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Evolution and host-specific adaptations of *Legionella pneumophila*

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Abstract

B. Moreno, A. 2022. Evolution and host-specific adaptations of *Legionella pneumophila*. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 1871. 42 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-513-1607-9.

How bacteria evolve pathogenic traits is shaped by their communities and environments. *Legionella pneumophila* is ubiquitous in aquatic habitats, where it persists by replicating within a broad range of protozoan hosts. Using the same mechanisms, *L. pneumophila* may also accidentally infect humans, causing a severe pneumonia known as Legionnaires' disease. As hosts, humans are evolutionary dead-ends, resulting in the loss of human-specific adaptations after infection. This thesis aims to identify and characterise these host adaptations.

In Paper I, we study the in-patient evolution of *L. pneumophila*. We collected a large set of strains from sporadic infections and outbreaks, pairing clinical isolates with their respective environmental sources. Using comparative genomic analyses, we identified two genes individually mutated in three independent infections. One gene encoded an outer membrane protein, a homolog from the OmpP1/FadL family, and the other an EAL domain-containing protein. These results suggest that convergent evolution may be at play and that these mutations are potential candidates for human-specific host adaptations.

In Paper II, we investigate host adaptation and the selective pressures that drive it using a long-term experimental evolution approach. We passaged *L. pneumophila* in *Acanthamoeba castellanii* and U937 macrophages, separately and in alternation, for over 800 generations. We found 49 fixed mutations across the 18 evolved populations: two distinct mutations in RpsL, which confers streptomycin resistance, as well as two additional mutations, each consistently associated with one of the former, in the chaperonin GroES or in RpsD, a known compensatory mutation. Mutations in the lipopolysaccharide synthesis operon were observed only in lineages passaged in *A. castellanii*, whilst mutations in LerC were fixed in six lineages passaged in U937, making these candidate mutations for host-specific adaptations.

In Paper III, we shift focus to *A. castellanii*, a natural host of *L. pneumophila*. We describe a novel method for high-efficiency transfection of this amoeba with a cationic polymer. Using a systematic approach to test different parameters, we found that widely available and inexpensive polyethylenimines can be used to transfect *A. castellanii* at a much greater efficiency than the currently used reagents.

In conclusion, these studies suggest that although *L. pneumophila* can infect humans, it is sub-optimally adapted for it, and offer potential determinants of host-specificity in *L. pneumophila*.

Keywords: Evolution, Legionella pneumophila, host-adaptation, experimental evolution, Acanthamoeba castellanii, transfection

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“Se podes olhar, vê. Se podes ver, repara.”

“If you can see, look. If you can look, observe.”

- José Saramago

List of Papers

This thesis is based on the following papers, referred to in the text by their Roman numerals.

I. Leenheer, D., **Moreno, A. B.**, Murray, S., Jarraud, S., Ginevra, S., Guy, L. (2022) Rapid, in-patient adaptations of *Legionella pneumophila* to the human host. *Under revision.*

II. **Moreno, A. B.**, Paranjape, K., Kay, E., Dobre Lereanu, C., Andersson, D.I., Guy, L. (2022) Host-specific determinants of *Legionella pneumophila* virulence passaged in single hosts and in alternation. *Manuscript*.

III. **Moreno, A. B.**, Ek, V., Eriksson, J., Sellin, M. E., Guy, L. (2022) High-efficiency transfection of *Acanthamoeba castellanii* using a cationic polymer. *Manuscript.*

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Other work by the author not included in this thesis:

Moreno, A. B., Brodin, E., Guy, L. Evolutionary and functional analysis of RavC, a *Legionellales*-wide conserved effector. *In preparation.*

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Contents

Abbreviations

®	Registered trademark
~	Approximately
A. castellanii	*Acanthamoeba castellanii*
CDC	Centers for Disease Control and Prevention
Dot/Icm	Defect in organelle trafficking/ intracellular multiplication
L. pneumophila	*Legionella pneumophila*
LCV	*Legionella*-containing vacuole
LD	Legionnaires disease
PCR	Polymerase chain reaction
PEI	Polytethylenimine
T4SS	Type IV secretion system
™	Unregistered trademark

Introduction

Historical perspective of Legionnaires' disease and *Legionella*

In July of 1976, over 4000 delegates of the American Legion gathered at the Bellevue-Stratford hotel in Philadelphia to attend the 58th Annual Legionnaires' Convention. Within days of the meeting ending, the American Legion's Pennsylvania chapter headquarters began receiving calls reporting several attendees with pneumonia-like symptoms and the death of others. As news spread, similar accounts of people who had not attended the convention but had been in the vicinity arrived. Ultimately, nearly 200 people contracted this severe pneumonia, most of which required hospitalisation, which resulted in the death of 34 people (McDade et al. 1977; Fraser et al. 1977).

Although the causative agent of "Legionnaires' Disease" was not immediately identified, after thorough epidemiological investigations, the hotel lobby was proposed as the point of exposure, and it was concluded that there was no person-to-person transmission (Fraser et al. 1977).

The CDC initiated one of its most extensive investigation campaigns at the time to find the "Philly killer", as dubbed by Time magazine (*Time Magazine* 1976; Fraser et al. 1977). After months of intense microbiological and epidemiological investigations under severe public scrutiny, the CDC researchers Joseph McDade and Charles Shepard were finally able to isolate and identify the causative pathogen: a Gram-negative, rod-shaped bacterium designated *Legionella pneumophila* (McDade et al. 1977).

This eponymous event, however, was not the first disease outbreak caused by *L. pneumophila*. One of the earlier reports of a similar epidemic occurred in Fort Brags, North Carolina, in 1943. Then, the isolated bacterium was thought to be a Rickettsia and became known as the "Tatlock strain" after the doctor who isolated it (Tatlock 1944; Daniels and Grennan 1943). Not until over 30 years later was it correctly identified as *Legionella micdadei* (Hébert, Steigerwalt, and Brenner 1980). Likewise, two strains isolated in 1947 and one in 1959 by Marilyn Bozeman, also believed to be Rickettsia, were later identified as *L. pneumophila, Legionella bozemannii,* and *L. micdadei* (Cordes et al. 1979; McDade, Brenner, and Bozeman 1979).

Chronic respiratory disease epidemics dating back to the 1950s have also been attributed to *L. pneumophila*. Notably, an outbreak where patients

suffered from a respiratory infection but no pneumonia in Pontiac, Michigan, in 1958. This milder form of legionellosis was named Pontiac fever and was thought to be spread via air conditioning systems (Glick et al. 1978; Armstrong and Miller 1985).

Since their discovery, *Legionella* species have remained a significant public health risk and are among the most common causes of community-acquired pneumonia. The most significant outbreaks infected over 800 people in Murcia, Spain, in 2001 (García-Fulgueiras et al. 2003) and 334 people in Vila Franca de Xira, Portugal, in 2014 (Shivaji et al. 2014).

The remarkable effort by scientists at the time to isolate and develop culturing and diagnostic techniques (McDade et al. 1977; Dumoff 1979; P H Edelstein and Finegold 1979; Feeley et al. 1979), discover the source dissemination (Shands 1985) and understand the importance of amoebae for *Legionella* species (Rowbotham 1980) set the foundations for current research (Winn 1988).

Figure 1. Covers of the American Legion convention program (left) and Time magazine from August 16th, 1976 (right)(*Time Magazine* 1976).

Legionellosis

> "Legionnaires' disease is an undercounted, underestimated, and underappreciated global threat to human health." – George Dehner (Dehner 2018)

Clinical manifestations

Legionellosis collectively encompasses the different infections caused by *Legionella* species (Kozak-Muiznieks, Mercante, and Raphael 2020). These infections may manifest as Legionnaires' disease (LD), Pontiac fever, or extrapulmonary syndromes. LD is considered an atypical pneumonia since it lacks distinct symptoms or signs. It has a 2-10 day period of incubation, and the condition can last for weeks; symptoms include high fever, muscle pain, difficulty in breathing, vomiting, diarrhoea, confusion, and delirium, (Stout and Yu 1997; Den Boer and Yzerman 2004; Bartram 2007). Risk factors contributing to individual susceptibility include age (>50 years old), cigarette smoking, alcohol abuse, lung disease, cancer, diabetes, renal failure, and immunosuppression (Dehner 2018; Mercante and Winchell 2015; Mondino et al. 2020). The fatality rate depends on the patient's susceptibility and the source of infection (community or nosocomial) and can go up to ~35%. Treatment of LD consists of antibiotic therapy (Mercante and Winchell 2015).

Pontiac fever is a milder form of legionellosis, with a short incubation period (24 -48 h) and infection duration (2 -5 days) (Bartram 2007). The symptoms include influenza-like illness, loss of strength, high fever, and muscle and joint pain. Treatment of Pontiac fever involves managing the symptoms, but it may also be resolved without treatment (Glick et al. 1978; Bartram 2007).

Legionella may, on rare occasions, cause severe extrapulmonary syndromes when they spread beyond the respiratory tract to other body parts, which can be fatal due to a challenging diagnosis (Paul H. Edelstein 2006; Bartram 2007).

Detection and diagnosis

Prompt laboratory diagnosis of LD is critical to positive patient outcomes; due to the absence of specific LD symptoms, different diagnostic methods can be used based on the type of sample obtained. Microbiological culture of sputum or respiratory secretions is considered the gold standard due to its high specificity; it can identify any species of *Legionella* independent of serogroup. This method also allows for subsequent antibiotic susceptibility and pathogenicity tests, as well as epidemiological studies; its significant drawbacks are the long incubation period of 3-10 days and the variability in sensitivity (11- 65%) (Lück et al. 2006; Dunne, Picot, and van Belkum 2017; Mondino et al. 2020).

Due to their easy sampling method, low cost, and readily available results, urinary antigen tests are used as a first-line screening method. Using enzyme immunoassays or immunochromatographic assays, this technique detects a lipopolysaccharide component in the bacterial cell wall and is highly sensitive (75-99%) and specific (99-100%). However, it can only detect *L. pneumophila* serogroup 1 (Mondino et al. 2020; Bartram 2007; Lück et al. 2006; Ceccarelli et al. 2020b).

Direct immunofluorescent assay (DFA) may also detect legionellae via antigens. DFA uses antibodies conjugated with a fluorochrome to detect *L. pneumophila* antigens in sputum samples. The sensitivity of this assay depends on the sample source (25-80%), and while it is highly specific (95-99%), it has not been validated for use with non-pneumophila species. Advantages of this method include the short staining procedure (2- 4 h), which allows for a rapid response time, and its use to detect antigens even after antibiotic treatment (Lück et al. 2006; Bartram 2007).

Serological diagnosis was once the preferred method to identify *Legionella* infections. This type of indirect immunofluorescence assay detects antibody levels from blood serum and has a high sensitivity (70-90%) and specificity rate (95-99%); however, because seroconversion of the antibodies can take 3-9 weeks and there is difficulty in obtaining samples from patients with a standardised immunological response this method has limited use in diagnostic laboratories. Still, it is a valuable tool for epidemiological studies (Stout and Yu 1997; Bartram 2007).

Detection of legionellae through nucleic acids was first performed in 1984 using a radioisotopically labelled RNA probe. Still, due to this assay's low sensitivity and specificity, it was later withdrawn from use (Fields, Benson, and Besser 2002). Currently, PCR assays are widely used to detect legionellae from environmental samples, but they may also be used for clinical diagnosis. The target genes are 16S ribosomal RNA to detect *Legionella* spp and the *mip* gene to detect *L. pneumophila*. For diagnostic tests, this rapid assay's sensitivity is sample dependent, with those from the respiratory tract being better (85-92%) than samples from urine serum (33-70%). The specificity is high regardless of the sample (94-98%) but may require other diagnostic tests to confirm positive results (Fields, Benson, and Besser 2002; Lück et al. 2006; Bartram 2007).

Treatment

With prompt and accurate diagnosis, LD can easily be treated with antibiotics. Due to the nature of the infection, the antibiotics chosen must be able to penetrate and reach a high concentration within alveolar macrophages. Macrolides and fluoroquinolones, such as azithromycin and levofloxacin, are the commonly prescribed antibiotics, but depending on the patient and diagnosis, tetracyclines and rifampicin may also be part of the therapy. Species-specific

diagnosis is not required for treating LD because all most legionellae that cause human infection are sensitive to these antibiotics (Mercante and Winchell 2015; Stout and Yu 1997; Bartram 2007; Ceccarelli et al. 2020a; Tan et al. 2021; Pedro-Botet and Yu 2006).

Natural antibiotic resistance is observed in *Legionella* spp: *L. pneumophila* (amongst others) is resistant to beta-lactams (e.g. penicillin) (Thornsberry and Kirven 1978; Marre, Medeiros, and Pasculle 1982), *Legionella drancourtii, Legionella dumoffii* and *Legionella fallonii* are resistant to erythromycin, and *L. fallonii* is also resistant to chloramphenicol (Gomez-Valero et al. 2014). Acquired antibiotic resistance, conversely, is more infrequent: a ciprofloxacin-resistant *L. pneumophila* strain has been isolated from a patient with LD, and there is a report of *in vivo* selection of fluoroquinolone resistance after patients were treated with these antibiotics (Bruin et al. 2014; Shadoud et al. 2015).

Transmission and epidemiology

Legionellosis has been termed a disease of affluence as the top niches in which *Legionella* propagate are built by humans (Dehner 2018; Bartram 2007). Although legionellae are ubiquitous in freshwater habitats in low abundance (Graells et al. 2018), most legionellosis occurs from inhalation of *Legionella*-containing aerosols spread by showers, faucets, air-conditioning, and fountains originating from contaminated artificial water systems such as plumbing networks, water reservoirs, and cooling towers, where legionellae find favourable conditions for replication: warm temperatures (25-45°C), biofilms and protozoans (Woo, Goetz, and Yu 1992; Falkinham et al. 2015). Legionellosis may also arise from contaminated water used to irrigate wounds, or from respiratory equipment; however, these cases seem to be particular to hospitals and care facilities (Dehner 2018; Falkinham et al. 2015). Transmission between humans is rare, with a single case described to date (Correia et al. 2016; Borges et al. 2016), but human-to-environment transmission has been suggested as the source of some particularly virulent strains of *L. pneumophila* (David et al. 2016).

Presently, over 65 species of *Legionella*, encompassing more than 70 serogroups, have been discovered (Mondino et al. 2020; Mercante and Winchell 2015). While almost half of these species have been isolated from clinical samples, all species are considered potential human pathogens. Despite this phylogenetic breadth, 80-90% of legionellosis worldwide is caused by *L. pneumophila*, except in Australia and New Zealand, where 50-60% of cases are caused by *L. longbeachae*. *L. micdadei* and *L. bozemanae* cause the remaining occurrences. Interestingly, even in infections caused by *L. pneumophila*, of its 15 serogroups, strains of serogroup one are overrepresented, comprising 90% of the cases. Furthermore, it has also been observed that clones from this serogroup are responsible for 50% of LD in northern Europe,

which suggests specific adaptations to anthropogenic environments (David et al. 2016; Den Boer and Yzerman 2004; Fields, Benson, and Besser 2002; Mondino et al. 2020; Kozak-Muiznieks, Mercante, and Raphael 2020; Mercante and Winchell 2015).

The worldwide occurrence of legionellosis is challenging to estimate due to misdiagnosis and underreporting, but it has been established as a critical contributor to both community- and hospital-acquired pneumonia. In the United States alone, legionellae account for 2-9% of all community-acquired cases of pneumonia, requiring about 8000-18000 hospitalisations every year. (Diederen 2008; Falcó et al. 1991; Lau and Ashbolt 2009; Mercante and Winchell 2015; Kozak-Muiznieks, Mercante, and Raphael 2020). In the past decade, the reported legionellosis cases have increased over two-fold in both the United States and Europe; this is thought to be due to a combination of factors, including environmental conditions, an increase in susceptible populations and better diagnostic testing. Most legionellosis cases are attributed to sporadic infections. There is also a widespread increase in cases during the summer months (Kozak-Muiznieks, Mercante, and Raphael 2020; Mondino et al. 2020).

Prevention and Control

Legionella species constitute a significant health burden to humans; to prevent their dissemination, risk management approaches must consider their ecological niches and the modes of transmission. To address the persistence of legionellae in anthropogenic water systems, frameworks have been devised to control the level of nutrients, prevent low flow and stagnation of water, control the temperature, and control the microorganisms present in these habitats. These measures can be implemented with a range of methods, from dosing the water with disinfectants, copper and silver ionisation, and thermal and UV disinfection, to point-of-use filters (Muraca, Stout, and Yu 1987; Biurrun et al. 1999; Kusnetsov et al. 2001; Darelid, Löfgren, and Malmvall 2002; Bartram 2007; Surman-Lee and Walker 2020).

Biology and pathogenesis of *Legionella pneumophila*

Overview

Soon after the identification of *L. pneumophila*, the *Legionellaceae* family was established (Brenner 1979). While at first *Legionellaceae* was comprised of three genera: *Legionella*, *Tatlock* and *Fluoribacter* (Garrity, Brown, and Vickers 1980), this was later contested by microbiologists due to the phenotypic similarities between them; *Legionella* is now the only recognised genus in this family. Species within the *Legionella* genus are all facultative intracellular bacteria, except for '*Candidatus* Legionella polyplacis', a symbiont of the louse *Polyplax serrata* (Říhová et al. 2017). Over 45 years since *L. pneumophila*, new species are still being discovered, with *Legionella antarctica* and *Legionella bononiensis* just in the past year (Shimada et al. 2021; Girolamini et al. 2022).

Bacteria of the genus *Legionella* are Gram-negative, pleomorphic, non-sporulated bacilli with dimensions ranging from 0.3-0.9 μm wide and 2-20 μm in length. Most have a polar flagellum (although some might have two) which makes them motile (Thomason 1979), except for *L. longbeachae* and *L. oakridgensis* (Appelt and Heuner 2017).

Life cycle and ecology

L. pneumophila exhibits a biphasic lifecycle alternating between replicative (avirulent) and transmissive (virulent) forms as it transitions between extracellular and intracellular growth, through highly regulated mechanisms (Oliva, Sahr, and Buchrieser 2018).

These forms are characterised by distinct morphogenic, metabolic, and gene expression patterns. When the environmental conditions are favourable for replication, as when it enters host cells, *L. pneumophila* upregulates the expression of genes for metabolism, amino acid degradation, cell division and biosynthetic processes. As the resources in the environment are spent, it arrests replication by upregulating transmissive traits such as motility, osmotic- and acid-resistance, and virulence, enabling it to escape the host. Once outside, they can find a new host or remain in the planktonic form (Oliva, Sahr, and Buchrieser 2018; Mondino et al. 2020; Chauhan and Shames 2021).

In liquid culture, *L. pneumophila* displays a similar life cycle where the replication phase corresponds to bacteria in exponential growth, and the transmissive phase corresponds to stationary growth phage (Oliva, Sahr, and Buchrieser 2018).

L. pneumophila can be found in nearly all natural and engineered aquatic environments, associated with biofilms and protists (Fields, Benson, and Besser 2002). These freshwater habitats may vary significantly in their composition of abiotic and biotic factors, which affect how well *L. pneumophila* can survive and replicate.

Abiotic factors that affect *L. pneumophila* in freshwater environments

The temperature has one of the most critical influences in regulating *L. pneumophila* density in the environment. *L. pneumophila* grows best at warm temperatures ranging from 30-40°C. It may tolerate higher temperatures, as evidenced by high levels of *L. pneumophila* in hot water tanks. At the colder end of the spectrum, although *L. pneumophila*has been isolated from water at 6°C, this does not indicate it can grow at these temperatures (Garduño 2020). In a study following *Legionella* from water reservoirs to a potable water distribution system, it was observed that the population at the source was more diverse and cold-adapted than the population in the potable water, which was less varied and thermotolerant (Lesnik, Brettar, and Höfle 2016). Adaptations to temperature are regulated at the gene level; thermotolerance is mediated by heat-stress proteins such as chaperonins and chaperone proteins. *L. pneumophila* has a more filamentous shape at high temperatures, linked to increased HtpB chaperonin levels (Piao et al. 2006).

L. pneumophila grown in culture media requires a narrow pH range, however, in laboratory-defined water cultures it has been shown to replicate at pH levels of 5.5-9.2 (Wadowsky et al. 1985). These parameters are more complex to establish in the environment because *L. pneumophila* grows intracellularly inside protozoan hosts, which shelter it (Garduño 2020).

The amino acid requirements for *L. pneumophila* were established during the formulation of the chemical-defined media for its growth (Warren and Miller 1979). More recently, *in silico* analysis of the *L. pneumophila* genome predicted that it cannot synthesise Arg, Ile, Leu, Met and Val (Price et al. 2014). In the environment, *L. pneumophila* gets access to amino acids by producing extracellular proteases, which digest proteins in the background, generating free amino acids. Inside host cells, *L. pneumophila* employs mechanisms to sequester amino acids into the LCV. The availability of amino acids affects *L. pneumophila*'s differentiation regulation (Molofsky and Swanson 2004).

For *L. pneumophila*, metal ions like calcium, magnesium, iron and sodium have a non-nutritional function. Calcium and magnesium affect the adherence of *L. pneumophila* to surfaces, which may have a role in biofilm formation (Koubar, Rodier, and Frère 2013). The impact of iron on *L. pneumophila*'s growth has been long known as it is an essential supplement to the media (Feeley et al. 1979). In artificial water systems, it is thought that *L. pneumophila* has access to iron and other metals from (corroded) pipes and tanks. *L. pneumophila* can acquire iron in the environment by direct uptake through

membrane proteins or siderophores (Cianciotto 2015). Sodium sensitivity in *L. pneumophila* is growth phase-dependent; it inhibits the growth of virulent *L. pneumophila*, although the mechanism has not been described (Byrne and Swanson 1998). This mechanism may also be temperature-regulated, as at low temperatures *L. pneumophila* is less sensitive to sodium (Garduño 2020).

Biotic factors that affect *L. pneumophila* in freshwater environments

The intracellular lifecycle of *L. pneumophila* inside amoebae was first described by Rowbotham in 1980 (Rowbotham 1980). Although it has been established that protists are integral for the persistence of *L. pneumophila* in freshwater niches, in natural environments interactions between the two seem to be much more infrequent than in engineered ones. These systems are thus considered "hot spots" for the evolution of *L. pneumophila* and emerging pathogens (Berk et al. 2006).

L. pneumophila can infect a range of protozoa such as *Acanthamoeba*, *Naegleria* and *Vermamoeba*, although this may be strain-specific, and temperature dependant (Buse and Ashbolt 2011). Equally, not all amoeba species and strains are permissive to *L. pneumophila*, such as *Naegleria lovanienesis* and some strains of *Willaertia magna* (Declerck et al. 2005; Dey et al. 2008). Amoebae that resist *L. pneumophila* infection do so by avoiding uptake, expulsing pellets of undigested, or by digesting *L. pneumophila* (Amaro et al. 2015). *L. pneumophila* also interacts with other protozoans such as ciliates and flagellates, the former does not digest *L. pneumophila*, but also does not support intracellular replication, and the latter consumes *L. pneumophila* as food (Garduño 2020).

Biofilms are responsible for the survival and persistence of *L. pneumophila* in man-made environments and have been associated with LD outbreaks. Planktonic *L. pneumophila* may colonise and survive in existing multi-species biofilms, which protects them from environmental stresses such as biocides, but it cannot replicate in biofilms without the presence of amoebae (Abu Khweek and Amer 2018).

Pathogenesis and virulence

The intracellular life cycle of *L. pneumophila*

L. pneumophila can replicate inside a range of host cells, from protists to macrophages, following a similar life cycle (Figure 2).

L. pneumophila enters host cells through phagocytosis, although it can also infect non-phagocytic cells. As soon as it enters, it loses its flagella and starts secreting effector proteins. Once entirely inside the cell and encapsulated in a phagosome, it must evade the traditional path of phagosome maturation and degradation. To achieve *L. pneumophila* creates a protective environment, the LCV. It hijacks ER-Golgi vesicles and mitochondria and recruits them to the

LCV membrane. While the association with the mitochondria is transient, the ER vesicles fuse with the LCV membrane. This process expands the LCV and makes it look like a host cell compartment, thereby protecting the *L. pneumophila* inside. Because of this appearance, the host decorates the LCV with ribosomes. In this safe replication-permissive niche, the *L. pneumophila* starts replication. Once the nutrient availability is exhausted, *L. pneumophila* differentiates into its transmissive phase, expressing virulence traits, including flagella, which allow it to escape the host cells (Chauhan and Shames 2021; Kay et al. 2020; Mondino et al. 2020). It's these free flagellated bacteria that if aerosolised, can infect human macrophages. Thus, through this process, amoebae prime *L. pneumophila* for infection (Molmeret et al. 2005).

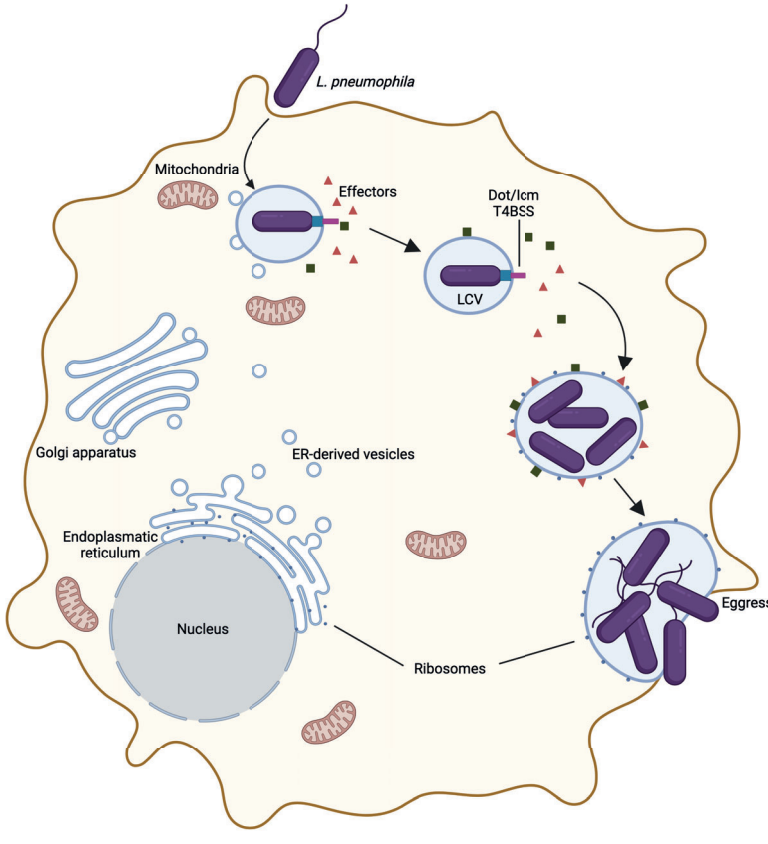

Figure 2. Intracellular life cycle of *L. pneumophila*. *L. pneumophila* enters the host cell. Once the phagosome, it starts secreting effector proteins through the Dot/Icm T4SS system that enable it to evade degradation by recruiting ER-derived vesicles to the LCV membrane, and transiently associating with mitochondria. The LCV becomes decorated with ribosomes and effector proteins and *L. pneumophila* safely replicates inside. Once there are no more nutrients, the transmissive stage is activated and *L. pneumophila* exits the cell. Created with BioRender.com.

The Dot/Icm type IV secretion system

The Dot/Icm T4SS translocates over 300 effector proteins involved in the intracellular replication of *L. pneumophila*. These effector proteins interfere with various host processes, such as vesicular trafficking, LCV formation and protein ubiquitination. It is the best-characterised secretion system of *L. pneumophila* due to its importance. The *dot/icm* gene loci are present in all *Legionella* species and are highly conserved, underlining their importance for intracellular replication of *L. pneumophila*.

The complex is made of 26 proteins, and although not all have been characterised, a working model proposes a core transmembrane subcomplex localised at the polar ends of the bacterium and a coupling protein subcomplex (Figure 3). In the transmembrane complex, DotH forms the outer membrane pore, is anchored by DotC and DotD, and receives energy from DotG. DotF interacts with DotG to regulate its energy-transducing activity. The coupling protein subcomplex recruits and carries substrates to the secretion channel; DotL in the inner membrane is an ATPase, bound to the DotM-DotN internal membrane/cytoplasm component, the two chaperones IcmS and IcmW, and the LvgA protein. There are 16 other proteins required for a functional system, particularly DotA, a cytoplasmic-membrane protein, without which effectors cannot be secreted. In the laboratory, Δ*dotA* mutants cannot replicate intracellularly (Gomez-Valero, Chiner-Oms, et al. 2019).

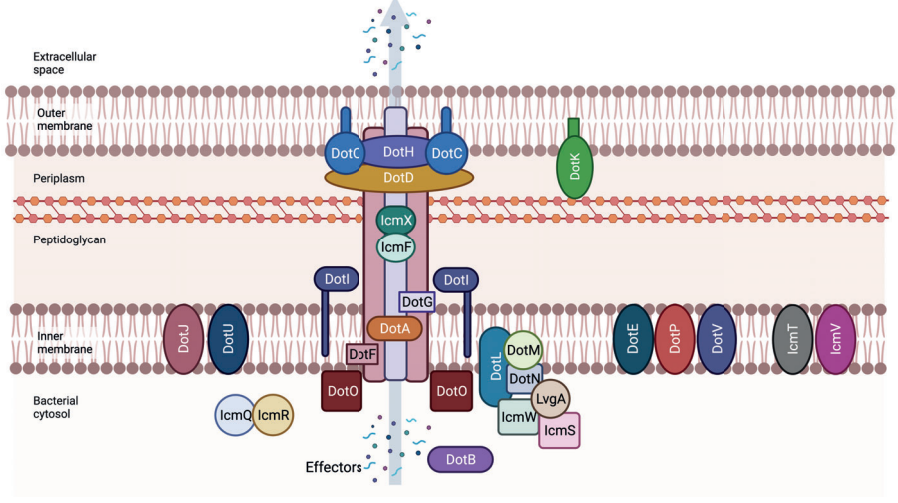

Figure 3. Model of the Dot/Icm type IV secretion system. Based on Gomez-Valero et al. 2019. Created with BioRender.com.

Regulation of effectors

The Dot/Icm T4SS is responsible for translocating proteins crucial for the intracellular survival of *L. pneumophila*. Thus, these effectors have to be tightly regulated at the gene expression level to insure the correct and timely expression of genes. Over 100 effector genes are regulated by three regulatory systems: the PmrAB two-component system (TCS), the CpxRA TCS, and the LetAS-RsmYZ-CsrA regulatory cascade (Figure 4).

The PmrAB TCS is composed of PmrB sensor histidine kinase, and PmrA DNA binding response regulator. The signals that activate PmrB are unknown, but there are some indications it could be triggered by low pH concentrations

(Al-Khodor et al. 2009). PmrA activates the expression of CsrA translational expression, resulting in higher levels of CsrA when the PmrAB TCS is active. PmrA additionally actives the connector protein LerC, which inhibits the LetAS regulatory cascade by binding to LetS.

The CpxRA TCS also comprises a sensor histidine kinase – CpxA, and a response regulator – CpxR. This dual regulator activates and inhibits the expression of Dot/Icm effectors, and activates the expression of Dot/Icm genes. CpxR also activates LetE, a connector protein that connects the CpxRA TCS to the LetAS-RsmYZ-CsrA regulatory cascade.

The LetAS-RsmYZ-CsrA regulatory cascade contains the LetAS TCS, two small RNAs (sRNAs) – RmsY and RmsZ, and the post-transcriptional repressor CsrA. LetS is activated during *L. pneumophila'*s differentiation into the stationary phase form. It then phosphorylates LetA, which in turn activates RmsYZ. These sRNAs bind and sequester CsrA molecules, which can longer interact with the mRNA, resulting in the inhibition of Dot/Icm effector expression. CsrA has an inhibitory effect on LerC expression (Kay et al. 2020; Feldheim et al. 2016; 2018; Linsky and Segal 2021).

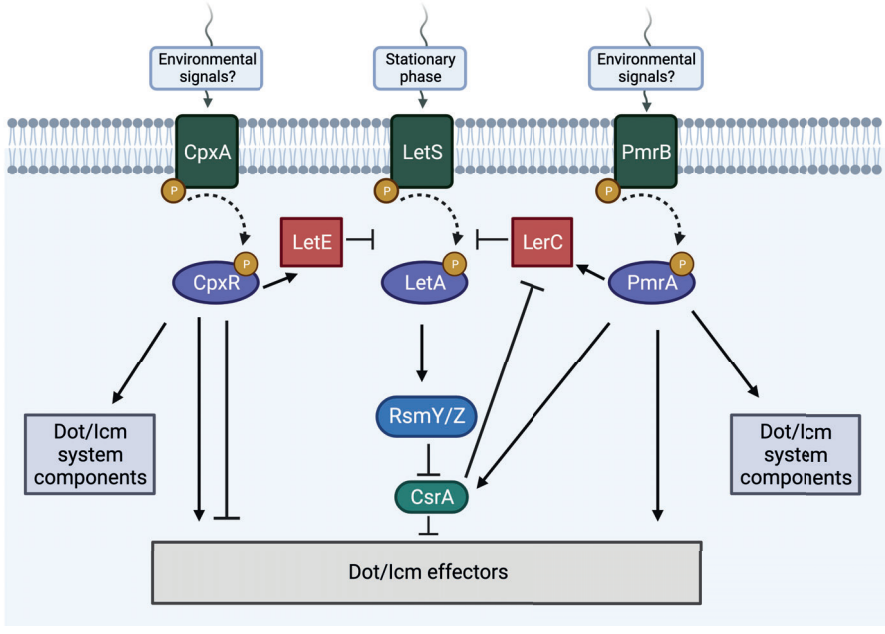

Figure 4. Model of the three regulatory systems that controls the expression of the Dot/Icm system: the CpxAR TCS, the letAS-RsmYZ-CrsA regulatory cascade, the PmrAB TCS, and the connector proteins LetE and LerC. Arrows indicate activation, T-shapped symbols indicate repression, dashed curved arrows indicate the phosphorelay mechanism between sensor kinase and response regulaor. Based on Linsky and Segal 2021. Created with BioRender.com.

The genome and evolution of *L. pneumophila*

The genome size of *Legionella* species ranges from 2.7- 4.9 Mb, with an average G+C content of 34-51%; for *L. pneumophila,* these values are 3.3-3.5 Mb and 38%, respectively (Cazalet et al. 2004; Chien et al. 2004). The presence of plasmids, episomal- and mobile elements, which can be integrated in the chromosome or carried by plasmids, varies between species and strains (Schroeder et al. 2010).

Analyses of the genus reveal a high level of genomic heterogeneity and plasticity. A study of 58 *Legionella* species and 80 strains found that the core genome of the genus comprises only 6% of all the genes, while within a species, it was 32% (Gomez-Valero, Rusniok, et al. 2019). These results differed from a previous study where 38 species were analysed, illustrating that the pan-genome of *Legionella* is still not defined (Burstein et al. 2016; Gomez-Valero, Rusniok, et al. 2019). The high variability in the *Legionella* genome has been attributed to a combination of different factors: (i) mobile genetic elements associated with genes encoding for transfer regions, (ii) high

recombination rates, and (iii) horizontal gene transfer (HGT) (Khodr et al. 2016; Gomez-Valero, Rusniok, et al. 2019). While DNA exchange between species is rare, there is a high number of eukaryotic-like genes and domains (Gomez-Valero, Rusniok, et al. 2019).

Not surprising, due to their essential role in intracellular replication, the *dot/icm* genes are highly conserved and a part of the core genome. While the Dot/Icm T4SS is highly conserved at the sequence level, the effector protein repertoire it translocates varies substantially. The effector pan-genome currently consists of over 18,000 putative proteins encoding over 200 eukaryotic-like proteins and 137 eukaryotic-like domains. The most abundant domains identified were ankyrin repeats, F- and U-box; a GTPase-like domain, unique in prokaryotes, has also been identified. Only eight effectors were found across all species, and seven others were in almost all. The core effector genome within a species is only 65% of all the effectors, showcasing high variability even at a species-level (Gomez-Valero, Rusniok, et al. 2019). These eukaryotic-like genes and domains are thought to have been acquired during *Legionella*'s co-evolution with protozoan hosts.

HGT plays a critical role in the evolution of bacteria, archaea and unicellular eukaryotes (Boto 2010). Studies have documented gene exchange within and between members of these different domains. However, exchanges between prokaryotes and eukaryotes are not as widely reported (Boto 2010; Gomez-Valero and Buchrieser 2019). The Dot/Icm system in *L. pneumophila* secretes over 10% of its proteome, the highest number of effectors secreted by any prokaryote. The second closest is *Coxiella burnetti*, an obligate intracellular pathogen also part of the *Legionellales* order, which secretes over 100 effectors, close to 5% of its proteome (Best and Abu Kwaik 2018). However, there is a high level of redundancy in the effector repertoire. Minimisation of one-third of *L. pneumophila*'s effectors had minimal effect on growth in mouse macrophages (O'Connor et al. 2011).

When looking at host-adapted bacteria, researchers found "some of the smallest, most stable, most deteriorated, most highly repeated and most highly recombined bacterial genomes" (Toft and Andersson 2010). Host adaptation is a dramatic ecological process which happens unidirectionally in stages and is governed by different selective forces (Toft and Andersson 2010). During integration, there are severe genomic changes in the bacterial genome (Figure). At first, as it transitions from the free-living to the facultative intracellular stage, the bacteria gain new genes through HGT, which it modifies through duplication and recombination events. The next stage, obligate intracellular, may be triggered by the loss and further modification of bacterial genes, making the bacteria unable to grow extracellularly and restricted to specific hosts. As their genome continues to be reduced through gene non-functionalisation and loss, they become endosymbionts and start co-evolving with the host, which leads to the further minimisation of the genome, eliminating genetic redundancy between the host and symbiont. In the final stage, the symbiont

becomes part of the host as an organelle, and its genes are transferred to the host or replaced by host nuclear genes(Moya et al. 2008; Toft and Andersson 2010).

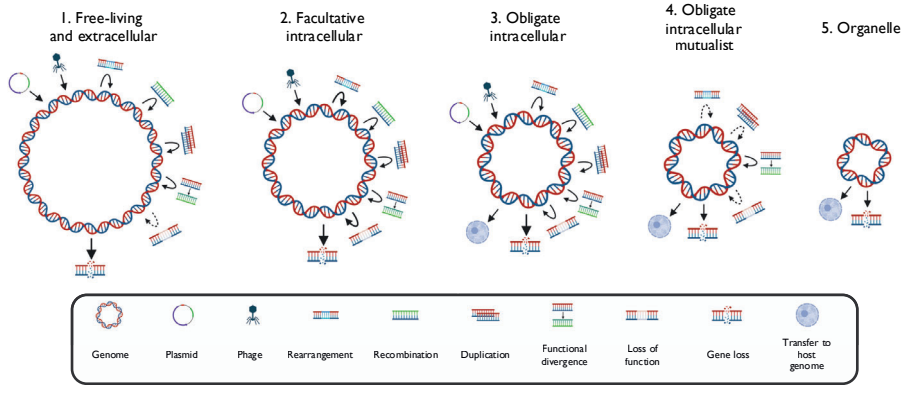

Figure 5. Genomic dynamics during different host-adaptation stages. Arrows pointing towards the genome indicate gene acquisition by HGT. Arrows looping towards the genome indicate changes within the genome. Arrows pointing away from the genome indicate gene loss or transfer to the host. The weight of the arrow indicates the influence of each type of process at the different stages. Based on Toft and Andersson (2010). Created with BioRender.com.

So, despite what is expected from a facultative intracellular pathogen, *Legionella* have preserved this high level of genomic redundancy.

The current hypothesis is that the diversity of effectors between species is what enables *Legionella* to replicate in such a broad range of hosts. *Legionella* use this arsenal of effectors in different combinations depending on the host. Selective pressures act on both their ability to replicate in a single host, and their ability to replicate in a variety of hosts, so they maintain and acquire new genes to add to their effector repertoire. A highly regulated differentiation between transmissive and replicative phases and expression virulence genes also has a significant role as it reduces the fitness costs of gene expression. Ultimately, it is this redundancy in effectors coupled with the similarities between protists and human phagocytic cells that permit *Legionella* to *accidentally* infect humans, despite not having an evolutionary history with these hosts.

Current investigations

Legionella pneumophila contains multitudes. Since it catapulted to fame after the eponymous Legionnaires' disease outbreak, it has become an increasingly important human pathogen. Its appeal, however, does not stem simply from the consequences of its interactions with humans – *L. pneumophila* is a remarkable and unique microorganism. With its life cycle, complex secretion systems, and interaction and co-evolution with other organisms, *L. pneumophila* is a valuable model organism.

The work in this thesis is primarily focused on studying the evolution of *L. pneumophila*. **Papers I** and **II** delve into this, asking: "How do potential pathogens adapt to cause disease in specific hosts?" and "What are the critical phenotypes and genes underlying these traits?". We try to answer these questions by looking at host-specific adaptations over short- and long-term timeframes, in natural (*in vivo)* and experimental (*in vitro*) settings. **Paper III** describes a novel method for the genetic manipulation of *Acanthamoeba castellanii*. While this may appear as a departure from the main topic, it illustrates the multitudes one faces when studying *L. pneumophila*. As a natural host of *L. pneumophila*, *A. castellanii* is a critical model for understanding host-pathogen interactions. Thus, it is crucial to develop more efficient and practical tools.

Paper I: Microevolution of *L. pneumophila* during human infection

L. pneumophila is ubiquitously found in natural and man-made aquatic habitats. In these environments, they encounter a range of protists which they use as hosts for replication. This intracellular life cycle is thought to have equipped *L. pneumophila* with the tools to infect human alveolar macrophages(Best and Abu Kwaik 2018). Thus, humans *accidentally* infected may develop a severe, often-fatal pneumonia termed Legionnaires' disease or a milder form of legionellosis, Pontiac fever.

Despite the shared similarities between protists and human macrophages, it is hypothesised that the different host-specific requirements result in high-selective value mutations in *L. pneumophila* that confer a significant fitness advantage in humans. However, due to limited person-to-person transmission,

these human-specific adaptations are not returned to the main *L. pneumophila* population after each accidental infection(Ensminger et al. 2012).

To identify these mutations, we collected *L. pneumophila* strains from independent infections, pairing patient isolates with their inferred environmental source samples. These sample pairs originated from sporadic cases of Legionnaires' disease and outbreaks. Additional strains were obtained from published work.

We implemented a system to filter through the samples and discard those caused by co-infections, or those from short incubation periods. After curation, we were left with 171 sample pairs, 24 of which were from two outbreaks and the remainder from independent cases. We whole genome sequenced 100 pairs and obtained the sequences from published data for the remaining sample sets.

Genomic variations between the clinical and environmental isolate sample pairs were assessed using the *de novo* assembled environmental strain sequences as a reference for the clinical strains. In nine genes, mutations were independently identified more than once, and for two of the genes, we identified three independent mutations. Our simulations suggest that the mutations in these two genes may have arisen from convergent evolution and not merely by chance.

One of these potentially adaptive in the human host (PAHH) genes encodes an outer membrane protein (lpg0707), an Omp1/FadL homolog. Two (out of three) mutations in this gene lead to premature stop codons, resulting in truncated proteins that are 3% or 40% the length of the full-size protein.

The other PAHH gene encodes an EAL domain-containing protein (lpg0891); all three mutations in this gene cause amino-acid substitutions, which are not predicted to result in a complete loss of function.

To evaluate the effect of these mutations *in vitro*, we performed extracellular and intracellular growth assays with both pairs of isolates and the *L. pneumophila* strain Paris as reference. Each pair's clinical and environmental isolates exhibited similar growth rates in the extracellular assays. There was no difference in growth rate in *A. castellanii* for the OmpP1/FadL-mutant (clinical isolate) compared with its environmental isolate; however, a significant relative growth rate increase (34.9%) was observed in U937 human macrophages. In contrast, the EAL-mutant exhibited a slight decrease in growth rate when grown in *A. castellanii* compared to its environmental isolate and an evident reduction in growth rate in U937 macrophages. Although these results seem promising and confounding, respectively, it is essential to note that experimental conditions, especially when using macrophages, do not account for the entire human immune system. Further studies are required to understand the role of these genes and their subsequent mutations in infection.

In **conclusion**, our comparative genomic analyses identified mutations in two genes in L. pneumophila that could potentially be responsible for human adaptation. One, encoding an OmpP1/FadL outer membrane protein, gave –

when knocked out – *L. pneumophila* a substantial increase in growth rate in human macrophages, consistent with the original hypothesis predicting the availability of high-selective value in-patient mutations; the other, an EAL-domain containing protein, which regulates cyclic-di-GMP, is involved in virulence and motility of *L. pneumophila*. In our assay, the mutated version of this gene displayed a surprising loss of fitness in macrophages. As these mutations repeatedly occurred in independent infections over a short period, there is a strong indication that they provide an evolutionary advantage in the human host. Together, these results suggest that *L. pneumophila*, despite being able to infect humans, is sub-optimally adapted to this host.

Future perspectives

i. Follow-up functional studies on the gene candidates in the context of different *L. pneumophila* hosts, including amoebae, human cell lines, and animal models (e.g., *Galleria mellonella* and *Caenorhabditis elegans*).
ii. Sequence more clinical and environmental isolate pairs to identify additional mutations, determine mutation frequency, and strengthen conclusions on whether the observed mutations result from convergent evolution or random mutation.

Paper II: Long-term experimental evolution of *L. pneumophila*

In **Paper I**, we looked at host-specific adaptations of *L. pneumophila* resulting from its short incubation period during human infection. In this study, we further explore the hypothesis that *L. pneumophila*'s environmental lifestyle governs its evolution as a *generalist* pathogen by selecting against mutations that may decrease fitness in the various hosts it encounters; we investigate whether, conversely, host restriction would drive *L. pneumophila* to become a *specialist*.

To answer this question, we set up a long-term experimental evolution system in which we cycled *L. pneumophila* in three different host settings: *A. castellanii*, U937 human macrophage-like cells, and alternating between the two hosts (Figure 2). With this system, we aimed to identify the critical mutations that drive the evolution of host adaptation in *L. pneumophila*.

The two fluorescently tagged isolates of *L. pneumophila* strain Paris (-SYFP2 and -dTomato) have now been passaged for over 1000 generations.

Each of the six lineages for all three host conditions was sequenced at the population level at two or three time points.

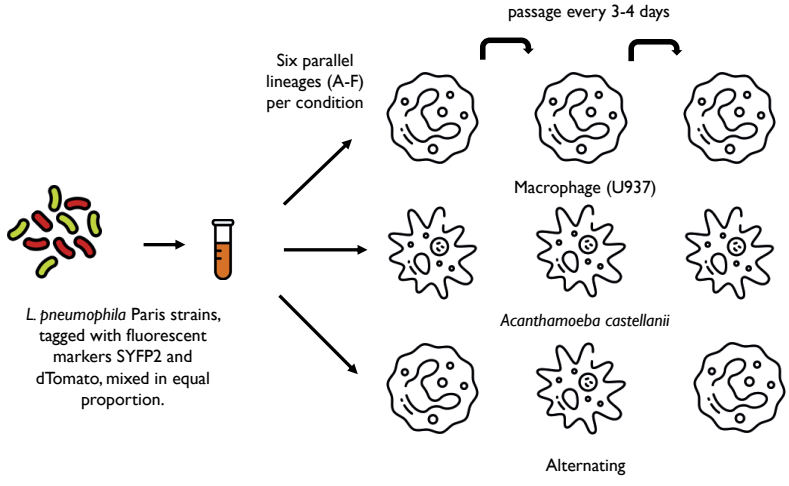

Figure 6. Long-term experimental evolution set-up.

Identifying mutations of interest proved to be more challenging than expected. In summary, the mutations found can be classified into four groups: (i) adaptations to laboratory conditions, specifically to streptomycin; (ii) compensatory mutations to restore the fitness losses caused by the previous group of mutations; (iii) mutations in the lipopolysaccharide (LPS) synthesis operon, which were only observed in lineages passaged in *A. castellanii*; and (iv) mutations in LerC, a regulator of effector expression, fixed in five lineages passaged in U937 macrophages.

Streptomycin resistance was found in most evolved lineages, with mutations in the ribosomal protein S12 RpsL (K43T and K88R), previously identified in *L. pneumophila*. Although we did not purposely expose the *L. pneumophila* to streptomycin, both hosts were grown in media supplemented with penicillin and streptomycin. We suspect that a fraction of the supplemented media was accumulated in the hosts' cytosol, which would come into contact with *L. pneumophila*.

In almost all instances, we found that each of the two RpsL mutations was accompanied by another mutation. RpsL43 was systematically found with a known compensatory mutation in RpsD (ribosomal protein S4), which restores efficient translation in cells with the former RpsL mutation. On the other hand, RpsL88 was often identified with a mutation in the co-chaperonin GroES, also known as HtpA in *Legionella*. The role of GroES is not well described, but chaperonins have been shown to be involved in different phases of the *Legionella* life cycle.

The LPS synthesis cluster contains close to 30 genes in *L. pneumophila*, however the specific function of each gene is not fully understood. In our evolved lines we found mutations in four of these genes (lpp0831, lpp0832, lpp0833, and lpp0835), which are part of the Paris strain-specific genes. The prevalence of independent mutations found in this gene cluster in the lineages cycled in *A.castellanii* suggest that these are pathoadaptive mutations.

The protein LerC is involved in the network regulation of effector proteins in *L. pneumophila*. Specifically, it has been described as a connector between the PmrAB and LetAS two-component systems. We identified six distinct mutations in the *lerC* gene, some fixed in five evolved populations. None of the LerC mutations were observed in the lineages cycled in *A. castellanii*, which suggests that these mutations could decrease fitness in this host. Conversely, these mutations increased fitness in U937 macrophages, making it a good candidate gene for human-specific adaptive mutation.

In **conclusion**, the function, fate and histories of the mutations in these 18 evolved lineages remain unclear. Additional studies of the candidate mutations in the LPS gene cluster and *lerC* gene are required to understand their role in *L. pneumophila* host adaptation. As mentioned in **Paper I**, it is challenging to comprehensively quantify fitness costs and advantages in an organism with a complex extra- and intracellular life cycle. Therefore, further methodological optimisation is also necessary to better characterise the effect of these mutations.

Future perspectives

Experimental evolution allows us to "turn back the evolutionary clock and replay life's tape" by trying to recreate in the lab what we hypothesise happens in nature. In some ways, this project is still at the early stages of realising its potential, as we have more questions than answers. As we continue to sequence the evolved populations and analyse the data, we will get a clearer picture of the evolutionary trajectories of the two original strains. Nevertheless, there are some steps to answer the more pressing questions.

i. Characterise the function and effect of the mutations described here.
ii. Optimise an assay for fitness measurements.
iii. Isolate and sequence colonies from specific populations.
iv. Sequence the remaining lineages at the latest time point.

Paper III: A method for highly efficient transfection of *A. castellanii*

A. castellanii is an emerging human pathogen of ecological, clinical, and evolutionary importance (Rayamajhee et al. 2022). Still, few genetic manipulation tools have been developed for this organism, which hinders its use as model organism(Moon et al. 2009; Swart et al. 2018).

The ability to introduce and express exogenous genes in eukaryotic organisms (i.e., transfection) has been paramount for studying gene function and expression in cells(Bono et al. 2020; Fus-Kujawa et al. 2021; Gam et al. 2019). Different transfection methods have been described for *A. castellanii*(Hu and Henney 1997; Kong and Pollard 2002; Lee et al. 2015; Leitsch et al. 2021; Rolland et al. 2020; Yin and Henney Jr. 1997), with commercial reagents presently being the most broadly used(Lee et al. 2015; Rolland et al. 2020). However, the reported transfection efficiency remains low, at around ~5% (Moon et al. 2009; Peng, Omaruddin, and Bateman 2005).

In this study, we describe the transfection of *A.castellanii* with polyethylenimine (PEI), a cationic polymer extensively used to transfect a range of eukaryotic cells(Bono et al. 2020; Boussif et al. 1995; Ponti et al. 2021). We systematically explored three forms of PEI and evaluated their effect on cell viability and transfection efficiency based on parameters established for other cell types.

We found that linear PEIs were less toxic to the cells and more effective than the branched form of PEI. Our high-throughput approach also revealed the importance of the DNA:PEI ratio and concentrations for optimal transfection conditions. Furthermore, we demonstrated that co-transfection of more than one plasmid per cell is common and can potentially be modulated by the concentration of PEI used. When comparing the PEIs to the commercial reagents – SuperFect® and ViaFect™, we found the PEIs to be at least ten times more efficient at transfecting *A. castellanii* with plasmid DNA.

In **conclusion**, we demonstrated that these readily available and inexpensive cationic polymers can be used to transfect A. castellanii with high efficiency. We also detailed the effect of different factors in optimising transfection conditions.

Future perspectives

While this work provides a sound basis for the transfection of *A. castellanii* with PEIs, there remain parameters of importance for the standardisation of this protocol that have not yet been investigated.

i. Understand the biophysical and biochemical properties of PEI and PEI-DNA complexes and how they interact with the *A. castellanii* at a molecular level.

ii. Explore the size and type of nuclei acid constraints on transfection efficiency, particularly siRNA.
iii. Establish this protocol with other *Acanthamoeba* species.

References

Abu Khweek, Arwa, and Amal O. Amer. 2018. 'Factors Mediating Environmental Biofilm Formation by Legionella Pneumophila'. *Frontiers in Cellular and Infection Microbiology* 8. https://doi.org/10.3389/fcimb.2018.00038.

Al-Khodor, Souhaila, Sergey Kalachikov, Irina Morozova, Christopher T. Price, and Yousef Abu Kwaik. 2009. 'The PmrA/PmrB Two-Component System of *Legionella Pneumophila* Is a Global Regulator Required for Intracellular Replication within Macrophages and Protozoa'. *Infection and Immunity* 77 (1): 374–86. https://doi.org/10.1128/IAI.01081-08.

Amaro, Francisco, Wen Wang, Jack A Gilbert, O Roger Anderson, and Howard A Shuman. 2015. 'Diverse Protist Grazers Select for Virulence-Related Traits in Legionella'. *The ISME Journal* 9 (10): 1607–18. https://doi.org/10.1038/ismej.2014.248.

Appelt, Sandra, and Klaus Heuner. 2017. 'The Flagellar Regulon of Legionella—A Review'. *Frontiers in Cellular and Infection Microbiology* 7. https://doi.org/10.3389/fcimb.2017.00454.

Bartram, Jamie, ed. 2007. *Legionella and the Prevention of Legionellosis*. Geneva: World Health Organization.

Berk, S. G., J. H. Gunderson, A. L. Newsome, A. L. Farone, B. J. Hayes, K. S. Redding, N. Uddin, et al. 2006. 'Occurrence of Infected Amoebae in Cooling Towers Compared with Natural Aquatic Environments: Implications for Emerging Pathogens'. *Environmental Science & Technology* 40 (23): 7440–44. https://doi.org/10.1021/es0604257.

Best, Ashley, and Yousef Abu Kwaik. 2018. 'Evolution of the Arsenal of Legionella Pneumophila Effectors To Modulate Protist Hosts'. Edited by Danielle A. Garsin. *MBio* 9 (5): e01313-18. https://doi.org/10.1128/mBio.01313-18.

Biurrun, Amaya, Luis Caballero, Carmen Pelaz, Elena León, and Alberto Gago. 1999. 'Treatment of a Legionella Pneumophila-Colonized Water Distribution System Using Copper-Silver Ionization and Continuous Chlorination'. *Infection Control & Hospital Epidemiology* 20 (6): 426–28. https://doi.org/10.1086/501645.

Bono, Nina, Federica Ponti, Diego Mantovani, and Gabriele Candiani. 2020. 'Non-Viral in Vitro Gene Delivery: It Is Now Time to Set the Bar!' *Pharmaceutics* 12 (2): 183. https://doi.org/10.3390/pharmaceutics12020183.

Borges, Vítor, Alexandra Nunes, Daniel A. Sampaio, Luís Vieira, Jorge Machado, Maria J. Simões, Paulo Gonçalves, and João P. Gomes. 2016. '*Legionella Pneumophila* Strain Associated with the First Evidence of Person-to-Person Transmission of Legionnaires' Disease: A Unique Mosaic Genetic Backbone'. *Scientific Reports* 6 (May): 26261. https://doi.org/10.1038/srep26261.

Boto, Luis. 2010. 'Horizontal Gene Transfer in Evolution: Facts and Challenges'. *Proceedings of the Royal Society B: Biological Sciences* 277 (1683): 819–27. https://doi.org/10.1098/rspb.2009.1679.

Boussif, O., F. Lezoualc'h, M. A. Zanta, M. D. Mergny, D. Scherman, B. Demeneix, and J. P. Behr. 1995. 'A Versatile Vector for Gene and Oligonucleotide Transfer into Cells in Culture and in Vivo: Polyethylenimine'. *Proceedings of the National Academy of Sciences* 92 (16): 7297–7301. https://doi.org/10.1073/pnas.92.16.7297.

Brenner, Don J. 1979. 'Classification of the Legionnaires' Disease Bacterium: Legionella Pneumophila, Genus Novum, Species Nova, of the Family Legionellaceae, Familia Nova'. *Annals of Internal Medicine* 90 (4): 656. https://doi.org/10.7326/0003-4819-90-4-656.

Bruin, J. P., T. Koshkolda, E. P. F. IJzerman, C. Luck, B. M. W. Diederen, J. W. Den Boer, and J. W. Mouton. 2014. 'Isolation of Ciprofloxacin-Resistant Legionella Pneumophila in a Patient with Severe Pneumonia'. *Journal of Antimicrobial Chemotherapy* 69 (10): 2869–71. https://doi.org/10.1093/jac/dku196.

Burstein, David, Francisco Amaro, Tal Zusman, Ziv Lifshitz, Ofir Cohen, Jack A. Gilbert, Tal Pupko, Howard A. Shuman, and Gil Segal. 2016. 'Genomic Analysis of 38 Legionella Species Identifies Large and Diverse Effector Repertoires'. *Nature Genetics* 48 (2): 167–75. https://doi.org/10.1038/ng.3481.

Buse, H. Y., and N. J. Ashbolt. 2011. 'Differential Growth of Legionella Pneumophila Strains within a Range of Amoebae at Various Temperatures Associated with In-Premise Plumbing'. *Letters in Applied Microbiology* 53 (2): 217–24. https://doi.org/10.1111/j.1472-765X.2011.03094.x.

Byrne, B, and M S Swanson. 1998. 'Expression of Legionella Pneumophila Virulence Traits in Response to Growth Conditions.' *Infection and Immunity* 66 (7): 3029–34.

Cazalet, Christel, Christophe Rusniok, Holger Brüggemann, Nora Zidane, Arnaud Magnier, Laurence Ma, Magalie Tichit, et al. 2004. 'Evidence in the Legionella Pneumophila Genome for Exploitation of Host Cell Functions and High Genome Plasticity'. *Nature Genetics* 36 (11): 1165–73. https://doi.org/10.1038/ng1447.

Ceccarelli, Giancarlo, Mario Venditti, Maria Scaturro, and Maria Luisa Ricci. 2020a. 'Clinical Symptoms and Treatment of Legionellosis'. In *Legionellosis Diagnosis and Control in the Genomic Era*. Caister Academic Press. https://doi.org/10.21775/9781913652531.05.

———. 2020b. 'Laboratory Diagnosis of Legionellosis'. In *Legionellosis Diagnosis and Control in the Genomic Era*. Caister Academic Press. https://doi.org/10.21775/9781913652531.06.

Chauhan, Deepika, and Stephanie R. Shames. 2021. 'Pathogenicity and Virulence of *Legionella* : Intracellular Replication and Host Response'. *Virulence* 12 (1): 1122–44. https://doi.org/10.1080/21505594.2021.1903199.

Chien, Minchen, Irina Morozova, Shundi Shi, Huitao Sheng, Jing Chen, Shawn M. Gomez, Gifty Asamani, et al. 2004. 'The Genomic Sequence of the Accidental Pathogen Legionella Pneumophila'. *Science* 305 (5692): 1966–68. https://doi.org/10.1126/science.1099776.

Cianciotto, Nicholas P. 2015. 'An Update on Iron Acquisition by *Legionella Pneumophila* : New Pathways for Siderophore Uptake and Ferric Iron Reduction'. *Future Microbiology* 10 (5): 841–51. https://doi.org/10.2217/fmb.15.21.

Cordes, L. G, G. W Gorman, HazelW Wilkinson, BonnieJ Fikes, and D. W Fraser. 1979. 'Atypical Legionella-like Organisms: Fastidious Water-Associated Bacte-Ria Pathogenic for Man.' *The Lancet*, Originally published as Volume 2, Issue 8149, 314 (8149): 927–30. https://doi.org/10.1016/S0140-6736(79)92623-0.

Correia, Ana M., Joana S. Ferreira, Vítor Borges, Alexandra Nunes, Bernardo Gomes, Rui Capucho, Jorge Gonçalves, et al. 2016. 'Probable Person-to-Person Transmission of Legionnaires' Disease'. *New England Journal of Medicine* 374 (5): 497–98. https://doi.org/10.1056/NEJMc1505356.

Daniels, Worth B., and H. Arthur Grennan. 1943. 'Pretibial Fever: An Obscure Disease.' *Journal of the American Medical Association* 122 (6): 361–65. https://doi.org/10.1001/jama.1943.02840230013004.

Darelid, J., S. Löfgren, and B. -E. Malmvall. 2002. 'Control of Nosocomial Legionnaires' Disease by Keeping the Circulating Hot Water Temperature above 55°C: Experience from a 10-Year Surveillance Programme in a District General Hospital'. *Journal of Hospital Infection* 50 (3): 213–19. https://doi.org/10.1053/jhin.2002.1185.

David, Sophia, Christophe Rusniok, Massimo Mentasti, Laura Gomez-Valero, Simon R. Harris, Pierre Lechat, John Lees, et al. 2016. 'Multiple Major Disease-Associated Clones of Legionella Pneumophila Have Emerged Recently and Independently'. *Genome Research* 26 (11): 1555–64. https://doi.org/10.1101/gr.209536.116.

Declerck, P., J. Behets, Y. Delaedt, A. Margineanu, E. Lammertyn, and F. Ollevier. 2005. 'Impact of Non-Legionella Bacteria on the Uptake and Intracellular Replication of Legionella Pneumophila in Acanthamoeba Castellanii and Naegleria Lovaniensis'. *Microbial Ecology* 50 (4): 536–49. https://doi.org/10.1007/s00248-005-0258-0.

Dehner, George. 2018. 'Legionnaires' Disease: Building a Better World for You'. *Environmental History* 23 (3): 522–44. https://doi.org/10.1093/envhis/emy046.

Den Boer, J. W., and E. P. F. Yzerman. 2004. 'Diagnosis of Legionella Infection in Legionnaires' Disease'. *European Journal of Clinical Microbiology and Infectious Diseases* 23 (12): 871–78. https://doi.org/10.1007/s10096-004-1248-8.

Dey, Rafik, Laurent Cavalié, Christine Vernet, Jacques Bodennec, and Pierre Pernin. 2008. 'The Free-Living Amoeba Willaertia Magna, Is Particularly Resistant to Infection by the Pathogenic Bacteria Legionella Pneumophila'. https://hal.archives-ouvertes.fr/hal-00121343.

Diederen, B. M. W. 2008. 'Legionella Spp. and Legionnaires' Disease'. *Journal of Infection* 56 (1): 1–12. https://doi.org/10.1016/j.jinf.2007.09.010.

Dumoff, Morris. 1979. 'Direct In-Vitro Isolation of the Legionnaires' Disease Bacterium in Two Fatal Cases'. *Annals of Internal Medicine* 90 (4): 694–96. https://doi.org/10.7326/0003-4819-90-4-694.

Dunne, W. Michael, Nathalie Picot, and Alex van Belkum. 2017. 'Laboratory Tests for Legionnaire's Disease'. *Infectious Disease Clinics of North America* 31 (1): 167–78. https://doi.org/10.1016/j.idc.2016.10.012.

Edelstein, P H, and S M Finegold. 1979. 'Use of a Semiselective Medium to Culture Legionella Pneumophila from Contaminated Lung Specimens'. *Journal of Clinical Microbiology* 10 (2): 141–43. https://doi.org/10.1128/jcm.10.2.141-143.1979.

Edelstein, Paul H. 2006. 'Clinical Features of Legionnaires' Disease: A Selective Review'. In *Legionella*, edited by Nicholas P. Cianciotto, Yousef Abu Kwaik, Paul H. Edelstein, Barry S. Fields, David F. Geary, Timothy G. Harrison, Carol A. Joseph, Rodney M. Ratcliff, Janet E. Stout, and Michele S. Swanson, 1–7. Washington, DC, USA: ASM Press. https://doi.org/10.1128/9781555815660.ch01.

Ensminger, Alexander W., Yosuf Yassin, Alexander Miron, and Ralph R. Isberg. 2012. 'Experimental Evolution of Legionella Pneumophila in Mouse Macrophages Leads to Strains with Altered Determinants of Environmental Survival'. Edited by Craig R. Roy. *PLoS Pathogens* 8 (5): e1002731. https://doi.org/10.1371/journal.ppat.1002731.

Falcó, Vicente, Tomás Fernández de Sevilla, José Alegre, Adelaida Ferrer, and José Manuel Martínez Vázquez. 1991. 'Legionella Pneumophila: A Cause of Severe Community-Acquired Pneumonia'. *Chest* 100 (4): 1007–11. https://doi.org/10.1378/chest.100.4.1007.

Falkinham, Joseph O., Elizabeth D. Hilborn, Matthew J. Arduino, Amy Pruden, and Marc A. Edwards. 2015. 'Epidemiology and Ecology of Opportunistic Premise Plumbing Pathogens: *Legionella Pneumophila* , *Mycobacterium Avium* , and *Pseudomonas Aeruginosa*'. *Environmental Health Perspectives* 123 (8): 749–58. https://doi.org/10.1289/ehp.1408692.

Feeley, J C, R J Gibson, G W Gorman, N C Langford, J K Rasheed, D C Mackel, and W B Baine. 1979. 'Charcoal-Yeast Extract Agar: Primary Isolation Medium for Legionella Pneumophila'. *Journal of Clinical Microbiology* 10 (4): 437–41. https://doi.org/10.1128/jcm.10.4.437-441.1979.

Feldheim, Yaron S., Tal Zusman, Anya Kapach, and Gil Segal. 2018. 'The Single-Domain Response Regulator LerC Functions as a Connector Protein in the *Legionella Pneumophila* Effectors Regulatory Network: The Legionella LerC Connector Protein'. *Molecular Microbiology* 110 (5): 741–60. https://doi.org/10.1111/mmi.14101.

Feldheim, Yaron S., Tal Zusman, Yariv Speiser, and Gil Segal. 2016. 'The *Legionella Pneumophila* CpxRA Two-Component Regulatory System: New Insights into CpxR's Function as a Dual Regulator and Its Connection to the Effectors Regulatory Network: The *Legionella* CpxRA Two-Component System'. *Molecular Microbiology* 99 (6): 1059–79. https://doi.org/10.1111/mmi.13290.

Fields, Barry S., Robert F. Benson, and Richard E. Besser. 2002. 'Legionella and Legionnaires' Disease: 25 Years of Investigation'. *Clinical Microbiology Reviews* 15 (3): 506–26. https://doi.org/10.1128/CMR.15.3.506-526.2002.

Fraser, David W., Theodore R. Tsai, Walter Orenstein, William E. Parkin, H. James Beecham, Robert G. Sharrar, John Harris, et al. 1977. 'Legionnaires' Disease: Description of an Epidemic of Pneumonia'. *New England Journal of Medicine* 297 (22): 1189–97. https://doi.org/10.1056/NEJM197712012972201.

Fus-Kujawa, Agnieszka, Pawel Prus, Karolina Bajdak-Rusinek, Paulina Teper, Katarzyna Gawron, Agnieszka Kowalczuk, and Aleksander L. Sieron. 2021. 'An Overview of Methods and Tools for Transfection of Eukaryotic Cells in Vitro'. *Frontiers in Bioengineering and Biotechnology* 9. https://www.frontiersin.org/articles/10.3389/fbioe.2021.701031.

Gam, Jeremy J, Breanna DiAndreth, Ross D Jones, Jin Huh, and Ron Weiss. 2019. 'A "Poly-Transfection" Method for Rapid, One-Pot Characterization and Optimization of Genetic Systems'. *Nucleic Acids Research* 47 (18): e106. https://doi.org/10.1093/nar/gkz623.

García-Fulgueiras, Ana, Carmen Navarro, Daniel Fenoll, José García, Paulino González-Diego, Teresa Jiménez-Buñuales, Miguel Rodriguez, et al. 2003. 'Legionnaires' Disease Outbreak in Murcia, Spain'. *Emerging Infectious Diseases* 9 (8): 915–21. https://doi.org/10.3201/eid0908.030337.

Garduño, Rafael A. 2020. 'Freshwater Ecology of Legionella Pneumophila'. In *Legionellosis Diagnosis and Control in the Genomic Era*. Caister Academic Press. https://doi.org/10.21775/9781913652531.02.

Garrity, G. M., A. Brown, and R. M. Vickers. 1980. ‘Tatlockia and Fluoribacter: Two New Genera of Organisms Resembling Legionella Pneumophila’. *International Journal of Systematic Bacteriology* 30 (4): 609–14. https://doi.org/10.1099/00207713-30-4-609.

Girolamini, Luna, Maria Rosaria Pascale, Silvano Salaris, Marta Mazzotta, Massimiliano Orsini, Antonella Grottola, Nicoletta Zini, and Sandra Cristino. 2022. ‘Legionella Bononiensis Sp. Nov., Isolated from a Hotel Water Distribution System in Northern Italy’. *International Journal of Systematic and Evolutionary Microbiology* 72 (9). https://doi.org/10.1099/ijsem.0.005512.

Glick, Thomas H., Michael B. Gregg, Bernard Berman, George Mallison, Wallace W. Rhodes, and Ira Kassanoff. 1978. ‘Pontiac Fever: An Epidemic of Unknown Etiology in a Health Department: I. Clinical and Epidemiologic Aspects’. *American Journal of Epidemiology* 107 (2): 149–60. https://doi.org/10.1093/oxfordjournals.aje.a112517.

Gomez-Valero, Laura, and Carmen Buchrieser. 2019. ‘Intracellular Parasitism, the Driving Force of Evolution of Legionella Pneumophila and the Genus Legionella’. *Genes & Immunity* 20 (5): 394–402. https://doi.org/10.1038/s41435-019-0074-z.

Gomez-Valero, Laura, Alvaro Chiner-Oms, Iñaki Comas, and Carmen Buchrieser. 2019. ‘Evolutionary Dissection of the Dot/Icm System Based on Comparative Genomics of 58 Legionella Species’. Edited by Ruth Hershberg. *Genome Biology and Evolution* 11 (9): 2619–32. https://doi.org/10.1093/gbe/evz186.

Gomez-Valero, Laura, Christophe Rusniok, Danielle Carson, Sonia Mondino, Ana Elena Pérez-Cobas, Monica Rolando, Shivani Pasricha, et al. 2019. ‘More than 18,000 Effectors in the Legionella Genus Genome Provide Multiple, Independent Combinations for Replication in Human Cells’. *Proceedings of the National Academy of Sciences* 116 (6): 2265–73. https://doi.org/10.1073/pnas.1808016116.

Gomez-Valero, Laura, Christophe Rusniok, Monica Rolando, Mario Neou, Delphine Dervins-Ravault, Jasmin Demirtas, Zoe Rouy, et al. 2014. ‘Comparative Analyses of Legionella Species Identifies Genetic Features of Strains Causing Legionnaires’ Disease’. *Genome Biology* 15 (11): 505. https://doi.org/10.1186/s13059-014-0505-0.

Graells, Tiscar, Helena Ishak, Madeleine Larsson, and Lionel Guy. 2018. ‘The All-Intracellular Order Legionellales Is Unexpectedly Diverse, Globally Distributed and Lowly Abundant’. *FEMS Microbiology Ecology* 94 (12). https://doi.org/10.1093/femsec/fiy185.

Hébert, G. Ann, Arnold G. Steigerwalt, and Don J. Brenner. 1980. ‘Legionella Micdadei Species Nova: Classification of a Third Species OfLegionella Associated with Human Pneumonia’. *Current Microbiology* 3 (5): 255–57. https://doi.org/10.1007/BF02601800.

Hu, Qianghua, and Henry R Henney. 1997. ‘An Acanthamoeba Polyubiquitin Gene and Application of Its Promoter to the Establishment of a Transient Transfection System’. *Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression* 1351 (1): 126–36. https://doi.org/10.1016/S0167-4781(96)00185-6.

Kay, Elisabeth, Virginie Lelogeais, Sophie Jarraud, and Christophe Gilbert and Patricia Doublet. 2020. ‘A “Secreted Army” for the Invasion and Survival of Legionella Pneumophila Within Host Cells’. In *Legionellosis Diagnosis and Control in the Genomic Era*. Caister Academic Press. https://doi.org/10.21775/9781913652531.03.

Khodr, A, E Kay, L Gomez-Valero, C Ginevra, P Doublet, C Buchrieser, and S Jarraud. 2016. 'Molecular Epidemiology, Phylogeny and Evolution of Legionella'. *MEEGID* 43: 108–22. https://doi.org/10.1016/j.meegid.2016.04.033.

Kong, Hyun-Hee, and Thomas D Pollard. 2002. 'Intracellular Localization and Dynamics of Myosin-II and Myosin-IC in Live Acanthamoeba by Transient Transfection of EGFP Fusion Proteins.' *Journal of Cell Science* 115 (Pt 24): 4993–5002. https://doi.org/10.1242/JCS.00159.

Koubar, Mohamad, Marie-Hélène Rodier, and Jacques Frère. 2013. 'Involvement of Minerals in Adherence of Legionella Pneumophila to Surfaces'. *Current Microbiology* 66 (5): 437–42. https://doi.org/10.1007/s00284-012-0295-0.

Kozak-Muiznieks, Natalia A., Jeffrey W. Mercante, and Brian H. Raphael. 2020. 'Epidemiology of Legionellosis and a Historical Perspective on Legionella Pneumophila Strains for the Genomic Era'. In *Legionellosis Diagnosis and Control in the Genomic Era*. Caister Academic Press. https://doi.org/10.21775/9781913652531.04.

Kusnetsov, Jaana, Eila Iivanainen, Nina Elomaa, Outi Zacheus, and Pertti J. Martikainen. 2001. 'Copper and Silver Ions More Effective against Legionellae than against Mycobacteria in a Hospital Warm Water System'. *Water Research* 35 (17): 4217–25. https://doi.org/10.1016/S0043-1354(01)00124-5.

Lau, H. Y., and N. J. Ashbolt. 2009. 'The Role of Biofilms and Protozoa in Legionella Pathogenesis: Implications for Drinking Water'. *Journal of Applied Microbiology* 107 (2): 368–78. https://doi.org/10.1111/j.1365-2672.2009.04208.x.

Lee, Yu-Ran, Byoung-Kuk Na, Eun-Kyung Moon, Su-Min Song, So-Young Joo, Hyun-Hee Kong, Youn-Kyoung Goo, Dong-Il Chung, and Yeonchul Hong. 2015. 'Essential Role for an M17 Leucine Aminopeptidase in Encystation of Acanthamoeba Castellanii'. *PLOS ONE* 10 (6): e0129884. https://doi.org/10.1371/journal.pone.0129884.

Leitsch, David, Alvie Loufouma Mbouaka, Martina Köhsler, Norbert Müller, and Julia Walochnik. 2021. 'An Unusual Thioredoxin System in the Facultative Parasite Acanthamoeba Castellanii'. *Cellular and Molecular Life Sciences* 78 (7): 3673–89. https://doi.org/10.1007/s00018-021-03786-x.

Lesnik, René, Ingrid Brettar, and Manfred G Höfle. 2016. 'Legionella Species Diversity and Dynamics from Surface Reservoir to Tap Water: From Cold Adaptation to Thermophily'. *The ISME Journal* 10 (5): 1064–80. https://doi.org/10.1038/ismej.2015.199.

Linsky, Marika, and Gil Segal. 2021. 'A Horizontally Acquired Legionella Genomic Island Encoding a LuxR Type Regulator and Effector Proteins Displays Variation in Gene Content and Regulation'. *Molecular Microbiology* 116 (3): 766–82. https://doi.org/10.1111/mmi.14770.

Lück, Paul Christian, Jürgen H. Helbig, Heike von Baum, and Reinhard Marre. 2006. 'Diagnostics and Clinical Disease Treatment: Usefulness of Microbiological Diagnostic Methods for Detection of *Legionella* Infections'. In *Legionella*, edited by Nicholas P. Cianciotto, Yousef Abu Kwaik, Paul H. Edelstein, Barry S. Fields, David F. Geary, Timothy G. Harrison, Carol A. Joseph, Rodney M. Ratcliff, Janet E. Stout, and Michele S. Swanson, 15–21. Washington, DC, USA: ASM Press. https://doi.org/10.1128/9781555815660.ch03.

Marre, R, A A Medeiros, and A W Pasculle. 1982. 'Characterization of the Beta-Lactamases of Six Species of Legionella'. *Journal of Bacteriology* 151 (1): 216–21. https://doi.org/10.1128/jb.151.1.216-221.1982.

McDade, Joseph E., Don J. Brenner, and F. Marilyn Bozeman. 1979. 'Legionnaires' Disease Bacterium Isolated in 1947'. *Annals of Internal Medicine* 90 (4): 659–61. https://doi.org/10.7326/0003-4819-90-4-659.

McDade, Joseph E., Charles C. Shepard, David W. Fraser, Theodore R. Tsai, Martha A. Redus, Walter R. Dowdle, and the Laboratory Investigation Team*. 1977. 'Legionnaires' Disease: Isolation of a Bacterium and Demonstration of Its Role in Other Respiratory Disease'. *New England Journal of Medicine* 297 (22): 1197–1203. https://doi.org/10.1056/NEJM197712012972202.

Mercante, Jeffrey W., and Jonas M. Winchell. 2015. 'Current and Emerging Legionella Diagnostics for Laboratory and Outbreak Investigations'. *Clinical Microbiology Reviews* 28 (1): 95–133. https://doi.org/10.1128/CMR.00029-14.

Molmeret, MaÃ¯Â¿Â½lle, Matthias Horn, Michael Wagner, Marina Santic, and Yousef Abu Kwaik. 2005. 'Amoebae as Training Grounds for Intracellular Bacterial Pathogens'. *Applied and Environmental Microbiology* 71 (1): 20–28. https://doi.org/10.1128/AEM.71.1.20-28.2005.

Molofsky, Ari B., and Michele S. Swanson. 2004. 'Differentiate to Thrive: Lessons from the Legionella Pneumophila Life Cycle'. *Molecular Microbiology* 53 (1): 29–40. https://doi.org/10.1111/j.1365-2958.2004.04129.x.

Mondino, Sonia, Silke Schmidt, Monica Rolando, Pedro Escoll, Laura Gomez-Valero, and Carmen Buchrieser. 2020. 'Legionnaires' Disease: State of the Art Knowledge of Pathogenesis Mechanisms of *Legionella*'. *Annual Review of Pathology: Mechanisms of Disease* 15 (1): 439–66. https://doi.org/10.1146/annurev-pathmechdis-012419-032742.

Moon, Eun-Kyung, Joung-Ok Kim, Ying-Hua Xuan, Young-Sun Yun, Se Won Kang, Yong Seok Lee, Tae-In Ahn, Yeon-Chul Hong, Dong-Il Chung, and Hyun-Hee Kong. 2009. 'Construction of EST Database for Comparative Gene Studies of Acanthamoeba'. *The Korean Journal of Parasitology* 47 (2): 103–7. https://doi.org/10.3347/kjp.2009.47.2.103.

Moya, Andrés, Juli Peretó, Rosario Gil, and Amparo Latorre. 2008. 'Learning How to Live Together: Genomic Insights into Prokaryote–Animal Symbioses'. *Nature Reviews Genetics* 9 (3): 218–29. https://doi.org/10.1038/nrg2319.

Muraca, P., J. E. Stout, and V. L. Yu. 1987. 'Comparative Assessment of Chlorine, Heat, Ozone, and UV Light for Killing Legionella Pneumophila within a Model Plumbing System.' *Appl. Environ. Microbiol.* 53 (2): 447–53.

O'Connor, Tamara J., Yewande Adepoju, Dana Boyd, and Ralph R. Isberg. 2011. 'Minimization of the *Legionella Pneumophila* Genome Reveals Chromosomal Regions Involved in Host Range Expansion'. *Proceedings of the National Academy of Sciences* 108 (36): 14733–40. https://doi.org/10.1073/pnas.1111678108.

Oliva, Giulia, Tobias Sahr, and Carmen Buchrieser. 2018. 'The Life Cycle of L. Pneumophila: Cellular Differentiation Is Linked to Virulence and Metabolism'. *Frontiers in Cellular and Infection Microbiology* 8 (January). https://doi.org/10.3389/fcimb.2018.00003.

Pedro-Botet, L., and V. L. Yu. 2006. 'Legionella: Macrolides or Quinolones?' *Clinical Microbiology and Infection* 12 (January): 25–30. https://doi.org/10.1111/j.1469-0691.2006.01394.x.

Peng, Zhihua, Romaica Omaruddin, and Erik Bateman. 2005. 'Stable Transfection of Acanthamoeba Castellanii'. *Biochimica et Biophysica Acta - Molecular Cell Research* 1743 (1–2): 93–100. https://doi.org/10.1016/j.bbamcr.2004.08.014.

Piao, Zhenyu, Chun Chau Sze, Oksana Barysheva, Ken-ichiro Iida, and Shin-ichi Yoshida. 2006. 'Temperature-Regulated Formation of Mycelial Mat-Like Biofilms by Legionella Pneumophila'. *Applied and Environmental Microbiology* 72 (2): 1613–22. https://doi.org/10.1128/AEM.72.2.1613-1622.2006.

Ponti, Federica, Matilde Campolungo, Clara Melchiori, Nina Bono, and Gabriele Candiani. 2021. 'Cationic Lipids for Gene Delivery: Many Players, One Goal'. *Chemistry and Physics of Lipids* 235 (March): 105032. https://doi.org/10.1016/j.chemphyslip.2020.105032.

Price, Christopher T. D., Ashley M. Richards, Juanita E. Von Dwingelo, Hala A. Samara, and Yousef Abu Kwaik. 2014. 'Amoeba Host- *Legionella* Synchronization of Amino Acid Auxotrophy and Its Role in Bacterial Adaptation and Pathogenic Evolution: Amino Acid Auxotrophy and Pathogenic Evolution of *Legionella Pneumophila*'. *Environmental Microbiology* 16 (2): 350–58. https://doi.org/10.1111/1462-2920.12290.

Rayamajhee, Binod, Mark D. P. Willcox, Fiona L. Henriquez, Constantinos Petsoglou, Dinesh Subedi, and Nicole Carnt. 2022. 'Acanthamoeba, an Environmental Phagocyte Enhancing Survival and Transmission of Human Pathogens'. *Trends in Parasitology*, September. https://doi.org/10.1016/j.pt.2022.08.007.

Říhová, Jana, Eva Nováková, Filip Husník, and Václav Hypša. 2017. 'Legionella Becoming a Mutualist: Adaptive Processes Shaping the Genome of Symbiont in the Louse Polyplax Serrata'. *Genome Biology and Evolution* 9 (11): 2946–57. https://doi.org/10.1093/gbe/evx217.

Rolland, Steven, Luce Mengue, Cyril Noël, Stéphanie Crapart, Anne Mercier, Willy Aucher, Yann Héchard, and Ascel Samba-Louaka. 2020. 'Encystment Induces Down-Regulation of an Acetyltransferase-Like Gene in Acanthamoeba Castellanii'. *Pathogens* 9 (5): 321. https://doi.org/10.3390/pathogens9050321.

Rowbotham, T J. 1980. 'Preliminary Report on the Pathogenicity of Legionella Pneumophila for Freshwater and Soil Amoebae.' *Journal of Clinical Pathology* 33 (12): 1179–83. https://doi.org/10.1136/jcp.33.12.1179.

Schroeder, Gunnar N., Nicola K. Petty, Aurélie Mousnier, Clare R. Harding, Adam J. Vogrin, Bryan Wee, Norman K. Fry, et al. 2010. 'Legionella Pneumophila Strain 130b Possesses a Unique Combination of Type IV Secretion Systems and Novel Dot/Icm Secretion System Effector Proteins'. *Journal of Bacteriology* 192 (22): 6001–16. https://doi.org/10.1128/JB.00778-10.

Shadoud, Lubana, Iyad Almahmoud, Sophie Jarraud, Jérôme Etienne, Sylvie Larrat, Carole Schwebel, Jean-François Timsit, Dominique Schneider, and Max Maurin. 2015. 'Hidden Selection of Bacterial Resistance to Fluoroquinolones In Vivo: The Case of Legionella Pneumophila and Humans'. *EBioMedicine* 2 (9): 1179–85. https://doi.org/10.1016/j.ebiom.2015.07.018.

Shands, Kathryn N. 1985. 'Potable Water as a Source of Legionnaires' Disease'. *JAMA: The Journal of the American Medical Association* 253 (10): 1412. https://doi.org/10.1001/jama.1985.03350340064018.

Shimada, Sho, Ryosuke Nakai, Kotaro Aoki, Sakae Kudoh, Satoshi Imura, Norifumi Shimoeda, Giichiro Ohno, et al. 2021. 'Characterization of the First Cultured Psychrotolerant Representative of *Legionella* from Antarctica Reveals Its Unique Genome Structure'. Edited by Adriana Lopes dos Santos. *Microbiology Spectrum* 9 (2): e00424-21. https://doi.org/10.1128/Spectrum.00424-21.

Shivaji, T, C Sousa Pinto, A San-Bento, L A Oliveira Serra, J Valente, J Machado, T Marques, et al. 2014. 'A Large Community Outbreak of Legionnaires' Disease in Vila Franca de Xira, Portugal, October to November 2014'. *Eurosurveillance* 19 (50). https://doi.org/10.2807/1560-7917.ES2014.19.50.20991.

Stout, Janet E., and Victor L. Yu. 1997. 'Legionellosis'. *New England Journal of Medicine* 337 (10): 682–87. https://doi.org/10.1056/NEJM199709043371006.
Surman-Lee, Susanne, and James T. Walker. 2020. 'Regulatory and Risk Management Strategies for Control of Legionella'. In *Legionellosis Diagnosis and Control in the Genomic Era*. Caister Academic Press. https://doi.org/10.21775/9781913652531.08.
Swart, A. Leoni, Christopher F. Harrison, Ludwig Eichinger, Michael Steinert, and Hubert Hilbi. 2018. 'Acanthamoeba and Dictyostelium as Cellular Models for Legionella Infection'. *Frontiers in Cellular and Infection Microbiology* 8 (March). https://doi.org/10.3389/fcimb.2018.00061.
Tan, Loh Teng-Hern, Wei Yu Tee, Tahir Mehmood Khan, Long Chiau Ming, and Vengadesh Letchumanan. 2021. 'Legionella Pneumophila — The Causative Agent of Legionnaires' Disease'. *Progress In Microbes & Molecular Biology* 4 (1). https://doi.org/10.36877/pmmb.a0000193.
Tatlock, Hugh. 1944. 'A Rickettsia-like Organism Recovered from Guinea Pigs.' *Proceedings of the Society for Experimental Biology and Medicine* 57 (1): 95–99. https://doi.org/10.3181/00379727-57-14711.
Thomason, Berenice M. 1979. 'Flagella on Legionnaires' Disease Bacteria: An Interim Report'. *Annals of Internal Medicine* 91 (2): 224. https://doi.org/10.7326/0003-4819-91-2-224.
Thornsberry, Clyde, and L. A. Kirven. 1978. 'β-Lactamase of the Legionnaires' Bacterium'. *Current Microbiology* 1 (1): 51–54. https://doi.org/10.1007/BF02601708.
Time Magazine. 1976. 'Disease Detectives Tracing the Philly Killer', 16 August 1976.
Toft, Christina, and Siv G. E. Andersson. 2010. 'Evolutionary Microbial Genomics: Insights into Bacterial Host Adaptation'. *Nature Reviews Genetics* 11 (7): 465–75. https://doi.org/10.1038/nrg2798.
Wadowsky, R M, R Wolford, A M McNamara, and R B Yee. 1985. 'Effect of Temperature, PH, and Oxygen Level on the Multiplication of Naturally Occurring Legionella Pneumophila in Potable Water'. *Applied and Environmental Microbiology* 49 (5): 1197–1205. https://doi.org/10.1128/aem.49.5.1197-1205.1985.
Warren, William J, and Richard D Miller. 1979. 'Growth of Legionnaires Disease Bacterium (Legionella Pneumophila) in Chemically Defined Medium'. *JOURNAL OF CLINICAL MICROBIOLOGY* 10 (1): 50–55.
Winn, W. C. 1988. 'Legionnaires Disease: Historical Perspective.' *Clinical Microbiology Reviews* 1 (1): 60–81. https://doi.org/10.1128/CMR.1.1.60.
Woo, Andrew H., Angella Goetz, and Victor L. Yu. 1992. 'Transmission of Legionella by Respiratory Equipment and Aerosol Generating Devices'. *Chest* 102 (5): 1586–90. https://doi.org/10.1378/chest.102.5.1586.
Yin, Jingwen, and Henry R. Henney Jr. 1997. 'Stable Transfection of *Acanthamoeba*'. *Canadian Journal of Microbiology* 43 (3): 239–44. https://doi.org/10.1139/m97-033.

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