Amoebae as a Host for *Legionella* Replication

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Doctor of Philosophy

March 2020

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The University of Melbourne
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Submitted in total fulfilment for the degree of

Doctor of Philosophy

ABSTRACT

Legionella pneumophila (*L. pneumophila*) is a gram-negative bacterium found ubiquitously in natural water sources where it replicates within amoebae, such as *Acanthamoeba castellanii* (*A. castellanii*). The evolution in amoebal hosts allows *L. pneumophila* to 'accidentally' infect human lungs after inhalation of aerosols containing *L. pneumophila.* In both human macrophages and amoebae, *L. pneumophila* replicates intracellularly within a vacuole known as the *Legionella* containing vacuole (LCV) that avoids fusion with the endocytic pathway. Fundamental to this process is the translocation of over 330 proteins by *L. pneumophila* into host cells by the Dot/Icm type 4 secretion system. To date, only a subset of Dot/Icm effector proteins has been characterised. These proteins are involved in manipulating host cellular processes such as ER vesicle recruitment, post-translational modifications and host cell survival. The majority of *L. pneumophila* effector proteins remain uncharacterised due to functional redundancy of effector proteins and complex effector protein regulation. In addition, despite the ecological and evolutionary significance of the intra-*A. castellanii* stage of the *L. pneumophila* life cycle, very little is known about this host-pathogen interaction.

In this study, we constructed in-frame markerless mutants in *L. pneumophila* 130b according to genomic regions enriched for putative Dot/Icm effector genes and revealed host-specific genomic regions that are required for optimal *L. pneumophila* replication in *A. castellanii*. Specifically, by identifying a putative glutamate transporter necessary for replication in *A. castellanii*, but not in macrophages, this study suggests an aspect of *L. pneumophila* metabolism adaptation in different hosts.

A. castellanii presents two life stages: active trophozoites and dormant cysts. Trophozoites act as reservoirs for *L. pneumophila* and are hijacked by *L. pneumophila* for bacterial replication. Cysts are resistant to anti-microbial treatments and were previously thought to facilitate *L. pneumophila* persistence and dissemination. However, in this study, we showed that the infection of *A. castellanii* with *L. pneumophila* led to inhibition of the transition from trophozoites to cysts in a Dot/Icm dependent manner. This inhibition partially required the gene *letA*, which encodes the regulator of two-component system LetAS. Pathogenic amoeba cysts prose a human health problem. This study provides a biological model for anti-cyst research and provides clues into critical encystment pathways.

L. pneumophila can interfere with a broad range of host cellular pathways to establish the replication niche. Here, we investigate *A. castellanii* transcriptional response with *L. pneumophila* infection by RNA sequencing analysis. As a result, *A. castellanii* genes associated with mitosis, DNA replication and cell wall synthesis were significantly downregulated at late infection phase, which might contribute to the inhibition of *A. castellanii* proliferation and encystment. Compared with transcriptional profile in macrophage, *L. pneumophila* displayed different regulation on host eEF1a expression and genes associated with ATP production. Furthermore, a gene encoding sirtuin family protein, Sir6f, was upregulated during *L. pneumophila* infection, and silencing of *sir6f* led to decreased bacterial replication, suggesting that Sir6f is a host factor that facilitates *L. pneumophila* replication in *A. castellanii*.

Evolution of *L. pneumophila* in amoebal hosts is hypothesized to allow its adaptations to survive in macrophages. Overall, this study highlights a number of strategies utilised by *L. pneumophila* during *A. castellanii* infection, including host-specific virulence factors, inhibition of *A. castellanii* encystment, affect host transcriptome and hijack of host factors. These microbial strategies will add valuable insights and provide potential targets regarding the development of new mechanisms for *L. pneumophila* control and prevention.

DECLARATION

This is to certify that:

i. This thesis comprises only my original work towards the Doctor of Philosophy except where indicated in the preface

ii. Due acknowledgement has been made in the text to all other material used

iii. This thesis is fewer than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices

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PREFACE

In accordance with the regulations of The University of Melbourne, I acknowledge that some of the work presented in this thesis was collaborative. Specifically:

In chapter 3, the collocation and annotation of effector protein genes to *L. pneumophila* 130b genome was carried out with the help of Shivani Pasricha.

In chapter 3, *L. pneumophila* mutants were constructed in collaboration with Raissa Wibawa. The growth curve and replication assay of *L. pneumophila* mutants in human macrophage THP-1 were carried out by Raissa Wibawa.

In chapter 3, whole-genome sequencing and SNPs characterisation of *L. pneumophila* strains was performed by Sacha Pidot and Tim Stinear.

In chapter 4, the electron microscopy was processed by Vicki Bennett Wood. The photos of *A. castellanii* generated from Imagestream were captured by Garrett Ng.

In chapter 5, Library preparation, RNA-sequencing was performed by Research Center for Zoonosis Control, Hokkaido University. Genome alignment was performed by Shivani Pasricha and Dane Vassiliadis. Phylogenetic analysis was performed by Shivani Pasricha.

The remainder of this thesis comprises only my original work.

LIST OF PUBLICATIONS ARISING FROM THIS THESIS

This thesis contains material that is currently in preparation for publication:

Legionella pneumophila infection of *Acanthamoeba castellanii* leads to the rewiring of amoeba transcription and highlights potential biocide targets.

Legionella pneumophila replication in *Acanthamoeba castellanii* leads to the inhibition of cyst formation.

ACKNOWLEDGEMENTS

Completion of this PhD will not be possible without the help, support and guidance of many people, all of whom needs to be properly acknowledged. I am full of gratitude for the past four years.

First of all, respect and thank my supervisor, Liz. Thank you for giving the opportunity to have a fantastic experience at Melbourne University. You are the one leading me on the road of scientific research and greatly expanded my academic horizons. Liz is not only a great scientist with a bright personality but also a kind and wisdom supervisor. Thank you for the patient guidance, encouragement and advice throughout my PhD. I have been fortunate to be your student. Your attitude to science is one of the most valuable things I learn here. This will be a valuable asset in my life and career.

I also would like to thank my co-supervisor Dr Shivani Pasricha, for her constant support, availability and constructive suggestions. Your creative methodology impresses me a lot. You gave me much guidance when I first started working in the lab. The regular meetings during the past four years were not always peaceful but are helpful and beneficial to me. Thank you so much for correcting my writing all the time. Even after a lot of repeated changes, you are still very kind to me. You are also an elder friend who taught me the balance of experiment and life. I still remember your suggestion 'Be a human', when I stay in the lab for too long. Also a special thank to your husband for the contribution of data analysis. May you continue to do what you love and have a wonderful family.

I would like to express my special thanks of gratitude to my committee Hayley Newton and Nichollas Scott for the support during my PhD. Your effort and constructive advice kept my PhD on the track. I would not forget to remember your encouragement and timely support and guidance until the completion of my project work.

Many thanks to my PhD mates: Raissa, Yoges, Jiyao and Linda. I am very grateful for your company. Thanks to you guys, we did barbeques and had a lot of fun, releasing the stress from PhD. Special thanks to Raissa. Together, we faced the difficulties occurred in this project and shared the happiness when we successfully made some mutants. Also, you taught me organ attack and many other board games. You are a very smart person as you are often the winner in the games.

A big thank you to all Hartland lab members past and present. You helped me to adapt to life in Melbourne. Thank you, Ying, Cristina, Gina, Ka Yee, Jac, Josh and Max. You were always patient to tell me new things. Parties, drinks and quizzes are always a good memory. Most importantly, you together made the lab a wonderful place to work in. You are responsible and always ready for help. So I was proud to tell CSC applicants that here is a paradise for academic research.

A tremendous thank you goes to my friends outside lab. It is really enjoyable to meet so many interesting people with different cultural background. I will cherish the memory of talking about literature, cooking and travelling with you.

Lastly, I am extremely grateful to my parents for their love and sacrifices for educating and preparing me for my future. Also, I express my thanks to my sister for giving me more joy. Finally, to my caring, loving, and supportive girlfriend, Xialin, my heartfelt thanks. Your encouragement when the times got rough is much appreciated and duly noted. Thank you for your understanding and support, as well as your company when I worked in late-night, although we were located north and south of the earth.

ABBREVIATIONS

The following abbreviations have been used throughout the thesis:

%	Percentage
~	Approximate
°C	Degrees Celsius
ACES	N-(2-acetamido)-2-aminoethanesulfonic acid
ADP	Adenosine diphosphate
AK	Acanthamoeba keratitis
AMP	Adenosine monophosphate
ARF1	ADP-ribosylation factor 1
ATP	Adenosine triphosphate
BCYE	Buffered charcoal yeast extract
BMDMs	Bone marrow-derived macrophages
bp	Base pair
CFU	Colony forming unit
CFW	Calcofluor white
DEGs	Differentially expressed genes
dH2O	Distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
eEF1α	Elongation factor 1-alpha
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
ER	Endoplasmic reticulum
GAE	Granulomatous Amoebic Encephalitis
GAP	GTPase activating protein

GEF	Guanine exchange factor
GFP	Green fluorescent protein
GO	Gene ontology
h	Hour
hMDMs	Human monocyte-derived macrophages
hpi	Hours post infection
IL	Interleukin
iMAD	Insertional mutagenesis and depletion
IκB	Inhibior of NF-κB
kb	Kilobase
LAMP-1	Lysosomal-associated membrane protein1
LB	Luria-Bertani
LCV	Legionella-containing vacuole
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MAPK	Mitogen-activated protein kinase
MCM	Mini chromosome maintenance protein
MFP	Membrane fusion protein
min	Minute(s)
MOI	Multiplicity of infection
mRNA	Messenger RNA
MyD88	Myeloid differentiation primary response gene 88
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B
NLR	Nod-like receptor
OD	Optical density

padj	Adjusted p-value
PCR	Polymerase chain reaction
PI(4)P	Phosphatidylinositol 4-phosphate
PRRs	Putative pattern recognition receptors
PYG	Peptone Yeast Glucose
qRT-PCR	Quantitative reverse transcription PCR
RQ	Relative quantitation
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RT	Room temperature
Sec	Eneral Secretion system
SEM	Standard error of the mean
siRNA	Small interfering RNA
SNARE	Soluble N-ethylmaleimide-sensitive factor
SNPs	Single Nucleotide Polymorphisms
T1SS	Type I secretion system
T2SS	Type II secretion system
T4SS	Type IV secretion system
Tat	Twin arginine translocation
TCS	Two-component systems
TraSH	Transposon site hybridisation
v/v	Volume/volume
WT	Wild type

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Chapter 1: Literature review

1.1 Historical perspective

In 1976, a pneumonia outbreak occurred during the American Legion convention in Pennsylvania, Philadelphia, and 29 out of 182 people died because of respiratory failure (1). The bacterium was later isolated from the lung tissues in four fatal cases by inoculation into guinea pigs and was termed as *Legionella pneumophila* (*L. pneumophila*) (1, 2). The transmission pattern of this new pathogen was air-borne with extremely rare reports of person-to-person transmission (1). Meanwhile, a new genus *Legionella* was established and earlier isolated bacterial strains, such as a bacterial strain isolated from soil amoebae in 1954, were also included in this genus (2-4). In the following years, buffered charcoal yeast extract (BCYE) agar was developed to culture and isolate *L. pneumophila* (5, 6).

The discovery of *L. pneumophila* allowed elucidation of the aetiological agent in earlier outbreaks. A Pontiac fever outbreak in 1968 was thus attributed to *Legionella* (7). An unsolved outbreak of infection at St. Elizabeth's Hospital that occurred in 1965 was also linked with *Legionella* (8).

1.2 Microbiology of *Legionella*

Legionella spp are gram-negative bacteria belonging to the *gamma (γ)-proteobacteria* (2). All *Legionella* bacteria have been isolated from aqueous and soil environments (9). The ideal conditions for *L. pneumophila* replication include temperatures between 25°C and 45°C, water stagnation and the presence of protozoa in the environment (10-15). In laboratory conditions, *Legionella* grows on various solid selective and non-selective media with strict growth requirements, including the addition of soluble iron and L-cysteine (16-18). Some *Legionella* spp can be isolated by amoebae enrichment but cannot be grown on routinely used media. These are termed *Legionella*-like amoebal pathogens (9, 19). Generally, *Legionella* is 0.3-0.9 μm in diameter and 2-20 μm in length (3, 20, 21). Negative stain electron microscopy has revealed pili and flagella in many *Legionella* species like *L. pneumophila* (22, 23). *L. longbeachae* differs in the absence of flagella and the presence of a capsule (24).

Several methods have been used for subtyping *Legionella* spp (25). Monoclonal antibody (mAb) subgrouping, pulsed-field gel electrophoresis (PFGE) and amplified

fragment length polymorphism (AFLP) are standardised and reproducible methods for genotyping all *Legionella* spp, while sequence-based typing (SBT) is available for *L. pneumophila* only (25). Non-nucleic acid based identification of *Legionella* at the species level requires sophisticated tests including phenotypic characteristics; growth requirements; serological identification by agglutination or fluorescent antibody technique; fatty acid, carbohydrate, or ubiquinone analysis and protein profiling (26-28). Hence, isolates of *Legionella* are generally characterised by comparing their 16S ribosomal RNA or *mip* gene sequence with known sequences (19, 29, 30). To date, more than 65 species and 70 serotypes have been identified in *Legionella* genus, and the numbers of species subspecies and serotypes are still increasing (9, 26, 31).

1.3. Legionellosis- Legionnaires' disease and Pontiac fever

1.3.1 Clinical features

Legionellosis refers to diseases caused by *Legionella* and presents as two clinical conditions: the more severe Legionnaires' disease and the comparatively mild, flu-like Pontiac fever (7). Smokers, the elderly and immunocompromised individuals have a higher risk of Legionellosis (32-34). However, some people who carry *Legionella* antibodies indicating exposure to this pathogen are entirely asymptomatic (35).

Legionnaires' disease is a severe multisystem disease involving pneumonia (3). The symptoms of Legionnaires' disease include malaise, muscle ache, fever, non-productive cough and headaches accompanied by chest pain (3, 32, 36). While the chest X-ray pattern is not different from other types of pneumophila, Legionnaire's disease is commonly associated with alveolar infiltrates (37). In contrast to typical community-acquired pneumonia, Legionnaires' disease can also be accompanied by hypophosphatemia in early-stages, diarrhea, elevation in serum creatine kinase levels and delirium (38, 39).

Pontiac fever is a mild and self-limited disease (7). The symptoms of Pontiac fever, similar to influenza, include fever, asthenia, myalgia, arthralgia, headache, cough, nausea and sore throat (40-42). Other symptoms such as dyspnea, thoracic pains, vomiting and diarrhoea are also described (40, 41, 43-45). Generally, patients recover in two to five days without any treatment (42, 46). The pathogenesis of Pontiac fever

remains obscure, and there is no agreed-on definition, nor any specific clinical findings or laboratory tests for its diagnosis (9). As a result, the occurrence of Pontiac fever is reported far less than Legionnaires' disease (42). The reason for two distinct syndromes caused by the same pathogen is still unclear, but might be associated with the number of inhaled bacteria or individual immune responses and susceptibility (43, 47, 48).

1.3.2 Diagnosis tools for *Legionella* infection

The clinical features and chest radiographic findings are not specific enough to distinguish *L. pneumophila* from other respiratory pathogens (49, 50). Instead, *Legionella* infection can be diagnosed by non-culture and culture techniques (50-54).

Urinary antigen detection is the first-line diagnostic test, but only detects serotype 1 *L. pneumophila* (50, 55). By detecting a component of a cell wall lipopolysaccharide, the specificity of urinary antigen test is very high (~100%) (9, 18, 56). However, some positive cases are missed as the sensitivity varies from 56-99% (18, 56).

Direct immunofluorescence microscopy can detect *Legionella* in lung tissues and respiratory samples, but the sensitivity depends on the sampling process, and this technique requires significant experimental skills (53, 55).

Several indirect ELISA tests have been developed for detecting *L. pneumophila* antibodies in serum samples (51, 52, 57-59). The reported sensitivity varies from 41% to 75%, which might be the result of variation of the antigen preparation, the reference strain or a cross-reaction (50). However, some patients develop antibodies against *Legionella* spp early during infection (50, 60). The antibodies last up to 48 months after disease onset in 33% of patients, making early-stage diagnosis inaccurate (50, 60).

Various PCR-based tests have been developed to recognise *L. pneumophila* DNA in sputum, urine and blood. Real-time PCR targeting the 23s-5s rRNA intergenic spacer region allows the detection of all *Legionella* species and accurate discrimination from *L. pneumophila* (61). Six PCR-associated assays have been developed, and one of them has been approved by the US Food and Drug Administration (9, 62). The sensitivities of PCR based tests are 80-100% for lower respiratory tract secretions, 30-80% for serum and 0-90% for urine samples (63).

Bacteriological diagnosis is predominantly based on culture of the pathogen isolated from sputum, pleural fluid, bronchoalveolar and sometimes from blood cultures (49, 50). Samples are pre-treated with an acid-wash solution to minimise commensal respiratory microbiota and diluted to restrain the growth inhibition by tissue and serum factors, or antibiotics (18). BCYE medium supplemented with 0.1% α-ketoglutaric acid and necessary antibiotics is used for isolation and growth of *Legionella* spp (18, 64). Samples in the acute infection phase need to be processed quickly before initiating antimicrobial therapy (9). The sensitivity of culturing respiratory samples is 20-80% and is reduced in patients with insufficient sputum, or undergoing previous antibiotic therapy (65, 66). Even though the yield varies with sample types and is influenced by infection severity, the bacteriological culture enables the diagnosis of all *Legionella* spp, outbreak investigation and further epidemiological studies (9, 18, 64, 67).

Overall, diagnosis based on culture of the lower respiratory tract is the gold standard for detecting Legionnaires' disease (18, 50). Optimal sensitivity for the diagnosis of *Legionella* infection is achieved by using a combination of bacteriological culture, serological investigation, and urinary antigen detection. For example, combining *L. pneumophila* respiratory PCR and urinary antigen testing results in increased sensitivity and specificity (68). For public health purposes, both cultural and serological investigation remain essential diagnostic tools (50).

1.3.3 Treatment

While the delay in early-stage therapy results in increased mortality, the overall mortality for Legionnaires' disease in the US has been declining since the 1990s, possibly attributed to the development of empirical therapy for community-acquired pneumonia (69, 70). Historically, erythromycin is commonly used to treat Legionnaires' disease (32, 71). However, because of side effects and therapeutic failure with erythromycin, quinolones (mainly levofloxacin) and macrolides (azithromycin, clarithromycin, roxithromycin) are commonly used as therapeutic choices and show better *in vitro* inhibition of growth of *L. pneumophila* (71-73). In fact, azithromycin and levofloxacin are licensed by the Food and Drug Administration for Legionnaires' disease and are now considered preferable to erythromycin (32). According to the British Thoracic Society, fluoroquinolone is recommended to use for

severe or life-threatening Legionnaires' disease (74). However, all fluoroquinolone drugs have a potential side-effect of peripheral neuropathy (75). Nonetheless, fluoroquinolones (ciprofloxacin, levofloxacin, or moxifloxacin) or macrolides (azithromycin) are still the recommended antimicrobial therapies (71). More investigation is required for a universally accepted advanced treatment plan for Legionnaires' disease.

1.4 Epidemiology of *Legionella*

1.4.1 Ecology

Legionella is found ubiquitously in aquatic bodies including rivers, lakes and even mud (76-79). Soil and sediment provide nutrients and shelter for the formation of biofilms in which *Legionella* can persist for decades (80). In addition, *Legionella* replicates in a broad range of protozoan hosts like amoebae, ciliated protozoa and slime moulds (10, 81-89). The free-living protozoa protect intracellular bacteria from harsh environmental conditions such as biocide exposure and supply nutrients for bacterial replication (80). In nutrient-depleted environments, *Legionella* enters a non-replicative viable but nonculturable (VBNC) state (90). However, the dormant bacteria are resuscitated by addition of free-living amoebae, such as *Acanthamoeba castellanii* (*A. castellanii*), suggesting a critical role of protozoan hosts in *Legionella* ecology (90).

Legionella can also be detected in human-made water systems such as cooling towers and potable water facilities in hotels and hospitals (80). As water temperature is a crucial feature, *Legionella* can be found in high numbers in biofilms and detritus at the bottom of hot water tanks (47). Bacteria and protozoa can also colonise water pipe surfaces, thereby promoting *Legionella* replication in closed water systems (91, 92). In fact, 50% of large building water systems and 10-30% of home water systems are estimated to contain *Legionella* in the US, and the percentage is increasing with more sensitive detection techniques (76, 93).

Around 30 species of *Legionella* are human pathogens, but not all are equally responsible for laboratory-confirmed cases of Legionnaires' disease worldwide (9, 31, 54). Of all the *Legionella* species, *L. pneumophila* causes approximately 90% of Legionnaires' disease cases in the US and 80-85% worldwide, while *Legionella*

longbeachae (*L. longbeachae*) is particularly associated with Legionnaires' disease in Australia and New Zealand (32, 54, 94, 95). Even within the same species, *L. pneumophila* serogroup1 is responsible for 90% of cases caused by *L. pneumophila* (31). Cases caused by *L. pneumophila* Sg3 and Sg6, *Legionella bozemanii*, and *Legionella micdadei*, are also reported in Europe and the United States (34, 54, 96). Infection with less common strains is rarely reported because of the infrequency and the lack of diagnostic reagents (94, 95).

1.4.2 Transmission

Legionnaires' disease is predominantly caused by the inhalation of *Legionella*-containing aerosols (97). Microaspiration of contaminated water systems or direct contact with surgical wounds are less common modes (98, 99). Notably, *Legionella* in natural water bodies does not usually cause outbreaks except hot springs, where the condition is ideal for bacterial replication (100). Many artificial systems producing aerosols are linked to cases of Legionnaires' disease and outbreaks, including cooling towers, hot tubs, industrial equipment, plumbing systems, water outlets, thermal spas, respiratory devices and nebulisers or nasogastric tubes in hospitals (26, 101). As *Legionella* is prevalent in home water systems, bacteria from showerheads and faucets can also be a source of infection (102, 103). Bacterial load, the infectivity of the colonising bacteria, aerosol type and the efficiency of dissemination determines the likelihood of bacterial infection (9). In addition, cumulative exposure to bacterial source leads to a higher risk for infection (101).

Specifically, Legionnaires' disease caused by *L. longbeachae* is linked to potting soil and soil conditioners containing the microorganism and being close to wet hanging flowers, but the transmission mode remains unclear (104, 105).

To date, only one probable person-to-person transmission case has been reported and this occurred during the Legionnaires' disease outbreak in Vila Franca de Xira, Protugal (106). One patient was infected with the *L. pneumophila* ST1905, the causative agent of the Legionnaires' disease outbreak, but the case was not geographically linked to the cluster epicentre (106, 107). Acquisition of disease was a result of 8 hours' direct contact with another patient showing severe Legionnaires' disease symptoms in a small area of a nonventilated room (106).

1.5 Infection cycle of *L. pneumophila*

L. pneumophila appears to apply the same mode of infection in mammalian and protozoan cells (94, 108, 109). After phagocytosis, *L. pneumophila* avoids endocytic maturation and phagolysosomal degradation. Instead, *L. pneumophila* establishes the Legionella-containing vacuole (LCV), which involves the recruitment of ER-derived vesicles, mitochondria and ribosomes to support bacterial replication and metabolism of host amino acids. Eventually, *L. pneumophila* replicates to high number and lyses host cells to infect other cells (Figure 1.1).

L. pneumophila is known to secret virulence factors by different secretion systems to subvert host cellular function. The type II secretion system (T2SS) and the Dot/Icm type IV secretion system (T4SS) are most studied as they play essential roles during infection. The T2SS translocates over 27 substrates, and nearly 20 have enzymatic activity (110-113). The *L. pneumophila* Dot/Icm T4SS is crucial for LCV biogenesis and the establishment of replicative niche (114-116). Over 330 effector proteins translocated by Dot/Icm secretion system into host cells and regulate every step of intracellular replication (117).

1.5.1 Entry into host cells

1.5.1.1 *L. pneumophila* attachment to host cells

The entry of *L. pneumophila* into host cells involves both bacterial virulence factors and host factors. Adherence to host cells is the first step for bacterial pathogenesis and usually has a direct impact on phagocytosis, as well as subsequent intracellular events (118). Mutagenesis studies have revealed that *L. pneumophila* genes *pilE$_L$*, *rtxA*, *enhC*, *lcI*, *laiA* and *pilY1* facilitate bacterial attachment to different hosts. Mutation of the type IV pilin gene *pilE$_L$*, also termed as CAP (competence- and adherence-associated pili), led to an approximate 50% decrease in attachment to human epithelial cells, macrophages U937 and *Acanthamoeba. polyphaga* (*A. polyphage*) without influencing the bacteria replication capacity (119). RtxA and EnhC are homologous to repeats in the structural toxin protein and the secreted Sel-1 protein from *Caenorhabditis elegans*, respectively (120). *L. pneumophila* mutants lacking *rtxA* or *enhC* displayed significantly reduced entry into *A. castellanii* and human monocytic cells, compared to wild type bacteria (120, 121). LcI is a polymorphic

adhesin that is conserved in *L. pneumophila* serogroups. It binds to sulphated glycosaminoglycans (GCGs) on the host extracellular matrix and subsequently mediates the interaction between *L. pneumophila* and lung epithelial cells (122). LcI contains large regions of collagen-like repeats encoded by the variable number of tandem repeats (VNTRs), and the number influences its adhesive characteristics (123). LaiA shares homology with an integrin analogue of *Saccharomyces cerevisiae* and is involved in *L. pneumophila* attachment to human epithelial A549 cells (124). *L. pneumophila* mutant lacking *pilY1* presented decreased adhesion to THP-1 macrophages and A549 epithelial cells (125). In addition, the *L. pneumophila* major outer membrane protein (MOMP) binds complement component C3 and C1q and serves as an adhesive molecular to human monocytes U937 cells (126, 127). A monoclonal antibody specifically binding to MOMP reduces bacterial attachment (128). Lastly, Hsp60 is enriched in virulent *L. pneumophila* strains and has been reported to mediate the attachment to Hela cells (129). Overall, the detailed functional mechanism of all these bacterial factors is still unknown.

Host factors have been identified for *L. pneumophila* attachment but vary in macrophages and amoebal hosts. In monocytes, the complement receptors CR3 (CD18/CD11b) and CR1 (CD35), which work as receptors for opsonins, play a role in *L. pneumophila* attachment, as blocking the monocyte receptors CR3 and CR1 using mAbs strongly inhibits the adherence of *L. pneumophila* to monocytes (130, 131). However, the presence of specific antibodies and a role for the Fc receptor recognising those antibodies is indispensable for the complement-mediated adherence of *L. pneumophila* (89, 132, 133). Non-complement mediated adhesion of *L. pneumophila* has also been reported, but the host cell receptors involved in this process remain unknown (131, 134, 135). In protozoan hosts, a Gal/GalNAc lectin is an amoebal receptor that aids *L. pneumophila* attachment to the protozoan, *Hartmannella. vermiformis* (*H. vermiformis*) (136). While Gal and GalNac blocked the attachment of *L. pneumophila to H. vermiformis*, these sugars only partially blocked bacterial attachment to *A. polyphaga* (137). Meanwhile, *L.pneumophila* mutants that are defective in *A. polyphaga* attachment exhibit only a minor reduction in the attachment to *H. vermiformis*, suggesting that different mechanisms are involved during attachment to different amoebal hosts (137). Hence, the attachment of *L. pneumophila* to host cells depends on the cell type. The detailed knowledge of the

molecular mechanisms that allow *L. pneumophila* host cell attachment is still not well understood.

1.5.1.2 *L. pneumophila* is phagocytosed by host cells

After attaching to the host cell surface, *L. pneumophila* is phagocytosed by human macrophages or amoebal hosts (109, 138-140). As well as the classical mechanism of phagocytosis, *L. pneumophila* can be engulfed by coiling phagocytosis in macrophages (141). In this process, *L. pneumophila* is enveloped by a unilateral pseudopod that rolls into itself rather than fusing with its stem (141). In the case of protozoan hosts, *L. pneumophila* is also internalised by coiling phagocytosis by *A. castellanii* but by classical phagocytosis by *H. vermiformis* (109, 139, 140). Notably, in *Dictyostelium discoideum* (*D. discoideum*), a species of soil-dwelling amoeba, *L. pneumophila* was internalised by macropinocytosis, a process that does not require particle binding to the cell surface (142). Micropinocytosis is less efficient than phagocytosis and is partially sensitive to IP3K inactivation (142). In summary, the different phagocytosis mechanisms are influenced by the bacterial strain, mammalian or amoebal host cell type and perhaps the experimental methods.

At the molecular level, phagocytosis of *L. pneumophila* is an actin-dependent process conserved in macrophage and amoeba (134, 143-147). Treatment of cells with cytochalasin-D, an actin polymerisation inhibitor, impairs the uptake of *L. pneumophila* by macrophages and lung epithelial cells (134, 143-146). In contrast, *L. pneumophila* entry into *Dictyostelium* is cytochalasin-A sensitive instead of cytochalasin-D sensitive, possibly due to the different depolymerisation mechanism of actin filaments in amoebal hosts (143, 148). In addition to actin, specific actin-associated proteins are also involved in the uptake of *L. pneumophila* in mammalian and amoebal hosts (149). In *D. discoideum*, the actin-binding protein coronin accumulates at the phagocytic cups of *L. pneumophila* containing phagosomes at very early infection stages and is released rapidly after phagocytosis (150). This process is conserved in U927 and U937 macrophage-like cells (145-147).

In addition to host factors, the uptake process is also enhanced by *L. pneumophila* (140, 151, 152). For example, the Dot/Icm effector protein VipA works as an actin nucleator to polymerise microfilaments and is required for efficient *L. pneumophila* invasion into lung epithelial cells (153). In summary, phagocytosis of *L. pneumophila*

is an actin mediated process that is relatively conserved in macrophage and amoebal hosts.

1.5.2 LCV establishment

1.5.2.1 Avoiding the endocytic pathway and acidification

Shortly after internalisation, phagosomes containing *L. pneumophila* escape the endocytic pathway and establishes membrane-bound replication niche known as the *Legionella*-containing vacuole (LCV). Typically, following phagocytosis, inert particles and non-pathogenic bacteria contained by phagosomes are delivered to early endosomes and go through an intracellular pathway known as the endocytic pathway. Endosomal carrier vesicles are transformed into a phagolysosome, which is highly acidic. The maturation or acidification of phagosomes is driven by v-ATPases, which function as ATP-driven proton pumps (154, 155). The acidic condition in phagosomes is required for lysosomal degradation.

Interestingly, six hours after uptake, *L. pneumophila* can maintain a neutral luminal pH, while phagosomes containing heat-killed *L. pneumophila* are acidic (156). Inhibition of LCV acidification and maturation by the proton ATPase inhibitor, bafilomycin A1 treatment, impairs bacterial replication, indicating that avoidance of phagosome acidification is important for *L. pneumophila* replication at an early infection stage (156). However, at 18 hours post infection in macrophages, the LCV displays an acidic pH and recruits endosomal markers such as the Lysosomal-associated membrane protein1 (LAMP-1) (156). Compared to bacteria cultured in vitro in broth, bacteria released after replication in macrophages are acid resistant, suggesting a final fusion of the replicating LCV with the lysosomal compartment (156, 157).

The *L. pneumophila* Dot/Icm secretion system is crucial for LCV maturation. In *D. discoideum*, VatM, which is a transmembrane subunit of the v-ATPase, is recruited to the phagosome in *L. pneumophila* mutants lacking a functional Dot/Icm secretion system leading to vacuole acidification. However, wild type *L. pneumophila* avoids the recruitment of VatM (158). Using the Dot/Icm effector protein SidK, which interacts with the proton pump component VatA, *L. pneumophila* inhibits ATP hydrolysis, proton translocation and subsequently vacuole acidification (159).

Another effector protein, WipB, is a lysosome-targeted phosphatase that interacts with the v-ATPase (160). Thus, the absence of v-ATPase in the LCV seems to be a key feature for the successful replication of *L. pneumophila* in both amoebae and macrophages (161). In addition, lipopolysaccharide is an independent factor that assists evasion of lysosomal degradation (162).

1.5.2.2 Recruitment of ER-derived vesicles

After phagocytosis by macrophages, *L. pneumophila* hijacks early secretory vesicles trafficking between the ER and the Golgi and incorporates their membranes and luminal content onto the LCV, leading to the establishment of an ER-like organelle that supports bacterial replication (108, 163-165). By intercepting early secretory vesicles, *L. pneumophila* acquires proteins residing in the transitional and rough endoplasmic reticulum (164). In macrophages, *L. pneumophila* acquisition of ER markers, such as calnexin, calreticulin or glucose-6-phosphatase involves the activity of small GTPases, including Sar1, Arf1 and Rab1 that influence membrane organelle fusion (164, 166, 167). The hijacking of secretory vesicles by *L. pneumophila* is also described when infecting amoebal hosts like *H. vermiformis* and *D. discoideum* (109, 147, 149, 167). Using magnetic immune-separation with an antibody against the LCV localised effector SidC, followed by density gradient centrifugation to purify intact LCVs, the proteome analysis of LCVs in macrophages RAW264.7 revealed the presence of multiple small GTPases, including Arf1, Rab7, Rab14, Rab21 (168). Similar proteome analysis of LCVs in *D. discoideum* revealed 566 host proteins, including Arf1, Rab1 and other Rab GTPases (169). These studies suggest similar biological events in mammalian and amoebal hosts.

Rab1, which regulates the trafficking between ER and Golgi complex, is recruited to the LCV within the first hour of infection, and at least six Dot/Icm effector proteins have been reported to target Rab 1, indicating a complex regulation of Rab1 during *L. pneumophila* infection (163, 166). SidM acts as a guanine exchange factor (GEF) for Rab1 to recruit it to the LCV and is synergised by another effector protein LidA, which attaches to the cytoplasmic face of the LCV (170). SidM also harbours an amino-terminal region which acts as a nucleotide transferase that covalently attaches an AMP moiety onto a conserved tyrosine residue of Rab1, a reaction known as AMPylation (171, 172). Another effector protein AnkX modifies Rab1 through the

addition of a phosphocholine (PC) moiety to a serine residue using CDP-choline as substrate, a reaction called phosphocholination (173). Phosphocholination by AnkX and AMPylation by SidM of Rab1 reduces its affinity for GAPs, stabilizing the Rab1 on the LCV and preventing its inactivation (171, 173). Interestingly, the effector protein SidD deAMPylases Rab1, while the effector protein Lem3 dephosphocholinates Rab1, reversing the activity of AnkX and SidM, respectively (174, 175). SidM-SidD, AnkX-Lem3 are thus termed metaeffectors, meaning an effector protein mediating the function of another effector protein. In addition, effector protein LepB harbours GAP activity that converts Rab1 GTP to Rabw1 GDP (176). Therefore, *L. pneumophila* controls Rab1 activity spatially and temporally during infection by a series of Dot/Icm effector proteins.

Unlike Rab1, Rab5, which mediates phagosomal maturation through heterotypic endosomal fusion does not appear on the LCV (177, 178). The Dot/Icm effector protein VipD localises to endosomes and can catalyse the removal of PtdIns(3)P from endosomal membranes, blocking endosomal fusion by depleting early endosomal antigen 1 (EEA1) and other fusion factors such as Rab5 and Rab 22 (178). Another Dot/Icm effectors, Lgp0393, was reported to target Rab5, Rab21 and Rab22 (179). These observations highlight the ability of *L. pneumophila* to recruit secretory vesicles to the LCV.

1.5.2.3 Recruitment of mitochondria and ribosomes

Mitochondria are recruited to the LCV in macrophages and amoebal hosts. As early as 15 mins post infection in monocytes, 30% of LCVs were surrounded by mitochondria (108). This proportion is even higher at one hour post infection and reduces after 4 hours (108). Mitochondrial recruitment to LCV is also reported in the amoeba hosts, *Hartmannella vermiformis* (*H. vermiformis*), *Nagleria fowleri* (*N. fowleri*) and *D. discoideum* (109, 180, 181). Recruitment of mitochondria to the LCV was thought to be Dot/Icm dependent. However, mitochondrial number or movement is not different between wild type and Δ*dotA* infected Drosophila S2 cells within 4 hours post infection (182).

After 4 hours post infection, fewer smooth vesicles and mitochondria are present, while the LCV becomes surrounded by ribosomes (108). LCV biogenesis is generally completed by 8 hours post bacteria uptake, and bacteria starts replication with a

doubling time of 2 hours during mid-log phase (183, 184). Replication of *L. pneumophila* within a ribosome-studded LCV has also been reported in amoebal hosts, *H. vermiformis*, *N. flowleri* and in the ciliated protozoa *Tetrahymena pyriformis* (109, 180, 185).

1.5.3 Egress of *L. pneumophila*

Host cell escape or egress is a fundamental step for *L. pneumophila* to exit depleted host cells and then infect other host cells (186). However, surprisingly, little is known about this process. At 12 hours post infection, 71% and 74% LCVs are disrupted within macrophages and *A. polyphaga* respectively (187). Formation of cytolysin pores less than 3 mm diameter in the host plasma membrane has been reported to be required for the lysis of macrophages U937 and *A. polyphaga*, and this is Dot/Icm system-dependent (188-191). The identification of mutants with less efficiency of bacterial release (*rib* mutants) showed that the Dot/Icm gene *icmT* was involved in bacterial release (188, 189, 191). The *rib* mutants, consistent with a Δ*icmT* mutant, displayed less pore-forming activity, but no intracellular replication defect in macrophages and *A. polyphaga* (188, 189, 191). However, release of the *rib* mutants from macrophage was likely due to host cell apoptosis rather than the pore-forming activity, while in *A. polyphaga*, failure of egressment led to reduced viability of the mutants (188, 189, 191). In addition, iron deficiency leads to early egress of *L. pneumophila*, suggesting that nutrient limitation might be a stimulus for *L. pneumophila* egressment (192).

In summary, several cellular processes subverted by *L. pneumophila* in macrophage and amoebae are similar, including remodelling of the LCV, recruitment of host secretory vesicles containing Arf1 and Rab1 to the LCV, anchoring of effector proteins to the LCV by PI(4)P metabolism and attachment of poly-ubiquitinated proteins to the LCV.

1.6 Protein secretion systems of *L. pneumophila*

Bacterial pathogens use a range of secretion systems to translocate virulence proteins during infection. These secreted proteins hijack host cellular processes to facilitate bacterial replication. At least seven secretion systems have been discovered in Gram-negative bacteria (193). *L. pneumophila* harbours Type I secretion system, Type II

secretion system and Type IV secretion system for efficient and rapid deliverance of substrates into the phagocytotic host cells.

1.6.1 The Type I secretion system of *L. pneumophila*

The Type I secretion system (T1SS) or ABC transport system in bacteria mediates the secretion of diverse protein substrates from the cytoplasm into the extracellular milieu (194). T1SS works as a tripartite double–membrane-spanning channel composed of an inner membrane component (IMC), a periplasmic adaptor membrane fusion protein (MFP) and an outer membrane protein channel which functions to export the substrate into the extracellular environment (TolC) (193, 195). To date, the best-studied T1SS is the hemolysin system in uropathogenic *Escherichia coli* (UPEC). A pore-forming Rtx toxin, HlyA, is secreted via this system. Despite great diversity in size and function, the most commonly found substrates of T1SS enzymes are toxins such as lipases and proteases, often associated with nutrient acquisition (196, 199) (193, 196).

L. pneumophila harbours a putative T1SS, which is composed of six genes *lssXYZABD* (197). LssB and LssD show significant similarity to the type I secretion system of *V. cholerae* and *E. coli*, respectively (197). Genome analysis indicates that TolC-like proteins and HlyD-Pfam (MFP) domain-containing proteins are also present in the *L. pneumophila* genome (197). LssB contains a C39 peptidase –like motif, which is a signature of the ABC transporter group (198). The functionality of the LssB-LssD-TolC T1SS was demonstrated recently, where *lssB*, *lssD* and *tolC* genes were required for RtxA secretion and RtxA-mediated pore-forming activity of *L. pneumophila* Paris strain (198). Absence of T1SS led to strongly attenuated virulence of *L. pneumophila* in *A. castellanii* and *D. discoideum*, but only to a moderate degree in macrophages U937 (198). Interestingly, the intracellular replication defect was not caused by lack of LCV biogenesis, but the entry of bacteria, suggesting that the *L. pneumophila* T1SS plays a role mainly in facilitating bacterial invasion to hosts (198).

1.6.2 The Type II secretion system *L. pneumophila*

Type II secretion system (T2SS) or general secretion pathway is a two step secretion process to secret folded proteins from the periplasm to extracellular environment

(199). Firstly, proteins to be secreted are delivered across the inner membrane and into the periplasm via the Sec translocation or Tat pathway (193, 200, 201). Proteins are then transported across the outer membrane by the T2SS apparatus after folding into a tertiary conformation (193, 200, 201). Accordingly, T2SS consists of four parts: an OM complex, a periplasmic pseudopilus, an IM platform and a cytoplasmic ATPase. Substrates of T2SS include toxins, proteases and diverse enzymes.

The T2SS, Lsp system, of *L. pneumophila*, was first discovered in 1999 based on the presence of PilD (202). Later, the full set of Lsp genes were confirmed by genome sequencing in strains Aalcoy, 130b, Corby, Lens, Paris and Philadelphia and 43290 (158, 203-207). Existence of Lsp system in other strains was further confirmed by Southern hybridisation and PCR analysis using Lsp- specific probes and primers (208, 209). The Lsp system is required for bacterial replication in both freshwater amoebae and human macrophages (202, 208, 210-214). And in an aquatic environment, the Lsp system is essential for *L. pneumophila* survival in temperatures as low as 4℃ in at least four genera of amoebae (202, 210-216). The Lsp is also involved in *L. pneumophila* biofilm formation and sliding motility (122, 217). *L.pneumophila* mutants lacking Lsp have a severe replication defect in the human macrophage-like cell line U937 and murine macrophages derived from A/J mice (208, 213). The growth defect is not from reduced phagocytosis or more degradation through the phagosome-lysosome pathway but due to a replication defect in LCV from 4 to 12 hours (218).

L. pneumophila triggers cytokine production during infection, and interestingly, Lsp has a dampening effect on the cytokine response in human U937 macrophages but not murine macrophages. (94). Higher cytokine levels like IL-6 were observed in *lsp* mutant infected U937 cells compared to that of wild type infected cells (219). This was due to the inhibiting effect on TLR2-MyD88 signalling, which subsequently influences both NF-KB and MAPK activation (219). However, the mechanism is still unknown.

Lsp secrets over 27 substrates, and nearly 20 harbour enzymatic activities (110-113). Lsp substrates like the acyltransferase PlaC, metalloprotease ProA, RNase SrnA, NttA and NttC are known to potentiate infection in protozoan hosts (211, 215, 220). However, the importance of those Lsp substrates varies in different amoebae hosts.

From mutagenesis studies, *srnA, nttC, plaC* and *proA* were all required for the optimal replication in *H. vermiformis* but not *A. castellanii* (211, 215). *L. pneumophila* lacking both *srnA* and *proA* displayed a more severe replication defect in *H. vermiformis* than that exhibited by mutants lacking *srnA* or *proA*, implying the combined effect of Lsp substrates in *L. pneumophila* infection (221). The Lsp substrate ChiA was the first protein with chitinase activity reported to promote bacterial infection in mammalian host cells (222). Mutants lacking *chiA* maintain the ability to replicate in U937 cells but are recovered less at 72 hours post infection, suggesting that ChiA is associated with bacterial persistence rather than replication (222).

In summary, Lsp substrates facilitate bacterial survival in water and amoebae hosts, as well as bacterial persistence in mammalian cells and a reduced activation of host cytokines. Lsp secretes various effector proteins to promote bacterial survival in the environment and disease progression.

1.6.3 The Type IV secretion system of *L. pneumophila*

Type IV secretion systems (T4SS) are present in both Gram-negative and Gram-positive bacteria, as well as in some archaea (223). Compared with other secretion systems, T4SSs have the special ability to translocate DNA (in addition to proteins) into bacterial or eukaryotic hosts. For pathogens like *Agrobacterium tumefaciens*, *Helicobacter pylori*, *Bordetella pertussis*, *Brucella spp*., *L. pneumophila*, *Coxiella burnetii*, T4SSs export effector proteins that are required for the intracellular replication niche. The T4SSs have been classified into two major groups: T4ASS and T4BSS. T4ASS has been well characterised and consists of the *Agrobacterium* VirB T4SS and *E. coli* conjugation apparatuses encoded by the R388 and pKM101 plasmids (224, 225). The VirB system consists of a lytic transglycosylase (VirB1), pilins (VirB2, VirB5), inner-membrane proteins (VirB3, VirB6, VirB28), ATPase (VirB4, VirB11, VirD4) and three factors (VirB7, VirB9, VirB210) that span the double membranes (226). The T4BSS consists of more than 25 proteins for transportation, of which only a few are homologous to the VirB subunits, and most are specific for the IVB machinery (225).

The *L. pneumophila* Dot/Icm T4BSS was characterised after two groups of genes were discovered independently to be important for LCV biogenesis: *dot* (for a defect in organelle trafficking) and *icm* (for intracellular multiplication) (114-116). Notably,

the Dot/Icm T4BSS is encoded in 5 loci in two distinct genomic regions (227). Region 1 comprises *dotDCB* and *dotA-icmVWX* while Region 2 comprises *icmTSRQPONMLKEGCDJBFH* (228-230). Gene order and orientation are highly conserved in the majority of the *Legionella* genus (231). The Dot/Icm core complex consists of at least five proteins: DotC, DotD, DotF, DotG, DotH, and form a ring-shaped structure (232, 233). Outer membrane lipoproteins DotC and DotD recruit DotH to the outer membrane, forming the DotC-DotD-DotH complex (224). DotG forms a central channel spanning inner and outer membranes with its C-terminal domain in the outer membrane complex (233). The embedment of DotG into the complex is promoted by DotF (224). Recently, electron cryotomography (ECT) revealed the *in situ* structure of the Dot/Icm secretion system (234). Despite little sequence similarity between the Dot/Icm secretion system and representative T4ASS like R388 plasmid in *E.coli*, the overall structures were remarkably similar (234). However, neither T4ASS nor T4BSS showed an obvious tube-like channel for substrate transport (234). In addition, the Dot/Icm secretion system localised at both poles of *L. pneumophila*, and this localisation was essential for the bacterial virulence (235).

Dot/Icm substrates have been identified using a variety of techniques (236, 237). Bioinformatics approaches have investigated protein sequences for the presence of C-terminal Dot/Icm translocation motif E block, eukaryotic domains and similar features to known effector proteins (207, 238-240). Several direct fusion assays such as the TEM-1 β-lactamase quantitative assay have been developed to measure the protein delivery of putative Dot/Icm effector proteins to infected host cytosol (238, 241, 242). Collectively, over 330 *L. pneumophila* Philadelphia-1 proteins are translocated into the host cells by the Dot/Icm system (117). However, due to a large number of effector proteins and functional redundancy among them, deletion of single effector protein-encoding gene does not always impair LCV biogenesis or bacterial intracellular replication (207, 243-245). So far, SdhA and MavN are the only two effector proteins that result in a bacterial replication defect when deleted (246-249). Even deletion of 31% of the Dot/Icm effector protein reservoir has little impact on the ability of *L. pneumophila* to replicate within mouse macrophages (250). Thus, the functional redundancy of effector proteins is a barrier to characterisation of the role of effector proteins during infection. Even so, Dot/Icm effector proteins are involved in

every stage of the infection cycle and are considered to be the most essential virulence factors of *L. pneumophila* (251).

1.7 Regulation of the Dot/Icm effector proteins by two-component systems

The translocation of over 330 effectors via the Dot/Icm secretion system is essential for *L. pneumophila* pathogenesis in different hosts. The cellular processes manipulated by *L. pneumophila* and the stepwise events that occurred during infection are highly regulated at the gene expression level (161, 252, 253). Two-component systems (TCSs) are comprehensive stimulus-response coupling systems that enable bacteria to respond to environmental stimuli mainly by altering gene expression (161). A typical two-component system consists of a membrane-embedded histidine kinase sensor and a corresponding cytoplasmic response regulator (RR) (254). Generally, the sensor histidine kinase is autophosphorylated after sensing the stimulus. The response regulator is activated through transfer of the phosphoryl group from a histidine residue to an aspartic acid residue in the regulator. So far, four TCSs have been discovered in *L. pneumophila* that control Dot/Icm effector genes expression. These are CpxRA, PmrAB, LetAS, and LqsRS (Table 1.2).

1.7.1 The CpxRA two-component system

The CpxR-CpxA TCS consists of a sensor histidine kinase CpxA, cytoplasmic response regulator CpxR and the accessory protein CpxP. Three phosphotransfer reactions are involved in this system, including autophosphorylation of a conserved histidine of CpxA, the transphosphorylation of an aspartate of CpxR and the dephosphorylation of phosphorylated RR to return the system to the restimulated state (255). The Cpx TCS was initially identified and characterised in *E. coli*. CpxA senses a broad range of stimuli including pH, misfolded lipids that cause stress to the bacterial envelope, leading to the phosphorylation of CpxR and transcription of downstream genes (256, 257). Genetic screening in *L. pneumophila* discovered that CpxR binds to GTAAA of the gene *icmR*, which is a component of the Dot/Icm secretion system, leading to its upregulation (258). The CpxR regulatory elements are located between 49 and 54 bp from the promoter element in these activated genes, whereas the CpxR regulatory elements are located from -24 to 98 bp to the promotor element in repressed genes (259). Any shift of the distance of regulatory regions to the promotor elements results in decreased gene activation by CpxR or even prevents

expression, indicating a specific narrow range required for efficient CpxR regulation (259). Furthermore, CpxR can be phosphorylated by the small phosphate donor molecule acetyl phosphate(Ac-P) independent of CpxA (259). As a result, deletion of *cpxA* only partially blocks the system, leading to moderate phenotype for the CpxR regulated genes (259).

Bioinformatic analysis of *L. pneumophila* genome to identify CpxR regulatory regions revealed 38 genes putatively regulated by CpxR, including 27 Dot/Icm effectors genes, four Dot/Icm components (*icmR*, *icmV*, *icmW* and *lvgA*) and two genes encoding regulatory components (*letE* and *oxyR*) (259-261). Gene expression studies and mobility shift assays confirmed the direct interaction between CpxR and the regulatory regions for controlled Dot/Icm genes as well as effector genes (261). Sixteen of the CpxR regulated genes were activated by CpxR, while the remaining 22 genes were repressed (259).

CpxR regulates a broad range of functions including pilus assembly, adherence, biofilm formation as well as host cell invasion in several species (262, 263). *L. pneumophila* CpxR can self-interact which, to some degree, guarantees the downstream gene activation (259). When examining intracellular replication in different hosts, deletion of *cpxR* leads to defects in intracellular replication in *A. castllanii*, while deletion of *cpxA* does not (257). However, the defect is not observed in the HL-60 and U937 human macrophages (257, 259). SidM/DrrA and SidD are effector proteins whose expression is regulated by this system (161, 261). But most of the CpxRA regulated effector proteins are uncharacterised. Furthermore, the environmental stimuli that activate the *L. pneumophila* CpxA sensor kinase are unknown (263).

1.7.2 The PmrAB two-component system

The PmrAB two-component system is well studied in *Salmonella enterica* and consists of sensor histidine kinase PmrB and cytoplasmic response regulator PmrA (264). This system is activated when sensing environmental stimuli such as low pH or high concentrations of Fe^{3+} (265-267). Bioinformatic analysis of the *L. pneumophila* genome revealed 13 effector genes harbouring the conserved binding sequence of the PmrA regulator, which is quite similar to the sequence characterised in *S. enterica* (264, 268). The gene encoding PmrA is required for bacterial replication in

amoeba, (240, 264). *L. pneumophila* PmrAB directly activates the expression of 45 Dot/Icm genes, and the existence of PmrAB regulatory elements in the upstream sequence of many hypothetical proteins has led to validation of novel Dot/Icm effector proteins (236, 264). The environmental stimuli activating *L. pneumophila* PmrB are unknown but possibly are related to the pH levels of the LCV, as *L. pneumophila* inhibits phagosome-lysosome fusion early during infection (108, 269).

Among the PmrAB regulated effectors, SdhA, SidF and AnkB are involved in the maintenance of LCV and anti-apoptosis activity (246, 247, 270, 271). SidL, SidI and Lgt3 interact with eukaryotic translation elongation machinery, leading to host protein synthesis inhibition (272-274). Interestingly, despite the similar functions of Lgt1 or Lgt2 to Lgt3, regulation of Lgt1 and Lgt2 by PmrAB is not confirmed (272, 273).

1.7.3 The LetAS two-component system

The *L. pneumophila* LetAS TCS consists of response regulator LetA and sensor kinase LetS (275). Orthologs of *letA* are present in several species of gamma subdivision of *Proteobacteria: gacA* in *Pseudomonas* species, *expA* in *Erwinia carotovora, uvrY* in *E. coli varA* in *Vibrio cholerae* (*V. cholerae*) and *sirA* in *S. enterica* (276, 277). This TCS plays an important role in the regulation of virulence genes (276). The LetA/S was first discovered to be involved in the expression of flagellin and later was characterised as stress regulator in stationary phase and is required for the efficient replication of *L. pneumophila* in *A. castellanii* (275, 278, 279). Subsequently, LetA was confirmed to regulate the Dot/Icm secretion system by influencing the expression of *dotA* and a regulatory cascade of signal transduction from LetS to individual effector encoding genes was also described (280). LetA is activated by LetS during stationary phase by a four-step phosphorelay (281). Activated LetA upregulates two non-coding small RNAs, RsmY and RsmZ, which jointly antagonise CsrA to relieve its repression on effector protein-encoding genes. Thus, effector proteins are post-transcriptionally regulated by the LetA-CsrA cascade (282). In addition, the gene encoding CsrA is essential for intracellular multiplication in amoebae and its expression is activated by the PmrA response regulator (282, 283).

Expression of at least four effector proteins is regulated by LetAS TCS in *L. pneumophila* Philadelphia-1 strain: RalF, LegC2, LegC7, VipA, while the number is highly likely to expand with more studies (282). The product of effector genes regulated by the LetAS-RsmYZ- CsrA cascade all appear to perform their function early during infection (282, 284).

1.7.4 The LqsRS two-component system

LqsRS TCS in *L. pneumophila* was initially identified as homologous to the *V. cholerae* CqsAS quorum sensing system (285). This system consists of a putative autoinducer synthase LqsA, a sensor kinase LqsS and a response regulator LqsR (285). The diffusible signalling molecule 3-hydroxypentadecan-4-one (*Legionella* auto induce-1 LAI-1) is catalysed by LqsA and is presumably recognised by the sensor kinase LqsS, which subsequently activates LqsR (286). LqsRS TCS regulates the transition from stationary phase to the replicative growth phase, *L. pneumophila* morphology, as well as virulence gene expression (285, 287). Inactivation of the *lqsR* gene impairs intracellular replication in both amoeba and macrophage host (287). DNA microarray analysis between *lqsR* mutant and wild type *L. pneumophila* revealed multiple genes regulated by LqsRS, including the Dot/Icm component LvgA and 12 effector protein genes (285). As a result, the protein level of the effector proteins is influenced by the absence of the LqsRS component (287, 288). The expression of LqsR requires the RpoS sigma factor and is modulated by the response regulator LetA (285). Additionally, the expression of LqsR is post-transcriptionally influenced by RsmY-RsmZ-CsrA cascade (289). However, as LqsR lacks any known DNA binding motif, how it affects target gene expression is still unclear.

Among the LqsRS TCS regulated effector proteins, the function of AnkX and SidK have been discovered. AnkX activates Rab1 on the LCV by catalysing the transfer of phosphocholine to Rab1 (175, 290, 291). SidK interacts with a VatA, contributing to the avoidance of phagosome acidification during the early stage of *L. pneumophila* infection (288).

1.8 Modulation of host cellular processes by *L. pneumophila* Dot/Icm effector proteins

1.8.1 Dot/Icm effector proteins affect host transcription

L. pneumophila Dot/Icm effector proteins can interfere with NF-κB in different ways to manipulate host cell proinflammatory transcriptional response and facilitate bacterial survival. Bacterial structures such as surface-exposed lipopolysaccharide (LPS), peptidoglycan, flagellin and bacterial DNA containing methylated CpG motifs, are recognised by pattern-recognition receptors including Toll-like receptors (TLRs) (292). The activation of TLRs induces an antimicrobial response through downstream signal transduction pathways, in particular the activation of NF-kB and MAPK, leading to increased transcription of proinflammatory cytokines (292, 293). *L. pneumophila* was reported to perform biphasic regulation on NF-κB pathways. *L. pneumophila* activated an early TLR5 and MyD88-dependent NF-κB activation, which is followed by a later Dot/Icm system-dependent activation (294). Indeed, *L. pneumophila* mutants with defective Dot/Icm system showed very low activation of host MAPK and NF-κB pathways (295, 296). Several Dot/Icm effector proteins have been reported to activate the late NF-κB response. The Dot/Icm effector protein, LegK1 is a serine/threonine protein kinase that directly phosphorylates the NF-κB inhibitor IκBα, leading to robust NF-κB activation (297). LnaB, another Dot/Icm effector protein without sequence similarity to known proteins, also activates NF-κB (298). Additionally, the Dot/Icm effector protein, EnhC, interferes with the degradation of peptidoglycan, allowing bacteria to control the production of pattern-recognition receptor ligands and evade immune recognition by suppressing Nod1-dependent NF-kB activation (299, 300). Furthermore, the activation of NF-κB signalling promotes host survival by inducing the transcription of anti-apoptotic genes, which also supports bacterial invasion. The Dot/Icm effector proteins, SidF and SdhA, have been reported to prevent host cell apoptosis during infection (301, 302). Current findings indicate a clear role for *L. pneumophila* Dot/Icm effector proteins in affecting the transcription of the proinflammatory cascade during infection.

In addition, *L. pneumophila* has been shown to induce epigenetic modification to affect host transcription. The *L. pneumophila* Dot/Icm effector protein RomA/LegAS4 contains a SET domain (suppressor of variegation, enhancer of zest, and trithorax) and methylates histones of host cells. RomA/LegAS4 of *L. pneumophila* Paris strain localizes to the infected cell nucleus where it promotes a

burst of H3K14 methylation, which plays a crucial role in epigenetic control of gene transcription (303). H3K14 methylation results in global gene transcriptional repression, including genes associated with innate immunity (303). RomA/LegAS4 from *L. pneumophila* Philadelphia stain, on the other hand, catalyses H3K4 methylation to promote increased transcription of rDNA genes through direct interaction with heterochromatin binding protein 1 and does not trigger a global host gene transcription (304). Another Dot/Icm effector protein, SnpL, localises to the nucleus and binds the eukaryotic transcription elongation factor SUPT5H, which is involved in regulating RNA polymerase II-dependent mRNA processing and elongation (305, 306). Ectopic expression of SnpL led to massive upregulation of host gene expression and macrophage cell death (306). Overall, the *L. pneumophila* Dot/Icm effector proteins can target the host nucleus and affect host transcriptional response directly.

1.8.2 Dot/Icm effector proteins inhibit host protein translation

Many pathogens are hypothesised to disrupt host translation to increase the availability of amino acid or to dampen the host response (307). *L. pneumophila* has been reported to inhibit host protein synthesis in mammalian cells (308). At least eight *L. pneumophila* Dot/Icm effector proteins have been reported to inhibit host cell translation in mammalian cells and most of these effector proteins interact with eEF1α (272, 274, 309, 310).

Lgt1–Lgt3 glucosylate eEF1α at residue S53, leading to blocking of protein biosynthesis and host cell death (272, 311, 312). SidI targets both eEF1α and eEF1β, two components of the elongation machinery of eukaryotic protein synthesis, and such interaction leads to the inhibition of host protein synthesis (313). The interaction between SidI and eEF1α also induces the expression of heat shock protein Hsp60, which is likely beneficial to *L. pneumophila* replication (274). Interestingly, the translation inhibition induced by SidI is regulated by a metaeffector protein, SusF, which binds to SidI and suppresses SidI-mediated inhibition of protein translation (314). Another effector protein, SidL, is toxic to mammalian cells and is capable of inhibiting protein translation *in vitro* via an unknown mechanism (307). However, deletion of five effector genes (*lgt1–lgt3*, *sidI*, *sidL*) only displayed compromised

ability to inhibit host translation, suggesting that *L. pneumophila* may encode additional inhibitors of host translation (307).

In addition, instead of interacting with eEF1α, effector protein LegK4 phosphorylates cytosolic Hsp70 and leads to reduced ATPase activity and protein refolding activities of Hsp70, resulting in inhibited global translation in mammalian host cells (315).

1.8.3 Dot/Icm effector proteins modulate host mitochondria dynamics

L. pneumophila Dot/Icm effector proteins have been reported to modulate host mitochondria dynamics to advantage bacterial replication (316). Mitochondria are highly dynamic organelles that move within eukaryotic cells and change morphology to meet the energy requirements of a cell. Single mitochondria can fuse to form the highly energised elongated mitochondrial networks, or can divide into single organelles with decreased activity (317). An *L. pneumophila* chaperonin that accumulates in the LCV, HtpB, has been suggested to mediate mitochondria recruitment and induce microfilament rearrangements in CHO cells (318). The Dot/Icm effector protein, MitF, induces mitochondrial fragmentation through accumulation of mitochondrial DNM1L,which is a fission associated GTPase (316). The Dot/Icm effector protein LncP resembles a mitochondrial central protein in the mitochondrial inner membrane during *L. pneumophila* infection *in vitro*, catalysing unidirectional transport and exchange of ATP across membranes, working as a putative ATP transporter (319). The Dot/Icm effector protein, Lpg1974 shows high homology with human voltage-dependent anion channels, suggesting a potential role in mitochondrial communication (320). In addition, although *L. pneumophila* infection induces mitochondrial fragmentation, the release of cytochrome c (CytC), a cell death signal, is not triggered until 12 hours post infection, nor is the Annexin-V or caspase-1 activation detected (316). The Dot/Icm effector protein, Lpg1137, is a serine protease secreted to host mitochondrial membranes (321, 322). Lpg1137 cleaves a soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE), syntaxin17, participates in the regulation of mitochondrial dynamics and autophagy. Thus, cleavage of syntaxin17 blocks autophagy and staurosporine-induced apoptosis, subsequently blocking ER-mitochondria communication. Overall, *L. pneumophila* actively interferes with host mitochondrial dynamics to facilitate bacterial replication by modulating host metabolism and inhibiting host cell death.

1.8.4 Dot/Icm effector proteins manipulate host ubiquitination system

As one of the most prevalent post-translational modifications, protein ubiquitination regulates multiple cellular processes in eukaryotes, including cell cycle, vesicle trafficking, cell signalling and DNA repair (323). Accordingly, hijacking the host ubiquitin system is an ideal strategy for pathogenic bacteria to control multiple host cell processes. Ubiquitin is a 76 residue protein that binds covalently to substrate protein. This catalytic reaction is through a ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3). E1 activates ubiquitin in an ATP-dependent manner, leading to the formation of a thioester bond between an active-site cysteine and the C-terminal glycine residue of ubiquitin. After a transesterification reaction, the active ubiquitin is transferred to the active cysteine of an E2 enzyme. Finally, an E3 enzyme bridges both the ubiquitin-charged E2 and the specific substrate protein, leading to the attachment of ubiquitin to substrate proteins (323).

So far, genetic screening and bioinformatic studies have identified several *L. pneumophila* effector proteins containing U-box or F-box like domains. The majority of the U-box and F-box proteins are known effectors of the Dot/Icm secretion system (271, 324, 325). Seven F-box containing effectors (LegU1, LicA, Lpg1975/Lpp1959, AnkB/LegAU13, PpgA/Log2224, Lpg2525, Lpp2486) have been identified in *L. pneumophila* strain Philadelphia 1 and Paris (Table 1.1) (324-328). F-box is the motif interacting with other proteins such as SKP1 (329). As bacteria do not produce SKP1, CUL1 or RBX1, *L. pneumophila* F-box proteins require a host SCF component to be functional (329). Effector proteins LubX, GobX and RavN, have been characterised as U-box proteins (324, 330). The effectors, LegU1, AnkB, GobX, LuxB and RavN have been shown to possess E3 ligase activity through biochemical studies (117, 271, 324, 325, 331, 332).

Some *L. pneumophila* Dot/Icm effector proteins execute novel E3 activities. SidC and its paralogue SdeA anchor to the LCV via phosphatidylinositol-4 phosphate and recruit ER vesicles and ubiquitinated proteins to the LCV (333-335). Structural studies reveal a canonical Cys-His-Asp catalytic triad (C46-H444-D446) that is required for SidC and SdcA to be functional (336, 337). The SidE family of effectors including SdeA, SdeB, SdeC and SdeE utilise an abnormal ubiquitination system that

is independent of host E1 and E2 enzymes (338). SidE effectors use NAD to activate ubiquitin with ADP-ribosylation at Arg 42 and form an intermediate ADP-ribose-ubiquitin (ADPR-Ub) (339). This process is catalysed by a mono-ADP ribosyltransferase (mART) motif harboured by this protein family, independent of E1and E2 and ATP (339). ADPR-Ub is subsequently cleavaged by phosphodiesterase (PDE) activity, which is also embedded in SidE protein sequence, resulting in the release of AMP (339). Interestingly, both ADPRUb and PR-Ub produced by SidE impairs the conventional ubiquitination cascade by preventing the activation of E1 and E2 enzymes, thereby interfering with a wide range of ubiquitination-dependent cellular events like mitophagy and TNF signalling (340). SidJ is a metaeffector protein that mediates the removal of SidE family proteins from the LCV during late infection and thus inhibits toxicity to the host cell (244, 341).

Strategies applied by *L. pneumophila* varies from mimicking E3 ligase to DUBs, accompanied with novel activity strategies. Despite the importance of LCV biogenesis, the exact consequences of effector mediated ubiquitin modification are still unclear. One limitation is the characterisation of host targets of these effector proteins. Hence, researches on these effectors will add further insights into *L. pneumophila* pathogenesis and eukaryotic signalling processes (338).

1.8.5 Meta-effectors, the self-regulation of Dot/Icm effector proteins

Aside from interacting with host targets, *L. pneumophila* Dot/Icm effector proteins present complex self-regulation framework. Some effector proteins, termed metaeffectors, regulate the function of other effector proteins (342). The first characterised metaeffector, LubX, is a U-box type E3 ligase that contains two U-box domains which are crucial for its E3 activity and substrate binding activity, respectively (324, 330). Despite targeting host kinase, Cdh1 (Cdc2-like kinase 1) and mediating its ubiquitination, LubX displays the ability to target another Dot/Icm effector, SidH, for ubiquitination in late infection phase (324, 330, 342, 343). SidH is a protein whose protein level is associated with bacterial intracellular replication in CHO-FcγRII cells (342). The regulation of SidH might be a strategy of self-management contributing to the overall control of LCV biogenesis. SidJ is another metaeffector protein that mediates the removal of SidE family proteins from the LCV during late infection and thus inhibits toxicity to the host cell (244, 341). Unlike the

direct ubiquitination of SidH by LubX, the mode of interaction between SidJ and SidE family proteins is unknown (117).

In addition to metaeffector regulation, another mode of regulatory complexity is that one effecter protein may reverse the activity of another. For example, AnkX catalyses the transfer of phosphorylcholine to Rab1, while Lem3 is a dephosphorylcholinase that abrogates AnkX-mediated modification on Rab1 (173, 175). Similarly, SidM AMPylates Rab1 by covalently adding adenosine monophosphate (AMP), whereas SidD deAmpylates Rab1 (176, 344). The presence of this regulatory framework allows spatiotemporal control of important activities during different infection stages in the host cell (117).

1.9 Functional redundancy of Dot/Icm effector proteins

L. pneumophila Dot/Icm effector proteins display high functional redundancy, which suggests compensatory roles for two effector proteins or set of effector proteins. Among bacterial pathogens studied to date, *L. pneumophila* carries the largest Dot/Icm effector protein arsenal, and extensive studies have been carried out to characterise the function of effector proteins in mammalian hosts (117, 158, 345). While the deletion of some effector genes has resulted in a modest reduction in *L. pneumophila* virulence, only the deletion of *sdhA* (essential for maintaining the integrity of LCV) and *mavN* (which is involved in ferrous iron transport) has resulted in defective intracellular replication (246-249). O'Connor *et al.* constructed a *L. pneumophila* strain with a minimised genome to distinguish effector proteins essential for intracellular replication versus effector proteins necessary for growth in culture (250). Despite lacking 31% of known effector genes, this strain of *L. pneumophila* replicated to the same level as wild-type *L. pneumophila* in mouse macrophages (250).

Functional redundancy of effector proteins can be partially attributed to the existence of multiple effector protein paralogues. For example, the SidE effector family includes four homologous effector proteins SidE, SdeC, SdeB and SdeA, which catalyse the ubiquitination of host reticulon 4 (Rtn4) and Rab33b (339, 346). Reduced intracellular replication in *D. discoideum* was observed only when the entire SidE family was deleted (152, 339). Functional redundancy can also occur when effector proteins mediate similar processes during infection, despite different biochemical

activities or targets (183, 347). In *Salmonella enterica*, an intracellular pathogen that infects cattle, poultry and humans, type III effector proteins SopE and SopB promote bacteria uptake via activating Rho-family GTPases independently (347). While SopE is a GFP that activates Rho-family GTPases directly, SopB is an inositol polyphosphate that indirectly activates endogenous GEFs for Rho GTPases (347). As a result, only by eliminating both effector proteins can decreased *S. enterica* uptake be observed (348). In *L. pneumophila*, the effector protein RalF functions as a GEF for ADP ribosylation factor 1 (ARF1), which is a small GTPase involved in retrograde vesicle transport from Golgi to ER (349, 350). Elimination of Arf1 function results in a severe defect in bacterial replication, whereas removal of RalF does not (183, 349). One hypothesis is that *L. pneumophila* strains lacking RalF might stimulate the activities of endogenous GEFs using effectors with unrelated functions (183, 349). Effector proteins mediating similar processes by independent pathways may also lead to effector redundancy (183). More generally, effector proteins that modulate more than one host cellular process to accomplish a common goal can lead to redundancy at the system level (351). The existence of paralogues and functional redundancy of host processes are said to allow *L. pneumophila* to infect multiple hosts in the environment.

1.10 Protozoan hosts as cellular models for *L. pneumophila* infection

As heterotrophic predators, free-living amoebae (FLA) prey on bacteria, fungi and other protozoa. After ingestion by amoebae, numerous bacteria can escape being lysed and survive or even proliferate intracellularly (352, 353). Among these are pathogenic microorganisms such as *Salmonella* spp and *L. pneumophila*. Thus, FLA act as reservoirs and carriers for pathogenic microorganisms to distribute, leading to potential threats to public health (354). *L. pneumophila* grown in amoebae shows more resistance to antibiotics, acid, osmotic and thermal stress and its infectivity for humans is predicted to increase (353, 355-358). *L. pneumophila* grown in different amoebae species also display different features (356, 359-361). Moreover, interactions of *L. pneumophila* with protozoa hosts select for the acquisition of virulence factors that allow the bacteria to adapt to infect mammalian phagocytes (362-364). Given the similarity of the infection process between amoebae and macrophages, and the role of amoebae in *L. pneumophila* transmission, *A. castellanii*

and *D. discoideum* has been established as model protozoa for *L. pneumophila* infection (365).

1.10.1 *D. discoideum* as a cellular model for *L. pneumophila* infection

D. discoideum replicates by mitotic division of a single-cell and undergoes a sophisticated developmental program to form a multicellular organism (360, 366). The genome of *D. discoideum* is ~ 34 Mb and comprises six chromosomes (367). It encodes about 12500 predicted proteins, including a large number of mammalian orthologs (367). The completed sequenced genome of *D. discoideum*, combined with the molecular genetic tools established in *D. discoideum* allows the study of many fundamental cellular processes (360, 366, 368). *D. discoideum* has been used for the study of genes that in mutant form cause disease in humans (369).

Gene manipulation in haploid amoeba, random insertion mutagenesis, multiple gene deletions, RNA interference techniques and ectopic expression in *D. discoideum* are all available (370-373). For example, studies with *D. discoideum* mutants lacking different autophagy genes showed a different role for *atg9* and *atg16* in *L. pneumophila* intracellular replication (374). An extensive library of plasmids allowing constitutive or inducible production of fluorescently labelled *D. discoideum* is also available. *D. discoideum* producing calnexin-GFP or GFP-α-tubulin was employed to measure LCV and microtubule dynamics by live-cell imaging during *L. pneumophila* infection (375, 376). Proteomic analysis of purified LCVs revealed 560–1150 host proteins existing on LCVs (167, 377). As a result, studies in *D. discoideum* revealed numerous host cell factors and bacterial virulence factors implicated *L. pneumophila* infection (95, 158-160).

1.10.2 *A. castellanii* as a natural host for *L. pneumophila* replication

Acanthamoeba ubiquitously exists in soil and aquatic habitats and has been found in *Legionella*-positive habitats (95, 378). Similar to many other protozoa, *Acanthamoeba* adopts bi-phasic life cycle: active trophozoites and dormant cysts (Figure 1.2) (379). As trophozoites, *Acanthamoeba* replicates by binary fission in optimal conditions with a doubling-time of 8- 18 hours (359, 380). The trophozoites have a highly dynamic cytoskeleton and produce very active acanthopodia which are essential for movement or locomotion (381). *Acanthamoebae* actively move towards the source of

chemical signals produced by bacteria (95, 382, 383). This movement speed can reach 0.1-0.2 µm/s (95). Another typical feature of *Acanthamoebae* is the existence of contractile vacuoles, which expel water for osmotic regulation (384).

Due to stresses encountered in the adverse environment like starvation, adverse pH, osmotic pressure, and low-temperature conditions, *Acanthamoeba* becomes double-layer walled cysts, and this process is termed encystment (385, 386). *Acanthamoeba* cysts are a dormant form and are resistant to long-term starvation, low temperature and disinfectant treatments (379, 387). It was reported that *L. pneumophila* replicates in trophozoites and utilises double-layer cysts to escape adverse environmental conditions (133, 388). Based on cyst morphology, *Acanthamoeba* was initially classified into four groups. Group I cysts are large (> 18 µm) and covered with stellate endocysts and smooth or wrinkled ectocysts; Group II cysts are smaller (<18 µm) with polyhedric, globular, ovoid, or stellate endocysts and wavy ectocysts; and Group III cysts (>19 µm) have globular or ovoid endocysts and smooth or wavy ectocysts (389). As the morphology of cysts changes with culture conditions and other factors, subsequently, *Acanthamoeba* spp were clustered into 20 different evolutionary lines, termed as T1-T20, based on the nuclear small subunit ribosomal RNA gene (390, 391). In axenic conditions, cysts can be induced to form trophozoites, which is termed excystation (392-394). Excystment is a process accompanied by the formation of endocytic channels, phagocytic structures and existence of pseudopodial projection (394). Subsequently, excystation occurs through the ostioles while leaving the cyst walls behind (394, 395). In addition, excystement efficiency is critically dependent on the age of cysts (396, 397).

Belonging to T4 genotype, *A. castellanii* has been used as a cellular model to study the function of *L. pneumophila* virulence factors. The genome of *A. castellanii* is polyoid and encodes around 15500 compact intron-rich genes (398). *A. castellanii* harbours several putative pattern recognition receptors (PRRs), many of which are homologous to the innate immune systems of higher organisms (398). However, the genomic complexity of *A. castellanii* is still a barrier for its utilisation as a model organism. Although approaches using RNA interference have been successfully established, knockout of genes of interest and transfection is difficult (399, 400). Nevertheless, the increasing number of available molecular tools together with the

known genome sequence make *A. castellanii* an indispensable tool for cellular research with *L. pneumophila*.

Overall, although co-isolation of *L. pneumophila* with *Dictyostelium* species is rare, the intracellular replication events of *L. pneumophila* in is highly corelated with that in *A. castellanii* (87). Apparantly, with the available cellular maker, gene manipulation tools and sequenced genome, *D. discoideum* is a valuable infection model for studying essential host factors during *L. pneumophila* infection (87). However, the capacity of *L. pneumophila* to infect a board range of hosts, rather than in a singl "natural host", is important for its pathogenesis in humans (401). *A. castellanii* was thought to be a comparative restrict host comparing with *H. vermiformis* and *D. discoideum*, as lacking of a group of genes led to bacterial replication defect in *A. castellanii*, but not in other hosts (250, 402). Thus, *A. castellanii* is a desirable infection model to elucidate the function of *L. pneumophila* virulence genes during *L. pneumophila* infection.

1.11. Aim of this study

As an opportunistic human pathogen, *L. pneumophila* harbours a large number of virulence factors, possibly resulting from thousands of years co-evolution with natural protozoan hosts. To date, only a subset of virulence factors such as selected Dot/Icm effector proteins has been elucidated in mammalian cells. Genetic redundancy and the complexity of virulence factor regulation continue to limit the pace of discovery. Considering the role of *A. castellanii* in *L. pneumophila* transmission and pathogenesis evolution, it is desirable to focus on host-specific virulence factors in *A. castellanii*. Additionally, how *L. pneumophila* influences *A. castellanii* in the environment is also not well studied. The broad aim of this study is to establish genetic tools in *L. pneumophila* and corresponding molecular assays in *A. castellanii* to investigate *L. pneumophila* virulence factors essential for bacterial pathogenesis, and potentially leading to strategies for *L. pneumophila* control in the environment. The specific aims are to:

1. Identify *L. pneumophila* genes required for optimal intracellular replication in *A. castellanii*.

2. Investigate how *L. pneumophila* infection affects *A. castellanii* morphology.

3. Investigate how *L. pneumophila* virulence factors affect the *A. castellanii* transcriptome during infection.

Table 1.1 *L. pneumophila* effector proteins involved in host ubiquitination system

Effector protein	Name	Interactor/substrates	Enzymatic activity	References
Lpg0171	LegU1	SKP1, Culin1, BAT3	E3 ligase activity, F box protein	(331)
Lpg1408	LicA	SKP1	F-box protein	(331)
Lpg2144/lpp2082	AnkB/LegAU13	SKP1, Culin1, Parvin B	E3 ligase activity, F box protein	(271, 331, 403)
Lpg2224	PpgA	Unknown	F-box protein	(331)
Lpg2525		Unknown	F-box protein	(331)
Lpg2486		Unknown	F-box protein	(338)
Lpg2455	GobX	Unknown	U-Box protein, E3 ubiquitin ligase	(404)
Lpg2830	LegU2/LubX	Clk1, SidH	U-Box protein, E3 ubiquitin ligase	(324, 342)
Lpg1111	RavN		U-Box protein, E3 ubiquitin ligase	
lpg2510	SdcA and SidC		E3 ubiquitin ligas	(336)
lpg2511				(338)
lpg0234	SidE	Rab1, Rab6a, Rab30, Rab33b, Rtn4	All-in-one ubiquitin conjugation enzyme; Deubiquitinase	
lpg2153	SdeC			
lpg2156	SdeB			
lpg2157	SdeA			
lpg1148	LupA	Unknown	Deubiquitinase	(405)
lpg2155	SidJ	Rab1, Rab6a, Rab30, Rab33b, Rtn4	Phosphodiesterase, Deubiquitinase	(406, 407)
Lpg2147	MavC	UBE2N	transglutaminase	(408)
Lpg2370		unknown	Predicted RING type E3 ligase	(328)
Lpg2577	MavM	unknown	Predicted RING type E3 ligase	(328)
Lpg2498	MavJ	unknown	Predicted HECT type E3 ligase	(328)
Lpg2452	SdcB, LegAU14	unknown	unknown	(328)

Table 1.2 *L. pneumophila* effector proteins regulated by two-component systems

Response regulator	Sensor kinase	Type of regulation	Additional components	Regulated effectors	References
CpxR (Lpg1438)	CpxA (Lpg1437)	Direct	-	CegC1, LegA10, CegC2, Ceg7, LegA11, Ceg18, CegC3, CegC4, SidH, SidM/DrrA, SidD, Ceg33	(258, 261)
PmrA (Lpg1292)	PmrB (Lpg1291)	Direct	-	Ceg2-7, SidE, Ceg8-10, SdhA, Ceg11, LegA9, Ceg14/SidL, Ceg15, Ceg17-21, SidG, Ceg22, LegC5, Ceg23, SidB, Ceg24-25, LegLC8, LegAU13, SdeC, SdeB, SdeA, Ceg28-30, LegA14, SdbB, LepB, Ceg32/ SidI, SidF, Ceg33, VipE, Ceg34	(240, 264)
LetA (Lpg2646)	LetS (Lpg1912)	Indirect	RsmY, RsmZ, CsrA, LetE	VipA, YlfB/LegC2, RalF, YlfA/ LegC7	(275, 276, 278, 281-283, 289, 409)
LqsR (Lpg2732)	LqsS (Lpg2734)	Indirect	LqsA, LqsT	Lpg0081, Lpg0294, Lpg0634 Lpg0967, SidK, RavN, Lpg1453, MavB, CegC4, SdbB, Lpg2844, Lpg3000	(285, 288)

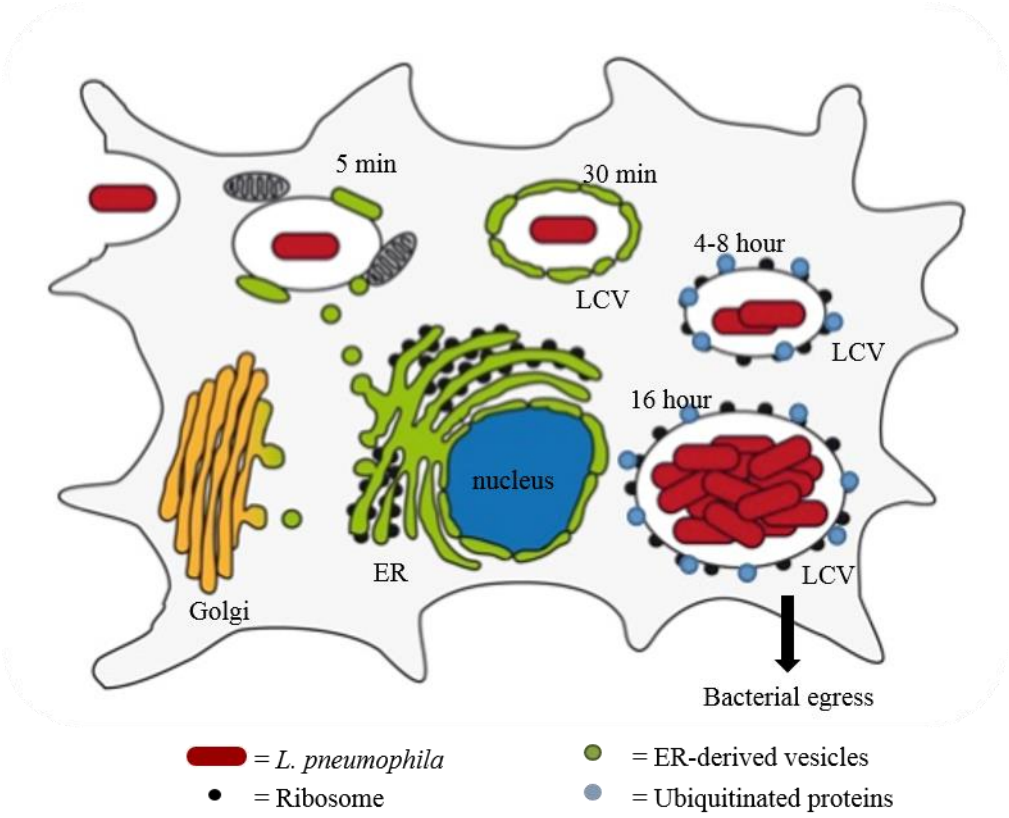


Figure 1.1 Infection strategy of *L. pneumophila*

Upon entry into alveolar macrophages or protozoan hosts via phagocytosis. Proteins and transport vesicles are recruited to LCV, which is slowly transformed into a ribosome-studded compartment that supports replication of *L. pneumophila*. Eventually, the host cell is lysed, and *L. pneumophila* are released to infect other macrophages. Image is generated by Ralf Schuelein.

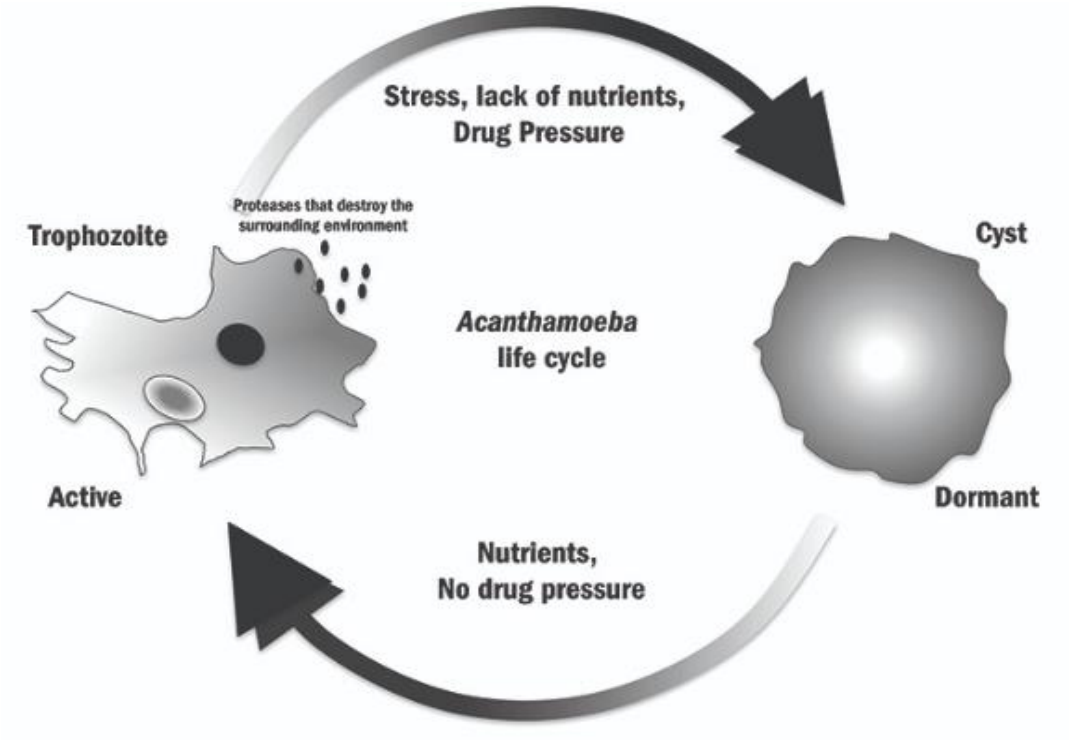


Figure 1.2 The life cycle of *A. castellanii*

Trophozoites are the active stage of *A. castellanii* with have highly dynamic acanthopodia, which are essential for movement or locomotion. *A.castellanii* turns to double-layer walled cysts in the adverse environment, like starvation or adverse pH. Cysts are the dormant stage and can be induced to trophozoites in axenic conditions *(410).*

Chapter 2: Materials and methods

2.1 Chemicals and reagents

Common chemicals and reagents used in this study were purchased from Sigma Aldrich, Merck, Amresco, Invitrogen, Astral, Bioline or BDH chemicals. Media components for routine culturing of bacteria and *A. castellanii* were purchased from Asia Pacific Solutions (APS), Sigma-Aldrich, BD or Oxoid. DNA restriction enzymes were purchased from New England Biolabs (NEB) or Promega, and antibiotics from Amresco, Boehringer Ingelheim or Invitrogen.

2.2 Bacteria strains and growth conditions

Bacteria strains and plasmids used in this study are listed in Table 2.1. Generally, *E. coli* was cultured in Luria-Bertani (LB) broth or agar with addition of antibiotics - 100 µg/mL kanamycin or 25 µg/mL chloramphenicol when necessary. *E. coli* cultured on LB agar was incubated at 37℃ for 16-24 h. *E. coli* cultured in LB broth was incubated at 37℃ for 16-24 h with agitation at 180 rpm. *L. pneumophila* was cultured in N-(2-acetamido)-2-aminoethanesulfonic acid (ACES) broth or on buffered charcoal yeast extract (BCYE) agar, supplemented with 25 µg /mL kanamycin or 6 µg/mL chloramphenicol when required. *L. pneumophila* grown on BCYE agar was incubated for 72 h at 37℃. *L. pneumophila* grown on ACES broth was incubated for 24 h at 37℃ with agitation at 180 rpm. Both *E. coli* and *L. pneumophila* strains were frozen in 50% glycerol broth at -80℃ for long-term storage.

2.3 Culture of *A. castellanii*

A. castellanii Neff strain (ATCC 30010) was cultured in Peptone Yeast Glucose medium (PYG) (2% peptone, 0.1% sodium citrate, 0.1% yeast extract) supplemented with 0.1 M glucose, 0.4 mM $CaCl_2$, 2.5 mM KH_2PO_4, 4 mM MgSO4, 2.5 mM Na_2HPO_4, 0.05 mM $Fe_4O_{21}P_6$. *A. castellanii* was maintained at room temperature and sub-cultured every 3 days to achieve optimal growth. Notably, during *L. pneumophila* infection, *A. castellanii* was incubated in minimal medium (PYG medium without glucose, yeast extract and tryptone) at 37℃ with 5% CO_2. All amoebae used in this study were passaged less than 30 times.

For long term storage, *A. castellanii* were adjusted to 1 10^6 cells/mL in PYG medium containing 10% (v/v) DMSO and stocked in 1mL aliquots in liquid nitrogen.

2.4 DNA manipulation

2.4.1 Genomic DNA isolation

A loopful of *L. pneumophila* harvested from BCYE agar was resuspended in 200 µL PBS and then treated with 1 mg/mL Proteinase K (NEB) at 37℃ for 10 min. After digestion, gDNA was isolated using Quick-gDNA™ MiniPrep kit (Zymo Research) following the manufacturer's instruction.

2.4.2 DNA purification

DNA fragments were purified from agarose gels or PCR products using Zymoclean™ gel DNA recovery kit or DNA clean & concentration™-5 kit (Zymo Research) following the manufacturer's instruction.

2.4.3 Oligonucleotide synthesis

All oligonucleotides used for DNA amplification and sequencing are listed in Table 2.2. Oligonucleotide primers were synthesised by Sigma GenoSys and diluted to the working concentration of 10 µM in nuclease-free water (NFW) and stored at – 20℃.

2.4.4 DNA amplification

All polymerase chain reaction (PCR) amplifications in this study were performed either in GS482 Thermal Cycler (G-Storm) or Life Touch Thermal cycler (Bioer). AmpliTaq Gold® Pfu ultra II DNA polymerase was used to amplify DNA fragments from *L. pneumophila* 130b genome according to manufacturer's recommendations. Generally, each reaction contains up to 200 µg DNA template, 20 nM of each primer, 0.7 mM dNTP, and 1 µL Pfu Ultra II enzyme. PCR cycling program generally involves denaturation at 95℃ for 30 sec, annealing at the appropriate melting temperature depending on primer sequences for 30 sec and extension at 72℃ for 15 s per kb of expected product. This cycle was repeated 30 times.

2.4.5 DNA ligation

Purified DNA fragments and vectors were digested with corresponding high-fidelity restriction enzymes (NEB or Promega), purified and then ligated together using T4 ligase (NEB), at the molecular ratio of between 3:1 to 10:1. Ligation reaction was incubated at 16℃ overnight or for 2 h at 25℃. Alternatively, two or more DNA fragments with overlap regions were ligated with linearised vector at 50℃ using the Gibson Assembly system (NEB).

2.4.6 Isolation of plasmid DNA

Plasmid DNA was isolated using the Qiaprep® Spin Miniprep Kit (Qiagen) following the manufacturer's instructions.

2.4.7 DNA sequencing

Purified DNA was sequenced by the Centre for Translational Pathology (The University of Melbourne, Victoria, Australia), Monash Health Translation Precinct Medical Genomics Facility (The Monash University, Victoria, Australia), or AGRF Sanger Sequencing services (Victoria, Australia) after Big-Dye reaction. The DNA sequences were analysed using Sequencher® version 5.0 software (Gene Code Corporation).

2.5 Bacteria transformation

2.5.1 Preparation of *E. coli* competent cells

Chemically competent *E. coli* pir2 or XL-1 Blue were used extensively in this study. Bacteria was cultured overnight on LB agar, and then a Single colony was transferred into fresh LB broth and cultured overnight at 37℃ with agitation of 200 rpm. This was then sub-cultured 1:100 in 1 L of SOB media and incubated at 16℃ with agitation of 200-250 rpm. Cells were harvest when the OD_{600} reached 0.4-0.8. Cells were incubated for 10 min on ice before they were pelleted at 4℃ and then washed with 40 mL pre-cold TB. Cells were incubated 10 mins on ice before resuspending in 4 mL ice-cold TB containing 7.5% (v/v) DMSO. The suspension was incubated on ice for 10 min and then snap-frozen in 50 µL aliquots and stored at -80℃ until ready for use Competent cells used in this study were prepared within 6 months.

2.5.2 Chemical transformation

Plasmids or ligation products were mixed well with chemically competent *E. coli* and incubated on ice for 10-20 min. The mixture was then heat-shocked at 42℃ for 90 s, followed by 5 min incubation on ice. After transformation, bacteria were recovered in SOC at 37℃ with agitation. To select for positive transformants, bacteria were plated on LB agar containing appropriate antibiotics.

2.5.3 Electroptransformation of *L. pneumophila*

Stationary phase *L. pneumophila* was harvested from BCYE agar, resuspended in ice-cold PBS and adjusted to OD_{600} of 3.2. Bacteria were washed twice with pre-cold dH_2O before resuspending in 100 µL10% (v/v) glycerol in distilled water. *L. pneumophila* electrocompetent cells were used immediately after preparation.

Four hundred-600 ng plasmid DNA was mixed well with 100 µL of electrocompetent *L. pneumophila* and then transferred to a pre-chilled 0.2 cm gap electroporation cuvette (Cell Project). Electroporation was performed using a Micropuler electroporator (brand) emitting an electric pulse of 2.3 kV at 200 Ω and 0.25 µF. Cells were recovered in ACES broth for at least 5 h at 37℃ with agitation before being plated on BCYE agar containing appropriate antibiotics to select for positive transformants.

2.6 Genetic manipulation of *L. pneumophila*

2.6.1 Construction of maker-less in-frame deletion *L. pneumophila* mutants

Suicide vector pSR47s which carries kanamycin antibiotic resistance cassette was used to generate *L. pneumophila* 130b strains carrying large genomic region deletions in this study (Figure 2.1). Briefly, flanking sequences of the target region were amplified from gDNA of *L. pneumophila* 130b and then fused using overlap PCR. This product was then ligated with pSR47. After confirmation of successful generation of suicide vector containing genomic region deletion cassette by Sanger sequencing, this was transformed into electrocompetent *L. pneumophila* 130b. Successful homologous recombination was selected for by plating transformants on BCYE agar containing 25 µg/mL kanamycin. The second screening on BCYE agar containing 5% sucrose resulted in either *L. pneumophila* WT or mutants where the

target region was excised. Primers locating outside the deleted region were used to confirm mutants. All oligonucleotides used in this study were listed in table 2.2.

2.6.2 Complementation of *L. pneumophila* mutants

L. pneumophila mutants were complemented via inducing the pMMB207c-HA×4 vector carrying the full-length gene. Target genes were amplified from gDNA of *L. pneumophila* 130b and ligated to pMMB207c-HA×4 vector. After confirmation using Sanger sequencing, this was transformed into *L. pneumophila* mutants.

Expression of target genes was confirmed by western blot prior to further studies. Complementation strains were cultured in ACES broth supplemented with 6 µg/mL chloramphenicol and 1 mM IPTG overnight at 37℃ with agitation. Cells were collected and lysed in LDS sample buffer (Life Technologies) containing 50mM DTT. Cell lysates were boiled for 10 min and loaded onto 10% SDS-PAGE gels.

After SDS-PAGE, proteins were transferred onto nitrocellulose membranes (Pall). The membrane was blocked in 5% skim milk (w/v) in TBST (20 mM Tris, 50 mM NaCl, pH8.0 supplemented with 0.1% Tween 20,). After washing with PBST, HAx4 tag antibody (BioLegend) diluted in 1;2000 was added to probe for the protein of interest. After 1h incubation with agitation at room temperature. Membranes were washed and HRP-conjugated anti-mouse or anti-rabbit (PerkinElmer) secondary antibodies were incubated with the membrane at 1:3000 in TBST for 1 h. Amersham ECL™ Western Blotting Detection Reagents (GE Healthcare) were used to develop immunoblots before detection using the DNR MF-ChemiBIS Bio Imaging System or Amersham imager 680 Imaging System.

2.7 *L. pneumophila* intracellular replication assay

2.7.1 Intracellular replication in *A. castellanii*

In general, 2 ×10^5 *A. castellanii* were seeded into 24-well plates in PYG media and incubated at RT for 24 h. *A. castellanii* was washed with minimal medium to remove non-adherent cells (411). *A. castellanii* was then infected with the stationary phase *L. pneumophila* with a MOI of 2 and left to incubate at 37℃ in presence of 5% CO_2. After 2 h, the media was replaced with minimal medium containing gentamicin (100 µg/mL) to kill off any extracellular bacteria for 1 h. Finally, gentamicin containing

buffer was removed and replaced with fresh minimal medium. At desired time-point, bacteria were released from amoeba by vigorous vortexing. Serial dilutions of the inoculum and bacteria recovered from lysed amoeba were plated on BCYE agar and incubated at 37℃ for 72 h. Bacterial colony-forming units (CFU) were then enumerated. Additionally, bacterial invation rate was calculated by comparing CFU at 3 h post infection and inoculum.

2.7.2 Intracellular replication in THP-1

Prior to infection, 5 x 10^5 THP-1 monocytes were differentiated into macrophages using phorbol 12-myristate 13-acetate (PMA) at 5 x 10^5 mg/mL for 3 days at 37℃, 5% CO2. Infection with *L. pneumophila* was then performed at a MOI of 10 for 2h at 37℃, 5% CO2, followed by gentamicin treatment (100 μg/mL) for 1 h. Gentamicin containing media was then replaced with fresh media and left to incubate till desired experimental time-point. To lyse cells at each time-point, 0.2 mL of 0.05% (w/v) digitonin (Sigma Aldrich, St Louis, USA) was added to the cells and left to incubate at room temperature for 5 min. Following this, 0.8 mL of ACES broth was added to each well and cells were scraped and transferred into sterile microfuge tubes. Serial dilution was then performed in PBS prior to plating on BCYE plates. At the time-points of 24 h and 48 h post-infection, culture media was first collected into sterile microfuge tubes and then the cells were lysed as described above. Cell lysates and culture media were pooled together, and serial dilution was performed in PBS prior to plating on BCYE plates. After 3 days of incubation at 37℃, CFU on BCYE plates was counted to enable enumeration of bacteria at each tested time points.

2.8 *A. castellanii* proliferation assay

A. castellanii proliferation was assessed using the protocol published previously (412). Briefly, 1 × 10^4 cells were seeded into 24 well plates and infected with *L. pneumophila* at MOI of 20 in minimal medium. After 2 h infection, the medium was replaced with PYG containing 20 μg/mL gentamicin. 16, 24 and 48 h post-infection, cells were collected and quantified using a counting chamber (Sigma). Cell numbers were relative to uninfected cells at 16 h. *A. castellanii* proliferation was calculated from three biological repeats.

2.9 Electron microscopy of *A. castellanii*

8×10^5 *A. castellanii* were seeded into 6-well plates and infected with *L. pneumophila* at MOI of 20 in amoeba infection buffer. After 2 h infection, the media was replaced with fresh PYG. Cells were collected, pelleted by centrifugation (10 000 g, 2 min) at 8 h, 16 h, 24 h post infection and resuspended in fixation buffer (2.5% glutaradehylate in 0.1 M sodium cacodylate) for 2 h at 25℃. Cells were collected and washed twice with 0.1 M sodium cacodylate before post-fixation in 1% osmium tetroxide for 2 h. Cell pellets were then washed with dH_2O and left overnight at 4°C in 0.3% uranyl acetate. Samples were rinsed in dH_2O before dehydration in a graded series of acetone and then infiltrated and embedded with Epon resin. Sections (70-80 nm thick) were cut, stained with uranyl acetate and lead citrate before viewing under a Phillips CM120 transmission electron microscope at 120Kv.

2.10 Transcriptomic analysis in *L. pneumophila* infected amoebae

A. castellanii (2×10^5) were seeded in technical and biological triplicate in PYG medium in 24-well plates (4×10^5 cells per mL) and incubated for 24 h at RT. *A. castellanii* was then washed with minimal medium to remove non-adherent cells followed by *L. pneumophila* infection with MOI of 50 (411). After 1 h incubation at 37℃ with 5% CO_2, cells were washed with minimal medium and incubated in fresh PYG medium at 37℃ with 5% CO_2. At 3 h, 8 h, 16 h and 24 h post infection, RNA was isolated from *A. castellanii* using TRIsure™ (Bioline) according to the manufacturer's instructions. RNAseq was then performed by Research Center for Zoonosis Control, Hokkaido University in Japan.

Principal component analysis (PCA) was performed to observe the variance between each sample. After data normalisation, volcano plot was generated using Excel to visualise $\log_2$ fold change expression data against the FDR (DESeq-PADJ) and a 5% FDR cut-off was set. The significant differentially expressed genes were determined if the $\log_2$ fold change was >2 or <-2. The Generic GO Term Finder developed within the Bioinformatics Group at the Lewis-Sigler Institute was used to identify significantly enriched functional gene groups in accordance with GO ontology (413).

2.11 Validation of RNA-seq via qRT-PCR

L. pneumophila infection was performed as above. Cells were collected, pelleted by centrifugation at 24 h post infection. RNA isolation was performed using Isolate II RNA Mini Kit (Bioline) following the manufacturer's guideline.

cDNA was synthesized by using iScript^{tm} cDNA synthesis kit (Bio-Rad) according to the manufacturer's instruction. Each reaction (20 µL) consists of up to 1 µg RNA, 1 µL iScript reverse transcriptase and 4 µL reaction buffer. The mixture was incubated at 25℃ for 5 min, followed by subsequent incubation at 42℃ for 30 min and 85℃ for 5 min. The product was then used as the template for qRT-PCR without dilution.

Each 10 µL qRT-PCR reaction consists of 2 µL cDNA template, 300 nM of each primer, 5 µLSso-Advanced Universal SYBR Green Supermix (BioRad). The mixture was load into MicroAmp® Optical 384-Well Reaction Plate (Life technologies$^{\#}$ 4309849) and run on QuantStudio^{tm} 6 q-RT-PCR system. Data were analysed by QuantStudio^{tm} real-time PCR program using 18s rRNA as the endogenous control and uninfected samples as the reference sample. Results were expressed as the mean of at least three independent biological repeats.

2.12 *A. castellanii* encystment

A. castellanii (8×10^5) were seeded into 6-well plates and infected with *L. pneumophila* expressing GFP at MOI of 50 in amoeba infection buffer. After 1 h infection, cells were washed twice with amoeba infection buffer and incubated in fresh amoeba infection buffer at 30℃ for 72 h. Cells were collected, pelleted by centrifugation at 24 h, 48 h and 72 h post infection. Amoebae were stained with calcofluor white solution (Sigma, 1:100) for 2 min at 25℃. Following washing by PBS, amoebae were fixed and permeabilized for 30 min at 4℃ using the eBiosciencetm Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher). Cells were resuspended in 100 µL FACS buffer and processed on LSR-Fortessa X20 at FlowCore MHTP. Data were analysed by Flowjo_V10 program. Results were expressed as the mean of at least three independent biological repeats.

Alternatively, after calcofluor white staining, 10 µL suspension was dropped on the glass slide and visualised under Olympus FV1200 confocal at MHTP.

2.13 siRNA induced knock-dnon of *sir6f* expression in *A. castellanii*

Small interfering RNA (siRNA) targeting the sirturin7 mRNA was synthesised by Bioneer. The duplex siRNA with sense (5'- GACAUCAAGGAGGUGGAGU) and antisense (5'-ACUCCACCUCCUUGAUGUC) was hydrated in sterile siRNA buffer to a final concentration of 10 µM. SuperFect from Qiagen was used for siRNA transfection in *A. castellanii*. For transfection in a 24 well format, 25 µL PYG was mixed with 5 µL SuperFect and 2.5 µL siRNA, and then incubated for 10 min at room temperature. The mixture was added to 8 $\times 10^4$ *A. castellanii* in a volume of 200 µL and incubated at room temperature for 48h for siRNA knockdown to occur.

Forty-eight later, *L. pneumophila* infection was carried out as described above but with slight modification. Generally, the siRNA transfected *A. castellanii* was infected with *L. pneumophila* with a MOI of 40. After 1 h incubation at 37℃, cells were treated with gentamycin (100 µg/mL) for another hour. Cells were harvested and washed with minimal medium, and then incubated in PYG. The knock-down of *sirtuin6f* was validated at 24 h post infection by qPCR. Non-targeting scramble siRNA (Bioneer) optimised for human and mouse cell lines was used as a knockdown control.

2.14 Statistical analysis

Statistical analysis was executed by GraphPad Prism 6.0 (GraphPad In Stat Software Inc.). When required, an unpaired t-test or one-way ANOVA with Dunnett post-test was applied to determine the results. Statistical significance was determined for P-values less than 0.05.

Table 2.1 Plasmids and bacterial strains used in this study

Strain/plasmid	Characteristics	Source/Reference
E. coli XL-1 Blue		Stratagene
E. coli pir2		Stratagene
L. pneumophila 130b	Serogroup 1, Clinical isolate (USA), ATCC BAA-74	(414)
L. pneumophila 130b Δ*dotA*	*dotA* in-frame deletion mutant of 130b	(415)
pSR47s	sacB based suicide vector for mutagenesis	(416)
pSR47s: ΔA	Vector carrying genomic region A deletion cassette	This study
pSR47s: ΔB	Vector carrying genomic region B deletion cassette	This study
pSR47s: ΔC	Vector carrying genomic region C deletion cassette	This study
pSR47s: ΔD	Vector carrying genomic region D deletion cassette	This study
pSR47s: ΔE	Vector carrying genomic region E deletion cassette	This study
pSR47s: ΔF	Vector carrying genomic region F deletion cassette	This study
pSR47s: ΔG	Vector carrying genomic region G deletion cassette	This study
pSR47s: ΔH	Vector carrying genomic region H deletion cassette	This study
pSR47s: ΔI	Vector carrying genomic region I deletion cassette	This study
pSR47s: Δ*lpw4831*	Vector carrying *lpw4831* deletion cassette	This study
pSR47s: Δ*lpw4841*	Vector carrying *lpw4841* deletion cassette	This study
pSR47s: Δ*lpw4851*	Vector carrying *lpw4851* deletion cassette	This study
pSR47s: Δ*lpw4861*	Vector carrying *lpw4861* deletion cassette	This study
pSR47s: ΔG1	Vector carrying genomic region G1 deletion cassette	This study
pSR47s: ΔG2	Vector carrying genomic region G2 deletion cassette	This study
pSR47s: ΔG3	Vector carrying genomic region G3 deletion cassette	This study
pSR47s: ΔG4	Vector carrying genomic region G4 deletion cassette	This study
pMMB207c	N-terminal 4XHA expression vector	(417)
pMMB207c:*lpw4831*	Vector expressing 4XHA tagged *lpw4831*	This study
pMMB207c:*lpw4841*	Vector expressing 4XHA tagged *lpw4841*	This study
pMMB207c:*lpw4851*	Vector expressing 4XHA tagged *lpw4851*	This study
pMMB207c:*lpw4861*	Vector expressing 4XHA tagged *lpw4861*	This study
pMip	pMMB207 with the promoter region of mip cloned into SacI/XbaI	(418)
pMIP:EGFP	pMIP carrying EGFP cloned into the MCS	Newton

Table 2.2 List of primers used in this study

Number	Sequence (5'-3')	Purpose
2072	AGCTATGACCATGATTACGC	F- sequence pSR47s
2073	GTGAACGGCAGGTATATGT	R- sequence pSR47s
2713	TTGACAATTAATCATCGGC	F- sequence pMMB207c-HAx4
2714	AGGCAAATTCTGTTTTATC	R- sequence pMMB207c-HAx4
6136	CGAATTCCTGCAGCCCGGGGGATCCCAGTTTGCGGCCTCCACT	F- amplify 5' homologous arm of genomic region F
6137	AGCAGCCTGTCGTCTATTATTTTAC	R- amplify 5' homologous arm of genomic region F
6138	GTAAAATAATAGACGACAGGCTGCTTTGGCGGAAGTTGATGTCG	F- amplify 3' homologous arm of genomic region F
6139	CTAAAGGGAACAAAAGCTGGAGCTCTGGCTTTCTCCACCAGGCT	R- amplify 3' homologous arm of genomic region F
6271	ATACGCCCTGATATGGGAAAT	F- confirm the deletion of genomic region F
6272	GGCAAGAATAGTTCCTGTGCC	R- confirm the deletion of genomic region F
6273	CAAGGTCGAGGCAGAAAATG	Sequencing primer for pSR47s-ΔF
6314	CGAATTCCTGCAGCCCGGGGGATCCTGTCGGTTTCATTCAAATCCTC	F- amplify 5' homologous arm of genomic region B
6315	TTCACCATATCCTGGCTCCAC	R- amplify 5' homologous arm of genomic region B
6316	TGAGGTGGAGCCAGGATATGGTGAATGGTAATGACTCGCTCTGGG	F- amplify 3' homologous arm of genomic region B
6317	CTAAAGGGAACAAAAGCTGGAGCTCCATGCTTTAGTTGCATAGGTTGTAT	R- amplify 3' homologous arm of genomic region B
6326	GAAGTTGGTATGCGGGGTGC	Sequencing primer for pSR47s-ΔB
6332	CTCGGTCAATATCGCTCATAGG	F- confirm the deletion of genomic region B
6389	CATCGCCCTTGTATCGTTTG	R- confirm the deletion of genomic region B
6461	CGGCGGCCGCCCCTGGCAATGTCACCTACC	F- amplify 5' homologous arm of genomic region G
6462	TACCTCCTGATTGTGGTTTTGTTT	R- amplify 5' homologous arm of genomic region G
6463	AAAACAAAACCACAATCAGGAGGTAATCCACTTCCCTATGTCATGATTT	F- amplify 3' homologous arm of genomic region G
6464	CGCGAGCTCGGCGTAAGGAAACGACAGG	R- amplify 3' homologous arm of genomic region G
6465	CGGGATCCGGAAAAGGAGCATCAAAACAGT	F- amplify 5' homologous arm of genomic region H
6466	ATTAAGGAAAACCGAGGCAAA	R- amplify 5' homologous arm of genomic region H

6467	ATTTTTTGCCTCGGTTTTCCTTAATTAACAACTGCCCTCATTGCTTT	F- amplify 3' homologous arm of genomic region H
6468	CGGCGGCCGCCCATAGGTGCTTGCGCTACA	R- amplify 3' homologous arm of genomic region H
6469	CGGGATCCAATGATTGTCACTTGCGATGG	F- amplify 5' homologous arm of genomic region I
6470	CGATTTGAGAATTATTTGAGGAGC	R- amplify 5' homologous arm of genomic region I
6471	AGCTCCTCAAATAATTCTCAAATCGTATTGCTTTTCCTCCTTGGTTT	F- amplify 3' homologous arm of genomic region I
6472	CGGCGGCCGCTCTTGCTGCTTTGGCTGTATC	R- amplify 3' homologous arm of genomic region I
6502	TTGAAGTCACATTTGCATATCC	Sequencing primer for pSR47s- ΔG
6503	TATCGATGAAAAGCACAGGAG	F- confirm the deletion of genomic region G
6504	AATTCCATCCATTTTGGCGATA	R- confirm the deletion of genomic region G
6505	GCATGCCATCAAAATCCAATT	Sequencing primer for pSR47s-ΔH
6506	GAGCTATATTTGCCCTTGGTA	F- confirm the deletion of genomic region H
6507	ATCAGACTTGGTTGAAATTGTG	R- confirm the deletion of genomic region H
6508	TATCACGAAATGAGCAGACC	Sequencing primer for pSR47s-ΔI
6509	AAAGAATAATTGGTTCGGCGA	F- confirm the deletion of genomic region I
6510	ATTTCTCGCTCTCATTTGCAA	R- confirm the deletion of genomic region I
6551	TTGCGGCCGCTGCTCGCAGCGCATCA	F- amplify 5' homologous arm of genomic region C
6552	TCCTCGCAAAACCACCCA	R- amplify 5' homologous arm of genomic region C
6553	TATGCATTGGGTGGTTTTGCGAGGAGTGAAAACAATAATTGCTTGAAACC	F- amplify 3' homologous arm of genomic region C
6554	CGGAGCTCTCCCAGATAATAGCCGAGTGC	R- amplify 3' homologous arm of genomic region C
6563	CGGGATCCCTGCTTTAGCGGGGAACC	F- amplify 5' homologous arm of genomic region D
6564	AGAATGTGATAGGACGGTAGAAAAT	R- amplify 5' homologous arm of genomic region D
6565	ATTTTCTACCGTCCTATCACATTCTAACAAGCGATTCGACTGATAAAA	F- amplify 3' homologous arm of genomic region D
6566	TTGCGGCCGCCTGAAGAGGTAGATGCTGGTCC	R- amplify 3' homologous arm of genomic region D
6575	GAGGGATCCGACCCTGTTGGCAGCTATC	F- amplify 5' homologous arm of genomic region E
6576	TATCCCTACCTCTTGTGGAG	R- amplify 5' homologous arm of genomic region E

6577	CTCCACAAGAGGTAGGGATAATGGCAGACAAATGTTAAGGAA	F- amplify 3' homologous arm of genomic region E
6578	AGCGGCCGCCGGTTGGAGTAGTTTGGACA	R- amplify 3' homologous arm of genomic region E
6678	AGCGGGTCCATATAAAATAGC	Sequencing primer for pSR47s-ΔE
6679	GAACACGCGCTTGTAAGAAAA	F- confirm the deletion of genomic region E
6680	AAATTGCAAAGATAAGATTGTACT	R- confirm the deletion of genomic region E
6767	CTGCTTTAGCGGGGAACC	F- confirm the deletion of genomic region D
6768	TGCGTGCCTTATGATTCGTT	R- confirm the deletion of genomic region D
6769	CAGCCATGTTGGGTGAC	Sequencing primer for pSR47s-ΔD
6773	GTGTCAGGACGGCATAGGG	F- confirm the deletion of genomic region C
6774	TCATCGCCTGCTTATGCTTG	R- confirm the deletion of genomic region C
6775	TTGCGGAACGAACCA	Sequencing primer for pSR47s-ΔC
6916	GGACTAGTCGACACTCAAACCTCCACCC	F- amplify 5' homologous arm of genomic region A
6917	AATAGAAGCCCCTTACCTCACAA	R- amplify 5' homologous arm of genomic region A
6918	TTTTGTGAGGTAAGGGGCTTCTATTAAATGCCCCGCTTTCTGA	F- amplify 3' homologous arm of genomic region A
6919	TTGCGGCCGCTTTTATCGGTGCTGTAATCTTGG	R- amplify 3' homologous arm of genomic region A
7027	AAATCTGACCAATAGCCCAAGC	F- confirm the deletion of genomic region A
7028	TTTGACGAAAAGGGAAACTGG	R- confirm the deletion of genomic region A
7263	CGGGGTACCTAAAGCATTTACCCATTTGTGTAAA	F- clone *lpw4831* to pMMB207c
7264	CGCGGATCCTTAATGGAACTCATGCCTATCCA	R- clone *lpw4831* to pMMB207c
7265	CGGGGTACCATGATTTATGTTTTAATTTGTATTATGCTA	F- clone *lpw4841* to pMMB207c
7266	CGCGGATCCCTATTTCATACCAAAACCAGGTTG	R- clone *lpw4841* to pMMB207c
7267	CGGGGTACCATGAGCAATAAAAAACATTCTCTCACT	F- clone *lpw4851* to pMMB207c
7268	TCTAGATTATACAGCAGGTTCCAAATCCG	R- clone *lpw4851* to pMMB207c
7269	CGGGGTACCATGGCCTATATTGACGAATTGTT	F- clone *lpw4861* to pMMB207c
7270	CGCGGATCCTCAGGATATTGCTCGATTGAGC	R- clone *lpw4861* to pMMB207c
7330	CGCGGATCCAGCAGGCGGTTGGGTTT	F- amplify 5' homologous arm of *lpw4831*

7331	CCATAGTAAAGATCGCTCTATTGGT	R- amplify 5' homologous arm of *lpw4831*
7332	ACCAATAGAGCGATCTTTACTATGGGTAAGCCTGACTTGTTTGGAGG	F- amplify 3' homologous arm of *lpw4831*
7333	CGCGAGCTCGGGATCTTGAGCAATACAGATTTT	R- amplify 3' homologous arm of *lpw4831*
7334	CGCGGATCCTTGGCTTTATGGTAGGCTTCG	F- amplify 5' homologous arm of *lpw4841*
7335	CAAACAAGTCAGGCTTACTGCTCT	R- amplify 5' homologous arm of *lpw4841*
7336	TAGAGCAGTAAGCCTGACTTGTTTGTCGGACCTGTACTGAAATGAAAA	F- amplify 3' homologous arm of *lpw4841*
7337	CGCGAGCTCCCCGCAACAAACGATAGAATC	R- amplify 3' homologous arm of *lpw4841*
7338	CGCGGATCCAAGACAGCAAGGGGAGAACG	F- amplify 5' homologous arm of *lpw4851*
7339	CAGGTCCGATGCGAAAACA	R- amplify 5' homologous arm of *lpw4851*
7340	CATATGATGTTTTCGCATCGGACCTGCAGGTTAAGTTTAATAACCCCAGTT	F- amplify 3' homologous arm of *lpw4851*
7341	CGCGAGCTCCGATTTCAGGATATTGCTCGA	R- amplify 3' homologous arm of *lpw4851*
7342	GGACTAGTTTCTCGGAGGGCTTGGTG	F- amplify 5' homologous arm of *lpw4861*
7343	CTGGGGTTATTAAACTTAACCTGTC	R- amplify 5' homologous arm of *lpw4861*
7344	GACAGGTTAAGTTTAATAACCCCAGGCCTGGACAATTCAAGATAACG	F- amplify 3' homologous arm of *lpw4861*
7345	CGCGAGCTCCAAAGCAAAGTTGGCAGAGC	R- amplify 3' homologous arm of *lpw4861*
7354	TTTATCGGTTGCTCATTAGGGT	F- confirm the deletion of *lpw4831*
7355	CGGGACGAGAACTCAAAGTAAG	R- confirm the deletion of *lpw4831*
7356	AGCCCTGCGAAAGTGGTTG	F- confirm the deletion of *lpw4841*
7357	AAATGACAATATGATGGCGGTTA	R- confirm the deletion of *lpw4841*
7358	TGACTGTGAATACCGATTTTGTTT	F- confirm the deletion of *lpw4851*
7359	GCCAACGCCATAGCGATTA	R- confirm the deletion of *lpw4851*
7360	CATTGGTTTGGGTGCTGTTT	F- confirm the deletion of *lpw4861*
7361	TTTCTCAAGTGGCTTTATTAGGG	R- confirm the deletion of lpw4861
7478	CAGGCCATGTCCACATCAGA	F-ACA1_084890 for qPCR

7479	CTCGTAGATGCCCATGCCAG	R-ACA1_084890 for qPCR
7482	CAAGAGCTTCCAGGCCATCA	F-ACA1_256400 for qPCR
7483	CTCCTCGGTCGAAATGACCC	R-ACA1_256400 for qPCR
7488	TGAGCTATCCGCAATCGGAC	F-ACA1_282330 for qPCR
7489	AGCTCAACCTCAAAGCTGGG	R-ACA1_282330 for qPCR
7490	GAGCTCAGTCTGTGGGACAC	F-ACA1_383370 for qPCR
7491	ACCAAGAACACGTCAGTCCC	R-ACA1_383370 for qPCR
7496	ATGCAGGTGGTCTTCGCTC	F-ACA1_095970 for qPCR
7497	GAATCCAATGTTGTCGCCCG	R-ACA1_095970 for qPCR
7502	TCGCCTCTCTTGCTTCCATC	F-ACA1_048640 for qPCR
7503	AGGATCACAGCCGAACACAG	R-ACA1_048640 for qPCR
7508	AAGTCCCTGTCCGCCATTTT	F-ACA1_078030 for qPCR
7509	CGGCAGTTTCACGTTGTTGA	R-ACA1_078030 for qPCR
7609	TGTATTCCTTTATTTGCTCAGAGC GATATT	Sequencing primer for pSR47s-ΔG1
7610	CACAATCTGCCTCCCTCTTTT	Sequencing primer for pSR47s- ΔG2
7611	TGACAAAAGGAATAAGCCAAGTA GA	Sequencing primer for pSR47s-ΔG3
7612	ACTCCCTTCTGTTGGCATTTC	Sequencing primer for pSR47s- ΔG4
7522	CCTCGGATCGTCACTTACCG	F-ACA1_153540 for qPCR; Pair 1
7523	AATGACTAGGTTGCCTCCGC	R-ACA1_153540 for qPCR; Pair 1
7524	ACCTTCGTTCCGCTTCACAT	F-ACA1_153540 for qPCR; Pair 2
7525	CACCTCCTTGATGTCCCACC	R-ACA1_153540 for qPCR; Pair 2

F: forward primer R: reverse primer

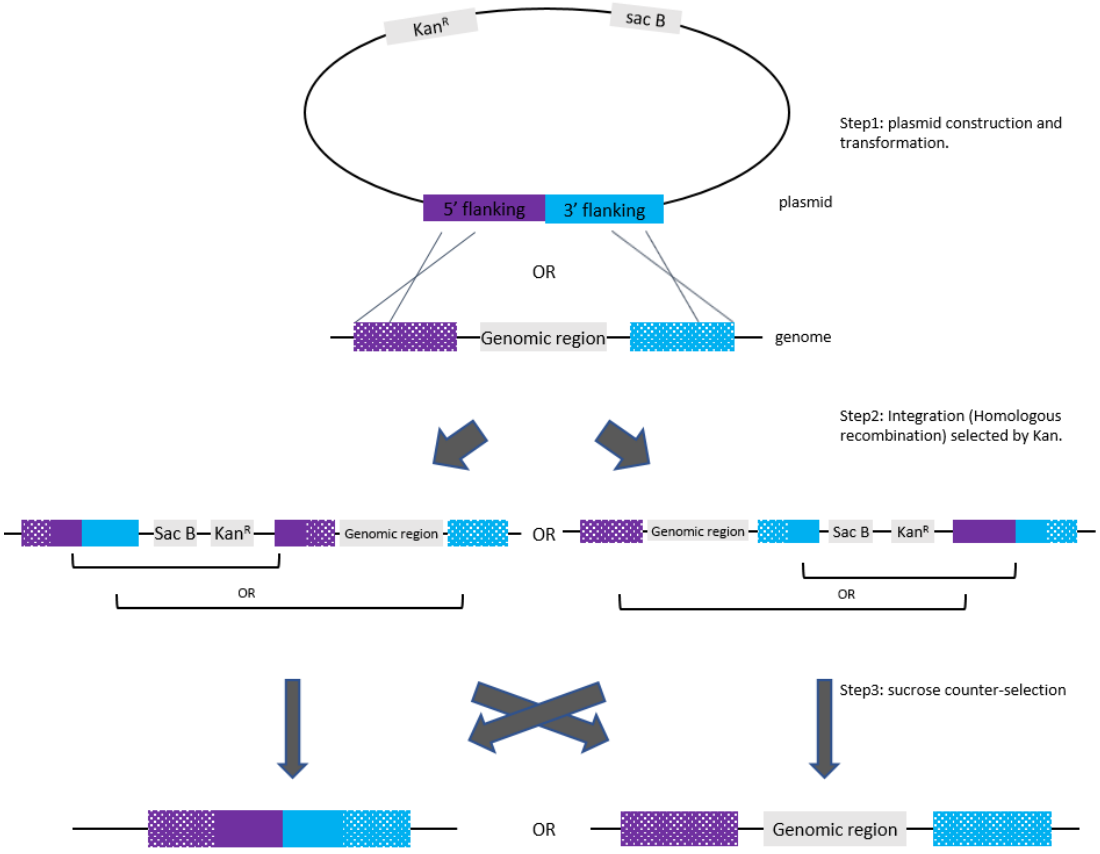


Figure 2.1 Schematic map of mutants construction

Suicide vector pSR47S carrying kanamycin-resistant cassette was used to construct mutants lacking large genomic regions. 1) Flanking regions (1-3 kb) of to be deleted genomic regions were cloned to suicide vector. 2) Vector sequence was integrated into chromosome after homologous recombination, followed by kanamycin screen. Crossing on both flanking regions was possible to occur. 3) Expected mutants were screened by sucrose counter-selection. 300 maximum colonies were screened on this step.

Chapter 3: Characterizing *L. pneumophila* genes essential for intracellular replication by constructing large genomic region deletions

3.1 Introduction

In the environment, *L. pneumophila* replicates in more than 20 species of amoebae, two species of ciliated protozoa, one species of slime mould and also exists as free bacteria in water or biofilms (10, 81-89). When *L. pneumophila* containing aerosols enter human lungs, the bacteria are rapidly engulfed by alveolar macrophages which *L. pneumophila* then hijacks to replicate within, leading to Legionellosis in immunocompromised individuals (133, 419). While the host range is diverse, the intracellular life cycle of *L. pneumophila* is similar in each case (89). Following uptake, *L. pneumophila* escapes the endocytic pathway and establishes the *Legionella*-containing vacuole (LCV), which involves the recruitment of ER-derived vesicles, ubiquitinated proteins and the host organelles (156, 167, 420, 421). Aside from the traditional virulence factors like lipopolysaccharide (LPS), flagella and pili, multiplication of *L. pneumophila* in hosts requires the Dot/Icm T4BSS effector proteins and two-component transcriptional regulators (PmrAB, CpxRA, LetAS, LqsRS) (114, 115, 223, 240, 258, 261, 265, 276, 280, 285). The Dot/Icm system translocates over 330 effector proteins, which are involved in all stages of bacterial intracellular trafficking and replication, and finally the two-component systems regulate expression of approximate 70 Dot/Icm effector proteins during infection (115, 231, 251, 284). Dot/Icm effector proteins are essential for pathogenesis in both protozoan and mammalian hosts (227, 422).

The function of effector proteins ranges from manipulating vesicle trafficking pathways, activation of NF-κB in mammalian cells to remodelling of the host epigenome (244, 423-425). However, the majority of *L. pneumophila* effector proteins remain uncharacterised and even fewer have been well elucidated in protozoan hosts (117, 250). Challenges to characterise the function of effector proteins include functional redundancy of effector protein families, the complexity of the effector protein regulation system and lack of systematic tools (117, 245, 250). This has made mutagenesis studies and phenotypic screening difficult as the deletion of a single or

even multiple effector genes does not necessarily result in impaired bacterial replication or altered LCV biogenesis (207, 243-245).

L. pneumophila is naturally competent and accessible for genetic manipulation (250, 426-429). More recent strategies for elucidating the role of effector proteins include the identification of genomic clusters defined by transposon site hybridisation (TraSH) mutant library and a genetic approach "insertional mutagenesis and depletion (iMAD)". The TraSH mutant library distinguished seven gene clusters that were not required for optimal growth in nutrient-rich media and thus were further mapped as host-specific genes by constructing mutants lacking large genomic regions in *L. pneumophila* strain Philadelphia (250). The mutant library facilitated phenotype screening but requires subsequent mutagenesis work. The cleavage of syntaxin 17, a soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein by *L. pneumophila* effector protein Lpg1137 was discovered based on this method (321). In contrast, iMAD can be applied to characterise the parallel pathways if utilised by *L. pneumophila* for the same purpose by constructing a bacterial transposon mutant library and depleting common host pathways (430, 431). iMAD has been developed in *Drosophila melanogaster* cells. Although one iMAD screen takes 4-8 weeks, it takes 6-18 month to establish and optimise experimental conditions (431). The lack of tools to systematically interrogate functions of *L. pneumophila* effector proteins is one of the reasons why most are still uncharacterised.

In this study, we applied a suicide vector approach to construct mutants lacking large genomic regions in *L. pneumophila* strain 130b and used them to screen for genes essential for intracellular replication in *A. castellanii* and human THP-1 macrophages respectively. This allowed us to compare genes required for intracellular replication in two different host types.

3.2 Results

3.2.1 Annotation of putative effector proteins in *L. pneumophila* 130b genome

In 2010, the *L. pneumophila* 130b genome was sequenced by performing paired-end 454 FLX pyrosequencing technology to approximately 11-fold coverage and assembled into four scaffolds (consisting of 145 contigs) and 14 small, unscaffolded contigs (206). The genome of *L. pneumophila* 130b consists of a single circular chromosome with an average G+C content of 38%, is approximately 3.5 Mb in size and harbours 3293 open reading frames and 42 tRNAs (206). The genome also encodes several secretion systems: Sec system, TAT system, T2SS, lvh1 T4ASS, lvh2 T4ASS, T4BSS Dot/Icm, Trb-1-like T4SSA, G1-T4SS 1 and G1-T4SS 1 (206). Secretion components encoding genes are listed in Appendix 1.

Notably, the Dot/Icm T4BSS is encoded in 5 loci in two distinct regions (227). Gene order and orientation are highly conserved in the majority of the *Legionella* genus (231). In this study, we first collated all characterised and putative *L. pneumophila* 130b effector genes found in published literature and annotated them to the genome (306, 432). As a result, 249 effector genes (listed in Appendix 2) were annotated in (Figure 3.1). Following this, 35 groups (referred to as genomic regions) were chosen based on the enrichment of effector-coding genes (red). Secretion system components (blue) and predicted essential metabolic genes were excluded from those genomic regions (black). Notably, the defined genomic regions also included genes encoding non-effector proteins.

3.2.2 Construction of *L. pneumophila* mutants using the suicide vector approach

Genomic regions were deleted in *L. pneumophila* 130b strain using homologous recombination with the suicide vector pSR47s. Details have been described in section 2.6.1. Briefly, the homologous arms of the genomic regions were ligated into pSR47s. After transformation into the *L. pneumophila* strain 130b, the construct was integrated into the chromosome by a homologous single recombination. Successful recombination was screened by kanamycin resistance. Subsequently, bacteria were screened on BCYE plates containing 5% sucrose to select for double recombination. This resulted in vector sequence being removed, resulting in either reversal to WT or the expected deletion mutant. Notably, mutants made using this method were

markerless as the kanamycin resistance marker was removed during double recombination, which benefited future manipulation. Notably, some non-effector genes were also deleted in several regions. These included genes involved in cellular processes such as lipid metabolism, carbohydrate metabolism, transcription regulation.

In some cases, the presence of essential non-effector genes likely influenced mutant yields. In addition, the recombination efficiency was influenced by the length of homologous arms but varied in each case. In this study, we aimed to delete 35 genomic regions, but 14 failed at the cloning stage; 6 failed at the recombination step even with the use of increased homologous arms; 6 reverted to WT only after homologous recombination, possibly due to essential metabolic genes located in those regions. Finally, nine mutants (annotated ΔA through to ΔI) were generated. Mutants lacking multiple genomic regions were also created, ΔQ and ΔP, which lack 41 and 46 effectors respectively. Table 3.1 details the deleted regions for each mutant. All deletion mutants were confirmed using whole-genome sequencing. Draft genomes were sequenced using an Illumina NextSeq platform with Nextera XT libraries, as per manufacturer's instructions. Single Nucleotide Polymorphisms (SNPs) within the draft genomes were detected by Snippy v3.2 using the *L. pneumophila* 130b genome sequence (GenBank Assembly accession: GCA_000211115.2) as a reference. When required, Illumina reads were assembled using SPAdes v3.7.1 (433).

While the whole genome sequencing confirmed the deletion of genomic regions, it also identified a number of SNPs in other regions of the genome (Table 3.2). For ΔA and ΔH, SNPs occurred in intergenic regions, presumably not affecting any coding sequences. However, we also identified SNPs in coding sequences, resulting in premature translation stop or amino acid alteration. For example, a SNP in ΔG led to a premature stop codon for *lpw28981*, which encodes a putative DNA-binding regulator homologous to the TCS component LetA. The SNPs found in the coding sequence added new mutations and were taken into account during the following phenotypic analysis.

3.2.3 Growth of *L. pneumophila* mutants in nutrient-rich media

Given the fact that deleting these genomic regions not only removed genes coding putative effector proteins but also deleted genes coding non-effector proteins, mutants

were cultured in nutrient-rich media to confirm viability prior to subsequent analysis (124, 126-128, 152). All the single mutants (ΔA – ΔI) and multiple mutants (ΔQ and ΔP) were capable of growing to the same density as *L. pneumophila* WT on BCYE plates (data not shown). In ACES broth, growth of mutants ΔC to ΔI were comparable to *L. pneumophila* 130b WT. ΔA grew slower in exponential phase (10-28 h) compared to WT (10-22 h) and thus reached the stationary phase later compared to WT. Meanwhile, ΔB displayed a longer lag phase (0-14 h) but took the same time to reach stationary phase as the *L. pneumophila* 130b WT after 30 h (Figure 3.2). Interestingly, the growth of ΔP, which lacks 4.6% of the whole genome, was still comparable to WT (Figure 3.2). In summary, the mutants constructed in this study were capable of growth *in vitro*, indicating their maintenance of essential genes for growth.

3.2.4 Intracellular replication of *L. pneumophila* mutants in different hosts

After establishing the *in vitro* growth rate of the mutant strains, we next screened them for attenuated intracellular replication phenotype in *A. castellanii*. The infection method has been described in 2.7. Briefly, *A. castellanii* was infected with *L. pneumophila* mutants at MOI 2 at 37℃, followed by gentamycin treatment. Cells were then lysed, serially diluted and plated on BCYE agar 3 h and 48 h post infection. Δ*dotA* mutant, which lacks the ability to replicate in both *A. castellanii* and macrophage THP-1, was used as a negative control. CFU at 3 h post infection and inoculum disclosed no difference in infection rate between mutants and *L. pneumophila* 130b WT, indicating that the decreased CFU was not caused by reduced bacteria invasion (Figure 3.4). At 48 h post infection mutants ΔB, ΔE, ΔF, ΔG, ΔI showed a significantly decreased CFU compared to the *L. pneumophila* 130b WT at 48 h post infection, while ΔA, ΔC, ΔD, ΔH showed no reduction in *A. castellanii* compared to wild type (Figure 3.3 A). The intracellular replication defect in *A. castellanii* varied for each mutant, ranging from ~ 10^3-10^5 lower in recovered bacterial CFU compared to wild type. ΔQ, which lacks genomic regions F, G, H and I had the most severe replication defect, consistent with Δ*dotA* mutant levels. This suggests that like Δ*dotA*, ΔQ may be unable to escape the endocytic pathway and establish the LCV. Overall, these results suggested that genes deleted in the mutants were critical, but had different effects for bacterial replication in *A. castellanii*.

We also screened mutants for attenuated intracellular replication in the human macrophage line THP-1 (Figure 3.3 B). The infection method has been described in section 2.7.2. Briefly, THP1 monocytes were differentiated into macrophages and seeded at $5x10^5$ cells per well in 24-well plates. Infection was carried out at a MOI of 10 at 37℃, followed by gentamycin treatment. Cells were lysed by 0.05% digitonin, serially diluted and plated on BCYE agar 3 h and 48 h post infection. Although a number of genes had been removed, none of the mutants showed attenuated replication in THP-1 compared to WT. Mutant ΔP lacking 46 effectors did not show any replication defect compared to WT, indicating that almost 20% of the effector protein reservoir was dispensable for replication in human macrophages.

3.2.5 *L. pneumophila* Lpw4851 is a putative glutamate transporter required for intracellular replication in *A. castellanii*

A significant decreased in CFU for mutant ΔB at 48 h post infection in *A. castellanii* was observed compared to WT. Four genes *lpw4831- lpw4861* were deleted in mutant ΔB (listed in table 3.3). We further investigated how the deleted genes contributed to the bacterial replication defect in *A. castellanii*. Using the pSR47s suicide vector approach, mutants lacking single genes were constructed. Surprisingly, while mutant ΔB entered the exponential phase later than WT, none of the single mutants showed this phenotype. Growth of the single mutants in the broth was comparable to WT (Fig 3.5 A).

Intracellular replication assays of all the single mutants in *A. castellanii* were performed as described above. Δ*lpw4851* showed significantly decreased CFU compared to the *L. pneumophila* 130b WT at 48 h post infection, while Δ*lpw4831*, Δ*lpw4841*and Δ*lpw4861* did not, indicating that *lpw4851* was the virulence factor responsible for the replication phenotype of ΔB. Plasmid pMMB207 carrying the promoter region and the full coding sequence of *lpw4851* was reintroduced into ΔB and Δ*lpw4851* respectively. As a result, the attenuated replication of both mutants was restored (Fig 3.5.B). According to homology detection by HMM-HMM comparison (https://toolkit.tuebingen.mpg.de/tools/hhpred), the secondary structure of Lpw4851 was similar to the *E. coli* glutamate-GABA transporter Gadc (E=1.9e-43), suggesting that *lpw4851* might contribute to bacterial intracellular replication by nutrient acquisition in *A. castellanii*.

3.3 Discussion

Many intracellular pathogens translocate effector proteins via different secretion systems to modulate host processes to facilitate bacterial survival. As a pathogen of amoebae and macrophages, *L. pneumophila* translocates a large number of effector proteins to hijack host cell biology for intracellular survival and replication. To characterise *L. pneumophila* effector proteins, ectopic expression libraries and phenotypic screening of mutants lacking single effector genes are commonly used methods (244, 423). To our knowledge, less than 25% of effector proteins have been well characterised. This low percentage highlights the challenges facing the characterisation of *L. pneumophila* virulence proteins and the requirement for new genetic tools (434).

In this study, we constructed in-frame markerless mutants in *L. pneumophila* 130b according to genomic regions enriched for putative Dot/Icm effector genes. All the mutants created were capable of growth in nutrient-rich media, while ΔA exhibited slower exponential phase growth and ΔB exhibit extended lag phase compared to WT. A selection of genes deleted in ΔA were predicted to encode multidrug-efflux proteins: *lpw00141* (EmrA), *lpw00171* (outer NodT) and *lpw00181* (RND transporter permease). Efflux systems in bacteria are involved in pumping out antibiotics and toxic substrates (435). The decreased capacity to export toxic metabolic substrates in ΔA might be associated with its slower exponential phase (Figure 3.2). In contrast, ΔB showed an extended lag phase in the nutrient-rich media. The lag phase is still poorly studied. It is predicted to allow the bacteria to adapt to the new culture environment (436). This process might include repair of macromolecular damage and preparation of cellular components for exponential growth (437). Interestingly, the subsequent mutants that lack single genes from genomic region B (Fig 3.5.A) did not show this extended lag phase, indicating a potential synergy mechanism applied by those genes for bacterial growth somewhat surprisingly. ΔP that lacked 4.6% of the whole genome was capable of growth in nutrient-rich media to WT levels (250). This indicates that the deletion of 106 genes had no adverse effect on *L. pneumophila* growth in nutrient-rich medium, suggesting that genes in those regions were designated for purposes other than fundamental biological processes.

In this study, we identified *L. pneumophila* genomic regions specifically required for optimal replication in *A. castellanii*. Pathogenicity islands or contiguous regions of DNA, genetic elements that enable the bacterium to colonise a host, have been well described in pathogens like *E. coli* and *Salmonella* (438-441). In *Salmonella*, pathogenicity islands are acquired from horizontal gene transfer and therefore, is always associated with an insertion site like tRNA genes (441). So far, 10 *Salmonella* pathogenicity islands have been discovered with diverse structure, function and contributions to pathogenesis (441). However, in *L. pneumophila*, bioinformatic-based analysis has failed to predict such genomic islands or organised clustering of genes required for intracellular growth other than Dot/Icm substrates. In our study, we checked intracellular replication of *L. pneumophila* mutants in *A. castellanii* and THP-1. Experimental conditions, including the MOIs, infection temperatures and lysis methods, were optimised in the lab to reflect infection capacity of *L. pneumophila*. As a result, ΔB, ΔE, F, ΔG, ΔI showed defects in replication in *A. castellanii*, even though all the mutants were still able to replicate in THP-1. The distinct replication phenotypes demonstrated the host-specific requirement of genes in those regions and defined genomic regions that are specifically essential for ideal pathogenicity in *A. castellanii*. Furthermore, deletion of 152 genes didn't affect replication in THP-1, but removal of 4 genes led to a severe growth defect in *A. castellanii*, suggesting that *A. castellanii* is a more restrictive host than human macrophages.

L. pneumophila uses host-specific amino acid transporters for nutrient acquisition. As *L. pneumophila* replicates specifically inside the LCV, numerous studies have been carried out to detect how *L. pneumophila* uses amino acids *in vivo*, but mainly in an indirect way or based on the transcriptional level (442). Host components of aerobic metabolism and amino acid catabolism, as well as the Entner-Doudoroff pathway, are expressed during infection, suggesting that *L. pneumophila* takes advantage of host carbohydrates and amino acids for nutrients during the replication phase (358). In human monocytes, *L. pneumophila* replication was blocked when SLC1A5, a neutral amino acid transporter, was inhibited, either by competitive inhibitor or siRNA silencing (443). Using ^{13}C isotopologue profiling to directly reflect metabolic pathway and fluxes, *L. pneumophila* was shown to acquire Ala, Asp, Glu, Ser, Phe, Tyr, Pro, and Gly from the *A. castellanii* cytosol surrounding the LCV and

incorporate these amino acids directly into bacterial proteins (442). Even though the bacteria were able to synthesis Ala, Asp, and Glu from other host derived carbon bodies, *L. pneumophila* incorporated host amino acid directly, probably for more efficient energy utilisation (442). The *L. pneumophila* Dot/Icm effector protein, AnkB, has been reported to anchor into the LCV membrane and recruit host degraded amino acids to support bacteria replication in LCV (444). In this study, we identified a putative glutamate transporter encoded by *lpw4851* to be required for bacterial replication specifically in *A. castellanii*. Subsequent complementation confirmed that Lpw4851 was required for *L. pneumophila* replication in *A. castellanii*.

Glutamate is a crucial amino acid for the lifecycle of *L. pneumophila in vitro* (445-447). However, it is not an indispensable amino acid for bacterial replication in human monocytes *Mono Mac 6*, suggesting a metabolism transition when replicating intracellularly (443). Also, previous metabolic profiling of *L. pneumophila* in *A. castellanii* and human monocytes, which is consistent with our finding of the distinct replication phenotype of Δ*lpw4851* in *A. castellanii* and THP-1, suggests that replication in different hosts requires the adaption of bacteria metabolism (442). Further studies will pursue the function of this protein, and hopefully provide deeper insight into bacterial metabolic routes used *in vivo* and perhaps inspires new strategies for bacterial control.

Recent work provides better knowledge of the expansion and evolution of *L. pneumophila* virulence factors like Dot/Icm effector protein (117, 231, 448). Previously, O'Connor generated *L. pneumophila* Philadelphia 1 mutants lacking gene clusters defined by transposon insertion mutant library screening. Gene clusters deleted in O'Connor's study varies from 77 kb-220 kb, which were much more larger than genomic regions deleted in this study. Homologous genes in C to I were also deleted in the Philadelphia 1 mutant library, but genomic regions A and B were unique to this study. Comparing with the corresponding mutants in O'Conner's study, mutant ΔC, ΔH, ΔI exhibited similar growth in nutrient-rich media and *A. castellanii*. However, ΔD, ΔE, ΔF and ΔG displayed different replication phenotypes in a protozoan host, probably due to the extra genes deleted by O'Conner or unrecognised SNPs.

Consistent with the minimised genome study in the Philadelphia 1 strain, the mutants constructed in this study mapped host-specific virulence factors to the genomic location (250). This aids phenotype screening and subsequent mutagenesis studies, as we can narrow down phenotype-associated virulence factors to a genomic region rather than investigating the whole genome. However, this method is time-consuming and can lead to unexpected genomic mutations. As host cellular pathways or factors are also critical for bacterial intracellular replication, new methods combining bacterial mutants and host cell pathway depletion will be a mounting solution to elucidate the function of bacterial virulence factors and host factors devoted to bacterial pathogenesis, especially in mammalian cells where the pathway depletion and genome-wide screening techniques are being well established (95, 431). By characterising a putative glutamate transporter necessary for replication in *A. castellanii*, this study reveals another aspect of *L. pneumophila* metabolism adaption in different hosts and provides new insight into host-pathogen co-evolution in the environment. Next, the mutants will be used as a tool to investigate the function of *L. pneumophila* virulence factors in influencing the *A. castellanii* life cycle and other cellular processes

Table 3.1 Genomic region deletion mutants

Region		Size (bp)	No. of effector genes	Percentage of total effectors	No. of Non-effector genes
Single-region deletion mutants					
ΔA	*lpw00071-lpw00221*	18017	3	1.2	13
ΔB	*lpw04831-lpw04861*	4764	3	1.2	1
ΔC	*lpw11451-lpw11581*	14387	5	2	9
ΔD	*lpw16861-lpw16971*	20752	5	2	7
ΔE	*lpw19951-lpw20201*	33112	10	4	16
ΔF	*lpw23101-lpw23561*	57568	13	5.2	32
ΔG	*lpw26241-lpw26431*	21878	8	3.2	11
ΔH	*lpw26521-lpw26861*	32458	8	3.2	27
ΔI	*lpw27241-lpw27551*	45088	12	4.8	20
Total			67	27	
Combination-region deletion mutants					
ΔF, G, H, I (Quadruple)		156992	41	16.5	90
ΔE, F, G, H, I (Penturple)		190104	46	18.5	106

Table 3.2 SNPs found in the constructed mutants

Mutant	Genes affected by SNPs	Consequence
ΔA	intergenic region	Unclear
	intergenic region	Unclear
ΔB	N/A	N/A
ΔC	*lpw31381*	Stop codon
ΔD	N/A	N/A
ΔE	*lpw20211*	Amino acid alteration
ΔF	N/A	N/A
ΔG	*lpw28981*	stop codon
ΔH	*lpw01551* (*lvh1D4*)	Amino acid alteration
	lpw31381 (*PtsP*)	Amino acid alteration
	intergenic region	Unclear
ΔI	*lpw31381*	Stop codon
ΔQ	N/A	N/A
ΔP	N/A	N/A

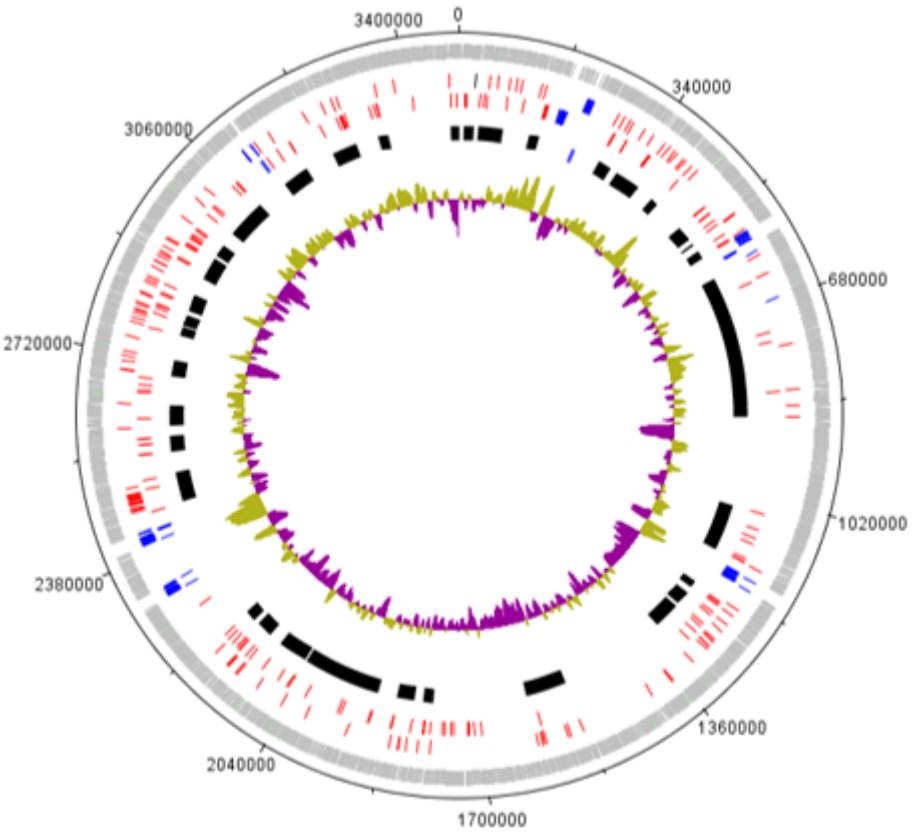


Figure 3.1 Schematic of the *L. pneumophila* 130b genome

L. pneumophila 130b genome harbours 3293 coding sequences (grey). Putative effector protein-encoding genes are shown in red; secretion system components-encoding genes are shown in blue, chosen genomic regions are shown in black. GC skewing (G−C/G+C) is shown in purple (< 0) and mustard (> 0). The map was constructed in DNAplotter (449).

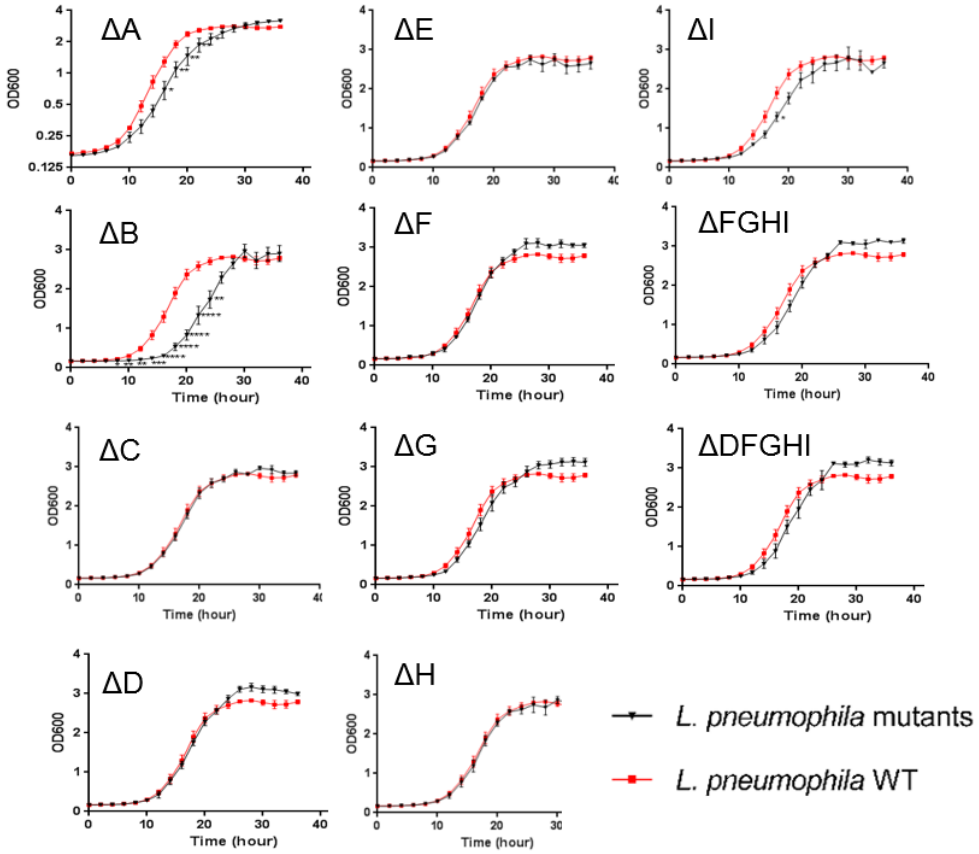
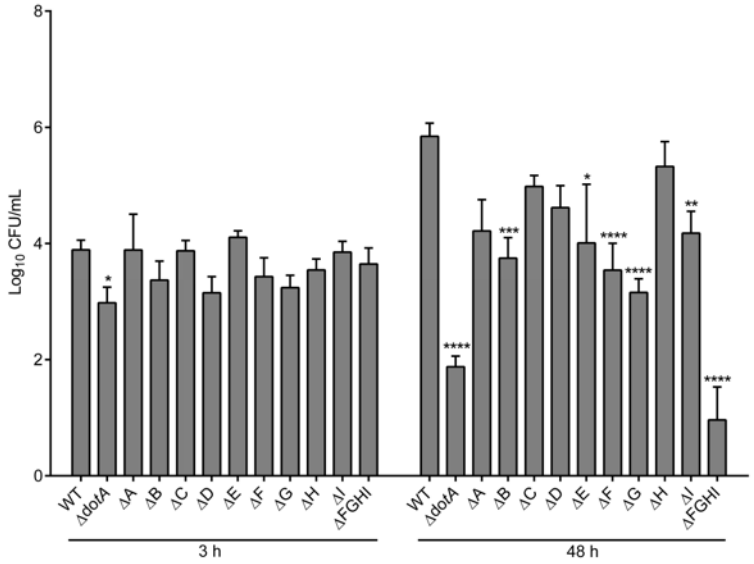


Figure 3.2 Growth of *L. pneumophila* in nutrient-rich media

Growth curves showing bacteria proliferation in nutrient-rich media ACES. Results are the mean ± SEM of four independent experiments carried out in triplicate. The asterisks indicate conditions that are significantly different compared to WT at the same time points (*$p < 0.05$, one-way ANOVA with Dunnett post test).

A.



B.

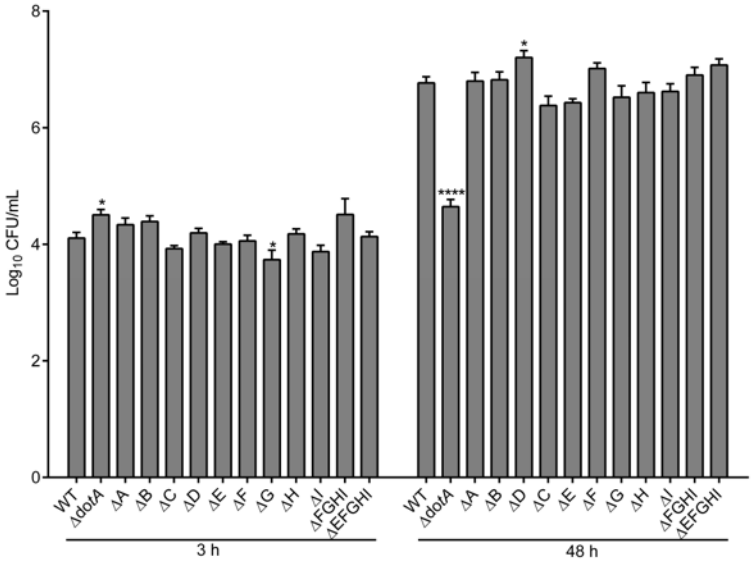


Figure 3.3 Intracellular replication assay of *L. pneumophila* mutants in different host cell types

Intracellular replication assay of *L. pneumophila* mutants in *A. castellanii* (**A)** and THP-1 **(B)**. Results are an average of five independents experiments, and error bars represent the standard error of the mean (±SEM). The asterisks indicate conditions that are significantly different compared to WT (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001, one-way ANOVA with Dunnett post test).

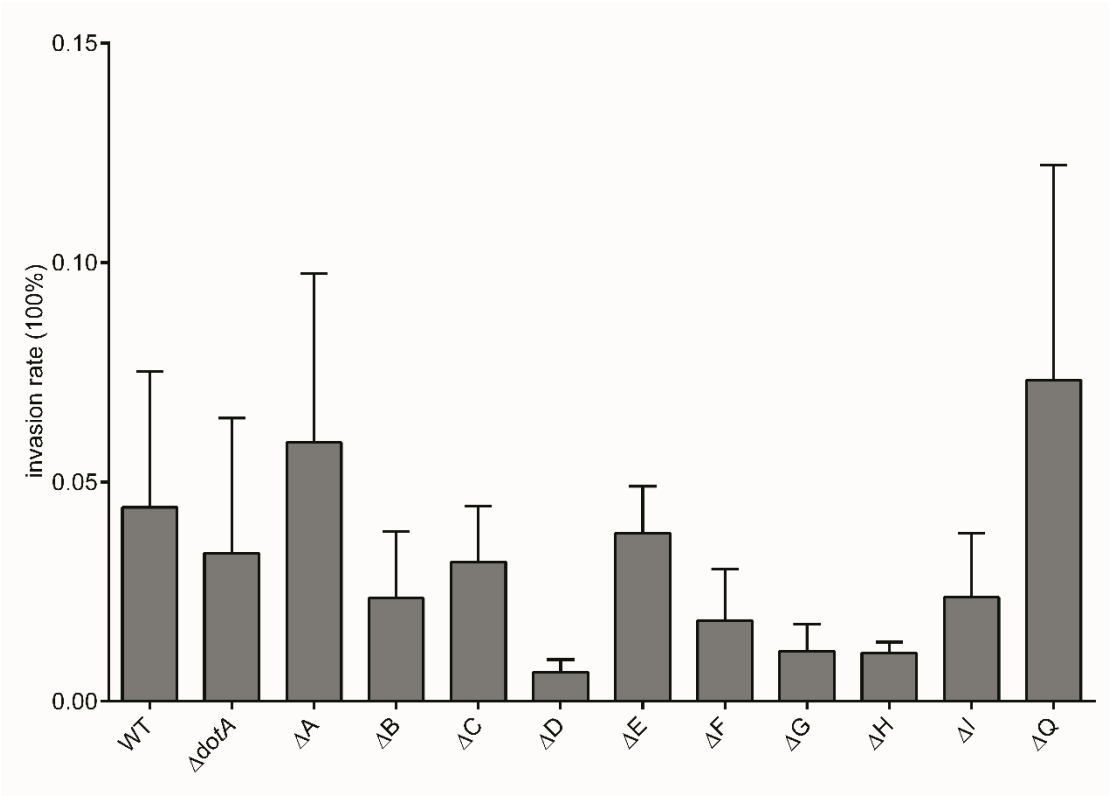


Figure 3.4 Invasion rate of *L. pneumophila* mutants

Invasion rate of *L. pneumophila* mutants in *A. castellanii*. Results are an average of five independents experiments, and error bars represent the standard error of the mean(±SEM). No significantly decreased (*$p < 0.05$) infection rate of muants was observed compared to WT using one-way ANOVA with Dunnett post test.

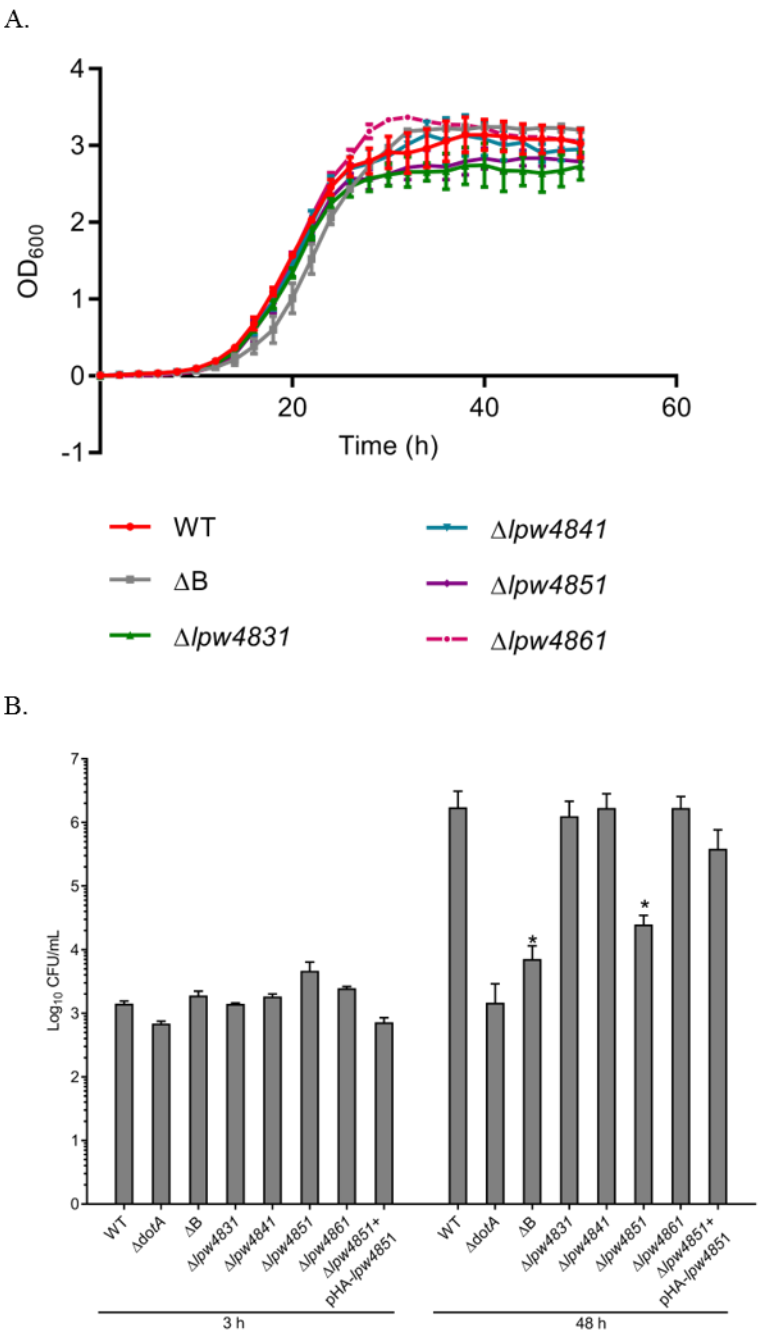


Figure 3.5. *lpw4851* is required for *L. pneumophila* replication in *A.castellanii*

A. Growth curve of *L. pneumophila* mutants Δ*lpw4831*-Δ *lpw4861*, ΔB and WT in nutrient-rich media ACES. Results are the mean ± SEM of four independent experiments carried out in triplicate. The asterisks indicate conditions that are significantly different compared to WT. (*p<0.05, one-way ANOVA with Dunnett post test). **B**. Intracellular replication assay of *L. pneumophila* mutants Δ*lpw4831*-Δ*lpw4861*, ΔB, Δ*lpw4861*:pHA-GadC and WT in *A. castellanii*. Results are an average of five independent experiments, and error bars represent the standard error

of the mean(±SEM). The asterisks indicate conditions that are significantly different to WT (*$p < 0.05$, one-way ANOVA with Dunnett post test).

Chapter 4. Investigating how *L. pneumophila* infection affects *A. castellanii* morphology

4.1 Introduction

In the environment, *L. pneumophila* is able to hijack the cellular processes of protozoan hosts such as *A. castellanii* to replicate intracellularly (354). Following replication in protozoan hosts, *L. pneumophila* displays increased resistance to antibiotics, acid, osmotic stress, thermal stress, as well as increased infectivity in humans (353, 355-358). The co-evolution of *L. pneumophila* with protozoa has led to the accumulation of virulence factors acquired from the protozoan host or from co-infecting pathogens (231). It is this interaction that amoebal hosts have allowed *L. pneumophila* to adapt to infect mammalian phagocytes (8-10). In both amoebae and human macrophages, *L. pneumophila* escapes the host lysosomal pathways and establishes the LCV to support intracellular replication (94, 108, 109).

The knowledge of host cellular processes influenced by *L. pneumophila* infection is poorly understood in the natural host, *A. castellanii*, as well as the *L. pneumophila* virulence factors involved in those cellular processes. To date, most studies on *L. pneumophila* pathogenesis are carried out in human macrophages. *A. castellanii* acts as a reservoir for intracellular bacteria like *L. pneumophila*, but the genomic complexity of *A. castellanii* and the restriction on phenotype screening tools in *A. castellanii* continue to limit the pace of discovery (398-400). The genome of *A. castellanii* is polyoid, encoding around 15500 intron-rich genes (398). Although approaches using RNA interference have been successfully established, knockout of genes of interest and transfection is difficult (399, 400).

Like many protozoan parasites, the life cycle of *Acanthamoeba* presents as two stages: trophozoites and cysts (379). Trophozoites are the active stage and replicate by binary fission (359, 380). Cysts are the dormant form and resistant to long-term starvation, low temperature and disinfectant treatments (379, 387). *A. castellanii* adopts bi-phasic life cycle for persistence and spread, but both trophozoites and cysts can be exploited by intracellular bacteria and giant viruses (450). *Legionella* spp, *Burkholderia pickettii, V. cholerae, Mycobacterium avium, Mycobacterium leprae* or methicillin-resistant *Staphylococcus aureus*, can all survive inside trophozoites and take advantage of the amoebae as vectors for dissemination (451-457). Meanwhile,

food-borne bacteria like *S. enterica*, *E. coli* and *Listeria monocytogenes* can survive during the encystment process and become significantly more resistance to various stressors after harvesting from *A. castellanii* cysts, suggesting cysts facilitate persistence of pathogenic bacteria in food-related environments (458, 459).

In addition to being environmental hosts of microorganisms, *Acanthamoebae*, including *A. castellanii*, are also opportunistic human pathogens causing Acanthamoeba keratitis (AK) and Granulomatous Amoebic Encephalitis (GAE) (460, 461). To date, cyst resistance to therapeutic agents, in addition to the recurrence of infection caused by excystment, remains a challenge that is yet to be addressed (379, 462). GAE occurs in immunocompromised individuals, such as those undergoing chemotherapy for cancer, immunosuppression for organ transplantation, HIV positivity or other clinical presentations (463-468). GAE is a rare infection but always fatal as *Acanthamoeba* enters the central nervous system (469, 470). *Acanthamoeba*, either trophozoites or cysts can enter the human body via nasal passages, lungs, skin lesions or olfactory neuroepithelium (471-473). The entry of *Acanthamoeba* into the central nervous system most likely occurs through the endothelial lining of cerebral capillaries (470, 474). Cutaneous and respiratory infections may develop over several months, but the involvement of the central nervous system can give fatal consequences within days or weeks (475). Unlike GAE, AK occurs in immunocompetent individuals, and most patients are direct contact lens users (476, 477). AK is a vision-threatening infection leading to 15% blindness in untreated patients (476, 478). In eye infections with *Acanthamoeba*, both the trophozoite and the cyst are considered infective (394). Improper contact-lens solutions like propylene glycol induce the encystment process, causing AK (479). While antimicrobial chemotherapy is the most commonly used method for *Acanthamoeba* infection, cysts are resistant to antibiotics, gamma radiation, anti-septics and other disinfectant treatment (480). The morbidity and mortality associated with AK and GAE are still high, partially due to incomplete knowledge of *Acanthamoeba* biology and pathogenesis. A large number of compounds that target membranes, intracellular processes, nucleic acid, protein synthesis have been explored, but their translational value remains unclear (461, 481-486). As a result, searching for potential targets during encystment processes is another approach to treating *Acanthamoeba* infection (379, 462).

The signals inducing encystment of free-living amoebae are various. Starvation, acidic pH, osmotic pressure and low-temperature are common environmental stimuli, while starvation is mostly used in lab conditions (386, 487). Bacterial infections, like *Francisella tularensis* type A strains, and toxin-producing *Pseudomonas aeruginosa* infection can also induce the encystment of *A. castellanii* (488, 489). Ras GTPases that activate the mitogen-activated protein kinase (MAPK) pathway might play a role in the encystment, as inhibition of Ras farnesylation reduces encystment (490). cAMP and the receptor that activates cAMP synthesis are thought to be involved in the encystment of *Hartmannella culbertsoni* and *Entamoeba histolytica* (491, 492). However, the knowledge of the signalling pathways involved in encystment is limited.

The encystment of *A. castellanii* involves morphological events and actin dynamics. Belonging to *Acanthamoeba* subgroup 3, *A. castellanii* cysts are round, smooth with ostioles distributed on the cell surface (381, 386, 493). Recent ultrastructural studies divided the encystment process into four morphologically stages: trophozoites; round precysts, in which the acanthapodia remain only as small protuberances; immature cyst with an ectocyst wall; and mature cysts with a double membrane cell wall (381). Encysting amoebae display cytoplasmic areas containing thin fibrillar materials and small vesicles containing electron-dense material (381). Mature cysts present pseudopodia projection toward the ostiole and hypothetical chromotoid bodies (394). Additionally, actin is in the cytoplasm of premature cysts and cysts undergo excystment but not in mature cysts, indicating a dynamic actin synthesis during encystment (381). In axenic conditions, cysts can transition to trophozoites, and this process is termed excystation (392-394). Excystation occurs through the ostioles and leaves the cyst walls behind, following the formation of endocytic channels, phagocytic structures and the pseudopodia projection (394, 395).

Protein turnover, cell wall synthesis and epigenetic modifications are important events occurring during *A. castellanii* encystment (490, 494-500). The outer-cyst wall, known as the ectocyst is composed of a high percentage of proteins and polysaccharides, while the inner endocyst is composed mainly of cellulose (394, 501, 502). The first described molecular change in amoebae during encystment is the expression of CSP21 (cyst-specific protein of 21 kDa). CSP21 is tightly repressed in trophozoites and highly induced in the early differentiation stage, suggesting an anti-

repression mechanism regulates the encystment process (503). Later, transcriptomic analysis had revealed a subtilisin-like serine protease and a cysteine protease which are induced during the encystment (504-506). siRNA targeting the catalytic domain of the proteases or treatment with chemical inhibitors efficiently reduced encystment in *A. castellanii* (507-509). An M17 leucine aminopeptidase, AcLAP, is highly expressed during the late phase of encystment and is essential for the morphology of mature cysts (500). The induction of proteases indicates the need for protein turnover, which is completed via lysosomes or ubiquitin-proteasome systems during encystment (387). Autophagy associated genes *atg8*, *atg12*, *atg16* are also upregulated during encystment (494-498, 510, 511). In addition to the presence of autolysosomes during encystment, it is likely that proteolysis plays a role (511).

Cellulose and xylose are two fundamental components of cyst walls (490, 501, 502). Pioneering work has demonstrated that *Acanthamoeba* encystment induces the incorporation of glucose into cellulose β-glucan synthetase activity (512-514). Chemical inhibition of cellulose synthesis or siRNA knockdown of glycogen phosphorylase, which are involved in glycogen hydrolysis to glucose and subsequently serve in cellulose synthesis, lead to defective encystment (515, 516). Gas chromatography/mass spectrometry (GC/MS), has revealed xylose as an important constituent and siRNA probes against xylose isomerase and its exogenous inhibitor, sorbitol, block *A. castellanii* encystation (490, 517). Surprisingly, exogenous xylose reduces *A. castellanii* encystation, possibly because exogenous xylose replaces cellulose biosynthesis with incorporation into the cyst wall, leading to immature cysts (517). These findings show that xylose isomerase and cellulose synthase are important mediators of encystation in *A. castellanii*.

Earlier studies of *A. castellanii* have mainly focused on its biochemistry, cell biology or pathogenesis (379). *A. castellanii* encystment is attracting more attention given its role in the pathogenesis of human infections. Recently, more studies have focused on the role of intracellular bacteria that could interfere with the *A. castellanii* life cycle. In this study, we investigated changes in *A. castellanii* morphology during *L. pneumophila* infection and identify *L. pneumophila* virulence factors involved in affecting *A. castellanii* encystment.

4.2 Results

4.2.1 *L. pneumophila* replication led to the inhibition of *A. castellanii* proliferation

The infection process of *L. pneumophila* in *A. castellanii* is reminiscent of what occurs in human macrophages. In order to evaluate how *L. pneumophila* infection affects *A. castellanii* biology, we first investigated *A. castellanii* proliferation during *L. pneumophila* WT or Δ*dotA* infection. *A. castellanii* was infected at MOI of 10 and 16, 24 and 48 hours after infection, amoebal cells were quantified. Amoebae numbers were similar to uninfected cells at 16 hours. Overall numbers of amoebae were significantly decreased upon infection with *L. pneumophila* WT compared to uninfected amoebae while the number of amoebae infected with ⊿*dotA* was not, indicating that inhibition of *A. castellanii* proliferation is Dot/Icm dependent (Figure 4.1 B).

To investigate the morphology of *A. castellanii* with *L. pneumophila* infection, we used electron microscopy to compare the morphology of *L. pneumophila* infected *A. castellanii*, trophozoites and mature cysts. *A. castellanii* was infected with *L. pneumophila* by a MOI of 10 and incubated 24 h in PYG at 37℃. Meanwhile, the transition from trophozoites to mature cysts was induced by incubating *A. castellanii* in amoebae buffer for three days at 30℃, without *L. pneumophila* infection. The mature cysts and *L. pneumophila* infected *A. castellanii* were then fixed and processed for electron microscopy (Materials and Methods). Under electron microscopy, the LCV is clearly observed in *L. pneumophila* WT infected *A. castellanii* from 8 h post infection (Figure 4.1 A). Numerous *L. pneumophila* was observed in LCV at 16 h post infection (Figure 4.1 C). *L. pneumophila* replicated to a high number and lysed cells at 24 h post infection (Figure 4.1 C). After 24 h post infection, *A. castellanii* displayed a different morphology from typical trophozoites. Cells became rounded without clear pseudopodia. Notably, neither pseudopodia nor double-layer cyst walls were visible on *L. pneumophila* infected amoebae (Figure 4.1 C-E). The distinct morphology of *L. pneumophila* infected *A. castellanii* compared to trophozoites and mature cysts suggests that *L. pneumophila* infection interferes with *A. castellanii* life cycle transition but does not induce *A. castellanii* encystment.

4.2.2 *L. pneumophila* infection inhibits starvation-induced encystment of *A. castellanii*

To investigate if *L. pneumophila* infection inhibits the encystment capacity of *A. castellanii*, we examined starvation-induced encystment of *A. castellanii* during *L. pneumophila* infection. We transformed a GFP-expressing vector pMip-GFP into *L. pneumophila* to quantify infection efficiency. *A. castellanii* was infected with GFP-expressing *L. pneumophila* and incubated in amoebae buffer at 30℃. After three days, *A. castellanii* was collected, stained with calcofluor white (CFW) stain to detect mature cysts, and processed for confocal microscopy (Materials and Methods). Under Confocal FV1200, two groups of cells were observed. Uninfected *A. castellanii* showed no GFP signal but strong CFW was detected, indicative of two-layer cyst walls of mature cysts (Figure 4.2 A). In contrast, *A. castellanii* harbouring *L. pneumophila* showed no CFW staining (Figure 4.2 B). This suggested that *A. castellanii* infected with *L. pneumophila* did not turn to cysts.

In order to confirm the inhibition of *L. pneumophila* infection on *A. castellanii* encystment, flow cytometry was subsequently utilised to quantify the percentage of cysts upon *L. pneumophila* infection. In this experiment, *L. pneumophila* expressing GFP was used to infect *A. castellanii* with MOI of 10. *A. castellanii* were then incubated in amoebae buffer 3 days at 30℃. At 24 h, 48 h, 72 h post infection, cells were harvested, stained with CFW, fixed and then processed on Fotesa II. X and Y axis indicated CFW and GFP signal density, respectively. The GFP^+/CFW^+ population was regarded as the infected cysts. Cyst percentage was denoted as the percentage of infected cysts from the infected cells. Meanwhile, the percentage of uninfected cysts among uninfected cells was quantified as a control. As a result, 20% of uninfected amoebae transitioned to cysts from trophozoites at 24 h post infection, and the percentage reached 50% at 72 h post infection. Notably, approximately 50% of uninfected *A. castellanii* were not stained by CFW after 72 h induction (Figure 4.2 C). According to the images captured by ImageStream, uninfected *A. castellanii* which did not turn to cysts at 72 h were probably dead in the nutrient-depletion medium (Figure 4.5). In contrast, the percentage of cysts in *L. pneumophila* infected *A. castellanii* was negligible (Figure 4.2 C). The percentage of infected *A. castellanii* that was CFW positive was less than 1% even after 72 h (Figure 4.2 C).

Altogether, the results confirm that *L. pneumophila* infection significantly inhibits starvation-induced encystment of *A. castellanii*.

4.2.3 *L. pneumophila* ΔG infected *A. castellanii* showed reduced encystment inhibition compared to wild type *L. pneumophila* infected *A. castellanii*

In order to determine which *L. pneumophila* virulent factors contribute to the inhibition on *A. castellanii* encystment, we assessed *A. castellanii* encystment upon infection with *L. pneumophila* mutants carrying large genome deletions. Firstly, mutants carrying large genomic deletions constructed in this study (Chapter 3) were transformed with pMip-GFP (Materials and Methods). The percentage of *A. castellanii* cells infected with *L. pneumophila* mutants that transitioned to cyst was quantified by flow cytometry. *L. pneumophila* infection and quantification of cysts percentage were carried out as described above. X and Y axis indicated CFW and GFP signal density, respectively. *A. castellanii* infected with ΔB and ΔF mutants exhibit less GFP positive cells at 24 h post infection, which might be caused by defective replication capacity of those mutants (Fig 4.3 A). Only a small number of GFP positive cells were detected in ΔQ infected samples, consistent with the reduced replication observed in replication assays (Fig 4.3 A). Among all the mutants examined in this study, ΔG displayed an attenuated ability to inhibit the encystment of *A. castellanii*, with a cyst percentage of 17% at 48 h post infection and 22% at 72 h post infection (Fig 4.3 B). Additionally, the attenuated replication capacity of *L. pneumophila* mutants does not appear to affect the inhibition of *A. castellanii* encystment as ΔB and ΔF still showed potent inhibition of *A. castellanii* encystment. Taken together, our results suggested that genes deleted in ΔG were associated with inhibition of *A. castellanii* encystment, and this process was not entirely dependent on bacterial numbers.

4.2.4 *L. pneumophila letA* contributed to encystment inhibition during *A. castellanii* infection

In order to determine which gene in ΔG was responsible for encystment inhibition of *A. castellanii*, we examined the genes deleted in ΔG (Table 4.1). According to whole-genome sequencing, the genomic region spanning *lpw26241 to lpw26431* was deleted in ΔG. In addition, SNPs occurred in *lpw28981* (*letA*) which led to its premature translation stop. Therefore, sub mutants ΔG1 -ΔG4 and Δ*letA* were constructed using

the pSR47s suicide vector approach and subsequently transformed with pMip-GFP (Materials and Methods). *L. pneumophila* infection and quantification of the percentage of cysts was carried out as described above. X and Y axis indicated CFW and GFP signal density, respectively. Δ*letA* displayed an attenuated capacity to inhibit the encystment of *A. castellanii*, while ΔG1- ΔG4 displayed strong inhibition on *A. castellanii* encystment, comparable to *L. pneumophila* WT (Figure 4.4 B). To confirm that the Δ*letA* infected *A. castellanii* could undergo encystment, we also investigated the morphology of Δ*letA* infected amoebae using Imagestream. Following *L. pneumophila* infection and cyst inducement, amoebae were subgrouped according to GFP and CFW signal density. Representative images were captured by Imagestream. As a result, encysting *A. castellanii* harbouring Δ*letA* displayed weak CFW staining, indicative of immature ectocyst cell walls, compared to uninfected cysts that displayed intense CFW staining, indicative of double cell walls. According to the representative images captured by Imagestream, three location patterns of *L. pneumophila* Δ*letA* was observed (Figure 4.5): 1). between the exocyst and the endocyst, 2). in the cytosol of fold cysts, 3) close to exocysts. These results suggested that *L. pneumophila letA* was required for complete inhibition of *A. castellanii* encystment.

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4.3 Discussion

To date, the impact of *L. pneumophila* infection on the life cycle of the natural host, *A. castellanii* has been poorly studied. Here, we developed a novel approach for quantifying cyst formation in the context of *L. pneumophila* infection. We developed a flow cytometry-based CFW staining method to quantify the encystment capacity of *A. castellanii*. CFW binds to cellulose and generates stable fluorescence, providing a convenient method for distinguishing cysts from trophozoites (518). We found that infection of *A. castellanii* with *L. pneumophila* led to inhibition of cell proliferation and the transition from trophozoites to cysts, including when amoebae were cultured under starvation conditions. These findings were consistent with previous studies, that revealed a putative CDK protein CDC2b could be related to proliferation inhibition in *L. pneumophila* infected *A. castellanii*, but its mechanism needs more investigation (412).

Previously, it was thought that cysts facilitate *L. pneumophila* persistence and dissemination (133). *L. pneumophila* has been observed in mature cysts of *A. polyphaga* by EM following chlorine exposure (388). However, mature cysts of *A. castellanii* containing *L. pneumophila* has not been observed, even in an experiment where *A. castellanii* was incubated in nutrient-depletion medium for six months with *L. pneumophila* infection (412, 519). In our study, wild type *L. pneumophila* created LCVs in trophozoites but not in cysts. As experimental conditions significantly influence the efficiency of amoebae encystment and the morphology of cysts, the different observations are possibly a result of different amoebae strains and different experimental conditions (386, 480, 520). However, here we confirmed that *L. pneumophila* strongly inhibited starvation-induced encystment of *A. castellanii*.

L. pneumophila may inhibit *A. castellanii* cyst formation in order to maximise its replication and transmission in the environment. Firstly, inactive metabolic cysts are not supportive of bacterial replication. As an example, *F. tularensis* expressing GFP was only detected at 24 h post infection in encysting *A. castellanii*, possibly due to metabolic inactivity of bacteria in cysts (488). Secondly, inhibition of encystment by *L. pneumophila* maintains the permissivity of amoebae to pathogens (521). *L. pneumophila* is taken up mainly by phagocytosis in *A. castellanii*, where the

pseudopodia are essential (141). Encysting *A. castellanii* and mature cysts lose pseudopodia and are less efficient in *L. pneumophila* internalisation. As a result, by inhibiting *A. castellanii* encystment, *L. pneumophila* maintains a host cell conducive for bacterial uptake and replication.

The ability of *L. pneumophila* to inhibit *A. castellanii* cyst formation was decreased when *L. pneumophila letA* was deleted under starvation conditions. The decreased encystment inhibition of Δ*letA* infected amoebae might be caused by less efficient second-round infection, which occurred 24 h post infection, as Δ*letA* could still form an LCV according to analysis via confocal microscopy (data not shown). LetA is the response regulator of the LetAS regulation system in *L. pneumophila* and forms LetAS-RsmYZ-CsrA regulation cascade (276). LetA is stress regulator during stationary phase and is required for efficient replication of *L. pneumophila* in *A. castellanii* (275, 278, 279). In addition to regulating the expression of Dot/Icm several effector proteins, including RalF, LegC2, LegC7, VipA, LetAS also regulates the expression of Dot/Icm secretion system encoding genes *icmT*, *icmP*, *icmR* (276, 282, 284). One hypothesis is that the effector proteins regulated by LetAS are expressed during the late infection phase, which is equivalent to stationary phase, and thus are translocated early in the next infection cycle to be functional (284). Dot/Icm effector proteins are secreted at the stationary phase when cultured in broth, to support successful early infection events (522). Δ*let* might be attenuated in effector secretion at the beginning of the second round of infection although this remains to be tested. Additionally, *icmT* is required for bacterial egress via pore-forming activity in *A. polyphaga* and macrophage (188, 189, 191). Absence of i*cmT* leads to less bacterial viability in *A. polyphaga*, which might influence bacterial infection capacity (188, 189, 191). Thus, *L. pneumophila* Δ*letA* might be less viable intracellularly and exhibit deficient translocation of early-phase effector proteins, leading to attenuated invasion and replication during the second round infection in *A. castellanii*. Accordingly, among the encysting *A. castellanii* with Δ*letA* infection, we only observed single *L. pneumophila* in encysting *A. castellanii*, instead of a bacterial replication vacuole. Investigation of effector protein expression profiles and translocation profiles might give more insights into how *letA* contributes to encystment inhibition.

Δ*letA* was shown to localise to the clear region between the ectocyst and the endocyst or in the cytosol of fold cysts. This localisation is reminiscent of *Simkania negevensis*

or *Mycobacterium avium* infection of *A. polyphaga* where the bacteria are found in mature cysts, specifically in the space of cyst walls (488, 523, 524). The particular location protects *Mycobacterium avium* from adverse environmental conditions and allows the bacteria to escape rapidly from *A. polyphaga* cysts under favourable conditions (525). The interaction between cellulase or cellulose-binding proteins with cellulose in cyst walls might play a role in *Mycobacterium avium* localisation to cyst walls (111, 525, 526). *L. pneumophila* encodes putative cellulase (Lpg1918/Lpw19571) and cellulose-binding proteins, which might be responsible for the special localisation of this localisation of Δ*letA* (110).

We also propose that encystment might be triggered as a self-protection mechanism with bacterial or virus invasion. Previous studies reported that infection with *Francisella tularensis*, an etiological agent of the zoonotic disease tularemia, induced *A. castellanii* encystment, although *A. castellanii* is not the natural host of *F. tularensis* (488, 520). *A. castellanii* undergoing encystment or mature cysts are resistant to infection with the *Acanthamoeba polyphaga mimivirus* (APMV) or marseillevirus, the giant viruses which are phagocytosed by *A. castellanii* (450, 521). Moreover, waterborne bacteria *V. cholerae* can replicate in trophozoites and lyse mature cysts of *A. castellanii* (453). Those observations suggest that *A. castellanii* encystment is advantageous to protect *A. castellanii* from bacteria or virus invasion, but the long-term coevolution allows some ‘passengers’ to accumulate enough virulence factors to overcome this strategy by lysing cysts or inhibiting encystment. The distinct fate of the “passengers” of *A. castellanii* also reflects different evolutionary directions for pathogenic bacteria in the same host.

Our study revealed that *L. pneumophila* rapidly inhibits *A. castellanii* encystment and suggested that *L. pneumophila* inhibits *A. castellanii* encystment for maximum replication and transmission in the environment. We found that the LetAS system, which regulates the expression of several Dot/Icm effector proteins was important for cyst inhibition. Future studies will investigate the LetAS regulated effector proteins that contribute to the inhibition of *A. castellanii* encystment directly. In addition, cysts from pathogenic amoebae pose a threat to human health as mature cysts are resistant to antibiotics, antiseptics and high levels of UV and gamma radiation (527, 528). Mature cysts also avoid attracting neutrophils or macrophages to the site of infection (529). Our study provides a biological model for research into cyst formation and

anti-cyst research. The cellular pathways differentially regulated in *A. castellanii* responding to *L. pneumophila* infection and Δ*letA* infection will be investigated further to provide insights into critical encystment pathways. Our study will add knowledge of *L. pneumophila* transmission and *A. castellanii* biology and may also inform new strategies to restrict *L. pneumophila*, and amoebal transmission in the environment.

Table 4.1 List of genes deleted in ΔG1-ΔG4

Subgroup	Gene	Name	description
G1	*lpw26241*	*ceg29*	hypothetical protein lpg2409
	lpw26251		sensor histidine kinase
	lpw26261	*vpdA*	patatin-like phospholipase
	lpw26271		
	lpw26281	*lem24*	hypothetical protein lpg2411
G2	*lpw26291*		transposase (ISmav2)
	lpw26301		beta-lactamase
	lpw26311		hypothetical protein *lpg2413*
	lpw26321		sulfate transporter
	lpw26331		hypothetical protein *lpg2414*
	lpw26341		transmembrane protein
G3	*lpw26351*	*legA1*	ankyrin repeat-containing protein
	lpw26361		hypothetical protein *lpg2417*
	lpw26371		penicillin-binding protein AmpH
	lpw26381		hypothetical protein *lpg2419*
	lpw26391		hypothetical protein *lpg2420*
G4	*lpw26401*	*lem25*	hypothetical protein *lpg2422*
	lpw26411		hypothetical protein *lpg2423*
	lpw26421	*mavG*	hypothetical protein *lpg2424*
	lpw26431	*mavH*	hypothetical protein *lpg2425*

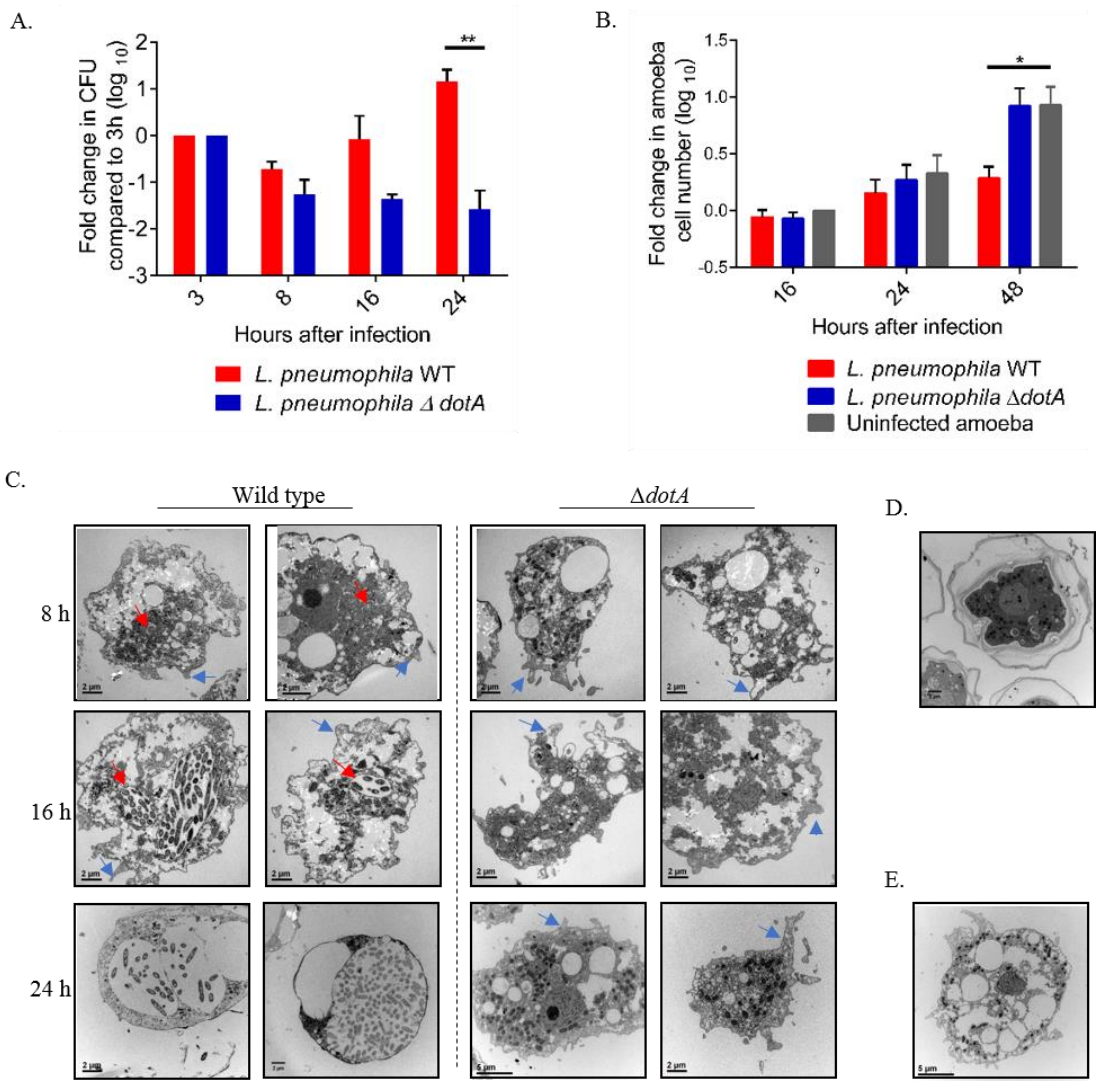


Figure 4.1 *L. pneumophila* infection leads to *A. castellanii* proliferation inhibition and morphological transition

A. Intracellular replication assay of *L. pneumophila* in *A. castellanii* within 24 h. **B.** *A. castellanii* proliferation with *L. pneumophila* infection. Results of proliferation and intracellular replication assay are an average of three independent experiments, and error bars represent the standard error of the mean (±SEM) (*$p < 0.05$, unpaired t-test). **C.** *A. castellanii* morphology with *L. pneumophila* WT or Δ*dotA* infection at indicated time points. **D.** Morphology of uninfected mature cysts after 3 days inducement. **E.** Morphology of uninfected trophozoite. Red and blue arrows indicate LCV and pseudopodia, respectively.

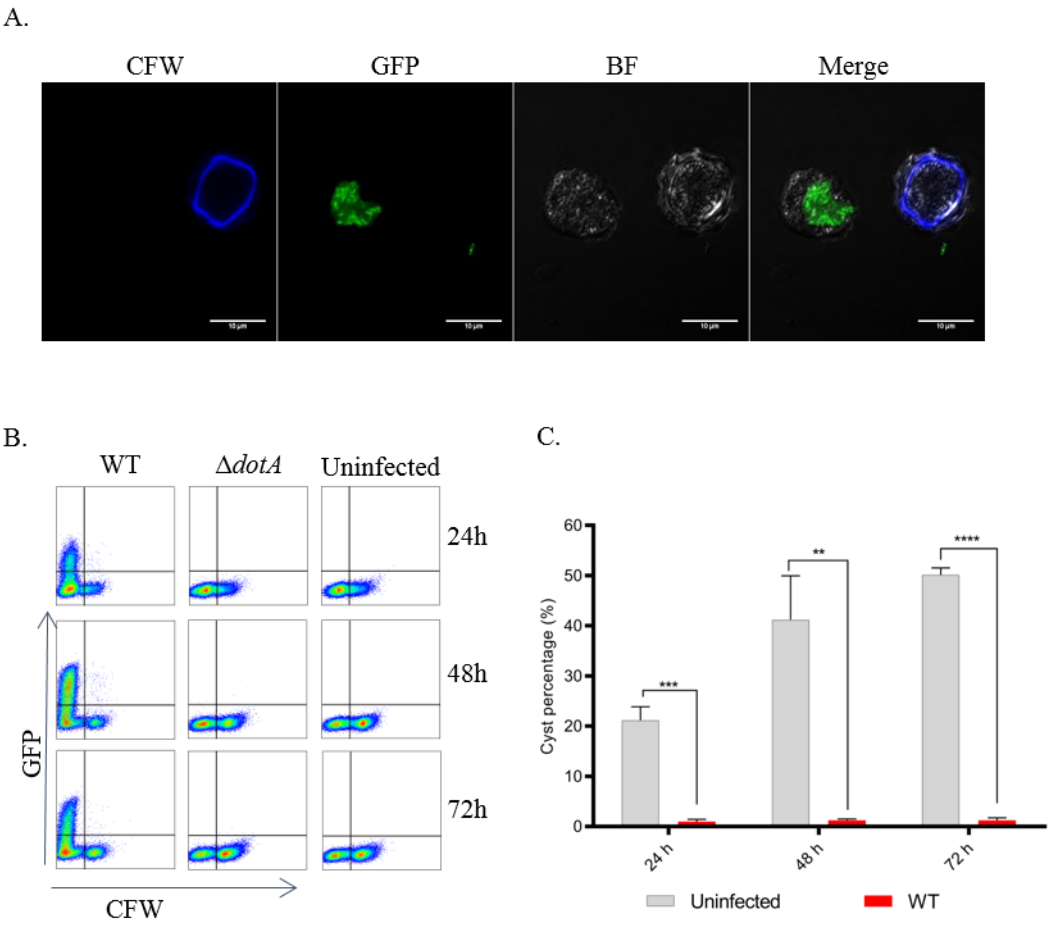
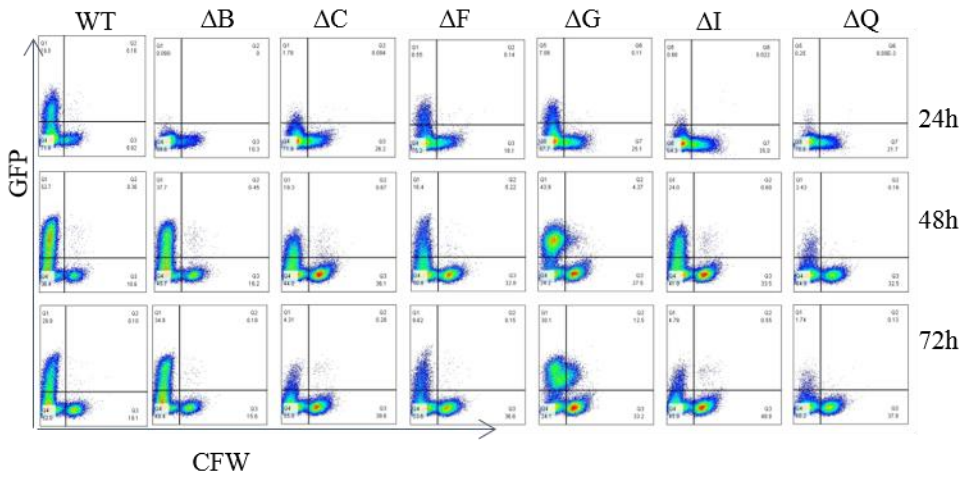


Figure 4.2 *L. pneumophila* infection inhibits *A. castellanii* encystment

A. Confocal fluorescence microscopy showing *A. castellanii* morphology. GFP and CFW staining indicate *L. pneumophila* and mature cysts, respectively. **B.** Detection of *A. castellanii* encystment with *L. pneumophila* infection by flow cytometry. X and Y-axis represent GFP and CFW density, respectively. **C.** Quantification of the percentage of cysts with *L. pneumophila* infection determined by flow cytometry. Results are the mean of three independent experiments and error bars represent the standard error of the mean(±SEM) (**p < 0.01, ***p < 0.005, ****p < 0.001, unpaired t-test)

A.



B.

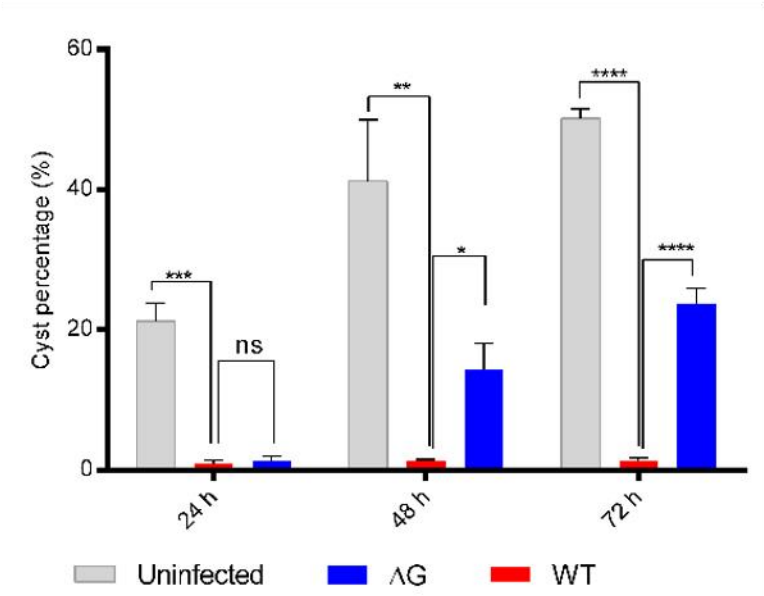
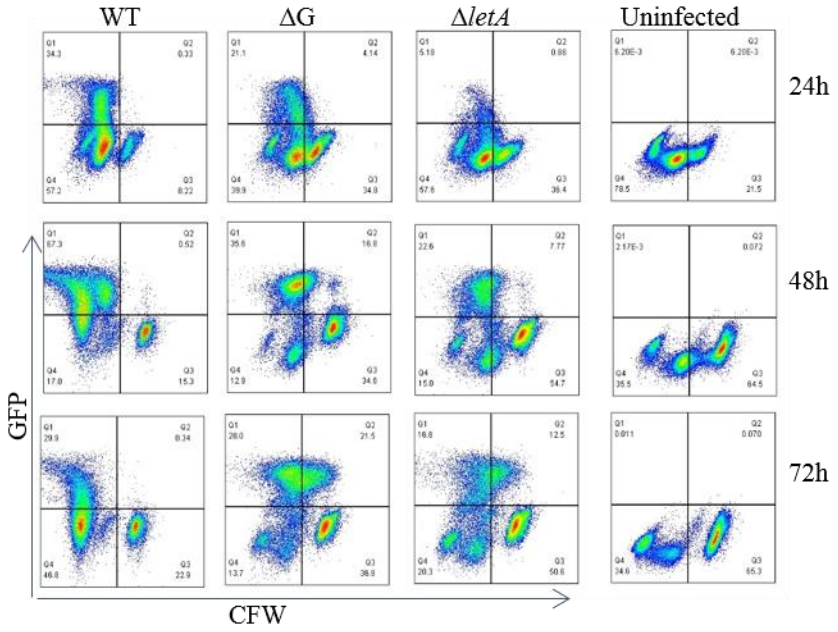


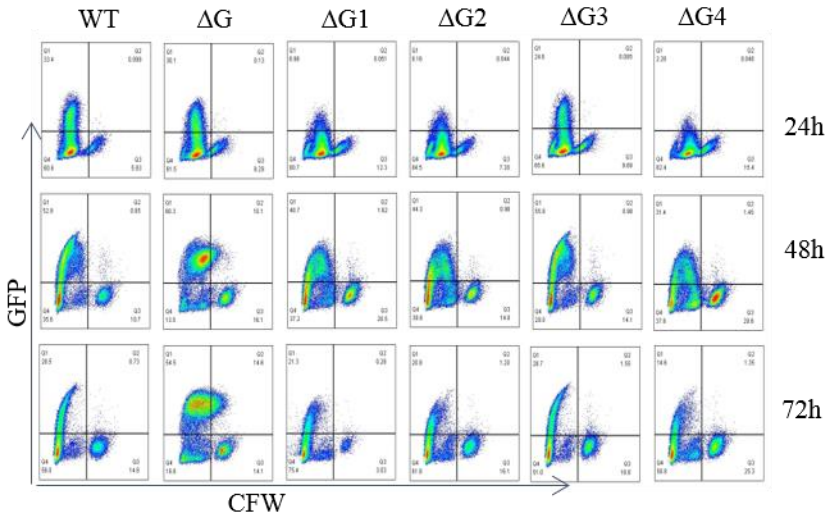
Figure 4.3 *A. castellanii* encystment is partially eliminated with *L. pneumophila* ΔG infection

A. Detection of *A. castellanii* encystment with *L. pneumophila* mutants infection by flow cytometry. X and Y-axis represent GFP and CFW density, respectively. **B.** Quantification of cysts percentage after *L. pneumophila* ΔG infection determined by flow cytometry. Results are the mean of three independent experiments, and error bars represent the standard error of the mean(±SEM) (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001, unpaired t-test).

A.


WT
ΔG
Δ*letA*
Uninfected
24h
48h
72h
GFP
CFW

B.


WT
ΔG
ΔG1
ΔG2
ΔG3
ΔG4
24h
48h
72h
GFP
CFW

C.

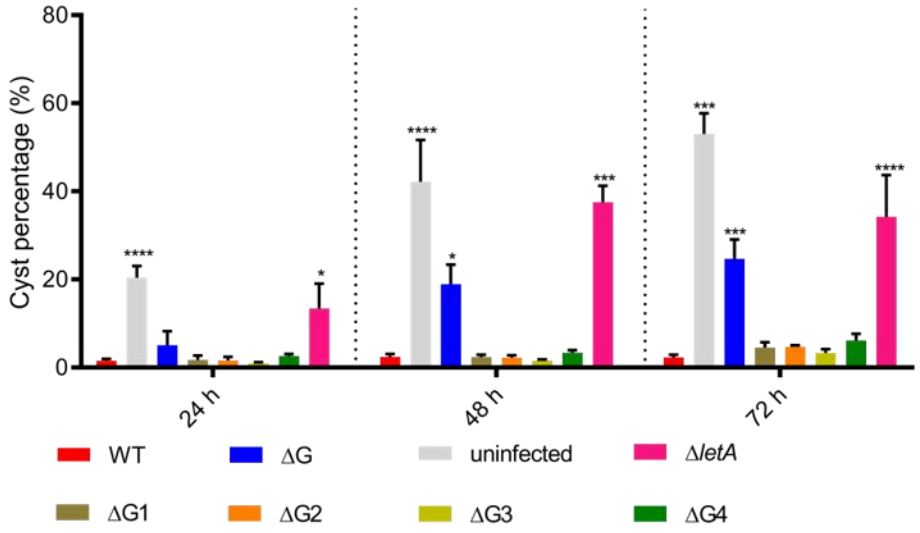

Cyst percentage (%)
80
60
40
20
0
24 h
48 h
72 h
WT
ΔG
uninfected
Δ*letA*
ΔG1
ΔG2
ΔG3
ΔG4

Figure 4.4 *L. pneumophila letA* is required for inhibition on *A. castellanii* encystment

A,B. Detection of *A. castellanii* encystment upon *L. pneumophila* WT, ΔG, ΔG1, ΔG2, ΔG3, ΔG4 and Δ*letA* infection by flow cytometry. X and Y-axis represent GFP and CFW density, respectively. **C.** Quantification of the percentage of cysts after infection with *L. pneumophila* WT, ΔG, ΔG1, ΔG2, ΔG3, ΔG4 and Δ*letA* determined by flow cytometry. Results are the mean of at least three independent experiments and error bars represent the standard error of the mean(±SEM). The asterisks indicate conditions that are significantly different compared to WT at the corresponding time point (*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, ****$p < 0.001$, unpaired t-test).

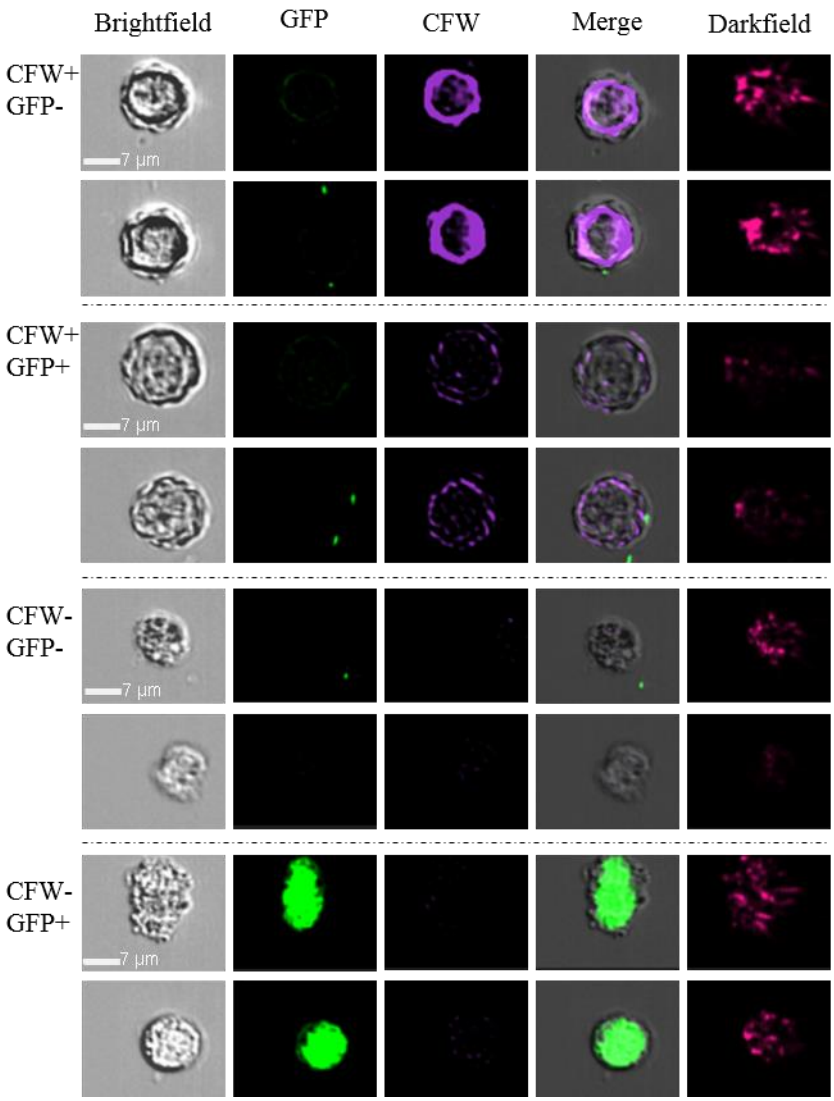


Figure 4.5 Morphology of different populations in Δ*letA* infected *A. castellanii*

Representative images displaying the morphology of Δ*letA* infected *A. castellanii* which were divided into four population based on the fluorescent density. Fixed amoebae were acquired on an Amnis Imagestream X at 60 × magnification with extended depth of field. IDEAS software was used to identify and collate images from populations based on CFW staining and GFP-*Legionella* burden.

Chapter 5: Investigating *L. pneumophila* virulence factors that affect the *A. castellanii* transcriptome during infection

5.1 introduction

Previously, we investigated *L. pneumophila* virulence factors essential for bacterial replication in *A. castellanii* (Chapter 3) and identified the life cycle of *A. castellanii* during *L. pneumophila* infection (Chapter 4). Underpinning these replicative and morphological changes is *L. pneumophila*'s ability to interfere with a broad range of host cellular pathways to establish the replication niche and host defence response. The complex interactions between *L. pneumophila* and different hosts, particularly between *L. pneumophila* and *A. castellanii* are not well understood.

Transcriptomic analysis using RNA sequencing or DNA microarrays has provided insights into how host cellular processes respond to *L. pneumophila* infection. In mammalian macrophages, *L. pneumophila* triggers a productive transcriptional response that includes many early mediators of immune and inflammatory responses, as well as regulators of cell survival and apoptosis (444, 530, 531). Specifically, *L. pneumophila* flagellin triggers a specific response from B6 bone marrow-derived macrophages (BMDMs) involving inflammasome effectors (Il-1α and Il-1β), chemokines associated with the attraction of neutrophils (Cxcl1, Cxcl2 and Ccl7) and modulation of the cell cycle progress (Gadd45a, Gadd45b and Cdk5r1) (531). Further transcriptome studies using *L. pneumophila* infected C57BL/6J BMDMs, has revealed the induction of a novel innate immune response termed the 'effector triggered response' (ETR) (307). BMDMs infected with *L. pneumophila* exhibit a MAP kinase response where genes involved in the pathway are transcribed but fail to be translated due to effector-dependent host protein synthesis inhibition. However, a few pro-inflammatory cytokines, such as IL-1α and IL-1β, can overcome suppression in these cells by a MyD88 dependent manner (532). Human monocyte-derived macrophage (HMDM) transcriptome profiling during *L. pneumophila* WT infection reveals upregulation of multiple inflammatory pathways such as IL-10 anti-inflammatory, interferon signalling, anti-apoptosis signalling pathways controlled by the transcriptional regulator NF-κB, and downregulation of protein synthesis pathways (530, 533).

L. pneumophila up-regulates transcription of various host transporters during infection including SLC1A5 and SLC7A5, enabling *L. pneumophila* to import amino acids from the cytoplasm into the LCV lumen (443, 530). In *D. discoideum*, genes associated with amino acid metabolism, amino-acyl tRNA synthetases and enzymes involved in nucleotide metabolism are upregulated (534). Moreover, changes in diverse lipid metabolic pathways were the most pronounced metabolic alterations observed in HMDMs infected *L. pneumophila*, regardless of bacterial replication (530). *L. pneumophila* also utilises inositol metabolism for promoting the intracellular growth, virulence, and fitness of the pathogen (535). Taken together, these results suggest that *L. pneumophila* likely hijacks host metabolism pathways for bacterial replication. Indeed, *L. pneumophila* infection leads to a rapid increase in intracellular concentration of amino acids to power rapid bacterial growth (444).

In *D. discoideum*, *L. pneumophila* triggers the regulation of host vesicle fusion. Arf1 and CopB encoding genes were modulated during the infection process, supporting interference of the pathogen with vesicle transport (534, 536). *D. discoideum* induces many stress protein-encoding genes, like the superoxide dismutase (*sodB*) or the alternative oxidase (*aoxA*) with *L. pneumophila* infection (534).

The previous study investigated the expression of lateral gene transfer candidates of *A. castellanii* under different conditions, and one condition was *L. pneumophila* infection within 14 hours (398). However, the global transcriptomic response of *A. castellanii* to *L. pneumophila* infection has not been well evaluated. In this study, we analysed the transcriptional response of *A. castellanii* to *L. pneumophila* WT infection compared to Δ*dotA* infection. Also, to gain insights into how genes deleted from ΔB influenced the host transcriptome, we also compared the transcriptional response of *A. castellanii* to ΔB infection.

5.2 Results

5.2.1. *L. pneumophila* infection leads to huge transcriptomic changes in *A. castellanii*.

The *A. castellanii* response to *L. pneumophila* infection was monitored by genome-wide transcriptional profiling at different time points. We compared the transcriptional response of *L. pneumophila* WT infected *A. castellanii* versus Δ*dotA* infected *A. castellanii* at 3 h, 8 h, 16 h and 24 h post infection (hpi). Differentially expressed genes (DEGs) in *A. castellanii* were presented as volcano plots (Figure 5.1 A). In this analysis, significantly upregulated or downregulated genes were determined $p < 0.05$ for cut off. DEGs that showed a fold change in expression > 4 or < 1/4 in *L. pneumophila* infected *A. castellanii* compared to Δ*dotA* infected *A. castellanii* were selected for further analysis (Fig 5.1 A).

Overall, we observed increased DEGs over time and more upregulated DEGs compared to downregulated DEGs. At 3 hpi, 60 upregulated DEGs were observed without any downregulated DEGs, illustrating the fast response that might be triggered by the release of *L. pneumophila* virulence factors into the host cell. The number of DEGs increased to 728 at 16 hpi and 779 at 24 hpi, suggesting a more active host response in WT infected *A. castellanii* compared to Δ*dotA* infected *A. castellanii* at late infection phase, which equals bacterial transmissive phase (Figure 5.1 A). DEGs at different infection phases were diverse, as only 21 DEGs were continuously expressed throughout the 24 h infection cycle (Figure 5.1 B). However, of the downregulated DEGs at 16 hpi, 48% also presented at 24 hpi, indicating that approximately 50% regulation maintained during late infection phase (Figure 5.1 B). In summary, RNA-seq between WT and Δ*dotA* infected *A. castellanii* showed a large number of DEGs, especially during the transmissive phase of bacteria. The distribution of DEGs at different infection phases likely reflects different host responses to *L. pneumophila* infection.

5.2.2 *L. pneumophila* infection downregulates cell cycle-associated genes in *A. castellanii* at late infection phase

In order to interpret the transcriptional response triggered by *L. pneumophila*, we performed gene set enrichment analysis (GO analysis) to identify significantly

enriched functional groups (P < 0.01) of *A. castellanii* altered by *L. pneumophila* WT infection compared to Δ*dotA* infection.

DEGs downregulated exclusively at 16 hpi in WT infected *A. castellanii* compared to Δ*dotA* infected *A. castellanii* were enriched for chromosome segregation (GO: 0007059) (Figure 5.2). DEGs downregulated at 16 hpi and 24 hpi were enriched mainly in cell cycle-associated Go terms: cell cycle (GO: 0007049), microtubule-based movement (GO: 0007018), spindle organisation (GO: 0007051), chromosome segregation (GO: 0007059), organelle fission (GO:0048285), DNA recombination (GO: 0006310), regulation of cell cycle phase transition (GO: 1901987) and DNA replication (GO: 0006260) (Figure 5.2). Specifically, chitin metabolic process (GO: 0006030) and cytoskeleton organisation (GO: 0007010) were enriched in the downregulated transcripts of WT infection *A. castellanii* compared to Δ*dotA* infected *A. castellanii* at 24 hpi (Figure 5.2). These results suggest that *L. pneumophila* infection interferes *A. castellanii* division, perhaps also cell wall synthesis, from 16 hpi.

Indeed, among the most significantly down-regulated transcripts in WT infected *A. castellanii* were genes encoding spindle organisation**,** PRC1, augmin complex and Hklp2/Kif15, which are the host molecules involved in mitosis cycle regulation. Notably, *A. castellanii* genome consists of 5 minichromosome maintenance protein (MCM) subcomplex (MCM2,3,4,6,7) genes, and 4 of them were downregulated from 16 hpi (Figure 5.3). Other DNA replication-associated genes that were downregulated at the late infection phase includes *ctf18*, *dcc1*, *ctf8* and *pcna* (proliferating cell nuclear antigen). These results suggest that MCM proteins and Ctf18-RFC complex could be responsible, at least in part, for the lowered proliferation observed in *L. pneumophila* WT infected *A. castellanii* compared to Δ*dotA* infected *A. castellanii* (Chapter 4).

5.2.3 *L. pneumophila* infection triggers the regulation of host metabolism associated pathways of *A. castellanii*

At 8 hpi, DEGs downregulated in *L. pneumophila* WT infected *A. castellanii* compared to Δ*dotA* infected *A. castellanii* were enriched for metabolism processes like oxidation-reduction process (GO:0055114) and fatty acid beta-oxidation (GO:0006635). On the other hand, at 3, 8 and 24 hpi, DEGs upregulated in

L. pneumophila WT infected *A. castellanii* compared to Δ*dotA* infected *A. castellanii* were enriched for respiratory chain complex III assembly (GO:0017062). In addition to respiratory chain complex III assembly, GO term Lipid glycosylation (GO:0030259) was also enriched in upregulated DEGs at 8 and 24 hpi. Go term purine ribonucleoside salvage (GO:0006166) was enriched in upregulated DEGs at 16 hpi exclusively. These results suggest that *L. pneumophila* activated genes associated with ATP generation in *A. castellanii* and interfere with multiple host metabolic pathways.

5.2.4 *A. castellanii sirtuin6f* upregulation is required for *L. pneumophila* replication

The largest number of genes were shown to be differentially regulated at the 24 hpi. Thus, we investigated the most upregulated genes in detail. Top 10 upregulated DEGs in *L. pneumophila* WT infected *A. castellanii* compared to Δ*dotA* infected *A. castellanii* at 24 hpi were list in Figure 5.5 A. Minus the unannotated genes, the most upregulated DEGs included the genes encoding elongation factor 1-alpha (eEF1α), NEDD8-conjugating enzyme Ubc12, NAD-dependent deacetylase ACA1_153540, glutathione transferase family, mitochondrial chaperone BCS1 and endonuclease exonuclease phosphatase family. We performed qRT-PCR to validate the upregulation of *ACA1_153540* and *eEF1α* in *L. pneumophila* WT infected *A. castellanii* compared to Δ*dotA* infected *A. castellanii* (Figure 5.5 B).

Next, we chose ACA1_153540 for further investigation as it was homologous to sirtuin family proteins, which function as class III histone deacetylases that participate in a board range of cellular processes (537). According to phylogenetic analysis, ACA1_153540 was closely related to the human protein Sirtuin6 and was termed Sir6f (Figure 5.6 A). *A. castellanii* genome encodes 17 putative sirtuin genes. Interestingly, the expression of most of sirtuins genes was not influenced in *L. pneumophila* WT infected *A. castellanii* compared to Δ*dotA* infected *A. castellanii*, especially in the early infection phase (3-16 hpi). Only *sir6f* was dramatically upregulated throughout *L. pneumophila* WT infection (Figure 5.6 B). According to qRT-PCR results, approximate 20-fold upregulated of *sirtuin6f* was observed in WT infected *A. castellanii* compared to Δ*dotA* infected *A. castellanii* at 24 hpi.

To elucidate the role of *sir6f* in *L. pneumophila* replication, we checked *L. pneumophila* replication when *A. castellanii sirtuin6f* was knocked down by siRNA. Briefly, 8×10^4 *A. castellanii* were seeded in 24-well plate. 25 µL PYG was mixed with 5 µL SuperFect and 2.5 µL siRNA and then incubated for 10 min at room temperature. The mixture was added to *A. castellanii* and incubated at room temperature for 48 h, followed by *L. pneumophila* infection (Materials and methods). The siRNA transfection resulted in approximately 30% knockdown of *sir6f* mRNA level at 24 hpi compared to samples treated with scramble siRNA. Accordingly, a mild but significant decrease of *L. pneumophila* CFU was observed after siRNA treatment compared to the scramble control. Altogether, our results suggest that *A. castellanii sir6f* was upregulated during *L. pneumophila* possibly to facilitate bacterial replication.

5.2.5 Deletion of *lpw4831* leads to decreased *A. castellanii arf1* expression during infection

As described above, *L. pneumophila* WT infection triggered significant and diverse transcriptomic changes in *A. castellanii* compared to Δ*dotA*, suggesting that Dot/Icm effector proteins were contributing to transcriptomic regulation in *A. castellanii*. To investigate the role of *L. pneumophila* virulence factors in regulating host cellular processes in *A. castellanii*, here we investigated the transcriptional response of *A. castellanii* infected with *L. pneumophila* WT compared to *A. castellanii* infected with ΔB, which lacks four genes (*lpw4831*, *lpw4841*, *lpw4851*, *lpw4861*). The ΔB mutant was selected for this study because it displayed attenuated replication but was still able to establish an LCV according to confocal microscopy inspection (Data not shown).

DEGs in WT infected *A. castellanii* compared to ΔB infected *A. castellanii* were present in volcano plots (Figure 5.8 A). Significantly upregulated or downregulated genes were determined $p < 0.05$ for cut off. DEGs that showed a fold change in expression > 4 or < 1/4 in *L. pneumophila* infected *A. castellanii* compared to ΔB infected *A. castellanii* were selected for further analysis. (Fig 5.8 A). Sixty-three and 85 significant regulated DEGs presented in WT infected *A. castellanii* compared to ΔB infected *A. castellanii* at 16 and 24 hpi, respectively (Figure 5.8 B). DEGs upregulated in WT infected *A. castellanii* were enriched for methylation (GO:

0032259) at 16 hpi and small GTPase mediated signal transduction (GO: 0007264) at 24 hpi (Figure 5.8 D). Genes associated with small GTPase signal transduction included *arf1*, *rab8b* and *rac1* (Fig 5.9).

In order to investigate which *L. pneumophila* gene was responsible for the downregulation of the small GTPase-encoding genes, the expression of *arf1*, *rab8a* and *rac1* in responding to WT, Δ*dotA*, Δ*lpw4831*, Δ*lpw4841*, Δ*lpw4851*, Δ*lpw4861* infection were checked by qRT-PCR at 24 hpi (Figure 5.9). The expression of *arf1* and *rab8a* in *L. pneumophila* WT infected *A. castellanii* was significantly higher than that of Δ*dotA* infected *A. castellanii*, while the upregulation of *rac1* was not validated (Figure 5.9). Notably, *arf1* expression in Δ*lpw4831* and Δ*lpw4851* infected *A. castellanii* was significantly less than that in *L. pneumophila* WT infected *A. castellanii*. Meanwhile, *arf1* expression in Δ*lpw4841* and Δ*lpw4861* infected *A. castellanii* showed no difference with WT infected *A. castellanii*. Of note was that, Δ*lpw4851* displayed attenuated replication in *A. castellanii* (Fig 3.4), which might contribute to the observed decrease in *arf1* expression. Deletion of *lpw4831* did not impair bacterial replication but led to decreased *arf1* expression, suggesting that the Dot/Icm effector protein Lpw4831 may influence *arf1* expression during the late infection phase in *A. castellanii*.

5.3 Discussion

L. pneumophila replicates within amoebae in the environment and 'accidentally' infects mammalian cells, indicating that the evolution of *L. pneumophila* primarily occurs during the interactions with amoebal hosts (538, 539). By analysing the transcriptional profile of *L. pneumophila* infected *A. castellanii*, we gained further insights into the global responses of *A. castellanii* to *L. pneumophila* infection. In this study, we analysed a time course of *A. castellanii* response to *L. pneumophila* infection. Time points we chose represented important infection events such as bacteria invasion, LCV establishment, bacterial replication and bacterial egress. As a result, we identified a broad range of host cellular processes modulated by *L. pneumophila* in *A. castellanii,* including cell cycle, cell wall synthesis and metabolic pathways.

Our study suggests that *L. pneumophila* infection led to the suppression of *A. castellanii* mitosis-associated genes, which might contribute to the inhibition of *A. castellanii* proliferation. *L. pneumophila* infection inhibits host cell proliferation in a Dot/Icm dependent manner (412, 530, 531). A previous study revealed that a putative CDK protein CDC2b could be related to proliferation inhibition in *A. castellanii*, but its mechanism needs more investigation (412). In this study, we observed the downregulation of mitosis associated genes in *L. pneumophila* WT infected *A. castellanii* compared to Δ*dotA* infected *A. castellanii* after 16 hpi. Genes associated with multiple cell cycle processes were also downregulated DEGs at 16 and 24 hpi. The products of downregulated DEGs mainly regulate two mitosis events in human: chromatin organisation and spindle organisation. Kinesin Kif18A, CENP-E, chromosome condensation factor Brn1p are required for chromatin organisation or dynamics in mitosis (540-542). Xkid provides the metaphase force that pushes chromosome arms toward the equator of the spindle and that its destruction is needed for anaphase chromosome movement (543). Mad2, an essential spindle checkpoint protein which is a regulatory system that restrains progression through the metaphase-to-anaphase transition [18]. Genes encoding augmin complex, Hklp2/Kif15, are involved in spindle organisation, contributing to mitotic progression (544, 545); Cernunnos/XLF promotes the ligation of mismatched and noncohesive DNA ends (546); PRC1 is required for interaction of the two half-spindles and for localisation of KIF4 and CENP-E [52]. Interestingly, these genes were all downregulated from 16

hpi, suggesting that downregulation of mitosis-associated genes occurred during the transmission phase of *L. pneumophila*.

Genes associated with DNA replication complexes were also downregulated from 16 hpi, including *mcms, ctf4*, *ctf18*, *dcc1*, and *ctf8* and *pcna*. Mcm proteins are required for the initiation of DNA replication and replicative chain elongation (547-552). MCM proteins present in all eukaryotes examined (553-556). In *A. castellanii*, orthologs of Mcm2, Mcm3, Mcm4, Mcm6, Mcm7 were discovered in the genome, while four of five were downregulated with *L. pneumophila* WT infection compared to Δ*dotA* infection. Ctf4, which is required for sister chromatid cohesion in yeasts, was also shown to be essential for the chromatin binding of DNA polymerase and the initiation of DNA replication in mammalian cells (557, 558). Ctf18, Dcc1, and Ctf8 form the Ctf18-RFC complex, which is required for the establishment of sister chromatid cohesion and may load the replication clamp PCNA onto DNA during DNA replication and repair (557-560). A recent study revealed that *L. pneumophila* blocks host DNA synthesis and prevents cell cycle progression to avoid host S phase, as S phase was shown to restrict *L. pneumophila* replication and LCV establishment (561, 562). These results highlight the necessity for *L. pneumophila* to interfere with host mitosis transition and DNA replication in *A. castellanii*.

The morphological transition of *A. castellanii* with *L. pneumophila* infection observed in this study might be caused by changes in the transcriptional regulation of cell wall synthesis. To date, it is thought that *A. castellanii* undergoes encystment to survive with bacterial or viral invasion (450, 488, 520, 521). However, as described in the previous chapter, *L. pneumophila* infection led to *A. castellanii* morphological changes. *L. pneumophila* infected *A. castellanii* lost cell pseudopodia and became round but displayed no features of cysts such as a double cell wall. Cellular processes linked to cell wall synthesis were downregulated at 24 hpi in *L. pneumophila* WT infected *A. castellanii* compared to Δ*dotA* infected *A. castellanii*, including cell wall macromolecular catabolic and chitin metabolic processes. As we proposed, *A. castellanii* undergoes the encystment process with a bacterial infection. The transcriptional suppression on cell wall synthesis associated gene might be utilised by *L. pneumophila* to interfere with this process.

While *L. pneumophila* utilises similar infection strategies in macrophages and protozoan hosts, the regulation of ATP production was different, possibly due to the adaption to different hosts. This study showed a constant upregulation of genes associated with respiratory chain complex III assembly by *L. pneumophila*. Chain complex III anchors in the inner membrane and plays a role in the biochemical generation of ATP. Previous studies measured the mitochondrial parameters during *L. pneumophila* infection and discovered the inhibition of mitochondria respiration during infection in HMDMs (563). *L. pneumophila* infection first up-regulated oxygen consumption and secondly impaired the mitochondrial respiration, ATP production and cellular ATP pool (316). The transition of metabolic activity was thought to promote bacterial replication in HMDMs, but the mechanism is unknown (316).

According to our transcriptomic analysis and previous studies, *L. pneumophila* possibly executes more than one mechanism to modulate the activity of host eEF1α (564). A previous study showed that expression of eEF1α was repressed in *L. pneumophila* WT infected HMDM (530). However, our transcriptional profile of *L. pneumophila* WT and Δ*dotA* infected *A. castellanii* revealed the upregulation of host *eEF1α* during infection. eEF1α is responsible for loading charged amino acids to the A site of the ribosome during protein synthesis (313). It has been reported that *L. pneumophila* leads to significant inhibition of host protein synthesis in mammalian hosts by the interacting with eEF1α (272, 274, 308-310). So far, at least eight Dot/Icm effector proteins (Lgt1–Lgt3 LegK4, Lpg1489, SidI, SidL, SusF) are involved in eEF1α modulation in mammalian cells (272, 274, 309, 310). The effector proteins, Lgt1–Lgt3, glucosylate at residue S53 of eEF1α, leading to the block of protein biosynthesis and causes death of target cells (272, 311, 312). Another effector protein, SidI, targets host eEF1α and induces expression of heat shock protein hsp60 (274). Furthermore, effector protein SusF binds to SidI and suppresses SidI-mediated inhibition of protein translation (314). However, inhibition of host protein synthesis seems not to be beneficial for *L. pneumophila* as host proteins and amino acids are important for bacterial carbon and energy source (448, 565-567). In mammalian cells, it is possible that the robust innate immune response to translation inhibition serves primarily to compensate for the decrease in translation (307). As evidence, deletion of five effector proteins (*lgt1, lgt2, lgt3, sidI, sidL*) did not impair the replication of *L.*

pneumophila in macrophages nor eliminate the host translation inhibition (307). In the scenario of protozoan hosts, inhibition of host protein biosynthesis has not been observed. In addition to its role in protein translation, eEF1α facilitates the expression of heat shock genes in the amoebal host (568). Previous observations have also demonstrated the importance of heat shock protein Hsp90 for *L. pneumophila* replication in *A. castellanii* (568). It was hypothesised that SidI modulates eEF1α to specifically amplify heat shock genes, which can enhance the survival of *L. pneumophila* infected cells (314). Moreover, deletion of *sidI* did not impair *L. pneumophila* replication in *D. discoideum*, but deletion of *lgt1, lgt2, lgt3, sidI, sidL* did, suggesting the redundancy of effector proteins on modulation host eEF1α (274, 307). In summary, our transcriptomic analysis added evidence that *L. pneumophila* modulation of host eEF1α leads to different consequences in different hosts; namely inhibition of host protein synthesis or activation of host heat shock proteins.

In this study, we identified the Sir6f to be a host factor that facilitating *L. pneumophila* replication in *A. castellanii*. The sirtuin proteins in mammalian cells are categorised as class III histone deacetylases that play complex and important roles in ageing-related pathological conditions such as cancer and the deregulation of metabolism. Seven isolates are found in human, and each has a unique subcellular localization and distinct function [59, 60]. *A. castellanii* genome encodes 17 sirtuin family genes, belonging to 4 different classes of sirtuins based on phylogenetic analysis (569-571). In a previous study, *D. discoideum* mutants lacking *sir2D* exhibited defected cell proliferation and development (537). Inhibition of *sirtuin1-3* by the novel sirtuin inhibitor KH-TFMDI, a 3-arylideneindolin-2-one, based on a 6,7-dichloro2-oxindole scaffold, in protozoan *Trypanosoma cruzi* led to its death by apoptosis and autophagy (572). Belonging to class IV, *sir6f* is distributed in metazoans, plants and vertebrates, but not prokaryotes (571). In humans, Sir6f is predominantly a nuclear chromatin-associated protein which aids in the protection of DNA damage and suppresses genomic instability through association with base-excision repair and DNA-end resection (573-575). In this study, we showed that inhibition of *sir6f* in *A. castellanii* impaired *L. pneumophila* infection at 24 hours post infection. Expression of the other sirtuin genes should be tested in the future. Nevertheless, this provided evidence that the suppression of one or more sirtuin genes influences *L. pneumophila* replication, but its mechanism needs further investigations.

A limitation to understanding the role of Sir6f in *A. castellanii* was the unavailability of gene knock out tools in *A. castellanii*. The polyploid genome organisation of *A. castellanii* prevents simple knockout of genes of interest (95). To investigate the role of *A. castellanii sir6f* for *L. pneumophila* replication, we applied a SuperFect-based siRNA transfection and examined *L. pneumophila* replication with the knockdown of *sir6f*. Chemicals that inhibit sirtuin activity are available but require high concentrations. However, the solution used to gain high concentration for this chemical induced *A. castellanii* encystment in this study, while its toxicity to *A. castellanii* or *L. pneumophila* was negligible (data are not shown). Using the siRNA-induced knockdown, we observed a mild but significant lowering of *sir6f* mRNA. As a result, bacterial CFU harvested from siRNA treated *A. castellanii* was modestly decreased compared to CFU harvested from scramble siRNA treated *A. castellanii*. A clearer phenotype might be achieved if the experimental conditions are developed including the gene knock-out of *sir6f* in *A. castellanii* by CRISPR.

We also employed RNA-sequencing strategies to understand how a subset of *L. pneumophila* genes, that contribute to replication efficiency affect the host transcriptome in the hope of identifying target processes. We found several GTPases were downregulated in ΔB infected *A. castellanii* compared to WT infected *A. castellanii*. Through the use of sub-deletions, we observed that deletion of *L. pneumophila* effector protein Lpw4831 alters host Arf1 expression during late infection phase in *A. castellanii*. Small GTPases hydrolyse GTP to GDP and activate downstream effectors when bound to GTP, thus functioning as a sophisticated regulator of a diverse range of cellular processes (576). Arf1 regulates the assembly of several types of vesicle coat complexes and is implicated in Golgi disassembly, chromosome segregation, and cytoskeletal rearrangement during mitosis (577). Due to their involvement in various critical cellular processes, small GTPases are direct targets for *L. pneumophila* effectors during LCV formation (578, 579). Arf1 is recruited to the LCV shortly after uptake in both mammalian and protozoan hosts (349, 580, 581). Effector protein RalF, which is a guanine nucleotide exchange factor (GEF) stimulates the release of GDP to allow binding of GTP and recruits Arf1 to the LCV (350). Arf1 disappears from the LCV 10 h post *L. pneumophila* up-take in iBMDM macrophages, suggesting that a cellular Arf1 GTPase-activating protein (GAP), or perhaps *L. pneumophila* GAP protein, counteracts RalF (582).

Furthermore, *L. pneumophila* cannot establish the LCV if host Arf1 is depleted, but the deletion of RalF does not impair bacterial intracellular replication in murine bone marrow-derived macrophages, human macrophage-like cell line U937 or *A. castellanii*, indicating the existence of an undiscovered regulatory mechanism of *L. pneumophila* on host Arf1 (350, 582). In this study, deletion of effector protein Lpw4831 had no impact on bacteria replication but reduced *arf1* expression in *A. castellanii* at 24 hpi, reflecting another aspect of systematic redundancy of transcriptional management of host Arf1 (164, 349).

In summary, the transcriptomic profiling of WT infected *A. castellanii* compared to Δ*dotA* infected *A. castellanii* provides us with insights into how *L. pneumophila* inhibits *A. castellanii* cell proliferation and morphological transition. Compared to the transcriptional profiles of *L. pneumophila* infected mammalian cells, we observed similar transcriptional regulation of cell cycle and lipid metabolism, and differential regulation of host ATP production. *L. pneumophila* also appeared to regulate host eEF1a for different purposes. We identified Sir6f to be a host factor facilitating *L. pneumophila* replication in *A. castellanii* and we observed that the *L. pneumophila* Dot/Icm effector protein Lpw4831 might play a role in regulating host Arf1 expression. Importantly, this study adds knowledge about the global response of *A. castellanii* to *L. pneumophila* and identifies potential targets for controlling *L. pneumophila* in the environment by disrupting bacterial replication in amoebae.

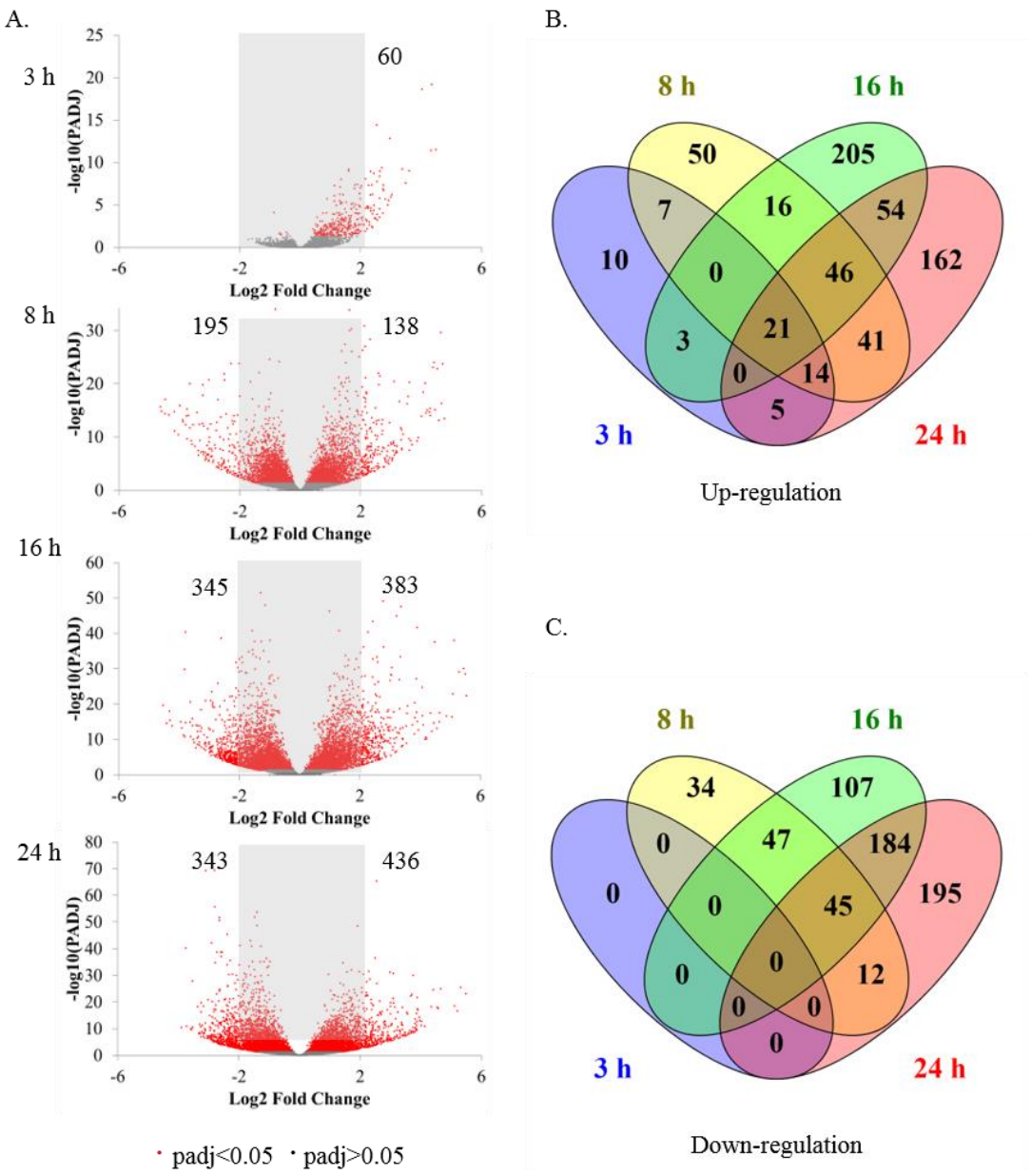


Figure 5.1 Transcriptional profiles of *A. castellanii* infected with *L. pneumophila* WT compared to *A. castellanii* infected with *L. pneumophila* Δ*dotA*

A. Differentially expressed genes (DEGs) in *L. pneumophila* WT infected *A. castellanii* compared to Δ*dotA* infected *A. castellanii* were presented in volcano blot. Grey dots represented the DEGs with padj < 0.05. Red dots represented DEGs with padj > 0.05. Grey dots represented significantly regulated DEGs with $\log_2$ fold change > 2 or < -2. **B-C.** Venn diagrams displaying the number of regulated DEGs that overlap between different time points in *L. pneumophila* WT infection *A. castellanii* compared to Δ*dotA* infected *A. castellanii*.

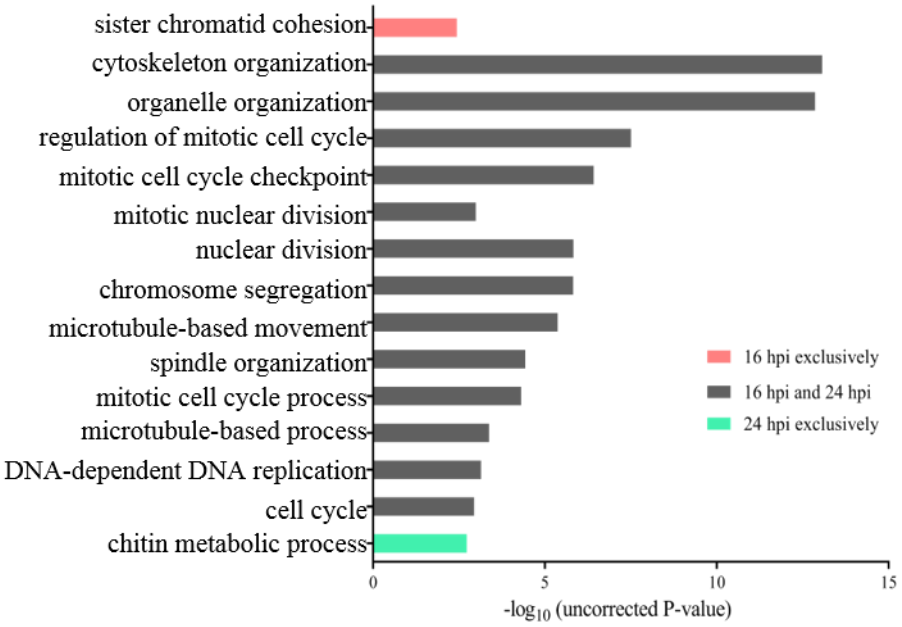


Figure 5.2 *L. pneumophila* downregulates cell cycle-associated genes in *A. castellanii* at late infection phase

Enriched GO terms in the biological processes (BP) category for the downregulated DEGs of WT infected *A. castellanii* compared to Δ*dotA* infected *A. castellanii* at 16 and 24 hpi. X axis represents statistical significance of enrichment (-log10 (uncorrected p-value)). Enrichment false discovery rate (FDR) value < 0.01 was used as a cut-off.

Figure 5.3 Heatmap of the top 45 downregulated DEGs at 24 hpi

The top 45 downregulated DEGs at 24 hpi were selected and presented as heatmap at indicated timepoints. Grey frames indicated the value with padj > 0.05.

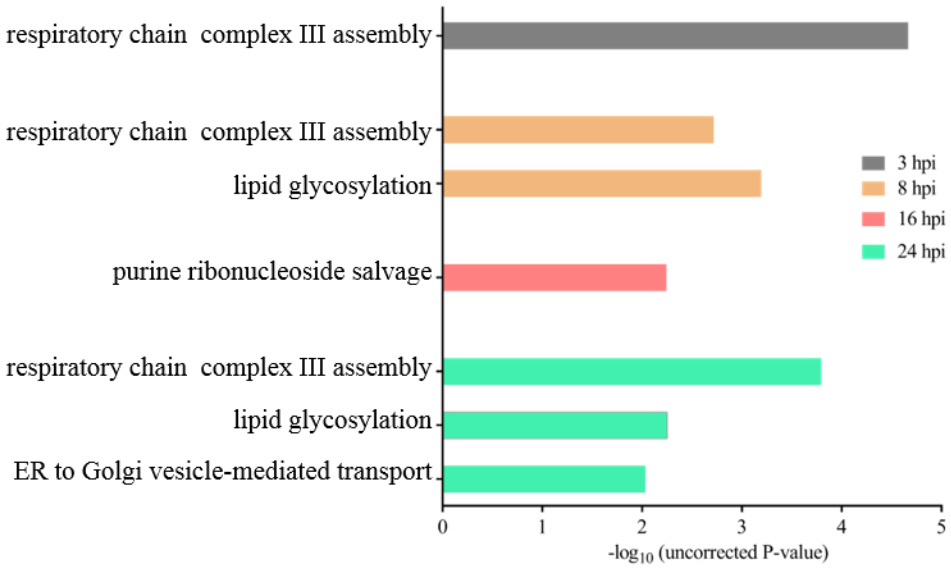


Figure 5.4 Gene ontology enrichment analysis of upregulated DEGs in *L. pneumophila* WT infected *A. castellanii* compared to Δ*dotA* infected *A. castellanii*

Enriched GO terms in the biological processes (BP) category for the upregulated DEGs of WT infected *A. castellanii* compared to Δ*dotA* infected *A. castellanii* at 16 and 24 hpi. X axis represented the value of $-\log_{10}$ (uncorrected p-value). Enrichment false discovery rate (FDR) value < 0.01 was used as a cut-off.

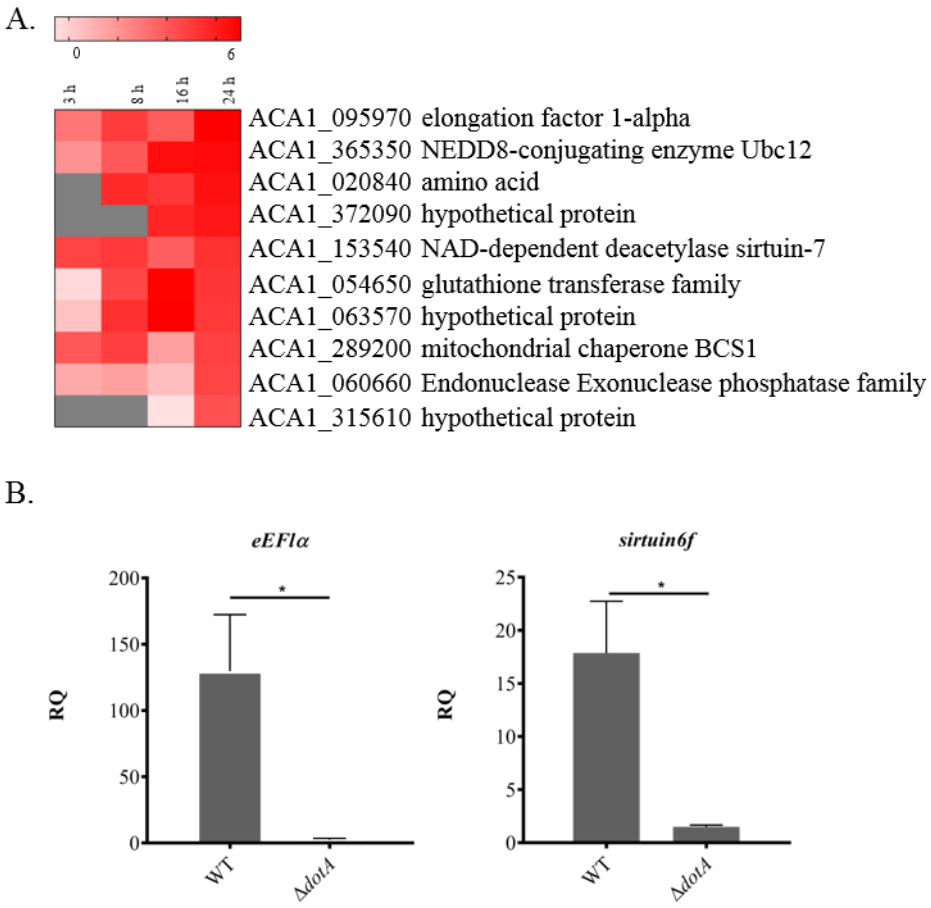


Figure 5.5 Genes upregulated in *L. pneumophila* WT infected *A. castellanii* at 24 hpi compared to Δ*dotA* infected *A. castellanii*

A. Top 10 DEGs at 24 hpi were selected and present as heatmap at indicated timepoints. Grey frames indicated the value with padj > 0.05. **B.** qPCR results indicating the expression of *sir6f* and *eEF1α* in WT infected *A. castellanii* compared to Δ*dotA* infected *A. castellanii*. Results were normalised to expression levels of 18s and expressed relative to uninfected infected samples. Results are the mean ± SEM of four independent experiments carried out in technical duplicates. The asterisks indicate conditions that are significantly different compared to WT infected samples (*$p < 0.05$, unpaired t-test).

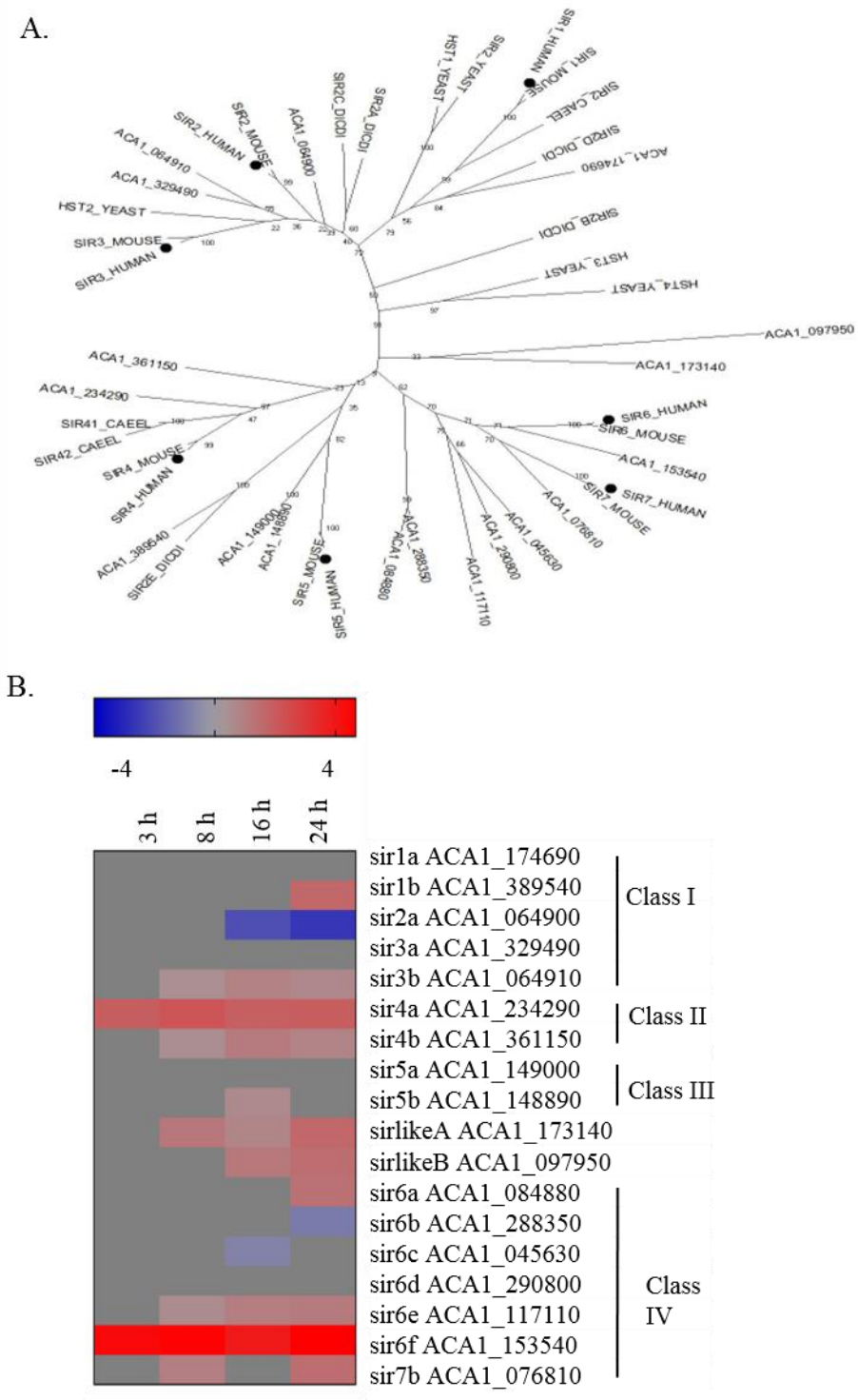


Figure 5.6 *L. pneumophila* WT upregulates *sir6f* in *A. castellanii*

A. The sirtuin protein relatedness tree created using the Maximum Likelihood method in MEGA7 (583). Numbers next to the branching points indicate the relative support from 1000 bootstrap replicates. **B.** Heatmap displaying the expression of sirtuin family genes at indicated timepoints. Grey frames indicated the value with padj > 0.05.

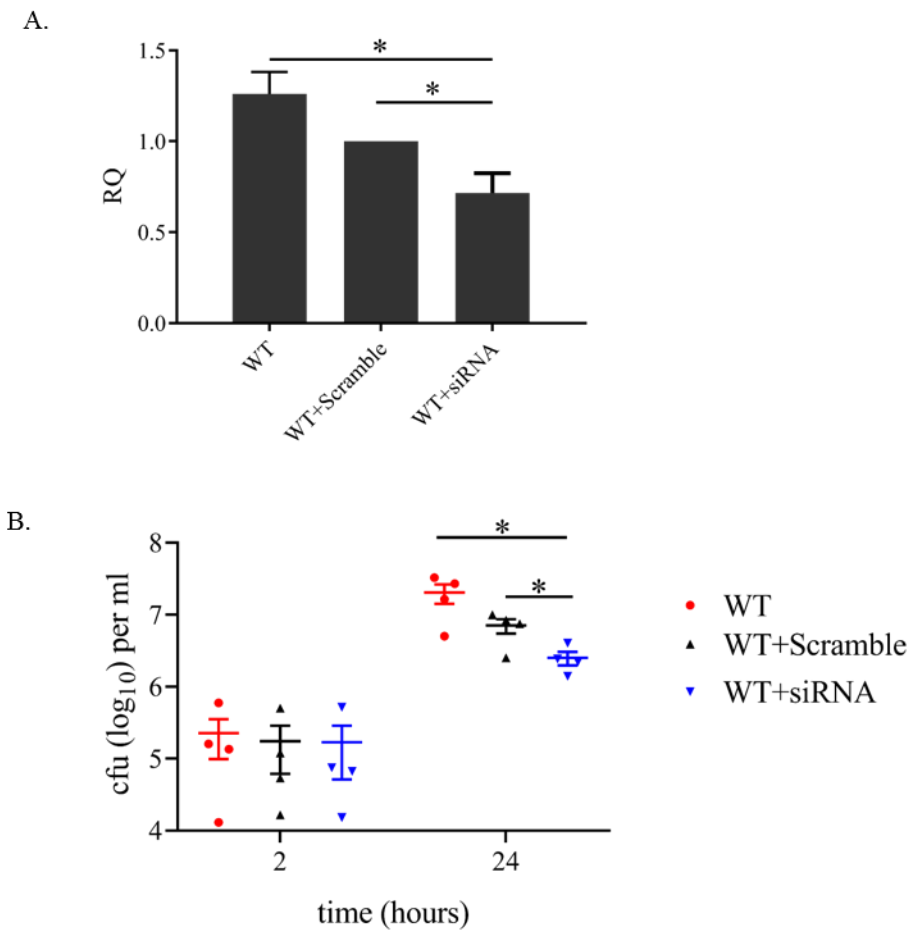


Figure 5.7 siRNA induced knockdown of *sir6f* leads to defect bacterial replication in *A. castellanii*

A. qRT-PCR of *sir6f* expression with siRNA transfection followed by *L. pneumophila* infection. Results were normalised to expression levels of 18s, and expressed relative to uninfected samples. Non-targeting scramble siRNA (Bioneer) optimised for human and mouse cell lines was used as a knockdown control. **B.** Intracellular replication assay of *L. pneumophila* in *A. castellanii* following siRNA transfection. All results were an average of four independents experiments, and error bars represent the standard error of the mean (±SEM) (*$p < 0.05$, unpaired t-test).

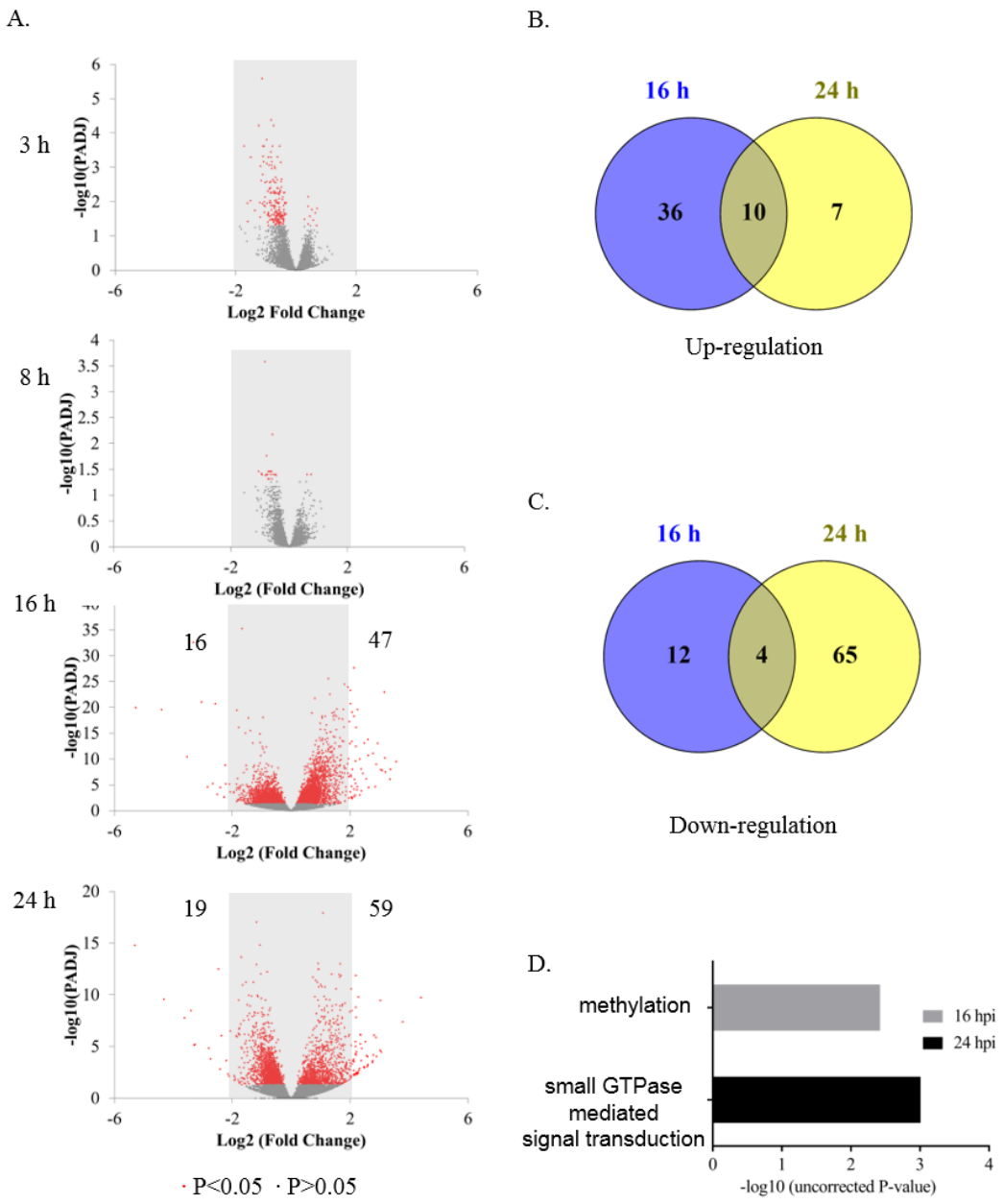


Figure 5.8 Transcriptional profiles of *L. pneumophila* WT and ΔB infected *A. castellanii*

A. Differentially expressed genes (DEGs) in WT infected *A. castellanii* compared with ΔB infected *A. castellanii* were present in volcano blot. Significantly upregulated or downregulated genes were determined using 4-fold change and $p<0.05$ for cut off. **B-C.** Venn diagrams displaying the number of regulated DEGs that overlap between different time points in WT infection *A. castellanii* compared to ΔB infected *A. castellanii*. **D.** Enriched GO terms in the biological processes (BP) category for the down- and up-regulated DEGs at the 16 and 24 hpi. No GO term was enriched at 24 h

post infection for upregulated DEGs. Enrichment false discovery rate (FDR) value < 0.01 was used as a cut-off

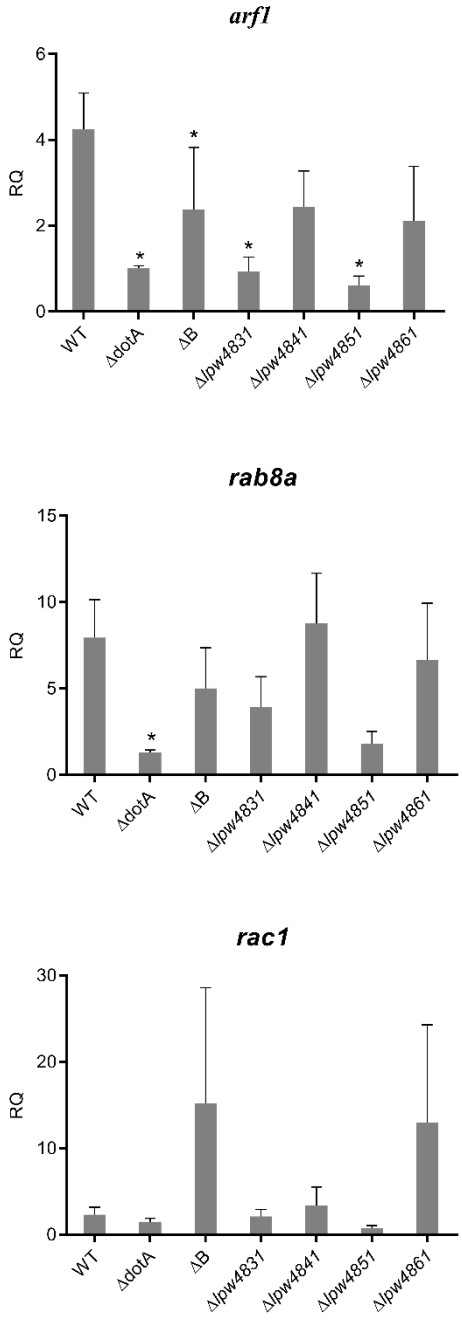


Figure 5.9 Gene expression analysis of chosen small GTPase genes

Expression levels of chosen small GTPase genes were quantified by qRT-PCR at 24 h post infection. Results were normalised to expression levels of 18s, and expressed relative to uninfected samples. Results are the mean ± SEM of four independent experiments carried out in technical duplicates. The asterisks indicate conditions that are significantly different compared to WT infected samples (*$p < 0.05$, unpaired t-test).

Chapter 6: Perspectives

Since the initial identification of *L. pneumophila* in 1977, regular outbreaks of Legionnaires' disease caused by *Legionella* species have highlighted the threat of the opportunistic pathogen to public health (1). Legionnaires' disease is predominantly caused by the inhalation of *Legionella*-containing aerosols, as only one probable person to person transmission case has been reported over the decades (97, 106). The burden of Legionnaires' disease is increasing each year worldwide (31, 584, 585). For example, in the United States, reported Legionnaires' disease cases have multiplied nearly nine times from 2000 to 2017 (9, 586). In New Zealand, the overall incidence of Legionnaires' disease cases in hospitals of Canterbury was 5.4 per 100 000 people per year, which was three times the average over the preceding three years (587). Even so, Legionnaires' disease cases may be underdiagnosed because of the limitations of commonly used diagnostic methods: The sensitivity of bacteriological diagnosis is weak, and urine antigen testing only detects serogroup 1 strains of *L. pneumophila* (584, 585, 588). It has been estimated that only 5% to 10.6% of real cases are reported or clinically diagnosed (9, 585, 589). The increase in Legionnaires' disease incidence might be associated with climate change, increasing proportion of susceptible people in the general population, the introduction of water-saving feature in commercial and domestic buildings and unhindered bacterial colonisation in artificial water systems (91, 92, 590).

The co-evolution of *L. pneumophila* with amoebae hosts has altered *L. pneumophila* virulence gene composition and capacity to cause disease in humans (10, 133, 363). *Legionella*–amoebae interactions were characterised shortly after the bacteria were identified and were thought to grant *L. pneumophila* the ability to infect mammalian macrophages, as the human infection is incidental and a dead-end for *L. pneumophila* (10, 31, 361, 402, 591, 592). *L. pneumophila* replicates in more than 20 species of amoebae, two species of ciliated protozoa, one species of slime mould (10, 81-89). Soil and sediment provide nutrients and shelter for the formation of biofilms in which *L. pneumophila* can persist for decades (80). While *L. pneumophila* can acquire nutrients by forming a synergistic relationship with other members of the biofilms, intracellular replication in protozoa is likely the predominant mechanism for *L. pneumophila* amplification in its natural habitat (10-15). The *L. pneumophila* genome exhibits a high degree of plasticity and the bacteria readily adapt to a

selective environment through the ability to take up exogenous DNA by natural transformation, acquire mobile genetic elements or to lose unrequired gene clusters. This adaptation is evident from *L. pneumophila* that has been passaged for hundreds of generations in macrophages and gained more efficient replication capacity in macrophages but displayed reduced replication in amoebae (593). In addition, 23 of 47 *Legionella* species can replicate in the human macrophage-like cell line THP1, but these do not cluster phylogenetically, indicating that the capacity to replicate in macrophages has been acquired independently many times from the interaction with natural hosts like amoebae (594). Amoebae hosts also work as Trojan horses to deliver intracellular microorganisms to new habitats and protect the microorganisms from hostile environmental conditions, as well as promote bacterial adaption to replication in macrophages (594, 595).

Interactions with amoebae allow intracellular organisms to acquire genetic material from and amoebal host and co-infecting organisms (595-597). Amoebal hosts are able to harbour bacteria, viruses, fungi or degraded microorganisms at the same time, resulting in a melting pot of genes from various origins that are integrated into those intracellular microorganisms (596). Phylogenetic analyses of *A. polyphaga* mimivirus genes showed that ~10% of the mimiviral ORFs are closely related to homologues found in different amoebas hosts, as well as intracellular pathogens (596, 598, 599). Indeed, a protein predicted in the *L. pneumophila* genome has homologs only in the *Acanthamoeba* polyphaga mimivirus, indicating genetic exchange with eukaryotic viruses (597). Another example is the *L. drancourtii* genome that possesses 28 genes coding for proteins with high sequence similarity to proteins from other intra-amoebal bacteria, including *Amoebophilus asiaticus*, *Protochlamydia amoebophila* and *R. bellii* (600). These horizontal gene transfers between intra-amoebal microorganisms and amoebae probably provide a genetic pool from which genes required for adaptation to intracellular replication in eukaryotic cells can be sourced, thereby increasing the risk of human infection.

It is known that transition from a free-living to intracellular life cycle is often associated with genome reduction and that extreme reduction is associated with extreme host specialisation of pathogens (601, 602). Whereas different *L. pneumophila* strains exhibit genomes from 3.3 Mbp to 3.5 Mbp, the closest phylogenetic relatives and obligate intracellular pathogens, *C. burnetii* and

F. tularensis, exhibit genomes of 1.9 Mbp and 1.8 Mbp, respectively and the genome of *A. polyphaga* mimivirus harbours 911 predicted ORFs (603-607). The larger genome size of *L. pneumophila* likely reflects its adaptation to parasitism of diverse protozoan and perhaps metazoan hosts (596). The absence of effective innate immune sensing pathways in protozoa together with the general plasticity of the *L. pneumophila* genome has likely allowed newly acquired genetic material to contribute to the total effector protein arsenal and hence the ability to survive predation by multiple protozoan species (117).

Over 18,000 secreted effector proteins have been predicted in the *Legionella* genus (594). However, virulence associated effector proteins among the *Legionella* species are mostly non-overlapping. Analysis of 38 *Legionella* species predicted 5885 Dot/Icm effector proteins, whereas, only seven core effectors were found in all the species studied (231). Members of the genus contain the species-specific effectors, which vary from 52 to 300 putative effectors (231). Even within *L. pneumophila*, only 65% of Dot/Icm effector proteins were conserved in 11 strains analysed (594). This conservation might be even lower when adding more strains for analysis, indicating the strain specific nature of effector protein repertoires (594). Between *L. pneumophila* strain Paris and *L. pneumophila* strain Lens, 2,664 genes are conserved, but 428 and 280, respectively, are strain-specific genes (207). Notably, evolutionarily close species tended to have similar sets of effectors, probably due to preferential horizontal gene transfers among those species in similar hosts.

The close coevolution with amoebae has shaped a large Dot/Icm effector protein repertoire that allows *L. pneumophila* to exploit many hosts and signalling pathways through protein secretion systems. Thus, increased knowledge about *Legionella*–amoebae interactions is necessary to enable the development of new strategies for disease control and prevention. Our study used *A. castellanii* as a cell model to map host-specific virulence factors of *L. pneumophila* and assess their contribution to intracellular replication in amoebae and macrophages. Subsequently, we identified the role of a putative glutamate transporter and TCS regulator, LetA, during bacterial infection in *A. castellanii*. Furthermore, by analysing the transcriptional profile of *A. castellanii* during *L. pneumophila* infection, we observed different host responses in *A. castellanii* compared to macrophages. Notably, *A. castellanii* Sir6f was apparently utilised by *L. pneumophila* for optimal intracellular replication

In aquatic habitats, *L. pneumophila* replicates to high numbers in *A. castellanii* , but the ability of other protozoa to serve as hosts for *L. pneumophila* replication and dissemination is different (608, 609). The maximum amount and rate of *L. pneumophila* growth can vary significantly between hosts (414). *L. pneumophila* can achieve up to 10,000-fold growth in *A. castellanii* but only 10-fold growth in *N. lovaniensis* over the same period (414). Similarly, *L. pneumophila* strain Paris grows robustly in *W. magna* strain T5[S]44 but is defective for growth in other *W. magna* strains (215, 610). This suggests that *L. pneumophila* strains might be more specifically adapted to local amoebae populations than previously appreciated.

The biological instinct of microbes is to acquire nutrients and proliferate (611). *L. pneumophila* intracellular replication requires multiple stages of nutrient acquisition. In addition to employing its own transport systems to take up and utilise exogenous amino acids, *L. pneumophila* exploits host cell transporters and host proteolytic processes to acquire nutrients (443, 444, 565). For example, Dot/Icm effector proteins are associated with degradation of polyubiquitinated proteins, modulation of phospholipid metabolism and purine and polyamine salvage (444, 612-614). *L. pneumophila* recruits Ala, Asp, Glu, Ser, Phe, Tyr, Pro, and Gly from the *A. castellanii* cytosol surrounding the LCV and incorporates these amino acids directly into bacterial proteins, even though the bacteria are able to synthesis Ala, Asp, and Glu from other host-derived carbon sources (442). In this study, we identified a putative *L. pneumophila* glutamate transporter that was explicitly required for bacterial replication in *A. castellanii* but not macrophages, suggesting that nutrient utilisation in different hosts requires the adaptation of bacterial metabolism (442).

The long term coevolution of *A. castellanii* and *L. pneumophila* has not only allowed *L. pneumophila* to replicate intracellularly but also to interfere with *A. castellanii* encystment. Encystment can be triggered as a self-protection mechanism upon bacterial or virus invasion or in response to nutrient starvation (133). *A. castellanii* cysts lose pseudopodia, which are essential for phagocytosis. Thus, *A. castellanii* undergoing encystment and mature cysts are resistant to infection by organisms such as APMV (450, 521, 615). In this study, we observed strong inhibition of *A. castellanii* starvation-induced encystment during *L. pneumophila* infection. *L. pneumophila* Δ*letA* and Δ*dotA* were defective for the inhibition of *A. castellanii* encystment, while other mutants carrying large genomic deletions were not. These

findings suggest that *L. pneumophila* actively blocks *A. castellanii* encystment in a Dot/Icm dependent manner, presumably to preserve the permissive trophozoite stage of the amoeba life cycle for *L. pneumophila* replication. Recently, a *L. pneumophila* Dot/Icm effector protein, LamA, was shown to subvert *A. pylyphaga* encystment by mediating glycogenolysis (616). The effectors responsible for this inhibition in *A. castellanii* have yet to be identified.

In summary, our study revealed several genomic regions of *L. pneumophila* that were important for bacterial intracellular replication in *A. castellanii* but not in macrophages. One of the genes *lpw4851*, which encodes a putative glutamate transporter, was shown to be responsible for the defect mutant ΔB. Notably, the infection of *A. castellanii* with *L. pneumophila* led to inhibition of cysts in a Dot/Icm dependent manner. This inhibition partially required *letA*, which encodes the regulator of two-component system LetAS. Finally, transcriptomic analysis of *A. castellanii* infected with *L. pneumophila* WT and Δ*dotA* identified that genes associated with mitosis, DNA replication and cell wall synthesis were significantly downregulated during the late infection phase, which may contribute to the inhibition of *A. castellanii* proliferation and encystment. Given that the evolution of *L. pneumophila* in amoebae has conferred the capacity of the pathogen to survive in macrophages, understanding the virulence mechanisms utilised by *L. pneumophila* in amoebae will add valuable knowledge to the development of new mechanisms for disease control and prevention. Furthermore, the broad range of hosts that support *L. pneumophila* replication provides a wide range of pathogenic potential. Future host-pathogen studies should take amoebae into consideration as well as human or mouse macrophages since all host-microbe interactions provide potential targets for novel approaches to treat or prevent disease.

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Appendix 1 Overview of the secretion systems in *L. pneumophila* 130b strain

Secretion system	Gene	Locus
General secretory (Sec) system	*secA, -B*	*lpw14821, lpw24951*
	secE, -Y	*lpw03951, lpw04281*
	yajC, secD, -F	*lpw20591 to lpw20611*
	secG, yidC	*lpw30451, lpw32881*
Twin arginine translocation (TAT) system	*tatA, -B, -C*	*lpw31791, lpw31801, lpw32171*
T1SS	*lssXYZABDE*	*lpw15351 to lpw15411*
T2SS	*pilD, lspK to -F*	*lpw15471, lpw13701 to lpw13651*
	lspE, -D, lspC	*lpw13281, lpw13291, lpw09771*
	lepB, lspL, lspM	*lpw19051, lpw19081, lpw19091*
T4SS		
Dot/Icm T4BSS	*dotA, icmV, -W, -X*	*lpw29401 to lpw29431*
	dotD, -C, -B	*lpw29281 to lpw29301*
	icmT, -S, -R, -Q, -P, -O, -N, -M, -L, -K, -E, -G, -C, -D, -J, -B, -F, -H	*lpw05221 to lpw05401*
	dotV, lvgA	*lpw05521, lpw06041*
Lvh1 T4ASS	*lvr1E to lvr1A*	*lpw01541 to lpw01691*
Lvh2 T4ASS	*lvr2A to lvr2E*	*lpw01881 to lpw02031*
Trb-1-like T4ASS	*lvhrI-traM1*	*lpw22591 to lpw22831*
GI-T4SS 1	*Lvr3R, -A to -C, lgi1A to -T*	*lpw10731 to lpw10961*
GI-T4SS 2	*Lvr4R, -A to -C, lgi2A to -T*	*lpw21631 to lpw21861*

Appendix 2 List of putative effector proteins in *L. pneumophila* 130b strain

Gene	Description	Gene	description	Gene	Description
lpw00071	*ravA*	*lpw04721*	*vipA*	*lpw12011*	Unknown
lpw00111	*cegC1*	*lpw04831*	*legA7/ceg11*	*lpw12061*	*ravP*
lpw00221	*Unknown*	*lpw04841*	*ankG/ankZ/legA7*	*lpw12081*	*ravQ*
lpw00311	*ravB*	*lpw04861*	Unknown	*lpw12121*	Unknown
lpw00381	*ankQ/legA10*	*lpw05041*	*legY*	*lpw12211*	*ravR*
lpw00441	Unknown	*lpw05181*	*ankJ/legA11*	*lpw12401*	*ravS*
lpw00451	Unknown	*lpw05191*	*ceg14*	*lpw12861*	*vpdB*
lpw00621	*ceg2*	*lpw05201*	*ceg15*	*lpw12871*	Unknown
lpw00781	*ceg3*	*lpw05631*	*ankC/legA12*	*lpw13261*	*legC1*
lpw00791	Unknown	*lpw05951*	*legD2*	*lpw14431*	*vpdC*
lpw00881	*lem1*	*lpw05981*	Unknown	*lpw14671*	Unknown
lpw00961	*ceg4*	*lpw06951*	*sidA*	*lpw14711*	Unknown
lpw01031	*vipF*	*lpw07081*	Unknown	*lpw15031*	*legK1*
lpw01261	*cegC2*	*lpw07161*	*wipB*	*lpw15041*	Unknown
lpw01311	Unknown	*lpw07721*	*ankN/ankX legA8*	*lpw15081*	*lgt3/legc5*
lpw01361	*sdhB*	*lpw07731*	*lem3*	*lpw15091*	*ravX*
lpw02541	*ravD*	*lpw07931*	Unknown	*lpw15181*	*lem10*
lpw02641	*ravC*	*lpw08111*	*ravH*	*lpw16011*	Unknown
lpw02651	*legU1*	*lpw09801*	*ceg18*	*lpw16131*	*legC6*
lpw02661	Unknown	*lpw10111*	*ravI*	*lpw16231*	*lem11*
lpw02761	Unknown	*lpw10251*	*lidA*	*lpw16241*	*legL2*
lpw02821	*ceg5*	*lpw10311*	*legL1*	*lpw16461*	*ceg23*
lpw02851	*ravE*	*lpw10491*	Unknown	*lpw16511*	*lem23*
lpw02861	*ravF*	*lpw10531*	Unknown	*lpw16651*	Unknown
lpw02981	*ravG*	*lpw10541*	*sidK*	*lpw16681*	*sidB*
lpw03151	*ceg7*	*lpw10551*	*ravK*	*lpw16861*	*legL3*
lpw03221	*sidE/laiD*	*lpw11451*	*lem4*	*lpw16871*	Unknown
lpw03291	*ceg8*	*lpw11501*	Unknown	*lpw16921*	Unknown
lpw03361	*ceg9*	*lpw11531*	*ravL*	*lpw16931*	Unknown
lpw03461	*sdeA*	*lpw11571*	*lem5*	*lpw16971*	Unknown
lpw03491	Unknown	*lpw11581*	*ravN*	*lpw17121*	*mavA*
lpw03641	*sdbA*	*lpw11681*	*lem6*	*lpw17141*	Unknown
lpw03651	*legG2*	*lpw11691*	*ceg19*	*lpw17231*	*ppeA/legC3*
lpw03741	*ceg10*	*lpw11741*	Unknown	*lpw17241*	*ppeB*
lpw03751	*lem2*	*lpw11801*	*ravO*	*lpw17391*	Unknown
lpw03861	Unknown	*lpw11901*	Unknown	*lpw17401*	Unknown
lpw04431	Unknown	*lpw11971*	*cegC3*	*lpw17411*	*ankI/legAS4*
lpw04441	Unknown	*lpw11981*	*lem7*	*lpw17761*	Unknown
lpw04591	*sdhA*	*lpw12001*	Unknown	*lpw17771*	Unknown

Gene	Description	Gene	Description	Gene	Description
lpw18031	Unknown	*lpw23561*	*legS2*	*lpw26861*	*sidD*
lpw18281	*marB*	*lpw23811*	*cegC4*	*lpw27041*	*sdbB*
lpw18331	Unknown	*lpw23821*	*cegC4*	*lpw27131*	*lepB*
lpw18691	*ceg25*	*lpw24011*	*legA2*	*lpw27241*	*mavJ*
lpw18871	*lem14*	*lpw24021*	*lem20*	*lpw27301*	*sidI/ceg32*
lpw19161	*ylfB/legC2*	*lpw24081*	*lpnE*	*lpw27311*	Unknown
lpw19211	Unknown	*lpw24091*	Unknown	*lpw27341*	*sdjA*
lpw19231	*legLC8*	*lpw24261*	Unknown	*lpw27351*	*sdeD*
lpw19461	Unknown	*lpw24371*	*lem21*	*lpw27371*	*sidC*
lpw19631	Unknown	*lpw24611*	Unknown	*lpw27461*	*snpL*
lpw19721	*lem15*	*lpw24841*	*ylfA/legC7*	*lpw27501*	*lem26*
lpw19951	*lem16*	*lpw24871*	*ankH/legA3/ ankW*	*lpw27521*	*mavL*
lpw19961	*lem17*	*lpw24981*	*ceg28/RidL*	*lpw27531*	Unknown
lpw19971	*ralF*	*lpw25121*	*ankK/legA5*	*lpw27551*	*lem27*
lpw20041	*legC4*	*lpw25181*	Unknown	*lpw27671*	Unknown
lpw20101	Unknown	*lpw25191*	*lem22*	*lpw27681*	Unknown
lpw20111	*lirA*	*lpw25371*	*mavE*	*lpw27701*	Unknown
lpw20131	*lirB*	*lpw25461*	*mavF*	*lpw27791*	Unknown
lpw20141	*pieC/lirE*	*lpw25561*	Unknown	*lpw27871*	Unknown
lpw20151	*pieD/lirF*	*lpw25841*	Unknown	*lpw27901*	Unknown
lpw20201	*pieE*	*lpw26021*	*sdbC*	*lpw27911*	*legK3*
lpw20291	*pieF*	*lpw26041*	*legL6*	*lpw28241*	*mavM*
lpw20351	Unknown	*lpw26121*	*legL6*	*lpw28321*	*sidF*
lpw20351	*pieG/legG1*	*lpw26191*	*lem23*	*lpw28361*	*legS1*
lpw20371	*setA*	*lpw26241*	*ceg29*	*lpw28391*	*ceg33*
lpw20431	Unknown	*lpw26261*	*vpdA*	*lpw28521*	*lem28*
lpw21141	Unknown	*lpw26281*	*lem24*	*lpw28781*	Unknown
lpw23101	*legK2*	*lpw26351*	*legA1*	*lpw28871*	Unknown
lpw23181	*ankB/legAU13*	*lpw26391*	Unknown	*lpw28891*	*mavV*
lpw23211	*mavC*	*lpw26401*	*lem25*	*lpw29461*	Unknown
lpw23221	Unknown	*lpw26421*	*mavG*	*lpw29481*	*legD1*
lpw23231	Unknown	*lpw26431*	*mavH*	*lpw29771*	*wipA*
lpw23271	*sdeC*	*lpw26521*	*ceg30*	*lpw29791*	*legN*
lpw23281	*sdeC*	*lpw26531*	Unknown	*lpw30031*	Unknown
lpw23291	*sidJ*	*lpw26641*	*mavI*	*lpw30041*	Unknown
lpw23301	*sdeB*	*lpw26701*	*ankF/legA14/ceg31*	*lpw30471*	*lepA*
lpw23331	*sdeC*	*lpw26751*	*ankD/legA15*	*lpw30591*	*lem29*
lpw23361	Unknown	*lpw26801*	Unknown	*lpw30711*	*mavN*
lpw23451	*lem19*	*lpw26851*	*sidM/drrA*	*lpw30831*	*ceg34*

Gene	Description	Gene	Description	Gene	Description
lpw30851	*Unknown*	*lpw31411*	*Unknown*	*lpw31931*	*Unknown*
lpw30861	*sidH*	*lpw31471*	*Unknown*	*lpw32251*	*Unknown*
lpw30881	*lubX/legU2*	*lpw31531*	*Unknown*	*lpw32851*	*legP*
lpw30891	*vipD*	*lpw31541*	*Unknown*	*lpw32861*	*Unknown*
lpw30921	*Unknown*	*lpw31571*	*Unknown*	*lpw32931*	*rvfA*