Triton X-100 (*A. polyphaga*). *L. pneumophila* CFUs were determined by plating serial dilutions onto BCYE agar.

Bioinformatics analysis of LamB

Protein domain analysis was performed using NCBI's Search for Conserved Domains (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) . Phylogenetic analysis was determined using amino acid sequences of LamB, with the Phyologeny.fr platform. Branch length was determined by aLRT [336, 337]. Predicted structures were generated via I-TASSER (https://zhanglab.ccmb.med.umich.edu/I-TASSER/) [320]. Structures generated from I-TASSER were aligned using TM-align to generate a TM-score of structural similarity (https://zhanglab.ccmb.med.umich.edu/TM-align/).

Translocation Assay

To assess translocation of LamB by *L. pneumophila* T4SS, during infection of host cells, an adenylate cyclase fusion [288] was generated using standard biology techniques with primers listed in Table S4-1. A total of 1 X 10^6 hMDMs were infected with wild type or *dotA* mutant *L. pneumophila* harboring plasmids expressing various adenylate cyclase fusions at an MOI of 20 for 1h, as previously described [97, 288]. Following infection, the cell monolayers were lysed and processed to assess cAMP concentration by ELISA using the Direct cAMP ELISA kit (Enzo) according to the manufacturer's protocol and measure with a Synergy H1 microplate reader (BioTek).

Amylase activity

To determine if LamB is a functional amylase, the *lamB* gene was cloned into the IPTG-inducible GST-fusion expression vector pGEX-6p-1 (Amersham) and expressed in *E. coli* BL21 using primers listed in Table S4-1. Additionally, residues within the predicted catalytic pocket were substituted to alanine using inverse PCR using primers listed in Table S4-1. *E. coli* cultures (5 ml) harboring either the empty vector, *lamB*, or the various catalytic inactive mutants were grown in LB broth at 37°C with shaking until

the OD_{600nm} reached 0.8. The cultures were spilt and one half was induced with 0.1 mM IPTG for 2.5h at room temperature. One ml of each culture was pelleted by centrifugation and subjected to lysis with 0.5 ml buffer (0.1% v/v Triton X-100, 150 nM NaCl, 10 mM Tris pH7.5), containing protease inhibitors. Insoluble material was pelleted by centrifugation (16000 x g, 10 min, 4°C) and the resulting supernatant was retained. Expression of fusion proteins was similar in all cultures (see Supplementary Fig. S4-3 online). To measure amylase activity, 25 µl of supernatant was analysed using an Amylase Assay Kit (Sigma), following the manufacturer's instructions. This kit utilizes an artificial substrate, ethylidene-pNP-G7, which when cleaved by an amylase generates a colorimetric product detectable at 405nm.

Mouse model

For testing the virulence of the *lamB* mutant, specific pathogen-free, 6-8 weeks old A/J mice were used, as previously described[97, 168]. Groups of 3 A/J mice, for each time point, were infected intratracheally with 1 x 10^6 CFUs. At 2, 12, 24, 48, and 72 h after infection mice were humanely sacrificed and lungs, liver, and spleen were harvested and homogenized in sterile saline (5ml) followed by cell lysis in distilled water. To determine CFUs, serial 10-fold dilutions were plated on BCYE agar and incubated at 37°C. For histopathology, lungs of infected mice were fixed in 10% neutral formalin and embedded in paraffin. Serial 5 µm sections were cut, stained with haematoxylin and eosin (H&E), for light microscopy analysis. Twenty random high-powered fields (HPFs) were assessed to grade inflammation severity including alveolar and bronchial damage, as well as percentage of parenchyma involved. The histology assessment included the number of the mononuclear cells and percent of parenchyma involved by using modification of double-blind scoring method at a magnification of

40x, as we described previously [338]. The inflammation process was graded normal (score of 0), when there were 0-19 monocular cells infiltrates per HPF with no alveolar and bronchial involvement; mild (score of 1), for 20 to 49 cells per HPF, including mild damage of alveolar and bronchial regions; moderate (score of 2), for 50 to 99 cells per HPF with moderate alveolar and bronchial inflammation; or severe (score of 3), for 100 to 200 mononuclear cells per HPF with severe effacement of alveolar and bronchial regions. The murine lung section was examined in sagittal direction and percent of parenchyma involved was scored as 0 when no area was compromised. The involvement of the parenchyma was scored as 1 when up to 25% of the total area was occupied by inflammatory exudate, or scored as 2 when 26 to 50% of parenchyma area was occupied with inflammatory cells, and 3 if comprised of more than 51% of the total area. The total histology score was calculated as an average of individual criteria scores. Uninfected tissue was used as a baseline score. All the experimental procedures were in accordance with National guidelines and were approved by the Institutional Animal Care and Use committee (IACUC) at Faculty of Medicine, University of Rijeka.

Data availability

All data generated or analysed during this study are included in this published article (and its Supplementary Information files).

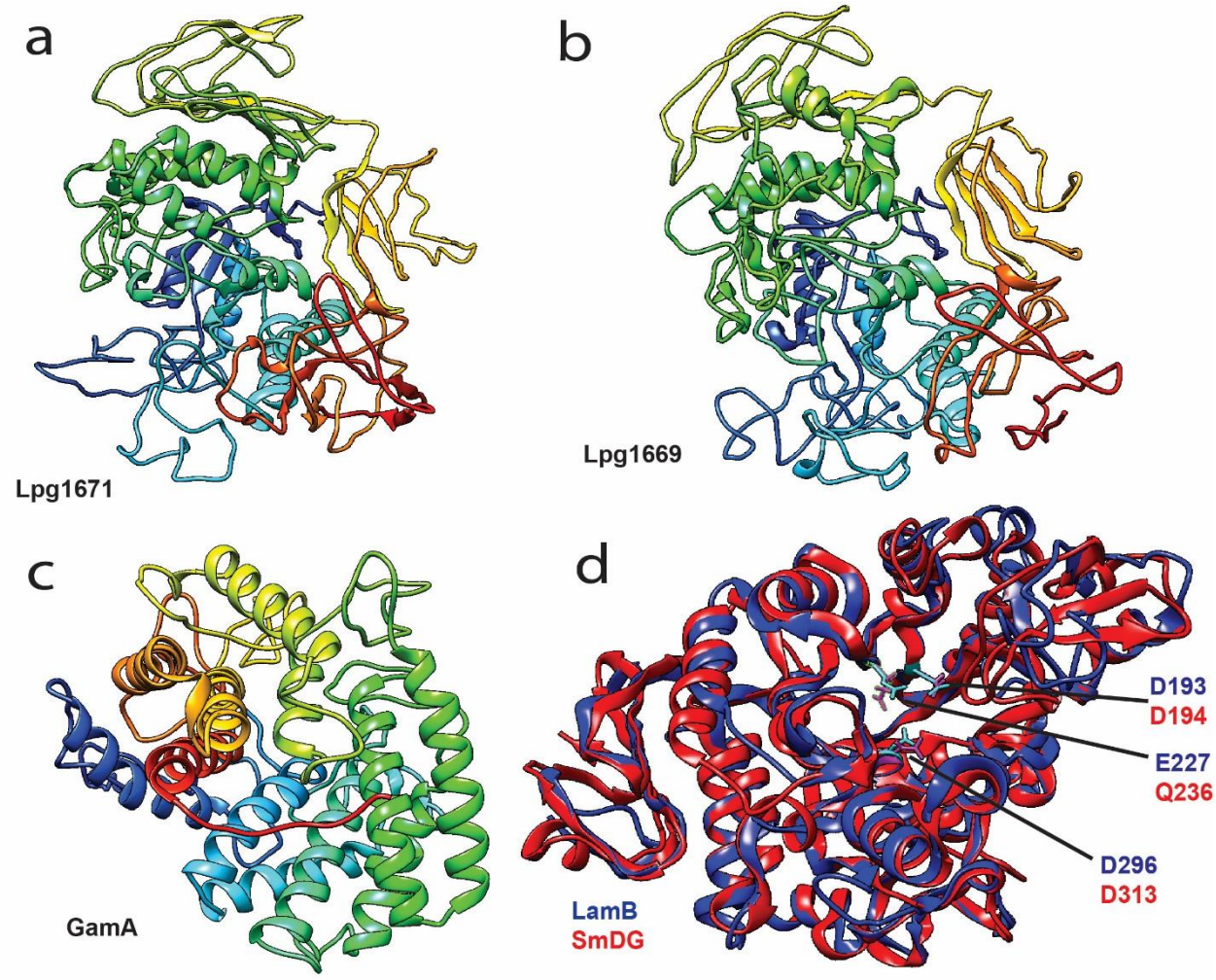

Figure S4-1. Putative structure of amylases in *L. pneumophila*. Two additional putative amylases, A) Lpg1672 and B) Lpg1669, were identified in *L. pneumophila* by domain sequence homology. C) The *L. pneumophila* GamA has been previously described as an amylase and substrate of type-II secretion. D) The predicted structure of LamB is similar to the crystalized structure of *Streptococcus mutans* dextran glucosidase, which share a conserved alpha amylase catalytic domain (NCBI domain: cl07893) and catalytic residues that are highlighted.

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Legionella pneumophila          ---------------------------MSPRICCYNQYPGLYQDFDAMREDLQRIQKMGF  33
Insolitispirillum peregrinum    ----------------------MSEAVGLRAPRIYNLFPPLTGSIPDWSRHILRVRDMGF  38
Rhodospirillum centenum         MSLTAQTRR--ESPGNARPTPATTTRPAGAGPRIYNLFPLLAGTVEDWKRHLPRIAAMGF  58
Methylobacterium variabile      MNVPRTSTKPAKRTREVTPLP-ATLAPLAAGPRIYNLFPLLVGRVSAWTAELPRIAALGF  59
Methylobacterium aquaticum      MNVPRTSTKPAKRTREVTPLP-ATLAPLAAGPRIYNLFPLLVGRVSAWTAELPRIAELGF  59

L.egionella pneumophila         KQVWVNPFYTPCQYNPVPNMANRIHSPYAMQDESIYPRYAKNDKSVQQYTAKANELGLMP  93
Insolitispirillum peregrinum    NWIYINPFHYPGFSGS----LYAVKDYYRLNPMLRGPAYASDDDLLRGFIESAGSHGLSV  94
Rhodospirillum centenum         DWVFLNPVHYPGFSGS----LYAVKDPYRLNELFRGDTDADTDTLLRDFFAEARRHGLTV 114
Methylobacterium variabile      DWVYLNPFHQTGGSRS----LYAVADPDRLDERLRDLDGTPDDEQIRRFCAAAAEHGLKI 115
Methylobacterium aquaticum      DWVYLNPFHQAGGSRS----LYAVADTDRLDERLRDLDGTPDDEQIRRFCAAAAGHGLKI 115

Legionella pneumophila          IFDLVARHLAVDHRFVNGDKFFLEKYNIDTKKWFKRHPNGNLVMHNMDENYNPLANNPWS 153
Insolitispirillum peregrinum    IMDLVINHTSRDSTLVH-----------EHPDWFQRDPDGNLVSPCASHPDDPDKRTVWG 143
Rhodospirillum centenum         MLDLVVNHTAKDALLAE-----------QHPDWYVRNRDGSLRSPGAVNPQDPTDVTVWG 163
Methylobacterium variabile      MTDLVVNHAANDGRLAH-----------ERPDFFVRDASGAPVSPHAVDPDDPSKITVWG 164
Methylobacterium aquaticum      MTDLVVNHAANDGRLAH-----------ERPDLFVRDASGAPVSPHAVDPDDPSKITVWG 164

Legionella pneumophila          DVATFNYDDPVICEQIIEYYWKPFIERNIEHLGFQGIRIDAPAMVNNKVLSRLTEIAKEA 213
Insolitispirillum peregrinum    DLAALDYHPRPQRAALVGY-FCSVV-RHYARLGMRAFRCDAAHRIPADVWLAIMAAAHQ- 200
Rhodospirillum centenum         DLAELDYSRQESREGLIRY-WADWV-RHQVGLGAAGFRCDAAYQVPAGVWRALIEAARG- 220
Methylobacterium variabile      DLAEFDYVAPGSRTALTEF-WNGYV-ERLQRLGVAGFRCDAAYKVPPDVWRSLIGAAKD- 221
Methylobacterium aquaticum      DLAEFDYVEAGPRTALTEL-WDGYI-ARLQALGVAGFRCDAAYKVPPDVWRSLIGAAKQ- 221

Legionella pneumophila           CQHKFQRDPLIIAETIGRGYMEENLALKGIVTHTMNSVFWMPGPEGGYGYS---YNLWQQ 270
Insolitispirillum peregrinum    ----ENPDILFFAETLGCTPDEVEAL-----------------RPARFDYLFNSSKWWDG 239
Rhodospirillum centenum         ----TDPEVLFFAETLGCTPEQVASL-----------------ADAGFDYLFNSAKWWDF 259
Methylobacterium variabile      ----RDHAALFAAETLGCTFEEARAT-----------------AEAGFDYLFNSFAWWDL 260
Methylobacterium aquaticum      ----RDHAALFAAETLGCTFEEARAT-----------------AEAGFDYLFNSFAWWDL 260

Legionella pneumophila          DDNWFTQNKGLLQQVGPTIGFPGSHDEPRYIQQLVEKGIHDDKLLAKRMREKLAVSAFCS 330
Insolitispirillum peregrinum    EAPWLLDHYEQFRDLAPSVSFPESHDTARLAAETEASGVSSVKLIAEVQRYRYLFSAMFS 299
Rhodospirillum centenum         RSDWFLEQYELYRSIAPTIAFPESHDTERLAAETGTGD---PRHLAALAKMRYLFSAAVS 316
Methylobacterium variabile      KAPWALEQYERLRILAPSIAFPENHDMPRLAASLPGGR----EAVARELKARYALAAFFS 316
Methylobacterium aquaticum      KAPWALEQYERLRILAPSIAFPENHDMPRLAASLEGGP----EAVAAELKSRYALAAFFS 316

Legionella pneumophila          DGGWILQYGDEYGATKPVNVFDPTPVEYHQQNHLRRFDLSDYISEINTTLAQLPNPHFPE 390
Insolitispirillum peregrinum    S-AVMMPMGYEFGFTRPMNVVTSRPEQWE----NARFDISPYVAEVNRLKASLSVLNREG 354
Rhodospirillum centenum         S-GVMLPAGFEYGFTRRLNVVETRPQDWQAEAAEPRLDLSGFIADVNAMKASVPALNREG 375
Methylobacterium variabile      A-GVLMPIGYEWGYAQPLHVVETTPESRE---NETGIDVSGYIAAINRLRADLPAANVEG 372
Methylobacterium aquaticum      A-GVLMPIGYEWGYAKPLHVVDTTPESRE---HHTGIDISGFVAAVNRLRADLPAANVEG 372

Legionella pneumophila          WAQRVFLPNHPDLVTFIVHQGWGYTGTSFVIATNTDPSQQIQLTEKELGEIMLANGRNNT 450
Insolitispirillum peregrinum    PQQRLASPSGAYALLRRNNHG----AEWVLTVINPPHNGTACLALD---RDPIGRRAA-- 405
Rhodospirillum centenum         PLRRITGAHDPVVALLRSD---PASGESALLLLNPETDGPRSLDPA---ILLA--AGDGG 427
Methylobacterium variabile      AQWRLSAPDAPYVALIRIDTGHPGSAENAVIVLYNPTDAMVAVDPA---PLIAGTGGEFG 429
Methylobacterium aquaticum      AQWRLSAPDAAYVALIRIDTGHTGSAENAVIVLHNPTDAPVAVDPA---PLIAGTGGEFG 429
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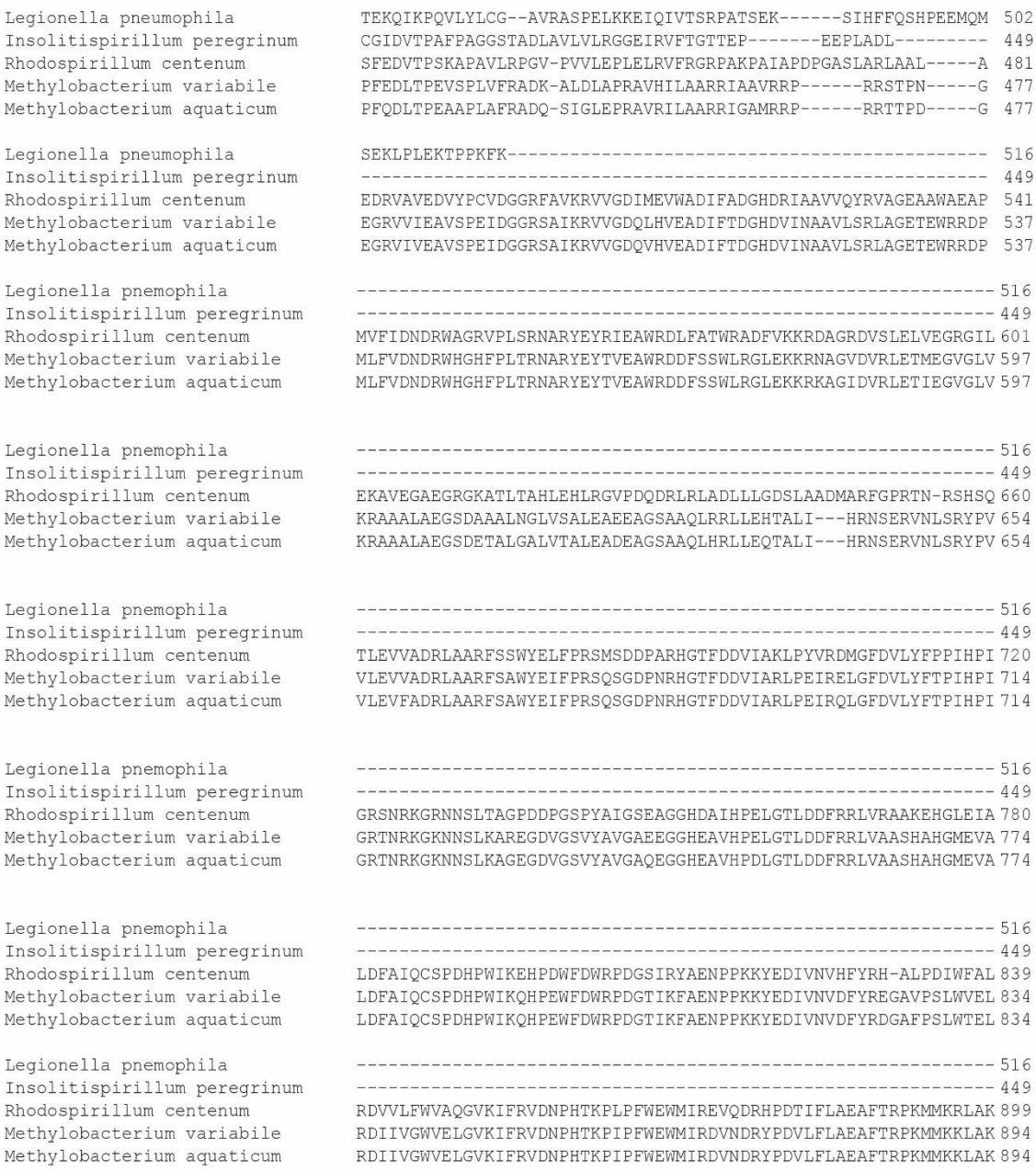
Figure S4-2. LamB-like proteins in intra-amoebal and environmental pathogens. Amino acid sequence homology of LamB shows alignment to other intra-amoebal and aquatic organisms like; *Insolitispirillum peregrinum*, isolated from a pond [339, 340]; *Rhodospirillum centenum*, isolated from the edge of a thermal spring [341]; *Methylobacterium variabile,* isolated from a drinking water [342]; and *Methylobacterium aquaticum*, also isolated from drinking water and capable of surviving and lysing amoebae [343, 344], but not other known pathogens.



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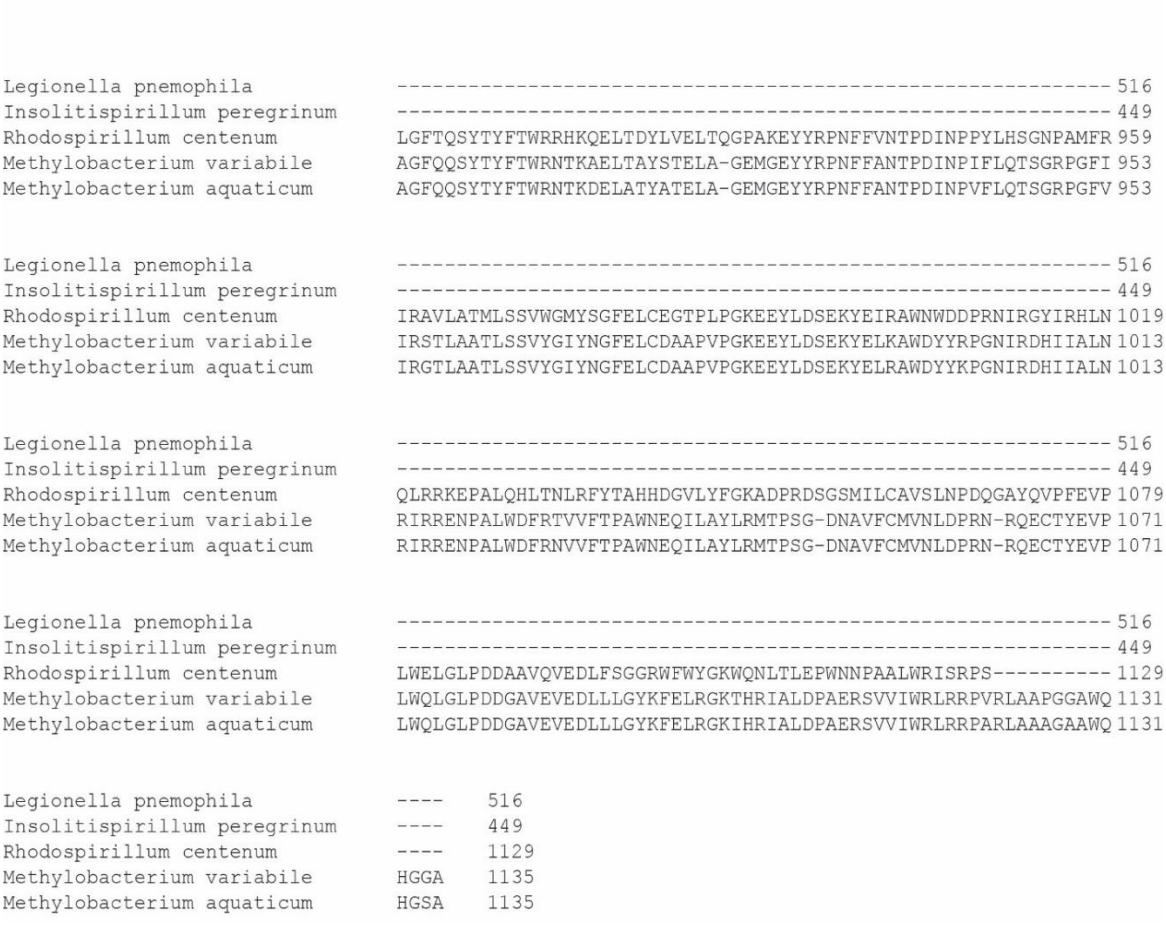

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Figure S4-3. The C terminal 100 amino acids of LamB. Bi-residues identified to be heavily enriched in the last 100 amino acids of T4SS effectors are highlighted in red for multiple strains of *L. pneumophila* LamB and *L. steigerwaltii* LamB. Seventeen bi-residues are found in LamB of the AA100 strain.

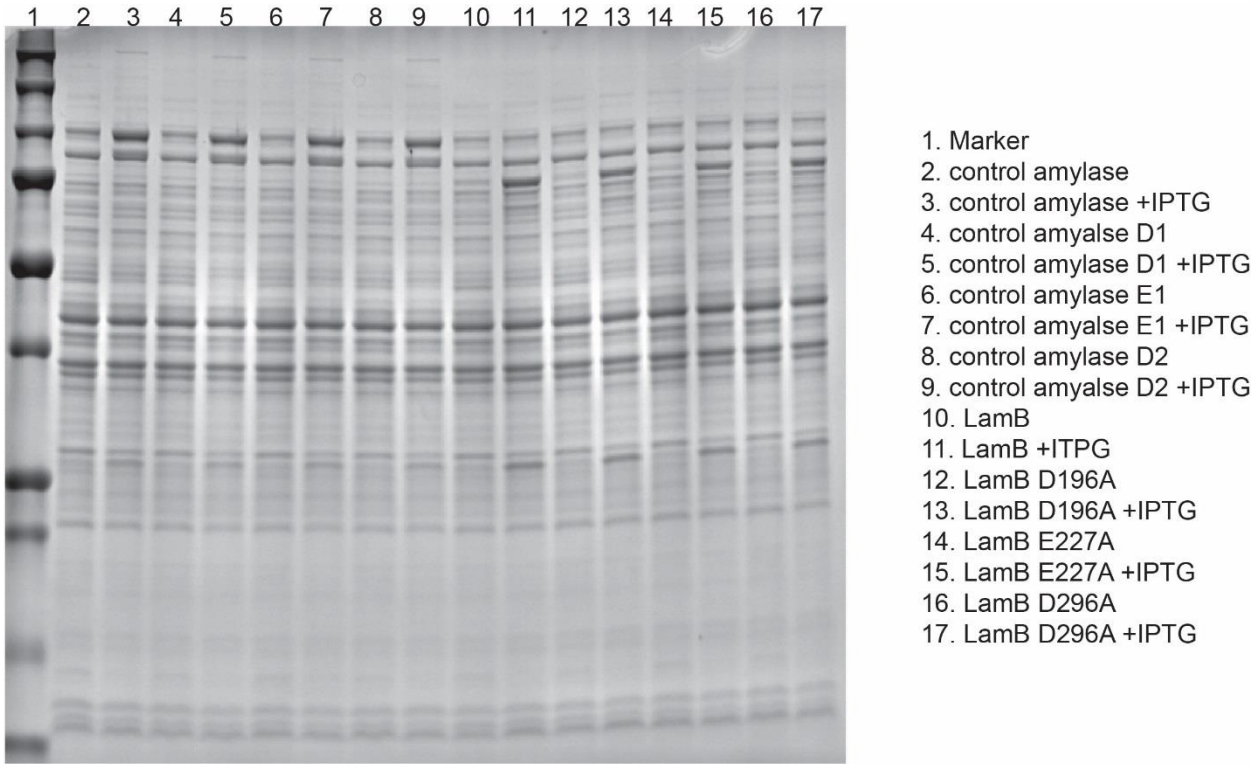

Figure S4-4. Induction of GST-LamB fusions in *E. coli.* *E. coli* BL12 harboring GST-LamB, GST-LamB D193A, GST-LamB E227E, or GST-LamB D296 constructs were grown to an OD_{600} 0.8 in LB broth before induction with 0.1mM IPTG at 37°C for 2.5h. Coomassie stain of uninduced and induced cultures shown.

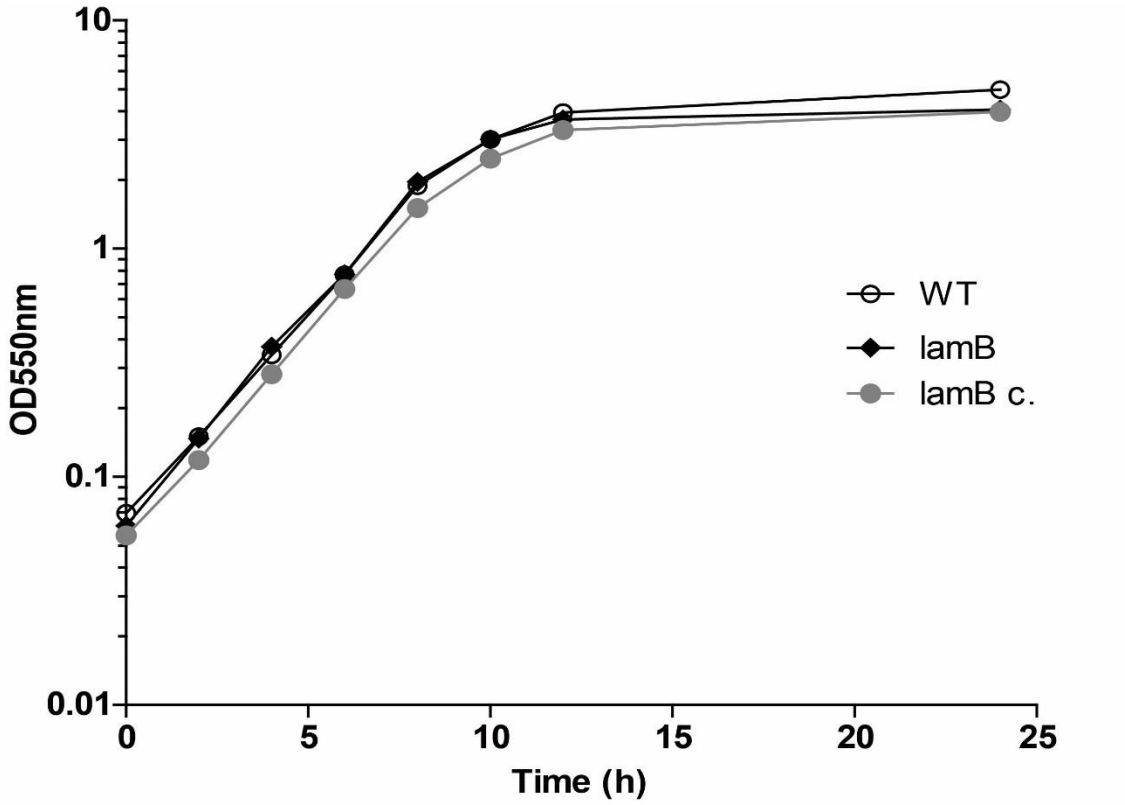

Figure S4-5. Growth of mutants *in vitro.* Overnight cultures of WT, Δ*lamB*, or catalytic mutants in BYE broth were grown overnight at 37°C then diluted to OD_{550} 0.05 and grown at 37°C for 24h. Growth rates were determined by measuring optical density at 550nm every two hours, for 12, then again at 24h post-inoculation. Data representative of three independent experiments.

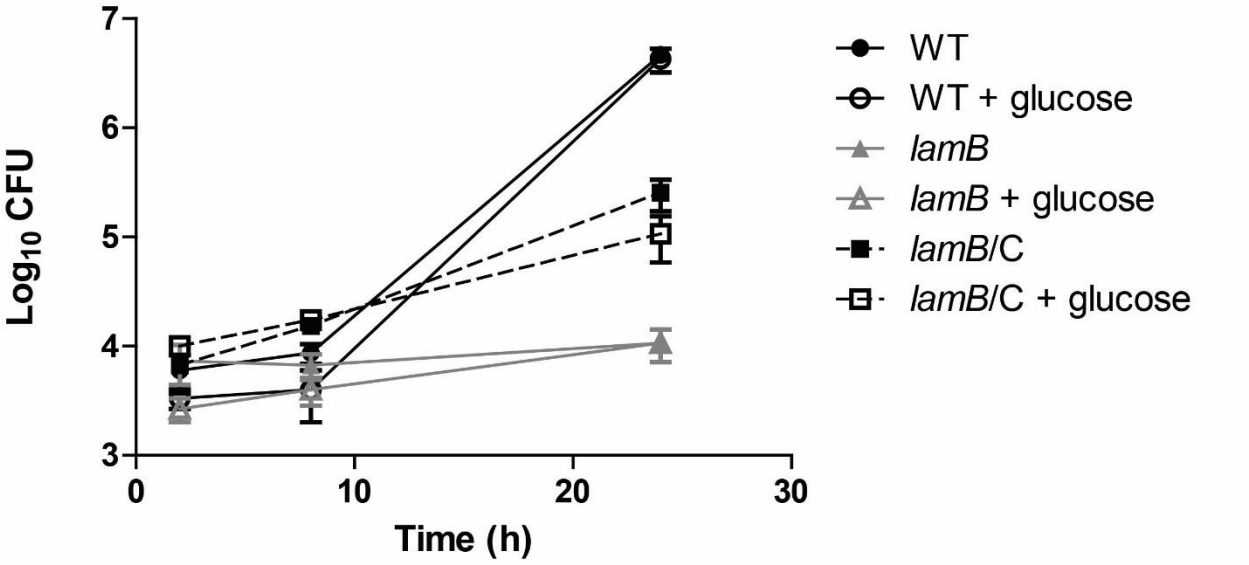

Figure S4-6. Supplementation of *A. polyphaga* with glucose does not compensate for the loss of *lamB*. Intra-vacuolar replication of the WT strain, the *lamB* mutant, and complemented *lamB* mutant (*lamB*/C) in *A. polyphaga* was determined upon glucose supplementation. The number of CFUs was determined at 2, 8, and 24h post-infection. Data points represent (mean CFUs ± SD, n=3) and are representative of two independent experiments.

Table S4-1. Primers used in this study

Primers	Sequence
lamB-KO F	GGATCCTAATGTCTTTATTACTTCAC
lamB-KO R	GTCGACTTTTCGTATCAAATAAACTA
lamB inverse F	TTTACCTGCAGGATAATAATATTTACCGGCT
lamB inverse R	TTTATTGATGCAGATCGCTGACTTCTCAATTTC
Pcr confirm KO F	GAACAGAAATTGAGAAGTCAGC
PCR confirm KO R	TATAAATGCAATATAGCCGGTAAATATTA
lamB COMP F	CTCGAGAGTAAATATGATGCCCATAA
lamB COMP R	GGATCCTTATTTAAATTTAGGTGGTGTT
Kan F, R	/5Phos/CTGTCTCTTATACACATCTCAA
lamB CYA F	GGATCCTTATGTCTCCACGGATTTGTTGC
lamB CYA R	AAGCTTTTATTTAAATTTAGGTGGTG
lamB-GST fusion (pGEX) F	GGATCCATGTCTCCACGGATTTGTTG
lamB-GST fusion (pGEX) R	GTCGACTTATTTAAATTTAGGTGGTG
lamB D193A F	/5Phos/AGCAATACGTATTCCCTG
lamB D193A R	/5Phos/GCCCCAGCCATGGTAAAT
lamB E227A F	/5Phos/TGCTGCAATAATAAGTGG
lamB E227A R	/5Phos/ACCATAGGCAGAGGTTAT
lamB D296A F	/5Phos/AGCATGAGATCCAGGAAA
lamB D296A R	/5Phos/GAGCCACGCTATATTCAG

CHAPTER 5:

CONCLUSIONS AND FUTURE DIRECTIONS

Growth within the vacuole allows for certain protections from the host and provides a replicative niche. However, that means that nutrients are out of reach in the cytosol [75]. In an effort to understand nutritional virulence in *L. pneumophila,* we sought to characterize how amino acids are acquired from the host cytosol, across the LCV membrane [176, 345]. This has been an open question in *L. pneumophila* biology. SLCs are the most likely candidate to be the transporters of metabolites across the LCV membrane as they have appeared in the proteome of many LCV mass-spectrometry studies and are the main metabolite transporters in eukaryotic cells [227-229, 231].

Given our interest in nutritional virulence, we sought to fill this important gap by examining SLC colocalization to the LCV membrane. Surprisingly, we could not confirm the presence of any amino acid-transporting SLCs. This does not rule out SLCs as potential transporters of amino acids across the LCV. Individual SLCs may not be present in large enough quantities on the LCV to be detect by confocal microscopy. Given that many SLCs for the same substrates exist, it is likely that families of transporters, rather just individual transporters are responsible for amino acid transport [235]. This indeed would make detection difficult when limited to detecting a single family member at time. If broad antibodies that detected all members within an SLC family existed, we may be able to detect LCV-colocalization. Alternatively, these transporters may function well enough to provide the bacterium with the nutrient it needs while still being under the detection threshold by confocal microscopy. New technologies in confocal microscopy,

such as an avalanche photodiode, which can reach single molecule detection, could allow us to overcome our current threshold of detection limitation [346].

Knock-down of individual SLC transporters poses a problem for the host cell and may not indicate that the transporter is required for transporter of amino acids across the LCV [347]. Functional redundancy of SLCs means that loss of one transporter would not prevent the import of any single amino acid across the LCV, unless only few and specific SLC transporters were utilized on the LCV. Wieland *et al.* showed that SLC1a5 is required for *L. pneumophila* intracellular replication by siRNA silencing of SLC1a5 [247]. Decreased amino acid uptake by the host cell from the extracellular milieu may have been responsible for the failure of *L. pneumophila* to replicate under SLC1a5-knockdown conditions [247]. Additionally, it was not confirmed that knockdown did not alter the downstream signaling to mTOR by the SLC1a5/SLC3a2/SLC7a5/SLC16a1 complex, which has been shown to occur during knockdown of SLC1a5 [247, 348]. Additionally, treatment with the inhibitor BCH affects the whole complex, not just SLC1a5, which would alter mTOR signaling, likely preventing *L. pneumophila* replication due to the initiation of host autophagy rather than the loss of SLC1a5 [247].

Understanding how SLCs are recruited to the LCV membrane may help elucidate which transporters are important for intracellular replication. SLCs are ubiquitous on the plasma membrane, since the LCV is formed from a plasma-membrane derived vacuole, it could be that SLCs maintained from uptake are sufficient to support replication. ER-derived vesicles containing SLCs that are in the process of being trafficked to the plasma membrane could be intercepted by LCV-localized Dot/Icm effectors [135, 349].

Alternatively, Dot/Icm effectors may directly intercept SLCs to incorporate them to the LCV membrane. PDZ domains have been shown to be involved in binding to SLCs and directing their trafficking [350, 351]. *L. pneumophila* contains at least five putative proteins with PDZ domains (Lpg0505, Lpg0499, Lpg1331, Lpg2333, and Lpg0903), none of which have been functionally characterized. Future studies looking at effectors with PDZ domains may elucidate how SLCs are potentially manipulated by *L. pneumophila*.

Finally, it is also possible that the semi-permeable LCV membrane may allow sufficient "leakage" of amino acids into the LCV lumen. As polyubiquitinated proteins are abundant on the LCV membrane and AnkB-dependent generation of amino acids occurs at the LCV membrane, the flow of nutrients would be skewed towards influx into the LCV lumen [75].

We did demonstrate that SLC2a1/Glut1, a glucose transporter, is recruited to the LCV in a Dot/Icm-dependent manner. This finding is interesting because *L. pneumophila* does not use glucose as a source of carbon and energy to replicate [220]. Unfortunately, we were not able to pursue this line of study due to termination in production of the antibody used in our experiments. If a new functional SLC2A1/Glut1 antibody can be found and this finding can be reproduced, it will be interesting to determine why and how SLC2a1/Glut1 is trafficked to the LCV membrane in a Dot/Icm-dependent manner. Knock-down of SLC2a1/Glut1 could cause harmful effects to the host cell that would alter *L. pneumophila* replication separate of the import of glucose. Therefore, if trafficking of SLC2a1/Glut1 to the plasma membrane could be inhibited, it would be interesting to see if this blocks colocalization of SLC2a1/Glut1 to the membrane.

Transporters with ~50% similarity to SLC2a1/Glut1 can be found in amoebae species using NCBI's BLAST, indicating that modulation of SLC2a1/Glut1 may be done through a conserved mechanism. Glucose is a large driver of the macrophage pro-inflammatory response and is used to generate ROS [253]. Sequestering glucose from the host could dampen this inflammatory response while providing the precursor for anabolism of the storage molecule, PHB.

Because we were still interested in how amino acids were crossing the LCV membrane, we chose to examine the possibility that bacterial transporters were responsible for this action. Many eukaryotic-like genes in *L. pneumophila* have been acquired by inter-kingdom horizontal gene transfer [284]. We identified a pool of SLC-like proteins in *L. pneumophila* with 42% - 56% amino acid similarity to SLCs. Structural homology also supports the idea that these transporters are SLC-like, as they share strong 3-dimensional homology with SLCs. These *L. pneumophila* transporters are likely members of the MFS family of evolutionarily conserved transporters, which can be found in other bacterial species as well [236, 237]. SLCs are described to be members of the MFS family [237].

As expected, there was functional redundancy among our pool of SLC-like proteins in *L. pneumophila*. Seven were predicted to be transporters of cationic amino acids. Most amino acid transporters show high-affinity for single amino acids, but still transport multiple amino acids [231, 235]. Varying substrate specificity is likely the difference amongst these seven transporters. Because of substrate overlap, mutations in individual genes are unlikely to result in a growth defect of *L. pneumophila*. Like much of the *L. pneumophila* genome, this functional redundancy likely is what allows the

organism to replicate within a variety of environmental hosts and ensures acquisition of amino acids needed for replication.

A single transporter of tricarboxylates was also identified, LstJ. Given that citrate can rescue the *ankB* mutant; this transporter may prove to be required for intracellular replication. Further studies on this transporter to confirm substrate-specificity and requirement for replication will provide more insight into nutrient acquisition of *L. pneumophila*.

We confirmed that LstA and LstB are transporters of glucose. Individual gene mutations are not sufficient to affect intracellular replication; whereas, a deletion in both genes causes a severe intracellular replication defect. Importantly, we identified functional redundancy within *L. pneumophila* as a mechanism to ensure nutrient acquisition.

Similarly to the potential recruitment of SLC2a1/Glut1 to the LCV membrane, LstA and LstB could be important, in part, for sequestering glucose from the host to dampen the pro-inflammatory response while also acquiring the precursor for PHB. Glucose supplementation does not enhance *in vitro* growth of *L. pneumophila*, therefore the need for glucose is solely during intracellular growth [220]. However, we cannot exclude the possibility that other important metabolites are imported by LstA and LstB. Future studies examining how *L. pneumophila* senses intracellular growth vs extracellular, thus regulating specific gene transcription, may provide insight as to what governs the intracellular need for glucose. It is known that global transcriptional changes occur in *L. pneumophila* within the host compared to *in vitro* growth, but the mechanism by which these changes occur is unknown [177, 181].

The SLC-like proteins in *L. pneumophila* do not contain any known C-terminal translocation sequence nor any known type-II secretion signal. By the CyaA reporter assay, we were not able to show translocation of LstA or LstB. This does not exclude the possibility that LstA-J are translocated and acting on the LCV membrane to transport amino acids, as the CyaA reporter has been shown to interfere with the transport of some effectors [127]. So while our initial goals behind identifying SLC-like transporters in *L. pneumophila* were to find out if the bacterium was utilizing bacterial transporters on the LCV membrane to acquire nutrients, it is more likely that LstA and LstB are localized only to the bacterial membrane to import glucose from the LCV lumen.

Continuing to characterize the role of glucose in *L. pneumophila*, we were interested in the identification of two new putative amylases that were also potentially putative effectors of the Dot/Icm translocation system. We chose to characterize, LamB, as it was only found in two *Legionella* species, *L. pneumophila* and *L. steigerwaltii* and thus unique. It was surprising to find putative amylase effectors as no amylase has ever been shown to be translocated by type-III-VII translocation systems of Gram-negative pathogens. However, we were unable to confirm the translocation of LamB using the CyaA reporter assay. Strong evidence exists that this protein is a potential Dot/Icm effector, as it contains a translocation signal on the C-terminus. However, the CyaA reporter can interfere with the translocation of effectors [127]. Future studies examining translocation using another reporter assay, such has the β-lactamase reporter assay, may show that LamB is translocated by the Dot/Icm.

The target of LamB is still unknown. Glucose supplementation in *A. polyphaga* does not rescue the *lamB* mutant, so it is unlikely that the generation of excess glucose

through glycogen or starch degradation is the function of LamB. Generation of large amounts of glucose could be detrimental to *L. pneumophila* as this would support the pro-inflammatory response of the host cell [253]. LamB may act to control the host response in a mechanism that requires the cleavage of glucan bonds. Alpha-amylases are known to act on the glycosylation of proteins [335]. Therefore, LamB could be acting on the post-translational modification of host proteins to control processes important for replication. Future studies to identify the target of LamB will help understand why this protein is import for intracellular replication.

LamB could be acting in one of three locations during intracellular growth: the bacterial cytosol, the LCV lumen, or the host cytosol. Because LamB lacks a type-II secretion signal, it is unlikely that LamB is type-II secreted to function in the LCV lumen. Additionally, glycogen, starch, or glycosylated proteins are too large to be imported into the LCV lumen by transporters, so it is unlikely that a target for LamB is present in the LCV lumen. No target in the bacterial cytosol is likely to exist for LamB during intracellular replication. *L. pneumophila* is not known to make stores of glycogen, and if did, it would likely be degrading glycogen to survive extracellular when nutrients are limited, not during intracellular growth. Therefore, the more likely and biologically relevant space for LamB to function within, is within the host cytosol.

Our interest in nutritional virulence led us to discover important roles for glucose in the intracellular replication of *L. pneumophila*. The host glucose transporter, SLC2a1/Glut1, may play a role during infection to import glucose into the LCV lumen, while *L. pneumophila* SLC-like transporters, LstA and LstB, import glucose in from the

LCV lumen and/or host cytosol. Additionally, *L. pneumophila* employs amylases to possibly increase the host levels of glucose within the cytosol.

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CURRICULUM VITAE

Ashley Best (née Richards)

NSF Graduate Research Fellow, University of Louisville
ashleymariebest@gmail.com

EDUCATION

Ph.D., Microbiology and Immunology, School of Medicine
University of Louisville, Louisville, KY – *May 2018*

Masters of Science, Microbiology and Immunology, School of Medicine
University of Louisville, Louisville, KY – May 2014

Bachelor of Science, Microbiology, College of Arts and Sciences
Bachelor of Arts, Spanish, College of Arts and Sciences
Miami University, Oxford, Ohio - May 2012

RESEARCH PROJECTS

Graduate Student – NSF Fellow ***August 2012 – Present***
Laboratory of Dr. Yousef Abu Kwaik, Bumgardner Chair in Molecular Pathogenesis, University of Louisville
The Abu Kwaik Lab focuses on looking at the modulation of the human host by the environmental pathogen, *Legionella pneumophila.*

- Developed and tested experimental designs and analytical methods.
- Trained and frequently use Olympus confocal microscopy as a tool to understand intracellular relationships between *L. pneumophila* and human macrophages.
- Performed genetic manipulation on *L. pneumophila* and *E. coli* in order to better understand mechanism of pathogenesis.

Undergraduate Research Assistant ***January 2010 - May 2012***
Laboratory of Dr. Luis Actis, Chair, Department of Microbiology, Miami University
The Actis Lab focuses on understanding the host-pathogen interactions between humans and the opportunistic bacteria, *Acinetobacter baumannii.*

- Designed and executed original research project based on personal observations and research on characterization of a knockout strain within *A. baumannii*.
- Worked to troubleshoot difficult to perform experiments.
- Assisted in the characterization of a large number of army isolates.

- **LEADERSHIP & SERVICE EXPERIENCE**

Founding Director, Science Policy and Outreach Group (SPOG), *University of Louisville*

- Founded new graduate student organization at the University of Louisville to advocate for the sciences and science funding. Our goal is to better connect the community to scientists, in order to educate adults and children about the importance of science research, the need for science funding, and the career opportunities available. We also wish to create a better local dialogue between elected officials and scientists and promote science-based policy and STEM education. This group also seeks to better prepare graduate students for careers outside of academia and make well-rounded and informed scientists.
- For more information visit our website: www.SPOGatUofL.weebly.com

Subcommittee Chair on Volunteering and Judging, *Louisville Regional Science and Engineering Fair*

- Developed collaboration with the Louisville Regional Science and Engineering Fair (LRSEF) and SPOG to connect students with graduate student mentors and assist in the judging of fair projects
- Guided the development of judges' student feedback procedures for the 2016 fair. Implementation of these continued for the 2017 fair.
- Collaborated on planning advocacy day for science fair winners in Frankfort, Kentucky

Student Representative for the School of Medicine Graduate Council, *University of Louisville*

- Provide feedback and student prospective on issues occurring with the Graduate programs in the School of Medicine

SIGS Ambassador, *University of Louisville*

- Assist in prospective student visits, new student orientations, and provide other services to the School of Interdisciplinary and Graduate Studies (SIGS).

College Ambassador for the College of Arts and Sciences, *Miami University*

- Volunteer at university-sponsored events to provide prospective and insights to life and education at Miami University
- Faculty-nominated program, in recognition of academic performance and noteworthy achievements.

Science Education and Advocacy Committee*, Kentucky Academy of Sciences*

- Created Speaker's Bureau, a resource for Kentucky teachers to connect with researchers in the state who are interested in presenting science/research in classrooms

Public Engagement Committee, *Kentucky Academy of Sciences*

- Planned public outreach events at the Kentucky Science Center

CRAFT Seminar Series Committee Graduate Student Representative, *University of Louisville*

PROFESSIONAL MEMBERSHIPS

American Association for the Advancement of Science (AAAS) Sept. 2016 - Present
Kentucky Academy of Science Jan. 2015 - Present
Founder and Director - Science Policy and Outreach Group July 2014 – 2016
American Society for Microbiology (ASM) - Student Member 2013 – Present
Golden Key National Honor Society 2013 – Present
Omicron Delta Kappa 2013 – Present

Integrated Programs in Biomedical Sciences (IPIBS) – Fellow Aug 2012 – July 2014

TEACHING & MENTORING EXPERIENCE

Guest Lecturer – Research Methods in Microbiology & Immunology 610, University of Louisville

- *Confocal Microscopy,* Fall 2016
- *Confocal Microscopy,* Fall 2017

Guest Lecturer - *Microbial Pathogenesis 687,* University of Louisville

- *Acinetobacter Infections,* Spring 2015

Mentor – Undergraduate Student ***January 2015 – January 2016***

- Trained and supervised student in laboratory safety/conduct, aseptic technique and other general practices.
- Educated on scientific methods, experimental methodology, and higher level biological processes.

PROFESSIONAL TRAINING

***Massive,* MassiveSci.com,** Nov 2017 – Present
***Friends of Joe's Big Idea (FOJBI)*, NPR,** Nov 2017 - Present
***Graduate Teaching Academy,* University of Louisville,** *2014-2015*

AWARDS

***Inaugural Trainee Spotlight*,** *University of Louisville Office of Graduate and Postdoctoral Studies,*
January 2016
Graduate Student Spotlight*, University of Louisville School of Interdisciplinary and Graduate Studies,*
August 2014
Graduate Research Fellowship, *National Science Foundation (NSFGRFP), 2014 – Present*
Excellence in Research Award, *University of Louisville, Department of Microbiology and Immunology,*
April 2014
Tuition Match Award, *University of Louisville,* 2014-2015
Undergraduate Presentation Award, *Miami University 2012*

GRANTS

National Science Foundation Graduate Research Fellowship

- Stipend and research funding support from the National Science Foundation 2014-2017

Doctoral Undergraduate Opportunities Scholarship (DUOS) Research Grant, *Miami University*

- Protein Interactions in *Acinetobacter baumannii*: Who Turned the Lights Out? 2011

Undergraduate Research Award (URA), *Miami University*

- Role of Cyclic di-GMP in Light-Mediated BIsA Regulon in *Acinetobacter baumannii, 2011*
- Role of Blue-Light Sensing in the Response of *Acinetobacter baumannii* to Oxidative Stress, *2012*

OUTREACH & ADVOCACY

Lead Organizer – March for Science, Louisville ***April 2017***

- Planned satellite March for Science in Louisville, KY featuring prominent Louisvillians as guest speakers to engage and rally the public behind the importance of the scientific enterprise.
- Spoke on the importance of scientists and the community forming meaning and lasting interactions.

Science Fair Mentoring ***Numerous events***

- Organized mentoring day for middle school and high school students participating in science fair. Mentors were graduate students of SPOG. Mentors aided students in understanding science concepts, how to design and execute experiments.

GEAR UP Pre-Professional Academy ***July 2015 & 2016***

- Assisted in organization and program development for a weekend summer camp in which students would be exposed to what it means to be a researcher through a variety of activities designed to education and enlighten students.

Louisville Urban League ***July 2015***

- Developed programming for students interested in learning about a variety of career options.
- Led half-day event for students visiting the campus, to learn about career opportunities available upon completion of graduate school.

UK Health Researchers Youth Academy ***July 2014 & 2015***

- Planned visit for students from University of Kentucky area, interested in STEM careers – assisted in hands on activities for students.
- Participated in student panel – to allow students time to ask a variety of questions related to STEM careers and our personal experiences.

Brain Awareness Week ***2015***

- Arranged visit of high school student class that is part of Project Lead the Way's biomedicalscience program, in conjunction with the Louisville Chapter of the Society for Neuroscience.
- Assisted in demonstrations of comparative neural anatomy.

Career Fairs & Guest Speakers (Educational Programs)

- Participated in various career fairs in the Louisville Metro region, as representative for careers with a PhD in the field(s) of biomedical science. As well as, spoken to students in various career development programs about PhD-track science careers and career readiness.

High School Visits

- Have organized, oversaw, and participated in many other student visits to the Clinical and Translation Research Building.
- Explained the purpose of research, discussed some of the work being done here and how it is helping to advance modern science, and demonstrated some of the tools we use in the lab such as confocal microscopy.

Posters on the Hill, Washington D.C ***March 2012***

- Invited to present original undergraduate research at the House Administration Conference Room in Washington, D.C. as part of STEM Advocacy Week.
- Met with Senators and Representatives to discuss the importance of research funding and opportunities it allows for undergraduate students to conduct research.

Undergraduate Research & Experiential Learning Statehouse Day, Columbus, Ohio ***March 2012***

- Participated in the Statehouse day, along with other public universities of Ohio.
- Interacted with Members of the Ohio General Assembly, to discuss the importance of undergraduate research through anecdotal evidence.

PUBLICATIONS - SCIENTIFIC

Best A.M., Abu Kwaik Y. (2018) Mammalian Solute Carrier (SLC)-like transporters of *Legionella pneumophila*. *Manuscript in review*

Best A.M., Price C.T., Jones S.C., Abu Kwaik Y. (2018) A *Legionella pneumophila* amylase essential for intracellular replication in human macrophages and amoebae. *Sci Rep*, 2018. 8(1): p. 6340

Price C.T., Merchant M., Jones S, **Best A.M.**, Von Dwingelo J.E., Lawrenz M.B., Alam N., Schueler-Furman O., Abu Kwaik Y. (2017) Host FIH-Mediated Asparaginyl Hydroxylation of Translocated *Legionella pneumophila* Effectors. *Front. Cell. Infect. Microbiol.* DOI: 10.3389

Richards A.M., Abu Kwaik Y., Lamont R.J. (2014) Code blue: *Acinetobacter baumannii*, a nosocomial pathogen with a role in the oral cavity. *Mol Oral Microbiol.* DOI: 10.1111/omi.12074

Price C.T., **Richards A.M.,** Abu Kwaik Y. (2014) Nutrient generation and retrieval from the host cell cytosol by intra-vacuolar *Legionella pneumophila. Front Cell Infect Microbiol*. doi: 10.3389/fcimb.2014.00111

Price C.D., **Richards A.M.,** Von Dwingelo J.E., Samara H.A. and Abu Kwaik Y. (2013*)* Amoeba host-*Legionella* synchronization of amino acid auxotrophy and its role in bacterial adaptation and pathogenic evolution. *EMI*. doi: 10.1111/1462-2920.12290

Richards A.M., Von Dwingelo J.E., Price C.T., Abu Kwaik Y. (2013) Cellular microbiology and molecular ecology of *Legionella*–amoeba interaction. *Virulence*; 4:307 - 314; PMID: 23535283; http://dx.doi.org/10.4161/viru.24290

Nwugo C.C., Arivett B.A., Zimbler D.L., Gaddy J.A., **Richards A.M.**, Actis L.A. (2012) Effect of Ethanol on Differential Protein Production and Expression of Potential Virulence Functions in the Opportunistic Pathogen *Acinetobacter baumannii. PLoS ONE* 7(12): e51936. doi:10.1371/journal.pone.0051936

PUBLICATIONS – OTHER

Best, A.M. "Natural disasters leave behind more than just physical damage." *Massive* 5 Jan 2018

Best, A.M. "Astronomy" Real Science for Real Life. *Kentucky Teacher* 26 Oct 2017

Best, A.M. and Steadman, C. "Time" Real Science for Real Life. *Kentucky Teacher*. 27 April 2017

Best, A.M. and Steadman, C. "Winter Weather" Real Science for Real Life. *Kentucky Teacher*. 23 Feb 2017

Best, A.M. and Fuller, A. "Buoyancy." Real Science for Real Life. *Kentucky Teacher*. 27 Oct 2016

Best, A.M. and Fuller, A. "Be a Forest Firebrand." Real Science for Real Life. *Kentucky Teacher*. 23 June 2016

Best, A.M. and Fuller, A. "So You Made it Through Flu Season." Real Science for Real Life. *Kentucky Teacher*. 28 April 2016.

PRESENTATIONS/ABSTRACTS

A.M. Best. Nutritional virulence of *Legionella pneumophila.* Dissertation Defense, Louisville, KY 2018

A.M. Best. Import of metabolites by the *Legionella*-containing vacuole. Departmental Seminar, Louisville, KY 2017

A.M. Best. Nutrition of intracellular *Legionella pneumophila*. Departmental Seminar, Louisville, KY 2016

A.M. Richards. Nutrition of intracellular *Legionella pneumophila*. Departmental Seminar, Louisville, KY 2014

A.M. Richards, B.A. Arivett. J.A. Gaddy, L.A. Actis. Light at the end of the tunnel or a train: blue-light induced virulence in *Acinetobacter baumannii.* Ohio Branch American Society of Microbiology, Athens, OH 2011

A.M. Richards, B.A. Arivett. J.A. Gaddy, L.A. Actis. Light at the end of the tunnel or a train: blue-light induced virulence in *Acinetobacter baumannii.* Undergraduate Research Forum, Oxford, OH 2011

A.M. Richards, B.A. Arivett. J.A. Gaddy, L.A. Actis. Light at the end of the tunnel or a train: blue-light induced virulence in *Acinetobacter baumannii.* Undergraduate Research Information Fair**,** Oxford, OH 2011

A.M. Richards, B.A. Arivett. J.A. Gaddy, L.A. Actis. Light at the end of the tunnel or a train: blue-light induced virulence in *Acinetobacter baumannii.* College of Arts & Science's Alumni Advisory Board, Oxford, OH 2011

A.M. Richards, B.A. Arivett. J.A. Gaddy, L.A. Actis. Light at the end of the tunnel or a train: blue-light induced virulence in *Acinetobacter baumannii.* 9th Annual Cell, Molecular, and Structural Biology (CMSB) Symposium, Oxford, OH 2011.

A.M. Richards, B.A. Arivett, D.L. Zimbler, C.C. Nwugo, L.A. Actis. A blue-light sensing protein in *Acinetobacter baumannii* mitigates oxidative stress and the role of glutathione**.** 18th Annual Midwest Microbial Pathogenesis Conference, Ann Arbor, MI 2011

A.M. Richards, B.A. Arivett, C.C. Nwugo, D.L. Zimbler, L.A. Actis. The oxidative response of the bacterial pathogen *Acinetobacter baumannii* depends on sensing blue light. Posters on the Hill, Washington D.C. 2012

J.T. Newman, J.G. McChesney, S.L. Distelhorst, R.F. Relich, D.A. Jurkovic, **A.M. Richards**, M.F. Balish. Fast-gliding mycoplasmas from rodents. 113th General Meeting of the American Society of Microbiology, Denver, CO 2013

Penwell, W.F., Zimbler, D.L., Beckett, A.C., **Richards, A. M**., Arivett, B. A., Fiester, S.E., Actis, L.A. Variability of virulence factors among *Acinetobacter Baumannii* isolates obtained from wounded military personnel. 20th Annual Midwest Microbial Pathogenesis Conference, Columbus, OH 2013

TRAININGS

Biosafety Training, Radiation Safety, Formaldehyde Training, Recombinant DNA Guidelines,
HIPAA (Health Insurance Portability and Accountability Act), Blood-Borne Pathogen training, Biohazard and Laboratory Safety Training