ANNUAL REVIEWS

Annual Review of Pathology: Mechanisms of Disease

Legionnaires' Disease: State of the Art Knowledge of Pathogenesis Mechanisms of *Legionella*

Sonia Mondino,[1] Silke Schmidt,[1,2] Monica Rolando,[1] Pedro Escoll,[1] Laura Gomez-Valero,[1] and Carmen Buchrieser[1]

[1]Institut Pasteur, Biologie des Bactéries Intracellulaires, CNRS UMR 3525, 75015 Paris, France; email: smondino@pasteur.fr, silke.schmidt@pasteur.fr, mrolando@pasteur.fr, pedro.escoll-guerrero@pasteur.fr, lgomez@pasteur.fr, cbuch@pasteur.fr

[2]Sorbonne Université, Collège doctoral, 75005 Paris, France

Annu. Rev. Pathol. Mech. Dis. 2020. 15:439–66

First published as a Review in Advance on October 28, 2019

The *Annual Review of Pathology: Mechanisms of Disease* is online at pathol.annualreviews.org

https://doi.org/10.1146/annurev-pathmechdis-012419-032742

Copyright © 2020 by Annual Reviews. All rights reserved

ANNUAL REVIEWS CONNECT

www.annualreviews.org

- Download figures
- Navigate cited references
- Keyword search
- Explore related articles
- Share via email or social media

Keywords

Legionella, Legionnaires' disease, free-living amoeba, eukaryotic-like proteins

Abstract

Legionella species are environmental gram-negative bacteria able to cause a severe form of pneumonia in humans known as Legionnaires' disease. Since the identification of *Legionella pneumophila* in 1977, four decades of research on *Legionella* biology and Legionnaires' disease have brought important insights into the biology of the bacteria and the molecular mechanisms that these intracellular pathogens use to cause disease in humans. Nowadays, *Legionella* species constitute a remarkable model of bacterial adaptation, with a genus genome shaped by their close coevolution with amoebae and an ability to exploit many hosts and signaling pathways through the secretion of a myriad of effector proteins, many of which have a eukaryotic origin. This review aims to discuss current knowledge of *Legionella* infection mechanisms and future research directions to be taken that might answer the many remaining open questions. This research will without a doubt be a terrific scientific journey worth taking.

1. INTRODUCTION

1.1. The History of *Legionella* and Legionnaires' Disease

Legionella species are gram-negative bacteria that were unrecognized until the summer of 1976 when an explosive outbreak of pneumonia in Philadelphia, Pennsylvania, United States, caught the attention of the US Centers for Disease Control and Prevention (CDC) and the media. An unusual respiratory disease affected 221 attendees of the 58th annual convention of the American Legion, and 34 fatal cases were reported (1). Due to the importance of the outbreak and the fact that the causative agent was not known, the CDC employed what at that time was the largest team in its history to identify the source of the infection. In December 1976, Joseph E. McDade and Charles C. Shepard identified a bacterium as the causative agent of Legionnaires' disease. They discovered a new rod-shaped gram-negative bacterium, named *Legionella pneumophila* after the American Legion, and the new genus named *Legionella*, which at that time had only one known species (1–3).

Once the organism was identified, further studies revealed that *Legionella* had been already isolated in 1947, but it was not further characterized at that time (4). It was also shown that *Legionella* were the cause of previously unexplained outbreaks of flu-like disease such as the one that occurred in 1968 in Pontiac, Michigan, a clinical condition subsequently named Pontiac fever (5). Today, the genus *Legionella* comprises more than 65 different species, and our understanding of the biology and pathogenicity of the different members of this genus continues to increase.

1.1.1. Ecology and epidemiology.

Legionella are gram-negative rod-shaped γ-proteobacteria that are ubiquitously found in freshwater environments, as well as in moist soil and composted material (6). *Legionella* were the first bacteria described that multiplied within protozoan hosts, primarily aquatic amoebae, which led to the idea that the capacity of the bacteria to infect protozoa may also allow them to replicate within human lung macrophages (7), a finding that was confirmed later through many different studies (reviewed in Reference 8). Today, it is established that *Legionella* are primarily found in the environment, either associated with their host or as free-living biofilm-associated bacteria (9) (**Figure 1**).

Human infection most commonly occurs as a consequence of inhaling *Legionella*-containing aerosols generated by contaminated manmade water sources, such as showers, hot tubs, plumbing networks, and air-conditioning systems. However, aspiration of contaminated water has been suggested as another route of transmission (10) (**Figure 1**). Although human-to-human transmission was not thought to occur, one case has been reported, suggesting that this form of transmission may exist, but it is rare (11). In general, human infection is incidental and a dead end for the bacteria. Individuals at higher risk for developing Legionnaires' disease are males older than 50 years, smokers, and people with an underlying medical condition such as diabetes, cancer, or immunosuppression; however, anybody can develop Legionnaires' disease (12). Summer and early fall are the most common times of the year for *Legionella* infection to occur.

The burden of Legionnaires' disease in Europe and in the United States is increasing each year, with both regions showing comparable notification rates and similar settings and epidemiology of infections. The increase in reported cases could be due to environmental conditions, such as changes in rainfall, temperature, and climate, that can affect the incidence (13); to the increasing proportion of more susceptible people, such as elderly people and those who are immunocompromised; and partly also to improvements in the surveillance systems in these regions during the past two decades (14).

To put Legionnaires' disease in perspective, from 2011 to 2015, the age-standardized rate of Legionnaires' disease in Europe showed an average annual increase of 0.09 cases per 100,000

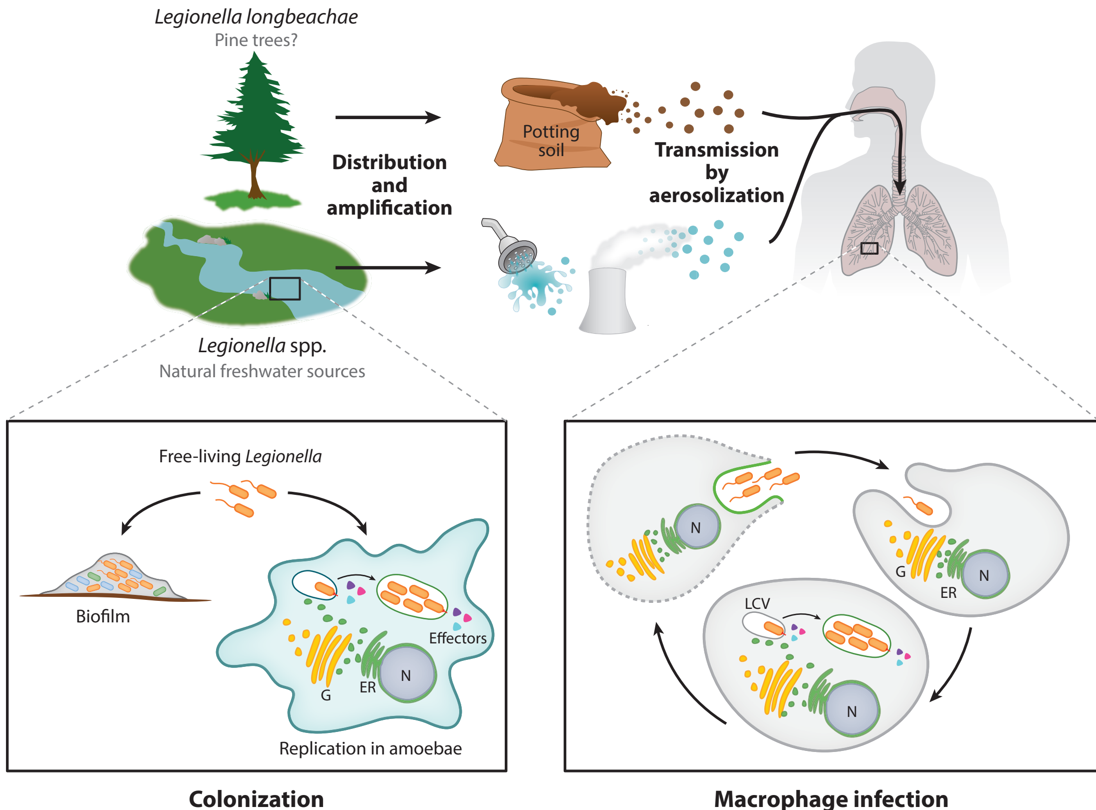

Figure 1

Transmission routes and life cycle of *Legionella pneumophila* and *L. longbeachae*. *Legionella* are commonly found in freshwater environments associated with biofilms or replicating inside amoebae. The development of manmade aquatic environments, such as showers, cooling towers, and fountains, allows for bacterial distribution and amplification in these artificial environments. Subsequent aerosolization from these sources exposes humans to inhalation or aspiration of contaminated water droplets. Through this means, *Legionella* can reach the human lungs, where they can infect alveolar macrophages using the same mechanisms that they utilize to survive within their amoebal hosts. Inside the host cell, *Legionella* reside in a separated compartment, from where they modulate diverse host signaling pathways through the secretion of effector proteins by a dedicated Dot/Icm type 4B secretion system. *L. longbeachae* is found in soil and potting mixes, some of which contain composted pine bark, and, presumably, is also associated with amoebae and biofilm communities. The route of human infection with *L. longbeachae* has not yet been established, but it may involve the inhalation of aerosolized particles generated after the manipulation of contaminated soil-derived products. Abbreviations: ER, endoplasmic reticulum; G, Golgi apparatus; LCV, *Legionella*-containing vacuole; N, nucleus.

individuals, reaching 1.30 cases per 100,000 individuals in 2015. The mortality rate fluctuated between 0.07 and 0.09 deaths per 100,000 individuals, with an overall case–fatality ratio continuously decreasing during the 2011–2015 period. The decreasing case–fatality ratio may be due to improvements in reporting completeness that may be correcting a former bias toward fatal outcomes of the disease (14). During this time, the source of infection was identified for 88% of reported cases. Of these, 70.7% of infections were community acquired; 19.9% were travel associated; and 7.3% were health-care related (14). In addition, a report by the European Centre for Disease Prevention and Control in 2016 showed 1.4 case notifications per 100,000 individuals, the highest ever observed for Europe, with a case–fatality ratio similar to the one observed in 2015 (15). In accordance with reports from Europe, active surveillance in the United States

described an incidence of 1.89 cases of Legionnaires' disease per 100,000 individuals in 2015, with a case–fatality ratio similar to that observed in Europe and with similar epidemiology and sources of infection (16).

To date, the genus *Legionella* comprises 65 species, but, interestingly, not all of them are equally responsible for the laboratory-confirmed cases of Legionnaires' disease worldwide, as *L. pneumophila* accounts for 80–90% of the cases in Europe and the United States (17). Furthermore, even within this species, disease-causing *L. pneumophila* strains are unevenly distributed, as strains of serogroup (Sg) 1 are responsible for approximately 90% of cases. Additionally, within the Sg1 strains, specific clones have recently emerged and already account for more than 50% of the reported cases of Legionnaires' disease in northern Europe, suggesting that these disease-related clones became adapted to manmade aquatic environments (18). *L. longbeachae* accounts for approximately 1% of cases worldwide, but, interestingly, for 50–60% of cases in Australia and New Zealand. However, during the past 10 years, cases caused by *L. longbeachae* infection have also been increasingly reported in Europe (19). Other species and serogroups, such as *L. pneumophila* Sg3 and Sg6, *L. bozemanii*, and *L. micdadei*, may also cause disease in Europe and the United States, but are rare (14, 17, 20).

1.1.2. Detection and treatment.

For *Legionella* infection, the time to detection remains critical for the final disease outcome, especially for at-risk populations. A patient with community-acquired Legionnaires' disease generally shows pneumonic as well as extrapulmonary findings, such as gastrointestinal and neurological symptoms, relative bradycardia, hypophosphatemia, or increased serum ferritin levels (21), or some combination of these. In addition to the clinical symptoms, laboratory confirmation is essential for diagnosis; thus, specific detection methods have been developed for assessing *Legionella* infection using sputum or respiratory secretions; tissue, blood, or serum samples; or urine samples (22). These methods include serological and antibody-based assays, bacterial culture, urinary antigen tests, and nucleic acid amplification testing (for detailed reviews see References 22, 23).

Initially, serology was the method of choice to assess infections with *Legionella*, but the use of this technique has dropped significantly because of the development of more user-friendly and rapid methods, such as the urinary antigen test and polymerase chain reaction (PCR)–based detection methods. However, serology remains relevant for retrospective epidemiological investigations and when the infectious agent cannot be isolated despite strong evidence of Legionnaires' disease (22). The urinary antigen test, which detects a component of the *Legionella* cell wall in urine samples, is now widely used as a first-line screening method because it is easy and low cost, and results are rapidly available. However, as it allows only for the detection of *L. pneumophila* Sg1, there is still a need to develop assays that identify different serogroups and *Legionella* species (22, 24). Recently, PCR-based methods, such as the ones developed to detect *L. pneumophila* Sg1 (25) or the emerging *L. pneumophila* ST47 clone (26), have become more commonly used in reference centers, but with the exception of New Zealand, they are still used only rarely for clinical diagnosis (27). The utility of PCR-based assays to complement other diagnostic methods has also been demonstrated by the development of a rapid and reliable multiplexed real-time PCR assay that allows for the detection of four clinically relevant non-*pneumophila* species from mock human sputum specimens (28, 29). Nevertheless, culture on defined growth medium remains the standard reference method for *Legionella* diagnosis and identification, as it allows for identification of different *Legionella* species and serogroups, and subsequent epidemiological studies of their distribution (23).

Fortunately, antibiotic resistance is not yet a problem for *L. pneumophila* infections. To date, one fluoroquinolone (ciprofloxacin)-resistant *L. pneumophila* strain has been isolated from a patient

with Legionnaires' disease in the Netherlands (30), and the in vivo selection of fluoroquinolone resistance mutations in *L. pneumophila* was reported in two infected patients treated with these antibiotics in France (31), suggesting that, overall, antibiotic resistance is rare. Nevertheless, the incidence of fluoroquinolone resistance might be underestimated, supporting the need for prompt identification of *Legionella* infection to ensure the rapid and accurate administration of antibiotic therapy (32). Related to this, a digital PCR assay used to detect fluoroquinolone-resistant mutants of *Legionella* in patients' samples has proven useful as a diagnostic tool to assess the effectiveness of antibiotic therapy (32). Given the rare instances of resistance reported, the recommended antimicrobial therapy still includes fluoroquinolones (ciprofloxacin, levofloxacin, or moxifloxacin) or macrolides (azithromycin) (33).

1.2. *Legionella longbeachae*: Similar but Different

L. longbeachae is a major cause of disease only in Australia and New Zealand (20). However, during the past decade, infections with this bacterium have also been increasingly reported from Europe (19, 34, 35), the United States (36), Canada (37), Thailand (38), and Taiwan (39), a phenomenon that might correlate with increased clinical awareness and the wider use of improved detection methods. A total of 15 serogroups are recognized for *L. pneumophila*, but only 2 are recognized for *L. longbeachae*, with Sg1 being responsible for the majority of reported cases. A comparison of the clinical features and outcomes of disease caused by *L. pneumophila* and *L. longbeachae* showed that both species cause a similar disease pattern, and similar risk factors apply, such as older age, being a smoker, and having immunosuppression or other preexisting medical conditions. However, the main seasons for disease caused by *L. longbeachae* are spring and summer, whereas *L. pneumophila* legionellosis occurs more frequently in late summer and early fall (40).

Legionella species are ubiquitously found in aquatic environments; however, *L. longbeachae* is found in moist soil and potting mixes, presumably also associated with protozoa. Thus, gardening and using potting soil are unique risk factors associated with *L. longbeachae* infections (41). This characteristic might partly explain the differences in the seasons of onset, as gardening activities usually occur more frequently in spring and summer. The route of transmission to humans is still not completely understood, but it may be that infection occurs through the inhalation of aerosolized, contaminated compost particles that are formed when the bags are opened, when the potting mix is handled, or when plants are watered (20, 41) (**Figure 1**). Yet the report of a recent outbreak suggested that waterborne transmission of *L. longbeachae* may also occur, as the bacterium was detected both in the water of a cooling tower and as cause of human infection. However, due to the lack of clinical isolates, the cooling tower could not be confirmed as the source of this infection (42).

L. pneumophila has a pronounced, so-called biphasic life cycle during which it switches between a replicative (avirulent) and a transmissive (virulent) form (43). This differentiation, in which metabolic as well as morphogenetic changes take place, occurs during the transition between intracellular and extracellular environments, and it is accompanied by a specific switch in the gene expression pattern (44). In a simple model, when conditions are favorable for replication (in a nutrient-rich environment), *L. pneumophila* represses the expression of the transmission traits (motility, osmotic- and acid-resistance, cytotoxicity) and expresses the genes necessary to replicate and multiply intracellularly and to use the resources available from the host. Conversely, when the bacteria density increases and nutrients become limited, *L. pneumophila* stops replicating, while inducing the coordinated expression of the transmission traits (45). Thus, the bacteria escape from the cell and spread to new hosts to resume the cycle. During bacterial growth in liquid medium, the replicative and transmissive phases are represented by, respectively, the exponential and

stationary growth phases (43, 46). As a consequence of this biphasic life cycle, the infection of a host cell and survival of *L. pneumophila* inside the cell depend on its metabolic state (47). A key regulator of the switch between these two phases is carbon storage regulator A (CsrA), an RNA-binding protein that is a global repressor of the transmission genes during the replicative phase (47, 48). Its repressive function is relieved under starvation conditions, as limited amino acid availability signals the production of the alarmone guanosine pentaphosphate [(p)ppGpp], which leads to the activation of the two-component system *Legionella* transmission activator and sensor (LetA/LetS) and the alternative sigma factor RNA polymerase sigma factor (RpoS). These regulators activate transcription of the small noncoding RNAs RsmX, -Y, and -Z that sequester CsrA, thereby releasing the repression of the transmissive traits (49, 50). A genome-wide analysis of CsrA targets provided evidence that this protein impacts the central carbon metabolism, motility, and infective capacity of *L. pneumophila* by controlling the expression of at least 40 Dot/Icm type 4B secretion system (T4SS) effector proteins (51). Comparable to *L. pneumophila*, *L. longbeachae* encodes the LetA/LetS two-component system and a CsrA protein that shows 98% amino acid similarity with the *L. pneumophila* CsrA; however, transcriptome analyses have shown that this species does not undergo as dramatic a switch between the two phases as does *L. pneumophila* (50, 52). These findings are in line with the observation that the infective capacity of *L. longbeachae* seems to be independent of its growth phase (53).

Further differences between *L. pneumophila* and *L. longbeachae* were identified when the genome sequence of *L. longbeachae* was analyzed (52, 54). Particularly interesting was the presence of a largely different T4SS effector repertoire, as only about 30% of the effectors present in *L. pneumophila* were also present in *L. longbeachae* (52, 55). Also, while *L. pneumophila* is non-encapsulated and flagellated, *L. longbeachae* encodes for a capsule but not for flagella (52). Actually, the presence of cytosolic flagellin leads to clearance of *L. pneumophila* from mouse macrophages due to the activation of the Naip5–Nlrc4 inflammasome and subsequent cell death by pyroptosis (56). Mice are more susceptible to *L. longbeachae* infection, even when compared with an *L. pneumophila* mutant lacking flagella, suggesting that the high lethality and the poor stimulatory activity of *L. longbeachae* could also be a consequence of the presence of a capsule as well as the different reservoir of effectors (52, 57). Overall, clear phenotypic differences are evident between these two species, and yet little is known about *L. longbeachae*'s biology and infection processes.

2. *LEGIONELLA*: AN ARMY WITH A LARGE ARSENAL OF WEAPONS

Legionella are able to replicate in a wide variety of phagocytic hosts, ranging from numerous amoeba species to mammalian cells (8), in which they form a distinct membrane-bound replicative niche known as the *Legionella*-containing vacuole (LCV) (**Figure 1**). This sophisticated intracellular compartment allows the bacteria to evade phagolysosomal degradation as well as to shelter from intracellular defenses and to intercept nutrients to support replication. In order to do these things, *Legionella* employ different secretion systems that deliver virulence-associated proteins across one or two cell membranes to the site of action. While the type 2 secretion system (T2SS) and T4SS are encoded by all *Legionella* strains, the type 1 secretion system (T1SS) is restricted to *L. pneumophila*, and the type 4A secretion system (Lvh type) is randomly distributed among different species (58, 59). The T2SS and T4SS have been extensively studied in *L. pneumophila* as they play essential roles during infection.

The delivery of effector proteins via T2SS is a two-step process in which proteins are first transported into the periplasm, where they are recognized by the T2SS apparatus, and then exit through a dedicated pore (60). Subsequently, T2SS effectors may be found associated with the LCV membrane after they escape into the host cytosol (61). This system translocates more than 25 effector proteins (62) that play major roles in intracellular replication in amoebae and also in

L. pneumophila pathogenesis in humans (63). One example is a chitinase that is secreted by T2SS that promotes bacterial persistence in the lungs (64).

The Dot/Icm T4SS is critical for LCV biogenesis and intracellular replication (65, 66). Recently, it was shown that the *L. pneumophila* T4SS is located at the bacterial cell poles, and effector delivery is triggered by phagocytosis (67, 68). Importantly, T4SS governs all steps of the intracellular life of *L. pneumophila* by secreting more than 330 effector proteins that target fundamental cellular processes conserved between protozoa and mammals (**Table 1**) (**Figure 2**).

2.1. *Legionella* Successfully Escape Host Cell Degradation

After bacterial uptake, *L. pneumophila* avoids endocytic maturation and phagolysosomal degradation. Instead, the bacterium modulates specific host cell signaling pathways through the secretion of a myriad of T4SS effector proteins, allowing for the formation of a safe niche where *Legionella* can efficiently replicate. During the past two decades, several of these effector proteins have been characterized functionally, leading to a better understanding of the mechanisms employed by *L. pneumophila* to subvert host cell functions. Among these mechanisms, several novel posttranslational modifications of host proteins induced by a bacterial pathogen were reported in *Legionella* for the first time.

2.1.1. *Legionella pneumophila* uptake and evasion of the endocytic maturation pathway. Although the Dot/Icm T4SS seems to promote bacterial uptake into phagocytic cells (69, 70), the entry mechanism itself depends on the host cell machinery. *L. pneumophila* is engulfed by host cells through a phagocytic and macropinocytic phosphatidylinositol (3,4,5) trisphosphate [PtdIns(3,4,5)P_3]-rich cup (71). Furthermore, it has been shown that a functional T1SS is also required for entry into the host cell (72). Shortly after internalization, phagosomes containing *L. pneumophila* evade endocytic maturation and prevent fusion with lysosomes (73). *L. pneumophila* prevents vacuolar acidification by blocking the host vacuolar ATPase (v-ATPase), a proton pump present throughout the membranes of the endocytic pathway. This process is driven by two secreted effectors: SidK and WipB. SidK binds the v-ATPase regulatory subunit VatA, resulting in the inhibition of ATP hydrolysis and proton translocation (74). WipB is a lysosome-targeted phosphatase that localizes to acidified LAMP1-positive lysosomal compartments where it interacts with the v-ATPase. SidK and WipB may converge to repress the activity of the host v-ATPase (75).

Phagosome maturation is tightly regulated by T4SS, as the Dot/Icm system appears to redirect vacuoles containing *L. pneumophila* away from the canonical endocytic pathway at an extremely early stage of infection. Small GTPases of the Rab family represent an important group of proteins involved in phagosome maturation, and the binding of specific Rab proteins to intracellular organelles enables specific targeting. Proteomic analyses of purified LCVs revealed the presence of several small GTPases anchored to the pathogen vacuole: Rab5, Rab7, Rab14, and Rab21 (76, 77). The GTPase Rab5 is an important regulator of the early endocytic pathway: GTP-bound Rab5 orchestrates the recruitment of several downstream ligands, resulting in PtdIns(3)P-mediated recruitment of early endosomal antigen 1 (EEA1) (78). Interestingly, a secreted effector named VipD has been shown to exhibit phospholipase A1 activity that is activated only upon binding to endosomal Rab5. VipD thus localizes to endosomes and can catalyze the removal of PtdIns(3)P from endosomal membranes. Consequently, EEA1 and other transport and fusion factors are depleted from endosomes, rendering them fusion incompetent (79). PieE, another secreted effector, has been shown to bind both Rab5 and Rab7, but its specific function remains unknown (80). Rab7 is a small GTPase protein that has a crucial role during phagosome maturation, as it gradually replaces Rab5 to induce the fusion between the degradative late endosomes and lysosomes (81). Rab5 and Rab7 also play roles in regulating

Table 1 Selected secreted effectors of *Legionella* with functions discussed in the review

Effector[a]	Gene[b]		Cellular target and function	Reference
	Paris strain	Philadelphia strain		
Bacterial uptake and evasion from the endocytic maturation pathway				
SidK	*lpp1030*	*lpg0968*	Blocks the host vacuolar ATPase to restrain vacuolar acidification	74
WipB	*lpp2775*	*lpg2718*		75
VipD	*lpp2888*	*lpg2831*	Depletes fusion factors from the endosomal membrane	79
PieE	*lpp1953*	*lpg1969*	Binds Rab5 and Rab7	80
RidL	*lpp2259*	*lpg2311*	Impairs retrograde trafficking	83
Bacterial interaction with the ER and LCV formation				
SidM (DrrA)		*lpg2464*	Binds the membrane	194
			Recruits Rab1 to the LCV	87, 88
			AMPylates Rab1	89, 90
SidD		*lpg2465*	DeAMPylates Rab1	91
LepB	*lpp2555*	*lpg2490*	Converts Rab1 GTP into Rab1 GDP	92
AnkX	*lpp0750*	*lpg0695*	Attaches a phosphocholine moiety to Rab1	93
Lem3	*lpp0751*	*lpg0696*	Removes phosphocholination	94
LidA	*lpp1002*	*lpg0940*	Enables the tethering of ER-derived vesicles	90
RalF	*lpp1932*	*lpg1950*	Recruits Arf1 to the LCV membrane	95
Ceg9	*lpp0316*	*lpg0246*	Interacts with Rtn4	97
LseA	Corby strain LPC_2110		Mediates membrane fusion	101
LegC3	*lpp1666*	*lpg1701*	Modulates membrane fusion events	102, 103
LegG1 (MitF)		*lpg1976*	Activates Ran GTPase; implicated in mitochondrial fragmentation	104, 105
Establishing a safe niche: hijacking the host cell response				
Autophagic response				
RavZ		*lpg1683*	Irreversibly deconjugates LC3	108
	lpp1139	*lpg1137*	Cleaves syntaxin 17	109
*Lp*SPL	*lpp2128*	*lpg2176*	Prevents autophagosome formation	110
Kinase signaling				
LeSHs	11 different effectors		Bind to phosphorylated Tyr	111
LegK7	*lpp1899*	*lpg1924*	Targets the Hippo pathway	112
LegK1	*lpp1439*	*lpg1483*	Activates NF-κB	114
LnaB	*lpp2592*	*lpg2527*	Activates NF-κB	115
MavC	*lpp2086*	*lpg2147*	Dampens NF-κB signaling	116
Lgt1	*lpp1322*	*lpg1368*	Decrease production of IκB, an inhibitor of the NF-κB pathway	117
Lgt2		*lpg2862*		
Lgt3	*lpp1444*	*lpg1488*		
SidI	*lpp2572*	*lpg2504*		
SidL	*lpp0504*	*lpg0437*		
Ceg4	*lpp0110*	*lpg0096*	Impacts MAPK signaling	121
Epigenetic regulation				
RomA	*lpp1683*	*lpg1718*	Changes histone marks	122
mRNA processing				
SnpL	*lpp2587*	*lpg2519*	Regulates mRNA processing	123

(*Continued*)

Table 1 (*Continued*)

Effector[a]	Gene[b] Paris strain	Gene[b] Philadelphia strain	Cellular target and function	Reference
Ubiquitin pathway				
LubX	*lpp2887*	*lpg2830*	E3 ligase; targets Clk1	124
GobX	*lpp2521*	*lpg2455*	E3 ligase; locates to Golgi membranes	125
RavN	*lpp1112*	*lpg1111*	E3 ligase	126
SidC	*lpp2579*	*lpg2511*	E3 ligase; phagosomal remodeling	128
LegU1	*lpp0233*	*lpg0171*	F-box domain	129
LicA	*lpp1363*	*lpg1408*		
AnkB	*lpp2082*	*lpg2144*	F-box domain; ubiquitinates ParvB and supplies nutrients to the vacuole	129–131
SidE	*lpp0304*	*lpg0234*	Ubiquitinate ER-associated Rab GTPases and target Rtn4 to control tubular ER dynamics	132, 133, 135
SdeA	*lpp2096*	*lpg2157*		
SdeB	*lpp2095*	*lpg2156*		
SdeC	*lpp2092*	*lpg2153*		
SidJ	*lpp2094*	*lpg2155*	Reverses SidE family activity	134
LotA (Lem 21)	*lpp2202*	*lpg2248*	Cleaves ubiquitin from the LCV	136
Modulation of cell death				
SidF	*lpp2637*	*lpg2584*	Antagonizes proapoptotic Bcl-rambo	138
SdhA	*lpp0443*	*lpg0376*	Prevents cell death	139
	lpp0782	*lpg0716*	Induce proapoptotic caspase-3 activity	140
Ceg18	*lpp0959*	*lpg0898*		
Lem12	*lpp1595*	*lpg1625*		
LegS2	*lpp2128*	*lpg2176*		
VipD	*lpp2888*	*lpg2831*		

Abbreviations: ER, endoplasmic reticulum; LCV, *Legionella*-containing vacuole; MAPK, mitogen-activated protein kinase.
[a]Empty cells in the Effector column indicate that no specific name was given to the effector other than the gene name.
[b]Empty cells in the Gene column indicate that there is no orthologous gene in the *Legionella* species.

retrograde trafficking, connecting the endosomal system with the trans-Golgi network (82). *L. pneumophila* affects this trafficking pathway through the secreted effector RidL (83).

2.1.2. *Legionella pneumophila* interaction with the endoplasmic reticulum and formation of *Legionella*-containing vacuoles. Intercepting vesicular traffic from endoplasmic reticulum (ER) exit sites and vesicle budding from the ER appear to be required for the establishment of the replication vacuole (84). In particular, it has been proposed that the LCV is localized in proximity to the ER exit sites, ideally suited to hijack vesicle trafficking from the retrograde secretory pathway on the route to the Golgi compartment (85). The small GTPases Arf1, Sar1, and Rab1 are important molecules that regulate host vesicular and membrane transport processes; during *L. pneumophila* infection they participate in the recruitment of ER-derived vesicles to the LCV membrane. Rab1 recruitment to the LCV is a well-orchestrated T4SS-dependent process that has been extensively studied. Indeed, Rab1 is a direct target of several different secreted effectors. (*a*) SidM (DrrA) is a protein containing three functional domains: a C-terminal domain that binds PtdIns(4)P (86), thereby also representing an LCV marker that accumulates on the membrane of the pathogen compartment; a guanine nucleotide exchange factor and a guanine

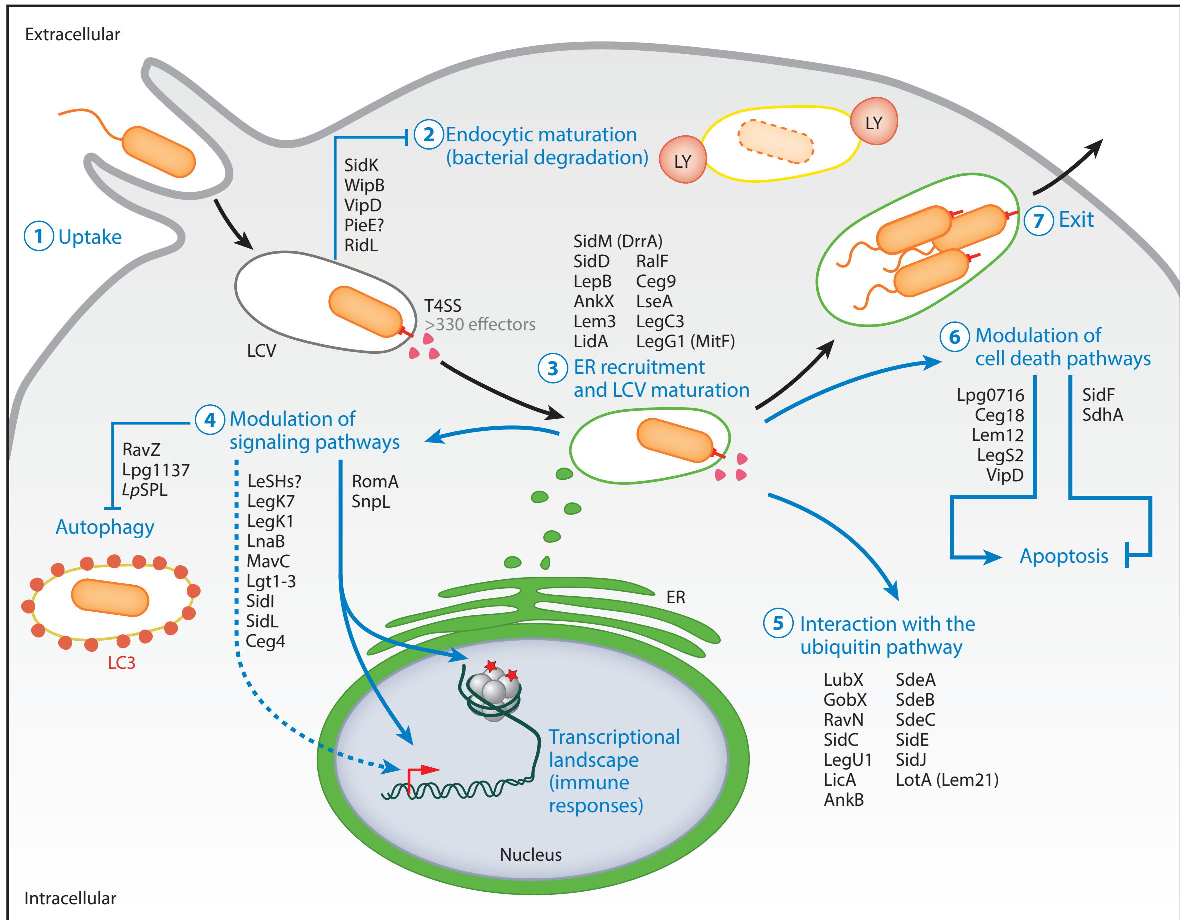

Figure 2

Intracellular pathways regulated by secreted *Legionella pneumophila* effectors: representation of the intracellular cycle of *L. pneumophila* and the effectors secreted by the type 4 secretion system that control the cellular response at each step of the cycle. After bacterial uptake (①), *L. pneumophila* avoids endocytic maturation (②) and instead recruits ER-derived vesicles to the LCV (③), allowing for the formation of a safe niche where (④–⑥) the bacterium replicates and modulates cell signaling pathways. Once the replication cycle is completed, *L. pneumophila* exits the cell (⑦) and infects a new host. Abbreviations: ER, endoplasmic reticulum; LCV, *Legionella*-containing vacuole; LY, lysosome.

nucleotide dissociation inhibitor displacement factor that recruit Rab1 to the LCV (87, 88); and an N-terminal enzymatic domain that catalyzes the addition of adenosine monophosphate to Rab1 (AMPylation) (89, 90). (*b*) SidD has been characterized as a deAMPylase that removes the adenosine monophosphate moiety from Rab1 (91). (*c*) LepB encodes for a GTPase-activating protein that converts Rab1 GTP into Rab1 GDP (92). (*d*) The phosphocholinase AnkX attaches a phosphocholine moiety to Rab1, thus disrupting secretory trafficking (93). (*e*) Lem3 removes this phosphocholination (94). And, finally, (*f*) LidA has Rab1-binding activity and facilitates the tethering of ER-derived vesicles (90). Thus, the recruitment and functional modifications of Rab1 facilitate the recruitment of ER-derived vesicles to the phagosome membrane.

The function of the small GTPases Arf1 and Sar1 is also important for the recruitment and tethering of ER vesicles to the LCV (84). Arf1 has been shown to play a critical role in coat protein

complex (COP) I–mediated retrograde trafficking in eukaryotic cells, whereas Sar1 is involved in intracellular COPII-mediated protein trafficking from the ER to the Golgi apparatus. The secreted effector RalF is a guanine nucleotide exchange factor that directly activates and recruits Arf1 to the LCV membrane (95). Interestingly, it has been suggested that because the bacteria enter at the cellular periphery, where the ER interacts with the plasma membrane (96), the first microbial encounter would be with the tubular peripheral ER. This was confirmed by the observation that the secreted effector Ceg9 directly associates with Rtn4, a protein that regulates ER tubule formation (97).

The LCV fuses with the ER by a noncanonical pairing of the vesicular membrane SNARE protein Sec22b on ER-derived vesicles with a plasma membrane target SNARE complex containing host syntaxins (98). SNAREs are host proteins that directly facilitate membrane fusion events (99). The SidM (DrrA) effector is sufficient to stimulate SNARE-dependent membrane fusion within Rab1 activation (100). Nonetheless, *L. pneumophila* also encodes for a secreted effector, LseA, that acts as a SNARE protein, which is suggested to mediate membrane fusion events in Golgi-associated pathways (101). Additionally, the LegC3 effector has also been referred to as a SNARE-like protein that can form a SNARE-like hybrid complex with VAMP4 and modulate membrane fusion events (102, 103).

The LCV is also able to move along microtubules, thanks to the activity of the secreted effector LegG1 (MitF), which activates Ran GTPase, thus promoting LCV formation, microtubule stabilization, and LCV motility (104). Interestingly, it has been recently shown that the T4SS effector LegG1 (MitF) is also implicated in mitochondrial fragmentation during infection that depends on the host factors DNM1L, Ran, and RanBP2 by a mechanism that, although not yet elucidated, has been suggested to involve WASP–Arp2/3-mediated recruitment of DNM1L to mitochondria. *Legionella*-induced mitochondrial fragmentation leads to a Warburg-like metabolism in the host cell that promotes pathogen replication (105, 106).

2.1.3. *Legionella pneumophila* modulation of host cell signaling pathways. The transformation of the nascent phagosome into a vacuole derived from the ER resembles an immature autophagosome. Indeed, it has been shown that the LCV carries markers associated with autophagosomes (107) and that several T4SS effectors play roles in inhibiting the autophagic response of the host cell to avoid the degradation of the vacuole by the autophagy machinery: (*a*) RavZ interferes with autophagy by irreversibly deconjugating an autophagy-related ubiquitin-like protein, LC3, from phosphatidylethanolamine (108); (*b*) Lpg1137 targets the mitochondria-associated ER membranes (MAMs) and cleaves syntaxin 17, a SNARE implicated in autophagy, via its Ser protease activity, thereby blocking the process (109); (*c*) *Lp*SPL, another MAM-located effector, prevents autophagosome formation by disturbing the host's sphingolipid metabolism (110).

During its intracellular replication cycle, *L. pneumophila* continuously interferes with different host cell signaling pathways to hijack the cellular response. An important role in signal transduction in mammalian cells is played by the tyrosine kinase machinery, and Src homology 2 domains, sequence-specific phosphotyrosine-binding modules, which are key actors required for substrate recruitment and catalytic activity. Interestingly, *L. pneumophila* encodes for Src homology 2 domain proteins that can translocate into host cells and bind phosphotyrosine (111). Furthermore, LegK7, a newly described effector kinase, promotes intracellular bacterial growth by targeting the host cell Hippo pathway (112). LegK7, like the Hippo kinase MST1, directly phosphorylates MOB1, thus triggering a signaling cascade that alters the transcriptional landscape of host cells.

Another preferential target of bacterial pathogens is the nuclear factor kappa–light-chain enhancer of activated B cells (NF-κB) pathway, due to its central role in transcriptional regulation and activation of host innate immune responses. It has been observed that *L. pneumophila* infection

impacts the NF-κB pathway in a differential way, depending on the stage of infection (113). LegK1 and LnaB are secreted *L. pneumophila* effectors that strongly activate the NF-κB transcription factor (114, 115). The activity of MavC—a transglutaminase that catalyzes monoubiquitination of the E2 enzyme UBE2N, thus inhibiting the formation of Lys63 polyubiquitinated chains—dampens NF-κB signaling, probably counteracting the effects of NF-κB activation at the initial phase of infection (116). Conversely, the Lgt family of cytotoxic glucosyltransferases, Lgt1, -2, and -3, together with SidI, and SidL specifically decrease the production of IκB, an inhibitor of NF-κB (117). Thus *L. pneumophila* secretes several different effectors to fine-tune NF-κB signaling to its advantage.

Similar to the NF-κB pathway, the mitogen-activated protein kinase (MAPK) pathway is also a central signaling cascade that is essential for the activation of immune responses. Indeed, *L. pneumophila* activates this pathway in a T4SS-dependent manner (118, 119) by secreting five effectors that inhibit host translation and lead to MAPK activation, thus shaping the transcriptional response of the host cell (120). Another secreted effector, Ceg4, can modulate the phosphorylation state of eukaryotic MAPKs through its haloacid dehalogenase–like phosphatase domain (121).

L. pneumophila is also able to directly modulate the host's transcriptional machinery by modifying histone marks. The T4SS-secreted *L. pneumophila* effector RomA methylates Lys14 of histone H3, a key residue usually acetylated at active promoters, to decrease cellular transcription (122). SnpL, another effector, also targets the host cell nucleus, where it binds the eukaryotic transcription elongation factor SUPT5H, which is involved in regulating RNA polymerase II–dependent mRNA processing and elongation (123).

2.1.4. *Legionella pneumophila* and interactions with the ubiquitin and apoptotic pathways. Ubiquitination is an important posttranslational modification in eukaryotic cells that regulates the activity and cellular localization of proteins and affects essential routes, for example, the immune response. Several T4SS effectors of *L. pneumophila* show similarities to eukaryotic E3 ubiquitin ligases, enzymes that actively participate in protein ubiquitination. LubX and GobX are U-box domain-containing E3 ligases: LubX, structurally similar to the RING E3 ligase domain, directly modifies Cdc2-like kinase 1 (Clk1) (124), whereas the targets of GobX remain to be determined, although its localization to Golgi membranes suggests that it functions at or in close proximity to this compartment (125). RavN encodes an atypical U-box-like motif and possesses E3 ubiquitin ligase activity (126), while SidC, an effector known to enhance ER recruitment to the LCV (127), defines a unique family of E3 ubiquitin ligases. SidC possesses atypical ubiquitin ligase activity as it uses a Cys–His–Asp triad to catalyze the formation of high-molecular-weight polyubiquitin chains through multiple ubiquitin Lys residues (128). LegU1, LegAU13, and LicA are F-box domain-containing proteins, translocated into the cytosol by T4SS, which specifically interact with components of the host ubiquitination machinery. In addition, LegU1 targets and ubiquitinates the host chaperone BAT3, a protein involved in apoptosis and ER stress response (129). AnkB is another F-box-containing secreted effector that interacts with Skp1 to form a Skp–Cullin–F-box complex that ubiquitinates ParvB (130). AnkB has also been suggested to play a role in supplying the replicative vacuole in amino acids through AnkB-dependent degradation of polyubiquitinated proteins that are used by *L. pneumophila* as nutrients (131).

Recently, the members of the SidE effector family (SdeA, SdeB, SdeC, and SidE) were shown to ubiquitinate ER-associated Rab GTPases by a novel ubiquitination mechanism that does not require E1 and E2 enzymes of the host ubiquitination machinery: Ubiquitin is first activated by Arg–ADP ribosylation by the mono-ADP-ribosyltransferase domain of SdeA; the intermediate is then cleaved by the phosphodiesterase domain within the same enzyme; and this occurs concomitantly with the attachment of ubiquitin to Ser residues of substrate proteins via a phosphoribosyl

linker (132, 133). Interestingly, the activity of SidE is affected by SidJ, an effector that reverses the ubiquitination of SidE-modified substrates (134). The members of the SidE family also transfer ubiquitin onto Rtn4 to control tubular ER dynamics (135). LotA (Lem21) is another deubiquitinase that was recently discovered and that possesses a Cys protease activity by which it is able to cleave ubiquitin from the LCV (136).

To preserve its replication niche, *L. pneumophila* modulates host cell-death pathways via the action of several T4SS substrates (137). SidF directly interacts with and neutralizes proapoptotic BNIP3 and Bcl-rambo (138), whereas SdhA contributes to the prevention of cell death by an unknown mechanism (139). Finally, *L. pneumophila* also possesses the ability to promote cell death: Several secreted effectors have been shown to induce proapoptotic caspase-3 activity (140). Therefore, fine-tuned control of the secretion of antiapoptotic and proapoptotic effectors might be necessary to support bacterial replication at the beginning of infection and to promote the release of the pathogen from the host cell at the end of the infection cycle.

2.2. Specific Features of the *Legionella* Dot/Icm T4SS Effector Repertoire

It is well established that *L. pneumophila* delivers more than 330 effector proteins into its host cells (141–144). Interestingly, a lack of phenotypes is often associated with genetic mutations in single effectors, and intracellular growth is completely abolished only when the Dot/Icm T4SS is inactivated. This observation, associated with the presence of multiple paralogs of the same protein, led to the concept of effector redundancy, which suggests there are compensatory roles for two proteins or set of proteins with the same biological activity or different activities that have an impact on the same pathway or cellular process. Transposon site hybridization was used to identify such so-called redundant proteins and allowed for the suggestion that there were several functional groups of effectors that concomitantly act on the same cellular pathway; consequently, their combined deletion altered *L. pneumophila* growth in host cells (145). Some examples are the many effectors that affect Rab1 activity or the Lgt family—Lgt1, Lgt2, and Lgt3—that are differentially regulated during bacterial growth and affect eukaryotic protein synthesis (146).

The *L. pneumophila* effector repertoire contains proteins that regulate the function of other bacterial effectors within the host cell, called metaeffectors. The first metaeffector described was the tandem U-box protein LubX, which ubiquitinates the host kinase Clk1 (124), and also exploits the host proteasome to temporally regulate SidH activity in the host cell (147). Since metaeffectors were first described, several others have been identified, such as SidJ, which modulates the function of SidE family proteins (148), Lpg2505, which inhibits SidI toxicity (149), and Lpg2149, which inhibits both MavC and MvcA (150). Recently, a systematic analysis of effector–effector regulation identified 14 additional metaeffectors whose functions can now be studied in detail (151).

One of the most intriguing features of the *Legionella* T4SS effectors, first identified during *L. pneumophila* genome sequencing analysis, is the presence of a large variety and high number of so-called eukaryotic-like proteins and eukaryotic domain-encoding proteins (152). This finding led to the hypothesis that *L. pneumophila* has acquired these proteins by horizontal gene transfer from its eukaryotic hosts (amoebae) and now uses them to subvert host functions (152). Indeed, further evolutionary analyses supported this hypothesis (153–155). One of the most evident examples is the sphingosine-1-phosphate lyase–encoding gene, which different evolutionary analyses have suggested was acquired from amoebae (156, 157). Furthermore, the protein encoded by this gene was shown to have the same activity as its eukaryotic counterpart, modulating the sphingolipid metabolism, and it is thus an excellent example of molecular mimicry, a main virulence strategy employed by *Legionella* (110, 158).

Interestingly, it is not only *L. pneumophila* but also all *Legionella* species that encode remarkably large effector repertoires, as the genus harbors more than 18,000 effectors that differ surprisingly among species (59, 159). **Figure 3** shows the distribution of the effectors discussed in this review (see also **Table 1**), clearly revealing that many of them are conserved only in *L. pneumophila* and rarely present in other *Legionella* species. All *Legionella* species show evidence of long-lasting coevolution with their protozoan hosts, as the analyses of the genus genome identified effector proteins encoding 137 different eukaryotic-like domains and more than 200 eukaryotic-like proteins (59). An interesting example constitutes the group of Rab-like proteins, which are uniquely present in the effector repertoire of certain *Legionella* species, including *L. longbeachae*, that clearly have been acquired from eukaryotic organisms, probably protists, as seen in the two examples in **Figure 4** (59). Like many bacterial pathogens, *L. pneumophila* also targets host Rab GTPases, for example, by recruiting Rab1 to the LCV to finally control vesicle trafficking from ER exit sites (160). The identification of bacterial Rab-like GTPases in the *Legionella* genome suggests that these bacteria are able to subvert host cell trafficking by secreting their own Rab proteins into the host cell, and these could interact or compete with certain host Rabs during infection.

Despite our increased knowledge about the function of the effectors secreted by *L. pneumophila*, little is known about the effectors of other *Legionella* species. When considering *L. longbeachae*, the second most frequent cause of Legionnaires' disease, more than 66% of the reported *L. pneumophila* Dot/Icm T4SS effectors are missing in this species, while 51 novel substrates have been identified (52). To date, only one *L. longbeachae* effector protein has been characterized. It was shown that SidC, similar to its homolog in *L. pneumophila*, is a PtdIns(4)P-binding protein that resides on the LCV and promotes ER recruitment (161). Previous reports suggested that trafficking of the *L. longbeachae* vacuole might be different from that of *L. pneumophila* because the *L. longbeachae* LCV may acquire early and late endosomal markers (53). However, a recent report suggests that both species may develop similar replicative niches, albeit through different mechanisms, probably correlated with the specific set of effectors each species secretes into the host cell (162). Therefore, gaining better knowledge about the effectors secreted by *L. longbeachae* should enrich our understanding of the diverse mechanisms *Legionella* species utilize to successfully infect their hosts.

3. *LEGIONELLA*–AMOEBAE INTERACTIONS: A NICHE FOR THE EMERGENCE OF HUMAN PATHOGENS

In the environment, *Legionella* replication within protozoa is likely the most common mechanism of bacterial proliferation (163). Free-living amoebae are a group of protozoa ubiquitously found in soil and natural or man-made aquatic environments. They feed on microorganisms, and interactions over millions of years gave rise to the ability of *Legionella* to overcome intracellular degradation and instead survive or even replicate inside protozoa. Thus, free-living amoebae can act as Trojan horses, delivering microorganisms to new habitats and hosts in the form of intact amoeba or expelled vesicles, while protecting the microorganisms from hostile environmental conditions (164). Indeed, many medically important environmental bacteria, viruses, and fungi are associated with and are able to survive inside amoebae (165). *Legionella*–amoebae interactions were characterized shortly after *Legionella* bacteria were identified (7), and since then, the similarities between the infection of amoebae and of human macrophages have become more evident (8, 166). Indeed, bacterial inactivation mechanisms are the same in amoebae and macrophages, as both consist of lysosomal degradation of the phagocytized material. Additionally, both functional outcomes (digestion and immunity, respectively) are related, as it has been proposed that they share a common evolutionary origin in metazoans (167).

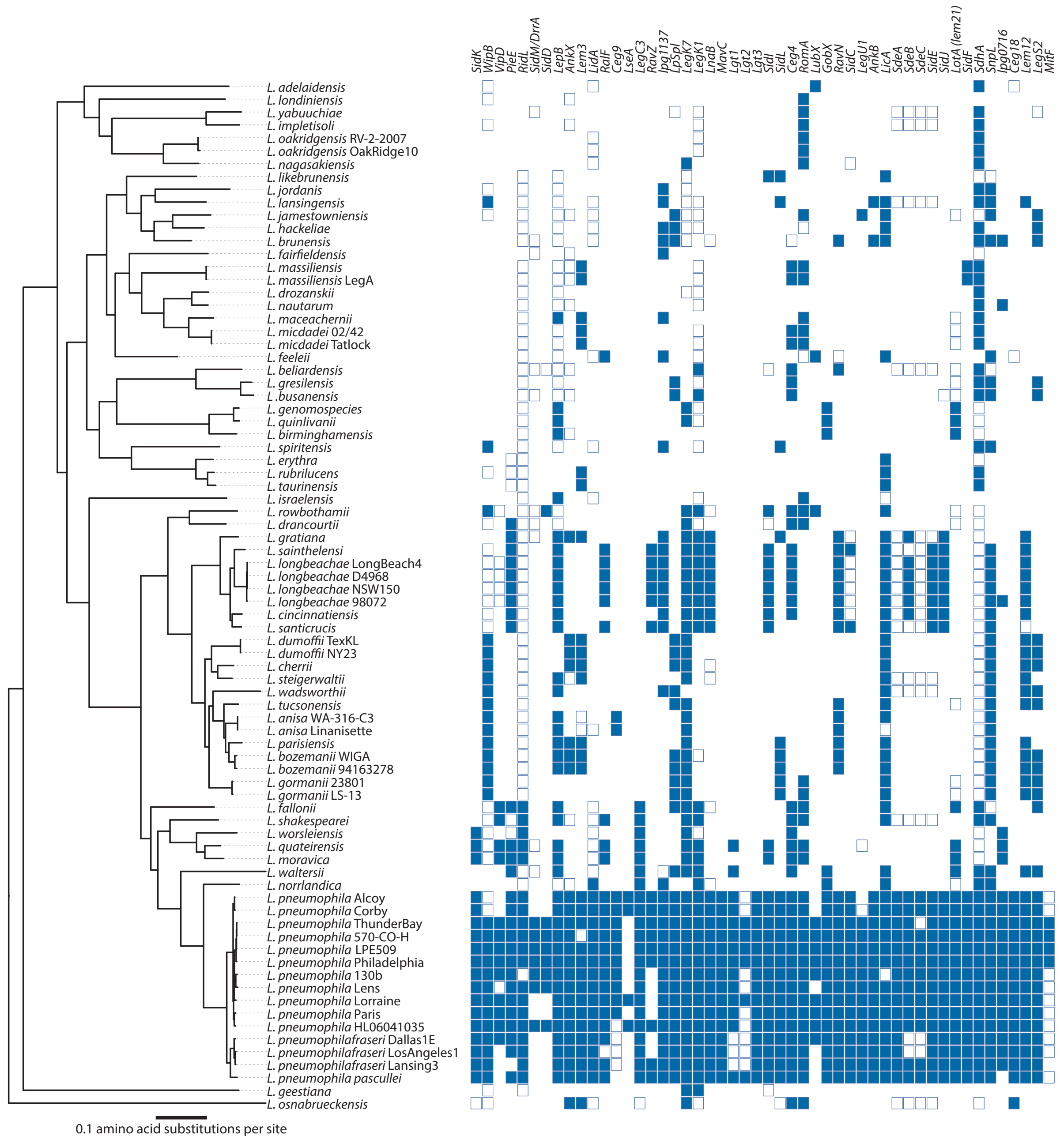

Figure 3

Distribution of 50 selected effectors from *Legionella pneumophila* in 58 different *Legionella* species and 80 *Legionella* strains. The sequence of the effector of *L. pneumophila* strain Philadelphia was used as reference to construct the table of orthologs to define their presence or absence in 80 *Legionella* strains previously analyzed (59). Blue-filled squares indicate the presence of the gene in the corresponding species based on predictions using *PanOCT* (the Pan-genome Ortholog Clustering Tool) with an identity cutoff of 30%, a BLAST (Basic Local Alignment Search Tool) Expect (*E*)-value cutoff of 10^{-5}, and a minimum percentage match length of subject and query of 65%. Blue-outlined squares indicate that an orthologous gene in the corresponding species is present, but the identity and/or the minimum percentage match length is under the cutoff selected for *PanOCT*. Empty spaces indicate that no orthologous gene was identified in the corresponding strain. The scale bar represents 0.1 amino acid substitutions per site.

Figure 4

(*Continued*)

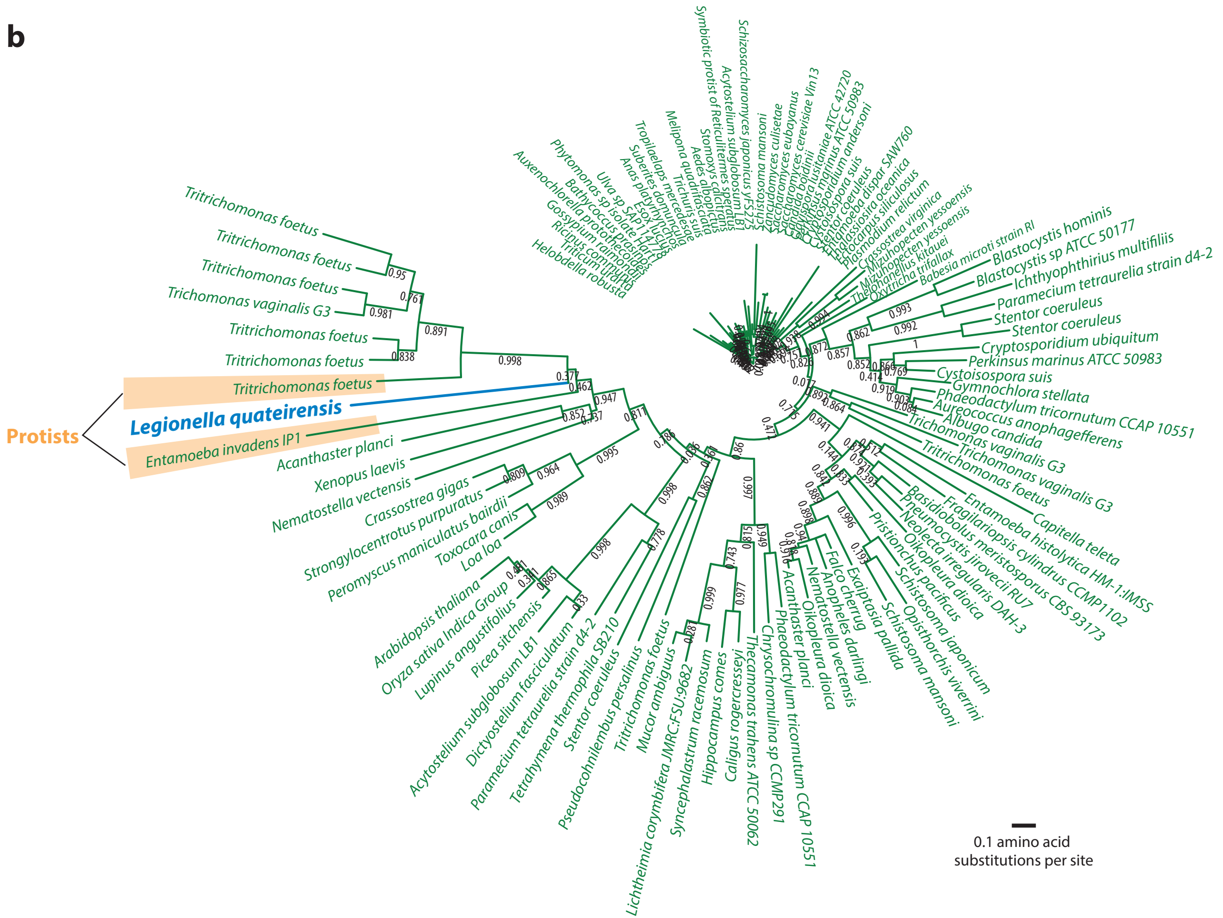

Figure 4

Phylogenetic trees of two Rab domain–containing proteins identified in the genus *Legionella*. (*Green* indicates eukaryotes; *blue* indicates *Legionella* species.) Blastp (protein–protein BLAST; Basic Local Alignment Search Tool) was used to search for homologs of these two proteins. Maximum likelihood was used for phylogenetic reconstruction. Local support values are represented by numbers on the corresponding branches. (*a*) *L. gratiana* protein Lgra3435 was used to recruit homologs. The scale bar represents 1 amino acid substitution per site. (*b*) *L. quateirensis* protein Lqua0234 was used to recruit homologs. The scale bar represents 0.1 amino acid substitutions per site. Figure adapted from Reference 59.

Among others, amoebae have been shown to be associated with bacteria from the genera *Legionella*, *Mycobacterium*, *Listeria*, and *Chlamydia*; soil fungi such as *Cryptococcus* species; and giant viruses belonging to the families *Mimiviridae* and *Marseilleviridae* (168). Pathogens that become specialized to infect hosts generally undergo genome reduction; however, this phenomenon is not observed in free-living amoebae-resistant bacteria (169). In contrast, it seems that *Legionella* bacteria undergo continuous genome expansion, with more gene gain events than losses, which is a consequence of gene acquisition by horizontal gene transfer, corroborated by the fact that the ancestral genomes were probably smaller (59).

Free-living amoebae seem to be melting pots of evolution in which giant viruses and bacteria can reside simultaneously, leading to gene fluxes in multiple directions and contributing to a so-called global mobilome (157, 169). One example is a protein identified in *L. pneumophila* that

has homologs only in the *Acanthamoeba polyphaga* mimivirus, indicating gene exchange involving eukaryotic viruses (155). Further examples involve other amoeba-associated bacteria, such as *Rickettsia* (170) and *Amoebophilus asiaticus* (171), in which recent genome sequence analyses have identified eukaryotic-like proteins in considerable numbers, similar to the *Legionella* species genomes. However, one of the enigmas of this genetic interchange remains to be resolved: What is the mechanism by which bacteria acquire and integrate the eukaryotic genes into their genome? One plausible explanation could be that the genetic transfer is related to RNA, which is subsequently retrotranscribed with the help of a *Legionella*-encoded reverse transcriptase. This would explain why no introns are present in the *Legionella* genes (157). Once integrated, these genes need to evolve to become specific, secreted effector proteins. Further, it has been proposed that a leaky delivery of these so-called proto-effectors to the host could allow for the selection of mutations to fine-tune protein function and to subsequently allow for the selection of an efficient C-terminal translocation signal (172).

Taken together, amoebae represent a niche allowing for the emergence of human pathogens. Thus, increased knowledge about *Legionella*–amoebae interactions is necessary to enable the development of new mechanisms for disease control and prevention.

4. NEW TECHNOLOGIES AND FUTURE DIRECTIONS

Since the identification of *Legionella* 40 years ago, the study of its biology has uncovered a vast arsenal of molecular tools that these bacteria use to modulate host pathways, and it has also provided insight into previously unknown mechanisms in eukaryotic cells. An example is RomA, a T4SS effector of *L. pneumophila* that methylates Lys14 of histone H3, a modification previously not known in mammalian cells (122). The recent finding that this epigenetic modification also occurs naturally in eukaryotic cells (173) highlights how advances in research achieved by studying mechanisms of bacterial infection can be valuable to further our understanding of basic cellular processes. The development of many new techniques in recent years has allowed for more detailed studies of eukaryotic processes and *Legionella*-induced alterations of host functions. We envisage that future epigenetic research will embrace genome-wide analyses of all known histone modifications during infection, and these will be combined with innovative genome-wide tools to perform precise epigenomic profiling, such as Internal Standard Calibrated Chromatin Immunoprecipitation, or ICeChIP (174). New tools will also ensure that the regulation of host microRNA during infection can be studied (175), as well as nucleosome positioning (176) in *Legionella*-infected cells. Seahorse technology (Agilent) allowed for the simultaneous analysis of oxidative phosphorylation and glycolysis in human primary macrophages infected with *L. pneumophila*, and this showed that specific T4SS-dependent metabolic shifts occur leading to metabolic reprogramming of the host cell (105). Future research using state-of-the-art methods—such as isotopolog profiling (177); integrated, stepwise, mass-isotopomeric flux analyses of the tricarboxylic acid cycle (178); or deep ^{13}C labeling (179)—will allow elucidation of detailed reprogramming of metabolic fluxes during infection.

For a long time, the lungs were thought to be sterile organs, but new sequencing technologies have shown that they harbor their own microbiome, like other body sites (180). Thus, next-generation sequencing of bronchoalveolar lavage fluids, sputum, and other clinical lung samples will allow sequencing analyses of the lung microbiome during *Legionella* infection (181). During disease development, *Legionella* might displace lung bacteria, similar to what is observed for the gut mucosa microbiome (180, 182). Notably, the results obtained from such studies will be relevant for the development of new strategies for disease diagnosis, prevention, and control and possibly for the development of new therapeutics.

The expansion of new technologies together with an increased interest in understanding cell biology have contributed to the elucidation of many previously unknown cellular processes, such as exosome production and cargo loading (183), the formation of phase-separated liquid droplets in the nucleus and cytoplasm (184), the formation of membrane nanotubes connecting cells (185, 186), the repertoire of interorganelle communication (187), and the mechanisms of cellular detoxification (188), including those of peroxisomes (189). Following these discoveries, questions arise, such as, what happens with these mechanisms during infection, and is *Legionella* manipulating these cellular processes?

Despite an increasing understanding of the biology and pathogenicity of *L. pneumophila*, there is still a lack of knowledge of the mechanisms of infection of *L. longbeachae*. The prediction of specific effector proteins in this species suggests that *L. longbeachae* is able to manipulate host cell pathways by means different from those used by *L. pneumophila* (52). The high incidence of *L. longbeachae* in Australia and New Zealand has been attributed to the presence of this bacterium in potting soils, which in these areas, in comparison to Europe, are mostly made from composted pine bark or sawdust. This suggests that *L. longbeachae* could be associated with trees and plants and that active multiplication of bacteria occurs during the composting process (190). The analysis of the *L. longbeachae* genome revealed that this species encodes for a set of enzymes probably devoted to the degradation of plant cell-wall components to be used as energy sources (52), thus supporting the hypothesis that *L. longbeachae* may also be associated with or infecting plants (190). This finding raises the question of whether organisms other than protozoa may also be hosts of different *Legionella* species. Indeed, *L. pneumophila* subverts well-established immune pathways in macrophages that are not conserved in amoebae, such as caspase-mediated apoptosis or the NF-κB pathway; thus, it is tempting to speculate that interactions between *L. pneumophila* and other susceptible hosts closer to higher eukaryotes were also relevant in shaping the repertoire of effectors of this bacterium (8). Some reports support this hypothesis, as it was shown that *L. pneumophila* can colonize and persist within the digestive tract of the nematode *Caenorhabditis elegans* (191); it can cause natural pneumonia in cattle (192); and it was also identified in the microbial community of the gastrointestinal tract in *Panaque nigrolineatus*, a tropical herbivorous freshwater fish (193). The future discovery of *Legionella* hosts other than protozoa will extend our knowledge and will open up new avenues for research into *Legionella*–host interactions.

Taken together, four decades of research on *Legionella* biology and Legionnaires' disease have brought important insights into the infection strategies and the mechanisms that these intracellular pathogens use to infect their hosts and to cause disease in humans. Despite these major advances, many open questions remain. Thus, the study of the intriguing ways that *Legionella* bacteria are exploiting their many hosts and signaling pathways is very exciting. Without doubt, it will teach us not only about the infection strategies of the bacteria but also about eukaryotic biology, thus this will continue to be a terrific scientific journey worth taking.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

Work in the C.B. laboratory is financed by the Institut Pasteur and funding has been received from the French Government (grants ANR-10-LABX-62-IBEID and ANR-15-CE17-0014-03 to C.B. and grant ANR-18-CE15-0005- 01 to M.R.) and the "Fondation de la Recherche Médicale" (grant

Erratum >

EQU201903007847 to C.B.). S.S. is a scholar in the Pasteur-Paris University (PPU) International Ph.D. program and received a stipend from the Institut Pasteur.

LITERATURE CITED

1. Fraser DW, Tsai TR, Orenstein W, Parkin WE, Beecham HJ, et al. 1977. Legionnaires' disease: description of an epidemic of pneumonia. *N. Engl. J. Med.* 297(22):1189–97
2. McDade JE, Shepard CC, Fraser DW, Tsai TR, Redus MA, Dowdle WR. 1977. Legionnaires' disease: isolation of a bacterium and demonstration of its role in other respiratory disease. *N. Engl. J. Med.* 297(22):1197–203
3. Brenner DJ, Steigerwalt AG, McDade JE. 1979. Classification of the Legionnaires' disease bacterium: *Legionella pneumophila*, genus novum, species nova, of the family Legionellaceae, familia nova. *Ann. Intern. Med.* 90(4):656–58
4. McDade JE, Brenner DJ, Bozeman FM. 1979. Legionnaires' disease bacterium isolated in 1947. *Ann. Intern. Med.* 90(4):659–61
5. Glick TH, Gregg MB, Berman B, Mallison G, Rhodes WW, Kassanoff I. 1978. Pontiac fever: an epidemic of unknown etiology in a health department: I. Clinical and epidemiologic aspects. *Am. J. Epidemiol.* 107(2):149–60
6. Newton HJ, Ang DKY, van Driel IR, Hartland EL. 2010. Molecular pathogenesis of infections caused by *Legionella pneumophila*. *Clin. Microbiol. Rev*. 23(2):274–98
7. Rowbotham TJ. 1980. Preliminary report on the pathogenicity of *Legionella pneumophila* for freshwater and soil amoebae. *J. Clin. Pathol.* 33(12):1179–83
8. Escoll P, Rolando M, Gomez-Valero L, Buchrieser C. 2013. From amoeba to macrophages: exploring the molecular mechanisms of *Legionella pneumophila* infection in both hosts. In *Molecular Mechanisms in* Legionella *Pathogenesis*, ed. H Hilbi, pp. 1–34. Berlin: Springer
9. Taylor M, Ross K, Bentham R. 2009. *Legionella*, protozoa, and biofilms: interactions within complex microbial systems. *Microb. Ecol.* 58(3):538–47
10. Blatt SP, Parkinson MD, Pace E, Hoffman P, Dolan D, et al. 1993. Nosocomial Legionnaires' disease: aspiration as a primary mode of disease acquisition. *Am. J. Med.* 95(1):16–22
11. Correia AM, Ferreira JS, Borges V, Nunes A, Gomes B, et al. 2016. Probable person-to-person transmission of Legionnaires' disease. *N. Engl. J. Med.* 374(5):497–98
12. Cunha CB, Cunha BA. 2017. Legionnaire's disease since Philadelphia: lessons learned and continued progress. *Infect. Dis. Clin. North Am.* 31(1):1–5
13. Walker JT. 2018. The influence of climate change on waterborne disease and *Legionella*: a review. *Perspect. Public Health* 138(5):282–86
14. Beauté J. 2017. Legionnaires' disease in Europe, 2011 to 2015. *Euro Surveill*. 22(27):506
15. ECDC (Eur. Cent. Dis. Prev. Control). 2018. Legionnaires' disease. In *ECDC: Annual Epidemiological Report for 2016*. Stockholm: ECDC
16. Shah P, Barskey A, Binder A, Edens C, Lee S, et al. 2018. *Legionnaires' Disease Surveillance Summary Report, United States: 2014–2015*. Atlanta, GA: CDC (Cent. Dis. Control Prev.)
17. Yu VL, Plouffe JF, Pastoris MC, Stout JE, Schousboe M, et al. 2002. Distribution of *Legionella* species and serogroups isolated by culture in patients with sporadic community-acquired legionellosis: an international collaborative survey. *J. Infect. Dis.* 186(1):127–28
18. David S, Rusniok C, Mentasti M, Gomez-Valero L, Harris SR, et al. 2016. Multiple major disease-associated clones of *Legionella pneumophila* have emerged recently and independently. *Genome Res*. 26(11):1555–64
19. Bacigalupe R, Lindsay D, Edwards G, Fitzgerald JR. 2017. Population genomics of *Legionella longbeachae* and hidden complexities of infection source attribution. *Emerg. Infect. Dis.* 23(5):750–57
20. Currie SL, Beattie TK. 2015. Compost and *Legionella longbeachae*: an emerging infection? *Perspect. Public Health* 135(6):309–15
21. Cunha BA. 2010. Legionnaires' disease: clinical differentiation from typical and other atypical pneumonias. *Infect. Dis. Clin. North Am.* 24(1):73–105

22. Mercante JW, Winchell JM. 2015. Current and emerging *Legionella* diagnostics for laboratory and outbreak investigations. *Clin. Microbiol. Rev.* 28(1):95–133
23. Dunne WM, Picot N, van Belkum A. 2017. Laboratory tests for Legionnaire's disease. *Infect. Dis. Clin. North Am.* 31(1):167–78
24. Fields BS, Benson RF, Besser RE. 2002. *Legionella* and Legionnaires' disease: 25 years of investigation. *Clin. Microbiol. Rev.* 15(3):506–26
25. Mérault N, Rusniok C, Jarraud S, Gomez-Valero L, Cazalet C, et al. 2011. Specific real-time PCR for simultaneous detection and identification of *Legionella pneumophila* serogroup 1 in water and clinical samples. *Appl. Environ. Microbiol.* 77(5):1708–17
26. Mentasti M, Cassier P, David S, Ginevra C, Gomez-Valero L, et al. 2017. Rapid detection and evolutionary analysis of *Legionella pneumophila* serogroup 1 sequence type 47. *Clin. Microbiol. Infect.* 23(4):264.e1–e9
27. Murdoch DR, Podmore RG, Anderson TP, Barratt K, Maze MJ, et al. 2013. Impact of routine systematic polymerase chain reaction testing on case finding for Legionnaires' disease: a pre–post comparison study. *Clin. Infect. Dis.* 57(9):1275–81
28. Cross KE, Mercante JW, Benitez AJ, Brown EW, Diaz MH, Winchell JM. 2016. Simultaneous detection of *Legionella* species and *L. anisa*, *L. bozemanii*, *L. longbeachae* and *L. micdadei* using conserved primers and multiple probes in a multiplex real-time PCR assay. *Diagn. Microbiol. Infect. Dis*. 85(3):295–301
29. Botelho-Nevers E, Grattard F, Viallon A, Allegra S, Jarraud S, et al. 2016. Prospective evaluation of RT-PCR on sputum versus culture, urinary antigens and serology for Legionnaire's disease diagnosis. *J. Infect.* 73(2):123–28
30. Bruin JP, Koshkolda T, IJzerman EPF, Lück C, Diederen BMW, et al. 2014. Isolation of ciprofloxacin-resistant *Legionella pneumophila* in a patient with severe pneumonia. *J. Antimicrob. Chemother.* 69(10):2869–71
31. Shadoud L, Almahmoud I, Jarraud S, Etienne J, Larrat S, et al. 2015. Hidden selection of bacterial resistance to fluoroquinolones in vivo: the case of *Legionella pneumophila* and humans. *EBioMedicine* 2(9):1179–85
32. Hennebique A, Bidart M, Jarraud S, Beraud L, Schwebel C, et al. 2017. Digital PCR for detection and quantification of fluoroquinolone resistance in *Legionella pneumophila*. *Antimicrob. Agents Chemother*. 61(9):e00628-17
33. Pedro-Botet L, Yu VL. 2006. *Legionella*: macrolides or quinolones? *Clin. Microbiol. Infect.* 12(Suppl. 3):25–30
34. Potts A, Donaghy M, Marley M, Othieno R, Stevenson J, et al. 2013. Cluster of Legionnaires' disease cases caused by *Legionella longbeachae* serogroup 1, Scotland, August to September 2013. *Euro Surveill.* 18(50):20656
35. de Bruin L, Timmerman CP, Huisman PM, Heidt J. 2018. *Legionella longbeachae*: Don't miss it! *Neth. J. Med*. 76(6):294–97
36. MMWR (Morbid. Mortal. Wkly. Rep.). 2000. Legionnaires' disease associated with potting soil—California, Oregon, and Washington, May–June 2000. *MMWR* 49(34):777–78
37. Picard-Masson M, Lajoie É, Lord J, Lalancette C, Marchand G, et al. 2016. Two related occupational cases of *Legionella longbeachae* infection, Quebec, Canada. *Emerg. Infect. Dis.* 22(7):1289–91
38. Phares CR, Wangroongsarb P, Chantra S, Paveenkitiporn W, Tondella M-L, et al. 2007. Epidemiology of severe pneumonia caused by *Legionella longbeachae*, *Mycoplasma pneumoniae*, and *Chlamydia pneumoniae*: 1-year, population-based surveillance for severe pneumonia in Thailand. *Clin. Infect. Dis.* 45(12):e147–55
39. Wei S-H, Tseng L-R, Tan J-K, Cheng C-Y, Hsu Y-T, et al. 2014. Legionnaires' disease caused by *Legionella longbeachae* in Taiwan, 2006–2010. *Int. J. Infect. Dis.* 19:95–97
40. Cameron RL, Pollock KGJ, Lindsay DSJ, Anderson E. 2016. Comparison of *Legionella longbeachae* and *Legionella pneumophil*a cases in Scotland; implications for diagnosis, treatment and public health response. *J. Med. Microbiol.* 65(2):142–46
41. Kenagy E, Priest PC, Cameron CM, Smith D, Scott P, et al. 2017. Risk factors for *Legionella longbeachae* Legionnaires' disease, New Zealand. *Emerg. Infect. Dis.* 23(7):1148–54

42. Thornley CN, Harte DJ, Weir RP, Allen LJ, Knightbridge KJ, Wood PRT. 2017. *Legionella longbeachae* detected in an industrial cooling tower linked to a legionellosis outbreak, New Zealand, 2015: possible waterborne transmission? *Epidemiol. Infect.* 145(11):2382–89
43. Molofsky AB, Swanson MS. 2004. Differentiate to thrive: lessons from the *Legionella pneumophila* life cycle. *Mol. Microbiol.* 53(1):29–40
44. Oliva G, Sahr T, Buchrieser C. 2018. The life cycle of *Legionella pneumophila*: Cellular differentiation is linked to virulence and metabolism. *Front. Cell. Infect. Microbiol.* 8:3
45. Brüggemann H, Hagman A, Jules M, Sismeiro O, Dillies M-A, et al. 2006. Virulence strategies for infecting phagocytes deduced from the in vivo transcriptional program of *Legionella pneumophila*. *Cell. Microbiol*. 8(8):1228–40
46. Byrne B, Swanson MS. 1998. Expression of *Legionella pneumophila* virulence traits in response to growth conditions. *Infect. Immun.* 66(7):3029–34
47. Molofsky AB, Swanson MS. 2003. *Legionella pneumophila* CsrA is a pivotal repressor of transmission traits and activator of replication. *Mol. Microbiol.* 50(2):445–61
48. Fettes PS, Forsbach-Birk V, Lynch D, Marre R. 2001. Overexpresssion of a *Legionella pneumophila* homologue of the *Escherichia coli* regulator *csrA* affects cell size, flagellation, and pigmentation. *Int. J. Med. Microbiol.* 291(5):353–60
49. Rasis M, Segal G. 2009. The LetA–RsmYZ–CsrA regulatory cascade, together with RpoS and PmrA, post-transcriptionally regulates stationary phase activation of *Legionella pneumophila* Icm/Dot effectors. *Mol. Microbiol.* 72(4):995–1010
50. Sahr T, Rusniok C, Dervins-Ravault D, Sismeiro O, Coppée J-Y, Buchrieser C. 2012. Deep sequencing defines the transcriptional map of *Legionella pneumophila* and identifies growth phase–dependent regulated ncRNAs implicated in virulence. *RNA Biol*. 9(4):503–19
51. Sahr T, Rusniok C, Impens F, Oliva G, Sismeiro O, et al. 2017. The *Legionella pneumophila* genome evolved to accommodate multiple regulatory mechanisms controlled by the CsrA-system. *PLOS Genet*. 13(2):e1006629
52. Cazalet C, Gomez-Valero L, Rusniok C, Lomma M, Dervins-Ravault D, et al. 2010. Analysis of the *Legionella longbeachae* genome and transcriptome uncovers unique strategies to cause Legionnaires' disease. *PLOS Genet*. 6(2):e1000851
53. Asare R, Abu Kwaik Y. 2007. Early trafficking and intracellular replication of *Legionella longbeachae* within an ER-derived late endosome-like phagosome. *Cell. Microbiol.* 9(6):1571–87
54. Kozak NA, Buss M, Lucas CE, Frace M, Govil D, et al. 2010. Virulence factors encoded by *Legionella longbeachae* identified on the basis of the genome sequence analysis of clinical isolate D-4968. *J. Bacteriol.* 192(4):1030–44
55. Gomez-Valero L, Rusniok C, Cazalet C, Buchrieser C. 2011. Comparative and functional genomics of *Legionella* identified eukaryotic like proteins as key players in host–pathogen interactions. *Front. Microbiol.* 2:208
56. Ren T, Zamboni DS, Roy CR, Dietrich WF, Vance RE. 2006. Flagellin-deficient *Legionella* mutants evade caspase-1- and *Naip5*-mediated macrophage immunity. *PLOS Pathog*. 2(3):e18
57. Massis LM, Assis-Marques MA, Castanheira FVS, Capobianco YJ, Balestra AC, et al. 2017. *Legionella longbeachae* is immunologically silent and highly virulent in vivo. *J. Infect. Dis.* 215(3):440–51
58. Qin T, Zhou H, Ren H, Liu W. 2017. Distribution of secretion systems in the genus *Legionella* and its correlation with pathogenicity. *Front. Microbiol.* 8:388
59. Gomez-Valero L, Rusniok C, Carson D, Mondino S, Pérez-Cobas AE, et al. 2019. More than 18,000 effectors in the *Legionella* genus genome provide multiple, independent combinations for replication in human cells. *PNAS* 116(6):2265–73
60. Cianciotto NP. 2005. Type II secretion: a protein secretion system for all seasons. *Trends Microbiol*. 13(12):581–88
61. Truchan HK, Christman HD, White RC, Rutledge NS, Cianciotto NP. 2017. Type II secretion substrates of *Legionella pneumophila* translocate out of the pathogen-occupied vacuole via a semipermeable membrane. *mBio* 8(3):e00870-17

62. Cianciotto NP. 2009. Many substrates and functions of type II secretion: lessons learned from *Legionella pneumophila*. *Future Microbiol*. 4(7):797–805
63. Cianciotto NP. 2013. Type II secretion and *Legionella* virulence. In *Molecular Mechanisms in Legionella Pathogenesis*, ed. H Hilbi, pp. 81–102. Berlin: Springer
64. DebRoy S, Dao J, Söderberg M, Rossier O, Cianciotto NP. 2006. *Legionella pneumophila* type II secretome reveals unique exoproteins and a chitinase that promotes bacterial persistence in the lung. *PNAS* 103(50):19146–51
65. Berger KH, Isberg RR. 1993. Two distinct defects in intracellular growth complemented by a single genetic locus in *Legionella pneumophila*. *Mol. Microbiol*. 7(1):7–19
66. Marra A, Blander SJ, Horwitz MA, Shuman HA. 1992. Identification of a *Legionella pneumophila* locus required for intracellular multiplication in human macrophages. *PNAS* 89(20):9607–11
67. Jeong KC, Ghosal D, Chang Y-W, Jensen GJ, Vogel JP. 2017. Polar delivery of *Legionella* type IV secretion system substrates is essential for virulence. *PNAS* 114(30):8077–82
68. Charpentier X, Gabay JE, Reyes M, Zhu JW, Weiss A, Shuman HA. 2009. Chemical genetics reveals bacterial and host cell functions critical for type IV effector translocation by *Legionella pneumophila*. *PLOS Pathog*. 5(7):e1000501
69. Hilbi H, Segal G, Shuman HA. 2001. Icm/Dot-dependent upregulation of phagocytosis by *Legionella pneumophila*. *Mol. Microbiol*. 42(3):603–17
70. Watarai M, Derre I, Kirby J, Growney JD, Dietrich WF, Isberg RR. 2001. *Legionella pneumophila* is internalized by a macropinocytotic uptake pathway controlled by the Dot/Icm system and the mouse *Lgn1* locus. *J. Exp. Med.* 194(8):1081–96
71. Weber S, Wagner M, Hilbi H. 2014. Live-cell imaging of phosphoinositide dynamics and membrane architecture during *Legionella* infection. *mBio* 5(1):e00839-13
72. Fuche F, Vianney A, Andrea C, Doublet P, Gilbert C. 2015. Functional type 1 secretion system involved in *Legionella pneumophila* virulence. *J. Bacteriol.* 197(3):563–71
73. Horwitz MA. 1983. The Legionnaires' disease bacterium (*Legionella pneumophila*) inhibits phagosome–lysosome fusion in human monocytes. *J. Exp. Med.* 158(6):2108–26
74. Xu L, Shen X, Bryan A, Banga S, Swanson MS, Luo Z-Q. 2010. Inhibition of host vacuolar H^+-ATPase activity by a *Legionella pneumophila* effector. *PLOS Pathog*. 6(3):e1000822
75. Prevost MS, Pinotsis N, Dumoux M, Hayward RD, Waksman G. 2017. The *Legionella* effector WipB is a translocated Ser/Thr phosphatase that targets the host lysosomal nutrient sensing machinery. *Sci. Rep.* 7(1):9450
76. Urwyler S, Nyfeler Y, Ragaz C, Lee H, Mueller LN, et al. 2009. Proteome analysis of *Legionella* vacuoles purified by magnetic immunoseparation reveals secretory and endosomal GTPases. *Traffic* 10(1):76–87
77. Hoffmann C, Finsel I, Otto A, Pfaffinger G, Rothmeier E, et al. 2014. Functional analysis of novel Rab GTPases identified in the proteome of purified *Legionella*-containing vacuoles from macrophages. *Cell. Microbiol.* 16(7):1034–52
78. Lawe DC, Chawla A, Merithew E, Dumas J, Carrington W, et al. 2002. Sequential roles for phosphatidylinositol 3-phosphate and Rab5 in tethering and fusion of early endosomes via their interaction with EEA1. *J. Biol. Chem.* 277(10):8611–17
79. Gaspar AH, Machner MP. 2014. VipD is a Rab5-activated phospholipase A1 that protects *Legionella pneumophila* from endosomal fusion. *PNAS* 111(12):4560–65
80. Mousnier A, Schroeder GN, Stoneham CA, So EC, Garnett JA, et al. 2014. A new method to determine in vivo interactomes reveals binding of the *Legionella pneumophila* effector PieE to multiple Rab GTPases. *mBio* 5(4):e01148-14
81. Rink J, Ghigo E, Kalaidzidis Y, Zerial M. 2005. Rab conversion as a mechanism of progression from early to late endosomes. *Cell* 122(5):735–49
82. Rojas R, van Vlijmen T, Mardones GA, Prabhu Y, Rojas AL, et al. 2008. Regulation of retromer recruitment to endosomes by sequential action of Rab5 and Rab7. *J. Cell Biol.* 183(3):513–26
83. Finsel I, Ragaz C, Hoffmann C, Harrison CF, Weber S, et al. 2013. The *Legionella* effector RidL inhibits retrograde trafficking to promote intracellular replication. *Cell Host Microbe* 14(1):38–50

84. Robinson CG, Roy CR. 2006. Attachment and fusion of endoplasmic reticulum with vacuoles containing *Legionella pneumophila*. *Cell. Microbiol*. 8(5):793–805
85. Kagan JC, Roy CR. 2002. *Legionella* phagosomes intercept vesicular traffic from endoplasmic reticulum exit sites. *Nat. Cell Biol.* 4(12):945–54
86. Schoebel S, Oesterlin LK, Blankenfeldt W, Goody RS, Itzen A. 2009. RabGDI displacement by DrrA from *Legionella* is a consequence of its guanine nucleotide exchange activity. *Mol. Cell* 36(6):1060–72
87. Murata T, Delprato A, Ingmundson A, Toomre DK, Lambright DG, Roy CR. 2006. The *Legionella pneumophila* effector protein DrrA is a Rab1 guanine nucleotide-exchange factor. *Nat. Cell Biol.* 8(9):971–77
88. Machner MP, Isberg RR. 2007. A bifunctional bacterial protein links GDI displacement to Rab1 activation. *Science* 318(5852):974–77
89. Müller MP, Peters H, Blümer J, Blankenfeldt W, Goody RS, Itzen A. 2010. The *Legionella* effector protein DrrA AMPylates the membrane traffic regulator Rab1b. *Science* 329(5994):946–49
90. Machner MP, Isberg RR. 2006. Targeting of host Rab GTPase function by the intravacuolar pathogen *Legionella pneumophila*. *Dev. Cell* 11(1):47–56
91. Neunuebel MR, Chen Y, Gaspar AH, Backlund PS, Yergey A, Machner MP. 2011. De-AMPylation of the small GTPase Rab1 by the pathogen *Legionella pneumophila*. *Science* 333(6041):453–56
92. Ingmundson A, Delprato A, Lambright DG, Roy CR. 2007. *Legionella pneumophila* proteins that regulate Rab1 membrane cycling. *Nature* 450(7168):365–69
93. Mukherjee S, Liu X, Arasaki K, McDonough J, Galán JE, Roy CR. 2011. Modulation of Rab GTPase function by a protein phosphocholine transferase. *Nature* 477(7362):103–6
94. Tan Y, Arnold RJ, Luo Z-Q. 2011. *Legionella pneumophila* regulates the small GTPase Rab1 activity by reversible phosphorylcholination. *PNAS* 108(52):21212–17
95. Nagai H, Kagan JC, Zhu X, Kahn RA, Roy CR. 2002. A bacterial guanine nucleotide exchange factor activates ARF on *Legionella* phagosomes. *Science* 295(5555):679–82
96. Hubber A, Arasaki K, Nakatsu F, Hardiman C, Lambright D, et al. 2014. The machinery at endoplasmic reticulum–plasma membrane contact sites contributes to spatial regulation of multiple *Legionella* effector proteins. *PLOS Pathog*. 10(7):e1004222
97. Haenssler E, Ramabhadran V, Murphy CS, Heidtman MI, Isberg RR. 2015. Endoplasmic reticulum tubule protein reticulon 4 associates with the *Legionella pneumophila* vacuole and with translocated substrate Ceg9. *Infect. Immun.* 83(9):3479–89
98. Arasaki K, Roy CR. 2010. *Legionella pneumophila* promotes functional interactions between plasma membrane syntaxins and Sec22b. *Traffic* 11(5):587–600
99. Jahn R, Scheller RH. 2006. SNAREs—engines for membrane fusion. *Nat. Rev. Mol. Cell Biol.* 7(9):631–43
100. Arasaki K, Toomre DK, Roy CR. 2012. The *Legionella pneumophila* effector DrrA is sufficient to stimulate SNARE-dependent membrane fusion. *Cell Host Microbe* 11(1):46–57
101. King NP, Newton P, Schuelein R, Brown DL, Petru M, et al. 2015. Soluble NSF attachment protein receptor molecular mimicry by a *Legionella pneumophila* Dot/Icm effector. *Cell. Microbiol.* 17(6):767–84
102. Bennett TL, Kraft SM, Reaves BJ, Mima J, O'Brien KM, Starai VJ. 2013. LegC3, an effector protein from *Legionella pneumophila*, inhibits homotypic yeast vacuole fusion in vivo and in vitro. *PLOS ONE* 8(2):e56798
103. Shi X, Halder P, Yavuz H, Jahn R, Shuman HA. 2016. Direct targeting of membrane fusion by SNARE mimicry: convergent evolution of *Legionella* effectors. *PNAS* 113(31):8807–12
104. Rothmeier E, Pfaffinger G, Hoffmann C, Harrison CF, Grabmayr H, et al. 2013. Activation of Ran GTPase by a *Legionella* effector promotes microtubule polymerization, pathogen vacuole motility and infection. *PLOS Pathog*. 9(9):e1003598
105. Escoll P, Song O-R, Viana F, Steiner B, Lagache T, et al. 2017. *Legionella pneumophila* modulates mitochondrial dynamics to trigger metabolic repurposing of infected macrophages. *Cell Host Microbe* 22(3):302–7
106. Escoll P, Buchrieser C. 2018. Metabolic reprogramming of host cells upon bacterial infection: Why shift to a Warburg-like metabolism? *FEBS J.* 285(12):2146–60

107. Amer AO, Swanson MS. 2005. Autophagy is an immediate macrophage response to *Legionella pneumophila*. *Cell. Microbiol.* 7(6):765–78
108. Choy A, Dancourt J, Mugo B, O'Connor TJ, Isberg RR, et al. 2012. The *Legionella* effector RavZ inhibits host autophagy through irreversible Atg8 deconjugation. *Science* 338(6110):1072–76
109. Arasaki K, Mikami Y, Shames SR, Inoue H, Wakana Y, Tagaya M. 2017. *Legionella* effector Lpg1137 shuts down ER–mitochondria communication through cleavage of syntaxin 17. *Nat. Commun.* 8:15406
110. Rolando M, Escoll P, Nora T, Botti J, Boitez V, et al. 2016. *Legionella pneumophila* S1P-lyase targets host sphingolipid metabolism and restrains autophagy. *PNAS* 113(7):1901–6
111. Kaneko T, Stogios PJ, Ruan X, Voss C, Evdokimova E, et al. 2018. Identification and characterization of a large family of superbinding bacterial SH2 domains. *Nat. Commun.* 9(1):4549
112. Lee P-C, Machner MP. 2018. The *Legionella* effector kinase LegK7 hijacks the host Hippo pathway to promote infection. *Cell Host Microbe* 24(3):429–438.e6
113. Bartfeld S, Engels C, Bauer B, Aurass P, Flieger A, et al. 2009. Temporal resolution of two-tracked NF-κB activation by *Legionella pneumophila*. *Cell. Microbiol.* 11(11):1638–51
114. Ge J, Xu H, Li T, Zhou Y, Zhang Z, et al. 2009. A *Legionella* type IV effector activates the NF-κB pathway by phosphorylating the IκB family of inhibitors. *PNAS* 106(33):13725–30
115. Losick VP, Haenssler E, Moy M-Y, Isberg RR. 2010. LnaB: a *Legionella pneumophila* activator of NF-κB. *Cell. Microbiol.* 12(8):1083–97
116. Gan N, Nakayasu ES, Hollenbeck PJ, Luo Z-Q. 2019. *Legionella pneumophila* inhibits immune signalling via MavC-mediated transglutaminase-induced ubiquitination of UBE2N. *Nat. Microbiol.* 4(1):134–43
117. Fontana MF, Banga S, Barry KC, Shen X, Tan Y, et al. 2011. Secreted bacterial effectors that inhibit host protein synthesis are critical for induction of the innate immune response to virulent *Legionella pneumophila*. *PLOS Pathog.* 7(2):e1001289
118. Welsh CT, Summersgill JT, Miller RD. 2004. Increases in c-Jun N-terminal kinase/stress-activated protein kinase and p38 activity in monocyte-derived macrophages following the uptake of *Legionella pneumophila*. *Infect. Immun.* 72(3):1512–18
119. Shin S, Case CL, Archer KA, Nogueira CV, Kobayashi KS, et al. 2008. Type IV secretion-dependent activation of host MAP kinases induces an increased proinflammatory cytokine response to *Legionella pneumophila*. *PLOS Pathog.* 4(11):e1000220
120. Fontana MF, Shin S, Vance RE. 2012. Activation of host mitogen-activated protein kinases by secreted *Legionella pneumophila* effectors that inhibit host protein translation. *Infect. Immun.* 80(10):3570–75
121. Quaile AT, Stogios PJ, Egorova O, Evdokimova E, Valleau D, et al. 2018. The *Legionella pneumophila* effector Ceg4 is a phosphotyrosine phosphatase that attenuates activation of eukaryotic MAPK pathways. *J. Biol. Chem.* 293(9):3307–20
122. Rolando M, Sanulli S, Rusniok C, Gomez-Valero L, Bertholet C, et al. 2013. *Legionella pneumophila* effector RomA uniquely modifies host chromatin to repress gene expression and promote intracellular bacterial replication. *Cell Host Microbe* 13(4):395–405
123. Schuelein R, Spencer H, Dagley LF, Li PF, Luo L, et al. 2018. Targeting of RNA Polymerase II by a nuclear *Legionella pneumophila* Dot/Icm effector SnpL. *Cell. Microbiol.* 20(9):e12852
124. Kubori T, Hyakutake A, Nagai H. 2008. *Legionella* translocates an E3 ubiquitin ligase that has multiple U-boxes with distinct functions. *Mol. Microbiol.* 67(6):1307–19
125. Lin Y-H, Doms AG, Cheng E, Kim B, Evans TR, Machner MP. 2015. Host cell-catalyzed *S*-palmitoylation mediates Golgi targeting of the *Legionella* ubiquitin ligase GobX. *J. Biol. Chem.* 290(42):25766–81
126. Lin Y-H, Lucas M, Evans TR, Abascal-Palacios G, Doms AG, et al. 2018. RavN is a member of a previously unrecognized group of *Legionella pneumophila* E3 ubiquitin ligases. *PLOS Pathog.* 14(2):e1006897
127. Ragaz C, Pietsch H, Urwyler S, Tiaden A, Weber SS, Hilbi H. 2008. The *Legionella pneumophila* phosphatidylinositol-4 phosphate-binding type IV substrate SidC recruits endoplasmic reticulum vesicles to a replication-permissive vacuole. *Cell. Microbiol.* 10(12):2416–33
128. Hsu F, Luo X, Qiu J, Teng Y-B, Jin J, et al. 2014. The *Legionella* effector SidC defines a unique family of ubiquitin ligases important for bacterial phagosomal remodeling. *PNAS* 111(29):10538–43

129. Ensminger AW, Isberg RR. 2010. E3 ubiquitin ligase activity and targeting of BAT3 by multiple *Legionella pneumophila* translocated substrates. *Infect. Immun.* 78(9):3905–19
130. Lomma M, Dervins-Ravault D, Rolando M, Nora T, Newton HJ, et al. 2010. The *Legionella pneumophila* F-box protein Lpp2082 (AnkB) modulates ubiquitination of the host protein parvin B and promotes intracellular replication. *Cell. Microbiol.* 12(9):1272–91
131. Price CTD, Al-Quadan T, Santic M, Rosenshine I, Abu Kwaik Y. 2011. Host proteasomal degradation generates amino acids essential for intracellular bacterial growth. *Science* 334(6062):1553–57
132. Qiu J, Sheedlo MJ, Yu K, Tan Y, Nakayasu ES, et al. 2016. Ubiquitination independent of E1 and E2 enzymes by bacterial effectors. *Nature* 533(7601):120–24
133. Bhogaraju S, Kalayil S, Liu Y, Bonn F, Colby T, et al. 2016. Phosphoribosylation of ubiquitin promotes serine ubiquitination and impairs conventional ubiquitination. *Cell* 167(6):1636–1649
134. Qiu J, Yu K, Fei X, Liu Y, Nakayasu ES, et al. 2017. A unique deubiquitinase that deconjugates phosphoribosyl-linked protein ubiquitination. *Cell Res*. 27(7):865–81
135. Kotewicz KM, Ramabhadran V, Sjoblom N, Vogel JP, Haenssler E, et al. 2017. A single *Legionella* effector catalyzes a multistep ubiquitination pathway to rearrange tubular endoplasmic reticulum for replication. *Cell Host Microbe* 21(2):169–81
136. Kubori T, Kitao T, Ando H, Nagai H. 2018. LotA, a *Legionella* deubiquitinase, has dual catalytic activity and contributes to intracellular growth. *Cell. Microbiol.* 20(7):e12840
137. Luo Z-Q. 2011. Striking a balance: modulation of host cell death pathways by *Legionella pneumophila*. *Front. Microbiol.* 2:36
138. Banga S, Gao P, Shen X, Fiscus V, Zong W-X, et al. 2007. *Legionella pneumophila* inhibits macrophage apoptosis by targeting pro-death members of the Bcl2 protein family. *PNAS* 104(12):5121–26
139. Laguna RK, Creasey EA, Li Z, Valtz N, Isberg RR. 2006. A *Legionella pneumophila*-translocated substrate that is required for growth within macrophages and protection from host cell death. *PNAS* 103(49):18745–50
140. Zhu W, Hammad LA, Hsu F, Mao Y, Luo Z-Q. 2013. Induction of caspase 3 activation by multiple *Legionella pneumophila* Dot/Icm substrates. *Cell. Microbiol.* 15(11):1783–95
141. Lifshitz Z, Burstein D, Peeri M, Zusman T, Schwartz K, et al. 2013. Computational modeling and experimental validation of the *Legionella* and *Coxiella* virulence-related type-IVB secretion signal. *PNAS* 110(8):E707–15
142. Zhu W, Banga S, Tan Y, Zheng C, Stephenson R, et al. 2011. Comprehensive identification of protein substrates of the Dot/Icm type IV transporter of *Legionella pneumophila*. *PLOS ONE* 6(3):e17638
143. Escoll P, Mondino S, Rolando M, Buchrieser C. 2016. Targeting of host organelles by pathogenic bacteria: a sophisticated subversion strategy. *Nat. Rev. Microbiol.* 14(1):5–19
144. Schroeder GN. 2017. The toolbox for uncovering the functions of *Legionella* Dot/Icm type IVb secretion system effectors: current state and future directions. *Front. Cell. Infect. Microbiol.* 7:528
145. O'Connor TJ, Adepoju Y, Boyd D, Isberg RR. 2011. Minimization of the *Legionella pneumophila* genome reveals chromosomal regions involved in host range expansion. *PNAS* 108(36):14733–40
146. Belyi Y, Jank T, Aktories K. 2013. Cytotoxic glucosyltransferases of *Legionella pneumophila*. In *Molecular Mechanisms in Legionella Pathogenesis*, ed. H Hilbi, pp. 211–26. Berlin: Springer
147. Kubori T, Shinzawa N, Kanuka H, Nagai H. 2010. *Legionella* metaeffector exploits host proteasome to temporally regulate cognate effector. *PLOS Pathog*. 6(12):e1001216
148. Jeong KC, Sexton JA, Vogel JP. 2015. Spatiotemporal regulation of a *Legionella pneumophila* T4SS substrate by the metaeffector SidJ. *PLOS Pathog*. 11(3):e1004695
149. Shames SR, Liu L, Havey JC, Schofield WB, Goodman AL, Roy CR. 2017. Multiple *Legionella pneumophila* effector virulence phenotypes revealed through high-throughput analysis of targeted mutant libraries. *PNAS* 114(48):E10446–54
150. Valleau D, Quaile AT, Cui H, Xu X, Evdokimova E, et al. 2018. Discovery of ubiquitin deamidases in the pathogenic arsenal of *Legionella pneumophila*. *Cell Rep*. 23(2):568–83
151. Urbanus ML, Quaile AT, Stogios PJ, Morar M, Rao C, et al. 2016. Diverse mechanisms of metaeffector activity in an intracellular bacterial pathogen, *Legionella pneumophila*. *Mol. Syst. Biol.* 12(12):893

152. Cazalet C, Rusniok C, Brüggemann H, Zidane N, Magnier A, et al. 2004. Evidence in the *Legionella pneumophila* genome for exploitation of host cell functions and high genome plasticity. *Nat. Genet.* 36(11):1165–73
153. de Felipe KS, Pampou S, Jovanovic OS, Pericone CD, Ye SF, et al. 2005. Evidence for acquisition of *Legionella* type IV secretion substrates via interdomain horizontal gene transfer. *J. Bacteriol.* 187(22):7716–26
154. Gomez-Valero L, Rusniok C, Buchrieser C. 2009. *Legionella pneumophila*: population genetics, phylogeny and genomics. *Infect. Genet. Evol.* 9(5):727–39
155. Lurie-Weinberger MN, Gomez-Valero L, Merault N, Glöckner G, Buchrieser C, Gophna U. 2010. The origins of eukaryotic-like proteins in *Legionella pneumophila*. *Int. J. Med. Microbiol*. 300(7):470–81
156. Degtyar E, Zusman T, Ehrlich M, Segal G. 2009. A *Legionella* effector acquired from protozoa is involved in sphingolipids metabolism and is targeted to the host cell mitochondria. *Cell. Microbiol.* 11(8):1219–35
157. Gomez-Valero L, Buchrieser C. 2013. Genome dynamics in *Legionella*: the basis of versatility and adaptation to intracellular replication. *Cold Spring Harb. Perspect. Med.* 3(6):a009993
158. Nora T, Lomma M, Gomez-Valero L, Buchrieser C. 2009. Molecular mimicry: an important virulence strategy employed by *Legionella pneumophila* to subvert host functions. *Future Microbiol*. 4(6):691–701
159. Burstein D, Amaro F, Zusman T, Lifshitz Z, Cohen O, et al. 2016. Genomic analysis of 38 *Legionella* species identifies large and diverse effector repertoires. *Nat. Genet.* 48(2):167–75
160. Weber MM, Faris R. 2018. Subversion of the endocytic and secretory pathways by bacterial effector proteins. *Front. Cell Dev. Biol.* 6:1
161. Dolinsky S, Haneburger I, Cichy A, Hannemann M, Itzen A, Hilbi H. 2014. The *Legionella longbeachae* Icm/Dot substrate SidC selectively binds phosphatidylinositol 4-phosphate with nanomolar affinity and promotes pathogen vacuole–endoplasmic reticulum interactions. *Infect. Immun.* 82(10):4021–33
162. Wood RE, Newton P, Latomanski EA, Newton HJ. 2015. Dot/Icm effector translocation by *Legionella longbeachae* creates a replicative vacuole similar to that of *Legionella pneumophila* despite translocation of distinct effector repertoires. *Infect. Immun.* 83(10):4081–92
163. Boamah DK, Zhou G, Ensminger AW, O'Connor TJ. 2017. From many hosts, one accidental pathogen: the diverse protozoan hosts of *Legionella*. *Front. Cell. Infect. Microbiol*. 7:477
164. Barker J, Brown MR. 1994. Trojan horses of the microbial world: protozoa and the survival of bacterial pathogens in the environment. *Microbiology* 140(6):1253–59
165. Scheid P. 2014. Relevance of free-living amoebae as hosts for phylogenetically diverse microorganisms. *Parasitol. Res.* 113(7):2407–14
166. Richards AM, Von Dwingelo JE, Price CT, Abu Kwaik Y. 2013. Cellular microbiology and molecular ecology of *Legionella*–amoeba interaction. *Virulence* 4(4):307–14
167. Broderick NA. 2015. A common origin for immunity and digestion. *Front. Immunol.* 6:72
168. Casadevall A, Fu MS, Guimaraes AJ, Albuquerque P. 2019. The 'amoeboid predator–fungal animal virulence' hypothesis. *J. Fungi* 5(1):10
169. Moliner C, Fournier P-E, Raoult D. 2010. Genome analysis of microorganisms living in amoebae reveals a melting pot of evolution. *FEMS Microbiol. Rev.* 34(3):281–94
170. Schulz F, Martijn J, Wascher F, Lagkouvardos I, Kostanjšek R, et al. 2016. A Rickettsiales symbiont of amoebae with ancient features. *Environ. Microbiol.* 18(8):2326–42
171. Schmitz-Esser S, Tischler P, Arnold R, Montanaro J, Wagner M, et al. 2010. The genome of the amoeba symbiont "*Candidatus* Amoebophilus asiaticus" reveals common mechanisms for host cell interaction among amoeba-associated bacteria. *J. Bacteriol.* 192(4):1045–57
172. Ensminger AW. 2016. *Legionella pneumophila*, armed to the hilt: justifying the largest arsenal of effectors in the bacterial world. *Curr. Opin. Microbiol.* 29:74–80
173. Zhao B, Xu W, Rong B, Chen G, Ye X, et al. 2018. H3K14me3 genomic distributions and its regulation by KDM4 family demethylases. *Cell Res*. 28(11):1118–20
174. Grzybowski AT, Chen Z, Ruthenburg AJ. 2015. Calibrating ChIP-Seq with nucleosomal internal standards to measure histone modification density genome wide. *Mol. Cell* 58(5):886–99
175. Bansal A, Singh TR, Chauhan RS. 2017. A novel miRNA analysis framework to analyze differential biological networks. *Sci. Rep.* 7(1):14604

176. Schones DE, Cui K, Cuddapah S, Roh T-Y, Barski A, et al. 2008. Dynamic regulation of nucleosome positioning in the human genome. *Cell* 132(5):887–98
177. Eylert E, Herrmann V, Jules M, Gillmaier N, Lautner M, et al. 2010. Isotopologue profiling of *Legionella pneumophila*: role of serine and glucose as carbon substrates. *J. Biol. Chem.* 285(29):22232–43
178. Alves TC, Pongratz RL, Zhao X, Yarborough O, Sereda S, et al. 2015. Integrated, step-wise, mass-isotopomeric flux analysis of the TCA cycle. *Cell Metab.* 22(5):936–47
179. Grankvist N, Watrous JD, Lagerborg KA, Lyutvinskiy Y, Jain M, Nilsson R. 2018. Profiling the metabolism of human cells by deep ^{13}C labelling. *Cell Chem. Biol.* 25(11):1419–1427
180. Dickson RP, Erb-Downward JR, Huffnagle GB. 2015. Homeostasis and its disruption in the lung microbiome. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 309(10):L1047–55
181. Pérez-Cobas AE, Buchrieser C. 2019. Analysis of the pulmonary microbiome composition of *Legionella pneumophila*-infected patients. *Methods Mol. Biol.* 1921(4):429–43
182. Pham TAN, Lawley TD. 2014. Emerging insights on intestinal dysbiosis during bacterial infections. *Curr. Opin. Microbiol.* 17:67–74
183. Hadifar S, Fateh A, Yousefi MH, Siadat SD, Vaziri F. 2019. Exosomes in tuberculosis: still terra incognita? *J. Cell. Physiol.* 234(3):2104–11
184. Uversky VN. 2017. Intrinsically disordered proteins in overcrowded milieu: membrane-less organelles, phase separation, and intrinsic disorder. *Curr. Opin. Struct. Biol.* 44:18–30
185. Onfelt B, Nedvetzki S, Benninger RKP, Purbhoo MA, Sowinski S, et al. 2006. Structurally distinct membrane nanotubes between human macrophages support long-distance vesicular traffic or surfing of bacteria. *J. Immunol.* 177(12):8476–83
186. Baidya AK, Bhattacharya S, Dubey GP, Mamou G, Ben-Yehuda S. 2018. Bacterial nanotubes: a conduit for intercellular molecular trade. *Curr. Opin. Microbiol.* 42:1–6
187. Gottschling DE, Nyström T. 2017. The upsides and downsides of organelle interconnectivity. *Cell* 169(1):24–34
188. Staerck C, Gastebois A, Vandeputte P, Calenda A, Larcher G, et al. 2017. Microbial antioxidant defense enzymes. *Microb. Pathog.* 110:56–65
189. Islinger M, Voelkl A, Fahimi HD, Schrader M. 2018. The peroxisome: an update on mysteries 2.0. *Histochem. Cell Biol.* 150(5):443–71
190. Whiley H, Bentham R. 2011. *Legionella longbeachae* and legionellosis. *Emerg. Infect. Dis.* 17(4):579–83
191. Brassinga AKC, Kinchen JM, Cupp ME, Day SR, Hoffman PS, Sifri CD. 2010. *Caenorhabditis* is a metazoan host for *Legionella*. *Cell. Microbiol.* 12(3):343–61
192. Fabbi M, Pastoris MC, Scanziani E, Magnino S, Di Matteo L. 1998. Epidemiological and environmental investigations of *Legionella pneumophila* infection in cattle and case report of fatal pneumonia in a calf. *J. Clin. Microbiol.* 36(7):1942–47
193. McDonald R, Schreier HJ, Watts JEM. 2012. Phylogenetic analysis of microbial communities in different regions of the gastrointestinal tract in *Panaque nigrolineatus*, a wood-eating fish. *PLOS ONE* 7(10):e48018
194. Schoebel S, Blankenfeldt W, Goody RS, Itzen A. 2010. High-affinity binding of phosphatidylinositol 4-phosphate by *Legionella pneumophila* DrrA. *EMBO Rep.* 11(8):598–604