Identification and characterisation of novel *Legionella pneumophila* virulence genes

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Abstract

Legionella pneumophila, the major causative agent of the severely pneumonic Legionnaires' disease, is an intracellular bacterial pathogen that is able to exploit aquatic protozoa as well as human alveolar macrophages as hosts for replication. After internalisation, the *Legionella* containing vacuole (LCV) avoids fusion with the endosomal pathway and intercepts endoplasmic reticulum (ER)-derived early secretory vesicles prior to their transport to the Golgi. This aids in the formation of a vacuole that supports bacterial replication and is driven by effector proteins translocated by the Dot/Icm type IV secretion system. Apart from the Dot/Icm system, few genes have been identified that play a major role in intracellular replication.

In this study, we constructed and screened a library of 10,006 *L. pneumophila* transposon mutants for attenuated intracellular replication within the amoebae *Acanthamoeba castellanii*. 34 mutants were identified, 21 of which carried insertions into the Dot/Icm Type IV secretion system. In addition to these, insertions into seven novel genes were discovered, including *lpw27511* and *sdeC*.

lpw27511 encodes a transcriptional regulator of the LuxR family. Its regulatory targets were identified in a DNA microarray, however, the majority of identified targets were weakly affected and there was no obvious explanation for the replication defect observed for the transposon mutant. Further analysis of an in-frame *lpw27511* deletion mutant showed that the defined mutant did not show the intracellular replication defect seen for the transposon mutant. Unfortunately subsequent investigations could not find an explanation for the replication defect originally observed for the transposon mutant.

sdeC encodes a Dot/Icm translocated effector and we investigated the contribution of this protein to *L. pneumophila* pathogenesis further. *sdeC* was required for full replication of *L. pneumophila* in amoebae, macrophages and in the lungs of A strain mice. HEK293T cells transfected with GFP-tagged SdeC showed that SdeC co-localised

with the ER. 4HA-tagged SdeC translocated by *L. pneumophila* during infection localised with the *Legionella* containing vacuole and the ER in macrophages and amoebae. Using multiple approaches no binding partners of SdeC were identified. Several imaging techniques were used to observe LCV biogenesis of the *sdeC* deletion mutant and wild type *L. pneumophila*, and, apart from a minor replication defect no differences between the two strains were observed. However given the localisation of SdeC, we suggest that SdeC promotes the interactions between the LCV and ER, thereby aiding intracellular replication of *L. pneumophila*.

During live cell imaging experiments multiple LCVs present in the same host cell were observed to fuse together. This was a novel observation of LCV development not previously reported in *Legionella* literature. Overall these studies have once again highlighted the importance of the Dot/Icm system for *L. pneumophila* intracellular replication and provided further insight into the molecular basis of this replication.

Declaration

This is to certify that

i. the thesis comprises only my original work towards the PhD except where indicated in the Preface,

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

iii. the 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:

Chapter 3:

Sequencing, annotation and analysis of the *L. pneumophila* 130b genome was a collaborative effort carried out by collaborators (Schroeder et al., 2010). The UK strains in the novel effector distribution screen were screened by the co-authors. The remainder of the strains were screened as part of this thesis work.

Half of the generation of the transposon mutant library and the transposon mutagenesis screen was performed by Aishah Zanuidin (University of Melbourne) as part of her PhD. The other half was performed here. Preliminary work on the screen formed part of my BSc (Hons) degree at The University of Melbourne.

Chapter 4:

Sequencing and analysis of the *lpw27511* transposon mutant genome was performed by Prof. Tim Stinear and Jessica Porter (University of Melbourne)

The DNA microarray was performed here under the supervision of Carmen Buchrieser and Tobias Sahr (Pasteur Institute, France). Differential analysis was performed by Tobias Sahr.

Chapter 5:

The A strain mouse infection was performed by Clare Oates (University of Melbourne)

Mass spectrometry was performed by Tom Nebl (WEHI)

Chapter 6:

Electron Microscopy was performed by Vicki Bennett-Wood (University of Melbourne)

The remainder of this thesis comprises only my original work.

List of publications arising from the thesis

G Schroeder, N Petty, A Mousnier, C Harding, **A Vogrin**, B Wee, N Fry, T Harrison, H Newton, N Thomson, S Beatson, G Dougan, E Hartland and G Frankel
Legionella pneumophila Strain 130b Possesses a Unique Combination of Type IV Secretion Systems and Novel Dot/Icm Secretion System Effector Proteins (2010)
J. Bacteriol. 192: 6001–6016

A Vogrin, A Mousnier, G Frankel and E Hartland
Subcellular localization of Legionella Dot/Icm effectors (2013)
Methods Mol Biol, 954: 333-44

A Vogrin, A Zainudin, M Aili, T Sahr, C Buchreiser, E Hartland
Identification of novel Legionella pneumophila virulence genes using the Amoebae Plate Test to screen a random transposon library
(Manuscript in preparation)

A Vogrin and E Hartland
Pathogenesis of Legionella pneumophila in humans.
In: Emerging and Re-Emerging Human Infections (2014)
John Wiley & Sons/Wiley Blackwell Press. (In press)

A Vogrin and E Hartland
Legionella and Legionnaires' disease
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Vicki Bennett-Wood (University of Melbourne) performing the electron microscopy conducted during this study.

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Chapter 1: Literature review

1.1. History

In July 1976, a convention of the American Legion was held at the Bellevue-Stratford Hotel in Philadelphia, USA. In the following days a large number of individuals present at the convention and other city visitors in the vicinity of the hotel presented with a severe form of pneumonia. In total there were 182 cases reported with 29 deaths and the illness was subsequently termed Legionnaires' disease (Fraser et al., 1977). The cause of the epidemic remained unknown for some months, inciting great public concern. Speculations were soon made about the cause, these ranged from nickel carbonyl poisoning to a CIA chemical warfare experiment gone wrong. Eventually in December 1976 Joseph McDade determined that the causative agent of the outbreak was a previously unknown bacterial species, which was subsequently named *Legionella pneumophila* (McDade et al., 1977).

L. pneumophila has since been identified as the aetiological agent of unexplained outbreaks of pneumonia prior to 1976. The earliest documented outbreak occurred in 1957 in Austin, Minnesota, USA where seventy eight townspeople were hospitalised with acute respiratory disease and two died. The source of the epidemic was not identified until 1979 when a study of survivors showed that they had elevated levels of antibodies to *L. pneumophila* in comparison to matched controls (Osterholm et al., 1983). This investigation along with clinical and epidemiological observations led the outbreak being attributed to *L. pneumophila*.

1.2. The microorganism

L. pneumophila is a gram-negative rod-shaped bacterium belonging to the gamma-subgroup of proteobacteria. The genus *Legionella* of the family *Legionellaceae* contains over 50 species belonging to over 70 serogroups, most of which are harmless to humans (Reviewed in (Fields et al., 2002)). Some species, however, are known to cause disease, with *L. pneumophila* being the most common infectious agent of Legionnaires' disease worldwide. *Legionella* species also cause the relatively less severe flu-like illness, Pontiac fever. Collectively, the diseases caused by *Legionella* are termed legionellosis.

Legionella species, including *L. pneumophila*, are found ubiquitously in freshwater environments, including man-made water supply systems, where they are able to parasitise various protozoan species. The ability of *L. pneumophila* to multiply intracellularly within amoebae was first described in 1980 (Rowbotham, 1980). This discovery led to a novel concept that bacteria able to parasitise protozoa may use the same mechanisms to infect human cells.

Technologies that use water at higher than ambient temperature and cause water aerosolization, such as large air conditioning systems, have contributed to the emergence of *Legionella* as a human pathogen. The inhalation of *Legionella*-contaminated water droplets enables the bacterium to enter the human lung, where it infects and replicates within alveolar macrophages. Outbreaks of Legionnaires' disease almost exclusively originate from contaminated man-made water supply systems and have caused worldwide public concern since the discovery of the pathogen.

1.3. Epidemiology

1.3.1. Incidence and risk factors

Annual reported legionellosis cases in the U.S. have increased 217 per cent, from 1,110 cases in the year 2000 to 3,522 in 2009 (CDC, 2011). This may be due to the fact that the reporting of legionellosis cases has been improving with many laboratories now routinely using a *Legionella* urinary antigen test in the diagnosis of pneumonia cases, rather than an increase in the incidence of the bacterial infection itself. In Europe, the number of reported cases has been steady over the years 2005 to 2008 (Joseph and Ricketts, 2010). However, as legionellosis is a largely under diagnosed disease, primarily due to symptomatic similarities to other respiratory illnesses, it is likely that the actual number of legionellosis cases is much higher in all countries. There are usually more cases of legionellosis reported in the summer and early autumn, but the disease can occur at any time of year.

While the mortality rate of Legionnaires' disease varies greatly from 5 to 30 per cent, during the period 1980 to 1998 the average case-fatality rate of Legionnaires' disease in the U.S. decreased from 34 to 12 per cent (Benin et al., 2002). Early recognition of Legionnaires disease from increased testing and rapid diagnosis has likely contributed to this decrease in mortality. Increased awareness of *Legionella* outbreaks, more timely and appropriate responses help to resolve infection by early treatment, thereby reducing mortality.

People most at risk of infection are the elderly, current or former smokers, people with a prior respiratory illness and immune-compromised individuals. Elderly people are at greatest risk because of the reduced capacity of their immune system to fight infection and their compromised pulmonary function. Many reported legionellosis cases are hospital-acquired, due to the prevalence of high-risk patients such as those receiving immunosuppressive treatments for various conditions including cancer.

1.3.2. Transmission

Legionella species are found naturally in most freshwater environments but also in damp soil. In Australia, New Zealand and Thailand, many cases of legionellosis can be attributed to *L. longbeachae*, which is primarily found in soil (Amodeo et al., 2010; Li et al., 2002; Phares et al., 2007). Consequently, compost and potting mixes now carry a mandatory warning describing the risk of infection from the inhalation of dust.

Legionella is commonly isolated from man-made aquatic environments where it is able to survive chlorination (Kuchta et al., 1983) and the bacteria are often found in biofilms (Declerck, 2010). *Legionella* exploit free-living protozoa as hosts for replication. These natural hosts provide nutrients and a protective niche for the bacteria that allows for abundant intracellular replication. Some protozoa have the ability to form protective cysts and this is especially beneficial to *Legionella* as it allows the bacteria to survive high temperatures, disinfection procedures and drying (Marciano-Cabral and Cabral, 2003). *Legionella* species grow best in warm water, and hence infection is associated with water held at higher than ambient temperature including in spas, showers and air conditioning cooling towers of large buildings (Breiman et al., 1990; Fallon and Rowbotham, 1990; Ferre et al., 2009). The water aerosols generated by these systems leads to transmission of *Legionella* to humans after inhalation of contaminated water droplets. The bacteria, however, do not spread from person to person.

The close biological association between *Legionella* and protozoa has pre-adapted the bacteria for human infection due to similarities in function between protozoa and human macrophages. *L. pneumophila*, in particular, is able to infect a wide range of protozoan species and it is widely accepted that *Legionella* has accumulated a diverse range of virulence factors in order to survive and replicate within distinctly different protozoa (Fields, 1996; Rowbotham, 1980; Valster et al., 2010). However, our knowledge of exactly what is required for bacterial survival within protozoan host cells

and the mechanisms involved in supporting *Legionella* replication is still developing. The inhalation of protozoa containing *Legionella* may also be an effective vehicle for transmission to humans, in addition to playing a crucial role in the continuing presence of *Legionella* in aquatic systems as a host for replication (Rowbotham, 1986).

Legionellosis is a disease that has emerged in the last half of the 20th century; this is thought to be primarily due to human alteration of the environment. Left in their natural environment, *Legionella* would be a very rare cause of human infection, as natural freshwater environments have not been implicated as significant reservoirs of outbreaks of legionellosis.

1.4. The *Legionella pneumophila* genome

1.4.1. Genome organization

The genomes of several clinical isolates of *L. pneumophila* have been sequenced and by comparison they are well conserved being 3.3-3.5 Mbp in length and possessing a GC content of approximately 38%. The genomes contain 2900-3200 protein-coding genes with an average length of 980-1080 bp and a coding density of 87-88% (Cazalet et al., 2004; Chien et al., 2004; Gomez-Valero et al., 2011; Schroeder et al., 2010).

A large study of 217 *L. pneumophila* strains showed a high degree of conservation among genes known to be associated with virulence, suggesting a strong selection pressure for their maintenance (Cazalet et al., 2008). No overall genomic profile distinguished clinical and environmental samples or strains of different serogroups. Interestingly, the genes responsible for the core and O side-chain synthesis of lipopolysaccharide (LPS) in serogroup one strains have a surprisingly significant level of genetic variation which suggests the likelihood of horizontal transfer of the LPS cluster (Cazalet et al., 2008).

Legionella possesses a Dot/Icm (Defective organelle trafficking/Intracellular multiplication) Type IVB secretion system (T4BSS) that injects a large number of bacterial virulence "effector" proteins into the host cell. The vast majority of *dot/icm* genes are conserved amongst all *Legionella* strains and are present in the same chromosomal location in *L. pneumophila* (Gomez-Valero et al., 2011; Morozova et al., 2004). More than 200 Dot/Icm secreted effectors are conserved in all strains of *L. pneumophila* with 95-100% nucleotide identity (Gomez-Valero et al., 2011). Despite an apparent high level of functional redundancy amongst these effectors, their conservation highlights their collective importance to the *L. pneumophila* life cycle and broad host range. In addition to the Dot/Icm T4BSS, the *L. pneumophila* genome encodes several T4ASSs and conjugative elements that likely contribute to genome plasticity as T4SSs are involved in DNA uptake and transfer as well as the spread of conjugative plasmids and protein translocation (Backert and Meyer, 2006).

Horizontal gene transfer of mobile genetic elements is the main source of genetic diversity amongst *L. pneumophila* strains (Cazalet et al., 2008; Gomez-Valero et al., 2011; Gomez-Valero et al., 2014; McAdam et al., 2014). In addition, plasmid excision and integration constitutes another source of genome plasticity in *L. pneumophila*. The gene cluster encoding a Lvh Type IVA secretion system (T4ASS) can be present on a plasmid or in an integrated form in the genome (Cazalet et al., 2004; Chien et al., 2004) and two mobile elements carrying a T4ASS have also been reported in *L. pneumophila* strain Corby (Glockner et al., 2008). A 100-kb region comprising several genes encoding efflux transporters for heavy metals and toxins has also been identified. Fringing this region are tRNA, phage-related and transposase genes indicating possible acquisition via horizontal transfer (Chien et al., 2004). This region may be responsible for protecting *Legionella* from toxins present in plumbing and man-made water supply systems. *Legionella* possesses more genes encoding efflux transporters for heavy metals and toxins relative to many other bacterial species, possibly because the

bacteria have to survive within protozoan hosts that accumulate heavy metals from the environment (Fernandez-Leborans and Herrero, 2000).

1.4.2. Evolution

The *L. pneumophila* genome encodes many homologs of eukaryotic proteins or proteins with motifs found primarily in eukaryotes and as a prokaryote possesses the widest variety of these (Cazalet et al., 2004; Chien et al., 2004). Some of these include a number of F-box and U box proteins, two CD39 ecto-nucleoside triphosphate diphosphohydrolases, a sphingosine-1-phosphate lyase, a SET domain protein, a Sec7 domain protein, a mitochondrial carrier protein and a SNARE protein (Hubber and Roy, 2010; Xu and Luo, 2013). The eukaryotic-like proteins were likely to have been obtained through the close association of *Legionella* with its protozoan host and presumably interfere with host cell processes by mimicking the function of eukaryotic proteins. An analysis of several *L. pneumophila* genomes found that more than 50% of genes encoding eukaryotic-like proteins are conserved amongst strains with a high level (89-100%) of nucleotide identity suggesting a high selection pressure for their maintenance (Gomez-Valero et al., 2011).

The majority of Legionnaires' disease cases worldwide are caused by *L. pneumophila* serogroup one. Multi-genome analysis of *L. pneumophila* serogroup one strains suggests a highly conserved core genome of housekeeping genes including many eukaryotic-like proteins, *dot/icm* genes and secreted effectors (Gomez-Valero et al., 2011). The core genome of the analysed strains includes 2434 genes, approximately 80% of the total number of predicted genes in each genome. The gene order in the strains is also highly conserved, except for a 260 kb inversion in the Lens strain.

Frequent horizontal gene transfer and recombination events have contributed to a diverse accessory genome. It has been suggested that numerous secretion systems of *L. pneumophila* facilitated the distribution of large chromosomal fragments of over 200

kb via conjugal transfer. In addition, *L. pneumophila* is naturally competent and possesses functional recombination machinery required for the integration of foreign DNA into the *Legionella* genome (Sexton and Vogel, 2004; Stone and Kwaik, 1999). This important evolutionary ability is permissive for *L. pneumophila's* dynamic accessory genome, allowing for frequent horizontal transfer and recombination events. Analysis of nucleotide polymorphisms amongst six strains identified numerous large fragments from different origins including eukaryotes, other prokaryotes as well as different strains and species of *Legionella* (Gomez-Valero et al., 2011). The diverse accessory genome of about 300 genes comprise mobile genetic elements, genomic islands and many genes of unknown function, including Dot/Icm effector proteins (Gomez-Valero et al., 2011). The dynamic genome of *L. pneumophila* reflects its capacity to survive in diverse range of environments and hosts.

1.5. Pathogenesis & Immunity

1.5.1. Pathogenesis

L. pneumophila is an intracellular pathogen that replicates within a host eukaryotic cell, utilizing it as a source of nutrients. The pathogen possesses a remarkable ability to manipulate multiple host cell processes to form a protective, membrane-bound compartment that supports intracellular replication known as the *Legionella*-containing vacuole (LCV).

After internalization by eukaryotic cells, ingested bacteria are normally destroyed via a degradative process known as the endocytic or lysosomal pathway. The LCV avoids the endocytic pathway and hence destruction in lysosomes by intercepting vesicles that are trafficking in the secretory pathway of the host cell (Roy et al., 1998). The recruitment and fusion of ER exit vesicles to the early LCV results in a vacuole positive for Rab1 and Sec22ba, which then matures into membrane that resembles

endoplasmic reticulum (ER) (Kagan and Roy, 2002). The cytoplasmic face of the LCV membrane becomes lined with ribosomes and resident ER markers are associated with the LCV membrane, including calnexin, BiP and Sec61 (Shin and Roy, 2008). Once the mature LCV is formed, *L. pneumophila* enters a replicative growth phase and after multiple rounds of replication will exit the host, usually via lysis of the host cell, allowing for infection of near-by cells where the infection process will begin anew (Shin and Roy, 2008).

L. pneumophila alternates between two growth states during infection, namely a non-motile, thin walled replicative form and a motile, thick-walled infectious form (Garduno et al., 2002; Rowbotham, 1986). Bacteria differentiate into the replicative state after entering a host cell and establishing the LCV. In this state the bacteria are avirulent and non-flagellated (Molofsky and Swanson, 2004). After numerous rounds of replication nutrients become limited, triggering the switch to the infectious form. The bacterial two-component gene regulators, LetA and LetS (*Legionella* transmission activator and sensor, respectively), govern this differentiation through activation of two small regulatory RNAs, RsmY and RsmZ (Rasis and Segal, 2009; Sahr et al., 2009). In this state the bacteria are highly virulent and flagellated allowing for release and transmission to a new host cell (Edwards et al., 2010; Hammer et al., 2002; Molofsky and Swanson, 2004). This switch between two different states allows *L. pneumophila* to focus on two distinct roles during the infection cycle.

1.5.2. The Dot/Icm type IV secretion system and protein translocation

Legionella possesses a Dot/Icm type IVB protein secretion system that is absolutely required for intracellular replication within protozoa and human macrophages as mutations in *dot/icm* genes abrogate intracellular replication (Andrews et al., 1998; Segal and Shuman, 1999). The Dot/Icm system is ancestrally related to DNA conjugation systems but transfers around 300 bacterial effector proteins into the host

cell, rather than conjugating plasmid DNA into a bacterial recipient. The Dot/Icm apparatus comprises around 27 proteins, including several bacterial inner and outer membrane components and presumably proteins that span LCV membrane (Qiu and Luo, 2013). The translocation of effector proteins requires recognition of a C-terminal secretion signal that varies in its amino acid composition among different effectors (Huang et al., 2011; Kubori et al., 2008; Nagai et al., 2005). Rather than the amino acid sequence itself, it is the physicochemical properties of the C-terminal 35 amino acids that are most important for recognition by the Dot/Icm system (Lifshitz et al., 2013). Effectors lacking a strong translocation signal require chaperones for their secretion (Lifshitz et al., 2013). The cytosolic Dot/Icm components, IcmS and IcmW, form a putative chaperone complex that is necessary for the translocation of a subset of effector proteins into the host cell (Cambronne and Roy, 2007). *icmS* and *icmW* knockout mutants still recruit early secretory vesicles and replicate to some extent, but the mutant vacuoles eventually fuse with lysosomes (Coers et al., 2000). This may indicate that effector proteins chaperoned by IcmS/IcmW are necessary for avoiding lysosomal fusion.

Given the essential contribution of the Dot/Icm system to *L. pneumophila* pathogenesis, there is much current investigation into the cellular processes that support intracellular survival and replication of the pathogen and the role of Dot/Icm effectors in modulating these pathways. The translocated Dot/Icm effector proteins contribute to formation of the LCV by interfering with multiple cellular operations (Albert-Weissenberger et al., 2007; Hubber and Roy, 2010; Xu and Luo, 2013). In fact *L. pneumophila* appears to influence almost every aspect of host cell physiology including vesicle trafficking, membrane fusion, gene transcription, protein translation and cell survival. Dissecting the function of individual effectors has proved difficult as many effectors are found in families of paralogs and even unrelated effectors can target the same cellular process. Nevertheless, research over the past few years has revealed the function of many newly identified *L. pneumophila* effectors. Some of the best-characterized effectors have novel enzymatic activities that introduce post-

translational modifications and act in concert to exert exhibit exquisite control over the function of their substrate (Mukherjee et al., 2011; Rolando and Buchrieser, 2012). Interestingly many Dot/Icm effectors also share amino acid sequence similarity with eukaryotic proteins or are predicted to carry a motif or domain that is found predominantly in eukaryotic proteins. Here, we provide an overview of several host processes that *L. pneumophila* interacts with.

1.5.3. Manipulation of host cell trafficking pathways

VipA is a Dot/Icm translocated effector that interferes with host organelle trafficking in the Multivesicular Body (MVB) pathway when ectopically expressed in yeast (Shohdy et al., 2005). It is an actin nucleator that was shown to bind actin in vitro and polymerized microfilaments without the aid of any additional proteins. VipA localized to actin-rich regions and components of the Multivesicular Body pathway such as endosomes in eukaryotic cells (Franco et al., 2012). Hence, VipA is a novel type of actin nucleator that may contribute to *Legionella* pathogenesis by utilising the host cytoskeleton to target host cell trafficking pathways.

The LCV intercepts ER-derived early secretory vesicles, which in part involves the recruitment of host factors Arf1 (ADP Ribosylation Factor-1) and Rab1 (a member of the RAS oncogene family), both of which are regulators of ER-Golgi vesicle traffic (Kagan and Roy, 2002). These host factors are found on wild-type but not *dot/icm* mutant LCVs (Kagan and Roy, 2002), indicating that Dot/Icm translocated effectors are responsible for their recruitment. Here we outline the function of several effectors identified to be involved in this process.

Arf1 is a host GTPase involved in the regulation of vesicle trafficking between the ER and the Golgi. Arf proteins are activated by Arf-specific guanine nucleotide exchange factors containing Sec7 protein domains. The Sec7 domain is required for the exchange

of GDP for GTP on Arf proteins, switching them to an active state (Jackson and Casanova, 2000). The Dot/Icm translocated effector RalF contains a Sec7 domain that is homologous to mammalian Sec7 domains (Amor et al., 2005). RalF functions as a guanine exchange factor that localizes to the LCV where it is able to recruit and activate Arf1 (Donaldson and Jackson, 2000; Nagai et al., 2002). A structural C-terminal capping domain regulates the activity and localization of RalF following translocation which confers guanine nucleotide exchange function (GEF) in the secretory pathway of the infected cell (Alix et al., 2012). This interaction will promote the recruitment of ER-derived vesicles to the LCV.

The host GTPase, Rab1, is involved in the recruitment of proteins that facilitate the transport, adherence and fusion of vesicles (Zerial and McBride, 2001). DrrA (or SidM) is a Dot/Icm translocated effector that recruits Rab1 to the LCV via the displacement of GDP association inhibitors that maintain inactive Rab1 in the cytosol (Machner and Isberg, 2006). Inactivation of Rab1 is mediated by the Dot/Icm effector LepB, which acts as a GTPase-activating protein (GAP). LepB binds activated Rab1-GTP and hydrolyses GTP to GDP, thereby converting Rab1 into an inactive form (Ingmundson et al., 2007). DrrA contains a second region that is responsible for the activation of Rab1, once it has been recruited to the LCV via the GEF activity of DrrA (Machner and Isberg, 2007). The GEF activity of DrrA is complemented by an N-terminal nucleotidyltransferase domain that mediates AMPylation of tyrosine 77 in the switch II region of Rab1 (Muller et al., 2010). This post-translational modification blocks access for GAP proteins, including LepB, and leads to constitutive activation of Rab1.

Rab1 activity is exquisitely regulated by another *L. pneumophila* effector, SidD, which deAMPylates Rab1 leading to the release of Rab1 from the LCV (Neunuebel et al., 2011; Tan and Luo, 2011). In addition, the *L. pneumophila* FIC domain protein, AnkX, modifies Ser76 in the switch II region of Rab1 with phosphocholine, which is also able to be removed by the *L. pneumophila* effector Lem3 (Mukherjee et al., 2011; Tan et al., 2011). Phosphocholination of Rab1 does not appear to affect binding by DrrA

suggesting that the modifications shape Rab1 interactions with distinct sets of proteins. Despite these seemingly important functions, deletion mutants of these effector genes are able to replicate at normal levels within macrophages and protozoa (Machner and Isberg, 2006; Nagai et al., 2002), indicating the likely existence of functional redundancy among translocated effectors targeting Rab1.

AMPylation of Rab1 by the nucleotidyltransferase domain of DrrA is important for accumulation and retention at the LCV membrane (Hardiman and Roy, 2014). Efficient localisation of Rab1 to the LCV occurred when Rab1 GEF activity and Rab1 AMPylation activity were provided by separate proteins, indicating both activities are important. The defect in Rab1 localization to the LCV of *Legionella* strains deficient in AMPylation was partially suppressed if the GTPase-activating protein LepB was removed. Rab1 phosphocholination by AnkX was not a substitute for Rab1 AMPylation, despite both phosphocholination and AMPylation of Rab1 having been shown in vitro to prevent deactivation by GAP proteins (Goody et al., 2012; Muller et al., 2010; Tan et al., 2011)

Various other Dot/Icm translocated effectors have also been linked to an interaction with ER-derived vesicles and the formation of the replicative vacuole. LidA is required for efficient formation of the replicative vacuole (Derre and Isberg, 2005) and enhances the activity of DrrA by promoting the tethering of ER-derived vesicles to the LCV via Rab1 association (Machner and Isberg, 2006). LidA has been shown to bind multiple Rab GTPases (Chen and Machner, 2013; Cheng et al., 2012; Schoebel et al., 2011). LidA may be recruited to the LCV via its association with Rab GTPases, where it could play a role in the tethering of ER vesicles to the LCV. Together these findings indicate a comprehensive capacity of *Legionella* to control the activity of Rab1 at several levels, dictating whether it is present in an active or inactive form on the LCV.

The fusion of ER-derived vesicles to the LCV is believed to require the attachment of target Soluble N-ethylmaleimide-sensitive factor Attachment protein REceptors (SNAREs) on the target membrane to a SNARE on the vesicle membrane (Kagan et al.,

2004). Sec22b is a SNARE located on ER-derived vesicles that is recruited to the LCV in a Dot/Icm-dependent manner, contributing to the formation of the replicative vacuole (Kagan et al., 2004). Vesicle fusion may be achieved via the plasma membrane-localised SNARE proteins syntaxin 2, syntaxin 3, syntaxin 4 and SNAP23, which localize to the LCV where they interact with Sec22b (Arasaki and Roy, 2010). It is also possible that a *Legionella* effector mimics SNARE function, facilitating the fusion of ER-derived vesicles to the LCV. For example, the effectors YlfA (LegC7) and YlfB (LegC2) possess a protein domain similar to the IncA protein family. IncA is a bacterial SNARE-like molecule involved in the fusion of vesicles in *Chlamydia* infected cells (Delevoye et al., 2004; Delevoye et al., 2008). YlfA/LegC7 associates with ER membranes and may target ER-derived vesicles (de Felipe et al., 2008).

DrrA was shown to stimulate SNARE association with the LCV and promote membrane fusion. The activation of Rab1 at the LCV by DrrA stimulates the tethering of ER-derived vesicles to the LCV resulting in vesicle fusion through the interaction of Sec22b with syntaxin proteins. This suggests that Rab1 activation by DrrA is sufficient to promote the recruitment and fusion of ER-derived vesicles at the LCV (Arasaki et al., 2012).

Another host GTPase implicated in LCV biogenesis is Sar1, which regulates the formation of coat protein II (COPII)-coated vesicles derived from the ER (Sato and Nakano, 2007). Sar1 plays several roles in the cell including COPII coat recruitment and cargo sorting. A siRNA knockdown of Sar1 and a dominant interfering Sar1 variant have been used to show that impeding Sar1 function interferes with the intracellular replication of *L. pneumophila* (Dorer et al., 2006; Kagan and Roy, 2002). The LCV in cells expressing Sar1H79G, a GTP-restricted Sar1 variant, does not associate with ER-derived vesicles (Robinson and Roy, 2006), suggesting that the vesicles destined to interact with the LCV are generated by a Sar1-dependent process and this may be driven by Dot/Icm effectors. Collectively, these examples demonstrate several complex

strategies employed by *L. pneumophila* to subvert the trafficking of ER-derived vesicles for the benefit of LCV maturation.

1.5.4. Phagosomal pH regulation

A phagosome undergoing endocytic maturation will become acidified by the vacuolar ATPase (vATPase) proton pump acquired at the late endosomal stage (Forgac, 2007). A low lumenal pH is important for lysosome maturation as well as the activity of lysosomal enzymes. In order to avoid lysosomal degradation many intracellular pathogens regulate pH in the phagosomal lumen (Huynh and Grinstein, 2007; Ohkuma and Poole, 1978). *L. pneumophila* and *L. longbeachae* are both able to maintain a vacuole of neutral pH. However reports differ on the association of vATPase with the LCV during infection. One report has stated that the majority of *L. pneumophila* and *L. longbeachae* containing vacuoles do not co-localise with the vATPase protein pump, in contrast to *dot/icm* mutants (Asare and Abu Kwaik, 2007). Proteomic analysis of LCVs purified from *Dictyostelium* and macrophages by immunomagnetic separation identified several components of the vATPase, despite neutral pH within the vacuole and the absence of other late endosomal features on the LCV (Shevchuk et al., 2009). This suggested that although the vATPase may be present on the LCV, it is not actively lowering pH. Later it was shown that the Dot/Icm translocated effector SidK specifically targets host vATPase via an interaction with VatA, the component of the vATPase that is responsible for hydrolysing ATP (Xu et al., 2010). Binding of SidK to VatA results in the inhibition of ATP hydrolysis and proton translocation into the LCV. The same study also demonstrated that microinjection of bone marrow-derived macrophages with SidK impaired their ability to digest non-pathogenic *E. coli* (Xu et al., 2010). Thus, SidK contributes to the protection of internalised *Legionella* by subverting the function of host vATPase thereby blocking the acidification of the LCV.

1.5.5. Manipulation of host cell ubiquitination and autophagy

Ubiquitination of proteins is a post-translational modification that regulates numerous host cell processes by altering the activity of proteins or directing proteins for degradation by the cell proteasome. The importance of this function to *Legionella* replication is illustrated by two early findings that poly-ubiquitinated proteins decorate the LCV shortly after infection and proteosome inhibitors limit *Legionella* intracellular replication (Dorer et al., 2006). During infection, *L. pneumophila* translocates a number of effectors with presumed or proven ability to interfere with or exploit the host cell ubiquitination machinery (Hubber et al., 2013). For example, the *L. pneumophila* genome encodes functional mimics of eukaryotic E3 ubiquitin ligases that act in concert with components of the host ubiquitination machinery to target both host and bacterial proteins for polyubiquitination. These include F-box and U-box containing proteins and proteins containing ankyrin repeat domains, a motif that has been implicated in numerous eukaryotic protein-protein interactions (Hubber et al., 2013; Mosavi et al., 2002). The identification of substrates for these E3 ligases is key to understanding their function. For example, Clk1 is a substrate of the U-box protein, LubX, and Clk family inhibitors limit intracellular growth, although the molecular role of Clk kinases in *Legionella* replication is not known (Kubori et al., 2008). Another proposal based on a severe replication defect of a mutant lacking the F-box protein, AnkB (LegAU13), is that protein ubiquitination and degradation promotes the supply of amino acids for *Legionella* replication in the LCV (Price et al., 2011). However this finding may be strain specific, or even mutant specific, as *ankB* mutants in other strains show little to no defect in intracellular replication.

Host proteins are not the only targets of *Legionella* U-box and F-box proteins and a mechanism has been suggested whereby Dot/Icm effector activity is regulated by targeted ubiquitination and degradation by the host cell proteasome. For example, the effector SidH is also a target of LubX, which has led to the description of LubX as a

"metaeffector", namely an effector that controls the activity of another subset of effectors (Kubori et al., 2010).

Another much studied aspect of *Legionella*-host cell interactions is the association of the LCV with the autophagy pathway. Eukaryotic cells may use autophagy to sequester cytosolic organelles, pathogens and pathogen modified vacuoles into a membrane-bound compartment termed the autophagosome (Joshi and Swanson, 2011). Autophagosomes are recognizable by a double membrane and various membrane markers such as Atg5, Atg7, Atg9 and LC3/Atg8. Autophagy is linked to the ubiquitin-conjugation system and is a means for the cell to dispose of non-functional organelles and to recycle components by fusing the autophagosome with lysosomes to promote degradation of their cargo machinery (Hubber et al., 2013; Joshi and Swanson, 2011). Autophagosomes also provide a defense mechanism against intracellular pathogens (Swanson, 2006).

Autophagy appears to limit *L. pneumophila* replication as mutants of the amoeba, *Dictyostelium discoideum*, that lack Atg9 are more permissive for bacterial replication (Tung et al., 2010) and depletion of Atg5 by siRNA treatment in macrophages promotes *L. pneumophila* replication (Matsuda et al., 2009). Likewise the induction of autophagy limits *L. pneumophila* replication and this depends on a functional Dot/Icm secretion system (Matsuda et al., 2009). The nascent LCV becomes positive for autophagy markers 2-4 h after infection but fusion with lysosomes is inhibited (Amer and Swanson, 2005; Joshi and Swanson, 2011). A key step in autophagosome development is the cleavage of cytosolic LC3, and its conjugation to phosphatidylethanolamine by Atg3 and Atg7. Recently the Dot/Icm effector, RavZ, was described as a cysteine protease that cleaves the amide bond between the carboxyl-terminal glycine residue and an adjacent aromatic residue in LC3 (Choy et al., 2012). This results in a form of LC3 that can no longer be conjugated by Atg3 and Atg7 (Choy et al., 2012). Hence, *L. pneumophila* irreversibly modifies LC3 to inhibit autophagosome development and fusion with lysosomes.

Despite these observations, another study has shown that *L. pneumophila* replicates normally in *D. discoideum apg1, apg5, apg6, apg7* and *apg8* autophagy mutants and produces LCVs that are morphologically indistinguishable from those in wild-type *D. discoideum* infections (Otto et al., 2004). This would indicate that autophagy overall is relatively dispensable for intracellular replication of *L. pneumophila* in *D. discoideum.*

1.5.6. Lipid metabolism

Phosphoinositide metabolism plays a role in membrane dynamics, actin remodelling and cell signalling (Di Paolo and De Camilli, 2006). Phosphoinositides will also anchor target proteins to specific membranes, contributing to the identity of subcellular compartments (Yeung et al., 2006). *L. pneumophila* exploits host phosphoinositides to localise secreted effector proteins to the cytoplasmic face of the LCV (Weber et al., 2009). There is evidence to suggest that phosphatidylinositol-4 phosphate (PtdIns(4)P), normally produced by the host PtdIns-4-kinase IIIβ, is enriched on the LCV membrane. Antibodies that bind PtdIns(4)P and a tagged PtdIns(4)P-binding protein, GST-FAPP1, both bind to vacuoles isolated from *L. pneumophila*-infected *Dictyostelium discoideum* and RAW 264.7 cells (Weber et al., 2009).

The Dot/Icm translocated effector SidC and its paralogue SdcA anchor to the LCV via phosphatidylinositol-4 phosphate (PtdIns(4)P) (Ragaz et al., 2008). *sidC-sdcA* deletion mutants are less efficient at recruiting ER-derived vesicles and establishing the LCV. Beads coated with SidC, or its 70 kDa N-terminal fragment, recruit ER vesicles in *Dictyostelium* and macrophage lysates suggesting that the effector plays an important role in formation of the ER-derived vacuole. SidC harbours a 20 kDa PtdIns(4)P-binding domain near the C-terminus for anchoring to the cytoplasmic face of the LCV and recruits ER vesicles to the LCV via a 70 kDa N-terminal fragment (Ragaz et al., 2008).

Other *L. pneumophila* effector proteins also exploit host phosphoinositides. SidM directly binds PtdIns(4)P, LpnE binds PtdIns(3)P and LidA binds PtdIns(3)P as well as PtdIns(4)P (Brombacher et al., 2009; Weber et al., 2009). These examples demonstrate the ability of *L. pneumophila* to exploit host cell phosphatidylinositol lipids, using them as a means to anchor various effector proteins to the membrane of the LCV. *Legionella* undoubtedly controls the composition and timing of PtdIns flux on the LCV membrane. For example, PtdIns(4)P is generated on the LCV by the effector, SidF, which is a phosphatidylinositol polyphosphate 3-phosphatase that specifically hydrolyzes the D3 phosphate of PI(3,4)P(2) and PI(3,4,5)P(3) (Hsu et al., 2012). The PI phosphatase activity of SidF is necessary for anchoring PI(4)P-binding effectors to the LCV.

Sphingolipid metabolism is involved in several physiological functions including proliferation, inflammation, cell survival and apoptosis (Bandhuvula and Saba, 2007). Recent evidence has suggested that *Legionella* may modulate sphingolipid metabolism resulting in the manipulation of one or more of these processes (Degtyar et al., 2009). The Dot/Icm secreted effector LegS2 is homologous to the conserved eukaryotic enzyme, sphingosine-1-phosphate lyase (SPL). SPL is an enzyme that is involved in the metabolism of sphingolipids and it is possible that *Legionella* manipulates sphingolipid metabolism to acquire a degradation product for its virulence. *L. pneumophila* translocates LegS2 into the host cytosol where it then localises to the mitochondria (Degtyar et al., 2009). This is in contrast to eukaryotic SPL, which localises to the ER. Exactly what function LegS2 performs at the mitochondria is yet to be determined. *L. pneumophila* encodes several other proteins putatively involved in sphingolipid metabolism including proteins highly similar to sphingomyelinase and sphingosine kinase (Gomez-Valero et al., 2009). While we don't completely understand why *Legionella* modulates sphingolipid metabolism, it serves as another example of the manipulation of a host process by *Legionella*.

1.5.7. Protein synthesis and the stress response

eEF1A and eEF1B*γ* are two eukaryotic elongation factors that are involved in polypeptide chain elongation. The Dot/Icm secreted effector SidI targets both of these elongation factors to inhibit host protein synthesis (Shen et al., 2009). Interaction with eEF1A simultaneously induces a stress response in host cells and it has been shown that eEF1A is required for the activation of heat shock factor 1 (HSF1), a major stress response regulatory protein (Shamovsky et al., 2006). *L. pneumophila* infection activates HSF1 in host cells but infection with a *sidI* deletion mutant leads to a reduction in the level of HSF1 activation (Shen et al., 2009). Various other host stress response genes are induced by *L. pneumophila* infection of both amoebae and mammalian cells (Farbrother et al., 2006; Losick and Isberg, 2006).

A family of effector proteins, Lgt1, Lgt2 and Lgt3, function as glucosyltransferases that are structurally similar to the large clostridial toxins and use UDP-glucose as a substrate (Belyi et al., 2013). The *L. pneumophila* effector protein Lgt1 glycosylates serine 53 in the GTP-binding domain of eEF1A, resulting in the inhibition of protein synthesis and induction of cell death (Belyi et al., 2006; Hurtado-Guerrero et al., 2010; Lu et al., 2010; Tzivelekidis et al., 2011). Lgt1 is also able to modify the heat shock protein 70 subfamily B suppressor Hbs1, adding to the induction of a stress response in host cells (Belyi et al., 2009). However, the toxicity induced by Lgt1 in yeast and mammalian cells seems to be due to its effect on eEF1A rather than Hbs1 (Belyi et al., 2012). Like many Dot/Icm effector mutants, deletion of all three *lgt* genes had no impact on intracellular replication (Ivanov and Roy, 2009) and hence the contribution of these effectors to *Legionella* infection is still unclear. It is possible that the induction of a stress response may make the host cell environment more favourable for *Legionella* replication, however the induction of cell death would seem to be counterproductive.

1.5.8. Cell death and cytotoxicity

The manipulation of host signalling in order to avoid untimely host cell apoptosis is important in preventing the premature termination of *L. pneumophila* replication. Microarray analysis of *L. pneumophila*-infected cells has shown that genes involved in NF-κB signalling and genes with anti-apoptotic function are transcriptionally upregulated (Abu-Zant et al., 2007; Losick and Isberg, 2006). The transcription factor NF-κB is involved in the promotion of host cell survival and *L. pneumophila*-infected cells activate NF-κB signalling in a Dot/Icm dependent manner (Abu-Zant et al., 2007; Losick and Isberg, 2006). This indicates that a Dot/Icm translocated effector may be responsible for the manipulation of NF-κB signalling, thus influencing host cell survival.

The activation of host NF-κB by *L. pneumophila* may also contribute to the inhibition of apoptosis (Losick and Isberg, 2006). NF-κB is a transcriptional regulator that contributes to inflammation and host cell survival and activates several anti-apoptotic genes. NF-κB is initially activated upon recognition of *Legionella* flagellin but activation is also sustained for several hours after infection in a flagellin-independent manner (Bartfeld et al., 2009). LegK1 is a substrate of the Dot/Icm T4SS that has a demonstrated ability to activate NF-κB by mimicking host IKK. LegK1 directly phosphorylates the IκB family of NF-κB inhibitor proteins resulting in their ubiquitination and degradation by the cell proteasome (Ge et al., 2009). Other Dot/Icm effectors have also been implicated in NF-κB activation. SdbA and LubX were both found to contribute to the sustained activation of NF-κB in A549 epithelial cells through an unknown mechanism (Bartfeld et al., 2009).

One effector putatively involved in preventing apoptosis is SdhA, a paralogue of SidH, a Dot/Icm T4SS secreted effector protein (Laguna et al., 2006). Macrophages infected with a *L. pneumophila sdhA* deletion mutant displayed increased nuclear degradation, membrane permeability, mitochondrial disruption and caspase activation (Laguna et al., 2006), suggesting a role for SdhA in the prevention of host cell death. Mutants

lacking *sdhA* display severe intracellular growth defects due to the rapid induction of host cell death and are attenuated *in vivo* in A/J mice as well as *Galleria melonella* (Harding et al., 2013b; Laguna et al., 2006). SdhA was also found to be critical for maintaining the integrity of the LCV (Creasey and Isberg, 2012). *sdhA* mutants become cytosolic as the LCV membrane is degraded and pyroptosis results from the recognition of cytosolic *Legionella* DNA by the AIM2 inflammasome (Ge et al., 2012). How SdhA maintains the LCV membrane is not known. Another *Legionella* effector, SidF, has been reported to inhibit apoptosis by interfering with the function of BNIP3 and Bcl-rambo, two pro-apoptotic members of the Bcl2 protein family (Banga et al., 2007). How SidF mediates this function is unknown, particularly given its activity as a phosphatidylinositol polyphosphate 3-phosphatase (Hsu et al., 2012). It is unusual that these Bcl2 protein family targets are not present in amoebae, the presumed evolutionary driving force of *Legionella*, hence the need to examine the precise function of SidF further. Hence it is evident that *L. pneumophila* manipulates multiple host pathways associated with apoptosis using an array of secreted effector proteins.

1.5.9. Immunity

L. pneumophila is an accidental pathogen of humans and to date human-to-human transmission has not been observed. Hence, people remain an evolutionary dead end for the bacteria and there has been no selective pressure from the mammalian immune system on evolution of the pathogen. In healthy individuals, *Legionella* infections are usually effectively cleared by the immune system with few symptoms (Palusinska-Szysz and Janczarek, 2010). A robust inflammatory response followed by cell-mediated immunity is the primary mechanism of host defence. Healthy individuals also generate anti-*Legionella* antibodies in their serum although their contribution to clearance is not clear (Rudbeck et al., 2009).

After inhalation, *Legionella* is phagocytosed by alveolar macrophages present in the lungs. *Legionella* poses a challenge to the human immune system as the bacteria have the ability to survive and replicate within the very immune cells whose role is to destroy bacteria (in addition to other roles in innate immunity, and integration with the adaptive immune response). In general, immune cells such as macrophages, natural killer (NK) cells and immature dendritic cells are primarily involved in the initial activation of the innate immune system triggered by bacterial surface antigens. Toll-like receptors (TLRs) present on these immune cells recognize components of bacteria such as flagellum, lipopolysaccharide (LPS) and peptidoglycan. The stimulation of TLRs leads to the production of pro-inflammatory cytokines and the expression of co-stimulatory molecules, recruiting lymphocytes to the site of infection and activating immune cells. Analysis of patient genotypes after a Legionnaires' disease outbreak showed that a common TLR5 polymorphism, which introduces a premature stop codon (TLR5392STOP), is associated with a small increased risk of Legionnaire's disease (Hawn et al., 2003). The dominant TLR5392STOP polymorphism likely increases an individual's risk of Legionnaires' disease, as TLR5 is no longer able to mediate flagellin signalling in lung epithelial cells, impairing the production of proinflammatory cytokines. Other innate immune signalling pathways associated with genetic susceptibility or resistance to Legionnaires' disease are TLR6 and TLR4. While the TLR6 polymorphism, 359T>C is associated with an elevated risk of Legionnaires' disease which is further enhanced by smoking (Misch et al., 2013), certain TLR4 alleles are associated with protection (Hawn et al., 2005).

Patients diagnosed with Legionnaires' disease have increased serum levels of interferon γ and IL-12, indicative of a Th1-type response (Tateda et al., 1998). *In vitro*, *L. pneumophila* infection assays of human monocytes and macrophages show that a Th1-type cytokine response ultimately inhibits *L. pneumophila* replication (Bhardwaj et al., 1986; Matsiota-Bernard et al., 1993; Nash et al., 1984).

T cells are also believed to be important for clearance of bacteria during infection given that depletion of CD4 and CD8 T cells results in a decreased survival rate for mice infected with *L. pneumophila* compared to non-treated mice (Susa et al., 1998). However, in humans the contribution of T-cells is not completely established as people with low CD4 T-cells counts, such as those infected with HIV, are not necessarily more susceptible to *Legionella* infection. Generally immune compromised people, such as those receiving immunosuppressive therapies, including anti-cancer chemotherapies, are at greater risk of serious infection (Ginevra et al., 2009).

There is no vaccine currently available for the prevention of legionellosis. However, several studies have demonstrated protective immunity in animal models using live avirulent bacteria, *Legionella* major secretory proteins, membrane fragments and flagellin (Blander et al., 1989; Blander and Horwitz, 1991; Blander and Horwitz, 1993; Ricci et al., 2005)

1.6. Clinical Features

The incubation period for Legionnaires' disease is from two to fourteen days. Patients with Legionnaires' disease commonly develop muscle aches, fever, chills, and a cough. As the illness progresses, pneumonia and severe respiratory distress develop. *Legionella* may disseminate to other organs in the body via the blood stream and lymphatic system and so non-pulmonary symptoms may arise. This leads to some patients experiencing headache, diarrhoea, tiredness, confusion and loss of appetite (Cunha, 2010; Ginevra et al., 2009; Jespersen et al., 2010). Pontiac fever on the other hand is an acute self-limiting illness that causes flu-like symptoms (fever, chills and malaise) without pneumonia. Incubation time is twenty four to forty eight hours.

The majority of patients presenting with legionellosis are elderly males who may be smokers, immunosuppressed and/or patients with a pre-existing illness. The major

cause of death in patients with Legionnaires' disease is respiratory failure. Many patients display abnormal chest x-rays, although an absence does not exclude *Legionella* infection (Jespersen et al., 2010). A comparison of pneumonia patients with Legionnaires' disease compared to non-Legionnaires' disease found that patients with Legionnaires' disease displayed an increased prevalence of central nervous system symptoms (headache, confusion and drowsiness) and diarrhoea in comparison to the non-Legionnaires' disease patients at the time of admission to hospital (Hugosson et al., 2007). Legionnaires' disease patients also displayed a higher fever and raised C-reactive protein levels. Hyponatraemia and elevated liver enzymes were also more frequent at the time of admission.

1.7. Diagnosis

Legionnaires' disease is difficult to diagnose without further testing, as symptoms are often identical to many other forms of pneumonia. Elevated erythrocyte sedimentation rates of over 90 mm/h have been shown to distinguish Legionnaires' disease from viral pneumonias (Cunha et al., 2010). Various abnormalities may be produced such as haematuria, hypophosphataemia, thrombocytopaenia, hyponatraemia and abnormal liver function tests. The absence of these symptoms does not exclude *Legionella* infection and so the use of laboratory-based diagnostic methods is important for early detection of legionellosis.

The *Legionella* urinary antigen test is a simple and rapid test now used by many diagnostic laboratories to detect antigens of *L. pneumophila* serogroup 1 (the most common causative agent of Legionnaires' disease worldwide) in urine. This test detects the presence of the *L. pneumophila* serogroup 1 antigen, present in urine during infection, using an enzyme immunoassay. It is highly specific for *L. pneumophila* serogroup 1 and so where possible is coupled with bacterial culture from respiratory secretions or pleural fluid to give a more definitive diagnosis of legionellosis. Although the urine antigen test will identify the most common cause of Legionnaires' disease, it

is sometimes used in conjunction with other diagnostic methods particularly in countries where non-*L. pneumophila* species are prevalent. For example, *L. longbeachae* is a common cause of legionellosis in Australia, Thailand and New Zealand and this organism will not be identified by the urine antigen test (Phares et al., 2007; Yu et al., 2002).

In vitro bacteriological culture of *L. pneumophila* requires specialised media, as the bacteria are fastidious in their growth requirements. Bacteria are cultured on buffered charcoal yeast extract (BCYE) media (Feeley et al., 1979) in the presence and absence of L-cysteine to determine if they belong to the genus *Legionella*. L-cysteine is needed for the growth of *Legionella* species and provides a mechanism to distinguish *Legionella* species from other bacterial genera in the same sample. *Legionella* spp. produce characteristic branched-chain fatty acids in their cell wall, giving them a distinct morphology helping to distinguish them from similar bacteria (Diogo et al., 1999). Although culture is specific for *Legionella*, sensitivity varies likely due to varying levels of bacteria present in patient samples. Culture of *Legionella* takes at least 3-5 days to obtain positive results (Jarraud et al., 2013).

Other diagnostic methods include serology, latex agglutination assays, direct immunofluorescence assays and various polymerase chain reaction (PCR) detection methods. Some rely on previous isolation of the strain by culture whereas other may be applied directly to a patient sample. Serology may be used to detect the presence of *Legionella* antibodies in patient serum. While being highly specific, sensitivity varies and it is not often used in clinical settings as results take several weeks to obtain due to the amount of time needed for seroconversion (Jarraud et al., 2013).

Direct fluorescence assays involve the staining of patient samples with fluorescent antibodies specific for individual *Legionella* strains. Samples are analysed using fluorescence microscopy to detect the presence of *Legionella*. This assay may be completed in less than 4 hours and is highly specific. Again, sensitivity varies likely due

to the number of bacteria present in the patient sample but also due to the skill of the laboratory staff performing the test as it is quite technically demanding (Murdoch, 2003; Sethi et al., 2007; She et al., 2007).

PCR is also used for rapid detection of *Legionella* DNA in patient samples and can be a highly effective diagnostic tool. PCR amplifies target regions of DNA and can be used to detect specific genes in samples. As *Legionella*-specific genes are targeted this test is highly specific with varying levels of sensitivity depending on the sample used (i.e. lower respiratory tract secretions are generally more reliable than serum or urine samples) (Benitez and Winchell, 2013; Diederen et al., 2008; Zhou et al., 2011). Quantitative real-time polymerase chain reaction (qPCR) has also been developed for rapid identification of *Legionella* in patient samples. One system targeting the macrophage infectivity potentiator (*mip*) gene of *L. pneumophila* demonstrated a degree of specificity. The test was able to positively identify all *L. pneumophila* subspecies and 16 serogroups tested (39/39 strains) with no cross-reaction from non-*pneumophila* strains (0/69 strains) and non-*Legionella* strains (0/58 strains). Culture positive patient samples were tested using this system and were also all found to be qPCR positive (81/81). Culture negative samples were also tested and 47/80 samples were found to be positive, indicating qPCR was the more sensitive method (Mentasti et al., 2012). With the wealth of genomic information now available, it is now possible to design rapid PCR tests to identify *Legionella* species and distinguish one species and even serogroup from another (Yong et al., 2010).

Multilocus sequence typing (MLST) uses PCR amplification and DNA sequencing of several specific genetic loci to identify distinct bacterial species. This sequence based typing can be highly sensitive and discriminatory in epidemiological typing of *Legionella* (Gaia et al., 2003). The cost and technical nature of the process may limit its widespread use in diagnostics. Diagnostic testing of clinical samples for *Legionella* has been extensively outlined in a recent methods handbook (Jarraud et al., 2013).

1.8. Treatment

Pontiac fever is a mild self-limited illness that is not treated with antibiotics. Supportive therapy may be used to treat symptoms if required but complete recovery will usually occur within one week (Glick et al., 1978; Remen et al., 2011). Legionnaires' disease can be treated successfully with antibiotics and healthy individuals usually recover from infection without complication, although recovery time varies and may take several weeks. β-lactam antibiotics such as penicillin are ineffective as they are unable to penetrate macrophages and most *Legionella* isolates produce β-lactamases (Marre et al., 1982). Instead, Levofloxacin (or other fluoroquinolones) or azithromycin are commonly used in the treatment of Legionnaires' disease (Reviewed in (Arora, 2012; Cunha, 2010)). Levofloxacin is a broad-spectrum antibiotic that inhibits the function of prokaryotic DNA gyrase and topoisomerase IV, preventing cell division. Azithromycin inhibits bacterial protein synthesis by binding the 50s ribosomal subunit thereby inhibiting the translation of mRNA into peptides. Azithromycin was found to be well tolerated and was effective in treating patients with community acquired Legionnaires' disease. The overall cure rate 10-14 days after therapy was 95% and 96% 4-6 weeks after therapy (Plouffe et al., 2003).

Combined antibiotic therapy can be used in severe unresponsive cases. Although there are few comprehensive studies looking at combined therapy, it was found that combined therapy reduced the ICU mortality rates in patients with severe community-acquired pneumonia with shock caused by *L. pneumophila* in comparison to monotherepy (Rello et al., 2013). Care must be taken when administering combined therapy as there is the risk of additional toxicity and drug interactions.

Antibiotic therapy should be administered intravenously for three to five days or until clinical stability is reached and then may be substituted with oral antibiotic therapy. Longer therapy duration of up to three weeks may be required for immunosuppressed individuals and patients with advanced disease (Arora, 2012; Cunha, 2010). Prognosis

of Legionnaires' disease depends largely on the patient's cardiopulmonary and immune function as well as the initial number of bacteria the patient was infected with and also the early administration of effective antibiotic therapy. Fatalities most often occur in individuals with a compromised immune system, prior respiratory conditions or in patients presenting with advanced disease prior to treatment. However if cardiopulmonary function is good, early treatment of Legionnaires' disease, even in compromised individuals, delivers a good prognosis.

1.9. Prevention and Control

There is no current commercially available vaccine for *Legionella* infection so control of the bacteria in the environment is extremely important for preventing disease. The key to the prevention of legionellosis is in the proper maintenance of water supply systems at risk. *Legionella* becomes a problem when the bacteria are allowed to grow to high numbers. This can be avoided with suitable maintenance, including routine cleaning and disinfection procedures as well as appropriate testing to ensure effective microbiological control (Kozak et al., 2013). Testing for the presence of *Legionella* has led to the identification of previously undetected potential sources of infection. The current gold standard for testing is bacteriological culture, which can take up to 10 days to deliver a result. This lengthy period is a problem in outbreak investigations where there is an urgent need for a more rapid test for environmental samples. Molecular based testing such as PCR is ineffective, as the test cannot reliably distinguish live from dead bacteria (Keer and Birch, 2003). The detection of live bacteria prompts intervention to prevent any (or additional) cases of legionellosis. In most countries legionellosis is a notifiable disease to the relevant national health authority. The surveillance and reporting of clinical legionellosis cases is extremely important for quick identification and control of outbreaks.

After detection, *Legionella* may be removed from aquatic systems via hyperchlorination treatment and superheating water above 70°C. Several other

eradication strategies exist including other chemical treatments and the continuous copper-silver ionization of water supply systems (Arora, 2012; Carson, 2010; Dupuy et al., 2011; Lin et al., 2011; Marchesi et al., 2012). Due to the exploitation by *Legionella* of environmental amoeba species as replicative and protective hosts, amoebicidal agents may also be considered in the control of legionellosis (Dupuy et al., 2011). Devices that continuously distribute chlorine dioxide into water supply systems have been used to control microbial contamination. However the use of a monochloramine alternative has proved more effective. A recent study found that over a one-year period a reduction in the number of *Legionella*-contaminated sites decreased from 97.0% to 13.3% with the use of monochloramine in comparison to a reduction of 100% to 56.7-60.8% with the use of chlorine dioxide devices (Marchesi et al., 2012).

To prevent the risk of human exposure to bacteria in water supply systems, conditions that produce aerosols and promote bacterial growth are minimised in modern heating systems. These controls may be achieved through the use of adiabatic cooling systems (occuring without transfer of heat or matter between a system and its surroundings), dry cooling plants, point of use heaters and routine disinfection protocols. Bacterial growth may also be reduced by avoiding temperatures between 20-45°C, designing pipework to prevent water stagnation and minimising organic contaminants (Reviewed in Fields et al., 2002; Carson, 2010).

Testing of water systems for the presence of *Legionella* is generally carried out at regular intervals to verify the maintenance protocols implemented are working effectively. If chemical disinfectants are used, concentrations are usually tested at multiple points in the system to evaluate effectiveness. It is clear that properly maintained systems reduce the risks of Legionnaires' disease (Dupuy et al., 2011; Reviewed in Fields et al., 2002; Flannery et al., 2006). Ideally, maintenance protocols for water supply are designed in conjunction with infection control practitioners to ensure the appropriate disinfection methods are implemented.

1.10. Aim of this study

Legionnaires' disease remains a significant public health concern, as it is impossible to eradicate the bacteria from the environment. Even with adequate monitoring and surveillance, sporadic cases and unexpected outbreaks still occur. Rapid diagnosis and early intervention with antimicrobial treatment is critical to achieving the best outcome for patients and the design of water supply systems, particularly in hospitals, needs to take *Legionella* infection risk into account. Independently of its importance as a human pathogen, *Legionella* has increased our understanding of many new and important aspects of cell biology and immunity, including mechanisms of vesicle trafficking and membrane fusion as well as inflammasome activation. Despite these recent advances in our knowledge of *L. pneumophila* host-pathogen interactions, many aspects of *L. pneumophila* intracellular survival and replication are still not well defined. Individually, Dot/Icm effector proteins are not essential for intracellular growth, with single effector mutants still replicating at equivalent levels to wild-type bacteria. This functional redundancy likely indicates that additional unidentified effector proteins are important and act in a complementary fashion to other effector proteins. It is clear that there is still a great deal to learn about the intracellular survival and replication mechanisms of *Legionella* that leads to Legionnaires' disease.

The broad aim of this study was to identify novel virulence genes of *L. pneumophila* that contribute to intracellular replication primarily using a transposon based genetic screen in amoebae and to determine the role of these genes in *Legionella* intracellular replication and survival.

Chapter 2: Materials and methods

2.1. Chemicals and reagents

All chemicals and reagents used in this study were of laboratory or analytical grade. Unless stated otherwise, chemicals and reagents were purchased from Sigma-Aldrich (USA), Merck (Germany), Chem Supply (Australia), Amresco (USA), BDH Chemicals (Australia), or Promega (USA). Media components were obtained from Invitrogen (USA), Oxoid (Cambridge,UK) or Sigma-Aldrich (USA), and antibiotics from Amresco (USA), Invitrogen (USA) or Boehringer Ingelheim (Germany). Tissue culture media components were obtained from Invitrogen (USA).

2.2. Strains and plasmids

Bacterial strains used in this study are listed in Table 2.1. Plasmids and primers used in this study are listed in Table 2.2. and Table 2.3. respectively.

E. coli cultures were grown in Luria-Bertani (LB) broth or on LB agar. Unless stated otherwise, broth cultures were incubated aerobically at 37 °C with shaking (180 rpm) for 24 h. Solid cultures were incubated aerobically overnight at 37 °C for 24 h. For storage at -80 °C, 600 µL of stationary phase *E. coli* LB broth culture was added to 300 µL of 50% glycerol solution.

Legionella strains were grown in ACES (N-(2-acetamido)-2-aminoethanesulfonic acid) buffered yeast extract (AYE) broth or on buffered charcoal yeast extract (BCYE) agar (Feeley et al., 1979). Unless stated otherwise, broth cultures were incubated aerobically at 37 °C with shaking for 24 h. Solid cultures were incubated aerobically for 72 h at 37

°C. For storage at -80 °C, *Legionella* was grown on BCYE agar plates and resuspended in 1.5 mL of 50% glycerol broth (3.7% Brain-heart infusion broth (Sigma-Aldrich), 50% glycerol).

When necessary, ampicillin, kanamycin and chloramphenicol were added to BCYE agar or ACES broth at 100 µg/mL, 25 µg/mL, and 6 µg/mL, respectively; and to LB broth or agar at 100 µg/mL, 100 µg/mL, and 12.5 µg/mL, respectively.

2.3. Culture and maintenance of *A. castellanii* and mammalian cell lines

A. castellanii ATCC50739 was routinely grown in PYG 712 media (2% (w/v) tryptone, 0.1% (w/v) yeast extract, 0.1% (w/v) trisodium citrate, 0.1 M glucose, 0.4 mM CaCl2, 2.5 mM KH2PO4, 4 mM MgSO4, 2.5 mM Na2HPO4, 0.05 mM ferric pyrophosphate, pH 6.9) at 25 °C in 75 cm^2 tissue culture flasks (Starstedt, Germany). Amoebae were passaged every three to four days into fresh media. All cell lines were obtained from the American Tissue Culture Collection and subjected to minimal passaging.

HEK293T cells were maintained in Gibco DMEM-High Glucose media (Invitrogen) with 10% foetal bovine serum (FBS), 2 mM L-glutamine (Invitrogen), 100 units/mL penicillin and 100 µg/mL streptomycin and incubated at 37°C and 5% CO_2 in 75 cm^2 tissue culture flasks (Starstedt). HEK293T cells were passaged every three to four days into fresh media.

THP-1 cells were maintained in RPMI 1640 (Invitrogen) media supplemented with 2 mM L-Glutamine (Invitrogen), 10% foetal bovine serum (heat inactivated), 100 U/mL penicillin (Invitrogen) and 100 µg/mL streptomycin (Invitrogen) at 37 °C, 5% CO_2 in 75 cm^2 tissue culture flasks (Starstedt). THP-1 cells were passaged every three to four days into fresh media.

Immortalised mouse bone marrow-derived macrophages from C57BL/6 mice, generated via J2 viral transformation (Blasi et al., 1989), were maintained in Gibco DMEM-High Glucose media (Invitrogen) with 10% foetal bovine serum (FBS), 2 mM L-glutamine (Invitrogen), 100 units/mL penicillin and 100 µg/mL streptomycin in 75 cm^2 tissue culture flasks (Starstedt) and incubated at 37°C and 5% CO_2. B6 BMDM cells were passaged every three to four days into fresh media.

HeLa cells were maintained in Gibco DMEM-High Glucose media (Invitrogen) with 10% foetal bovine serum (FBS), 2 mM L-glutamine (Invitrogen), 100 units/mL penicillin and 100 µg/mL streptomycin in 75 cm^2 tissue culture flasks (Starstedt) and incubated at 37°C and 5% CO_2. HeLa cells were passaged every three to four days into fresh media.

2.4. General DNA techniques

2.4.1. Isolation and purification of genomic DNA

Legionella strains were grown on BCYE agar plates for three days and resuspended in PBS (0.08% (w/v) NaCl, 0.002% (w/v) KCl, 0.0144% (w/v) Na2HPO4, 0.0024% (w/v) KH2PO4, pH 7.4). Genomic DNA was extracted using a DNeasy Blood and Tissue kit (Qiagen) as per the manufacturer's instructions.

2.4.2. Oligonucleotide synthesis

Oligonucleotides used for PCR amplification and sequencing are listed in Table 2.2. All oligonucleotide primers used in this study were designed from *L. pneumophila* genome sequence data (Philadelphia-1 and 130b strains) and synthesised by Sigma®Genosys (Australia), diluted to 100 ng/µL in dH20 and stored at minus 20 °C.

2.4.3. DNA-modifying enzymes

Restriction endonucleases (Roche (USA) and New England Biolabs Inc. (USA)) were used to digest DNA as per the manufacturer's guidelines. Restriction endonucleases

were inactivated by incubation at 65 °C for 15 min or via DNA purification using the Perfectprep Gel Cleanup Kit (Eppendorf, Germany).

Ligations of digested genomic DNA were performed using T4 DNA Ligase (Promega, USA) as per the manufacturer's instructions. When required, the T4 DNA ligase was inactivated via incubation at 65 °C for 10 minutes.

2.4.4. Plasmid construction and sequencing

Plasmids used and constructed in this study are listed in Table 2.3. Plasmid DNA was purified using the Qiaprep Spin Miniprep Kit (Qiagen, Germany) as per the manufacturer's instructions and plasmid concentration was measured using a NanoDrop 1000 Spectrophotometer with Nanodrop 1000 program version 3.7.1 software. Plasmid DNA was prepared for nucleotide sequencing using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Australia) in a Px2 Thermal Cycler (Integrated Sciences, Australia) as per the manufacturer's specifications and automated DNA sequencing was performed with an Applied Biosystems 3730S Genetic analyser by Micromon (Monash University, Australia). Sequencher 4.7 software (Gene Codes Corp., USA) was used to analyse the subsequent DNA sequences.

2.5. Polymerase Chain Reaction (PCR)

PCR amplification was performed using a Px2 Thermal Cycler (Integrated Sciences, Australia) with 0.25 µg of each primer and approximately 200 ng of template DNA or a single small bacterial colony per 25 µL reaction. Reaction conditions were a standard cycle including a denaturing step at 95 °C for 30 sec, an annealing step for 30 sec (with annealing temperatures adjusted for the oligonucleotide melting temperatures) and an extension step at 72 °C (one min for every 1000 bp of PCR product). This cycle was repeated 30 times. PCR enzymes used include PCR Extender System (5 Prime,

Germany) and Biotaq DNA polymerase (Bioline, Australia) according to manufacturer's specifications.

2.6. Agarose Gel Electrophoresis

Agarose gel electrophoresis was utilized for separation and analysis of DNA fragments. 1% (w/v) Agarose gel (Molecular Biology Grade Agarose, Scientifix, Australia) was made up in TAE buffer (40 mM Tris, 1 mM EDTA disodium salt, 0.114% glacial acetic acid (v/v), pH 8.3) containing 0.01% (v/v) SYBR Safe DNA gel stain (Invitrogen). DNA samples were combined with 6X Bromophenol Blue loading dye (0.25% (w/v) Bromophenol blue, 30% (w/v) sucrose, 100 mM EDTA) before loading onto the gel. 500 ng of Lambda phage DNA (Promega) digested with HindIII or Quick-Load 1 kb DNA ladder (New England Biolabs) was used to provide a standard for band size and concentration. Electrophoresis of gels was performed in TAE buffer at 100 V for 20-30 mins. Gels were photographed using GeneSnap 7.07.01 with a G:BOX HR Gel Documentation System (Syngene, UK) using an ultraviolet transilluminator. DNA fragments were excised from the gel and purified using the Perfectprep Gel Cleanup Kit (Eppendorf).

2.7. Sequencing the genome of *L. pneumophila* strain 130b

Chromosomal DNA was prepared for sequencing by proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation (Thomson et al., 2008). The whole genome of 130b was sequenced at the Wellcome Trust Sanger Institute using paired-end 454 FLX pyrosequencing and assembled using the 454/Roche Newbler assembly program into 274 contigs (N50 contig size, 35,584 bp) from 248,625 sequence reads with an average read length of 157 bp. The contigs were scaffolded using paired reads, with an average pair distance of 3,028 bp, into 11 scaffolds (N50 scaffold size, 2,421,541 bp). The *lvh* collapsed repeat region was reassembled into two distinct *lvh*

regions by first separating reads that mapped to the previously sequenced 130b lvh region (accession no. AF410854), differentiating *lvh* reads according to microheterogeneity (single-nucleotide polymorphism [SNP] content), and then using Newbler (with stringent cutoffs) to generate individual *lvh* assemblies that could be unambiguously positioned within a scaffold in the main assembly. Several contig gaps were closed using PCR and Sanger sequencing in conjunction with manual examination of the individual 454 reads in Consed (Gordon et al., 1998). Unassembled contigs of less than 300 bp were removed from the end of the assembly. The 130b genome was aligned with the other sequenced *L. pneumophila* genomes to aid whole-genome comparisons, making the first gene *dnaA*. An automated annotation was performed on the genome sequence using SUGAR, as previously described (Wilkinson et al., 2010). Artemis (Rutherford et al., 2000) was used to facilitate the manual curation of the sequence and annotation of the effectors and T4SSs. Bioinformatic analysis of domains and motifs of individual effector protein candidates was performed using the Pfam database (Pfam release 24, HMMER3.0 beta 3 (Finn et al., 2008), SMART (version 6 (Letunic et al., 2009)), and the NCBI Conserved Domain Database (version 2.18 (Marchler-Bauer et al., 2009))

2.8. Novel *L. pneumophila* 130b effector prevalence screen

Genomic DNA for 54 clinical and environmental *L. pneumophila* isolates was obtained from the Respiratory and Systemic Infection Laboratory, Health Protection Agency Centre for Infection, London, United Kingdom. These isolates were obtained from a range of locations across the United Kingdom and were previously characterized by serogroup, monoclonal antibody (MAb) subgroup, allelic profile, and sequence type (Harrison et al., 2009; Helbig et al., 2002; Ratzow et al., 2007). Thirty-three isolates were obtained from the Microbiological Diagnostic Unit at the University of Melbourne, as described previously (Newton et al., 2006), and from the collection of Stacey Yong, Taylor's University College, Malaysia. The PCR primers were designed to

amplify 400- to 700-bp sequences of the putative effector genes and controls. PCR screening using standard conditions was performed three times, and the results were analysed by agarose gel electrophoresis. PCR-negative strains from the Australian and Malaysian collections were, in addition, confirmed by Southern hybridization.

2.9. Southern blot hybridization

0.3-3 µg of digested genomic DNA was separated by agarose gel electrophoresis. A Digoxigenin (DIG) Labelled DNA Molecular-Weight Marker II (Roche) was used to provide a standard for band size and concentration. The gel was depurinated with 250 mM Hydrochloric acid for 10 min at room temperature with shaking. The gel was rinsed with H_20 and submerged in denaturation solution (0.5M NaOH, 1.5M NaCl and 0.1% w/v Sodiumdodecylsulphate) twice for 15 min at room temperature with shaking. The gel was rinsed with H_20 and submerged in neutralization solution (1M Tris and 1.5M NaCl. pH to 7.4 with HCl) twice for 15min at room temperature with shaking. DNA was transferred from the gel to a Biodyne nylon membrane (Pall, Germany) in a container of 20 x SSC (3M NaCl and 300mM Sodium Citrate. pH to 7 with NaOH or HCl) overnight at room temperature.

A Spectrolinker XL-1000 UV cross-linker (Spectronics Corporation, USA.) was used to crosslink DNA to the membrane. The membrane was submerged in prehybridization solution (5 x SSC, 0.1% w/v N-lauroylsarcosine, 0.02% w/v SDS and 5% skim milk powder) for 2 h at 68 °C. 20 µL of the DNA probe (Table 2.3.), made via PCR using 50% dNTPs and 50% DIG DNA labelling mix (Roche), was added to the prehybridization solution and hybridization was achieved overnight at 68°C with shaking. The membrane was washed with 2 x SSC, 0.1% SodiumDodecylSulphate (SDS) (w/v) for 2 x 5min at room temperature and then with 0.5 x SSC, 0.1% SDS (w/v) for 2 x 15min at 68 °C. The membrane was equilibrated with maleic acid buffer (0.1 M maleic acid and 0.15 M NaCl. pH to 7.5 with NaOH) for 1 min a room temperature and blocked with

maleic acid buffer, 5% (w/v) skim milk powder for 45 min at room temperature. An Anti-Digoxigenin-AP antibody (1 µL/mL) (Roche) was added to the membrane for 30min at room temperature. The membrane was washed with maleic acid wash buffer (0.1 M maleic acid, 0.15 M NaCl and 0.3% v/v Tween 20. pH to 7.5 with NaOH) for 2 x 15 min at room temp and equilibrated with 1 x detection buffer (1M Tris and 1M NaCl used to make 10x solution. pH to 9.5 with HCl) for 1 min at room temperature with. 5 µL of CDP-star (Roche) was mixed with 0.5mL of 1 x detection buffer and this was added to the membrane. The membrane was placed between two transparent, plastic sheets and luminescence detected with a Kodak image station 4000 mm (Kodak, USA).

2.10. Preparation and electroporation of electrocompetent *L.pneumophila*

L. pneumophila strains were grown on BCYE plates for three days at 37 °C. A wire loop was used to resuspend three to four loops of bacteria in 1 ml of 20 mM $MgCl_2$, 2% sucrose at 4 °C and OD 600nm was adjusted to approximately 2. The cells were pelleted by centrifugation (10 min, 5000 x g, 4 °C) and the pellet was washed with 1 ml of 2% sucrose at 4 °C and resuspended in 1 ml of 2% sucrose at 4 °C.

200 µL of electrocompetent bacteria was added to 10 ng of plasmid DNA or 1 µg of plasmid DNA for pC6 and transferred to 0.2 cm Gene Pulser electroporation cuvettes (Bio-Rad) on ice. Cuvettes were subjected to an electric pulse using a Bio-Rad MicroPulser (Bio-Rad) at 2.4 kV at 200 Ω and 25 µF and cells were resuspended in 1 ml of ACES broth. Cells were recovered aerobically at 37 °C for 5 h before plating on to BCYE agar plates supplemented with the appropriate antibiotics to select for transformants.

2.11. Construction of *L. pneumophila* transposon mutant library and the amoebae plate test

A *L. pneumophila* transposon mutant library was created by transforming *L. pneumophila* strain 130b with the plasmid pC6 via electroporation, as described previously, introducing the IS903-derived transposon randomly into the *L. pneumophila* genome. Transposon mutants were selected for by plating on BCYE agar plates containing 25 µg/mL kanamycin.

The amoebae plate test was adapted for screening from (Albers et al., 2005). A single colony of bacteria was added to 150 µL of ACES broth per well of a U-bottom 96-well plate. *L. pneumophila pMMB207:kanR* was added to the first row of the 96-well plate. A *L. pneumophila dotA* deletion mutant was added to the second row. Single colonies of *L. pneumophila* IS903 transposon mutants were added to the remaining wells. The 96-well plate was incubated overnight at 37 °C. BCYE agar plates containing 25 µg/mL kanamycin were spread with 4 x 10^6 *A. castellanii* cells and incubated overnight at 25 °C.

A 10x serial dilution of each column was performed six times in a separate 96-well plate using PBS. These dilutions were replica-plated on BCYE agar plates containing 25 µg/mL kanamycin and *A. castellanii*, BCYE agar plates containing 25 µg/mL kanamycin and BCYE agar plates containing 100 µg/mL ampicillin. Plates were incubated for three days at 37 °C.

A second Amoebae Plate Test was performed on transposon mutant strains that tested positive in the first Amoebae Plate Test where bacteria were grown overnight at 37 °C in 5 ml of ACES broth. Bacterial cultures were adjusted to an identical OD_{600nm} and then diluted and replica-plated as described above.

2.12. *L. pneumophila* and *A. castellanii* liquid co-culture assay

A. castellanii was cultured in PYG medium at 25 °C for 72 h prior to harvesting and seeding into 24-well tissue culture trays (Starstedt) in PYG media at a density of 2.5 x 10^5 cells/well. Stationary phase *L. pneumophila* resuspended in PBS was added at a multiplicity of infection (MOI) of 0.01 to the amoebae and trays were incubated at 37 °C, 5% CO_2. At the specified time points, the entire contents of each well was collected and appropriate dilutions were plated onto two BCYE agar plates containing 25 µg/mL kanamycin for viable counts *of L. pneumophila*. Each strain tested was added to duplicate wells and results are expressed as the mean log_{10}CFU, ± standard error of the mean, of at least three independent experiments. An unpaired, 2-tailed t-test was used to determine statistically significant differences ($P<0.05$) between infections at each time point.

2.13. *A. castellanii* co-culture plate reader assay

An *A. castellanii* intracellular replication assay was performed as described in (Coil et al., 2008) with the following changes. The assay was performed in a Fluostar Omega microplate reader (BMG Labtech) in 24-well tissue culture plates (Thermo Scientific). OD 600nm was measured over 70 h at 5 h intervals. Each strain was tested in duplicate in at least three independent assays. An unpaired, 2-tailed t-test was used to determine statistically significant differences ($P<0.05$) between infections at each time point.

2.14. Y-linker formation and identification of transposon insertion site

Oligonucleotides 1672 and 1683 (Table 2.3.) were used to form the Y-linker. 3.15 µg of Y-Linker 2 was phosphorylated at the 5’ end with T4 polynucleotide kinase (New

England Biolabs) as per the manufacturer's instructions. The T4 polynucleotide kinase was inactivated at 65 °C for 20 min and 9 µL of Y Linker 1 (350 ng/µL) was added. The sample was heated to 95 °C for 2 min and then slowly allowed to cool down to room temperature, forming the Y-linker. gDNA of strains containing the IS903 derived transposon was digested with *Sau*3A and the resulting DNA fragments were subsequently ligated to the Y-linker. PCR with primers 1670 and 1671 was used to amplify transposon flanking regions which were then sequenced to identify the location of the transposon in the strain.

2.15. Preparation and heat shock of chemically competent *E. coli*

Chemically competent *E. coli* XL-1 Blue cells were produced as described by Inoue et al (195). A single *E. coli* colony was inoculated into 5 ml of LB broth and cultivated overnight at 37°C. 1 ml of overnight culture was added to 100 ml of SOB (2% tryptone, 0.5% yeast extract, 10 mM NaCl and 2.5 mM KCl in dH_20) and 1 ml of 2M Mg2+ and cultivated at 18 °C for two days or until OD_{600nm} reaches approximately 0.45. Cells were chilled on ice for 10 min and harvested by centrifugation in a Sorvall RC-5C centrifuge at 1000 x g for 15 min at 4 °C. Cells were resuspended in 40 ml of chilled Transformation buffer (10 mM PIPES, 15 mM $CaCl_2.2H_2O$, 250 mM KCl, 55 mM $MnCl_2.4H_2O$, pH 6.7) and placed on ice for 15 min. Cells were once again harvested by centrifugation at 1000 x g for 15 min at 4 °C and resuspended in 4 ml of Transformation buffer and 300 µL of Dimethyl sulfoxide (DMSO). 100 µL aliquots were snap-frozen in a dry ice and 80 % ethanol bath before being stored at minus 70 °C

Aliquots of chemically competent *E.coli* XL1-Blue were thawed on ice for 20 min and mixed with either plasmid DNA or a ligation reaction and incubated on ice for 20 min. Cells were subsequently heat shocked for 1 min at 42 °C and incubated on ice for 5 min. 1 ml of LB was added to each sample and cells were recovered aerobically at 37 °C for 1 h. Cells were plated onto LB agar plates containing the appropriate antibiotics.

2.16. Blue/white selection of E. coli XL1- Blue containing pGEM- T Easy

DNA fragments to be sequenced were ligated into the pGEM-T Easy vector and transformed into *E. coli* XL1-Blue. Bacteria were plated onto LB agar plates containing 100 µg/mL ampicillin, 4 µL of 1 M IPTG and 40 µL of 1 M X-gal. Blue colonies expressing β-galactosidase possess a plasmid with no insert. White colonies will possess pGEM-T Easy with an insert and were patched onto LB agar plates containing 100 µg/mL ampicillin. PCR amplification was used to confirm the presence and size of the insert and the insert of positive strains was sequenced as described above using primers 4420 and 4421 (Table 2.3.).

2.17. *L. pneumophila* and THP-1 cell liquid co-culture assay

48 h prior to infection, THP-1 cells in RPMI media were seeded into 24-well tissue culture trays at a density of 5 x 10^5 cells/well. Differentiation into adherent, macrophage-like cells, was achieved by pre-treating cells with 10^{-8} M phorbol 12-myristate 13-acetate (PMA) and incubated at 37 °C, 5% CO_2 for 48 h.

Each well was subsequently washed three times with warm PBS and 500µL of warm RPMI assay media (RMPI 1640, 10% foetal bovine serum, 2 mM L-Glutamine) was added to each well. Stationary phase *L. pneumophila* strains in RPMI assay media were used to infect cells at a MOI of 5 and trays were incubated for two hours at 37 °C, 5% CO_2. Viable counts for the bacteria from the inocula were spread onto BCYE agar with appropriate dilutions in PBS. Cells were washed once in warm RPMI assay media and 100 µg/ml gentamicin (Invitrogen) was added to kill extracellular bacteria (Newton et al., 2006; Newton et al., 2007; Sansom et al., 2007). Trays were incubated for 1 h at 37 °C, 5% CO_2 and then washed three times with warm PBS. 500 µL of warm RPMI assay media was added to each well and trays were incubated at 37 °C, 5% CO_2. At each time point cell supernatant was collected and THP-1 cells were washed three times with

PBS. Cells were lysed by treatment with 200 µL of 0.05% Digitonin (Sigma-Aldrich) in PBS. Cell lysate was pooled with cellular supernatant and bacterial numbers enumerated by plating onto BCYE agar with appropriate dilutions in PBS. For assessment of bacterial uptake, THP-1 lysis and bacterial recovery were performed immediately following gentamicin treatment and washing. Results are expressed as the mean ± standard error of the mean $\log_{10}$CFU of at least two independent experiments with each strain in duplicate wells.

2.18. Infection of HEK293T cells with *L. pneumophila* strains

3 days prior to infection, *L. pneumophila* strains were plated on BCYE agar with appropriate antibiotics and incubated at 37 °C. 10^5 HEK293T cells in DMEM were seeded onto 12 mm round coverslips in 24-well tissue culture plates with duplicate wells prepared for each sample. Plates were incubated overnight at 37 °C and 5% CO_2. Media was removed before DMEM at 37 °C with 1 mM IPTG was added to each well. Wells were infected with *L. pneumophila* strains at an MOI of 100 and incubated at 37 ° C and 5% CO_2 for 2 h. Media was aspirated and cells were washed twice with DMEM to remove extracellular bacteria. DMEM at 37 °C with 1 mM IPTG was added to each well and plates were incubated at 37 °C and 5% CO_2.

2.19. Construction and complementation of defined mutants

L. pneumophila deletion mutants were created by amplifying gene-flanking regions via PCR. PCR primers were constructed so that an EcoRI restriction enzyme site was created in between the two fragments when they were combined during a successive PCR. The resulting fragment was ligated into the *L. pneumophila* suicide vector pPCRScript and a kanamycin resistance cassette from pUC4-KISS was ligated into the

EcoRI site. The plasmid was introduced into *L. pneumophila* 130b strain by natural transformation (see 2.20.) with the gene being subsequently replaced with the kanamycin resistance cassette via allelic exchange.

Deletion mutants were complemented *in trans* by introducing full length genes from *L. pneumophila* 130b (including predicted promoter and ribosome binding site) on the *Legionella* expression vector pMIP (Newton et al., 2006). Full length genes were amplified by PCR with the primers used creating a BamHI/HindIII fragment that was cloned into pMIP. The resulting construct was introduced into *L. pneumophila* deletion mutant strains by electroporation (refer to 2.10).

2.20. Natural Transformation in *L. pneumophila*

L. pneumophila strains were cultured on BCYE agar for 3 days and resuspended in ACES broth at an OD_{600nm} of 0.3. 10 µg/ml of plasmid was added to 2 mL cultures and incubated stationary at 30 °C until $OD_{600nm} > 1.5$ (usually 1-2 weeks). When $OD_{600nm} > 1.5$ Cultures were plated onto BCYE agar with appropriate antibiotics and incubated at 37 °C for 3-4 days. Cultures derived from single colonies were stored at -80 °C for further analysis.

2.21. DNA microarray analysis.

L. pneumophila 130b strain and the *L. pneumophila* 130b *lpw27511* deletion mutant were grown in ACES broth at 37 °C and harvested for RNA isolation at the exponential (OD_{600nm} at 1.5) or post-exponential (OD_{600nm} at 3.0) growth phase. RNA was reverse transcribed and indirectly labelled with Cy5 or Cy3 dye (Amersham Biosciences). A DNA microarray containing gene-specific 70-mer oligonucleotides based on all predicted genes of the genomes of *L. pneumophila* strains Paris, Lens, and Philadelphia was used,

and hybridizations were performed as described previously (Bruggemann et al., 2006). As controls, biological replicates as well as dye swap experiments were carried out. For normalization and differential analysis, the R software program (http://www.r-project.org/) was used. Loess normalization (Yang et al., 2002) was performed on a slide-by-slide basis, and differential analysis was carried out separately for each comparison, using the VM method (VarMixt package) (Delmar et al., 2005), together with the Benjamini and Yekutieli P value adjustment method (Reiner et al., 2003).

2.22. β-Lactamase TEM-1 translocation assay

2.22.1 *Construction of β-Lactamase TEM-1 Fusions*

RalF N-terminal translational fusions to TEM-1 β-lactamase lacking the signal peptide for secretion were generated in pXDC61 (Table 2.2.). The coding region, without the stop codon, of *L. pneumophila ralF* was amplified by PCR and an NdeI/EcoRI fragment was cloned into pXDC61. The construct was introduced by electroporation, as described previously, into *L. pneumophila*, a *dotA* deletion mutant and the *lpw27511* transposon mutant for β-Lactamase TEM-1 translocation assays.

2.22.2 *β-Lactamase TEM-1 translocation assay*

Translocation of RalF into immortalised B6 mouse bone marrow derived macrophages was assayed with a TEM-1 β-lactamase translocation assay (Charpentier and Oswald, 2004; de Felipe et al., 2008). Macrophages were propagated in DMEM containing 10% FCS and were seeded in 96-well plates at 1×10^6 cells/well 24 h before infection. *L. pneumophila*, a *dotA* deletion mutant and the *lpw27511* transposon mutant strains carrying N-terminal translational fusions of RalF to TEM-1 β-lactamase were grown in ACES broth containing 1 ug/mL IPTG before infection of B6 mouse bone marrow derived macrophages at a MOI of 30 for 3 h in 96-well tissue culture trays at 37 °C and

5% CO_2. After infection, cells were washed, loaded with CCF2/AM (Invitrogen) and incubated at 20 °C for an additional 2 h. β-Lactamase activity in infected cells was detected by measuring cleavage of the CCF2/AM substrate with a microplate reader and is recorded as a fluorescence emission ratio of 450/520 nm after excitation at 410 nm. Data presented are mean values of the results from triplicate wells from at least two independent experiments.

2.23. Sequencing of the *L. pneumophila lpw27511* transposon mutant genome

Whole-genome sequencing of the *L. pneumophila lpw27511* IS903-derived transposon mutant was performed using Ion Torrent semiconductor sequencing on the PGM platform. The genome was sequenced following the manufacturer's instructions (Life Technologies) with a single 316 chip with 100-bp or 200-bp chemistry. The resulting sequence was aligned, using Nesoni (Victorian Bioinformatics Consortium; http://vicbioinformatics.com/), to the *L. pneumophila* 130b strain reference genome. A genome-wide variant analysis was performed using Nesoni, and allelic variability at any nucleotide position in the transposon mutant relative to the 130b reference strain was tallied to generate a list of differences for the transposon mutant genome.

2.24. Quantitative real-time PCR

Overnight cultures of *L. pneumophila* strains were grown in ACES broth to an optical density at 600 nm of 0.6. 10 mL of culture was incubated with 20 ml of RNAprotect solution (Qiagen, USA) at room temperature for 10 min, after which cells were pelleted and RNA was purified by using a FastRNA pro blue kit (MP biomedical, USA) as per manufacturer's instructions. The samples were treated with DNase I (Qiagen) before further purification using an RNeasy MinElute kit (Qiagen). First-strand cDNA synthesis

was performed with 5 µg of total RNA, SuperScript II reverse transcriptase (Invitrogen), and random primers (Invitrogen) according to the manufacturer's instructions. Real-time PCR was performed with an MxPro-Mx3005P multiplex quantitative PCR system (Agilent Technologies, USA). Each 25-µl reaction mixture contained 10 ng cDNA, 300 nM of each specific primer (Table 2.3.), and 12.5 µl 2× SYBR green master mix (Applied Biosystems, USA). All reverse transcription-PCR (RT-PCR) data were normalized with the results for the 16s rRNA gene, and the relative expression ratio of the target gene was calculated as described by Pfaffl (Pfaffl, 2001).

2.25. SDS-PAGE and Western blot analysis

Proteins were separated and analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in a Mini-PROTEAN Tetra Cell (Bio-Rad) using a 12% acrylamide gel. Equal volumes of 2X SDS-PAGE sample buffer (0.75% (w/v) Tris, 10% (v/v/) glycerol, 1% (w/v) SDS, 1% (v/v) β-mercaptoethanol, 0.001% (w/v) bromophenol blue, pH 6.8) and samples were mixed and boiled for 5 min before being run adjacent to a Broad Range Protein Marker (New England Biolabs) at 120 V through the stacking gel (4.55% (w/v) Tris, 0.1% (w/v) SDS, 12% (v/v) acrylamide, 0.05% (v/v) ammonium persulfate (APS), 0.2% (v/v) N,N,N’,N’-tetramethylethylenediamine (TEMED), pH 8.8) and 200 V through the resolving gel (1.53% (w/v) Tris, 0.1% (w/v) SDS, 4% (v/v) acrylamide, 0.05% (v/v) APS, 0.2% (v/v) TEMED, pH 6.8) in SDS-PAGE running buffer (2.5 mM Tris, 25 mM glycine, 0.1% (w/v) SDS). Proteins were visualised using Coomassie Blue solution (10% (v/v) acetic acid, 45% (v/v) methanol, 0.25% (w/v) Coomassie Brilliant Blue) for 60 min, followed by immersion in destain solution (10% (v/v) glacial acetic acid, 30% (v/v) methanol) for 45 min.

Immunoblot analysis was carried out by transferring samples to nitrocellulose blotting membrane (Pall Corporation, USA) in transfer buffer (4.8 mM Tris, 3.9 mM glycine, 10% (v/v) methanol) using a Semi-Dry Blotting System (DKSH, Switzerland) at 50 mA

per membrane for 75 min. Following transfer, membranes were blocked in TBS containing 5% (w/v) skim milk powder for 30 min at room temperature. The membranes were probed using the appropriate antibodies and conditions as listed in Table 2.4 in TBS containing 0.05% (v/v) Tween-20 (MP Biomedicals, USA) The membrane was washed four times in TBS for 5 min after application of either the primary or secondary antibodies and colorimetric detection was achieved using BCIP/NPT alkaline phosphatase substrate (Moss Inc., USA).

2.26. *L. pneumophila* infection of A strain Mice

6-8 week old male and female mice were sourced from the Department of Immunology and Microbiology Animal Facility, the University of Melbourne. All mouse procedures were approved by the University of Melbourne Animal Ethics Committee (University of Melbourne Animal Ethics Approval Number 1112061). 3 days prior to infection, *L. pneumophila* strains were plated on BCYE agar with appropriate antibiotics and incubated at 37 °C. On day of infection, strains were resuspended in sterile PBS and adjust $OD_{600\ nm}$ to 0.1. Inoculum was diluted in PBS to 5 x 10^7 cfu/mL and 50 µl added intranasally, drop-wise ensuring each drop is inhaled, to each anesthetized mouse. Inoculum diluted in PBS was plated on BCYE agar and incubated at 37 °C for 3 days to confirm bacterial numbers.

After 72 h infection, mice were euthanized with carbon dioxide asphyxiation and lungs were harvested. Lung tissue was homogenized in PBS for 60 s and saponin was added to homogenate at a final concentration of 0.1% saponin. Homogenate was incubated at 37 °C for 30 min to lyse host cells before plating samples diluted in PBS on BCYE agar. Plates were incubated at 37 °C for 3 days for enumeration of viable bacteria.

2.27. Construction of 4HA-SdeC fusion protein

SdeC N-terminal translational fusions to a 4x hemagglutinin (HA) tag were generated in pICC562, a *Legionella* expression vector derived from pMMB207C and used for the generation of 4x HA-tagged effector proteins (Dolezal et al., 2012). The coding region of *L. pneumophila* 130b *sdeC* was amplified by PCR with primers 2717 and 2718 and the resulting fragment ligated into the BamHI and HindIII restriction enzyme sites of the MCS of pICC562. The construct was introduced into *E. coli* XL1-Blue (Novagene) by heat shock transformation of chemically competent *E. coli* and plasmid DNA was purified from LB broth culture incubated overnight at 37 °C using a QIAprep Spin Miniprep kit (Qiagen) according to the manufacturer's instructions. Recombinant plasmids were analysed by restriction enzyme digestion to confirm the correct size of the inserted fragment before DNA sequencing of positive clones to ensure correct nucleotide sequence and successful gene fusion. The construct was then transformed into *L. pneumophila* via electroporation for localisation studies.

2.28. Sample preparation for fluorescence microscopy

When the infection time point was reached, cells were washed with DMEM and fixed with 400 µL of 4% paraformaldehyde (diluted in PBS from 16% stock solution) for 20 min at room temperature. Cells were washed and permeabilised with 400 µL 0.1% Triton X-100 (Sigma-Aldrich) for 20 min and then cells were washed three times with PBS before adding 200 µL of primary antibody (refer to Table 2.4.) diluted with 0.2% BSA in PBS to each well and incubating for 1 h at room temperature. Cells were again washed three times with PBS and 200 µL of fluorescent secondary antibody (refer to Table 2.4.) diluted with 0.2% BSA in PBS was added to each well and incubated at room temperature with no light exposure for 30 min. 1 µL of 50 µg/mL DAPI (Invitrogen) diluted in PBS was added to stain nucleic acid and incubated for 5 min at room temperature. Cells were then washed three times with PBS before mounting coverslips

onto microscope slides using ProLong Gold anti-fade mountant (Invitrogen) and drying overnight at room temperature with no light exposure. Samples were analysed with a LSM 700 laser scanning confocal microscope with Zen 2010 software (Zeiss, Germany) using the appropriate fluorescent filters.

2.29. Generation of EGFP-SdeC fusion protein

SdeC N-terminal translational fusions to a EGFP tag were generated in the pEGFP-C2 (Clontech, USA), a eukaryotic expression vector used for the generation of EGFP-tagged effector proteins. The coding region of *L. pneumophila* 130b *sdeC* was amplified by PCR using primers 1943 and 1944 and the resulting fragment ligated into the MCS of pEGFP-C2. The construct was introduced into *E. coli* XL1-Blue (Novagene) by heat shock transformation of chemically competent *E. coli* and plasmid DNA was purified from LB broth culture incubated overnight at 37 °C using a QIAprep Spin Miniprep kit (Qiagen) according to the manufacturer's instructions. Recombinant plasmids were analysed by restriction enzyme digestion to confirm the correct size of the inserted fragment before DNA sequencing of positive clones to ensure correct nucleotide sequence and successful gene fusion.

2.30. Transfection of HEK293T cells and expression of EGFP-SdeC fusion proteins

1 x 10^5 HEK293T cells in DMEM were seeded onto 12 mm round coverslips in 24-well tissue culture plates with duplicate wells prepared for each sample. Plates were incubated overnight at 37 °C and 5% CO_2 before removing media. 4 µL Lipofectamine 2000 (Invitrogen) was added to 96 µL DMEM and incubated at room temperature for 5 min before 1 µg of plasmid in 100 µL DMEM was added. The mixture was then incubated at room temperature for 30 min. Cells were washed gently with 500 µL warm DMEM and the Lipofectamine/plasmid mixture was added to each well. Cells

were incubated at 37 °C and 5% CO_2 for 20 min before 300 µL DMEM was added to each well and plates were then incubated at 37 °C and 5% CO_2 for 24 h. Cells were washed with warm PBS before fixing and staining as described above before analysis by laser scanning confocal microscopy using the appropriate fluorescent filters.

2.31. GFP-Trap co-immunoprecipitation of HEK293T cells transfected with pEGFP-C2:SdeC

4 x 10^6 HEK293T cells in a T-75 tissue culture flask were transfected with pEGFP-C2:SdeC as described above with the following changes. 36 µg of plasmid and 72 µL of Lipofectamine 2000 were used in a transfection complex with a total volume of 20 mL made up with DMEM media.

A GFP-Trap (Chromotek, Germany) co-immunoprecipitation was performed as per the manufacturer's instructions. Immunoprecipitation samples were analysed by separating proteins on an SDS-PAGE gel before staining with a Coomassie Brilliant Blue R-250 (Thermo Scientific) protein stain as per the manufacturer's instructions.

2.32. HA Co-immunoprecipitation of proteins in B6 mouse bone marrow-derived macrophages infected with *L. pneumophila* pICC562:SdeC and LC-MS/MS mass spectrometry

4 x 10^7 B6 mouse bone marrow-derived macrophages in T-175 tissue culture flasks were infected with *L. pneumophila* pICC562:SdeC as described above.

Co-immunoprecipitation of cell lysate was performed with Pierce Anti-HA Magnetic Beads (Thermo Scientific) as per the manufacturer's instructions. Immunoprecipitation samples were analysed by separating proteins on an SDS-PAGE gel before staining with

a SYPRO Ruby Protein Gel Stain (Life Technologies) as per the manufacturer's instructions.

After separation by SDS-PAGE gel, proteins were excised and then analysed by multi-dimensional LC-MS/MS on an QStar Pulsar i MALDI-quadrupole time-of-flight mass spectrometer (AB Sciex) by the WEHI Proteomics Laboratory (Walter and Eliza Hall Institute, Australia), according to published protocols (Washburn et al., 2001). Data was analysed using Mascot against all Eubacteria or Eukaryotic protein entries in the latest release of the LudwigNR database.

2.33. Electron microscopy

2×10^6 mouse bone-marrow derived macrophages were seeded in 60 mm Petri dishes (Sarstedt) and infected with *L. pneumophila* strains at a MOI of 10. Cells were infected at 37 °C and 5% CO_2 for 2 or 24 h before cells were washed with PBS and fixed in 2.5% glutaraldehyde in PBS for 1 h. Cells were washed in PBS with 5% sucrose and scraped from the tray surface with cell scrapers before being pelleted and washed with PBS and 5% sucrose. This was followed by a post-fixation step in 2.5% osmium tetroxide for 1 h. Following dehydration in a graded acetone series, the cell pellet was embedded in Epon-Araldite epoxy resin. Thin sections were stained with 10% uranyl acetate and 2.5% lead citrate before viewing under a CM12 electron microscope (Phillips) at 60 kV.

2.34. Live cell imaging of *A. castellanii* and HeLa cells infected with *L. pneumophila* strains

A. castellanii or HeLa cells were seeded and infected as above in 30 mm μ-Dish (Ibidi, Germany) at a MOI of 10 and 50 respectively, for 2 h before extracellular bacteria were washed off with media three times. Samples were then incubated at 37 °C and 5% CO_2

and stained with ER-Tracker™ Red (Life Technologies) live cell imaging stain when required as per the manufacturer's instructions. Cells were imaged in a heated chamber of a LSM700 laser scanning confocal microscope at 37 °C and 5% CO_2.

2.34.1. Changes in LCV size over time in L. pneumophila-infected A. castellanii cells

A. castellanii cells were infected with *L. pneumophila* strains expressing EGFP and were imaged as described above. Images of a single dish were taken at 2, 6, 9, 12 and 15 h post-infection and a minimum of 40 LCVs were measured using ZEN2010 (Zeiss) to observe the change in average LCV size over time.

2.35. Bioinformatics

Sequence homology between DNA/protein sequences and the Genbank database was compared using BLASTX, BLASTN or BLASTP on the NCBI website (www.ncbi.nlm.nih.gov). The NCBI genome viewer (www.ncbi.nlm.nih.gov) was used to view the location of genes and their surroundings. Protein domain information was accessed via Interpro (http://www.ebi.ac.uk/interpro/).

2.36. Statistical analysis

Statistical analysis of results was achieved using GraphPad Prism 4.0 (GraphPad In Stat Software Inc., USA). When required, an unpaired, two-tailed student t-test, for normal distributions, or an unpaired, two-tailed Mann Whitney test, for non-normal distributions, was applied to analyse the results. Statistical significance was determined for P-values less than 0.05.

Table 2.1. Bacterial strains used in this study

Strain	Characteristics	Source
***E. coli* XL-1 Blue**	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [*F' proAB lacIqZΔM15 Tn10* (*Tet*^{R})]	Stratagene
***L. pneumophila* CT3C**	Serogroup 1, Environmental isolate, Malaysia	S Yong
***L. pneumophila* CT67**	Serogroup 2-14, Environmental isolate, Malaysia	S Yong
***L. pneumophila* CT4b**	Serogroup 2-14, Environmental isolate, Malaysia	S Yong
***L. pneumophila* C7**	Serogroup 2-14, Environmental isolate, Malaysia	S Yong
***L. pneumophila* C1**	Serogroup 2-14, Environmental isolate, Malaysia	S Yong
***L. pneumophila* C102**	Serogroup 2-14, Environmental isolate, Malaysia	S Yong
***L. pneumophila* C42**	Serogroup 2-14, Environmental isolate, Malaysia	S Yong
***L. pneumophila* PN2**	Serogroup 1, Environmental isolate, Malaysia	S Yong
***L. pneumophila* B6**	Serogroup 1, Environmental isolate, Malaysia	S Yong
***L. pneumophila* CT1**	Serogroup 1, Environmental isolate, Malaysia	S Yong
***L. pneumophila* PN1**	Serogroup 1, Environmental isolate, Malaysia	S Yong
***L. pneumophila* 02/40**	Serogroup 1, Clinical isolate, Australia	(Newton et al., 2006)
***L. pneumophila* 02/41**	Serogroup 1, Environmental isolate, Australia	(Newton et al., 2006)
***L. pneumophila* 03/41**	Serogroup 1, Environmental isolate, Australia	(Newton et al., 2006)
***L. pneumophila* 03/43**	Serogroup 1, Environmental isolate, Australia	(Newton et al., 2006)
***L. pneumophila* 03/42**	Serogroup 1, Environmental isolate, Australia	(Newton et al., 2006)
***L. pneumophila* 03/47**	Serogroup 1, Clinical isolate, Australia	(Newton et al., 2006)

***L. pneumophila* 03/45**	Serogroup 1, Environmental isolate, Australia	(Newton et al., 2006)
***L. pneumophila* 03/46**	Serogroup 1, Clinical isolate, Australia	(Newton et al., 2006)
***L. pneumophila* 03/49**	Serogroup 1, Clinical isolate, Australia	(Newton et al., 2006)
***L. pneumophila* 03/48**	Serogroup 1, Clinical isolate, Australia	(Newton et al., 2006)
***L. pneumophila* 03/53**	Serogroup 3, Clinical isolate, Australia	(Newton et al., 2006)
***L. pneumophila* 03/54**	Serogroup 3, Environmental isolate, Australia	(Newton et al., 2006)
***L. pneumophila* 03/55**	Serogroup 4, Environmental isolate, Australia	(Newton et al., 2006)
***L. pneumophila* 03/50**	Serogroup 1, Clinical isolate, Australia	(Newton et al., 2006)
***L. pneumophila* 03/58**	Serogroup 5, Environmental isolate, Australia	(Newton et al., 2006)
***L. pneumophila* 03/59**	Serogroup 6, Environmental isolate, Australia	(Newton et al., 2006)
***L. pneumophila* 03/56**	Serogroup 4, Environmental isolate, Australia	(Newton et al., 2006)
***L. pneumophila* 03/57**	Serogroup 5, Environmental isolate, Australia	(Newton et al., 2006)
***L. pneumophila* 03/64**	Serogroup 8, Environmental isolate, Australia	(Newton et al., 2006)
***L. pneumophila* 03/60**	Serogroup 6, Environmental isolate, Australia	(Newton et al., 2006)
***L. pneumophila* 03/61**	Serogroup 7, Environmental isolate, Australia	(Newton et al., 2006)
***L. pneumophila* 03/63**	Serogroup 8, Environmental isolate, Australia	(Newton et al., 2006)
L. pneumophila H075020013	Serogroup 1, Environmental isolate, Bellingham	(Schroeder et al., 2010)
L. pneumophila H075020015	Serogroup 1, Environmental isolate, Oxford/OLDA	(Schroeder et al., 2010)

L. pneumophila H075020016	Serogroup 4, Environmental isolate, Portland	(Schroeder et al., 2010)
L. pneumophila H080160263	Serogroup 6, Environmental isolate, NA	(Schroeder et al., 2010)
L. pneumophila H080200554	Serogroup 1, Environmental isolate, Heysham	(Schroeder et al., 2010)
L. pneumophila H080200555	Serogroup 1, Environmental isolate, Oxford/OLDA	(Schroeder et al., 2010)
L. pneumophila H080200559	Serogroup 6, Environmental isolate, NA	(Schroeder et al., 2010)
L. pneumophila H080200562	Serogroup 1, Environmental isolate, Oxford/OLDA	(Schroeder et al., 2010)
L. pneumophila H080200565	Serogroup 3, Environmental isolate, NA	(Schroeder et al., 2010)
L. pneumophila H080200566	Serogroup 1, Environmental isolate, Oxford/OLDA	(Schroeder et al., 2010)
L. pneumophila H080200568	Serogroup 1, Environmental isolate, Oxford/OLDA	(Schroeder et al., 2010)
L. pneumophila H080200573	Serogroup 6, Environmental isolate, NA	(Schroeder et al., 2010)
L. pneumophila H080200574	Serogroup 10, Environmental isolate, NA	(Schroeder et al., 2010)
L. pneumophila H080200575	Serogroup 1, Environmental isolate, Oxford/OLDA	(Schroeder et al., 2010)
L. pneumophila H080200576	Serogroup 1, Environmental isolate, Bellingham	(Schroeder et al., 2010)
L. pneumophila H080200577	Serogroup 1, Environmental isolate, Oxford/OLDA	(Schroeder et al., 2010)
L. pneumophila H080340093	Serogroup 1, Environmental isolate, Benidorm	(Schroeder et al., 2010)
L. pneumophila H080340097	Serogroup 1, Environmental isolate, Philadelphia	(Schroeder et al., 2010)
L. pneumophila RR07000773	Serogroup 3, Environmental isolate, NA	(Schroeder et al., 2010)
L. pneumophila RR07000774	Serogroup 1, Environmental isolate, Bellingham	(Schroeder et al., 2010)

L. pneumophila RR07000788	Serogroup 6, Environmental isolate, NA	(Schroeder et al., 2010)
L. pneumophila RR07000789	Serogroup 5, Environmental isolate, Cambridge	(Schroeder et al., 2010)
L. pneumophila RR07000790	Serogroup 6, Environmental isolate, NA	(Schroeder et al., 2010)
L. pneumophila RR07000791	Serogroup 1, Environmental isolate, Benidorm	(Schroeder et al., 2010)
L. pneumophila RR07000792	Serogroup 1, Environmental isolate, Oxford/OLDA	(Schroeder et al., 2010)
L. pneumophila RR08000123	Serogroup 1, Environmental isolate, Bellingham	(Schroeder et al., 2010)
L. pneumophila RR08000136	Serogroup 1, Environmental isolate, Oxford/OLDA	(Schroeder et al., 2010)
L. pneumophila H082940035	Serogroup 1, Clinical isolate, Philadelphia	(Schroeder et al., 2010)
L. pneumophila H082980021	Serogroup 1, Clinical isolate, Philadelphia	(Schroeder et al., 2010)
L. pneumophila H082680013	Serogroup 1, Clinical isolate, NA	(Schroeder et al., 2010)
L. pneumophila H081340222	Serogroup 1, Clinical isolate, Allentown/France	(Schroeder et al., 2010)
L. pneumophila H081760005	Serogroup 1, Clinical isolate, Benidorm	(Schroeder et al., 2010)
L. pneumophila H083120262	Serogroup 1, Clinical isolate, Allentown/France	(Schroeder et al., 2010)
L. pneumophila H083120268	Serogroup 1, Clinical isolate, Allentown/France	(Schroeder et al., 2010)
L. pneumophila H083080428	Serogroup 1, Clinical isolate, Benidorm	(Schroeder et al., 2010)
L. pneumophila H083140015	Serogroup 1, Clinical isolate, Allentown/France	(Schroeder et al., 2010)
L. pneumophila H083220038	Serogroup 1, Clinical isolate, Knoxville	(Schroeder et al., 2010)
L. pneumophila H083260176	Serogroup 1, Clinical isolate, Allentown/France	(Schroeder et al., 2010)

L. pneumophila H083480003	Serogroup 1, Clinical isolate, Allentown/France	(Schroeder et al., 2010)
L. pneumophila H083580455	Serogroup 1, Clinical isolate, Allentown/France	(Schroeder et al., 2010)
L. pneumophila H083620580	Serogroup 1, Clinical isolate, Allentown/France	(Schroeder et al., 2010)
L. pneumophila H083660004	Serogroup 1, Clinical isolate, Philadelphia	(Schroeder et al., 2010)
L. pneumophila H083840063	Serogroup 1, Clinical isolate, Benidorm	(Schroeder et al., 2010)
L. pneumophila H083920177	Serogroup 1, Clinical isolate, Philadelphia	(Schroeder et al., 2010)
L. pneumophila H083920180	Serogroup 1, Clinical isolate, Bellingham	(Schroeder et al., 2010)
L. pneumophila H083960064	Serogroup 1, Clinical isolate, Allentown/France	(Schroeder et al., 2010)
L. pneumophila H084060004	Serogroup 1, Clinical isolate, Allentown/France	(Schroeder et al., 2010)
L. pneumophila H084120109	Serogroup 1, Clinical isolate, Oxford/OLDA	(Schroeder et al., 2010)
L. pneumophila H084160068	Serogroup 1, Clinical isolate, Benidorm	(Schroeder et al., 2010)
L. pneumophila H084240079	Serogroup 1, Clinical isolate, Allentown/France	(Schroeder et al., 2010)
L. pneumophila H084340334	Serogroup 1, Clinical isolate, Knoxville	(Schroeder et al., 2010)
L. pneumophila H084380129	Serogroup 1, Clinical isolate, Benidorm	(Schroeder et al., 2010)
L. pneumophila H084680106	Serogroup 1, Clinical isolate, Philadelphia	(Schroeder et al., 2010)
L. pneumophila H084780287	Serogroup 1, Clinical isolate, Allentown/France	(Schroeder et al., 2010)
***L. pneumophila* 130b**	Serogroup 1, Clinical isolate (USA)	(Engleberg et al., 1984)
***L. pneumophila* 130b (pMIP)**	130b carrying pMIP	(Sansom et al., 2007)

***L. pneumophila* 130b IS903-derived transposon mutant 3E**	*dotA* transposon insertion mutant of 130b	This study
***L. pneumophila* 130b IS903-derived transposon mutant 19A**	*dotA* transposon insertion mutant of 130b	This study
***L. pneumophila* 130b IS903-derived transposon mutant 13C**	*icmB* transposon insertion mutant of 130b	This study
***L. pneumophila* 130b IS903-derived transposon mutant 13D**	*icmB* transposon insertion mutant of 130b	This study
***L. pneumophila* 130b IS903-derived transposon mutant 15C**	*icmB* transposon insertion mutant of 130b	This study
***L. pneumophila* 130b IS903-derived transposon mutant 8L**	*icmE* transposon insertion mutant of 130b	This study
***L. pneumophila* 130b IS903-derived transposon mutant 14D**	*icmE* transposon insertion mutant of 130b	This study
***L. pneumophila* 130b IS903-derived transposon mutant 16A**	*icmE* transposon insertion mutant of 130b	This study
***L. pneumophila* 130b IS903-derived transposon mutant 16B**	*icmE* transposon insertion mutant of 130b	This study
***L. pneumophila* 130b IS903-derived transposon mutant 16C**	*icmE* transposon insertion mutant of 130b	This study
***L. pneumophila* 130b IS903-derived transposon mutant 16D**	*icmE* transposon insertion mutant of 130b	This study
***L. pneumophila* 130b IS903-derived transposon mutant 16E**	*icmE* transposon insertion mutant of 130b	This study
***L. pneumophila* 130b IS903-derived transposon mutant 13F**	*icmH* transposon insertion mutant of 130b	This study
***L. pneumophila* 130b IS903-**	*icmK* transposon insertion mutant of 130b	This study

derived transposon mutant 13G		
***L. pneumophila* 130b IS903-derived transposon mutant 17A**	*icmK* transposon insertion mutant of 130b	This study
***L. pneumophila* 130b IS903-derived transposon mutant 17B**	*icmK* transposon insertion mutant of 130b	This study
***L. pneumophila* 130b IS903-derived transposon mutant 17C**	*icmK* transposon insertion mutant of 130b	This study
***L. pneumophila* 130b IS903-derived transposon mutant 13A**	*icmL* transposon insertion mutant of 130b	This study
***L. pneumophila* 130b IS903-derived transposon mutant 13B**	*icmL* transposon insertion mutant of 130b	This study
***L. pneumophila* 130b IS903-derived transposon mutant 18A**	*icmL* transposon insertion mutant of 130b	This study
***L. pneumophila* 130b IS903-derived transposon mutant 18B**	*icmL* transposon insertion mutant of 130b	This study
***L. pneumophila* 130b IS903-derived transposon mutant 8E**	*fabF* transposon insertion mutant of 130b	This study
***L. pneumophila* 130b IS903-derived transposon mutant 15A**	*lpw07571* transposon insertion mutant of 130b	This study
***L. pneumophila* 130b IS903-derived transposon mutant 13E**	*lpw27511* transposon insertion mutant of 130b	This study
***L. pneumophila* 130b IS903-derived transposon mutant 3G**	*lpw29561* transposon insertion mutant of 130b	This study
***L. pneumophila* 130b IS903-derived transposon mutant 4A**	*potC* transposon insertion mutant of 130b	This study
***L. pneumophila* 130b IS903-derived transposon mutant 4B**	*potC* transposon insertion mutant of 130b	This study

***L. pneumophila* 130b IS903-derived transposon mutant 4C**	*potC* transposon insertion mutant of 130b	This study
***L. pneumophila* 130b IS903-derived transposon mutant 4D**	*potC* transposon insertion mutant of 130b	This study
***L. pneumophila* 130b IS903-derived transposon mutant 4E**	*potC* transposon insertion mutant of 130b	This study
***L. pneumophila* 130b IS903-derived transposon mutant 4F**	*potC* transposon insertion mutant of 130b	This study
***L. pneumophila* 130b IS903-derived transposon mutant 3F**	*sdeC* transposon insertion mutant of 130b	This study
***L. pneumophila* 130b IS903-derived transposon mutant 5A**	*vacB* transposon insertion mutant of 130b	This study
***L. pneumophila* 130b IS903-derived transposon mutant 5B**	*vacB* transposon insertion mutant of 130b	This study
L. pneumophila* 130b Δ*dotA	*dotA* in-frame deletion mutant of 130b	This study
L. pneumophila* 130b Δ*sdeC	*sdeC* in-frame deletion mutant of 130b	This study
***L. pneumophila* 130b Δ*sdeC* (pMIP)**	*sdeC* in-frame deletion mutant of 130b carrying pMIP	This study
***L. pneumophila* 130b Δ*sdeC* (pMIP:*sdeC*)**	*sdeC* in-frame deletion mutant of 130b carrying pMIP:*sdeC*	This study
L. pneumophila* 130b Δ*lpw27511	*lpw27511* in-frame deletion mutant of 130b	This study
***L. pneumophila* 130b Δ*lpw27511* (pMIP)**	*lpw27511* in-frame deletion mutant of 130b carrying pMIP	This study
***L. pneumophila* 130b Δ*lpw27511* (pMIP:*lpw27511*)**	*lpw27511* in-frame deletion mutant of 130b carrying pMIP:*lpw27511*	This study
***L. pneumophila* 130b *lpw27511*:IS903 (pMIP:*lpw27511*)**	IS903 transposon insertion mutant of *lpw27511* in 130b carrying pMIP:*lpw27511*	This study
***L. pneumophila* 130b (pXDC61:*ralF*)**	130b carrying pXDC61:*ralF*	This study
***L. pneumophila* 130b Δ*dotA* (pXDC61:*ralF*)**	*dotA* in-frame deletion mutant of 130b carrying pXDC61:*ralF*	This study
***L. pneumophila* 130b *lpw27511:IS903* (pXDC61:*ralF*)**	IS903 transposon insertion mutant of *lpw27511* in 130b carrying pXDC61:*ralF*	This study
***L. pneumophila* 130b (pICC562)**	130b carrying pICC562	This study

***L. pneumophila* 130b (pICC562:*sdeC*)**	130b carrying pICC562:*sdeC*	This study
***L. pneumophila* 130b (pMIP:EGFP)**	130b carrying pMIP:EGFP	This study
***L. pneumophila* 130b Δ*sdeC* (pMIP:EGFP)**	*sdeC* in-frame deletion mutant of 130b carrying pMIP:EGFP	This study
***L. pneumophila* 130b (pMIP:mCherry)**	130b carrying pMIP:mCherry	This study

Table 2.2 Plasmids used in this study

Plasmid	Characteristics	Source
pC6	11700 bp, transposon mutagenesis vector, *ori R6K*, *mobRP4*, bla, *Km*^{R}, *tpnA*.	(Edelstein et al., 1999)
pGEM-T Easy	3015 bp, high copy cloning vector with a multiple cloning region containing the a-peptide coding region of the enzyme β-galactosidase, flanked by T7 and SP6 RNA polymerase promoters, Amp^R	Promega
pGEM-T Easy:SdeC-GFPN1	Plasmid for sequencing of pEGFP-N1:SdeC fragment	This study
pGEM-T Easy:SdeC-GFPC2	Plasmid for sequencing of pEGFP-C2:SdeC fragment	This study
pGEM-T Easy:pMIP-SdeC	Plasmid for sequencing of pMIP-SdeC fragment	This study
pGEM-T Easy:*sdeC*-ABCD	Plasmid for sequencing of fragments used in creation of *sdeC* deletion mutant	This study
pGEM-T Easy:pMIP-Lpw27511	Plasmid for sequencing of pMIP-Lpw27511 fragment	This study
pGEM-T Easy:*lpw27511*-ABCD	Plasmid for sequencing of fragments used in creation of *lpw27511* deletion mutant	This study
pGEM-T Easy: pICC562-SdeC	Plasmid for sequencing of pICC562-SdeC fragment	This study
pGEM-T Easy:3E	Plasmid for sequencing of *L. pneumophila* 130b transposon insertion mutant 3E	This study
pGEM-T Easy:19A	Plasmid for sequencing of *L. pneumophila* 130b transposon insertion mutant 19A	This study
pGEM-T Easy:13C	Plasmid for sequencing of *L. pneumophila* 130b transposon insertion mutant 13C	This study
pGEM-T Easy:13D	Plasmid for sequencing of *L. pneumophila* 130b transposon insertion mutant 13D	This study
pGEM-T Easy:15C	Plasmid for sequencing of *L. pneumophila* 130b transposon insertion mutant 15C	This study
pGEM-T Easy:8L	Plasmid for sequencing of *L. pneumophila* 130b	This study

	transposon insertion mutant 8L	
pGEM-T Easy:14D	Plasmid for sequencing of *L. pneumophila* 130b transposon insertion mutant 14D	This study
pGEM-T Easy:16A	Plasmid for sequencing of *L. pneumophila* 130b transposon insertion mutant 16A	This study
pGEM-T Easy:16B	Plasmid for sequencing of *L. pneumophila* 130b transposon insertion mutant 16B	This study
pGEM-T Easy:16C	Plasmid for sequencing of *L. pneumophila* 130b transposon insertion mutant 16C	This study
pGEM-T Easy:16D	Plasmid for sequencing of *L. pneumophila* 130b transposon insertion mutant 16D	This study
pGEM-T Easy:16E	Plasmid for sequencing of *L. pneumophila* 130b transposon insertion mutant 16E	This study
pGEM-T Easy:13F	Plasmid for sequencing of *L. pneumophila* 130b transposon insertion mutant 13F	This study
pGEM-T Easy:13G	Plasmid for sequencing of *L. pneumophila* 130b transposon insertion mutant 13G	This study
pGEM-T Easy:17A	Plasmid for sequencing of *L. pneumophila* 130b transposon insertion mutant 17A	This study
pGEM-T Easy:17B	Plasmid for sequencing of *L. pneumophila* 130b transposon insertion mutant 17B	This study
pGEM-T Easy:17C	Plasmid for sequencing of *L. pneumophila* 130b transposon insertion mutant 17C	This study
pGEM-T Easy:13A	Plasmid for sequencing of *L. pneumophila* 130b transposon insertion mutant 13A	This study
pGEM-T Easy:13B	Plasmid for sequencing of *L. pneumophila* 130b transposon insertion mutant 13B	This study
pGEM-T Easy:18A	Plasmid for sequencing of *L. pneumophila* 130b transposon insertion mutant 18A	This study
pGEM-T Easy:18B	Plasmid for sequencing of *L. pneumophila* 130b transposon insertion mutant 18B	This study
pGEM-T Easy:8E	Plasmid for sequencing of *L. pneumophila* 130b transposon insertion mutant 8E	This study
pGEM-T Easy:15A	Plasmid for sequencing of *L. pneumophila* 130b transposon insertion mutant 15A	This study
pGEM-T Easy:13E	Plasmid for sequencing of *L. pneumophila* 130b	This study

	transposon insertion mutant 13E	
pGEM-T Easy:3G	Plasmid for sequencing of *L. pneumophila* 130b transposon insertion mutant 3G	This study
pGEM-T Easy:4A	Plasmid for sequencing of *L. pneumophila* 130b transposon insertion mutant 4A	This study
pGEM-T Easy:4B	Plasmid for sequencing of *L. pneumophila* 130b transposon insertion mutant 4B	This study
pGEM-T Easy:4C	Plasmid for sequencing of *L. pneumophila* 130b transposon insertion mutant 4C	This study
pGEM-T Easy:4D	Plasmid for sequencing of *L. pneumophila* 130b transposon insertion mutant 4D	This study
pGEM-T Easy:4E	Plasmid for sequencing of *L. pneumophila* 130b transposon insertion mutant 4E	This study
pGEM-T Easy:4F	Plasmid for sequencing of *L. pneumophila* 130b transposon insertion mutant 4F	This study
pGEM-T Easy:3F	Plasmid for sequencing of *L. pneumophila* 130b transposon insertion mutant 3F	This study
pGEM-T Easy:5A	Plasmid for sequencing of *L. pneumophila* 130b transposon insertion mutant 5A	This study
pGEM-T Easy:5B	Plasmid for sequencing of *L. pneumophila* 130b transposon insertion mutant 5B	This study
pPCRScript	3000 bp, cloning vector, ori f1 (+), ori pUC, *bla*	Stratagene
pPCRScript:*sdeC*	pPCRScript carrying *sdeC* cloned into the MCS	This study
pPCRScript:*lpw27511*	pPCRScript carrying *lpw27511* cloned into the MCS	This study
pMIP	9523 bp, pMMB207 with the promoter region of mip cloned into SacI/XbaI, Cm^R	(Newton et al., 2006)
pMIP:*sdeC*	pMIP carrying *sdeC* cloned into the MCS	This study
pMIP:*lpw27511*	pMIP carrying *lpw27511* cloned into the MCS	This study
pMIP:EGFP	pMIP carrying EGFP cloned into the MCS	This study
pUC4-KISS	4000bp, Km^R, Amp^R	Pharmacia
pXDC61:*ralF*	pXDC61, plasmid facilitating β-lactamase TEM-1 fusion proteins, carrying *ralF* cloned into the MCS	This study
pICC562	Plasmid facilitating 4x HA fusion proteins	(Dolezal et al., 2012)
pICC562:*sdeC*	pICC562 carrying *sdeC* cloned into the MCS	This study
pEGFP-C2	4700 bp, plasmid facilitating EGFP-C2 fusion proteins,	Addgene

	EGFP, Kan^r, SV40 ori, pUC ori, f1 ori, SV40 poly A	
pEGFP-N1	4700 bp, plasmid facilitating EGFP-N1 fusion proteins,	Addgene
	EGFP, Kan^r, SV40 ori, pUC ori, f1 ori, SV40 poly A	
pEGFP-C2:*sdeC*	pEGFP-C2 carrying *sdeC* cloned into the MCS	This study

Table 2.3. Oligonucleotide sequences used in this study

Oligonucleotide number	Sequence	Use
1611	cgg gat cct cta gag at	Primer 1 used for sequencing pC6 transposon
1612	ctc tag agg atc ccg tc	Primer 2 used for sequencing pC6 transposon
1621	cgg tgc cgc tgg cga	Primer 3 used for sequencing pC6 transposon
1622	ggt cgc cag cgg cac	Primer 4 used for sequencing pC6 transposon
1641	gct acc tgg aga gac g	Primer 5 used for sequencing pC6 transposon
1644	cgt ctc tcc agg tag c	Primer 6 used for sequencing pC6 transposon
1646	gta cgc gta gtg caa cc	Primer 7 used for sequencing pC6 transposon
1647	ggt tgc act acg cgt ac	Primer 8 used for sequencing pC6 transposon
1652	gtc aga cat gta tac ccc	Primer 9 used for sequencing pC6 transposon
1669	gca tca tca gga gta cg	Primer 10 used for sequencing pC6 transposon
1670	gat cct cta gag att tat tc	pC6 transposon specific primer. For amplifying transposon-flanking regions
1671	cga att caa gct tct	Y-linker specific primer. From non-complementary region. For amplifying transposon-flanking regions
1672	ttt ctg ctc gaa ttc aag ctt cta acg atg tac ggg gac a	Y-linker 1. (use with Sau3A)
1683	gat ctg tcc ccg tac	Y-linker 2 (use with Sau3A)

	atc gtt aga act act cgt acc atc cac at	
1750	ctt tta gaa att ttt gcg gat a	forward primer for mavK. start of gene. Used as southern blot probe
1751	ctc tat ggt ttc atc ctg gtt	reverse primer for mavK. end of gene. Used as southern blot probe
1752	gaa tgc cca atc cag aat	forward primer for lpw27511. start of gene. Used as southern blot probe
1753	aat caa cgc agg gat tat tt	reverse primer for lpw27511. end of gene. Used as southern blot probe
1927	cct cga cgc ata gtt tat atc aat g	Forward primer used to generate a 711 bp PCR-product in the N-terminus of leg2771. For use in PCR screen
1928	gca aat aaa acc agc tgc atc ttc t	Rev primer used to generate a 711 bp PCR-product in the N-terminus of leg2771. For use in PCR screen
1929	aac tgc agt ggg agt tga cgc agc ta	(A) primer for mutagenesis of lpw27511. Pst1 site
1930	gaa gac agg ttt tca gca acg aat tcg	(D) primer for mutagenesis of lpw27511. EcoR1 site
1931	gga att cgt tgc tga aaa cct gtc ttc	(C) primer for mutagenesis of lpw27511. EcoR1 site
1932	ccc tcg agc tct agt aat cct ggc tca t	(B) primer for mutagenesis of lpw27511. Xho1 site
1933	aac tgc agt cga gaa tgg aac aca tac c	(A) primer for mutagenesis of SdeC. Pst1 site
1936	ccc tcg agc ttt tta agg tct tcc aca ctc	(B) primer for mutagenesis of SdeC. Xho1 site
1941	gga att ctt ggg aga aag taa aat gcc t	Forward primer to clone SdeC into pEGFP-N1. EcoRI site
1942	gcg tcg acc cta gac cat acc tta tat cat ca	Reverse primer to clone SdeC into pEGFP-N1. Sal1 site
1943	ccc tcg agc ttg gga gaa agt aaa atg cct	Forward primer to clone SdeC into pEGFP-C2. Xho1 site
1944	aac tgc agc ctt ata gac cat acc tta tat cat c	Reverse primer to clone SdeC into pEGFP-C2. Pst1 site
1973	ttg tga tgg tga att	(D) primer for mutagenesis of SdeC. EcoR1 site 500bp

	cct acc gct ggt gat atc gcc	
1974	cca gcg gta gga att cac cat cac aat ggt tga gga ag	(C) primer for mutagenesis of SdeC. EcoR1 site 500bp
2041	ggg ccc gat cga gga cct c	forward primer for sequencing of pGEM:SdeC-GFP-N1/C2 ~900bp from start
2042	ggg tct tac tac aga tac tgt ac	forward primer for sequencing of pGEM:SdeC-GFP-N1/C2 ~450bp from end
2043	cga gag gtt ccg tgg atg ttc	reverse primer for sequencing of pGEM:SdeC-GFP-N1/C2~900bp from end
2045	cgt gct ggt taa gaa aat ctc tgc	reverse primer for sequencing of pGEM:SdeC-GFP-N1/C2~2000bp from end
2046	cct gat gtg cag caa acc tac cc	forward primer for sequencing of pGEM:SdeC-GFP-N1/C2~2000bp from start
2047	gaa gtc gat ttc tat gcc gcg	forward primer for sequencing of pGEM:SdeC-GFP-N1/C2~1300bp from start
2093	gca atg gat aga att cta gtt	forward *sdeC* primer ou tside deletion region. For confirmation of deletion
2094	ctc gct ttc tcg tcc agc cac	reverse *sdeC* primer outside deletion region. For confirmation of deletion
2099	cat ccc gat gtg aac aaa cca	forward lpw27511 primer outside deletion region. For confirmation of deletion
1717	ctt ggt cga cag gaa ctt ggt aat tc	reverse lpw27511 primer outside deletion region. For confirmation of deletion
2119	gca gct ata aca tcg acg aac aac	For primer for southern of sdeC mutant. Deleted region. 65
2120	cac ccg tga ttt gcc ctc tta ac	Rev primer for southern of sdeC mutant. Deleted region. 65
2121	gtg tga ata ttc gta tcc att ag	For primer for southern of sdeC mutant. Kanamycin insert region.
2122	cta aca agc act taa atg caa cc	Rev primer for southern of sdeC mutant. Kanamycin insert region.
2134	att agc tat tac ggt cct cct ttg	for dotA primer 650bp
2135	gag tag gat tac ccc	rev dotA primer 650bp

	cac aag	
2136	atg gca tcg ggg aaa caa gat a	for leg130b_0090 primer 604bp. For use in PCR screen
2137	cct cct cca tcg tat ctt caa aa	rev leg130b_0090 primer 604bp. For use in PCR screen
2138	tcc att taa tgg att acc caa aga c	for leg130b_0338 primer 732bp. For use in PCR screen
2139	gca gca aaa gca gaa cct aat tta a	rev leg130b_0338 primer 732bp. For use in PCR screen
2140	ggc aag gga gcg tac ggt att g	for leg130b_1440 primer 509bp. For use in PCR screen
2141	cga cga taa tcc tgt tct gac g	rev leg130b_1440 primer 509bp. For use in PCR screen
2142	aaa tga gct gat taa gcg gat taa c	for leg130b_1581 primer 635bp. For use in PCR screen
2143	tta gaa ttt tac cca ggc cat ca	rev leg130b_1581 primer 635bp. For use in PCR screen
2144	tgc ttt agt gta tgg att tga acc a	for leg130b_1965 primer 563bp. For use in PCR screen
2145	ggt tga ggt tat atg cac tcg c	rev leg130b_1965 primer 563bp. For use in PCR screen
2146	cta tgc gtc tct ttt cag tgt tgg	for leg130b_1976 primer 514bp. For use in PCR screen
2147	ggg tta gta gcc gta att tac tct ttc ac	rev leg130b_1976 primer 514bp. For use in PCR screen
2148	ctg gtt cca gtc agt ccg cat	for leg130b_2266 primer 475bp. For use in PCR screen
2149	gaa atg gct aac tcc att ttg tct g	rev leg130b_2266 primer 475bp. For use in PCR screen
2150	ctg aat tag ctc aag ctt tt	for leg130b_2570 primer 521bp. For use in PCR screen
2151	tga att acc gat tct ttg agc	rev leg130b_2570 primer 521bp. For use in PCR screen
2152	cgc ttc tgt tta tcc tgt tac g	for leg130b_2740 primer 575bp. For use in PCR screen
2153	ctc ttc cca ttg aaa	rev leg130b_2740 primer 575bp. For use in PCR screen

	aaa tat cag c	
2154	tcc tta aaa aat tct gat gtg cct c	for leg130b_3311 primer 512bp. For use in PCR screen
2155	ctt gtc tat att gct cgg gag taa	rev leg130b_3311 primer 512bp. For use in PCR screen
2192	ggg gtc tag aca tcc cga tgt gaa caa acc a	forward primer lpw27511 complementation. XbaI site
2194	ggg gct gca gga gct tta atc aac agg cta cc	reverse primer lpw27511 complementation. PstI site
2195	ggg ggg atc cga tga agc gac gtc agc ctt ag	forward primer *sdeC* complementation. BamHI site
2196	ggg gct gca gca gcg ata gtg cgt ccg tca g	reverse primer *sdeC* complementation. PstI site
2272	ttt ttc ttt cct tcg ccg caa g	leg0246 FW 660bp. For use in PCR screen
2273	cgt aaa gcc tca tgg cgt tc	leg0246 RV 660bp. For use in PCR screen
2274	caa atg tta aga gca gca gct g	leg1990 FW 660bp. For use in PCR screen
2275	tca taa aat aga gca gta gga cca	leg1990 RV 660bp. For use in PCR screen
2276	ggt acc cct ttt caa ctt cct ttt gaa aaa	leg2960 FW 460bp. For use in PCR screen
2277	tct aga tta cat gaa tct tac aga atg gct	leg2960 RV 460bp. For use in PCR screen
2717	cccc gga tcc ttg gga gaa agt aaa atg cct aaa tac g	for primer *sdeC* with BamHI site for cloning into pICC562
2718	cccc aag ctt tta tag acc ata cct tat atc atc ac	rev primer *sdeC* with HindIII site for cloning into pICC562
2892	cag ctc gtg tcg tga gat gt	16S rRNA *Legionella pneumophila*. 80bp region for qrt pcr

2893	cct tag agt ccc cac cat ca	16S rRNA *Legionella pneumophila*. 80bp region for qrt pcr
2894	aag agg gca aat cac ggg tgt caa gca	*sdeC L. pneumophila* 130b 80bp region for qRT-pcr
2895	caa cag ggg tag tga ctg cgc gtt gc	*sdeC L. pneumophila* 130b 80bp region for qRT-pcr
4420	cacgacgttgtaaaacga c	M13 forward. For sequencing/confirmation of inserts in pGEM T-easy
4421	ggataacaatttcacaca gg	M13 reverse. For sequencing/confirmation of inserts in pGEM T-easy
883	cat ccg cca aaa cag cc	Reverse primer for sequencing/confirmation of inserts in pMIP and pICC562
1105	cgg ttc tgg caa ata ttc	Forward primer for sequencing/confirmation of inserts in pMIP and pICC562

Table 2.4. Antibodies used in this study

Antibody	Experimental Conditions	Source
Anti-HA.11	Primary antibody 1:200 dilution; 60 min incubation	(Covance)
Anti-calnexin	Primary antibody 1:200 dilution; 60 min incubation	(Life Technologies)
Anti-Golgi-97	Primary antibody 1:200 dilution; 60 min incubation	(Life Technologies)
Anti-β-Cop	Primary antibody 1:100 dilution; 60 min incubation	(Sigma Aldrich)
Anti-LAMP-1	Primary antibody 1:50 dilution; 60 min incubation	(Life Technologies)
Anti-caspase 3 (cleaved)	Primary antibody 1:100 dilution; 60 min incubation	(Abcam)
Anti-actin	Primary antibody 1:500 dilution; 60 min incubation	(Life Technologies)
Anti-mouse-AP	Secondary antibody 1:5000 dilution; 60 min incubation	(Sigma Aldrich)
Anti-rabbit-AP	Secondary antibody 1:5000 dilution; 60 min incubation	(Sigma Aldrich)
Alexa Fluor 488 (Anti-Rabbit or Mouse IgG)	Secondary antibody 1:600 dilution; 30 min incubation	(Life Technologies)
Alexa Fluor 568 (Anti-Rabbit or Mouse IgG)	Secondary antibody 1:600 dilution; 30 min incubation	(Life Technologies)
Alexa Fluor 635 (Anti-Rabbit or Mouse IgG)	Secondary antibody 1:600 dilution; 30 min incubation	(Life Technologies)

Chapter 3: A screen to identify genes of *L. pneumophila* required for intracellular replication

3.1. Introduction

Sequencing the *L. pneumophila* genome has uncovered the genetic basis for many aspects of the pathogenic lifestyle of *Legionella* spp. A multitude of eukaryotic-like proteins and genes encoding proteins with eukaryotic domains have been uncovered. *Legionella* secretes many of these proteins into the host cell during infection, subverting host processes to create a vacuole permissive for replication and thereby ensure its own survival. Many of these proteins are conserved amongst the different strains of *L. pneumophila* but a large number are present in smaller subsets of strains (Gomez-Valero et al., 2011).

Diverse genome content amongst *L. pneumophila* strains frequented with horizontal transfer events presumably allows for survival within a large number of host species, including humans. Due to this diversity the genomes of only a handful of *L. pneumophila* strains may not be representative of the population as a whole. Here we sequenced the *L. pneumophila* clinical isolate strain 130b (also known as ATCC BAA-74, Wadsworth or AA100) of serogroup one and compared it to the currently sequenced *L. pneumophila* strains. *L. pneumophila* strain 130b is a commonly used laboratory strain that originated from a transtracheal aspirate isolated from a patient in the United States in 1978. Sequencing the 130b genome will facilitate the analysis of *L. pneumophila*'s eukaryotic-like genes, secretion systems and the secreted effector repertoire when comparing these features to other sequenced strains.

Our knowledge of the pathogenesis of Legionnaires' disease at the molecular level is still somewhat basic. The identification of novel effectors and determining their function is important for our understanding of how *Legionella* causes disease.

As *Legionella* research expands, the number of secreted effectors identified and characterised has increased dramatically. The number of validated effector proteins now exceeds 300, an impressive number and one of the highest in comparison to other bacterial pathogens that translocate effectors into a host cell. As this this number has been steadily increasing there remains the potential for novel effectors to be identified and characterised. A high degree of functional redundancy exists amongst many effectors where there is no detectable difference in intracellular survival and replication if they are deleted; suggesting multiple effectors are responsible for targeting similar processes.

Several studies have used large scale screening approaches to identify and characterise novel *Legionella* virulence genes, the majority focusing on the identification on Dot/Icm secreted effectors. Machine learning algorithms were used in one study to define a set of features distinguishing known Dot/Icm effectors which was subsequently used to identify 40 novel effectors (Burstein et al., 2009). Another study used a Cre/*loxP* translocation system to confirm Dot/Icm translocated effectors after identifying *Legionella* substrates that interacted with DotF, a predicted inner-membrane component of the Dot/Icm translocation system, using a bacterial two-hybrid screen (Luo and Isberg, 2004).

In addition to determining the genome sequence of *L. pneumophila* 130b, here we create a large *L. pneumophila* transposon mutant library using an IS903-derived transposon (Derbyshire, 1995) and screened for attenuated intracellular replication within the natural protozoan host, *A. castellanii*. For the screen we developed a high throughput amoebae plate test based on a previous study (Albers et al., 2005). Due to the high level of functional redundancy in *L. pneumophila* effectors we did not expect

to identify many *dot/icm* effector genes. However, our overall goal was to identify any gene that was singularly important for intracellular replication and survival.

3.2. Results

3.2.1. Sequencing and analysis of the *L. pneumophila* 130b genome

The *L. pneumophila* 130b strain is a commonly used laboratory strain used for *L. pneumophila* studies. Sequencing the genome of this strain was intended to aid subsequent investigations and expand our knowledge of the differences between *L. pneumophila* strains at the genetic level. The 130b genome was sequenced using paired-end 454 FLX pyrosequencing and assembled using the 454/Roche Newbler assembly program in a collaborative effort (Schroeder et al., 2010).

Analysis of the assembled genome revealed a circular chromosome of 3.5 Mb with a G+C content of 38% and 3293 predicted coding sequences, correlating well with the previously sequenced *L. pneumophila* Sg1 genomes (Cazalet et al., 2004; Chien et al., 2004; Gomez-Valero et al., 2011). The 130b genome is also highly syntenic with the genomes of other sequenced strains. A number of secretion systems were identified including the Dot/Icm T4SS, a Trb-1-like T4SS, two Lvh T4SS gene clusters, two GI-T4SS, a general secretory (Sec) system, a twin arginine translocation (TAT) system, a T1SS and a T2SS.

With the aim to uncover novel secreted effector proteins, the 130b genome was analysed for genes encoding proteins with characteristics of known T4SS effectors, such as eukaryotic-like domains, that are absent from the *L. pneumophila* strain Philadelphia (Analysis performed by the Frankel lab, Imperial College London). The Philadelphia strain was chosen as most current analysis of secreted effectors has been

done using this strain. Our screen identified 16 new putative effector proteins using this method (Table 3.1).

Table 3.1. Putative novel 130b secreted effectors and their homologues (>80% identity) in other *L. pneumophila* strains

130b	Alcoy	Lens	Paris	Corby	Domain/motif
Lpw00581 (LtpA)	Lpa00080, Lpa04086	Lpl0057		Lpc0063, Lpc3099	Radial spoke head protein
Lpw02251					Calcineurin-like phosphoesterase domain
Lpw02301 (LtpB)					Peptidase C58, Ankyrin repeat
Lpw02381 (LtpC)					Ankyrin repeat, RasGEF domain
Lpw03701 (LtpD)					-
Lpw04551 (LtpE)					-
Lpw16311 (LtpF)					Leucine-rich repeats

Lpw20091 (LtpG)	Lpa02853	Lpl1931		Lpc1435	FIC domain
Lpw20341 (LtpH)					Ankyrin repeat
Lpw21901					Coiled coil
Lpw25791 (LtpI)		Lpl2297	Lpp2417		-
Lpw25801			Lpp2418	Lpc2130	Internal repeat
Lpw25861	Lpa03449			Lpc2106	Host cell attachment protein
Lpw26201 (LtpJ)		Lpl2330			Leucine-rich repeats
Lpw28181					Pentapeptide repeat
Lpw28221					NTPase (NACHT family)

A fluorescence-based β-lactamase TEM1 translocation assay was used to determine whether the identified effector candidates were substrates of the Dot/Icm T4SS (Schroeder et al., 2010). Raw264.7 macrophages were infected with *L.*

pneumophila 130b expressing N-terminal fusions of TEM1 to putative effector proteins. Translocation of TEM1 fusion proteins into host cells results in cleavage of the TEM1 substrate CCF2 when added. The shift in the fluorescence emission wavelength of CCF2 after cleavage can be measured and used to detect the translocation of fusion proteins. Comparisons to infections with *L. pneumophila dotA* mutant expressing the TEM1 effector fusion proteins are used to determine if fusion proteins are translocated by the Dot/Icm T4SS. Here, 10 novel effector proteins, termed *Legionella* translocated proteins (LtpA to LtpJ) (Table 3.1), were validated as novel Dot/Icm translocated effectors (Schroeder et al., 2010).

To determine the prevalence of these identified effectors outside the currently sequenced strains, in a collaborative effort we performed a PCR screen of a large set of environmental and clinical *L. pneumophila* isolates from a range of serogroups (Schroeder et al., 2010) (Appendix 1.1. and Appendix 1.2.). The results for representative PCR-negative strains were confirmed by Southern blotting (data not shown). The set of isolates consisted of 87 environmental and clinical *L. pneumophila* strains of different serogroups originating from Malaysia, Australia and the United Kingdom.

Five genes*, ltpB, ltpC, ltpE, ltpF, and ltpJ,* were detected in less than 15% of the analyzed strains. In contrast, the remaining five genes, *ltpA, ltpD, ltpG, ltpH,* and *ltpI*, were found in 34% to 62% of the isolates screened (Appendix 3.1.). There was no correlation between the presence of the novel effectors and whether the isolates were clinical or environmental in origin. This data supports the growing evidence that *L. pneumophila* is a highly diverse species, with the entire effector repertoire of the species being larger than any single strain (Cazalet et al., 2004; Chien et al., 2004; Gomez-Valero et al., 2009; Gomez-Valero et al., 2011; Steinert et al., 2007).

3.2.2. Generation of random mutants by transposon mutagenesis and sequencing of the IS903 transposon

The aim of this work was to screen a library of random *L. pneumophila* 130b mutants for an attenuated ability to replicate within host cells. This was performed to identify novel genes important for intracellular replication and survival in amoebae. A large library of over 10,000 transposon mutants was screened to cover the majority of the L. pneumophila genome, assuming random insertion of the IS903 transposon.

IS903 was used to generate a library of insertion mutants in *Legionella pneumophila* strain 130b. IS903 transposes efficiently, singly, and randomly into *L. pneumophila* (Wiater et al., 1994). A constitutively expressed kanamycin resistance cassette is present within the transposon for easy selection of successful transformants. The plasmid harbouring this transposon, pC6 (Figure 3.1) (Edelstein et al., 1999) is a suicide vector where the transposase gene (*tnp*) is placed outside of the transposon region, ensuring the stability of the transposon after insertion. Here the IS903 transposon was sequenced, with the initial primer, obtained from a previously reported partial sequence (Derbyshire, 1995). The complete sequence of the pC6 encoded IS903-derived transposon was ascertained via primer walking (Primers used listed in Table 2.2.). The IS903 transposon was 4373 bp in length and contained a kanamycin resistance gene within two inverted repeats (Figure 3.1.).

The pC6 suicide vector carrying ampicillin resistance was introduced into *L. pneumophila* strain 130b via electroporation and kanamycin resistant colonies were selected on BCYE agar. A library of 10,006 *L. pneumophila* transposon mutants was created in this manner.

3.2.3. Growth of transformants in the presence of *A. castellanii*

In order to identify genetic regions required for intracellular replication, the *L. pneumophila* transposon mutant library was screened for attenuated intracellular replication in the presence of the aquatic protozoan species *A. castellanii* with a revised version of an amoebae plate test (Albers et al., 2005).

Here, serial dilutions of transformants were plated onto BCYE agar plates spread with *A. castellanii* to observe replication in the presence of amoebae (Figure 3.2.). A BCYE plate containing no amoebae was included to observe growth in the absence of amoebae. Another plate was included containing 100 µg/mL ampicillin. Transformants that were able to grow in the presence of ampicillin (less than 5% of transformants, indicating a generally effective suicide vector) were discarded as ampicillin resistance indicated persistence of the pC6 plasmid.

Recombinants that displayed normal growth on BCYE agar plates lacking amoebae, but attenuated growth, relative to wild type *L. pneumophila* 130b, on BCYE agar plates containing *A. castellanii*, and no growth on BCYE agar plates containing ampicillin were resuspended in glycerol broth and stored at -80 °C for further study. A *L. pneumophila dotA* mutant is severely attenuated for intracellular replication and was used here as a control strain for comparison to the transposon mutant strains. A total of 10,006 recombinants were screened and the 227 recombinants that displayed initial attenuated growth in the presence of *A. castellanii* were rescreened to confirm attenuated intracellular replication within *A. castellanii* for selected mutants. To achieve this, a second amoebae plate test was performed on the identified recombinants where the initial $OD_{600\ nm}$ of each strain used was standardised. This step was not possible in the first high throughput amoebae plate test as the number of strains being tested was too great. Following the second amoebae plate test, 50 transposon mutants were identified as attenuated for replication in *A. castellanii*. Of the 10,006 transposon mutants screened, this represents a hit rate of 0.5%.

3.2.4. *L. pneumophila* - *A. castellanii* co-culture assay to confirm intracellular growth attenuation of recombinants

Recombinants that displayed only a modest decrease in intracellular growth within *A. castellanii* were analysed in two quantitative *L. pneumophila* - *A. castellanii* co-culture assays to confirm attenuated intracellular replication (Figure 3.3.). The assay was performed in duplicate over 72 hours post-infection and colony-forming units (CFUs) was determined at 0, 24, 48 and 72 h. Five transposon mutants were analysed using this method. Four transposon mutants, designated 8H, 14C, 7U and 14F, displayed intracellular growth at a similar level to wild type *L. pneumophila* and were not pursued further. One transposon mutant, 13D, displayed significantly attenuated intracellular replication compared to wild type *L. pneumophila* (unpaired, 2-tailed t-test) and was included for further study.

3.2.5. Confirmation of the presence of the IS903-derived transposon

Southern blot analysis was used to confirm the presence of the transposon in all mutants of interest (Figure 3.4). In addition to this, southern blotting would reveal multiple insertions within a single recombinant, which is not desired as determination of the genetic element responsible for the observed defect would be complicated. Genomic DNA was isolated from mutants of interest and digested with restriction enzymes HindIII, EcoRI and BamHI. The southern blot was performed using a probe corresponding to a 500 bp section of the IS903 transposon (Table 2.3.).

As expected, the transposon was detected in the majority of the recombinant; however three contained two inserts and were not studied further. The transposon-

flanking regions of the remaining 28 recombinants that contained a single transposon insertion were sequenced in order to reveal the identity of the disrupted gene.

3.2.6. Identification of transposon insertion site

A Y-linker method for specific amplification of transposon flanking sequences (Kwon and Ricke, 2000) was used to identify the location of the transposon insertion in each of the 28 recombinants.

Genomic DNA from the recombinants was digested with the restriction enzyme Sau3A or NlaIII and the resulting fragments were ligated to a Y-linker. The Y-linker has a region of non-complementary sequence at one end, creating a Y shape. The opposite end contains a region identical to the sticky end created by the appropriate restriction enzyme digestion.

Samples were used as templates for PCR amplification using a transposon-specific primer and a Y-linker primer. The resulting amplified fragments were ligated into pGEM-T Easy and transformed into chemically competent *E. coli* XL1-Blue. Blue/white selection and ampicillin resistance was used to select for strains that contained the pGEM-T Easy vector with a fragment ligated into its multi cloning site (MCS).

The pGEM- T Easy vector contains an ampicillin resistance gene and a *lacZ* gene. A functional *lacZ* gene will result in blue bacterial colonies when in the presence of IPTG and the β-galactosidase substrate 5-Bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal). The MCS is located within the *lacZ* gene and the insertion of a DNA fragment here will disrupt the gene, resulting in white colonies after blue/white selection using LB agar containing 100 µg/mL ampicillin and 10 mM IPTG, allowing for easy selection of vectors with inserted DNA fragments. White colonies were patched onto LB agar plates containing 100 µg/mL ampicillin. Colony PCR was used to confirm the presence and

correct size of the fragment within the MCS of pGEM- T Easy. Positive clones were sequenced to identify transposon-flanking regions.

Although the majority of genes identified were *dot/icm* genes, seven non-*dot/icm* genes were identified; *fabF*, *lpw07571, lpw27511, lpw29561, potC, sdeC* and *vacB* (Table 3.3).

Table 3.3. Identity of the genes disrupted by IS903 in transposon mutants displaying attenuated intracellular replication in *A. castellanii*. The number after the gene name indicates the number of times it was identified.

Mutant ID	Disrupted gene	Degree of attenuation in amoebae plate test (Fold-change less than *L. pneumophila*)	Gene function/motif
3E, 19A	*dotA (x2)*	10^5	Component of Dot/Icm T4SS (Roy et al., 1998; Vogel et al., 1998)
13C, 13D, 15C	*icmB (x3)*	10^2 - 10^4	Component of Dot/Icm T4SS (Segal et al., 1998)
8L, 14D, 16A, 16B, 16C, 16D, 16E	*icmE (x7)*	10^4 - 10^5	Component of Dot/Icm T4SS (Segal et al., 1998)

13F	*icmH*	10^3	Component of Dot/Icm T4SS (Segal et al., 1998)
13G, 17A, 17B, 17C	*icmK (x4)*	10^4 - 10^5	Component of Dot/Icm T4SS (Segal et al., 1998)
13A, 13B, 18A, 18B,	*icmL (x4)*	10^3 - 10^5	Component of Dot/Icm T4SS (Segal et al., 1998)
8E	*fabF*	10^2	fatty acid biosynthesis (Moche et al., 2001; von Wettstein-Knowles et al., 2006)
15A	*lpw07571*	10^5	putative peptidase
13E	*lpw27511*	10^2	putative transcriptional regulator
3G	*lpw29561*	10^2	putative sugar kinase

4A, 4B, 4C, 4D, 4E, 4F	*potC (x6)*	10^2	Component of PotABCD polyamine transporter (Nasrallah et al., 2011)
3F	*sdeC*	10^2	Dot/Icm translocated effector (Luo and Isberg, 2004)
5A, 5B	*vacB (x2)*	10^3	putative exoribonuclease

The *lpw27511, potC, sdeC* and *vacB* transposon mutants were chosen for further study as they produced significant growth defects in the presence of *A. castellanii* or seemed likely to be involved in virulence. The *lpw27511* and *sdeC* transposon mutants displayed approximately 10^2 fold growth attenuation in the presence of *A. castellanii* in the amoebae plate test, while the *potC* and *vacB* transposon mutants displayed approximately 10^4 fold growth attenuation (Figure 3.5.).

3.2.7. Co-culture of *lpw27511*, *potC*, *sdeC* and *vacB L. pneumophila* transposon mutants with *A. castellanii*.

Intracellular replication of *L. pneumophila* in *A. castellanii* was examined further in a quantitative co-culture viable count assay for *lpw27511, potC, sdeC* and *vacB L.*

pneumophila transposon mutants to determine the defect in intracellular replication (Figure 3.6.). The assay was performed in triplicate over 72 hours post-infection by determining CFU at 0, 24, 48 and 72 hours.

The *lpw27511, potC* and *vacB* transposon mutants were significantly attenuated at 48-72 hours post-infection, producing approximately 10^4 fold less CFU in comparison to wild type *L. pneumophila* in the presence of *A. castellanii*. The *sdeC* transposon mutant was also significantly attenuated at 48 hours post-infection producing approximately 10^2 fold less CFU in comparison to wild type *L. pneumophila* in the presence of *A. castellanii*. However, replication of the *sdeC* transposon mutant recovered to wild type levels by 72 hours post-infection.

3.2.8. Replication of *lpw27511 and sdeC L. pneumophila* transposon mutants in THP-1 cells

The *lpw27511* and *sdeC L. pneumophila* transposon mutants were deemed good targets for further study. Both displayed strong attenuated intracellular replication, in particular *lpw27511,* and SdeC is a known translocated Dot/Icm secreted effector of unknown function. We aimed to determine whether these genes were important for intracellular replication in human macrophages in addition to amoebae. Here THP-1 cells, a human monocytic cell line, were differentiated into macrophages and infected with the transposon mutants to evaluate intracellular replication up to 72 h post-infection (Figure 3.7.). The assay was performed in duplicate.

The *lpw27511* transposon mutant appeared attenuated at 48 and 72 hours post-infection, producing approximately 10^3 to 10^4 fold less CFU in comparison to wild type *L. pneumophila* in the presence of THP-1 cells, with statistical significance at 48 h. The *sdeC* transposon mutant also appeared attenuated at 48 and 72 hours post-infection producing approximately 10^2 to 10^3 fold less CFU in comparison to wild type *L.*

pneumophila. Despite these observed strong trends, to conclusively state that the *sdeC* mutant was attenuated for intracellular replication in THP-1 cells additional assays would need to be performed to achieve statistical significance.

Overall, this screen identified two *L. pneumophila* transposon insertion mutants that appeared attenuated for intracellular replication in both amoebae and human macrophages. Further characterisation of these genes was pursued, as described in subsequent chapters, to determine their role in *Legionella* pathogenesis in these host cells.

3.3. Discussion

Analysis of the *L. pneumophila* strain 130b genome revealed an extensive set of secretion systems and Dot/Icm T4SS effectors. A set of new effector candidates were identified and 10 were determined to be translocated by the Dot/Icm T4SS. An examination of the prevalence of these genes among 87 environmental and clinical *L. pneumophila* isolates revealed that half were only present in less than 15% of the strains tested, whilst the other five were relatively well conserved, present in 34-64% of isolates.

These effectors add to a growing list of Dot/Icm T4SS secreted proteins and also to a diverse repertoire of accessory effectors not present in all *L. pneumophila* strains. This reflects the ability of *L. pneumophila* to survive in a variety of different hosts and may account for differences in the virulence of particular *L. pneumophila* strains.

The effectors LtpA, LtpG and LtpJ, identified here, have homologues in the *L. pneumophila* Lens strain. The prevalence of these homologues was investigated in a multi-genome DNA array screen comparing over 200 *L. pneumophila* and other *Legionella* species (Cazalet et al., 2008). The gene *lpl0057* (an *ltpA* homologue) was

found in 36% of screened isolates, *lpl1931* (an *ltpG* homologue) in 40%, and *lpl2330* (an *ltpJ* homologue) in 8%. These results correlate well with the findings from our own screen of different *L. pneumophila* isolates.

LtpD was subsequently found to play a role in intracellular bacterial replication in THP-1 macrophages, the larvae of *Galleria mellonella* and mouse lungs. The secreted effector binds phosphatidylinositol 3-phosphate and inositol monophosphatase IMPA1, indicating a possible role in the avoidance of the endosomal pathway due to interactions with these host factors (Harding et al., 2013a). The role of the remaining newly identified effectors is yet to be determined and further investigation is required to determine their contribution to *Legionella* pathogenesis.

This study created a library of *L. pneumophila* transposon mutants and screened it for attenuated growth within the aquatic protozoan species *A. castellanii* in order to identify novel genes involved in intracellular replication and survival. 10,006 *L. pneumophila* transposon mutants were screened using an amoebae plate test and 34 transposon mutants were found to be attenuated for intracellular replication but not growth on BCYE agar *in vitro*. Thirteen different genes involved in *L. pneumophila* intracellular growth within *A. castellanii* were identified, with many of these identified multiple times.

Six of the identified genes, *dotA, icmB, icmE, icmH, icmK* and *icmL* encode components of the Dot/Icm T4SS. As the Dot/Icm T4SS is known to be essential for intracellular replication it was expected that the genes encoding it would be identified in this screen. The identification of genes already known to be involved in intracellular replication validates the screening method used in this study.

Seven non-*dot/icm* genes (*fabF, lpw07571, lpw27511, lpw29561, potC, sdeC* and *vacB*) were identified here, with the majority not being previously implicated in *Legionella* pathogenesis. *fabF* encodes a protein involved in lipid metabolism and is located adjacent to other fatty acid biosynthesis genes on the genome. *lpw07571* encodes a

putative peptidase of the esterase-lipase superfamily. *lpw29561* encodes a putative sugar kinase possessing a carbohydrate kinase protein domain. The three genes are present in all currently sequenced *L. pneumophila* strains. These metabolic genes may be important for the synthesis of nutrients after being phagocytosed by a host cell.

vacB encodes a putative exoribonuclease that may be involved in RNA degradation. *L. pneumophila* possesses other RNases that are secreted by a Type II secretion system. The targets for these RNases have not been identified however they were found to be important for optimal growth in another amoeba species *Hartmannella vermiformis* (Rossier et al., 2009). Secreted RNases may target specific host RNA subverting an unknown function to the benefit of the bacteria. Alternatively the nucleosides and/or phosphates produced from degraded RNA may be utilised nutritionally by *Legionella*. It is not known if VacB is secreted and/or translocated into the host cell. If this is the case it may function in such a manner. This work is the only known study to implicate VacB in *L. pneumophila* intracellular replication in *A. castellanii*.

potC is a member of the *pot* operon and encodes a part of the PotABCD polyamine transporter. PotC is a cytoplasmic membrane permease, containing six transmembrane α-helical hydrophobic domains that form a polyamine specific channel in the cytoplasmic membrane. *Legionella* requires polyamines for optimal intracellular growth (Nasrallah et al., 2011) and so lack of PotC may impair intracellular growth due to a non-functioning polyamine transporter. This work is the only known study to implicate PotC itself in *L. pneumophila* intracellular replication in *A. castellanii*.

sdeC encodes a paralogue of the *L. pneumophila* protein SidE, a Dot/Icm T4SS translocated effector. It exhibits high similarity (>67%) to SidE and other SidE paralogues SdeA and SdeB and is located upstream from *sdeB* and *sdeA,* while *sidE* and *sdeD* are located on a separate part of the genome. *sdeC* is present in all sequenced strains of *L. pneumophila*. SdeC was also shown to be translocated by the Dot/Icm T4SS and the same study created a *sdeC* deletion mutant that displayed a significant albeit minor growth defect in *Dictyostelium discoideum* and mouse bone marrow-derived

macrophages (Luo and Isberg, 2004). Interestingly despite a high degree of similarity *sidE*, *sdeA* and *sdeB* single deletion mutants did not display a detectable growth defect in either cell type. One SidE paralog, designated LaiA (SdeA), was identified as an integrin-like protein and was reported to be involved in adhesion to, and invasion of, human lung alveolar epithelial cells (Chang et al., 2005). Although SdeC and LaiA have been implicated in virulence, their specific function and the function of their paralogues remains unknown.

In addition to the previously described growth defect in *Dictyostelium discoideum* and mouse bone marrow-derived macrophages, our *sdeC* transposon mutant displayed a replication defect in *A. castellanii* and THP-1 cells. In *A. castellanii,* although there is a defect at 48 hours post infection, bacterial numbers are similar to wild type levels after 72 hours. This recovery and the relatively minor defect observed in *Dictyostelium discoideum* and mouse bone marrow-derived macrophages is not surprising as many *Legionella* effector proteins are functionally redundant after gene knockout studies (Luo and Isberg, 2004; Shin and Roy, 2008). Hence, the presence of several SidE paralogues in *L. pneumophila* may indicate the existence of functional redundancy amongst these proteins. Nevertheless our results and the findings of a previous study (Luo and Isberg, 2004), suggest that deletion or interruption of *sdeC* alone results in intracellular growth attenuation. It is possible that each SidE paralogue has evolved to promote replication only in specific host cells, although this is not supported by the fact that SdeC seems to promote growth in four different cell types including evolutionarily distant species.

lpw27511/lpg2524 encodes a putative transcriptional regulator of the LuxR family and a BLAST analysis of the amino acid sequence displays partial similarity to various other LuxR family transcriptional regulators from *Legionella* and other bacterial species. LuxR transcriptional regulators regulate a wide variety of biological processes including bioluminescence in *Vibrio fischeri* (Qin et al., 2007) and the expression of virulence factors in *Bordetella pertussis* (Beier and Gross, 2008). In addition to *L. pneumophila* strain 130b, the *lpw27511/lpg2524* full length gene was only present in the *L.*

pneumophila strain Philadelphia prior to this study, with a truncated homologous gene also present in the strain Lens. Microarray analysis of *L. pneumophila* strain Philadelphia-infected *A. castellanii* showed that during the transmissive phase the *lpw27511* homolog, *lpg2524*, was upregulated (Jules and Buchrieser, 2007), suggesting a possible role during transmission.

lpw27511/lpg2524 is located upstream of *lpg2525 (mavK)*, a gene encoding a protein that contains an F-BOX domain. F-BOX proteins are receptors that target their substrates for proteolysis by an ubiquitin-ligase complex and are largely a eukaryotic protein family (Schulman et al., 2000). This implicates MavK as a potential virulence factor that may modulate eukaryotic ubiquitination machinery and interfere with host cell processes. Lpw27511/Lpg2524 may regulate the expression of *mavK* and if MavK is involved in virulence this may explain the observed attenuated intracellular growth when *lpw27511/lpg2524* is disrupted, or the transposon insertion has affected expression of *mavK*. However, a *L. pneumophila* Philadelphia strain *mavK* mutant displayed no changes in ubiquitin accumulation at the LCV in comparison to wild type *L. pneumophila* so Lpw27511/Lpg2524 may influence more global gene expression (Ensminger and Isberg, 2010).

To date, this is the only study implicating *lpw27511/lpg2524* in the promotion of intracellular replication in *A. castellanii* and THP-1 cells, with the observation that the *lpw27511 L. pneumophila* transposon mutant displayed attenuation of intracellular replication compared to wild type *L. pneumophila*. It is worth noting that the intracellular replication defect seen in the co-culture viable count assay was more severe than that observed in the amoebae plate test. This may be due to differences between the two assays, such as the different use of solid and liquid media in the two assays, possibly indicating a role for factors such as motility in attenuation of the transposon mutant.

This screen aimed to saturate the *L. pneumophila* genome in the search of genes involved in intracellular replication and survival. The screening of 10,006 transposon

mutants will achieve approximately 95% saturation of the *L. pneumophila* genome using the IS903-derived transposon, assuming 3293 genes and random mutagenesis (Himpsl et al., 2008). According to this analysis, the majority of genes solely required for intracellular replication in *A. castellanii* should have been identified here, as long as random insertion of the transposon is achieved. This may not be the case if false negatives were present in the initial amoebae plate test due to differing bacterial numbers present in initial samples (only accounted for in the second amoebae plate test standardising initial bacterial numbers of all strains).

The creation of site-directed, non-polar mutants and the subsequent complementation of these mutants would be desirable for the genes identified in this study to discount the possibility of polar effects downstream of the transposon insertion. The deletion mutants and complemented strains need to be retested for replication in both *A. castellanii* and THP-1 cells to confirm that these genes are involved in intracellular survival and replication within these host cells. Here we chose to focus on two genes of interest. *sdeC* was of particular interest as it has been previously identified as encoding a secreted effector important for efficient replication in host cells. Further study of SdeC to determine its function after being translocated into a host cell includes an investigation of the localisation of SdeC after translocation and the identification of host cell binding partners. In addition, the identification of Lpw27511/Lpg2524 as a possible global regulator of intracellular replication is of great interest. In order to find out the regulatory targets of Lpw27511/Lpg2524, a microarray analysis comparing wild type *L. pneumophila* with an *L. pneumophila lpw27511* deletion mutant should be performed.

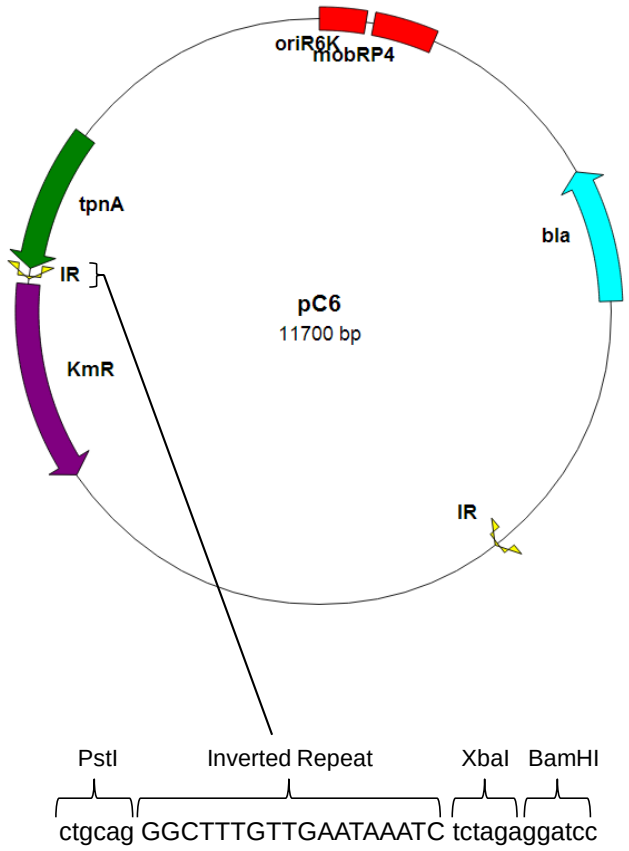


Figure 3.1. The structure of pC6. A representative scale vector map of pC6. *oriR6K*- R6K origin of replication, *mobRP4*- RP4 mobilization element, *bla*- ampicillin resistance gene, *KmR*- kanamycin resistance gene, *tpnA*- transposase gene. IR- two inverted repeats flanking the transposon. IR sequence and flanking restriction enzyme sites are shown. Plasmid map created using Savvy (http://bioinformatics.org/savvy/)

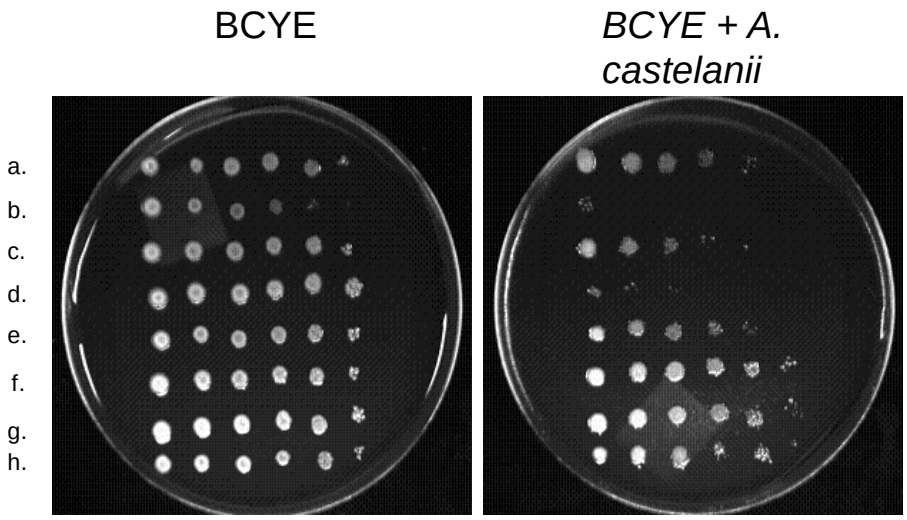


Figure 3.2. Screening of *L. pneumophila* transposon mutants in an amoebae plate test. Images of representative BCYE agar plates in the presence and absence of *A. castellanii* spotted with six 10-fold serial dilutions of *L. pneumophila* 130b (a), *L. pneumophila dotA* deletion mutant *(b), L. pneumophila* transposon mutant 8H *(c)*, *L. pneumophila* transposon mutant 8L *(d)*, *L. pneumophila* transposon mutant 8M *(e)*, *L. pneumophila* transposon mutant 8N *(f)*, *L. pneumophila* transposon mutant 8O *(g)* and *L. pneumophila* transposon mutant 8P *(h).* All plates were incubated for 72 hours at 37 °C. Strains replicate at different rates in the presence of *A. castellanii.* Those with attenuated replication in comparison to wild type *L. pneumophila* 130b were isolated and the gene disrupted by the transposon was identified.

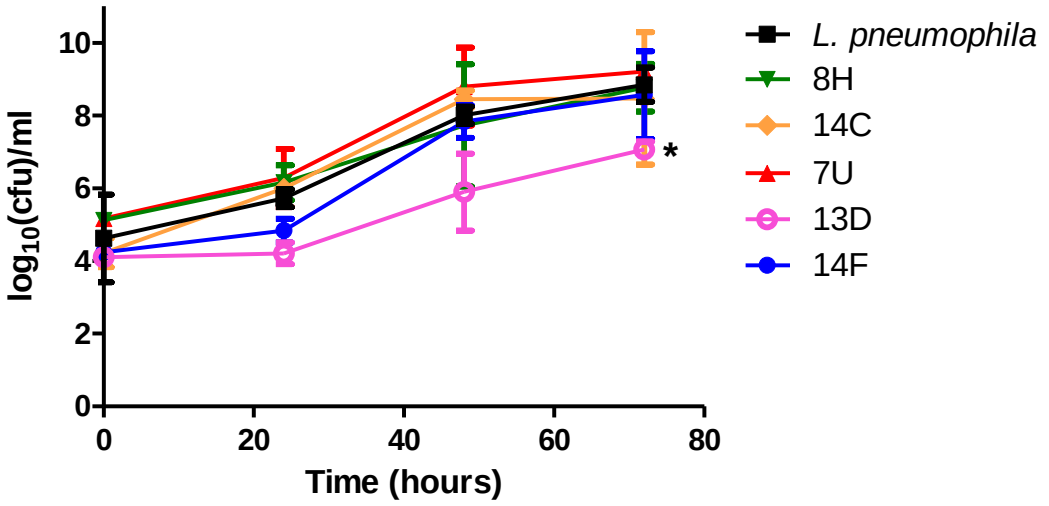


Figure 3.3. Co-culture of *L. pneumophila* with *A. castellanii* to confirm intracellular growth attenuation of recombinants. Line graph plotting log_{10}(cfu)/mL vs time in hours. *A. castellanii* was infected with wild type *L. pneumophila* 130b and *L. pneumophila* transposon mutants 8H, 14C, 7U, 13D and 14F. Cells were incubated at 37 °C and 5% CO_2. The assay was performed in duplicate over 72 h post-infection, plating colony forming units (CFU) at 0, 24, 48 and 72 h on BCYE agar. * indicates statistically significantly different value (t-test, unpaired, two tailed, $P < 0.05$) in comparison to *L. pneumophila* 130b at indicated time point.

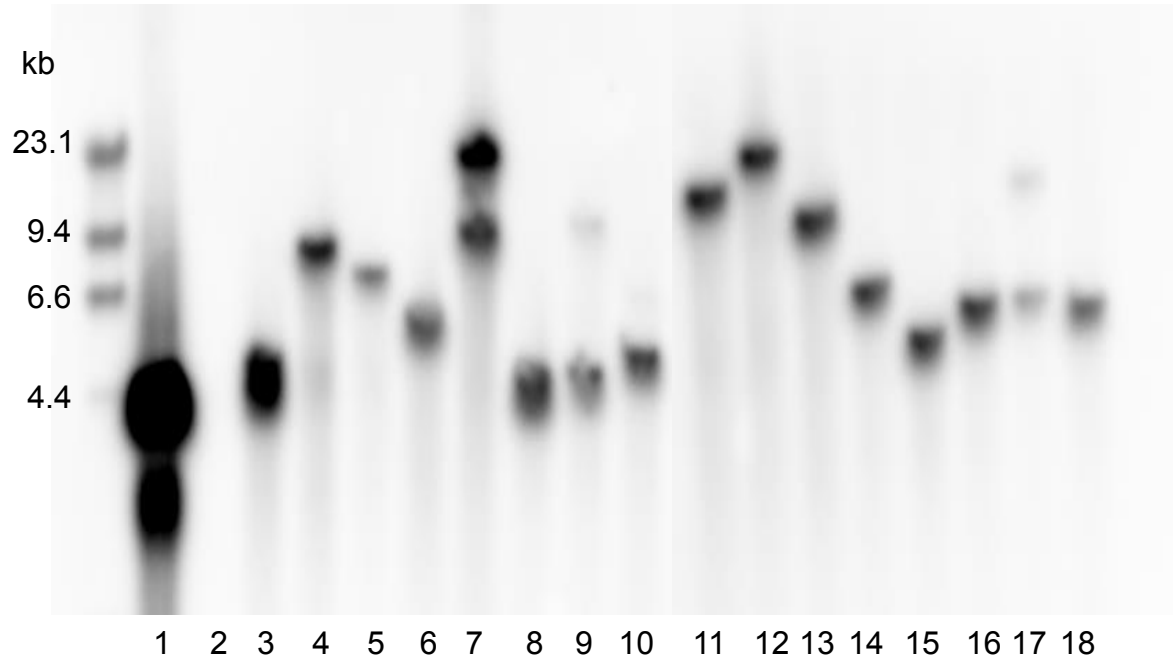


Figure 3.4. Presence of the IS903-derived transposon in *L. pneumophila* recombinants identified with attenuated growth in the presence of *A. castellanii*. Southern blot analysis probing for the presence of the IS903-derived transposon in pC6 plasmid DNA (1), wild type *L. pneumophila* 130b (2), *L. pneumophila* transposon mutants 13D, 13E, 13F, 13G, 14B, 14D, 14E, 15A, 3E, 3F, 3G, 8E, 8L, 13A, 13B and 13C (3-18 respectively). DNA ladder with known fragment sizes, in kb, added for comparison.

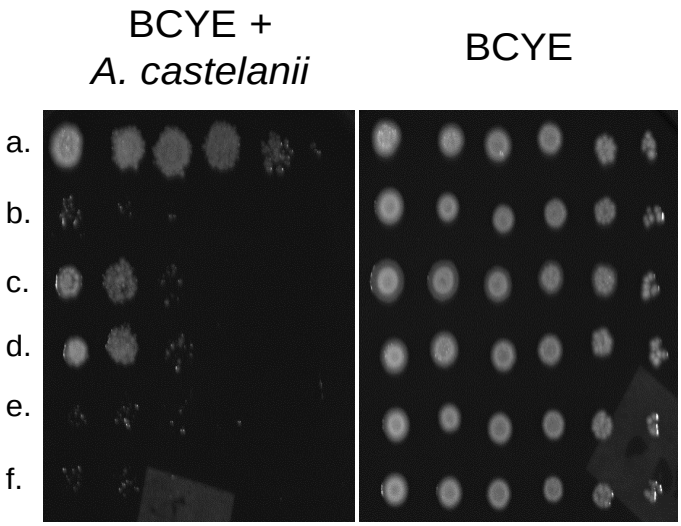


Figure 3.5. Comparison of *lpw27511, potC, sdeC* and *vacB L. pneumophila* transposon mutants in an amoebae plate test. Images of BCYE agar plates in the presence and absence of *A. castellanii* spotted with six 10-fold serial dilutions of *L. pneumophila* 130b (a), *L. pneumophila ΔdotA (b), L. pneumophila lpw27511:IS903 (c), L. pneumophila sdeC:IS903 (d), L. pneumophila potC:IS903 (e)* and *L. pneumophila vacB:IS903 (f)* and incubated for 72 hours at 37°C.

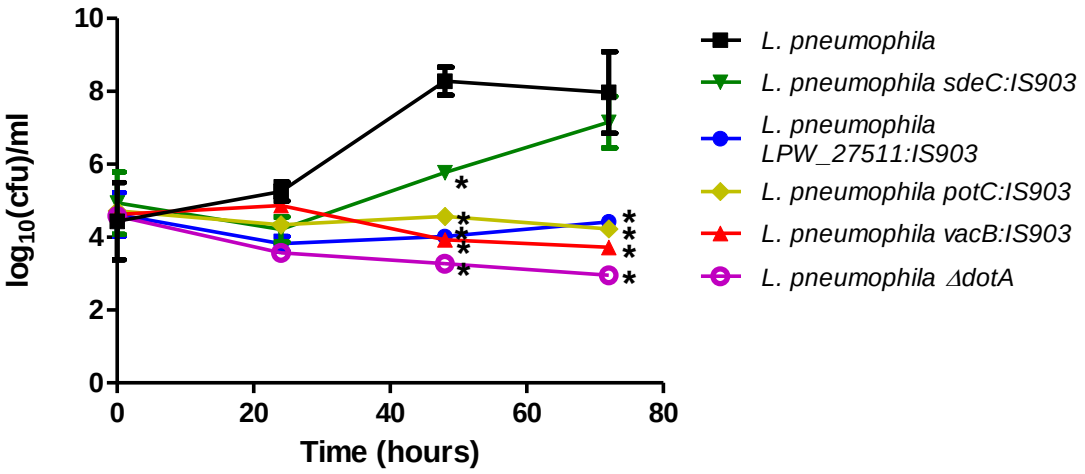


Figure 3.6. Co-culture of *L. pneumophila and A. castellanii* comparing replication of transposon mutants of interest. Line graph plotting log_{10}(cfu)/mL vs time in hours. *A. castellanii* was challenged with the *L. pneumophila* strains indicated. The assay was performed in triplicate over 72 h post-infection and CFU were plated on BCYE agar at 0, 24, 48 and 72 hour time points. Cells were incubated at 37 °C and 5% CO_2. * indicates statistically significantly different value (t-test, unpaired, two tailed, $P < 0.01$) in comparison to *L. pneumophila* 130b at indicated time point.

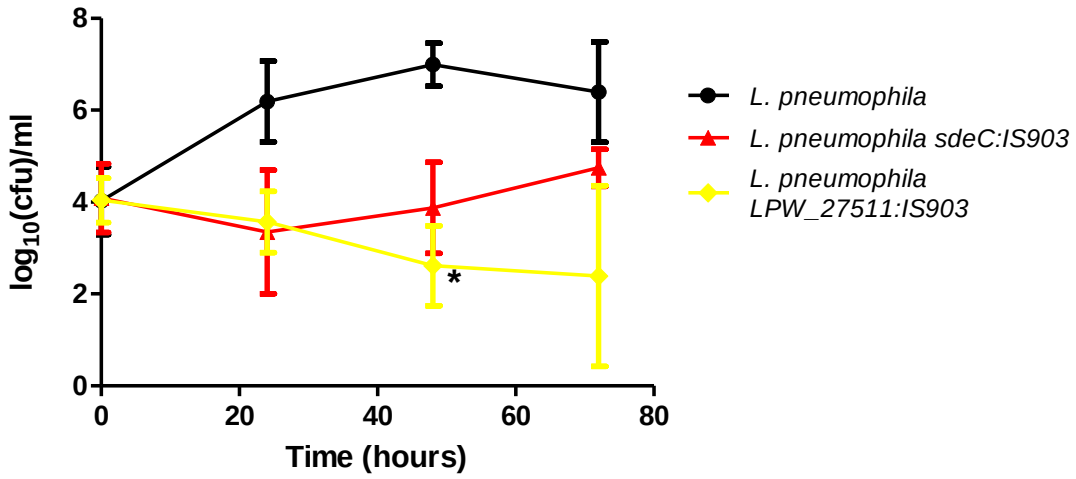


Figure 3.7. Replication of *L. pneumophila sdeC* and *lpw27511* transposon mutants in THP-1 macrophages. Line graph plotting log_{10}(cfu)/mL vs time in hours. *A. castellanii* was challenged with the *L. pneumophila* strains indicated. Cells were incubated at 37 °C and 5% CO_2. The assay was performed in duplicate over 72 h post-infection, plating CFU on BCYE agar at 0, 24, 48 and 72 h. * indicates statistically significantly different value (t-test, unpaired, two tailed, $P < 0.05$) in comparison to *L. pneumophila* 130b at indicated time point.

Chapter 4: Analysis of the putative LuxR transcriptional regulator encoded by *lpw27511*

4.1. Introduction

The *lpw27511* transposon mutant displayed a severe growth defect in *A. castellanii* suggesting an important role in intracellular survival and/or replication. However, the full length *lpw27511* gene is not present in all of the sequenced *L. pneumophila* strains, which suggests that the gene is not essential for intracellular replication and survival.

lpw27511 encodes a putative LuxR family transcription regulator. Other *L. pneumophila* genes that possess a sequence similarity with members of the LuxR family of transcriptional regulators have been identified in the *L. pneumophila* ATCC 33152 strain (Lebeau et al., 2004). LpnR2 (*L. pneumophila* regulator 2) and LpnR3 were both found to positively affect flagellin expression. LpnR2 is necessary for efficient invasion of *A. castellanii* and LpnR3 is necessary for efficient intracellular replication in the same organism. Lpw27511 may act in a similar manner, however, the regulatory targets of Lpw27511 are not currently known.

Apart from the possible regulation of flagellin expression, Lpw27511 may regulate other potential virulence factors. Transcriptional regulators often regulate nearby genes. Surrounding *lpw27511* is *lem26*, a gene encoding a Dot/Icm translocated effector of unknown function, and other hypothetical proteins of unknown function. Downstream of *lpg2524*, the Philadelphia strain *lpw27511 homolog,* is *lpg2525 (mavK)*, a gene encoding a Dot/Icm translocated effector that possesses an F-BOX domain. MavK is of particular interest as F-BOX domain containing proteins are largely a eukaryotic protein family involved in targeting proteins for ubiquitination and subsequent degradation by the cell proteasome. MavK may modulate eukaryotic

ubiquitination machinery and subsequently interfere with host cell processes. While a *mavK* homolog is not present downstream of *lpw27511* in the 130b strain, and so would not be responsible for the defect observed in the transposon mutant, the effector remains an interesting candidate for further study.

Here we analysed the distribution of *lpw27511* and *mavK* amongst a wide range of *Legionella* isolates of different serotypes. We also aimed to identify the regulatory targets of Lpw27511 and identify the cause of the major intracellular replication defect observed for the transposon mutant.

4.2. Results

4.2.1 Distribution of *lpw27511* and *mavK* in various *Legionella* isolates

Analysis of all sequenced *L. pneumophila* strains at the time of this study, found that full length *lpw27511* was only present in the 130b and Philadelphia strains and *mavK* was only present in the Philadelphia strain. In order to get a better idea of the distribution of these genes amongst different *Legionella* strains a southern blot was performed probing for *lpw27511* and *mavK/lpg2525* in several different clinical and environmental *Legionella* isolates of varying serogroups and *Legionella* species (Figure 4.1.). Southern hybridisation was chosen over PCR to account for nucleotide sequence variation. The full length gene was used as the southern blot probe in each case. These large probes will detect genes with complete sequence similarity and may also detect partial sequence similarity.

In addition to *L. pneumophila* Philadelphia and 130b strains, *lpw27511* was present in seven of the nine *L. pneumophila* isolates tested, indicating a relatively high level of conservation amongst the strains screened. However, it was not present in any of the six *L. longbeachae* or *L. micdadei* strains tested. In addition to the *L. pneumophila*

Philadelphia strain, *mavK* was present in four of the nine *L. pneumophila* isolates tested. It was not present in the six *L. longbeachae* or *L. micdadei* strains tested (Figure 4.1.), indicating a relatively moderate to low level of conservation amongst the strains screened.

4.2.2. Construction and complementation of defined *lpw27511* mutant

To confirm that the loss of *lpw27511* impaired *L. pneumophila* intracellular replication and to study the function of *lpw27511* further, an in-frame deletion mutant of *lpw27511* was created by replacing the full-length gene with a kanamycin resistance cassette (Figure 4.2.).

The genomic regions flanking *lpw27511* were cloned into the pPCRScript suicide vector using primers 1929-1932, creating an EcoRI restriction enzyme site in between the two regions. The kanamycin resistance cassette from pUC4-KISS was then cloned into the EcoRI site. This construct was added to naturally competent *L. pneumophila* 130b in liquid AYE broth allowing for natural uptake of the DNA plasmid and allelic replacement to replace *lpw27511* with the kanamycin resistance cassette. After seven days static culture at 30 °C samples were spread onto BCYE agar plates and kanamycin resistant colonies were selected. The deletion of *lpw27511* was confirmed via PCR using primers 2099 and 1717, binding to regions outside of the gene, to determine if it had been replaced with the kanamycin resistance cassette by determining the size of the amplified fragment (Figure 4.3.). The deletion was then also confirmed by sequencing the fragment generated using primers 2099 and 1717.

For complementation studies, full length *lpw27511* was cloned into the *Legionella* expression vector pMIP (Figure 4.3.). pMIP contains the *mip* promoter that will constitutively express the gene under its control (Wieland et al., 2002). The correct sequence was confirmed via sequencing. pMIP:*lpw27511* was then electroporated

into the *L. pneumophila lpw27511* deletion mutant and the presence of the correct construct was confirmed via PCR (Figure 4.3.).

4.2.3. DNA microarray of *L. pneumophila lpw27511* deletion mutant

To determine the regulatory targets of *lpw27511* a DNA microarray was performed comparing the gene expression profiles of the *L. pneumophila lpw27511* deletion mutant and wild-type *L. pneumophila* strain 130b. RNA of both strains was isolated at the exponential ($OD_{600\ nm}$ at 1.5) and post-exponential ($OD_{600\ nm}$ at 3.0) growth phases, corresponding to the replicative and infectious *L. pneumophila* growth phases respectively. RNA was then reverse transcribed and indirectly labelled with Cy5 or Cy3 dye. A DNA microarray containing gene-specific 70-mer oligonucleotides based on all predicted genes of the genomes of *L. pneumophila* strains Paris, Lens, and Philadelphia was used (Bruggemann et al., 2006), as these were the fully sequenced and annotated *L. pneumophila* strains available at the time. Results were normalised and differential analysis was performed using the R software program (http://www.r-project.org/). The results of three DNA microarrays, each using two biological replicates of each sample with a Cy3-Cy5 dye swap, were averaged and differences in gene expression between the two strains were calculated.

Differential analysis was carried out separately for each comparison, using the VM method (VarMixt package), together with the Benjamini and Yekutieli P value adjustment method. Differential expression with a P value greater than 0.05 and/or a fold change less than 2 were deemed insignificant.

In the exponential growth phase, corresponding to the replicative form of *L. pneumophila*, no significant differences between the two expression profiles were detected. In the post-exponential growth phase, corresponding to the infectious form

of *L. pneumophila*, expression levels of several genes of varying function were increased or decreased in the *lpw27511* mutant (Table 4.2.).

Table 4.1. Summary of differences in gene expression between *L. pneumophila* strain 130b and *L. pneumophila lpw27511* deletion mutant in the post-exponential growth phase. A fold-change above one indicates increased expression in the deletion mutant; conversely, a negative fold-change indicates reduced expression in the mutant.

Gene	Fold-change	Homologues	Function/motif
lpp2263	-5.26	*lpl2235, lpg2315, lpc1781, lpa03320, lpw25041*	
lpp2636	-3.70	*lpa03778, lpc0562, lpl2506, lpw28311*	
lpp1454	-2.56	*lpl1532, lpc0913, lpa02179, lpg1494, lpw15161*	Putative UDP-N-acetylmuramate--alanine ligase
EnhA	-2.13		Enhanced entry protein
lpp2032	-2.08	*lpa02991, lpc1535, lpg2049, lpl2027, lpw21131*	
lpp1025	2.02	*lpa01453, lpc2324, lpg0963, lpl0992, lpw10491*	Similar to substrate of Dot/Icm T4SS
sdeD	2.08		Substrate of Dot/Icm T4SS
cspC	2.08		Stress protein, member of the CspA-family

letE	2.09		Transmission trait enhancer protein
lpp0688	2.12	*lpa00996, lpc2660, lpg0634, lpl0671, lpw07081*	Similar to substrate of Dot/Icm T4SS
hisB	2.12		Histidine biosynthesis
lpp2169	2.15	*lpa03182, lpc1682, lpg2217, lpl2142, lpw24031*	Fragment of putative chitinase
hisA	2.16		Histidine biosynthesis
lpp1030	2.19	*lpa01460, lpc2319, lpg0968, lpl0997, lpw10541*	
lpp1409	2.26	*lpa02119, lpc0868, lpg1453, lpl1591, lpw14711*	
lpp0959	2.32	*lpc2395, lpg0898, lpl0929, ceg18*	Substrate of Dot/Icm T4SS
hisC1	2.36		Histidine biosynthesis
sidD	2.45		Substrate of Dot/Icm T4SS, deAMPylase
lpp2275	2.52	*lpa03335, lpc1794, lpg2327 lpl2247, lpw25181*	Similar to substrate of Dot/Icm T4SS
ppeB	2.56		Substrate of Dot/Icm T4SS
lpp1893	2.67	*lpa02774, lpc1372, lpg1918, lpl1882, lpw19571*	

fis	2.90		Transcriptional regulator
lpp2433	2.91	*lpw25701*	

The differences observed in the majority of genes detected were relatively low. Of most significance was a gene of unknown function, *lpp2263*, with a 5.26-fold decrease in expression levels in the *lpw27511* mutant. *lpp2263* encodes a hypothetical 8.5kDa protein with no known functional domains.

Unless the unstudied *lpp2263* is alone essential for *A. castellanii* intracellular replication, these results would not likely explain the extreme growth defect observed for the *lpw27511* transposon mutant during *A. castellanii* co-culture assays. Therefore, the defined mutant was retested in an *A. castellanii* co-culture plate reader assay to confirm the replication defect.

4.2.4. Co-culture of *L. pneumophila* Δ*lpw27511* with *A. castellanii* evaluating replication over 70 hours.

Similar to previous *A. castellanii* co-culture assays, here *A. castellanii* was challenged with the *lpw27511* deletion mutant to evaluate intracellular replication over 70 hours post-infection. Each of three independent assays were performed in duplicate and OD was measured at 600 nm every 5 h in a Fluostar Omega incubated plate reader at 37 °C (Figure 4.4). This measures the optical density of bacteria, giving an indication of the concentration of bacteria in the liquid sample. The microplate reader used possesses a controlled temperature incubation chamber so the microplate could be housed in the

microplate reader for the duration of the experiment with no need to disturb the sample.

In contrast to the severe defect displayed by the *lpw27511* transposon insertion mutant, the *lpw27511* deletion mutant did not differ from wild type *L. pneumophila*, although there was a slight trend towards reduced bacterial numbers at the later stages of infection (Figure 4.4.). Complementation with pMIP:*lpw27511* had no effect on intracellular replication. Given the discrepancy between the defined deletion mutant and the transposon insertion mutant, we sought an explanation for the significant differences in intracellular replication.

4.2.5. Complementation of *lpw27511* transposon mutant

To verify that the interruption of *lpw27511* was the reason for the intracellular replication defect of the *lpw27511* transposon mutant, this mutant was complemented with full length *lpw27511* gene in the *Legionella* expression vector pMIP and tested in an amoebae plate test. Four colonies of the complemented *lpw27511 L. pneumophila* transposon mutants were tested and growth was compared to wild-type *L. pneumophila* strain 130b containing the empty pMIP vector and the *lpw27511 L. pneumophila* transposon mutant containing the empty pMIP vector (Figure 4.5). Growth levels of the complemented mutant were similar to that of the transposon mutant indicating that reintroduction of *lpw27511* into the *lpw27511* transposon insertion mutant did not restore replication to that of wild levels. Hence, the intracellular replication defect of the *lpw27511* transposon insertion mutant was not due to the loss of a functional *lpw27511* gene alone.

4.2.6. Evaluation of the effector translocation capabilities of the *L. pneumophila lpw27511* transposon mutant

To understand further the intracellular replication defect of the *lpw27511* transposon insertion mutant we hypothesised that the mutant had additional mutations or defects outside the *lpw27511* gene itself. In order to identify transcription start sites, upstream regions of genes were scanned for putative SigmaA binding sites using PPP (http://bioinformatics.biol.rug.nl/websoftware/ppp). This revealed that *lpw27511* was not part of an operon and so the transposon insertion would not have had any polar effects on downstream genes. The defect seen in the transposon mutant may have been due to a spontaneous mutation in another area of the genome.

The observed defect in the transposon mutant is similar to strains lacking a functional Dot/Icm T4SS. Therefore, a TEM-1 translocation reporter system was used to determine if the transposon mutant had functioning Dot/Icm secretion machinery (Charpentier and Oswald, 2004; de Felipe et al., 2008). RalF is a known Dot/Icm T4SS translocated effector and so a TEM-1-RalF fusion was used to test translocation capacity of the *lpw27511* transposon insertion mutant (Nagai et al., 2002). The *Legionella* expression vector pXDC61 carrying the TEM-1-RalF fusion was electroporated into the transposon mutant. Wild type *L. pneumophila* and a *L. pneumophila dotA* mutant, both also expressing the RalF-TEM fusion, were used to compare Dot/Icm translocation efficiency upon 5 h infection of B6 mouse bone marrow-derived macrophages. β-Lactamase activity of translocated protein was detected by measuring cleavage of the fluorescent CCF2/AM substrate using a Fluostar Omega microplate reader. After excitation at 410 nm cleaved and uncleaved CCF2/AM will emit light at wavelengths of 450 and 520 nm respectively, with the difference recorded as an emission ratio of 450/520 nm. Data presented are mean values of the results from triplicate wells from at least two independent experiments (Figure 4.6.).

The *lpw27511* transposon mutant translocated RalF at a similar rate to wild type *L. pneumophila* indicating that the mutant possessed a functional Dot/Icm T4SS. A non-

functional Dot/Icm T4SS was therefore not responsible for the defect seen in the transposon mutant.

4.2.7. Sequencing the *L. pneumophila lpw27511* transposon mutant genome

We proposed that the *lpw27511* transposon mutant may have acquired a random mutation in another region of the genome essential for replication within host cells. If this is the case, it may reveal new information about genes essential for intracellular replication. In order to detect a random mutation, the genome of the transposon mutant was sequenced using Ion Torrent sequencing and fragments were assembled using the published *L. pneumophila* strain 130b genome as a reference genome.

After PCR amplification and sequencing to correct sequencing error present in the genome, the transposon mutant genome was compared to the 130b reference genome. Apart from the transposon insertion there were no detectable differences in the transposon mutant genome compared to the 130b reference genome. Hence, an explanation for the attenuation of the *lpw27511* transposon insertion mutant could not be definitively found.

4.3. Discussion

At the time of this study, of the sequenced *L. pneumophila* strains only the Philadelphia and 130b strains possessed a full length *lpw27511* gene. A current search revealed the gene is also present in the *L. pneumophila* strains ATCC 43290, LPE509, Thunder Bay and Lorraine. In addition to these strains, other *Legionella* species *Legionella massiliensis* and *Legionella sainthelensi*, possess LuxR-like regulators that share more than 80% amino acid sequence similarity to Lpw27511.

Southern blot analysis of the distribution of *lpw27511* revealed that seven of nine *L. pneumophila* Australian isolates possessed *lpw27511* and the gene was not present in any of the non-*pneumophila* strains tested (Figure 4.1). *lpw27511* also does not have any nucleotide sequence similarity to the other identified LuxR-like transcriptional regulators including *lpnR1, lpnR2, lpnR3* and *lpnR4* (or *letA*). This data, in addition to the search of currently available sequenced *Legionella* strains, indicated a relatively high level of conservation among *L. pneumophila* strains.

At the time of this study, *mavK/lpg2525* was found in one sequenced *L. pneumophila* isolate, the Philadelphia strain. A current search revealed the gene is also present in the *L. pneumophila* strains ATCC 43290, LPE509 and Thunder Bay. Southern blot analysis of additional *Legionella* strains revealed that four of nine *L. pneumophila* Australian isolates possess *mavK* (Figure 4.1). However, *mavK* was not present in any of the non-*pneumophila* strains tested. A moderate level of conservation amongst the strains tested may indicate that *mavK* does not play a central role in *Legionella* survival or the presence of functional redundancy with other *Legionella* proteins. It was interesting to note that all of the strains that possessed *mavK* also possessed *lpw27511*, suggesting a possible link between the two. The opposite, however, was not the case.

The Dot/Icm T4SS mediates the formation of K48 and K63 poly-ubiquitin conjugates to proteins associated with the LCV in macrophages and dendritic cells (Ivanov and Roy, 2009). The same study also found that *L. pneumophila* is able to suppress ubiquitin-rich dendritic cell aggresome-like structures (DALIS) in macrophages and dendritic cells in a Dot/Icm dependent manner. A *mavK* deletion mutant was found to have no defect in DALIS suppression or the ability to recruit ubiquitin conjugates to the LCV when compared with wild-type *L. pneumophila*, indicating MavK is not involved in these processes. At this stage the function of MavK during infection is unknown.

To determine the contribution of *lpw27511* to *L. pneumophila* pathogenesis, here we created a defined non-polar *lpw27511* mutant by replacing the gene with a kanamycin

resistance cassette. The gene expression profile of the deletion mutant was compared to that of wild type *L. pneumophila* in order to determine the regulatory targets of *lpw27511* (Table 4.2.). No significant changes in expression levels were detected in the exponential growth phase but in the post-exponential growth phase, corresponding to the infectious phase of *Legionella*, several changes were detected. The expression levels of several genes were found to be weakly increased or decreased by Lpw27511 with a low 2-3 fold change in expression. Many of genes identified were hypothetical proteins of unknown function. The most significant result was a 5.26 fold decrease in expression of *lpp2263* (Paris strain homolog of *lpw25041* in *L. pneumophila* 130b) in the mutant, indicating up-regulation of this gene when Lpw27511 is present. Lpp2263 is a hypothetical protein of unknown function and hence this does not inform us about the function of Lpw27511.

There was no obvious explanation in the microarray results for the major replication defect seen in the *lpw27511* transposon mutant during *A. castellanii* infection. Expression levels of *sdeD*, a translocated effector of unknown function, *letE*, a transmission trait enhancer, genes similar to Dot/Icm translocated effectors and various metabolic genes were changed in the *lpw27511* mutant but at low levels. Confirmation of these changes in expression via qRTPCR experiments is needed before any conclusions can be made as to whether these genes are regulated by Lpw27511. In addition, the defined mutant used for microarray analysis was not found to be significantly attenuated for intracellular replication.

The DNA microarray was only able to detect expression of genes from the *L. pneumophila* Philadelphia, Paris, Lens and Corby strains as these were the only annotated genomes available at the time. If Lpw27511 regulated genes that are present in the 130b strain but absent in the Philadelphia, Paris, Lens and Corby strains these genes would not have been detected in this microarray. The microarray was also performed using bacterial cultures in ACES broth and not during infection. There may be changes in the gene expression profile during infection that was not detected here in the DNA microarray performed only in culture.

Despite a slight trend of reduced bacterial numbers at later stages of infection, intracellular replication of the *lpw27511* deletion mutant in the presence of *A. castellanii w*as not significantly attenuated compared to wild type *L. pneumophila* (Figure 4.4.). This is in contrast to the severe defect seen with the *lpw27511* transposon mutant in the presence of *A. castellanii*. A lack of severe attenuation of intracellular replication upon deletion is not uncommon in the other studied LuxR-like transcriptional regulators. Of the LpnR LuxR-like regulators, only *lpnR2* and *lpnR3* deletion mutants display a mild defect in *A. castellanii* intracellular replication (Lebeau et al., 2004). We took several approaches to explain the large differences in intracellular replication displayed between the *lpw27511* mutants.

To confirm whether the severe defect of the *lpw27511* transposon mutant was due to the disruption of the *lpw27511* gene alone the transposon mutant was complemented with full length *lpw27511* and replication was compared to the transposon mutant and wild type *L. pneumophila* in an amoebae plate test (Figure 4.5.). Replication of the complemented strain was similar to that of the transposon mutant indicating that *lpw27511* alone was not responsible for the growth defect seen during intracellular replication. We postulated that the differences in intracellular replication could be explained by random mutations in other parts of the genome.

Since the defect of the transposon mutant was similar to that of strains with non-functioning Dot/Icm T4SS, we hypothesised that the transposon mutant may have acquired a random mutation in a *dot/icm* gene. Therefore, a TEM-1 translocation reporter system was used to determine if the transposon mutant had a functioning Dot/Icm T4SS by testing the strains ability to translocate the known effector RalF (Figure 4.6.). However, the transposon mutant was able to translocate RalF at similar rates to wild type *L. pneumophila*, indicating that the defect observed in the transposon mutant was not due to a non-functioning Dot/Icm T4SS. Moreover, sequencing the entire genome of the *lpw27511* transposon insertion mutant did not reveal any additional mutations when compared to wild type *L. pneumophila* strain 130b. Although, sequencing error, particularly in areas of repeat sequences such as

HPTs, and the gaps present after assembly of the genome may mask the presence of a random mutation.

The reason for the defect in the *lpw27511* transposon mutant remains unknown. *lpw27511* is not part of an operon and there should not be polar effects on any downstream genes, however transcription start and stop sites would need to be confirmed experimentally. Another possible explanation for the observed defect in the transposon mutant may be the production of a dominant negative phenotype with the gene-transposon product. Although unlikely, it remains possible that the transposon insertion created an Lpw27511-transposon fusion product that produced the replication defect observed. This may be analysed experimentally by expressing the fusion product in the *lpw27511* deletion mutant and testing intracellular replication in an *A. castellanii* co-culture assay. Given the lack of a direct role for Lpw27511 in intracellular replication of *L. pneumophila* strain 130b, we did not pursue further characterisation of this gene.

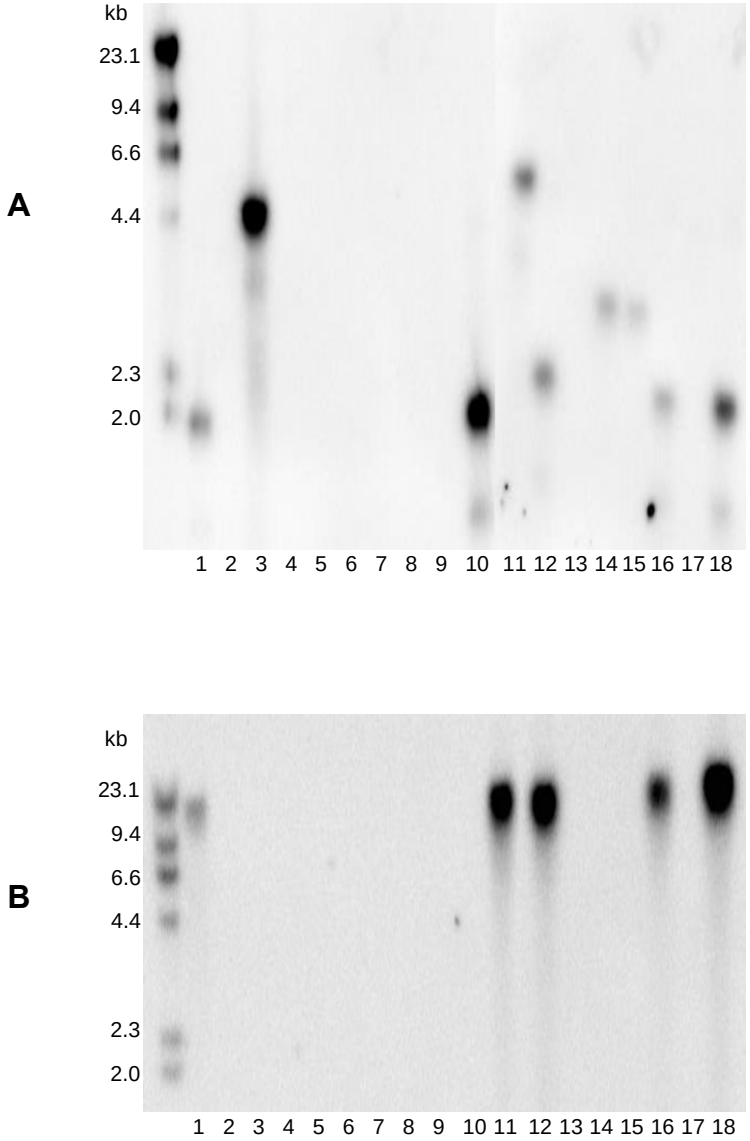


Figure 4.1. Southern blots investigating the prevalence of *lpw27511 and lpg2525/mavK* in various *Legionella* strains of different species. Southern blots of Genomic DNA of the strains indicated was probed with the full length genes *lpw27511* (**A**) and *lpg2525* (**B**). 1- *L. pneumophila* Philadelphia, 2- *L. pneumophila* Paris, 3- *L. pneumophila* 130b, 4- *L. longbeachae* A5H5, 5- *L. longbeachae* LA-24, 6- *L. longbeachae* L6C9, 7- *L. longbeachae* K8B9, 8- *L. longbeachae* NSW150, 9- *L. micdadei*, 10- *L. pneumophila* 02/41, 11- *L. pneumophila* 03/44, 12- *L. pneumophila* 03/46, 13- *L. pneumophila* 03/54, 14- *L. pneumophila* 03/55, 15- *L. pneumophila* 03/57, 16- *L. pneumophila* 03/59, 17- *L. pneumophila* 03/61, 18- *L. pneumophila* 03/63. DNA ladder with known fragment sizes, in kb, added for comparison.

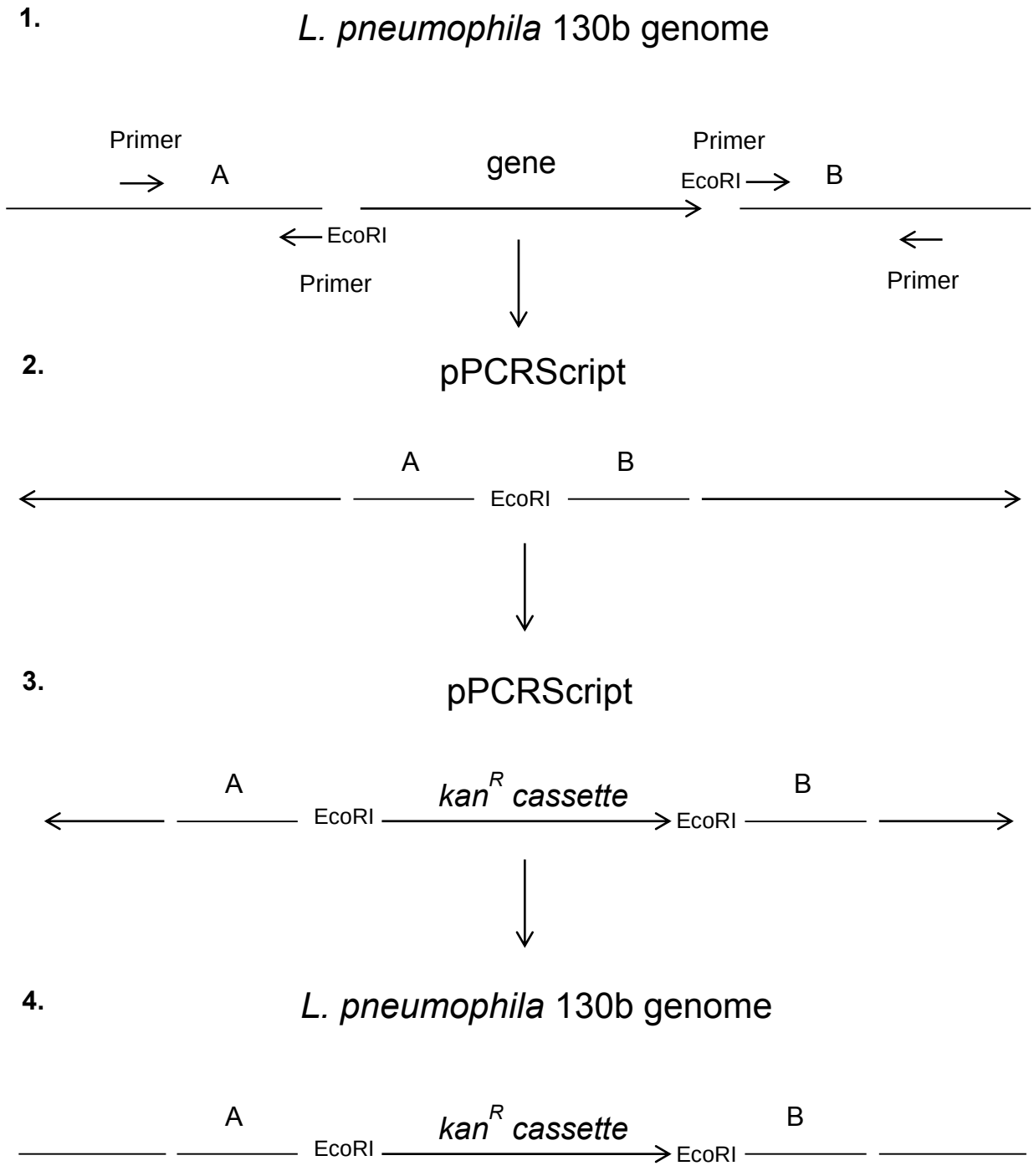

1.
L. pneumophila 130b genome
Primer
A
gene
Primer
EcoRI
B
EcoRI
Primer
Primer
2.
pPCRScript
A
B
EcoRI
3.
pPCRScript
A
kan^R cassette
B
EcoRI
EcoRI
4.
L. pneumophila 130b genome
A
kan^R cassette
B
EcoRI
EcoRI

Figure 4.2. Strategy for construction of *L. pneumophila* deletion mutants. Primers are used to amplify areas A and B via PCR (**1.**). A second round of PCR is performed, combining the two fragments. The primers were constructed so that an EcoRI restriction enzyme site is created in between the two fragments. The fragment is then ligated into the MCS of pPCRScript, a suicide vector in *L. pneumophila* (**2.**). A kanamycin resistance cassette is ligated into the EcoRI site between regions A and B (**3.**). The plasmid is introduced into *L. pneumophila* strain 130b and the gene is replaced with the kanamycin resistance cassette via allelic exchange (**4.**).

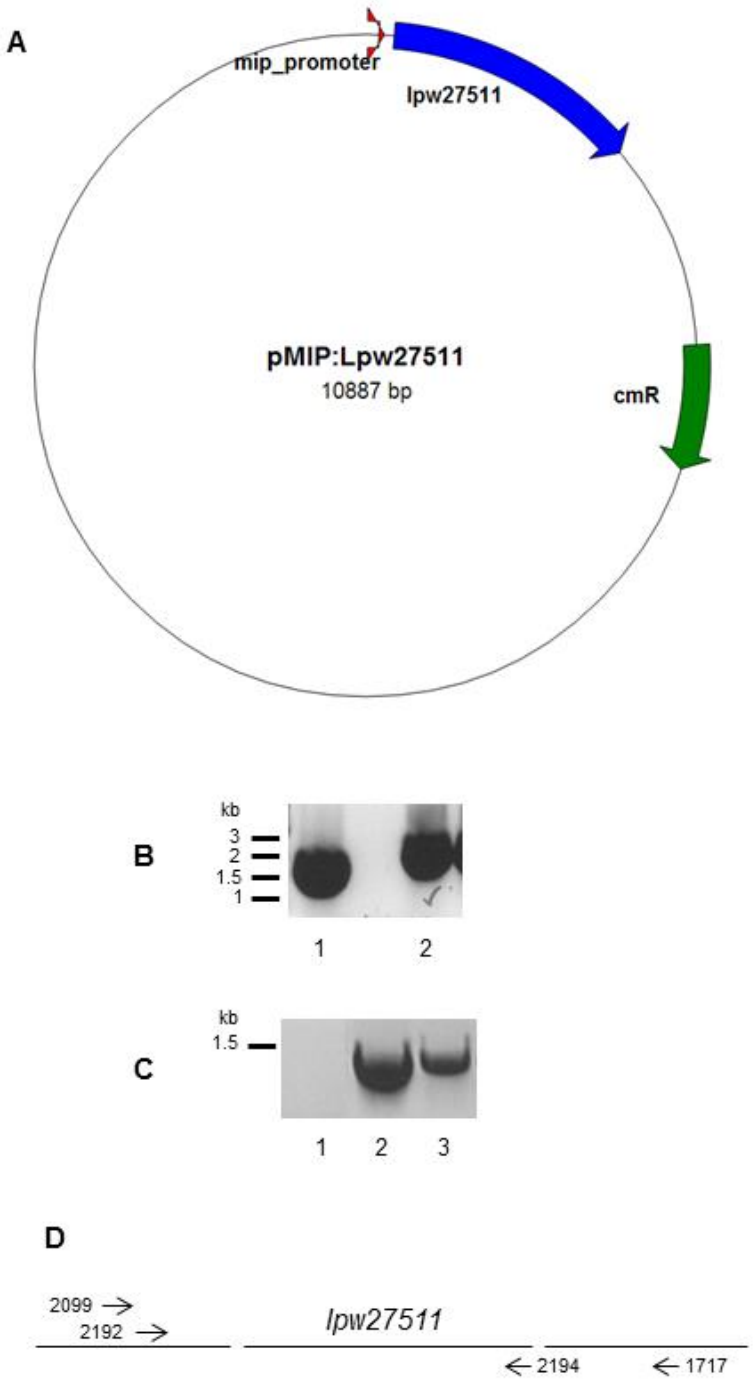

A
mip_promoter
lpw27511
pMIP:Lpw27511
10887 bp
cmR
B
kb
3
2
1.5
1
1
2
C
kb
1.5
1
2
3
D
2099
2192
lpw27511
2194
1717

Figure 4.3. The organisation of pMIP:Lpw27511 and PCR analysis of the *L. pneumophila lpw27511* deletion mutant and the complemented *L. pneumophila lpw27511* mutant. A) Organisation of pMIP:Lpw27511. Representative scale vector map. *cmR*- chloramphenicol resistance gene. Plasmid map created using Savvy (http://bioinformatics.org/savvy/). **B) Agarose gel electrophoresis of PCR products using primers 2099 and 1717 (located outside of the deleted region) to confirm insertion of the kanamycin resistance cassette in the *L. pneumophila lpw27511* deletion strain.** If *lpw27511* is replaced with the kanamycin resistance cassette a fragment of approximately 2.3 kb will be produced. An intact gene will produce a fragment of approximately 1.8 kb 1- *L. pneumophila* 130b strain, 2- *L. pneumophila lpw27511* deletion mutant. DNA ladder with known fragment sizes, in kb, added for comparison. **C) Agarose gel electrophoresis of PCR products using primers 2192 and 2194 to confirm the presence of *lpw27511* in pMIP:Lpw27511 in the complemented *L. pneumophila lpw27511* deletion strain**. 1- *L. pneumophila lpw27511* deletion mutant, 2- *L. pneumophila* 130b strain (native *lpw27511*), 3- *L. pneumophila lpw27511* deletion mutant (pMIP:Lpw27511). DNA ladder with known fragment sizes, in kb, added for comparison. **D) Location of primers used in B and C**.

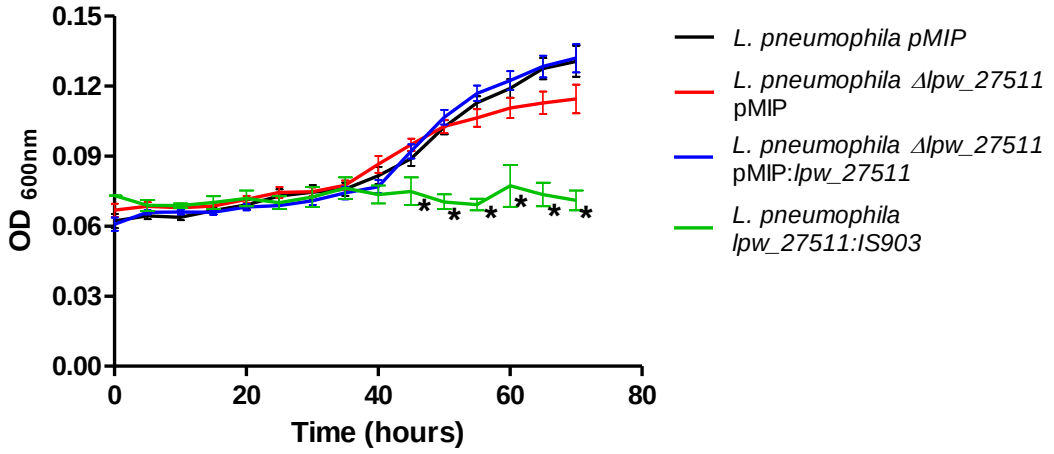


Figure 4.4. *L. pneumophila-A. castellanii* co-culture plate reader assay comparing the growth of the *lpw27511* defined mutant to various other strains. Line graph plotting OD $_{600nm}$ vs time in hours. *A. castellanii* was challenged with the *L. pneumophila* strains indicated. The OD_{600nm} was measured every five hours for 70 hours in a Fluostar platereader (BMG labtech). Cells were incubated at 37 °C. * indicates a statistically significantly different value ($P < 0.05$, t-test, unpaired, two tailed) in comparison to *L. pneumophila* (pMIP) at indicated time points.

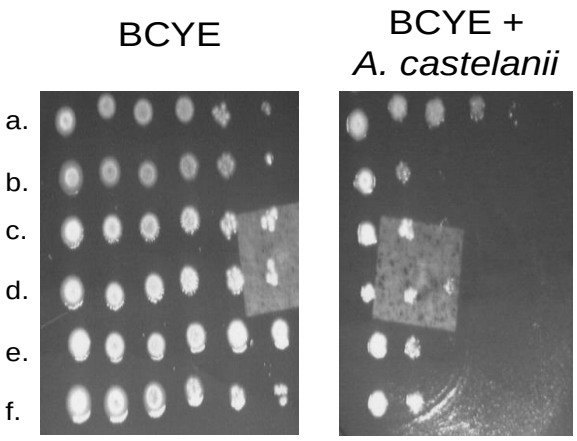


Figure 4.5. Amoebae plate test comparing the growth of *lpw27511 L. pneumophila* transposon mutant and the *lpw27511* complemented transposon mutant. Images of BCYE agar plates in the presence and absence of *A. castellanii* spotted with six 10-fold serial dilutions of *L. pneumophila* 130b (pMIP) (a), *L. pneumophila lpw27511:IS903 (b), L. pneumophila lpw27511:IS903 (pMIP: lpw27511) (c-f)* and incubated for 72 hours at 37 °C.

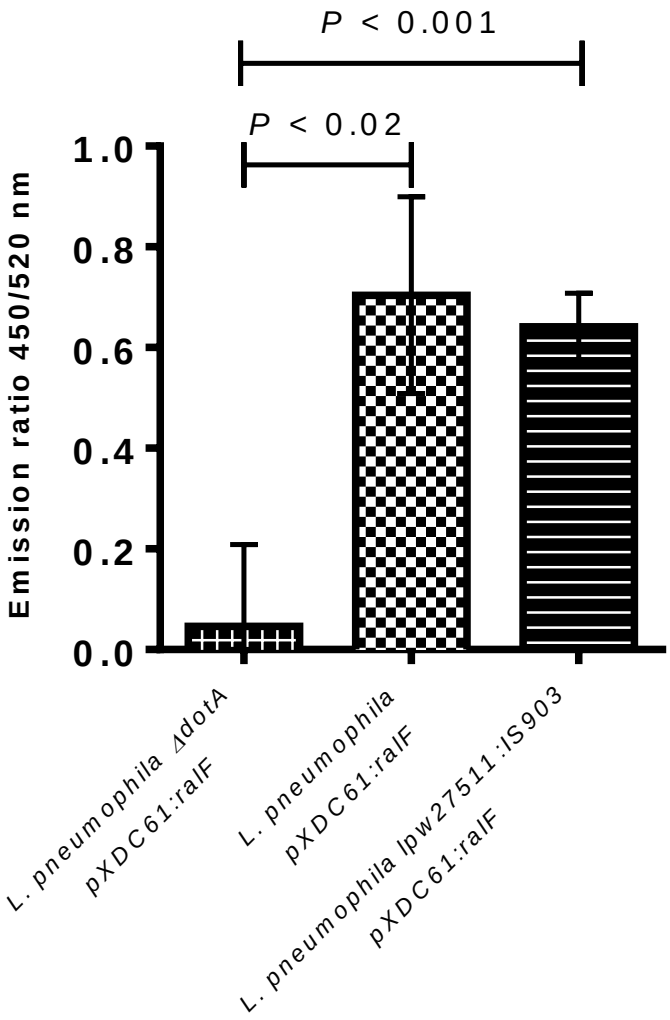


Figure 4.6. Analysis of the Dot/Icm T4SS translocation efficiency of the *L. pneumophila lpw27511* transposon mutant. Bar graph of emission ratio of 450/520 nm measured after infection vs bacterial strains. B6 mouse bone marrow derived macrophages were infected with the bacterial strains indicated. All strains expressed the TEM-RalF fusion protein. After infection, cells were washed and loaded with the CCF2/AM substrate. β-Lactamase activity in cells was detected by measuring cleavage of the CCF2/AM substrate with a fluorescence microplate reader and is recorded as an emission ratio of 450/520 nm. A t-test (unpaired, 2-tailed, $P < 0.05$) was used to determine statistically significant differences between infections.

Chapter 5: Role of SdeC in biology of *L. pneumophila*

5.1. Introduction

The transposon mutagenesis screen performed as part of this work also identified *sdeC* as necessary for efficient intracellular replication. SdeC has also been shown to be important during infection in a previous study (Luo and Isberg, 2004). Despite this, current knowledge of the mechanism of action of SdeC is extremely limited with the only information known being that it is translocated into host cells during infection via the Dot/Icm T4SS (Luo and Isberg, 2004).

sdeC is a large gene of approximately 4.6 kb in length. The gene is present in all sequenced strains of *L. pneumophila* and is located near other *sidE* paralogues, *sdeB* and *sdeA*, as well as *lpg2154*, a gene coding for a Sid-related protein, and other genes coding for hypothetical proteins of unknown function. The secondary structure of SdeC displays no similarity to protein motifs predictive of function (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

One paralogue, SdeA has been shown to interact with IcmS, a chaperone protein of the Dot/Icm T4SS (Bardill et al., 2005). A polyclonal antibody that recognises members of the SidE family (SdeA, SdeB and SdeC) was used in the same study to observe localisation after infection using immunofluorescence microscopy. Thirty minutes post-infection the antibody detected the presence of SidE related proteins on the phagosomal membrane adjacent to the bacterial cell poles. However due to the cross reactivity of the antibody to multiple SidE paralogues, it is not known whether only one of the proteins localised here at this time or all of them. It is also unclear whether the detection of the protein/proteins at the phagosomal membrane adjacent to the bacterial cell poles is due to secretion at this location or if they localise here for a

functional role. Localisation at later time points post-infection was not analysed in this study (Bardill et al., 2005).

As a proven Dot/Icm translocated effector of unknown function, SdeC, and the other members of the SidE family, are exciting candidates for further characterisation. Translocation into the host cell, retention of numerous paralogues in the *L. pneumophila* genome and the intracellular replication defect observed when SdeC is absent indicate an important function during infection, warranting further investigation.

Here we created a defined *sdeC* deletion mutant and re-tested it in intracellular replication assays. We also created a *L. pneumophila* strain expressing 4xHA-tagged SdeC and used it to observe SdeC localisation in macrophages and amoebae during all stages of infection. EGFP-tagged SdeC was used in transfection studies to observe localisation of the singular protein in the absence of infection. These epitope tags were used with SdeC for immunoprecipitation studies in order to identify any binding partners. Any results obtained via these experiments will aid in elucidating the function of SdeC during infection of host cells.

5.2. Results

5.2.1. Construction and complementation of defined *sdeC* deletion mutant

To confirm the replication defect seen with the transposon mutant an in-frame *sdeC* deletion mutant was created for retesting in co-culture replication assays. The deletion mutant was created via allelic exchange where the full-length gene was replaced with a kanamycin resistance cassette as described for *lpw27511* in 4.2.2.

The genomic regions flanking *sdeC* were cloned into a pPCRScript plasmid using primers 1933, 1936, 1973 and 1974 (Table 2.3), creating an EcoRI restriction enzyme site in between the two regions. The kanamycin resistance cassette from pUC4-KISS was then cloned into the EcoRI site. This construct was added to the naturally competent *L. pneumophila* 130b in liquid culture allowing for natural uptake of the DNA plasmid and facilitating an allelic exchange, replacing *sdeC* with the kanamycin resistance cassette. After seven days of static culture at 30 °C samples were spread onto BCYE agar plates and kanamycin resistant colonies were selected. The deletion of *sdeC* was confirmed via PCR using primers 2093 and 2094 (Figure 5.1.).

Full length *sdeC* was amplified using primers 2195 and 2196 and was cloned into the *Legionella* expression vector pMIP for complementation studies. The correct sequence was confirmed with sequencing and a positive complementation vector was then electroporated into the *sdeC* deletion mutant and kanamycin\chloramphenicol resistant colonies were selected. The presence of the correct vector in the complemented strain was confirmed by PCR (Figure 5.1.). The created *sdeC* deletion mutant and the complemented *sdeC* mutant strains were then used for subsequent infection studies.

5.2.2. Intracellular replication of *L. pneumophila sdeC* deletion mutant in amoebae and macrophages

To determine if the in-frame *L. pneumophila sdeC* deletion mutant possessed the same intracellular replication defect seen with the *sdeC* transposon mutant, the strain was tested in an *A. castellanii* co-culture replication assay.

Here *A. castellanii* was infected with the *sdeC* deletion mutant, the complemented mutant and wild type *L. pneumophila* strain 130b to evaluate intracellular replication over 70 hours post-infection. Each of three independent assays were performed in

duplicate and OD was measured at 600 nm every 5 h in a FLUOstar Omega microplate reader incubating at 37 °C (Figure 5.2).

Analysis of the assay data revealed that while the *sdeC* deletion mutant displayed a significant replication defect after 50 h post-infection, at levels similar to that of the previously analysed *sdeC* transposon mutant, the complemented mutant did not replicate at wild type levels as expected. A lack of complementation may indicate that SdeC alone is not responsible for the intracellular replication defect observed for the mutant or that the complemented strain was not expressing SdeC.

Replication of the *sdeC* deletion mutant, the complemented mutant and wild type *L. pneumophila* strain 130b was also tested over 72 h in the presence of B6 mouse bone marrow-derived macrophages and THP-1 cells (Figure 5.3. and Figure 5.4. respectively). Viable count intracellular replication assays were conducted in both cell lines and CFU were plated at 0, 24, 48 and 72 h. Similar results to the *A. castellanii* replication assay were observed where the deletion mutant displayed a replication defect but the complemented mutant was again unable to restore replication to wild type levels in THP-1 cells. However, while not reaching wild type levels, the complemented *sdeC* mutant displayed significantly higher CFU at 72 h post infection in murine BMDMs in comparison to the *sdeC* deletion mutant, indicating partial complementation at this late time point.

PCR amplification and subsequent DNA sequencing was used to reconfirm that the integrity of the complementation plasmid was not compromised and qRT-PCR was used to confirm expression of *sdeC* in the complemented mutant (Figure 5.5.). DNA sequencing revealed that the sequence of the *sdeC* gene on the complementation plasmid was identical to that of wild type *sdeC*. mRNA was isolated and reverse transcribed into cDNA, which was then exponentially amplified using gene specific primers and detected using a SYBRGreen based quantitative PCR detection system in a Stratagene Mx3005P Real Time PCR machine. *sdeC* expression levels of the complemented mutant and the *sdeC* deletion mutant were compared to that of wild

type *L. pneumophila* strain 130b. The *16s rRNA* gene from *L. pneumophila* 130b was used as a reference gene for data normalisation. This was included to correct for errors in sample quantification and sample to sample variations in qRT-PCR efficiency.

Analysis of the qRT-PCR results revealed that *sdeC* in the complemented mutant strain was expressed at levels 8.69-fold higher than the wild type *L. pneumophila* strain 130b (Figure 5.5.). The *sdeC* deletion mutant displayed expression levels 32-fold less than wild type *L. pneumophila*, with amplification plots on par with the no template negative control sample (data not shown). This data indicated that *sdeC* was indeed expressed in the complemented *sdeC* deletion mutant strain at levels exceeding wild type *L. pneumophila* strain 130b and so lack of expression was not the cause of the lack of functional complementation seen during the intracellular replication assays.

5.2.3. Analysis of lung colonisation ability in A strain mice

To determine if SdeC was also important for colonisation and replication in a mouse lung a lung colonisation assay was performed in A strain mice. This assay was also performed to determine if the lack of complementation observed in the complemented *sdeC* deletion mutant strain during the previous intracellular replication assay would also be present in a mammalian *in vivo* setting.

An established A strain mouse model was used to investigate *L. pneumophila* lung colonisation. Anaesthetised mice were inoculated intranasally with approximately 2.5 x 10^6 CFU of each strain under investigation. Here the ability of the *L. pneumophila sdeC* deletion mutant to colonise a mouse lung was compared to wild type *L. pneumophila* strain 130b and the *L. pneumophila sdeC* complemented mutant (Figure 5.6.). At 72 h post-infection, lungs were harvested and homogenised. Bacteria were isolated from lung tissue by host cell lysis with saponin, a detergent that does not lyse bacterial cells.

Viable bacterial numbers were determined by plating homogenate on BCYE agar plates for counts of CFU.

Bacterial numbers of the *sdeC* deletion mutant were two orders of magnitude lower than wild type *L. pneumophila* strain 130b. Interestingly, in this mouse model numbers of the complemented *sdeC* mutant were significantly higher than the *sdeC* deletion mutant and not significantly different from wild type *L. pneumophila,* indicating that complementation under these conditions was successful (Figure 5.6.).

Overall the *sdeC* deletion mutant was found to be significantly less fit than both wild type *L. pneumophila* and the complemented *sdeC* mutant during lung infection in A strain mice over 72 hours.

5.2.4. Analysis of SdeC localisation in the host cell

Since we observed that SdeC was important for efficient infection of several different host species, further analysis of SdeC during infection was warranted. Here we began characterisation of SdeC initially by performing effector localisation studies. The location of SdeC during infection may indicate a potential interaction with a host or bacterial component. Here we performed localisation studies using epitope tagged SdeC and immunofluorescence microscopy techniques.

SdeC was epitope tagged at the N-terminus with a 4x hemagglutinin (HA) tag by cloning full length *sdeC* into the MCS of the plasmid pICC562 (Dolezal et al., 2012). The correct gene fusion sequence was confirmed via PCR and DNA sequencing using primers 883 and 1105 (Table 2.3.). The confirmed plasmid was electroporated into *L. pneumophila* strain 130b. Expression of the 4HA-tagged SdeC was visualised by immunoblot (Figure 5.7A) and while expression of SdeC was confirmed, a large amount of protein degradation was also observed.

HEK293T cells (chosen for their ease of use and their ER biogenesis being the same as other human cell lines) and *A. castellanii* were infected with *L. pneumophila* (pICC562:*sdeC*) for 24 h at 37 °C and localisation of SdeC was observed after fluorescent immunostaining. Nucleic acid was visualised with 4',6-diamidino-2-phenylindole (DAPI), *L. pneumophila* was visualised using an anti-*L. pneumophila* serogroups 1 antibody and 4xHA-tagged SdeC was detected with an anti-HA antibody both coupled to fluorescent secondary antibodies (Figure 5.7.B).

In both HEK293T cells and *A. castellanii,* SdeC localised to the LCV and was present throughout the LCV membrane network. Within the same host cell multiple LCVs were observed, presumably arising from infection by multiple bacteria. Interestingly, only some of the vacuoles within the same cell were positive for the presence of SdeC.

Overall SdeC displayed an interaction with the LCV upon infection with two localisation patterns. In addition, expression was not observed in all infected cells. These different observations may have indicated that the localisation of SdeC was time dependent.

5.2.5. Time course of SdeC localisation during infection of HEK293T cells

Our investigation of the localisation of SdeC revealed that when SdeC is expressed during infection of a host cell it localised predominantly with the LCV membrane. To determine if this localisation pattern was time dependent, we performed a time course observing SdeC localisation during infection. HEK293T cells were infected with *L. pneumophila* expressing 4HA-tagged SdeC and localisation of SdeC was observed at 2, 7, 10 and 13 hour time points post-infection (Figure 5.8.).

SdeC was seen to localise to the LCV at every observed time point. At 2 hours post-infection SdeC was primarily localised at the poles of the LCV containing a single bacterium. At 7 and 10 hours post-infection SdeC was mainly localised throughout the

LCV membrane. At 13 hours post-infection SdeC localised throughout the host cell in addition to a major localisation at the LCV membrane.

5.2.6. SdeC localisation during transfection of HEK293T cells

Ectopic expression of SdeC in a host cell separate to infection has the potential to reveal an interaction with a host component when observing localisation. Enhanced Green Fluorescent Protein (EGFP) is a fluorescent tag often used in localisation studies. Cells transfected with the EGFP expressing vector pEGFP-C2 do not display any particular EGFP localisation pattern, with the expressed protein being distributed throughout the transfected cell, including the nucleus (Figure 5.9.).

EGFP-tagged SdeC was constructed for expression in mammalian cells via transfection. If SdeC only interacts with the LCV it should display no specific localisation pattern upon singular expression. *sdeC* was cloned into the pEGFP-C2 and pEGFP-N1 mammalian expression plasmids, placing SdeC at the C- and N-terminus respectively of the EGFP protein. These constructs were transfected into HEK293T cells to observe localisation. EGFP-N1:SdeC was not expressed by the host cell but EGFP-C2:SdeC was expressed by HEK293T cells. EGFP-C2:SdeC expressed ectopically in HEK293T cells did not display uniform distribution throughout the cell and resembled a perinuclear localisation pattern similar to the distribution of ER in a mammalian cell (Figure 5.9). Transfected cells were therefore stained with the ER marker calnexin using immunofluorescence to determine co-localisation with SdeC (Table 2.4.; Figure 5.10.). As hypothesised EGFP-C2:SdeC co-localised with calnexin upon expression, indicating a possible interaction with the host ER.

pEGFP-C2:SdeC transfected cells were stained with other cell markers to determine any additional effects on other cellular organelles. pEGFP-C2:SdeC transfected HEK293T cells were stained with TRITC-labelled phalloidin to visualise the host cell

cytoskeletal protein actin (Figure 5.11.) but no change in host cell actin was observed indicating that SdeC did not affect host cell actin on its own.

pEGFP-C2:SdeC transfected HEK293T cells were stained for Golgi structure by immunofluorescence using an anti-Golgi-97 monoclonal antibody (Table 2.4.; Figure 5.12.). No change in the host cell golgi apparatus was observed after transfection with pEGFP-C2:SdeC indicating that SdeC also did not affect golgi structure. SdeC primarily localised to the region surrounding the Golgi apparatus, likely due to the close proximity of the Golgi to the ER.

pEGFP-C2:SdeC transfected HEK293T cells were also stained for LAMP-1 (Figure 5.13.), a lysosomal marker, and β-COP (Figure 5.14.), a subunit of coatamer proteins involved in ER to Golgi transport, via immunofluorescence using the appropriate antibodies (Table 2.4.). However, no change in host cell LAMP-1 or β-COP markers were observed indicating that SdeC likely did not interact with host cell lysosomes or host coatamer proteins.

5.2.7. Activation of host cell apoptosis by SdeC

We noted during transfection of cells with pEGFPC2:SdeC that transfection rates were lower than with pEGFPC2 alone. We therefore hypothesised that transfected cells may have been undergoing apoptosis and that SdeC expression was cytotoxic. Cleaved caspase 3 leads to apoptosis in mammalian cells and so to test for the induction of apoptosis by SdeC, immunofluorescence was used to visualise activated, cleaved caspase 3 in EGFP-SdeC expressing HEK293T cells (Figure 5.15.). The number of transfected cells displaying cleaved caspase 3 was comparable to that of cells transfected with the pEGFP-C2 vector alone. Hence there was no significant difference in the number of cells with cleaved caspase 3 between the EGFP-SdeC expressing cells and cells expressing EGFP alone (There was also no evidence of substantial cell loss).

Therefore we concluded that SdeC alone did not trigger significant apoptosis in host cells.

5.2.8. Identification of binding partners of SdeC

SdeC localised to the ER and LCV indicating possible interactions at these sites and perhaps a role in LCV biogenesis, which relies on the recruitment of ER membrane. To determine the host binding partner of SdeC, we aimed to identify a host interacting protein by co-immunoprecipitation with SdeC. Multiple epitope tags including polyhistadine and a 2x hemagglutinin were used to generate tagged recombinant SdeC for use in pull-down experiments. These recombinant tagged-SdeC proteins however were unable to be produced in *E. coli* once cloned into the appropriate *E. coli* expression vectors. This may have been due to the large size of the protein (approximately 173 kDa) or the use of *E. coli* in producing the protein.

Instead, EGFP-SdeC used previously for localisation studies was used here in conjunction with a GFP-Trap to identify possible binding partners during transfection. HEK293T cells were transfected overnight with pEGFP-C2:SdeC and a cell pellet was prepared as per the GFP-Trap kit instuctions. Eluted fractions after immunoprecipitation were separated by SDS-PAGE and stained with Coomassie Brilliant Blue protein stain (Figure 5.16.).

Despite EGFP-tagged SdeC being present in the final elution of the immunoprecipitation, no other proteins were identified in the elution fraction that were not present in the empty pEGFP-C2 vector negative control (based on comparable protein band sizes) (Figure 5.16.). However a high molecular weight smear was present in the control sample in the approximate region of SdeC which may have contributed to false positives when identifying the presence of SdeC.

As an alternate approach, the 4xHA tag introduced on the *Legionella* expression vector pICC562 was used to epitope tag SdeC for expression in *L. pneumophila* during infection for co-immunoprecipitation studies. B6 mouse bone marrow-derived macrophages were infected with *L. pneumophila* (pICC562:*sdeC*) and *L. pneumophila* (pICC562) for 24 h at 37 °C and 5% CO_2. Co-immunoprecipitation of cell lysate was performed post-infection with Pierce Anti-HA Magnetic Beads as per the manufacturer's instructions and the final eluate was analysed by separating proteins by SDS-PAGE before staining with SYPRO Ruby Protein Gel Stain (Figure 5.17.). Using this approach, several unique protein bands were detected in the SdeC sample compared to the empty vector control. These bands were excised and the proteins identified via LC-MS/MS (Appendix 1.3.). Several common protein contaminants were identified in most of the samples, including ribosomal proteins, heat shock proteins, ATP synthases, actin and other cytoskeletal proteins, elongation and initiation factors. In addition to these, degraded SdeC was also identified. However, as actin was identified in multiple immunoprecipitation samples and corresponded to the molecular weight of the co-immunoprecipitated band, an immunoblot was performed probing for the presence of actin in immunoprecipitated samples to rule it out as a binding partner (data not shown). As expected, actin was not detected in the SdeC immunoprecipitation sample indicating that the actin identified previously by the LC-MS/MS was a likely contaminant. In summary, no binding partner of SdeC was able to be reliably identified in the immunoprecipitation experiments performed here.

5.3. Discussion

Further characterisation of SdeC was performed here, as a deletion mutant in a previous study (Luo and Isberg, 2004) and our *sdeC* transposon mutant displayed a significant defect during intracellular replication in several host cell types. Functional redundancy is often observed amongst *Legionella* effectors, however, here SdeC alone seemed to be important for full virulence even with the presence of multiple

paralogues of high similarity. This distinguishing feature and the lack of information pertaining to the specific function of SdeC at a molecular level shaped our decision to characterise this effector further.

To allow further characterisation, an *sdeC* deletion mutant was constructed in *L. pneumophila* strain 130b and complemented with full length *sdeC* on the pMIP plasmid. The *sdeC* deletion mutant displayed a significant defect during intracellular replication in *A. castellanii* (Figure 5.2), similar to the defect seen for the *sdeC* transposon mutant (Figure 3.6.). Despite expression of *sdeC* from the pMIP complementation vector, complementation did not restore growth of the *sdeC* mutant to wild type levels in this assay. Similar results were seen in THP-1 cells and immortalised murine bone marrow-derived macrophages (BMDM) at 24 and 48 h post-infection. However, at 72 h post infection in murine BMDMs the complemented *sdeC* mutant was significantly different to the *sdeC* deletion mutant, indicating partial complementation and possible recovery to a wild type levels after 72 hours.

When replication of the *sdeC* mutant and the complemented mutant were retested in an A strain mouse infection model (Figure 5.6.), the *sdeC* mutant again displayed a significant defect in comparison to wild type *L. pneumophila* and complementation with *sdeC* successfully restored replication of the mutant to wild type levels at 72 h, thereby correlating with the complementation in BMDMs. The reasons for differences in phenotype observed between different host cells are unknown but overall this confirmed the importance of *sdeC* in *L. pneumophila* infection. It is possible the expression of *sdeC* on the bacterial chromosome rather than on a plasmid is more important in some host cells than others.

In this study, the function of SdeC was initially characterised using cellular localisation studies. SdeC was epitope tagged for easy detection and the localisation of tagged SdeC after infection of *A. castellanii* and HEK293T cells was observed to be at the LCV (Figure 5.7.). SdeC was observed throughout the LCV membrane, which correlated well with previously reported localisation of SidE family proteins to the LCV during the early

stages of infection (Bardill et al., 2005). Our results suggested that SdeC is evenly distributed along the LCV membrane at the later stages of infection, indicating a possible role in LCV membrane biogenesis.

It was previously reported that SidE family proteins are predominantly expressed early after infection of a host cell (Bardill et al., 2005). This may indicate that SdeC and its paralogues are important during the initial stages of LCV development post infection. We observed that SdeC was present at the LCV throughout all stages of infection, although this may be due to the tagged protein being constitutively expressed from the plasmid vector introduced, as opposed to expression being under control of the natural promoter on the bacterial chromosome.

Some host cells contained multiple LCVs arising from infection by multiple bacteria (Figure 5.7.). We observed that only some of the vacuoles, within the same cell, were positive for the presence of SdeC. This may have been due to the loss of the plasmid expressing the HA-tagged SdeC or SdeC may not have been expressed at sufficient levels for detection or the conditions for the translocation of SdeC may not have been attained. This observation may also indicate that SdeC localises to the LCV immediately after translocation rather than acting at a separate location within the cell before localising to the LCV, where it would be distributed evenly on all LCVs. However, SdeC may localise to the LCV via an interaction that is only possible at a specific time after infection. If this is the case, SdeC may still be able to act in another part of the cell before localising only to LCVs that possess a specific interaction partner, be it bacterial or host-derived protein or lipid, and not LCVs that lack the interaction partner. Hence SdeC localisation may reflect the maturation state of the LCV.

A time course of a *L. pneumophila* expressing 4HA-tagged SdeC infection of *A. castellanii* was performed to determine any changes in SdeC localisation over time (Figure 5.8.), which may have explained the different localisation patterns observed in the previous localisation experiment. However, SdeC localised to the LCV at each of the time points observed (2, 7, 10 and 13 hours post-infection). It was noted that at

two hours post-infection SdeC was primarily localised at the poles of the internalised bacterium, which is consistent with the findings of Bardill et al. (2005). The observed localisation at the bacterial poles is likely due to secretion of SdeC via the Dot/Icm T4SS located at the poles of the bacterium (Weber et al., 2006).

To understand potential interaction partners of SdeC, a transfection experiment was performed in mammalian HEK393T cells using SdeC tagged with the fluorescent EGFP protein (Figure 5.9.). EGFP alone is distributed throughout the transfected cell, including the nucleus. SdeC was distributed throughout the cell, excluding the nucleus, after ectopic expression, however not in a uniform pattern. The localisation pattern was similar to that of the ER in a mammalian cell and to confirm this transfected cells were stained with the ER marker calnexin by immunofluorescence to determine co-localisation (Figure 5.10.). EGFP-SdeC co-localised with calnexin, indicating a possible interaction with the host ER. Hence, SdeC may be involved in LCV interactions with the host ER, possibly including the recruitment of ER-derived vesicles to the LCV.

EGFP-SdeC transfected cells were stained with other cell markers to determine any additional effects on other cellular components (Figure 5.11., Figure 5.12., Figure 5.13. and Figure 5.14.). EGFP-SdeC expressing HEK293T cells were stained with fluorescent stains to visualise host cell actin, golgi, lysosomes and coatomer proteins. No qualitative changes in these host components were observed after expression of SdeC.

During these experiments we noticed that transfection rates with pEGFP-SdeC were lower than cells transfected with pEGFP-C2 alone, particularly in transfected HeLa cells (data not shown). We hypothesised that expression of SdeC in mammalian cells may be toxic, leading to a reduced visible rate of transfection. To test for the induction of apoptosis by SdeC the detection of cleaved caspase 3, an indicator of apoptosis in mammalian cells, was performed on EGFP-SdeC expressing HEK293T cells via an immunofluorescence assay (Figure 5.15.). Only a small number of cells stained for cleaved caspase 3 in both the EGFP-SdeC expressing cells and the cells transfected with

pEGFPC2 alone. This did not indicate any significant induction of cell death via apoptosis upon expression of SdeC.

In summary, we observed SdeC localising to the ER during transfection and the LCV during infection of host cells indicating possible interactions at these sites. It may act in a similar fashion to other *L. pneumophila* effectors that are involved in ER-LCV interactions such as RalF and DrrA (outlined in 1.5.3.). Further experiments may focus on any differences in individual proteins, including host factors such as Rab1, SNARE proteins, Sec61 and BiP and *L. pneumophila* proteins such as DrrA and SdhA, recruited to the LCVs of wild type and *sdeC* mutant *L. pneumophila* via immunolabelling. To identify possible interaction partners of SdeC, various strategies were used to identify any protein binding partners. Different epitope tags were used to tag SdeC for use in immunoprecipitation experiments with many initial constructs proving unsuccessful (data not shown).

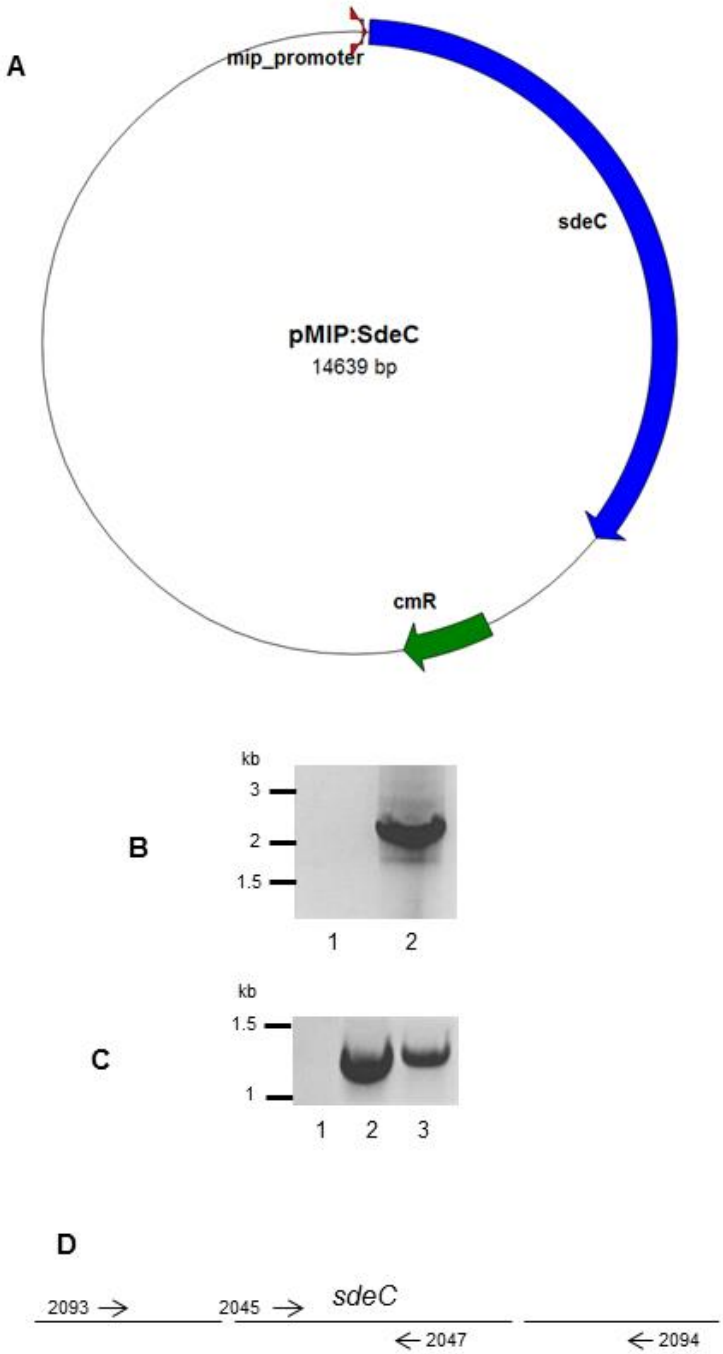
Other intracellular pathogens, such as *Chlamydia*, secrete proteins that localise to the pathogen-containing vacuole (inclusion). *Chlamydia* possesses a number of Inc proteins that are expresses early or mid-infection cycle and are important for establishing the inclusion and nutrient acquisition (Reviewed in Moore and Ouellette, 2014.). IncA is expressed mid-infection cycle and contains two SNARE-like motifs that are likely responsible for IncA's ability to bind several host SNARE proteins, found to be recruited to the inclusion (Delevoye et al., 2008; Ronzone and Paumet, 2013). *C. pneumoniae* proteins Cpn0585 and CT229 localise to the inclusion where they interact with host Rab GTPases (Cortes et al., 2007; Rzomp et al., 2006). SdeC may promote similar membrane fusion events at the LCV. Like some of *Chlamydia's* secreted proteins the time dependant expression of *sdeC* may be important during infection. This may account for the complementation issues observed with the *sdeC* deletion mutant and is may affect the results of localisation experiments where constitutive promoters were used in both constructs.

A GFP-Trap immunoprecipitation was performed on cells transfected with pEGFP-SdeC for identification of binding partners of SdeC (Figure 5.16). Based on the localisation experiments via transfection with this construct it was predicted that a component associated with the ER would be identified. However, no visible binding partners were identified after the immunoprecipitation. SdeC may have a binding partner that was unable to be identified due to conditions of the immunoprecipitation used, which may not have been permissible for its detection. This may be due to the binding partner being non-protein, harsh wash conditions or transient binding.

As an alternative approach a 4x HA tag was added to SdeC and the protein was expressed in *L. pneumophila* and used for immunoprecipitation experiments of infected cells (Figure 5.17.). Several unique protein bands were observed during immunoprecipitation of 4HA-SdeC in comparison to an empty vector control, indicating possible binding partners. In addition to degraded SdeC, several common contaminants of immunoprecipitation/mass spectrometry experiments including cytoskeletal proteins, heat shock proteins, initiation factors and elongation factors were identified and discounted. These proteins are often encountered due to their high abundance in biological samples.

Although actin is a common contaminant, since this band was identified multiple times in both immunoprecipitation experiments an immunoblot probing for the presence of actin in immunoprecipitation samples was performed. If actin just bound to the immunoprecipitation beads as an abundant weakly binding protein it should be detected in both immunoprecipitation samples of cells infected with *L.* pneumophila that possessed HA-tagged SdeC and *L. pneumophila* carrying empty vector control. Additional care was taken to avoid any contact with the cell pellet during preparation of cell lysate, a likely source of insoluble cytoskeletal proteins. However, actin was not detected in the SdeC immunoprecipitation sample by immunoblot, indicating the actin previously identified was likely to be a contaminant.

Unfortunately no protein binding partner of SdeC was able to be successfully identified using the methods performed here. If SdeC has a host protein interaction partner it may not be detectible using the methods used here possibly due to a weak interaction or factors needed for binding that are not present during experimental condition, although the addition of the crosslinking step should have accounted for this. Alternatively if SdeC is a novel enzyme then it may form only a transient interaction with a substrate that would not be detected by protein interaction methods. Moreover SdeC may interact with lipids and this would not be detected here. It should be emphasised that SdeC is a large hydrophobic protein and so there were technical limitations to what could be achieved biochemically. Further work could focus on generating significant amounts of recombinant protein for further biochemical characterisation.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2093
2045
sdeC
2047
2094

Figure 5.1. The organisation of pMIP:SdeC and PCR analysis of the *L. pneumophila sdeC* deletion mutant and the complemented *L. pneumophila sdeC* mutant. A) Organisation of pMIP:SdeC. Representative scale vector map. *cmR*-chloramphenicol resistance gene. Plasmid map created using Savvy (http://bioinformatics.org/savvy/). **B) Agarose gel electrophoresis of PCR products using primers 2093 and 2094 (located outside of the deleted region) to confirm insertion of the kanamycin resistance cassette in the *L. pneumophila sdeC* deletion strain.** If *sdeC* is replaced with the kanamycin resistance cassette a fragment of approximately 2.3 kb will be produced. 1- *L. pneumophila* 130b strain, 2- *L. pneumophila sdeC* deletion mutant. DNA ladder with known fragment sizes, in kb, added for comparison. **C) Agarose gel electrophoresis of PCR products using primers 2045 and 2047 to confirm the presence of *sdeC* in pMIP:SdeC from the complemented *L. pneumophila sdeC* deletion strain**. 1- *L. pneumophila sdeC* deletion mutant, 2- *L. pneumophila* 130b strain, 3- *L. pneumophila sdeC* deletion mutant (pMIP:SdeC). DNA ladder with known fragment sizes, in kb, added for comparison. **D) Location of primers used in B and C.**

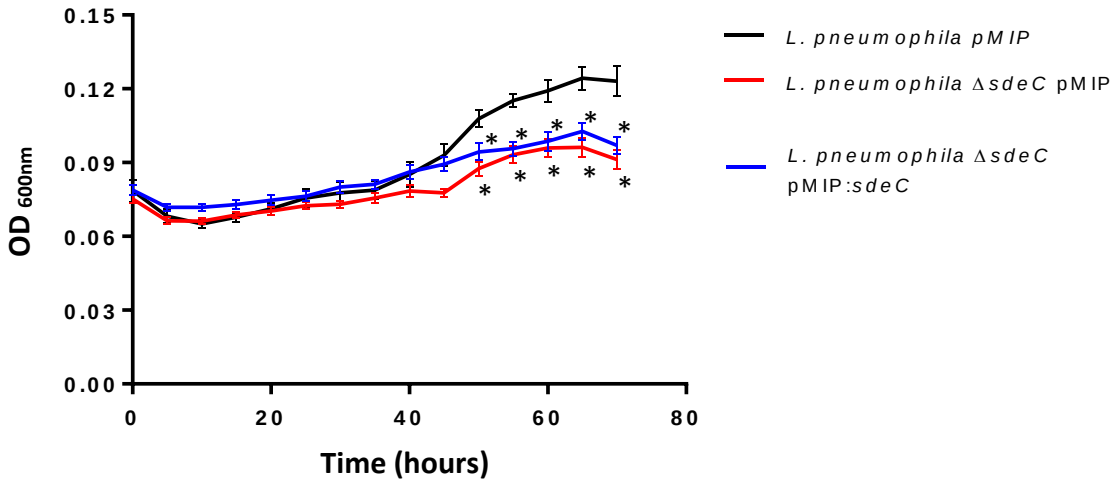


Fig 5.2. *L. pneumophila* - *A. castellanii* co-culture plate reader assay comparing the growth of the *sdeC* defined mutant to wild type *L. pneumophila* and complemented mutant strains. Line graph plotting OD_{600nm} vs time in hours. *A. castellanii* was challenged with the *L. pneumophila* strains indicated. OD_{600nm} was measured every five hours for 70 hours in a Fluostar platereader (BMG labtech). Cells were incubated at 37 °C. ***** indicates a statistically significant difference in comparison to wild type *L. pneumophila* (t-test, unpaired, 2-tailed, $P < 0.001$).

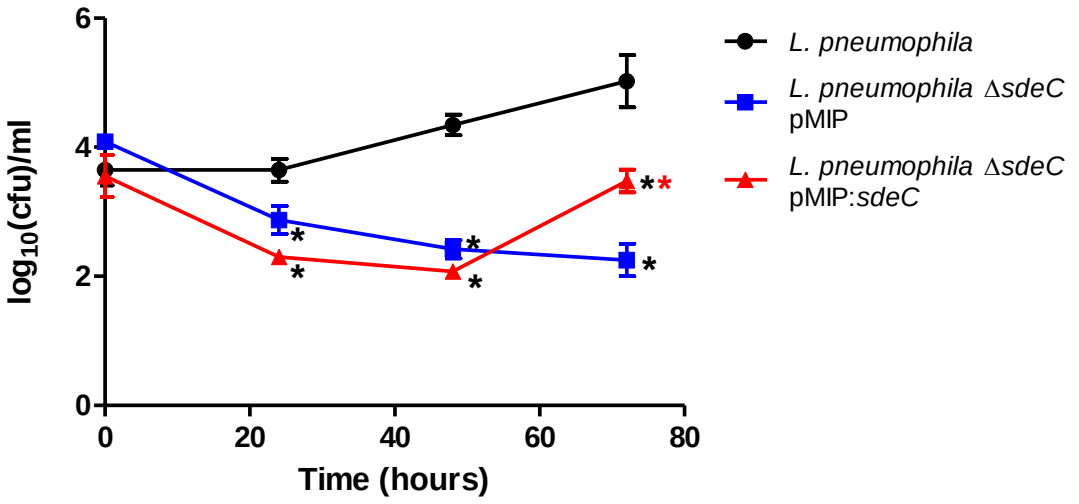


Figure 5.3. *L. pneumophila and B6 BMDM* co-culture assay comparing the growth of the *sdeC* defined mutant to wild type *L. pneumophila* and complemented mutant strains. Line graph plotting log_{10}(cfu)/mL vs time in hours. *B6 BMM* was challenged with the *L. pneumophila* strains indicated. Cells were incubated at 37 °C and 5% CO_2. The assay was performed twice in duplicate over 72 h post-infection, plating CFU on BCYE agar at 0, 24, 48 and 72 h. A t-test (unpaired, 2-tailed, P <0.05) was used to determine statistically significant differences between infections at each time point. ***** indicates a statistically significant difference in comparison to wild type *L. pneumophila.* ***** indicates a statistically significant difference in comparison to *L. pneumophila ΔsdeC* (pMIP).

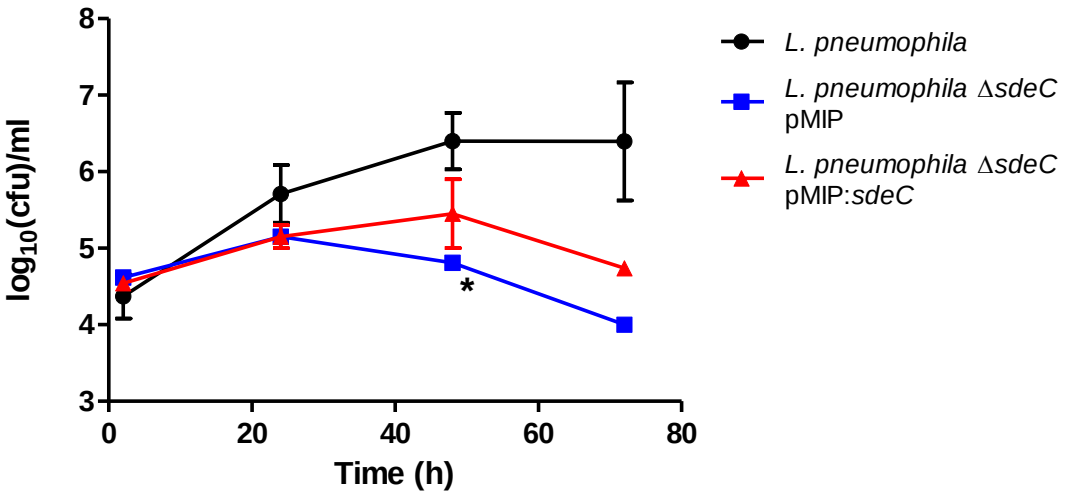


Figure 5.4. *L. pneumophila* - THP-1 co-culture assay comparing the growth of the *sdeC* defined mutant to wild type *L. pneumophila* and complemented mutant strains. Line graph plotting log_{10}(cfu)/mL vs time in hours. THP-1 *cells* were challenged with the *L. pneumophila* strains indicated. Cells were incubated at 37 °C and 5% CO_2. The assay was performed in duplicate over 72 h post-infection, plating CFU on BCYE agar at 0, 24, 48 and 72 h. A t-test (unpaired, 2-tailed, $P < 0.05$) was used to determine statistically significant differences between infections at each time point. ***** indicates a statistically significant difference in comparison to wild type *L. pneumophila.*

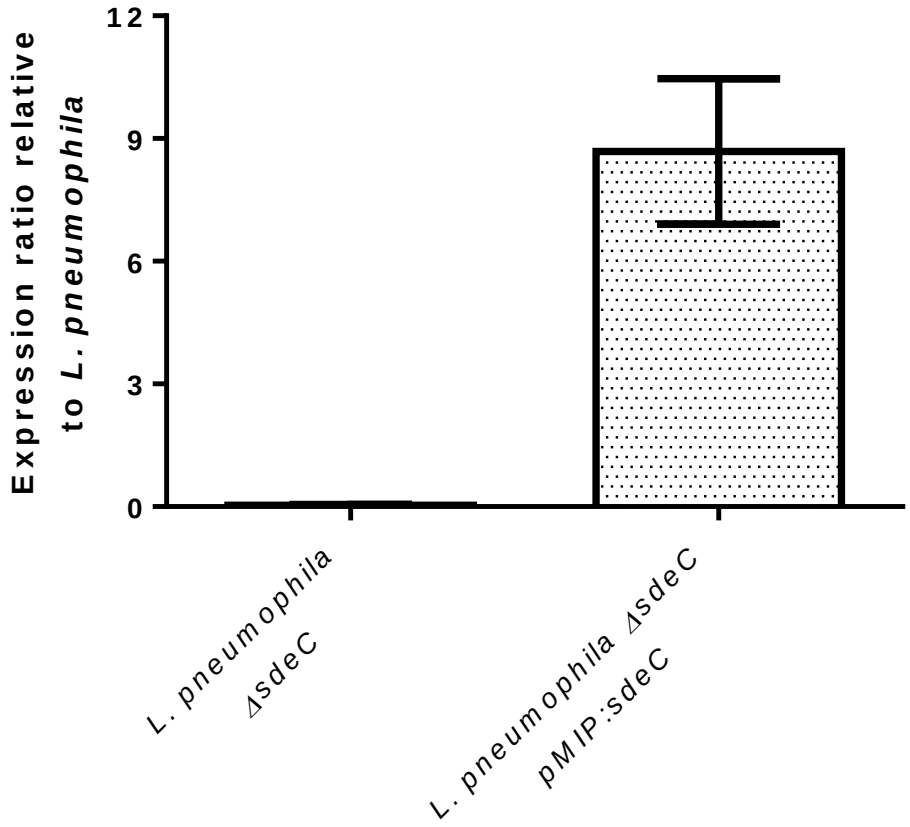


Figure 5.5. *sdeC* expression ratios of *L. pneumophila sdeC* deletion mutant and the complemented *sdeC* mutant relative to *L. pneumophila* strain 130b. Bar graph plotting the *sdeC* expression ratio of each strain relative to wild type *L. pneumophila*. cDNA was exponentially amplified using gene specific primers and detected using a SYBRGreen based quantitative PCR detection system in a Stratagene Mx3005P Real Time PCR machine. *sdeC* expression levels of overnight AYE broth cultures, induced with 1 mM IPTG, of the *sdeC* deletion mutant and the complemented *sdeC* mutant were compared to that of wild type *L. pneumophila* strain 130b. All RT-PCR data were normalized with the results for the *L. pneumophila* 16s rRNA gene and the relative expression ratio of *sdeC* was calculated as described by Pfaffl (Pfaffl, 2001). The *sdeC* deletion mutant had a mean expression ratio of 0.03 and the complemented *sdeC* mutant had a mean expression ratio of 8.69 relative to wild type *L. pneumophila*.

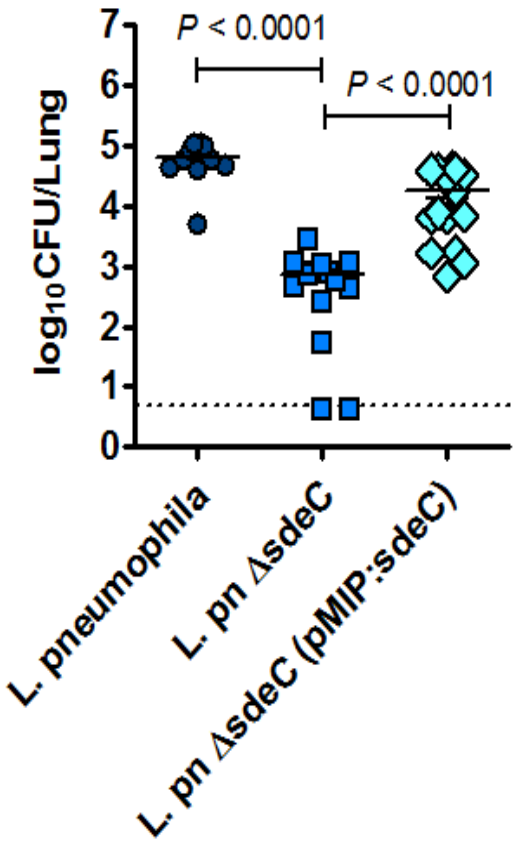


Figure 5.6. Comparison of bacterial CFU harvested from the lungs of A strain mice 72 h post-infection. Dot plot of $\log_{10}$(cfu)/lung for each indicated strain. A strain mice were infected with the *L. pneumophila* strains indicated. Each data point represents one animal. Lungs were harvested at 72 h post infection and homogenised. Viable bacterial numbers were determined by plating homogenate on BCYE agar for CFU counts. Mann-Whitney tests were used to determine statistically significant differences between infections (unpaired, two-tailed, P-value < 0.0001). Infection performed by Clare Oates (University of Melbourne).


A
175 kDa
4HA-SdeC
1
2
3
4

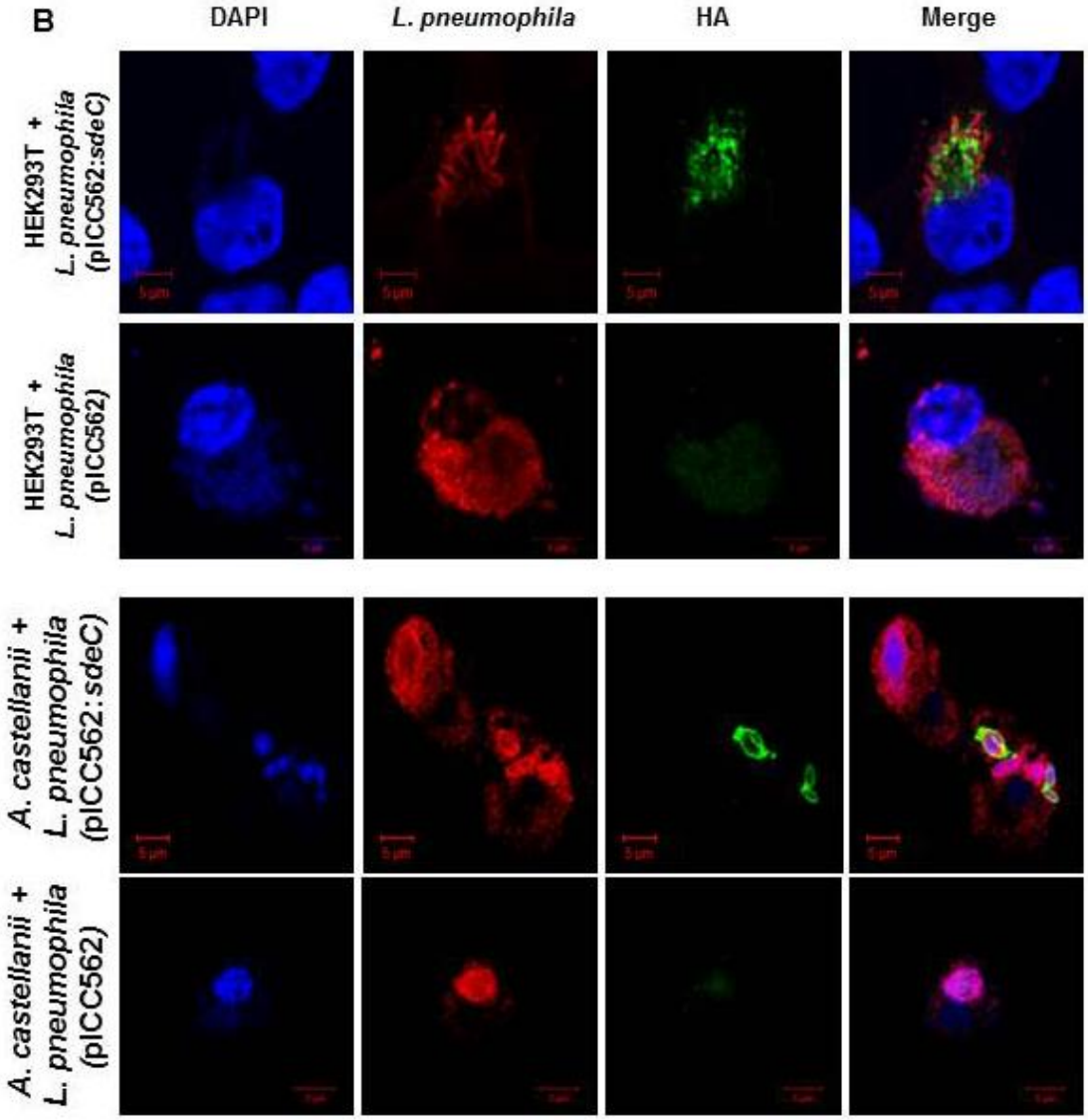

B
DAPI
L. pneumophila
HA
Merge
HEK293T + L. pneumophila (pICC562:sdeC)
HEK293T + L. pneumophila (pICC562)
A. castellanii + L. pneumophila (pICC562:sdeC)
A. castellanii + L. pneumophila (pICC562)

Figure 5.7. A) Immunoblot analysis of SdeC expression in *L. pneumophila* (pICC562:*sdeC*). Western blot probing indicated strains for HA-tagged SdeC. Bacteria were incubated overnight in AYE broth in the presence and absence of 1mM IPTG for induction of protein expression. Proteins in a whole cell lysate sample were separated via SDS-PAGE gel. A Western blot was performed using anti-HA antibodies to detect the fusion protein. 1- *L. pneumophila* pICC562 uninduced. 2- *L. pneumophila* (pICC562) induced with 1 mM IPTG. 3- *L. pneumophila* (pICC562:*sdeC*) uninduced. 4- *L. pneumophila* (pICC562:*sdeC*) induced with 1 mM IPTG. Protein ladder with known fragment sizes, in kDa, added for comparison. **B) Localisation of SdeC after infection of *A. castellanii* and HEK293T cells**. Fluorescent microscopy images of *A. castellanii* and HEK293T cells infected with *L. pneumophila* (pICC562:*sdeC*) and *L. pneumophila* (pICC562) for 24 h at 37 °C and 5% CO_2. Cells were fixed, permeabilized and stained for nucleic acid with DAPI, *Legionella* stained with an anti-*Legionella* antibody and Alexafluor 568 secondary antibody and 4HA-tagged SdeC stained with an anti-HA antibody and Alexafluor 488 secondary antibody. The localisation of HA-tagged SdeC was observed with a Zeiss LSM700 laser scanning confocal microscope using DAPI, Texas Red and FITC fluorescent filters.

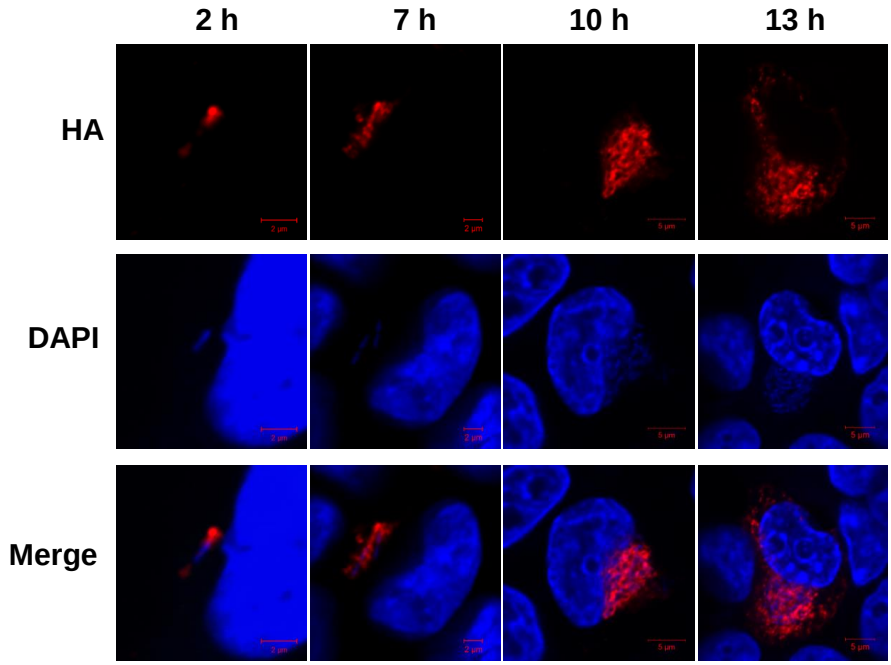


Fig 5.8. Time course of SdeC localisation during infection of HEK293T cells.

Fluorescent microscopy images of HEK293T cells infected with *L. pneumophila* (pICC562:*sdeC*) for indicated times at 37°C and 5% CO_2. Cells were fixed, permeabilized and stained for nucleic acid with DAPI and 4HA-tagged SdeC stained with an anti-HA antibody and Alexafluor 568 secondary antibody. The localisation of HA-tagged SdeC was observed with a Zeiss LSM700 laser scanning confocal microscope using DAPI and Texas Red fluorescent filters.

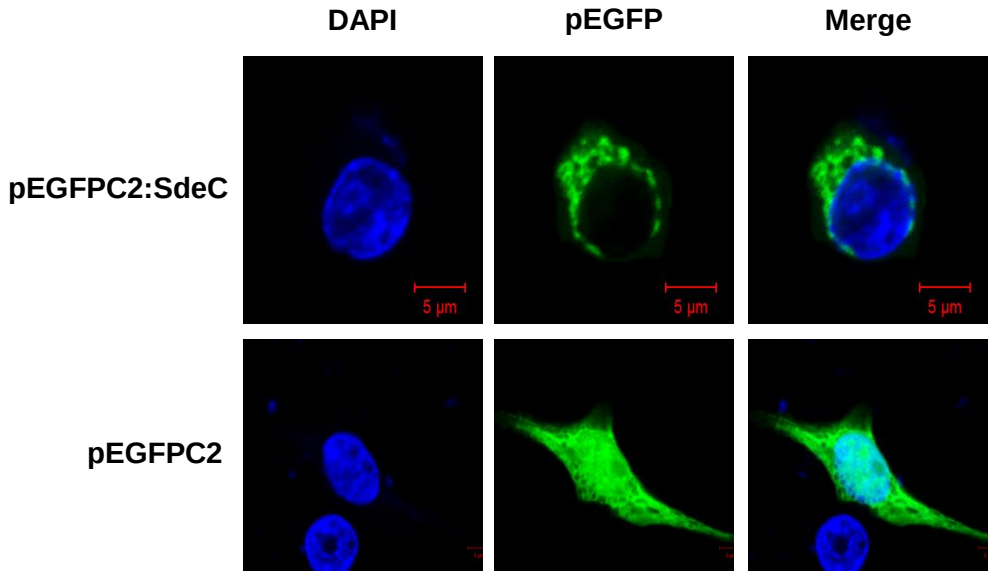


Fig 5.9. pEGFPC2:SdeC transfection of HEK293T cells. Fluorescent microscopy images of HEK293T cells transfected overnight with pEGFPC2:SdeC and pEGFPC2. Cells were fixed and stained with DAPI to visualise nucleic acid. Localisation was observed with a Zeiss LSM700 laser scanning confocal microscope using DAPI and FITC fluorescent filters.

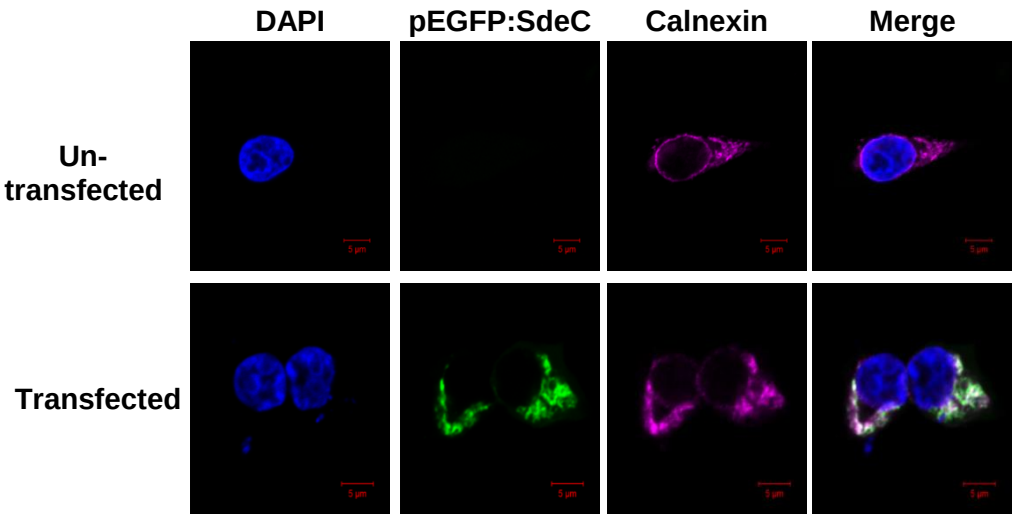


Fig 5.10. Observation of host calnexin upon pEGFPC2:SdeC transfection of HEK293T cells. Fluorescent microscopy images of untransfected and HEK293T cells transfected with pEGFPC2:SdeC. Cells were fixed, permeabilised and stained with DAPI to visualise nucleic acid and an anti-calnexin antibody and Alexafluor 647 secondary antibody to visualise the ER . The localisation of GFP-tagged SdeC was observed with a Zeiss LSM700 laser scanning confocal microscope using DAPI, Cy5 and FITC fluorescent filters.

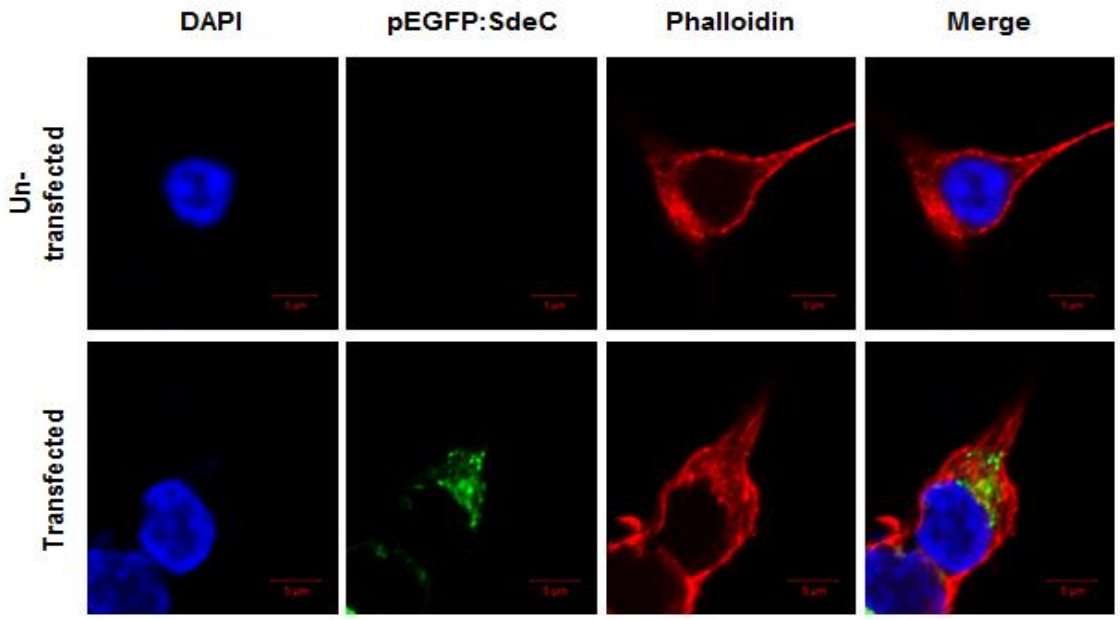


Fig 5.11. Localisation of host actin upon pEGFP-SdeC transfection of HEK293T cells. Fluorescent microscopy images of un-transfected HEK293T cells and HEK293T cells transfected with pEGFP-SdeC. Cells were fixed, permeabilised and stained with DAPI to visualise nucleic acid and TRITC-labelled phalloidin to visualise actin filaments. The localisation of EGFP-tagged SdeC was observed with a Zeiss LSM700 laser scanning confocal microscope using DAPI, Texas Red and FITC fluorescent filters.

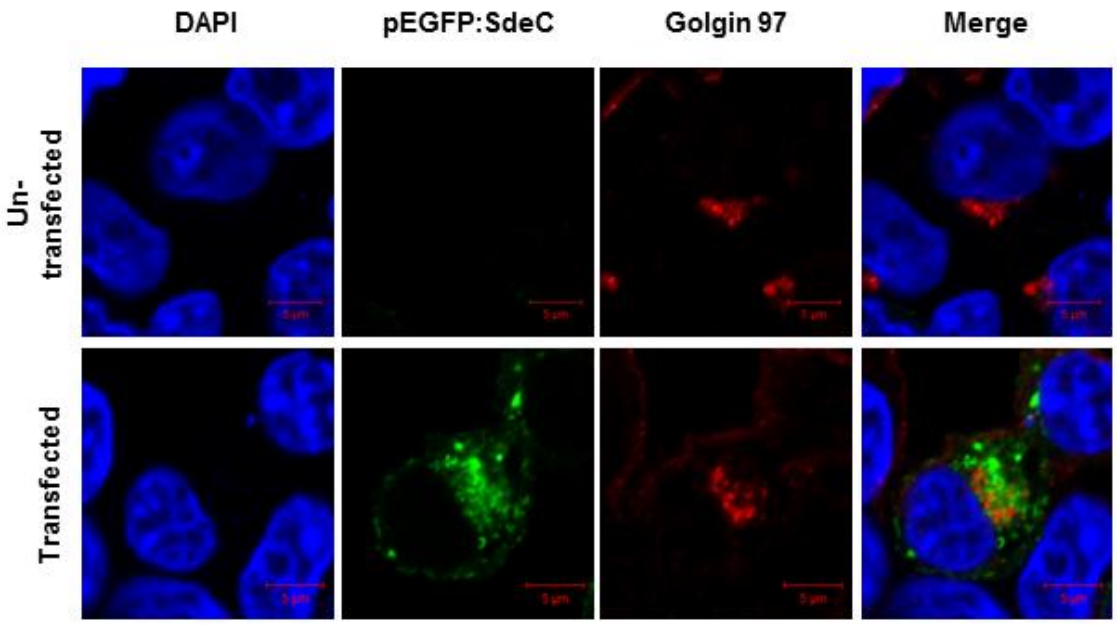


Fig 5.12. Localisation of host Golgi upon pEGFP-SdeC transfection of HEK293T cells. Fluorescent microscopy images of un-transfected HEK293T cells and HEK293T cells transfected with pEGFP-SdeC. Cells were fixed, permeabilised and stained with DAPI to visualise nucleic acid and an anti-golgi 97 antibody and Alexafluor 568 secondary antibody to visualise host Golgi. The localisation of EGFP-tagged SdeC was observed with a Zeiss LSM700 laser scanning confocal microscope using DAPI, Texas Red and FITC fluorescent filters.

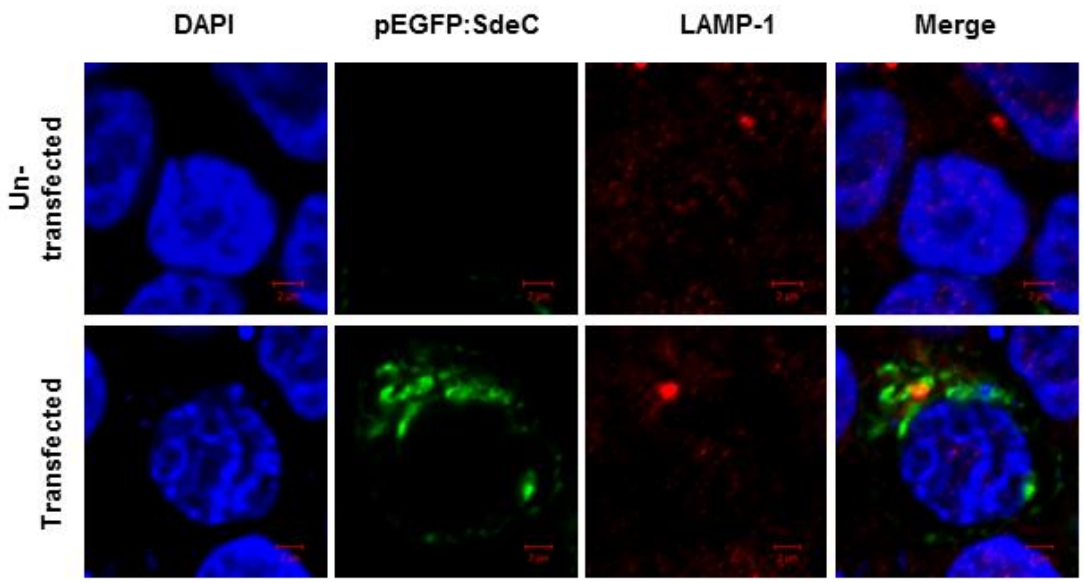


Fig 5.13. Localisation of host LAMP-1 upon pEGFP-SdeC transfection of HEK293T cells. Fluorescent microscopy images of un-transfected HEK293T cells and HEK293T cells transfected with pEGFP-SdeC. Cells were fixed, permeabilised and stained with DAPI to visualise nucleic acid and an anti-LAMP-1 antibody and Alexafluor 568 secondary antibody to visualise host LAMP-1. The localisation of EGFP-tagged SdeC was observed with a Zeiss LSM700 laser scanning confocal microscope using DAPI, Texas Red and FITC fluorescent filters.

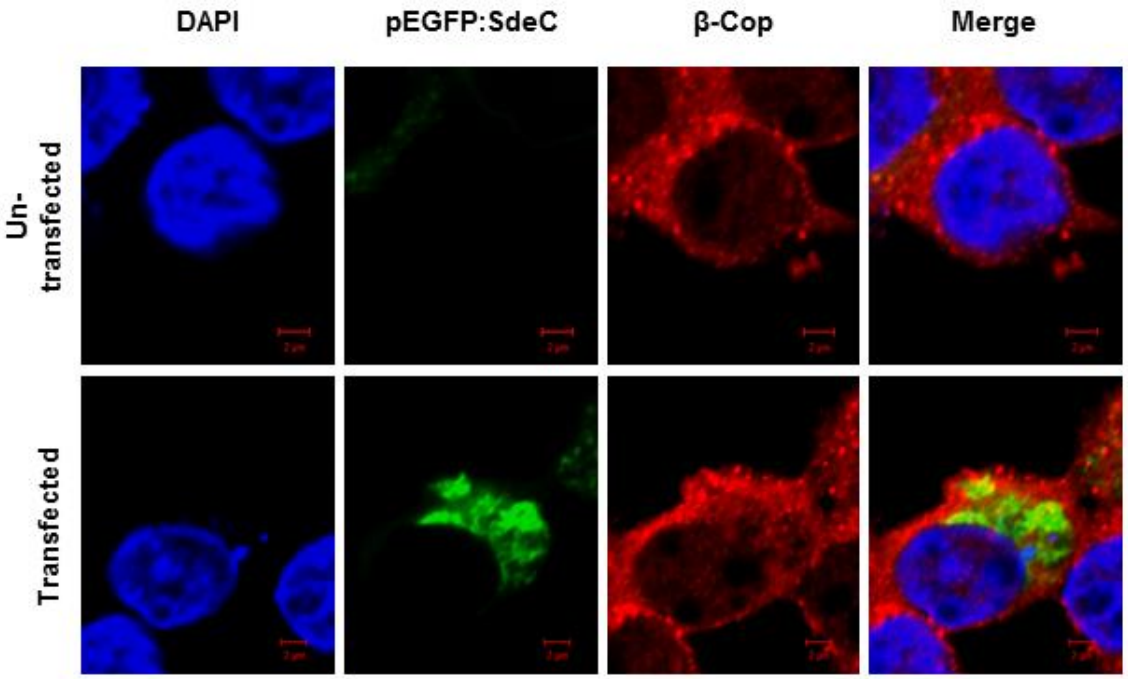


Fig 5.14. Localisation of host β-Cop upon pEGFP-SdeC transfection of HEK293T cells. Fluorescent microscopy images of un-transfected HEK293T cells and HEK293T cells transfected with pEGFP-SdeC. Cells were fixed, permeabilised and stained with DAPI to visualise nucleic acid and an anti-β-cop antibody and Alexafluor 568 secondary antibody to visualise host β-cop. The localisation of EGFP-tagged SdeC was observed with a Zeiss LSM700 laser scanning confocal microscope using DAPI, Texas Red and FITC fluorescent filters.

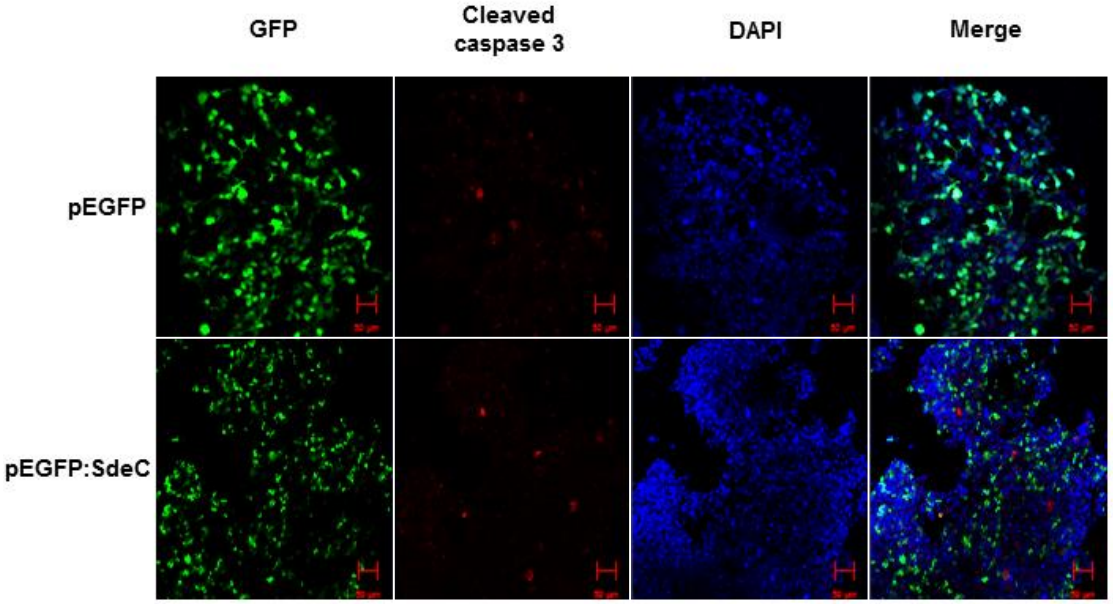


Fig 5.15. Activation of host cell apoptosis upon pEGFP-SdeC transfection of HEK293T cells. Fluorescent microscopy images of HEK293T cells transfected with pEGFP-SdeC or pEGFP-C2. Cells were fixed, permeabilised and stained with DAPI to visualise nucleic acid and an anti-cleaved caspase 3 antibody and Alexafluor 568 secondary antibody to visualise the activation of host cell apoptosis. Stained transfected cells were observed with a Zeiss LSM700 laser scanning confocal microscope using DAPI, Texas Red and FITC fluorescent filters.

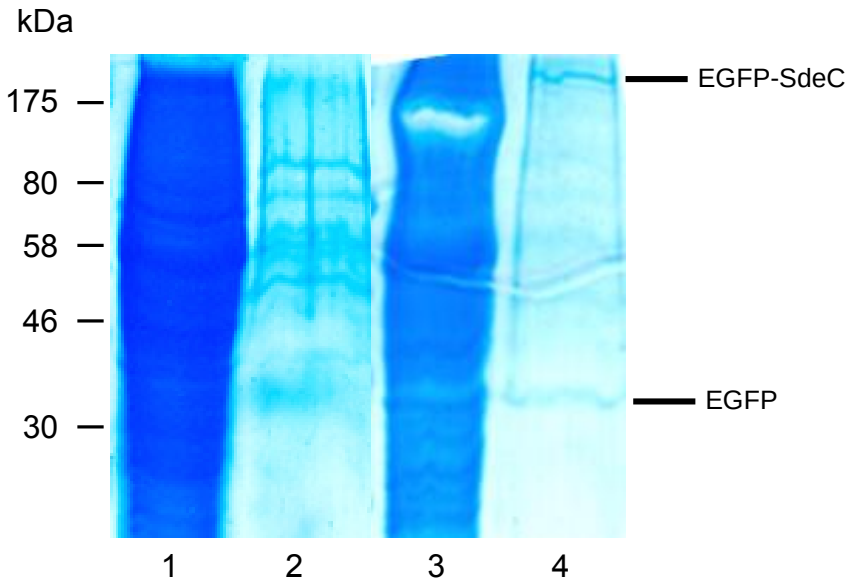


Figure 5.16. GFP TRAP co-immunoprecipitation of HEK293T cells transfected with pEGFP-SdeC. SDS-PAGE gel of stained with a Coomassie Brilliant Blue protein stain. HEK293T cells were transfected overnight with pEGFP-SdeC or pEGFP-C2 and a co-immunoprecipitation was performed as per the GFP-Trap kit instructions. Whole cell lysate of pEGFP-C2 and pEGFP-SdeC transfected cells (1 and 3 respectively) and co-immunoprecipitation elution fractions of pEGFP-C2 and pEGFP-SdeC transfected cells (2 and 4 respectively) were run on an SDS-PAGE gel and stained with a Coomassie Brilliant Blue protein stain to visualise all proteins present. Protein ladder with known fragment sizes, in kDa, added for comparison.

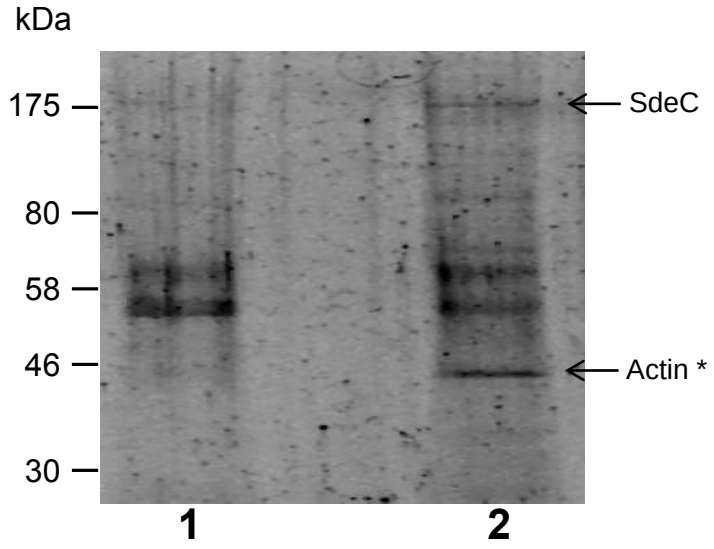


Figure 5.17. Hemagglutinin co-immunoprecipitation of immortalised B6 mouse bone marrow-derived macrophages infected with *L. pneumophila* (pICC562:*sdeC*) and *L. pneumophila* (pICC562). SDS-PAGE gel stained with a SYPRO Ruby Protein Gel Stain. B6 mouse bone marrow-derived macrophages were infected with *L. pneumophila* (pICC562) (1) and *L. pneumophila* (pICC562:*sdeC*) (2) for 24 h at 37 °C and 5% CO_2. Co-immunoprecipitation of cell lysate was performed post-infection with Pierce Anti-HA Magnetic Beads as per the manufacurer's instructions and the final eluate was analysed by separating proteins on an SDS-PAGE gel before staining with a SYPRO Ruby Protein Gel Stain. * Actin identified by LC-MS/MS. Protein ladder with known fragment sizes, in kDa, added for comparison.

Chapter 6: Microscopic observation of the *L. pneumophila sdeC* deletion mutant LCV

6.1. Introduction

Strategies we used to elucidate the function of an unknown effector protein included localisation studies and the identification of binding partners. Other strategies may include bioinformatic prediction of function based on an amino acid sequence, function prediction via protein structure using actual or predicted structures and experimental investigation of specific *in vivo* phenomena. *In vitro* assays to determine protein function are often developed based on the information obtained from the strategies described above.

In chapter 5, we observed that SdeC localised to the LCV during infection and the ER upon transfection. No binding partners were confirmed for the protein and we are yet to determine the specific function of SdeC. However to learn more about the ER and LCV association of SdeC, here we used electron microscopy and live observation of bacterial infection over time to detect any visible, qualitative differences between an *sdeC* deletion mutant and wild type *L. pneumophila*.

After infection of host cells, *Legionella* avoids destruction by the endocytic pathway and modifies the *Legionella*-containing vacuole via interactions with host components to create a mature vacuole permissive for bacterial replication. The LCV fuses with ER-derived vesicles prior to their transport to the Golgi and possibly directly with ER membrane. Our previous work pointed to a possible function for SdeC in *Legionella* vacuole biogenesis given localisation of SdeC with the ER. In order to detect any differences in LCV biogenesis between wild type *L. pneumophila* and the *sdeC* deletion mutant, live cell imaging was used to assess qualitative differences in LCV biogenesis.

In particular since we previously recorded a replication defect in the *sdeC* deletion mutant compared to wild type *L. pneumophila*, here we aimed to use microscopy to measure LCV size over time to potentially determine the point during infection that the observed defect occurs. The overall intracellular replication defect of the *sdeC* deletion mutant may result from slower replication once a mature replicative vacuole is established or the mutant may take longer to establish a mature vacuole prior to intracellular replication.

Live cell imaging to observe LCV development over time, had not been previously established in the laboratory. For this technique, live amoebae or HeLa cells were infected with derivatives of *L. pneumophila* and after infection the sample was placed on a microscope for imaging. The sample was contained within a sealed chamber where temperature and CO_2 are regulated. An image series was then recorded for the amount of time desired to obtain a time course of LCV biogenesis. Here we aimed to study the time course of LCV development for wild type *L. pneumophila* and the *sdeC* deletion mutant to observe any differences in LCV biogenesis. Live tracking dyes were used to stain cellular components to view any changes in cellular behaviour during a live infection setting.

We anticipated that visual investigation of the *sdeC* deletion mutant LCV via live cell imaging may show differences in LCV biogenesis over time in comparison to wild type *L. pneumophila*. The main advantage of live cell imaging is that it allows researchers to get a better understanding of the dynamics of cellular processes in real time. This captures the dynamic nature of live cells in comparison to the static image of fixed samples.

6.2. Results

6.2.1. Comparison of LCV biogenesis between wild type and *L. pneumophila sdeC* deletion mutant-infected cells

To compare the overall ultrastructure of the LCV from wild type *L. pneumophila* and *sdeC* deletion mutant infected cells, we used electron microscopy of mouse bone-marrow derived macrophages infected with *L. pneumophila* 130b and the *sdeC* deletion mutant at 2 and 24 h post-infection (Figure 6.1.). Here macrophages were used as a model system as they are biologically relevant to Legionnaires' disease. Imaging at 2 h post-infection allowed observation of small LCVs in the early stages of development. Imaging at 24 h post-infection allowed observation of LCVs of various sizes in various stages of development, including large established LCVs containing multiple replicating bacteria.

Overall there were no obvious ultrastructural differences in cells infected with the *L. pneumophila sdeC* deletion mutant compared to wild-type *L. pneumophila*. At 2 h post-infection, both strains were present in small vacuoles as a single bacterium. At 24 h post-infection, the LCV of both strains was large and contained multiple bacteria. The size and shape of the LCVs formed by both strains were similar. No differences in the qualitative structure of other cell organelles were observed between the two strains during infection.

6.2.2. Live cell imaging of cells infected with the *L. pneumophila sdeC* deletion mutant

As no differences between the *sdeC* deletion mutant and wild-type *L. pneumophila* were detected via electron microscopy, live cell imaging of infected cells was used to examine LCV biogenesis over time to examine possible differences in LCV formation

between the two strains. In this instance, *A. castellanii* were cultured in 30mm tissue culture dishes as a monolayer and infected with *L. pneumophila* (pMIP:EGFP) and *L. pneumophila ΔsdeC* (pMIP:EGFP) for six hours at 37 °C and 5% CO_2. The pMIP:GFP plasmid is a *Legionella* expression vector encoding EGFP constitutively expressed under the *mip* promoter. Bacteria possessing this vector will produce EGFP which will remain in the cytoplasm, with the now fluorescent bacteria easily identifiable during fluorescent imaging. After 6 h infection, phase contrast and fluorescent images of cells infected with *L. pneumophila* (pMIP:EGFP) and *L. pneumophila ΔsdeC* (pMIP:EGFP) were taken with a confocal microscope every 30 min for 5.5 hours (Figure 6.2. and Figure 6.3. respectively. Refer to .avi files of Figure_6_2 and Figure_6_3).

Live cell imaging of infection showed the development of large LCVs six to eleven hours post infection. This time period displayed a progression from small vacuoles containing few bacteria to large vacuoles that occupied the majority of the host cell. Separate smaller vacuoles in the same host cell were observed to fuse together into larger vacuoles in both strains from approximately 8 h post-infection. This is a previously unreported phenomenon in the current literature.

The development of the LCV was generally similar at each time point for both strains. Although wild type LCVs may have been slightly more developed at the 6 hour time point compared to *sdeC* mutant LCVs, the sizes of *sdeC* mutant LCVs were comparable to that of wild type *L. pneumophila* from 7 to 8 h post infection.

6.2.3. Changes in *L. pneumophila* vacuole size over time

We proposed that quantifying LCV sizes over time may reveal a defect in the development of the LCVs in a single round of infection with *L. pneumophila sdeC* mutant-infected cells. This could then explain the intracellular replication defect observed in the co-culture assays of previous chapters.

To determine when during the *Legionella* infection and replication process the *sdeC* deletion mutant displayed attenuated replication, the size of the mutant LCV was measured over time during infection of *A. castellanii* and compared to wild type *L. pneumophila*. *A. castellanii* were cultured in 30 mm tissue culture dishes as a monolayer and infected with *L. pneumophila* (pMIP:GFP) and *L. pneumophila ΔsdeC* (pMIP:GFP) at a temperature of 37 °C with 5% CO_2. Phase contrast and fluorescent images were taken with a confocal microscope at 2, 6, 9, 12 and 15 h post-infection (Figure 6.4.). LCVs, despite not being completely uniform in shape, were generally roughly circular and their diameter was measured.

There was no significant difference in average vacuole size of the *L. pneumophila sdeC* deletion mutant compared to wild type *L. pneumophila* at 2, 6 and 9 h post-infection. Vacuoles of both strains at 2, 6 and 9 h remained small containing single or few bacteria at 1-6 µm.

However, at 12 and 15 h post-infection sizes of *sdeC* mutant LCVs were significantly smaller compared to wild type *L. pneumophila* ($P < 0.02$, Mann-Whitney test, unpaired, 2-tailed), even though vacuole sizes of both strains at 12 and 15 h were highly variable at 3-22 µm.

While overall both strains behaved similarly, there were significantly smaller sized vacuoles of the *L. pneumophila sdeC* deletion mutant at 12 and 15 h post-infection. This reflects the previously observed intracellular replication defect of the *sdeC* mutant.

6.2.4. Investigation of changes in interactions with the ER of the *sdeC* mutant LCV

Previous SdeC transfection experiments suggested a link between SdeC localisation and the ER of host cells. Therefore, we performed a live cell imaging experiment using

HeLa cells and ER-Tracker Red (Life Technologies) to visualise ER recruitment to the LCV. HeLa cells, chosen for their ease of use and their ER biogenesis being the same as other human cell lines, were cultured in 30 mm tissue culture dishes as a monolayer and infected with *L. pneumophila* (pMIP:GFP) and *L. pneumophila ΔsdeC* (pMIP:GFP) at 37 °C and 5% CO_2. Infected cells were stained with ER-Tracker Red and phase contrast and fluorescent images were taken with a confocal microscope at 2.5, 4 and 7 h (Figures 6.5. and Figure 6.6.). Uninfected cells stained with ER-Tracker Red were also observed for comparison (Figure 6.7.).

Both *L. pneumophila* (pMIP:GFP) and *L. pneumophila ΔsdeC* (pMIP:GFP) vacuoles associated with the ER after infection. Bacteria were often located within the ER stained region of the HeLa cell. Occasionally the ER staining was more intense around the location of the bacteria, and this seemed to be slightly more prevalent in cells infected with wild type *L. pneumophila* (pMIP:GFP). Apart from this infrequent co-localisation, ER-Tracker was unable to clearly reflect the interaction of the LCV with ER. The stain also exhibited severe photobleaching over time and so may not have been the most ideal way to observe any changes in ER interactions during live infections.

6.2.5. Fusion of multiple *Legionella*-containing vacuoles

Upon observation of *Legionella*-containing vacuole biogenesis, separate small vacuoles were observed to fuse together into larger vacuoles (Figure 6.2. and Figure 6.3.). To confirm this we used *L. pneumophila* expressing two different fluorophores (EGFP and mCherry) and observed fusion between the two populations over time. *A. castellanii* were cultured in 30 mm tissue culture dishes as a monolayer and infected with *L. pneumophila* (pMIP:GFP) and *L. pneumophila ΔsdeC* (pMIP:mCherry) at a temperature of 37°C and 5% CO_2 for 24 hours. Cells infected with both fluorescent strains were identified and phase contrast and fluorescent images were then taken with a confocal microscope every 12 minutes for 96 minutes (Figure 6.8.; Refer to .avi file Figure_6_8).

Vacuoles of the two distinct populations of fluorescent *L. pneumophila* were observed to fuse together into a single large vacuole (Figure 6.8.). After initial infection, the two differentially labelled strains were present in distinct vacuoles. Over time the red and green fluorophores were co-localised in the same vacuole, indicating fusion of their separate vacuoles.

Here we confirmed the earlier observation from live cell imaging experiments that the fusion of multiple LCVs occurs during the later stages of *L. pneumophila* infection of amoebae. The bacterial factors directing this fusion are unknown.

6.3. Discussion

Here various microscopy techniques were used to try and observe differences between a *L. pneumophila sdeC* deletion mutant and wild type *L. pneumophila* to explain the replication defect of the *sdeC* mutant. Electron microscopy using a Phillips CM12 transmission electron microscope and live cell imaging techniques using a Zeiss LSM700 laser scanning confocal microscope were useful for the observation of infected cells, focusing on development of the *Legionella*-containing vacuoles.

Electron microscopy was used to visualise the LCV during infection. We would have expected the *L. pneumophila sdeC* deletion mutant to have an impaired ability to develop mature LCVs due to the defect in intracellular replication observed earlier. However, when we compared the vacuole of the *sdeC* deletion mutant to that of wild type *L. pneumophila*, no differences between the two strains were detected.

By electron microscopy, the *L. pneumophila sdeC* mutant behaved in the same way as wild type *L. pneumophila*, multiplying within the replicative vacuole over 24 hours. The size and shape of the LCVs of both strains were qualitatively similar. No differences in the structure of cell organelles were observed between the two strains during

infection. In fact, the absence of SdeC did not seem to affect the formation of mature LCVs in any observable way using electron microscopy. We hypothesised that observing different time points during LCV development may reveal subtle differences between the two strains that were not apparent at the time points we chose to investigate by electron microscopy.

Comparison of biogenesis of LCVs in cells infected with wild type *L. pneumophila* and the *sdeC* deletion mutant was performed over time using live cell imaging. However, again, no significant differences between the two strains were observed. The development of LCVs 6 to 11.5 h post-infection displayed a progression from small vacuoles containing few bacteria to large vacuoles with numerous bacteria that in many cases took up the majority of the host cell cytoplasm. The development of the LCVs for both strains was generally similar at each time point observed. Although, the LCVs in the wild type *L. pneumophila*-infected cells may have been slightly more developed at the 6 hour time point in comparison to the *L. pneumophila sdeC* deletion mutant-infected cells, this phenotype was subtle. In addition, the sizes of the *L. pneumophila sdeC* deletion mutant LCVs were comparable to that of wild type *L. pneumophila*-infected cells 7 to 8 h post infection. This may have indicated that the mutant has a slight delay in LCV development seen at 6 h post infection, but the delay is not detectable at later time points. However, the subtlety of this phenotype means more investigation is required.

To more quantitatively determine when during LCV development there may be a delay in bacterial replication, the change in LCV size over time for wild type *L. pneumophila* infected cells was compared to that of the *sdeC* deletion mutant infected cells.

There was no significant difference in average vacuole size of the *L. pneumophila sdeC* deletion mutant compared to wild type *L. pneumophila* at 2, 6 and 9 h post-infection. However, at 12 and 15 h post-infection, *sdeC* mutant LCVs were significantly smaller compared to wild type *L. pneumophila* LCVs.

Vacuole sizes of both strains at 12 and 15 hours were highly variable at 3-22 µm. While overall both strains behaved similarly, the significantly smaller sized vacuoles of the *L. pneumophila sdeC* deletion mutant at 12 and 15 h post-infection may reflect the previously observed intracellular replication defect of the *sdeC* mutant. It is unclear exactly when during the infection cycle the initiation of replication lies, with a sharp increase in LCV size between 9 and 12 h post-infection. More time points may need to be assessed in order to ascertain this, identifying where the delay in the formation of a mature LCV and the initiation of replication is between the 9 and 12 h time points.

A link between SdeC and the ER of host cells was suggested in previous SdeC transfection experiments. Therefore a live cell imaging experiment was conducted in HeLa cells with a fluorescent ER live cell imaging stain to visualise the ER during infection. We hoped this would highlight any differences in ER trafficking during infection when comparing infections of wild type *L. pneumophila* and the *L. pneumophila sdeC* deletion mutant. This live cell imaging experiment was not a continuous experiment conducted at the microscope in a specialised heated chamber as the previous live cell imaging experiment was. Instead, at each time point, cells were removed from the incubator and microscopic images were taken before returning the cells to the incubator. This was done to obtain higher resolution images of single cells as we were able to focus in on a single cell at each time point. This was not practical in the previous experiment with amoebae due to the dynamic nature of the live cells.

Both *L. pneumophila* (pMIP:GFP) and *L. pneumophila ΔsdeC* (pMIP:GFP) associated with the ER after infection. Bacteria were often located within the ER stained region of the host HeLa cell. Occasionally the ER staining was seen to be more intense around the location of the bacteria. This phenomena seems to be slightly more prevalent in cells infected with wild type *L. pneumophila* (pMIP:GFP).

To quantify this observation, future experiments may study multiple infected cells counting any displaying intense ER staining at the LCV and comparing this percentage

between wild type *L. pneumophila* and the *L. pneumophila sdeC* deletion mutant. A co-infection with both strains, differentially fluorescently labelled, in the one sample may also display a bias for ER recruitment to wild type *L. pneumophila* LCVs in cells infected with both strains simultaneously. It was noted that ER-tracker may not have been the most ideal stain to use due to extreme photobleaching. Different microscopy techniques and other superior stains may be investigated to improve the results. Host cells expressing fluorescently labelled ER markers would also be another way to ensure optimum fluorescent levels over long periods of time.

During live cell imaging experiments it was observed that separate LCVs within the one cell fused together during development. We used two *Legionella* strains expressing different fluorophores in a live cell imaging experiment to confirm this and observe vacuole fusion in more detail. Vacuoles of the two distinct populations of fluorescent *L. pneumophila* were observed to fuse together from approximately 8 h post-infection. After initial infection, the two differentially fluorescently labelled strains were present in distinct vacuoles. Over time, the two different fluorophores were co-located in the same vacuole, indicating fusion of their separate vacuoles.

Legionella is able to promote fusion with ER-derived vesicles when developing a replicative vacuole (Derre and Isberg, 2005; Kagan and Roy, 2002; Kagan et al., 2004; Liu and Luo, 2007; Ragaz et al., 2008; Robinson and Roy, 2006). However, fusion of multiple LCVs present in the same host cell has not previously been reported. It is possible that the same mechanisms *Legionella* uses to fuse the LCV with host cellular compartments are involved here. The receptors targeted by *Legionella,* present on the membranes of cellular compartments, will likely still be present on the LCV after fusion. A separate LCV in the same host cell may then use the same fusion mechanisms to fuse with the other LCV. There is also the possibility that quorum sensing is involved in the triggering of this event, which may be confirmed by observation of *L. pneumophila* with deletions in quorum sensing genes.

Our previous experiments with the *L. pneumophila sdeC* deletion mutant also exhibited fusion of multiple LCVs. However, this may still be an avenue of investigation in the future if the number of fusion events and their timing during the infection process is studied and compared to that of wild type *L. pneumophila*. Due to the possible link between SdeC and ER membrane and the LCV, this may uncover a defect in the mutant's vacuole fusion ability.

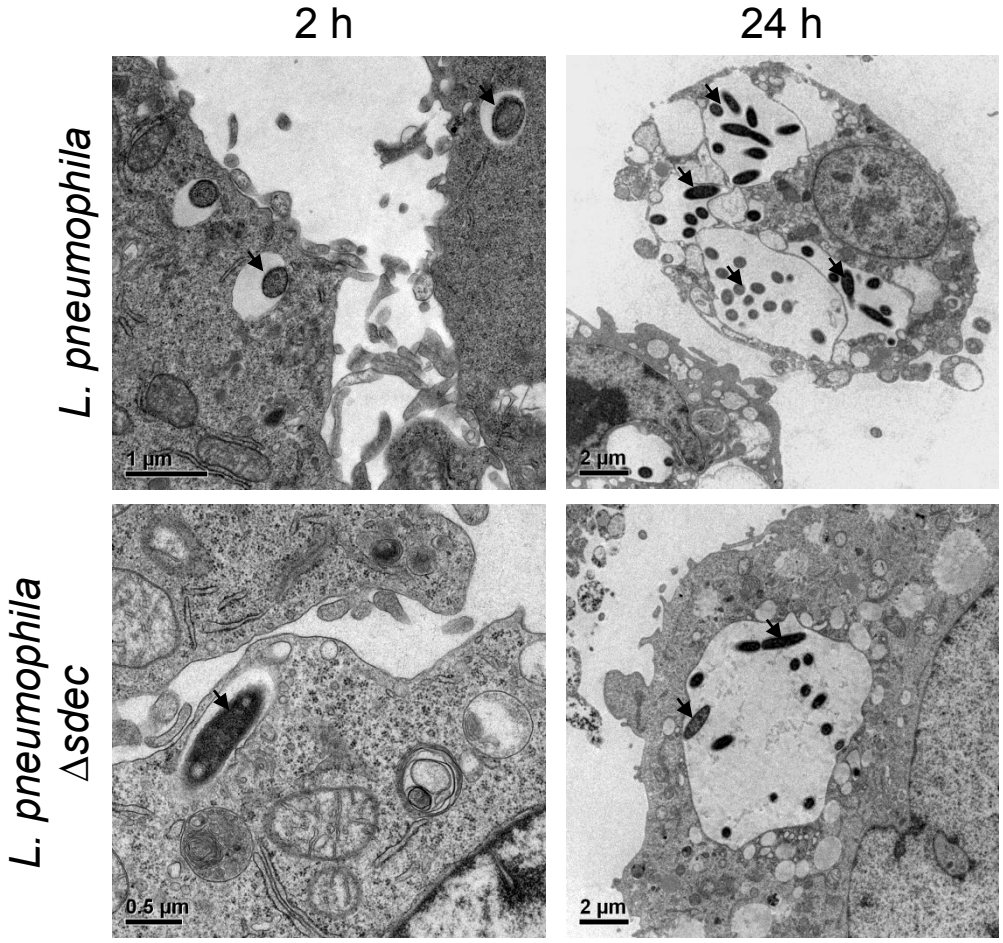


Figure 6.1. Electron micrograph of mouse bone-marrow derived macrophages infected with *L. pneumophila* and *L. pneumophila Δsdec.* Macrophages were infected with the strains indicated for 2 and 24 h at 37 °C and 5% CO_2 and prepared for electron microscopy. At two hours post-infection both strains are present in small vacuoles as a single bacterium (arrows). At 24 h post-infection LCVs of both strains are large and contain multiple bacteria (arrows). Representative images from at least 20 examined cells. Electron Microscopy was performed by Vicki Bennett-Wood (University of Melbourne).

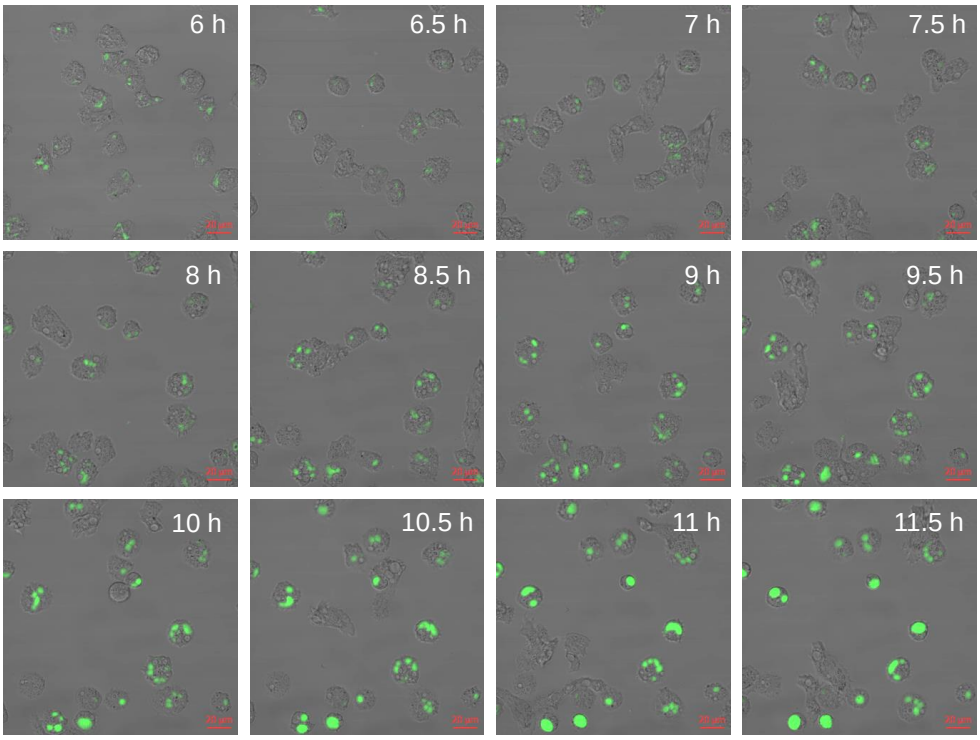


Figure 6.2. Live cell imaging of *A. castellanii* infected with *L. pneumophila* (pMIP:GFP). Fluorescent and phase contrast microscopy images of *A. castellanii* infected with *L. pneumophila* (pMIP:GFP) for 6 h at 37 °C and 5% CO_2. Phase contrast and fluorescent images were then taken with a Zeiss LSM700 confocal microscope every 30 min for another 5.5 h with a FITC fluorescent filter.

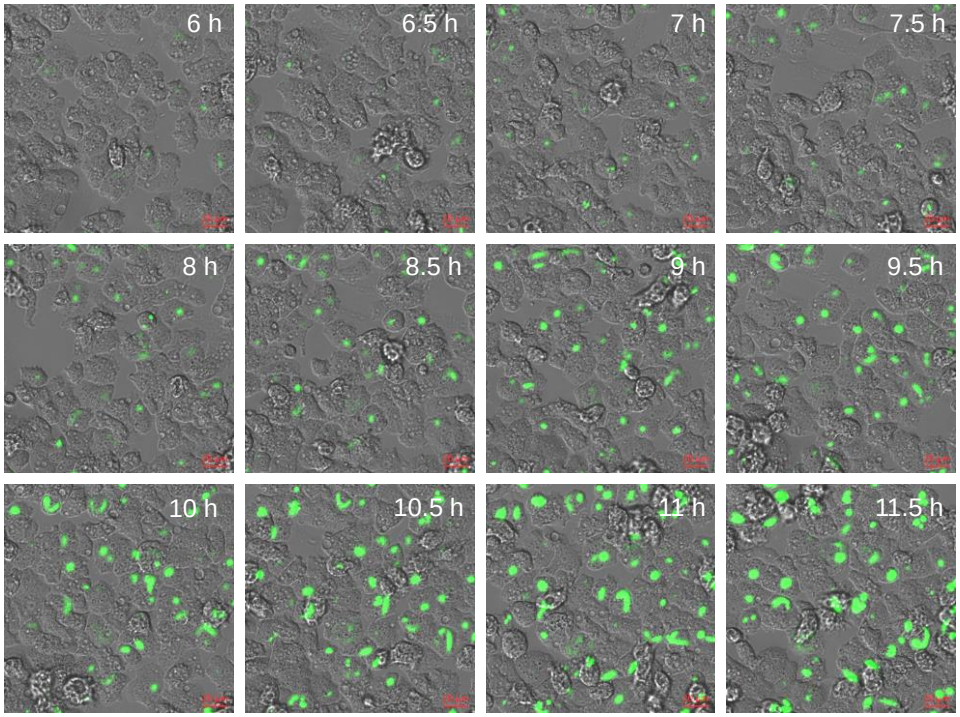


Figure 6.3. Live cell imaging of *A. castellanii* infected with *L. pneumophila ΔsdeC* (pMIP:GFP). Fluorescent and phase contrast microscopy images of *A. castellanii* infected with *L. pneumophila ΔsdeC* (pMIP:GFP) for 6 h at 37 °C and 5% CO_2. Phase contrast and fluorescent images were then taken with a Zeiss LSM700 confocal microscope every 30 min for another 5.5 h with a FITC fluorescent filter.

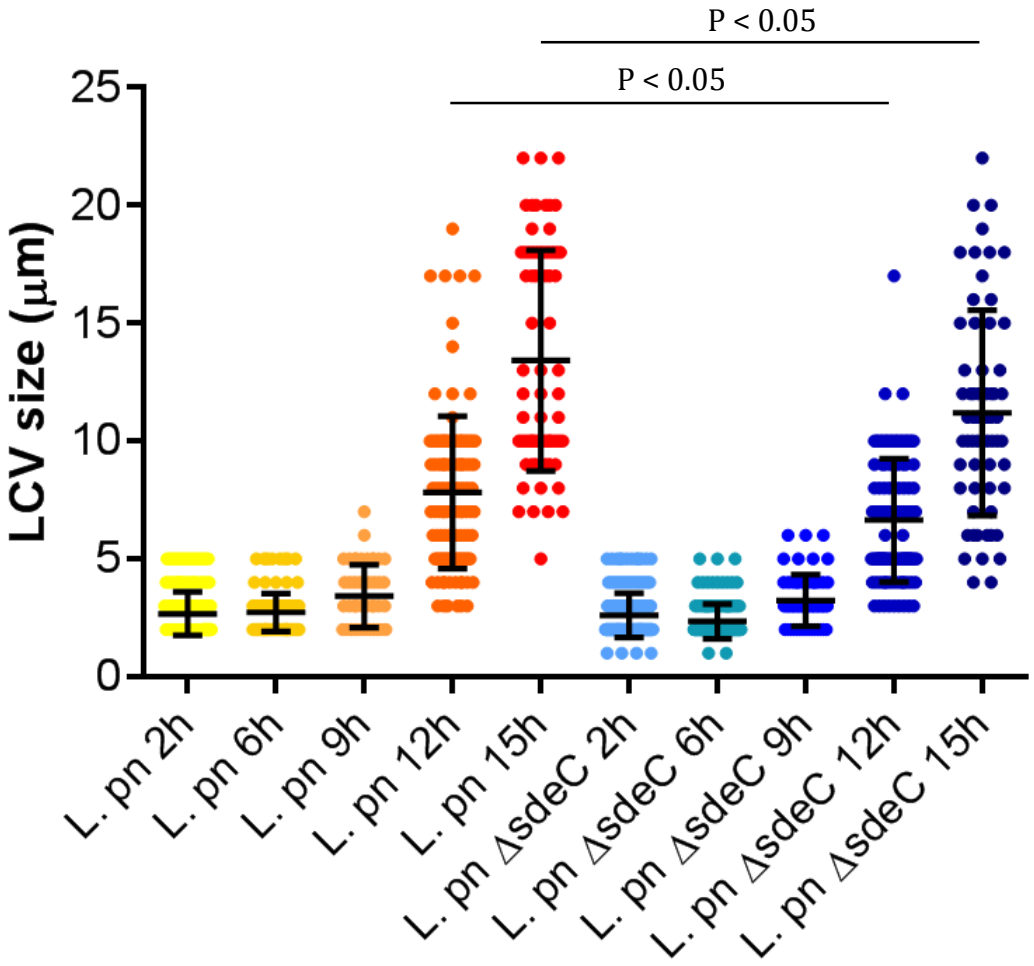


Figure 6.4. Changes in LCV size over time of *L. pneumophila* and *L. pneumophila sdeC* deletion mutant. Dot plot of LCV size for each indicated strain at the indicated time points. *A. castellanii* infected with *L. pneumophila* and *L. pneumophila sdeC* deletion mutant strains, both carrying the (pMIP:GFP) construct, at 37 °C and 5% CO_2. Phase contrast and fluorescent images were taken with a Zeiss LSM700 confocal microscope at 2, 6, 9, 12 and 15 h post-infection with the FITC fluorescent filter and LCV size of multiple vacuoles were measured using ZEN2010 software. Mann-Whitney tests were used to determine statistically significant differences between infections at each time point (unpaired, two-tailed, p-value < 0.05).

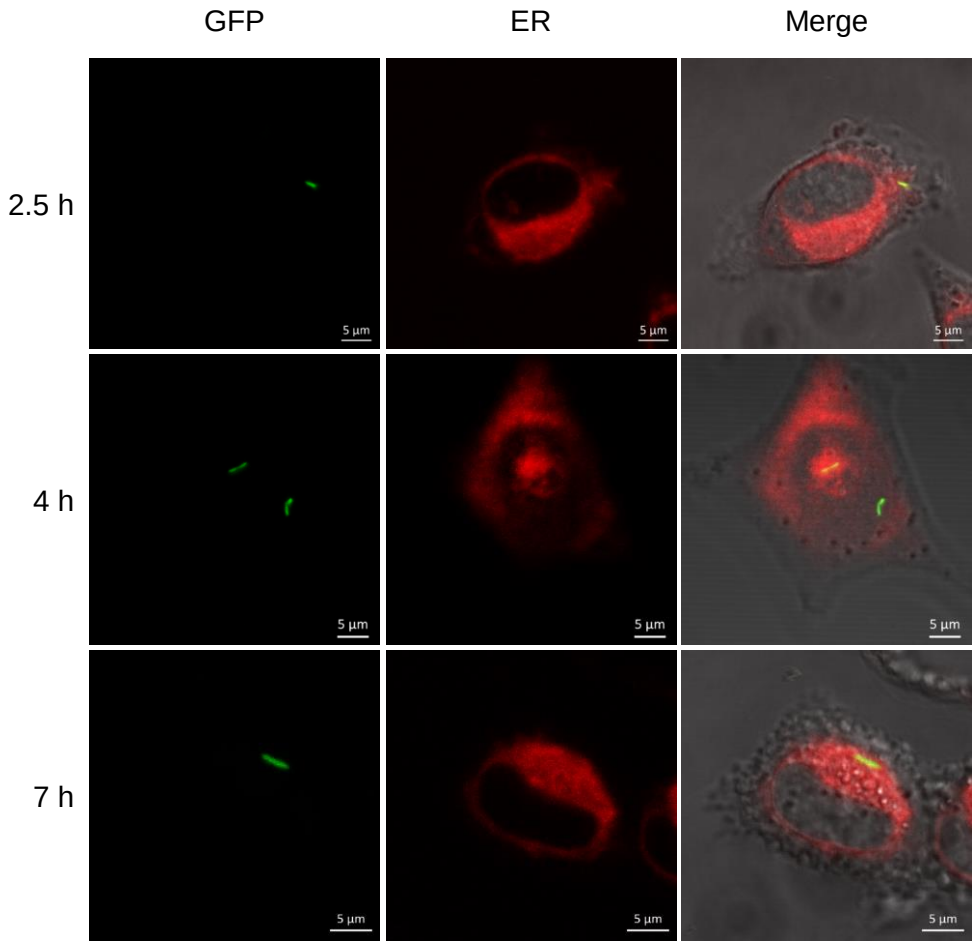


Figure 6.5. Live cell imaging of ER stained HeLa cells infected with *L. pneumophila* (pMIP:GFP). Fluorescent and phase contrast microscopy images of HeLa cells infected with *L. pneumophila* (pMIP:GFP). Cells were stained with ER-Tracker Red to visualise ER at 37 °C and 5% CO_2. Phase contrast and fluorescent images were taken with a Zeiss LSM700 laser scanning confocal microscope at 2.5, 4 and 7 h post-infection with the FITC and Texas Red fluorescent filters.

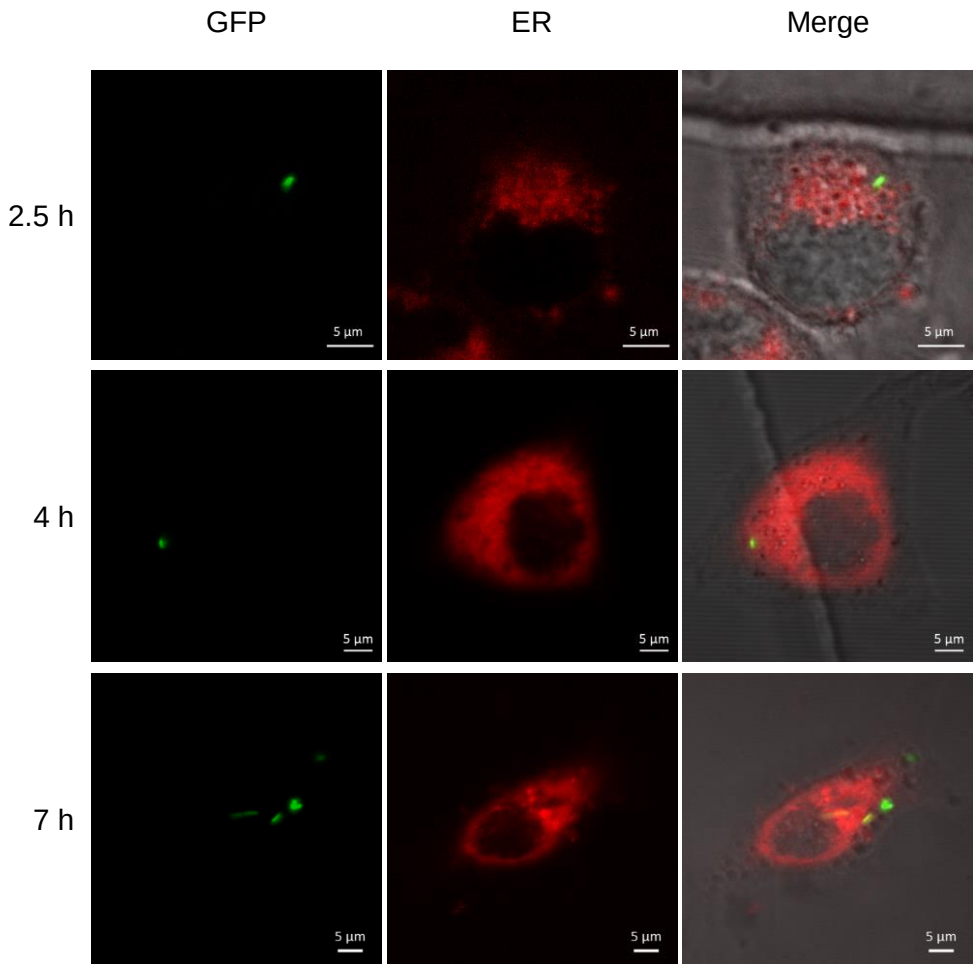


Figure 6.6. Live cell imaging of ER stained HeLa cells infected with *L. pneumophila ΔsdeC* (pMIP:GFP). Fluorescent and phase contrast microscopy images of HeLa cells infected with *L. pneumophila ΔsdeC* (pMIP:GFP). Cells were stained with ER-Tracker Red to visualise ER at 37 °C and 5% CO_2. Phase contrast and fluorescent images were taken with a Zeiss LSM700 laser scanning confocal microscope at 2.5, 4 and 7 h post-infection with the FITC and Texas Red fluorescent filters.

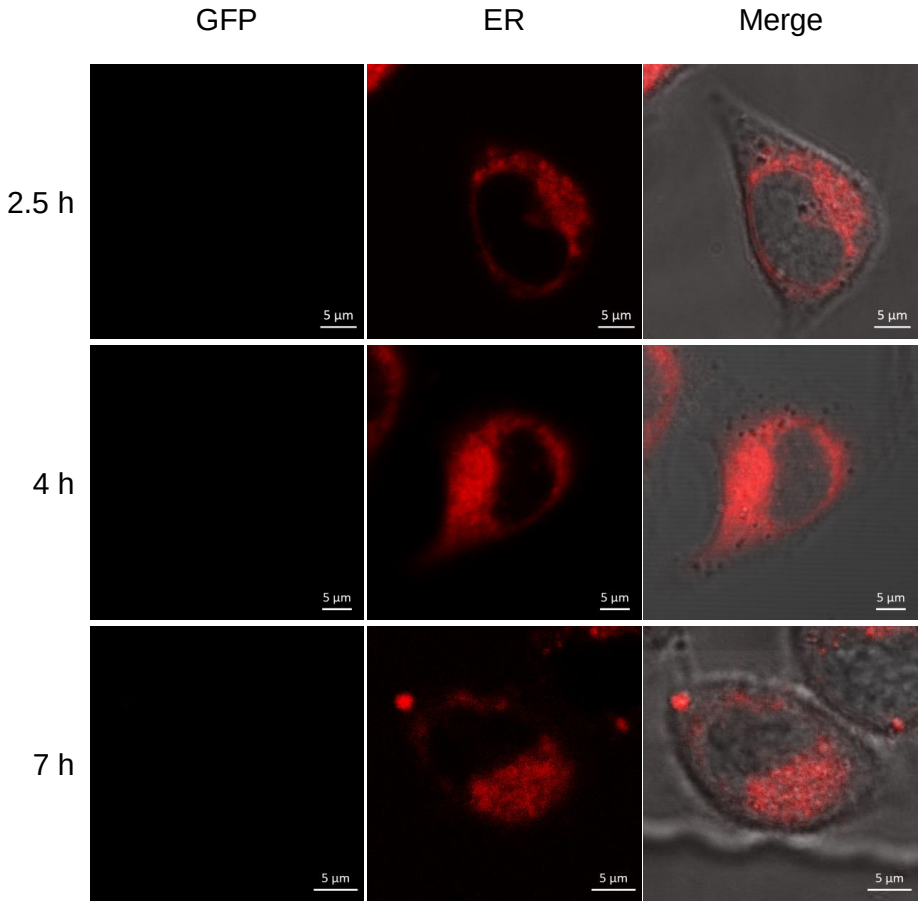


Figure 6.7. Live cell imaging of ER stained uninfected HeLa cells. Fluorescent and phase contrast microscopy images of uninfected HeLa cells stained with ER-Tracker Red to visualise ER cultured at 37 °C and 5% CO_2. Phase contrast and fluorescent images were taken with a Zeiss LSM700 laser scanning confocal microscope at 2.5, 4 and 7 h post-infection with the FITC and Texas Red fluorescent filters.

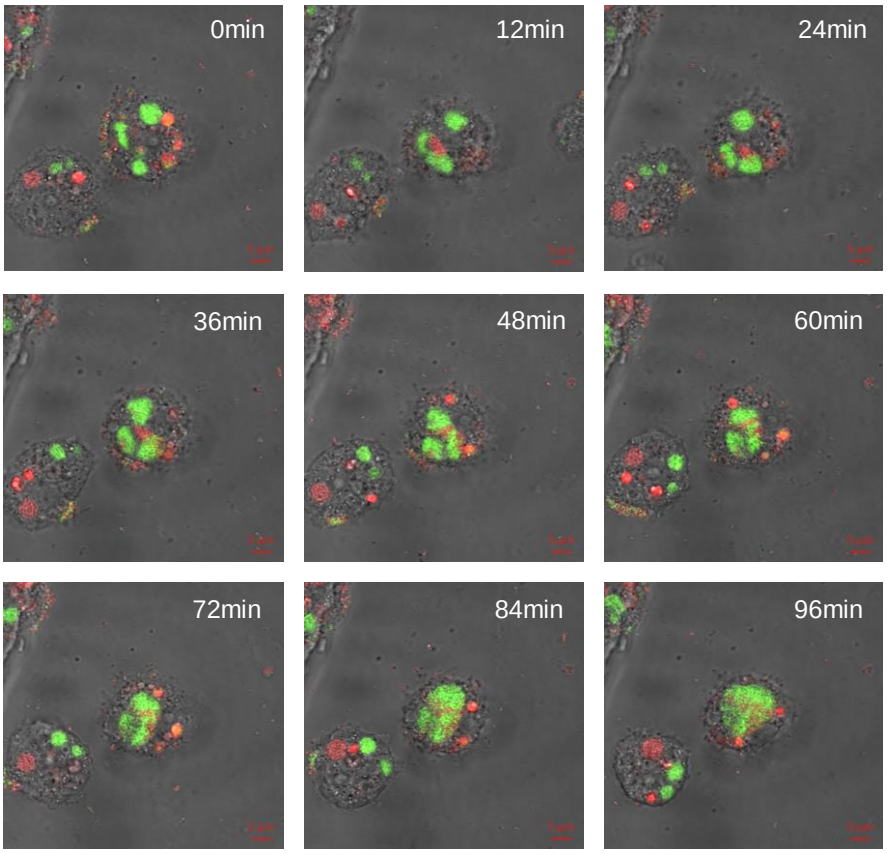


Figure 6.8. Fusion of multiple *L. pneumophila*-containing vacuoles during infection of *A. castellanii*. Fluorescent and phase contrast microscopy images of *A. castellanii* infected with *L. pneumophila* (pMIP:GFP; green bacteria) and *L. pneumophila* (pMIP:mCherry; red bacteria) for 24 h at 37 °C and 5% CO_2. A cell containing multiple LCVs of both colours was located and phase contrast and fluorescent images were then taken with a Zeiss LSM700 laser scanning confocal microscope with the FITC and Texas Red fluorescent filters every 12 min for 96 min.

Chapter 7: Perspective

Legionnaires' disease is an emerging illness that can become a major problem when *Legionella* is permitted to grow to large numbers in man-made water supply systems. Proper treatment of the disease and adequate control of *Legionella* in the environment relies on a solid understanding of ecological needs in addition to pathogenesis at the molecular level. Here we have facilitated advancement of this knowledge by identifying novel factors involved in pathogenesis of host cells with a focus on a host commonly identified in *Legionella*-contaminated aquatic environments, *A. castellanii*. Several genes that are potentially important for intracellular replication in *A. castellanii* were identified using a transposon mutagenesis screen. A few were subsequently selected for further study and characterisation.

The genome of *L. pneumophila* strain 130b was sequenced and annotated here. Ten new Dot/Icm translocated effectors were identified and their prevalence amongst various different *L. pneumophila* isolates was examined. Many of the identified effectors were only present in a few of the strains examined. These proteins add to a growing list of accessory effectors not present in all *L. pneumophila* strains and reinforce the diverse nature of *L. pneumophila* genomes existing between different strains. A diverse accessory genome may aid the ability of *L. pneumophila* to survive in a large variety of different hosts, reflecting its evolution in a variety of aqueous environments, and may account for differences in virulence seen in different *L. pneumophila* strains. The role of the newly identified effectors is yet to be determined. Further investigation is required to determine their contribution to *Legionella* pathogenesis.

Here a library of 10,006 transposon mutants in *L. pneumophila* strain 130b was created and screened for attenuated replication within *A. castellanii* in order to identify novel

genes involved in intracellular replication and survival. 13 unique genes were confirmed to negatively affect intracellular replication in *A. castellanii*. Six of these, *dotA, icmB, icmE, icmH, icmK* and *icmL,* encode components of the well-known Dot/Icm T4SS. The identification of these *dot/icm* genes was expected as the secretion system is essential for intracellular replication in host cells. Many of the remaining identified genes (*fabF, lpw07571, lpw27511, lpw29561, potC, sdeC* and *vacB*) had not previously been implicated in *Legionella* pathogenesis.

Our screen of 10,006 transposon mutants covered approximately 95% of the *L. pneumophila* 130b strain genome, potentially identifying the majority of genes singularly required for intracellular replication in *A. castellanii*. However, this may not be entirely accurate as completely random mutagenesis may not have been achieved as the IS903 transposon can show preferential binding to a particular sequence, albeit a highly degenerate one (Hu and Derbyshire, 1998; Hu et al., 2001). The fact that not all of the *dot/icm* genes, most of which have previously been reported as essential for intracellular replication (Andrews et al., 1998; Segal and Shuman, 1999), were identified in the screen may indicate non-random insertion. It is also worth noting that like *lpw27511*, some of the identified genes may be false positives and would require the retesting of independent deletions to verify.

As mentioned previously, a high level of functional redundancy exists in *L. pneumophila* virulence factors and so this screen would also not identify important groups of proteins with similar functions due to only one gene being disrupted at a time after transposon mutagenesis. It is also worth noting that the screen was specific to genes important for replication in *A. castellanii*. There may be genes important for intracellular replication in other host cells and not *A. castellanii* that were not identified here.

Of the genes identified, *fabF* produces a protein that is involved in fatty acid biosynthesis. Two other putative metabolic genes, *lpw07571* and *lpw29561,* a putative peptidase of the esterase-lipase superfamily and a putative sugar kinase respectively,

were also identified. It is possible that the transposon insertion into the putative sugar kinase gene, *lpw29561*, may result in the alteration of LPS structure, producing less fit bacteria in the presence of amoebae. These genes may be important for metabolism in an intracellular setting, hence their identification in this assay. However, the intracellular replication defect must still be confirmed via the construction of in-frame deletion mutants before deeming them important.

Two other genes *vacB*, a putative exoribonuclease, and *potC*, a component of a polyamine transporter, were also identified in the screen. The transposon mutants of both genes displayed severe intracellular replication defects in *A. castellanii*. The importance of these genes in intracellular replication must also be confirmed via the construction of in-frame deletion mutants.

This study focused on the characterisation of only two of the genes identified in the transposon mutagenesis screen, *sdeC* and *lpw27511*. The *dot/icm* genes that were identified encode components of the Dot/Icm T4SS already known to be essential to intracellular replication. The other genes identified are potentially required for intracellular replication and may also be good candidates for further study. The creation of in-frame deletion mutants and the subsequent complementation and retesting of these mutants in intracellular replication assays is important for the confirmation of their involvement in intracellular replication and survival in host cells.

sdeC encodes a highly similar paralogue of the Dot/Icm T4SS translocated effector, SidE. A previous study found that SdeC is also translocated by the Dot/Icm T4SS and unlike SidE and other SidE paralogues, was found to be important for efficient intracellular replication in *Dictyostelium discoideum* and mouse bone marrow-derived macrophages (Luo and Isberg, 2004). Our work added to this, with the *sdeC* mutant displaying intracellular replication defects in *A. castellanii* and THP-1 cells.

lpw27511 encodes a putative transcriptional regulator of the LuxR family. The *lpw27511* transposon mutant displayed a severe intracellular replication defect

compared to wild type *L. pneumophila* in *A. castellanii* and THP-1 cells and so was an exciting finding as it was thought to be a major uncharacterised global regulator important during infection for a subset of strains, as the full length *lpw27511* gene is not present in all of the sequenced *L. pneumophila* strains. To get a better understanding of the distribution of the gene amongst *L. pneumophila* strains, a southern blot was performed to detect the presence of the gene in a variety of strains. *lpw27511* was present in seven of nine *L. pneumophila* Australian isolates, indicating a relatively high level of conservation among *L. pneumophila* strains in this group. However, *lpw27511* was not present in any of the non-*pneumophila* strains tested. While the gene was identified in strains of several different serogroups (1, 4, 5, 6 and 8) and in both environmental and clinical strains, a larger sample size would be required to determine any preference to any group.

Downstream of *lpg2524,* the Philadelphia strain *lpw27511* homolog*,* is the gene *lpg2525 (mavK)*. *mavK* encodes a protein that contains an F-BOX domain. F-BOX proteins are primarily a eukaryotic protein family but several F-BOX proteins have been identified in *L. pneumophila*. MavK has not been previously studied and as a potential virulence factor warranted further study.

As *mavK* is also not present in all of the sequenced *L. pneumophila* strains, a southern blot was performed to get a better understanding of the distribution of the gene amongst various *L. pneumophila* strains. *mavK* was present in four of nine *L. pneumophila* isolates tested but was not present in any of the non-*pneumophila* strains tested. This moderate level of conservation amongst the strains tested may indicate that *mavK* does not play a central role in the *L. pneumophila* life cycle or that other proteins performing similar functions are present in the genome, leading to a low level of *mavK* conservation due to a functional redundancy. This eukaryotic-like protein, however, may still be an interesting candidate for further study and characterisation.

An in-frame *lpw27511* deletion mutant was created have by replacing the gene with a kanamycin resistance cassette. This deletion mutant was used for further study of *lpw27511*. To determine the regulatory targets of *lpw27511,* the gene expression profile of the deletion mutant was compared to that of wild type *L. pneumophila* using a microarray. Analysis during the replicative phase of *L. pneumophila* did not reveal any significant differences in gene expression levels between the two strains. This was not surprising as *lpw27511* is known to be active during the transmissive phase (Jules and Buchrieser, 2007). In the transmissive phase, the expression levels of several genes were increased or decreased at relatively low levels (2-3 fold changes in expression). The most significant result was a 5.26 fold up-regulation of expression of *lpp2263* (Paris strain homolog of *lpw25041* in 130b) when Lpw27511 was present. Like the majority of the genes identified in the microarray *lpp2263/lpw25041* encodes an unstudied hypothetical protein of unknown function. Although the changes in expression levels were low, other genes of note included *sdeD*, a Dot/Icm translocated effector of unknown function, several metabolic genes and *letE*, a protein that enhances differentiation of *L. pneumophila* to a transmissible form. Interestingly, *letE* transcripts were abundant in exponential growth phase bacteria but were rare in the post-exponential growth phase (Bachman and Swanson, 2004). Lpw27511 may be involved in this change as *letE* transcripts are more abundant in the post-exponential growth phase of the *lpw27511* deletion mutant in comparison to wild type *L. pneumophila*, indicating that when Lpw27511 is present *letE* expression is down-regulated. However, confirmation of changes in expression via qRTPCR experiments is needed before any conclusions can be made as to whether any of these genes are truly regulated by Lpw27511.

It is worth noting that the microarray was only able to detect expression of genes from the *L. pneumophila* Philadelphia, Paris, Lens and Corby strains as these were the only fully annotated genomes available at the time. The fully sequenced and annotated 130b genome, in particular, was unavailable. If Lpw27511 regulates genes that are present only in the 130b strain these genes would not have been detected in this

microarray. Although with an Lpw27511 homolog in the Philadelphia strain, a strain included in the microarray, the chances of this were likely reduced assuming the homolog affects similar genes. In addition, the DNA microarray was performed in culture with bacteria alone. It is possible that the results may be different if a microarray was performed during infection of a host cell. Unfortunately, no obvious explanation for the major intracellular replication defect seen for the *lpw27511* transposon mutant during *A. castellanii* infection was seen for the defined mutant.

To determine if the *lpw27511* deletion mutant still possessed the same intracellular replication defect as the *lpw27511* transposon mutant, bacterial numbers were compared to that of wild type *L. pneumophila* during a 72 h *A. castellanii* co-culture assay. Despite a slight trend of reduced bacterial numbers of the *lpw27511* deletion mutant at later stages of infection, after statistical analysis intracellular replication of the deletion mutant in the presence of *A. castellanii* is not significantly attenuated in comparison to wild type *L. pneumophila* at any time point. This is in contrast to the severe defect seen for the *lpw27511* transposon mutant in the presence of *A. castellanii*.

Bioinformatic analysis of transcriptional start and stop sites revealed that *lpw27511* is not part of an operon and so the transposon insertion should not have had polar effects on downstream genes, which may have been responsible for the difference in intracellular replication levels between the transposon insertion mutant and the deletion mutant. In addition, the results of the *A. castellanii* co-culture assay indicated that *lpw27511* alone was not responsible for the severe intracellular replication defect seen for the transposon mutant. To confirm this, the transposon mutant was complemented with full length *lpw27511* and intracellular replication in the presence of *A. castellanii* was compared to the transposon mutant and wild type *L. pneumophila* in an amoebae plate test. Growth of the complemented strain was similar to that of the transposon mutant.

The severe intracellular replication defect of the transposon insertion mutant could have been explained by random mutations in other parts of the genome. The level of attenuation observed was similar to that of strains with non-functioning Dot/Icm T4SS and we hypothesised that there may be a random mutation in a *dot/icm* gene. However, the ability of the transposon mutant to translocate the Dot/Icm effector RalF, tested via a TEM-1 translocation reporter system, was found to be still intact. While the Dot/Icm T4SS was still functioning in the transposon insertion mutant, there may have been random mutations in another part of the genome, explaining the severe defect. To determine if this was the case the entire genome of the transposon insertion mutant was sequenced and compared to that of wild type *L. pneumophila*. Although there were gaps present in the genome coverage, giving rise to the possibility that a single polymorphism went undetected, no differences were detected between the genomes of the transposon insertion mutant and wild type *L. pneumophila* strain 130b, apart from the transposon insertion itself.

While we determined that loss of Lpw27511 was not responsible for the severe intracellular replication defect seen in the transposon insertion mutant, a reason for the defect was not identified. The transposon mutant possesses a functioning Dot/Icm T4SS and there were no detected differences in genomic comparisons with wild type *L. pneumophila*. *lpw27511* is not part of an operon and therefore there should not be polar effects on any downstream genes, however transcription start and stop sites should be confirmed experimentally.

It is not uncommon for transcriptional regulators to affect intracellular replication in *Legionella*. In addition to LuxR-like transcriptional regulators, *L. pneumophila* possesses other regulators that have displayed varying effects on intracellular replication upon deletion. The PmrA/PmrB two-component system is a global regulator of 279 genes including genes encoding the Dot/Icm T4SS and secreted effectors, eukaryotic-like proteins, stress response genes, flagellar biosynthesis genes and metabolic genes. A *pmrB* mutant was more sensitive to low pH than wild type *L. pneumophila*, suggesting that acidity may trigger the two-component system. A *pmrB* mutant also exhibited a

significant intracellular replication defect within human macrophages (approximately 100-fold reduction in CFU relative to wild type *L. pneumophila*) and *Acanthamoeba polyphaga* (approximately 10-fold reduction) (Al-Khodor et al., 2009).

The CpxR/CpxA two-component system regulates the expression of several *dot/icm* genes and genes encoding Dot/Icm translocated substrates (Altman and Segal, 2008; Gal-Mor and Segal, 2003). However, CpxR and CpxA are dispensable for intracellular replication within *A. castellanii* and HL-60-derived human macrophages (Gal-Mor and Segal, 2003).

The LetA/LetS two component system regulates expression of flagella and pigment, and is required for resistance to oxidative stress and low pH in the post exponential phase. *letA* and *letS* were necessary for efficient invasion of A/J mouse bone marrow derived macrophages (Hammer et al., 2002). A *letA* mutant also displayed reduced infectivity in *A. castellanii* but in addition was attenuated for intracellular replication (approximately 100-fold reduction in CFU relative to wild type *L. pneumophila*) (Lynch et al., 2003).

csrA is an essential *L. pneumophila* gene and is involved in the regulation of motility related genes and the switch from the replicative to the transmissible form of *L. pneumophila*. A *csrA* mutant strain with drastically reduced production of CsrA displayed reduced intracellular replication within *Acanthamoeba castellanii* (approximately 100-fold reduction in CFU relative to wild type *L. pneumophila*) (Forsbach-Birk et al., 2004).

While not as profound as the intracellular replication defect seen with the *lpw27511* transposon mutant, the *sdeC* transposon mutant displayed a significant replication defect in the presence of *A. castellanii*. Along with its paralogues, SdeC is a known translocated effector of the Dot/Icm T4SS, which made it an exciting candidate for further characterisation as nothing is known about how this protein or any SidE homologues contribute to *Legionella* pathogenesis.

An *sdeC* deletion mutant was constructed in *L. pneumophila* strain 130b and was also complemented with the full length gene on the pMIP plasmid. While the *sdeC* deletion mutant displayed a defect during intracellular replication in *A. castellanii,* THP-1 cells and B6 BMDMs, similar to the defect seen with the *sdeC* transposon mutant, the complemented mutant did not restore growth to wild type levels in all of the assays. However in the BMDM host, partial successful complementation was observed at 72 h post infection. Future experiments may extend the duration of the infection to determine if full recovery can be achieved.

qRT-PCR was used to check if the complemented *sdeC* deletion mutant was still able to express SdeC at wild type levels. It was found that the complemented mutant did indeed express *sdeC* at 8.69 times the levels of wild type *L. pneumophila.* While we determined the lack of wild type phenotype restoration in the *A. castellanii* co-culture replication assay was not due to a lack of *sdeC* expression in the complemented mutant, it is possible that the high level of expression may have been detrimental to intracellular replication in *A. castellanii.* This may be tested by constructing another complemented *sdeC* deletion mutant using an expression vector with a less active promoter and retesting this strain in the *A. castellanii* co-culture assay. It is also possible that the complemented *sdeC* deletion mutant lost the complementation plasmid during the relatively long 72 h assay, reverting the strain back to the *sdeC* deletion mutant. This may be tested by recovering the bacteria after the assay and testing for the presence of the plasmid in the complemented mutant. Appropriate antibiotic selection can also be used to constantly select for bacteria possessing the complementation vector during infection to counteract this.

To observe the ability of the mutant to replicate in an animal model the *sdeC* deletion mutant and the complemented mutant strains were tested in an A strain mouse infection model and compared to wild type *L. pneumophila*. The *sdeC* deletion mutant again displayed a significant replication defect in comparison to wild type *L. pneumophila*. Interestingly, here the complemented mutant strain successfully restored replication to wild type levels. At this stage it is unclear why the

complemented *sdeC* deletion mutant restored replication to wild type levels in mice but not in the single-cell line replication assays. Although it is worth noting that the only time point analysed here was 72 h post infection, the same time point that displayed significantly higher CFU for the complemented *sdeC* mutant in the BMDM infection in comparison to the deletion mutant. Different factors associated with a single gene may be more or less important for different hosts. Factors such as differences in regulatory elements, the amount of protein produced, growth phase-sensitive expression levels and the alteration of small RNAs may be different between the complemented mutant and wild type strains.

The existence of several SidE paralogues with similar sequences in *L. pneumophila* may confer functional redundancy among the multiple paralogues. However our results and the findings of a previous study (Luo and Isberg, 2004), suggest that an *sdeC* deletion mutant alone results in intracellular replication attenuation in various host species and cell types, indicating that SdeC alone is required for efficient intracellular replication and the defect is not rescued by the other members of the SidE family. This is significant as deletions of single effectors usually results in no significant phenotype, making SdeC an exciting candidate for further characterisation.

After infection of *A. castellanii* and HEK293T cells, HA-tagged SdeC localised at the LCV. Most often SdeC was observed throughout the LCV but was also rarely observed around the perimeter of the vacuole. This correlates well with a previous report that SidE family proteins predominantly accumulated at the phagosomal membrane adjacent to the bacterial cell poles 30 min post infection (Bardill et al., 2005). This may be due to secretion of proteins via the Dot/Icm T4SS at this location or the proteins may localise at the cell poles for a functional role.

Our localisation experiments after infection showed that SdeC primarily localised to the LCV and was most often evenly distributed throughout. This indicates a role for SdeC at the LCV after infection. Here it may potentially aid in the development of the

LCV in several ways possibly including the recruitment of ER-derived host vesicles and their fusion with the LCV.

It was reported that SidE family proteins are predominantly expressed early on after infection of a host cell (Bardill et al., 2005). This may indicate that SdeC and its paralogues are important during the initial stages of LCV development post infection. Although our data found that SdeC was present at the LCV throughout the entire infection process, suggesting that SdeC plays a continuous role through to the latter stages of infection, this may only be the case because SdeC is constitutively expressed on our expression vector during our localisation studies, in contrast to the early expression seen when *sdeC* was present on the bacterial chromosome.

Some infected cells contained multiple LCVs presumably arising from infection by multiple bacteria. We observed that only some of the vacuoles within the same cell were positive for the presence of SdeC. This may have been due to the loss of the SdeC expression vector in some bacteria or SdeC may not be expressed at sufficient levels for detection. Also, the conditions for the translocation of SdeC may have not been attained in some of the LCVs, although this is unlikely as SdeC was seen to be expressed soon after establishing an LCV. The observation is important as it may indicate that SdeC localises to the LCV immediately after translocation rather than acting at a separate location within the cell. If SdeC acted at a separate location prior to accumulating at the LCV it would likely be present on all LCVs in the cell. However, it is possible that SdeC localises to the LCV via an interaction that is only possible on the LCV at a specific time after infection. If this is the case, SdeC may still be able to act in another part of the cell before only localising to LCVs that possess the specific interaction partner, be it bacterial or host-derived, and not LCVs that lack the interaction partner. This may be investigated further by a more in depth analysis of different time points post-infection, possibly staining for different host markers that are present on the LCV at specific time points.

When detected at two hours post-infection SdeC was primarily localised at the poles of the internalised bacterium, consistent with the findings of Bardill et al. (2005). This is likely due to secretion of SdeC via the Dot/Icm T4SS at the poles of the bacterium rather than SdeC localising at the cell poles for a functional role at this time point. At 13 h post-infection, in addition to the majority of SdeC localised at the LCV, the protein was often observed throughout the host cell away from the LCV in a non-uniform pattern. This may be due to overexpression of SdeC leading to a saturation of LCV binding and hence the location of SdeC outside of the LCV. If this was the case SdeC would be expected to be distributed evenly throughout the cell or, in light of the transfection experiments performed in this study, localise at the ER. Upon transfection in HEK393T cells SdeC was co-localised with an ER marker suggesting SdeC was associated with the ER. pEGFP-C2:SdeC transfected cells were also stained with other cell markers to determine any additional effects on other cellular components but no changes to host actin, golgi, lysosomes and coatomer proteins were detected. The potential interaction of SdeC with the ER seen during transfection may indicate a role for SdeC in the interaction the LCV has with the host ER. This may include membrane fusion, trafficking or other associated roles. It is worth noting that wild type SdeC and the epitope-tagged SdeC proteins may not be functionally equivalent. This may be verified by testing the *sdeC* deletion mutant carrying pICC562:*sdeC* in the A strain mouse infection assay in order to determine if the tagged SdeC complements the deletion.

Since SdeC was observed localising to the ER during transfection and the LCV during infection of host cells indicating possible interactions at these sites, several strategies were used to determine what host proteins SdeC may interact with at the molecular level. SdeC was tagged with different epitope tags for use in pull-down experiments to identify binding partners. Several constructs, including GST and His epitope tags, were produced but were unsuccessful in the production of the SdeC fusion protein (data not shown). This may have been due to the large size of SdeC and the fact that these constructs were expressed in a different bacterial species (*E. coli*). The mammalian

cells during our transfection experiments were able to express EGFP-tagged SdeC and a distinct ER localisation pattern was observed. Therefore these transfected cells were chosen for identifying a potential ER binding partner via an immunoprecipitation experiment. Unfortunately, no visible binding partners were able to be identified after the immunoprecipitation.

Instead, we proposed that an immunoprecipitation after infection rather than transfection of host cells may yield results where the previous GFP-Trap experiment failed. *L. pneumophila* expressing 4HA tagged SdeC, which was used in the localisation experiments, was used here in immunoprecipitation experiments of infected cells. Several unique protein bands were identified during the immunoprecipitation of SdeC compared to an empty vector control, indicating possible binding partners, including a common contaminant of immunoprecipitation experiments, actin. While the presence of actin in the immunoprecipitation sample could indicate a binding partner, it is also possible that it is present as a highly abundant protein in the cell lysate that weakly binds to the immunoprecipitation beads, although steps were taken to reduce the likelihood of this. To determine if the interaction of SdeC with actin was real, a western blot probing for the presence of actin was performed on the immunoprecipitation samples. The western blot did not detect any actin in the SdeC immunoprecipitation samples, indicating the actin previously identified in the mass spectrometry was likely a contaminant or that it is present in too small an amount in the immunoprecipitation sample to detect via western blotting.

No binding partner of SdeC was able to be successfully identified using the methods performed in this study. While a distinct localisation pattern SdeC displays may indicate a specific binding partner, it may not be identifiable using the immunoprecipitation methods used here. The strength of the bond may be too weak to overcome the stresses of the immunoprecipitation or the conditions for binding may be lost upon lysis of cells and purification of proteins. In an attempt to account for this, a crosslinking step was added to the immunoprecipitation after infection, however, this did not affect the outcome. Other methods such as a yeast 2-hybrid

screen or another co-immunoprecipitation with enhanced experimental conditions conducive to the conservation of any protein-protein interactions (i.e. less stringent wash steps) may be attempted in the future to identify a protein interaction partner. However the large size of SdeC and its hydrophobicity confer technical limitations in these approaches.

Various microscopy techniques were also used to observe any differences between the *sdeC* deletion mutant and wild type *L. pneumophila*, with a particular focus on the *Legionella*-containing vacuole due to SdeC localising there during infection. Electron microscopy was first used to observe infected cells. The LCVs in both the deletion mutant and wild type *L. pneumophila* strains at both 2 and 24 h time points behaved as expected and no differences between the two were observed. At 2 h post-infection bacteria of both strains were observed within small vacuoles usually with a single bacterium in each vacuole. At 24 h post-infection bacteria of both strains were present in high numbers in large vacuoles within host cells. The size and shape of the LCVs of both strains were similar. No differences in the behaviour of cell organelles were observed between the two strains during infection. The absence of SdeC did not seem to produce any observable differences in comparison to wild type *L. pneumophila* at the time points observed using electron microscopy.

We thought it was possible that a difference may be observed at other time points during live cell imaging of infected cells. However, no significant differences were observed between the two strains during live cell imaging of infected cells. The development of the LCVs of both the *sdeC* deletion mutant and wild type *L. pneumophila* strains was observed to be generally similar at each time point observed apart from a possible slight reduction in LCV size at 6 h post infection for the deletion mutant. Although from 7 to 8 h post-infection the LCVs of both strains seem comparable. While only slight, this difference may reflect the defect seen during the co-culture replication assays and mouse infection study.

To quantify the replication defect further, the change in LCV size over time of wild type *L. pneumophila* infected cells was compared to that of the *sdeC* deletion mutant infected cells at 2, 6, 9, 12 and 15 h post-infection. This allowed us to observe replication within LCVs during a single replication cycle. At 12 and 15 h post-infection sizes of *sdeC* mutant LCVs were significantly smaller in comparison to wild type *L. pneumophila,* although vacuole size was highly variable at these time points for both strains. This reflected the defect observed in previous replication assays.

In live cell imaging experiments both *L. pneumophila* (pMIP:GFP) and the *sdeC* deletion mutant (pMIP:GFP) were often located within the ER stained region of the host HeLa cell. Occasionally the ER staining was seen to be more intense around the location of the bacteria. This phenomena seemed to be slightly more prevalent in cells infected with wild type *L. pneumophila* (pMIP:GFP), however this was not encountered often enough to be considered a significant result.

Several further microscopy techniques were utilised here to observe *Legionella* infections at several time points post-infection. We aimed to observe the infection cycle in full during our experiments however, this was not always able to be achieved for a variety of reasons, including time and cellular staining restraints, resolution limitations and the dynamic nature of live cells during live cell imaging. Expanding our microscopy experiments to encompass additional time points would be ideal and may uncover unobserved behaviour not present at the time points selected here.

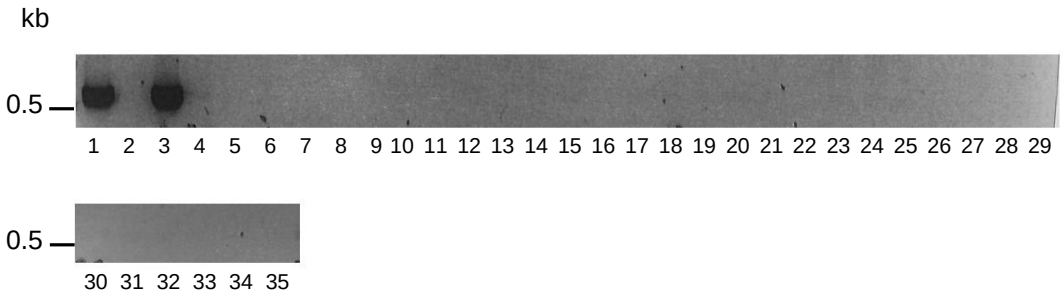
During live cell imaging experiments we observed that separate LCVs within the one cell fused together during development. This has not been recorded in current literature and so was studied in more detail. Two *Legionella* strains expressing different fluorophores in a live cell imaging experiment were used to confirm and observe vacuole fusion over the course of an infection. Vacuoles of the two distinct populations of fluorescent *L. pneumophila* were observed fusing together, with the different fluorophores observed mixing together in the same vacuole.

It is possible that the same mechanisms *Legionella* uses to fuse the LCV with cellular compartments such as ER-derived vesicles are involved in the homotypic fusion between multiple LCVs. The receptors targeted by *Legionella,* present on the membranes of cellular compartments, may still be present on the LCV after fusion. A separate LCV in the same host cell may then use the same mechanisms to fuse with the other LCV. The resulting larger vacuole could be more efficient than multiple smaller vacuoles due to reduced surface area to volume ratios and therefore reduced membrane requirements, in addition to the sharing of scavenged resources. This may also be advantageous if only one LCV is required to reach maturity before fusing with other less developed LCVs, decreasing the work required as a whole and reducing any competition for nutrients within the host. This process may be investigated experimentally via the deletion/inhibition/knock-down of bacterial or host factors involved in membrane fusion and determining if multiple LCV fusion events still occur.

While homotypic LCV fusion was observed with *L. pneumophila* in live cell imaging experiments, this may still be important for characterising SdeC further in the future if the number of fusion events and their timing during the infection process is found to be different to that of wild type *L. pneumophila*. Due to a possible link between SdeC and the ER this may uncover a defect in the *sdeC* deletion mutant's vacuole fusion ability.

This study has deepened our understanding of *Legionella* pathogenesis in several ways. The transposon mutagenesis screen identified multiple potential virulence genes in addition to reinforcing the importance of the Dot/Icm T4SS. The regulatory targets of Lpw27511 were recorded, however, the gene was found to be unimportant for intracellular replication upon creation of an in-frame deletion mutant. The Dot/Icm T4SS secreted effector SdeC was found to be important for intracellular replication and localised to the LCV during infection and the host ER upon transfection of mammalian cells. The fusion of multiple LCVs in the same host cell, a novel phenomenon, was observed here upon live imaging of *A. castellanii*. This event may open up a new avenue for investigation into LCV membrane fusion and development during infection.

Appendix



Appendix 1.1. Prevalence of *ltpF* in a collection of *L. pneumophila* isolates.
Representative PCR investigating the prevalence of one of the identified novel 130b Dot/Icm T4SS effector proteins in a collection of *L. pneumophila* isolates. Genomic DNA of the strains indicated was used as a template for PCR using primers 2142 and 2143 (Table 2.3.) amplifying a 635 bp fragment of *ltpF*. 1- *L. pneumophila* 130b, 2- *E. coli*, 3- *L. pneumophila* 02/40, 4- *L. pneumophila* 02/41, 5- *L. pneumophila* 03/41, 6- *L. pneumophila* 03/43, 7- *L. pneumophila* 03/42, 8- *L. pneumophila 03/47, 9- L. pneumophila 03/45, 10-L. pneumophila 03/46, 11- L. pneumophila 03/49, 12- L. pneumophila 03/48, 13- L. pneumophila 03/53, 14- L. pneumophila 03/54, 15- L. pneumophila 03/55, 16- L. pneumophila 03/50, 17- L. pneumophila 03/58, 18- L. pneumophila 03/59, 19- L. pneumophila 03/56, 20- L. pneumophila 03/57, 21- L. pneumophila 03/64, 22- L. pneumophila 03/60, 23- L. pneumophila 03/61, 24- L. pneumophila 03/63, 25- L. pneumophila PN1, 26- L. pneumophila CT1, 27- L. pneumophila B6, 28- L. pneumophila PN2, 29- L. pneumophila C42, 30- L. pneumophila C102, 31- L. pneumophila C1, 32- L. pneumophila C7, 33- L. pneumophila CT4b, 34- L. pneumophila CT67, 35- L. pneumophila CT3C.*

Appendix 1.2. Prevalence of the identified novel *L. pneumophila* strain 130b Dot/Icm T4SS effector proteins in a collection of *L. pneumophila* isolates. Strain information listed in Table 2.1. + indicates the presence of the gene in the *L. pneumophila* strain. - indicates the absence of the gene in the *L. pneumophila* strain.

Strain	Serogroup	*dotA*	*ltpA*	*ltpB*	*ltpC*	*ltpD*	*ltpE*	*ltpF*	*ltpG*	*ltpH*	*ltpI*	*ltpJ*
***L. pneumophila* 02/40**	1	+	+	+	+	+	+	+	+	+	+	+
***L. pneumophila* 02/41**	1	+	+	+	-	+	+	+	+	+	+	+
***L. pneumophila* 03/41**	1	+	+	-	-	+	-	-	-	+	+	-
***L. pneumophila* 03/43**	1	+	+	-	-	+	-	-	-	+	+	-
***L. pneumophila* 03/42**	1	+	-	-	-	-	-	-	-	+	+	-
***L. pneumophila* 03/47**	1	+	+	-	-	+	-	-	-	+	+	-
***L. pneumophila* 03/45**	1	+	-	-	+	-	-	-	-	+	+	-
***L. pneumophila* 03/46**	1	+	+	+	-	+	+	-	+	+	+	+
***L. pneumophila* 03/49**	1	+	-	-	-	+	-	-	-	-	+	-
***L. pneumophila* 03/48**	1	+	-	-	-	+	-	-	-	+	+	-
***L. pneumophila* 03/53**	3	+	+	-	-	+	-	-	-	+	+	-
***L. pneumophila* 03/54**	3	+	+	+	-	+	+	-	+	+	+	-
L. pneumophila	4	+	+	+	-	+	+	-	+	+	+	-

Strain	Serogroup	*dotA*	*ltpA*	*ltpB*	*ltpC*	*ltpD*	*ltpE*	*ltpF*	*ltpG*	*ltpH*	*ltpI*	*ltpJ*
03/55												
L. pneumophila	1	+	-	-	-	+	-	-	-	-	+	-
03/50												
L. pneumophila	5	+	+	-	-	+	-	-	-	-	+	-
03/58												
L. pneumophila	6	+	+	+	-	+	+	-	-	+	+	-
03/59												
L. pneumophila	4	+	+	-	-	+	-	-	-	+	+	-
03/56												
L. pneumophila	5	+	+	+	-	+	+	-	-	+	+	-
03/57												
L. pneumophila	8	+	+	-	-	+	-	-	+	+	+	-
03/64												
L. pneumophila	6	+	+	-	-	+	-	-	-	-	+	-
03/60												
L. pneumophila	7	+	+	+	-	+	+	-	-	+	+	-
03/61												
L. pneumophila	8	+	+	+	-	+	+	-	-	+	+	-
03/63												
L. pneumophila	1	+	+	-	-	-	-	-	-	+	+	-
PN1												
L. pneumophila	1	+	+	-	-	+	-	-	-	-	+	-
CT1												
L. pneumophila	1	+	+	-	-	+	-	-	-	+	+	-
B6												
L. pneumophila	1	+	+	-	-	-	-	-	-	+	+	-
PN2												
L. pneumophila	2-14	+	-	-	-	+	-	-	-	-	+	-
C42												
L. pneumophila	2-14	+	+	-	-	+	-	-	-	-	+	-
C102												
L. pneumophila	2-14	+	+	-	-	+	-	-	-	+	+	-
C1												
L. pneumophila	2-14	+	+	-	-	+	-	-	-	-	+	-
C7												

Strain	Serogroup	*dotA*	*ltpA*	*ltpB*	*ltpC*	*ltpD*	*ltpE*	*ltpF*	*ltpG*	*ltpH*	*ltpI*	*ltpJ*
L. pneumophila **CT4b**	2-14	+	+	-	-	+	-	-	-	+	+	-
L. pneumophila **CT67**	2-14	+	+	-	-	+	-	-	-	+	+	-
L. pneumophila **CT3C**	1	+	+	-	-	+	-	-	-	+	+	-
L. pneumophila ***H075020013***	1	+	-	-	-	+	-	+	+	+	-	-
L. pneumophila ***H075020015***	1	-	-	-	-	-	-	-	-	-	+	-
L. pneumophila ***H075020016***	4	+	-	-	-	-	-	-	+	+	-	-
L. pneumophila ***H080160263***	6	+	+	-	-	-	-	-	+	-	-	-
L. pneumophila ***H080200554***	1	+	-	-	-	-	-	-	-	+	+	-
L. pneumophila ***H080200555***	1	+	-	-	-	-	-	-	-	+	+	-
L. pneumophila ***H080200559***	6	+	-	-	-	-	-	-	-	-	-	-
L. pneumophila ***H080200562***	1	+	-	-	-	-	-	-	-	+	+	-
L. pneumophila ***H080200565***	3	+	+	-	-	-	-	-	+	-	-	-
L. pneumophila ***H080200566***	1	+	-	-	-	-	-	-	-	+	+	-
L. pneumophila ***H080200568***	1	+	-	-	-	-	-	-	-	-	+	-
L. pneumophila ***H080200573***	3	+	-	-	-	+	-	-	+	+	-	-
L. pneumophila ***H080200574***	10	+	-	-	-	-	-	-	-	+	+	-
L. pneumophila ***H080200575***	1	+	-	-	-	-	-	-	-	+	+	-
L. pneumophila	1	+	-	-	-	+	-	-	+	-	-	-

Strain	Serogroup	*dotA*	*ltpA*	*ltpB*	*ltpC*	*ltpD*	*ltpE*	*ltpF*	*ltpG*	*ltpH*	*ltpI*	*ltpJ*
H080200576												
L. pneumophila	1	+	-	-	-	-	-	-	-	+	+	-
H080200577												
L. pneumophila	1	+	-	-	-	-	-	-	-	+	+	-
H080340093												
L. pneumophila	1	+	-	-	-	-	-	-	-	+	-	-
H080340097												
L. pneumophila	3	+	+	-	-	-	-	-	+	-	-	-
RR07000773												
L. pneumophila	1	+	+	-	-	+	-	-	-	-	-	-
RR07000774												
L. pneumophila	6	+	-	-	-	-	-	-	+	-	-	-
RR07000788												
L. pneumophila	5	+	-	-	-	+	-	-	+	-	-	-
RR07000789												
L. pneumophila	6	+	+	-	-	-	-	-	+	-	+	-
RR07000790												
L. pneumophila	1	+	+	+	+	+	+	+	+	+	-	+
RR07000791												
L. pneumophila	1	+	+	-	-	-	-	-	+	+	-	-
RR07000792												
L. pneumophila	1	+	-	-	-	+	-	-	-	-	+	-
RR08000123												
L. pneumophila	1	+	-	-	-	+	-	-	-	-	+	-
RR08000136												
L. pneumophila	1	+	-	-	-	-	-	-	-	-	+	-
H082940035												
L. pneumophila	1	+	-	-	-	-	-	-	-	-	-	-
H082980021												
L. pneumophila	1	+	-	-	-	-	-	-	+	-	-	-
H082680013												
L. pneumophila	1	+	+	-	-	-	-	-	-	-	-	-
H081340222												
L. pneumophila	1	+	+	+	+	+	+	+	+	+	+	+
H081760005												

Strain	Serogroup	*dotA*	*ltpA*	*ltpB*	*ltpC*	*ltpD*	*ltpE*	*ltpF*	*ltpG*	*ltpH*	*ltpI*	*ltpJ*
L. pneumophila H083120262	1	+	+	-	-	+	-	-	+	-	-	-
L. pneumophila H083120268	1	+	-	-	-	+	-	-	-	-	-	-
L. pneumophila H083080428	1	+	-	-	-	+	-	-	+	-	-	-
L. pneumophila H083140015	1	+	+	-	-	+	-	-	+	+	-	-
L. pneumophila H083220038	1	+	+	-	-	+	-	-	+	+	-	-
L. pneumophila H083260176	1	+	-	-	-	+	-	-	+	-	-	-
L. pneumophila H083480003	1	+	-	-	-	+	-	-	-	-	-	-
L. pneumophila H083580455	1	+	-	-	-	+	-	-	+	-	-	-
L. pneumophila H083620580	1	+	-	-	-	+	-	-	-	-	-	-
L. pneumophila H083660004	1	+	-	-	-	-	-	-	-	-	+	-
L. pneumophila H083840063	1	+	-	-	-	+	-	-	-	-	-	-
L. pneumophila H083920177	1	+	-	-	-	-	-	-	-	-	-	-
L. pneumophila H083920180	1	+	+	-	-	+	-	-	-	-	-	-
L. pneumophila H083960064	1	+	-	-	-	+	-	-	-	+	-	-
L. pneumophila H084060004	1	+	-	-	-	+	-	-	-	-	-	-
L. pneumophila H084120109	1	+	+	-	+	+	-	-	+	-	+	-
L. pneumophila H084160068	1	+	+	-	+	-	-	-	+	-	-	-
L. pneumophila	1	+	+	-	-	-	-	-	-	-	-	-

Strain	Serogroup	*dotA*	*ltpA*	*ltpB*	*ltpC*	*ltpD*	*ltpE*	*ltpF*	*ltpG*	*ltpH*	*ltpI*	*ltpJ*
H084240079												
L. pneumophila	1	+	+	-	-	-	-	-	+	-	-	-
H084340334												
L. pneumophila	1	+	+	+	+	+	+	+	+	+	+	+
H084380129												
L. pneumophila	1	+	-	-	-	-	-	-	-	-	-	-
H084680106												
L. pneumophila	1	+	+	-	-	+	-	-	-	-	-	-
H084780287												

Appendix 1.3. Representative proteomics analysis summary of SdeC co-immunoprecipitation results of mouse bone marrow-derived macrophages infected with *L. pneumophila* pICC562:*sdeC.* After separation by SDS-PAGE gel, proteins were excised and then analysed by multi-dimensional LC-MS/MS on an QStar Pulsar i MALDI-quadrupole time-of-flight mass spectrometer (AB Sciex) by the WEHI Proteomics Laboratory (Walter and Eliza Hall Institute, Australia), according to published protocols (Washburn et al., 2001). Data was analysed using Mascot against all Eubacteria or Eukaryotic protein entries in the latest release of the LudwigNR database. In Mascot, the Mascot score for an MS/MS match is based on the absolute probability that the observed match between the experimental data and the database sequence is a random event.

Protein(s) inferred	Mol Wt (kDa)	Peptide Sequence(s)	Mascot score	Mascot Expect value
tr|A1L3K7|LOC100037034 LOC100037034 protein Tax_Id=8355 [*Xenopus laevis*]	47	(K)VNQIGSVTESLQAcK(L) (R)GNPTVEVDLYTAK(G) (K)GVSQAVEHINK(T) (R)SGKYDLDFK(S)	98.3 58.3 55.7 41.3	1.73E-06 0.0177 0.0262 0.477
sp|A5A6N4|EIF4A1 Eukaryotic initiation factor 4A-I Tax_Id=9598 [*Pan troglodytes*]	46	(K)GYDVIAQAQSGTGK(T) (K)mFVLDEADEmLSR(G) (R)VLITTDLLAR(G) (K)LQmEAPHIIVGTPGR(V)	91.6 65.1 62.5 43.7	7.4E-06 0.00102 0.00308 0.477
tr|B0YJC4|VIM Vimentin Tax_Id=9606 [*Homo sapiens*]	49	(R)QDVDNASLAR(L) (K)FADLSEAANR(N)	53.7 50.5	0.0308 0.0757

tr|B6T433|Elongation factor 1-alpha Tax_Id=4577 [*Zea mays*]	49	(K)IGGIGTVPVGR(V) (R)LPLQDVYK(I)	56.3 45.7	0.00869 0.338
tr|A1XSX3|Beta-actin Tax_Id=89462 [*Bubalus bubalis*]	41	(K)AGFAGDDAPR(A) (R)GYSFTTTAER(E) (K)DSYVGDEAQSKR(G) (K)DSYVGDEAQSKR(G) (K)DSYVGDEAQSK(R) (K)QEYDESGPSIVHR(K)	58.1 50.4 49.3 45.9 41.3 40.7	0.0074 0.0477 0.0659 0.151 0.213 0.477

Appendix 2. Abbreviations

± Plus-minus

Abs Absorbance

ACES N-(2-acetamido)-2-aminoethanesulfonic acid

Amp Ampicillin

Ank Ankyrin repeat domains

AYE ACES-buffered yeast extract

BCYE Buffered charcoal yeast extract

BLAST Basic Local Alignment Search Tool

bp Base pair(s)

cDNA complementary DNA

CFU Colony-forming unit(s)

cm Centimeter(s)

cm2 Centimeter(s) squared

Cm Chloramphenicol

° Degrees

°C Degrees Celsius

dH2O Distilled water

DNA Deoxyribonucleic acid

Dot/Icm Defective organelle trafficking/intracellular multiplication

EDTA Ethylenediaminetetraacetic acid

EGFP Enhanced green fluorescent protein

ER Endoplasmic reticulum

h Hour(s)

HEK293T Human Embryonic Kidney 293T

IPTG Isopropyl β-Dthiogalactoside

Kan Kanamycin

kb Kilobase(s)

kDa Kilodalton(s)

kg Kilogram(s)

kV Kilovolt(s)

L Litre(s)

LB Luria-Bertani

LCV *Legionella*-containing vacuole

Mb Megabase(s)

mRNA messenger RNA

µF Microfarad(s)

µg Microgram(s)

µL Microlitre(s)

µm Micrometer(s)

mA Milliamps(s)

mg Milligram(s)

min Minute(s)

mL Millilitre(s)

mM Millimolar

M Molar

MOI Multiplicity of infection

nm Nanometer(s)

PBS Phosphate buffered saline

PCR Polymerase chain reaction

pH Potential of hydrogen

PMA Phorbal 12-myristate 13-acetate

RNA Ribonucleic acid

rRNA ribosomal RNA

RT Room temperature

SDS Sodium dodecyl sulphate

SDS-PAGE Sodium dodecyl sulphatepolyacrylamide gel electrophoresis

sec Second(s)

SidM/DrrA Substrate of Icm/Dot/Defect in Rab1 recruitment

SOB Super optimal broth

SOC Super optimal broth with catabolite repression

TAE Tris-acetate electrophoresis

TBS Tris-buffered saline

TE Tris-EDTA

TEMED N,N,N’,N’-tetramethylethylenediamine

V Volt(s)

v/v Volume/volume

w/v Weight/volume

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