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Review

Legionella pneumophila – a human pathogen that co-evolved with fresh water protozoa

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Abstract. The bacterial pathogen *Legionella pneumophila* is found ubiquitously in fresh water environments where it replicates within protozoan hosts. When inhaled by humans it can replicate within alveolar macrophages and cause a severe pneumonia, Legionnaires disease. Yet much needs to be learned regarding the mechanisms that allow *Legionella* to modulate host functions to its advantage and the regulatory network governing its intracellular life cycle. The establishment and publication of the complete genome sequences of three clinical *L. pneumophila* isolates paved the way for major breakthroughs in understanding the biology of *L. pneumophila*. Based on sequence analysis many new putative virulence factors have been identified foremost among them eukaryotic-like proteins that may be implicated in many different steps of the *Legionella* life cycle. This review summarizes what is currently known about regulation of the *Legionella* life cycle and gives insight in the *Legionella*-specific features as deduced from genome analysis.

Keywords. *Legionella pneumophila*, Legionnaires disease, pathogenesis, eukaryotic-like proteins

Introduction

The genus *Legionella* comprises over 48 species with 65 serogroups [1, 2], the majority of which are relatively slow-growing, harmless, ubiquitous, aquatic saprophytes. *Legionellae* are gram-negative, non-spore-forming bacilli belonging to the gamma-subgroup of proteobacteria. Natural freshwater environments are the major reservoirs [3]; however, *Legionella* are widespread in man-made hot-water systems [1]. The demonstration of the ability of *Legionella* to multiply intracellularly in freshwater protozoa as well as in human macrophages [4] has resulted in a new precept in microbiology: bacteria can parasitize protozoa and utilize the same process to infect humans [5–7].

A minority of *Legionella* species are human pathogens and foremost amongst these is *Legionella pneumophila,* the causative agent of Legionnaires' disease, an often fatal pneumonia, if not promptly and correctly diagnosed. *L. pneumophila* was first recognized as a pathogen during a large outbreak at an American Legion convention in Philadelphia in 1976 [8]. Since then many outbreaks and sporadic cases of nosocomial and community-acquired legionellosis have been identified worldwide. Most susceptible to legionellosis are elderly, smokers and immunocom-

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promised persons. The species *L. pneumophila* is responsible for more than 98% of the Legionnaires' disease cases, and about 95% are due to serogroup 1 [9]. In contrast, other species such as *L. anisa*, *L. micdadei*, *L. dumoffii* or *L. feeleii* are rarely pathogenic in humans, although they relatively frequently colonize water distribution systems [9–11].

Legionellosis emerged in the second half of the 20th century partly due to human alterations of the environment. Growth within a broad protozoal spectrum plays a major role in the transmission of *Legionella* in natural and man-made aquatic environments (for a review see [12]). Thus, the presence of *Legionella* in the aquatic environment also appears to depend on the spectrum of host-protozoa present. Whether the adaptation to a specific host or the multiplication capacity in the different protozoal hosts correlates with virulence and epidemiological prevalence of *L. pneumophila* remains to be answered.

L. pneumophila enters the human body through inhalation of contaminated aerosols. It can then reach the alveolar parts of the lungs where it is engulfed by macrophages. Within minutes a phagosome with a single membrane is formed that does not enter the classical endolysosomal pathway. *L. pneumophila* remains in the phagosome, and inhibits phagolysosomal fusion and acidification of the phagosome [13, 14]. Instead, *L. pneumophila* establishes phagosomes that are completely isolated from the endosomal pathway but surrounded by endoplasmic reticulum (ER). Within this protected vacuole, *L. pneumophila* proliferates and, after about 16–20 h, the pathogen vacuoles merge with lysosomes, which provide a nutrient-rich replication niche. However, the ability of *L. pneumophila* to stall or block progression to lysosomes also depends on the host cell, as in primary mouse macrophages the fusion of the *L. pneumophila* vacuole with the lysosomes is readily detected by 15 h after infection [15] but, in human monocytes, fusion with lysosomes is not detected at any time after infection [16]. Finally, a decline of nutrients leads to the transition to the transmissive-phase bacteria [17] that express many virulence-associated traits that promote the release of the bacteria and transmission to a new host cell [17–20]. However, infection of human macrophages appears to be a dead end since transmission of *Legionella* among humans has not been observed so far.

In this review we focus on *Legionella* life cycle-related functions and their regulation as well as on known and putative virulence factors. Emphasis is given on the analysis and comparison of the three genome sequences of *L. pneumophila* strain Paris, Lens and Philadelphia 1 published recently [21, 22], by highlighting characteristic features putatively related to virulence and the *Legionella* life cycle. Finally, some of the known virulence factors, particularly secreted and surface proteins, are discussed and future perspectives with respect to *Legionella* genomics are presented.

The biphasic life cycle of *L. pneumophila*

In vitro studies in broth culture demonstrated that the life cycle of *L. pneumophila* consists of at least two discrete phases: post-exponential phase bacteria, which evade from the host cell and which are flagellated, highly motile and virulent, and replicative bacteria that are found in the replicative vacuole that are sodium resistant and unflagellated [17–20]. These two phases also exist *in vivo* during infection of *Acanthamoeba castellanii*, as judged from intracellular gene expression profiles of *L. pneumophila* [23]. However, under certain conditions, intracellular *L. pneumophila* may differentiate to a spore-like 'mature intracellular form' [24] and *L. pneumophila* can also reside in biofilms [25–27]. Thus, the *L. pneumophila* life may be more sophisticated depending on the conditions studied.

A complex regulatory network governs the *Legionella* life cycle switch

Regulation of the transition from the replicative to the transmissive phase in *L. pneumophila* requires complex regulatory networks, which are only partially understood. By analogy to *Escherichia coli,* where the regulator of glycogen accumulation [28], the small RNA-binding protein carbon storage regulator A (CsrA) and the non-coding repressor RNA *csrB* are known to function as a global post-transcriptional regulatory system, it is thought that a CsrA homolog of *E. coli* present in *L. pneumophila* represses the expression of transmission traits [29, 30]. For the transition to the transmissive phase the CsrA repression must be relieved [29, 30]. Csr systems control gene expression post-transcriptionally and are found in several bacteria [28, 31]. In *E. coli*, CsrA regulates translation initiation of several genes by binding to the mRNA. Two regulatory RNAs, *csrB* and *csrC* have been identified that sequester CsrA and compensate its regulatory activity [28, 31]. In *E. coli*, like in a variety of other gram-negative bacteria, a two-component signal transduction system named BarA/UvrY regulates expression of *csrB* [28, 31, 32].

L. pneumophila possesses the two-component system LetA/LetS that has homology to BarA/UvrY or GacA/GacS in *Pseudomonas aeruginosa* [31, 33, 34].

It has been demonstrated that LetA is necessary for intracellular multiplication in *Acanthamoeba castellanii*, a natural host of *L. pneumophila* [34]. Furthermore, *letA* mutants show a marked defect in macrophage infection; however, intracellular replication in A/J mouse bone marrow-derived macrophages of *letA* mutants that survived infection was normal. Thus, replication in A/J macrophages appeared to be independent of the putative LetA/LetS regulon [33]. Molofsky and colleagues [30] showed that, during nutritional deprivation, LetA/LetS induces the expression of transmissive traits by relieving CsrA repression. Accordingly, *L. pneumophila* LetA/LetS is predicted to induce the expression of a regulatory RNA that alleviates CsrA binding to mRNAs. This putative regulatory RNA has not yet been identified, likely because the nucleotide sequence of these RNAs has not been conserved in evolution, whereas the structure and function has. Given the critical role played by CsrA in several bacterial species, considerable effort has been made to identify these putative regulatory RNAs in different bacteria. Recently, Kulkarni and colleagues [35] have developed a computer program (CSRNA_FIND) designed to locate potential CsrA-regulating small RNAs in bacteria. This approach allowed the prediction of two putative CsrA-regulating sRNA genes for *L. pneumophila*, named *rsmY* and *rsmZ*. Future experiments will determine whether these are interacting with CsrA or not. Furthermore, differentiation in *L. pneumophila* caused by LetA/LetS is enhanced by the *letE* locus, predicted to encode for a 12-kDa protein [33, 36]. As deduced from transcriptome analysis and motif search, *letE* expression might be regulated by RpoN, as the *letE* promoter region shows an RpoN consensus sequence motif [23].

Two-component systems continuously monitor external conditions and rapidly respond to extracellular and cytoplasmic signals reflecting changes in the environment. The *L. pneumophila* LetA/LetS two-component system probably responds to ppGpp [30, 33, 36]. This is in accordance with the observation that the entry into the transmissive phase in *L. pneumophila* is initiated by a mechanism called 'stringent response' [37]. Under conditions of nutrient starvation, signaled probably by low amino acid levels [38], RelA synthesizes the alarmone molecule (p)ppGpp [33, 37, 39]. However, this may not be the only signal and regulatory mechanism governing the biphasic life cycle. Another factor involved in this regulation is the alternative sigma factor RpoS, although its implication and exact role is not yet understood, as contradictory results are reported. RpoS seems to be involved in replicative phase regulation cascades, as it was shown to be essential for intracellular survival and replication [40, 41]. Conversely, the expression of RpoS increases during the stationary phase, and RpoS was shown to coordinate the expression of several transmissive phase traits of *L. pneumophila* [18, 30, 42–45]. By analogy to *E. coli*, it has been speculated that the accumulation of ppGpp increases the amount of the alternative sigma factor RpoS, but recent reports did not find a link between the stringent response and RpoS in *L. pneumophila* [41]. However, a link between LetA/LetS and RpoS may exist [34, 43, 44, 46], but the mode of interaction remains speculation (Fig. 1).

As described above, the *L. pneumophila* life cycle switch to transmissive phase correlates with the expression of many virulence traits and high motility. The regulatory pathways of most of the virulence genes of *L. pneumophila* are not yet well understood. Probably the best characterized is the regulation of motility, the flagellum regulatory cascade, in part due to the fact that these regulatory mechanisms in gram-negative bacteria are highly conserved [47].

The flagella regulatory cascade in *L. pneumophila*

Flagellation of *L. pneumophila* is dependent on its life cycle, as full motility occurs only in the transmissive phase. Thus, synthesis of flagella is dependent on the above-described regulatory cascade allowing the switch from the replicative to the transmissive phase. The proposed model of the flagella regulatory cascade is based on the expression profiles of flagella genes during intracellular growth of *L. pneumophila* in *A. castellanii* and the literature. When the bacteria enter transmissive phase, the first level of the flagellum regulatory cascade is governed by the alternative sigma factor RpoN (σ^{54}) in a common action with the transcriptional activator protein FleQ. Together, they positively regulate expression of *fleN*, *fliM*, *fleSR* and presumably 27 additional genes encoding proteins involved in flagellum biogenesis. Most of these proteins participate in the biogenesis of the flagellum basal body and hook. In analogy to *P. aeruginosa,* the *L. pneumophila* homolog FleN may be the anti-activator of FleQ [48], and FleS/FleR a two-component system involved in the flagellum regulation cascade [23, 49]. The sensor kinase FleS probably responds to an external signal subsequently activating FleR by phosphorylation. Because FleR possesses an RpoN-interacting domain, it is supposed that $FleR^{P}$ triggers, together with RpoN, the expression of *motAB*, *flgMN* and *fliA* (encoding the alternative σ^{28} factor FliA). FliA controls the last step of the flagellum synthesis, including flagellin (FlaA) expression [23, 50]. As in *Salmonella typhimurium*, the

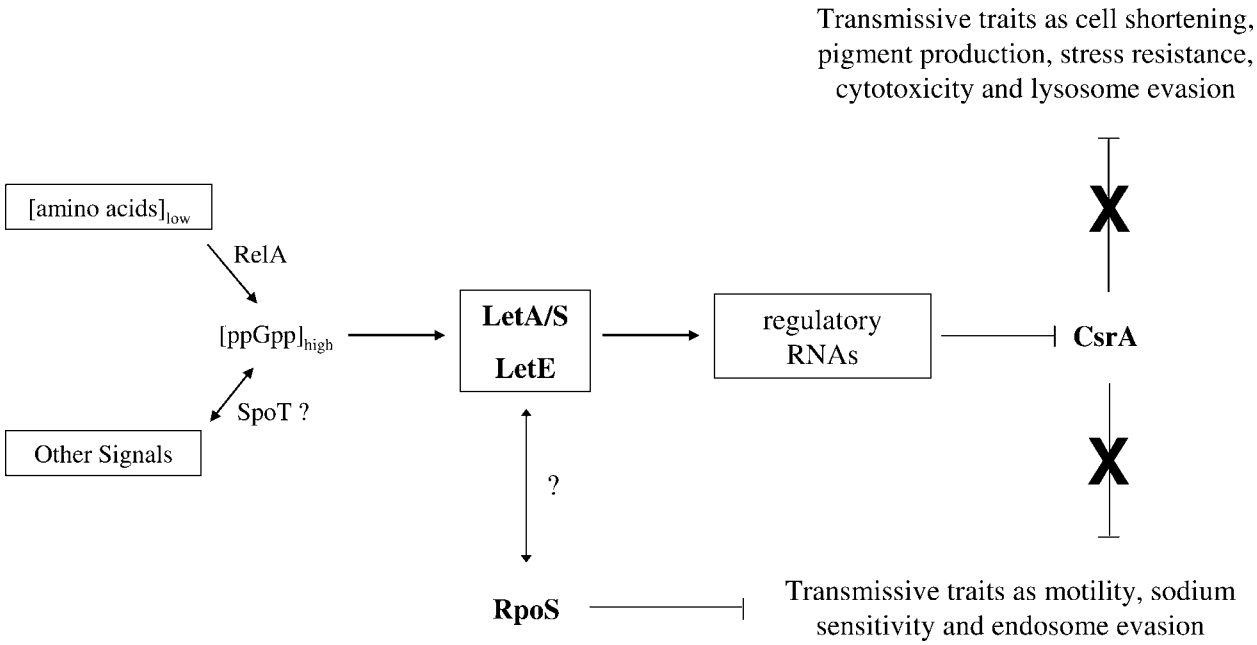

Figure 1. Model for the transition from replicative to the transmissive phase in *Legionella pneumophila*. Nutrition starvation causes synthesis of the alarmone ppGpp. A high ppGpp level probably leads, mediated by the two-component system LetA/S, to transcription of one or several regulatory RNAs. These regulatory RNAs are thought to bind and antagonize the activity of CsrA. Thus, repression of transmissive traits is abolished. Additionally, RpoS is involved in the expression of some transmissive phase traits (adapted from [30, 49]). The contribution of the stringent response signal transduction pathway to the *L. pneumophila* life cycle has been established in broth models, but its role during intracellular growth has not been tested rigorously, yet.

activity of FliA may be controlled by a post-transcriptional mechanism. FlgM, an anti-σ^{28} factor may bind to FliA and in this way prevent activation of FliA-dependent gene expression. Additionally, FlgN (the chaperone protein of FlgKL) may activate *flgM* mRNA in the absence of FlgKL. Consequently, once the flagellum hook-basal body structure is built, FlgM will be exported and relieve repression of FliA so that the flagellum can be completed [51] (Fig. 2).

The flagellum of *L. pneumophila* mediates motility necessary for the transmission to a new host, but flagellin, encoded by the gene *flaA,* also mediates cytotoxicity and induces macrophage death [52]. Furthermore, flagellin is responsible for growth restriction of *L. pneumophila* in mouse macrophages, as cytosolic flagellin is recognized by the macrophages that responds by a Naip5 and a caspase 1-dependent mechanism to restrict bacterial replication and release proinflammatory cytokines. In agreement, flagellin-deficient *Legionella* mutants are able to evade caspase-1- and Naip5-mediated macrophage immunity [53, 54]. Moreover, other virulence-associated traits might be regulated by the different regulators involved in the flagella regulatory cascade, given that mutants not expressing flagella have lower virulence [50, 52, 55]. Some hints as to which factors are involved, come from a transcriptome study, since a number of FliA-dependent genes identified are predicted to affect the first steps of cell invasion [23].

Genomics of *Legionella*

Understanding the complex functional mechanisms and regulatory networks that govern the *L. pneumophila* life cycle requires the global and parallel analysis of different stages in the *Legionella* life cycle. Genome analysis and subsequent application of DNA microarrays have already yielded an enormous amount of new knowledge about many bacterial pathogens, and has also brought first insights into intracellular gene expression of *L. pneumophila* [23]. The completion, analysis and publication of three different *L. pneumophila* genomes [21, 22] provide the basis for the application of new powerful approaches for understanding of the biology of this organism.

The *Legionella pneumophila* genome sequence

The complete genome sequences of *L. pneumophila* strains Paris, Lens and Philadelphia 1 comprise 3503610 base pairs (bp), 3345687 bp and 3397754 bp, respectively, with an average G+C content of 38%. The genomes contain ~3000 genes of which

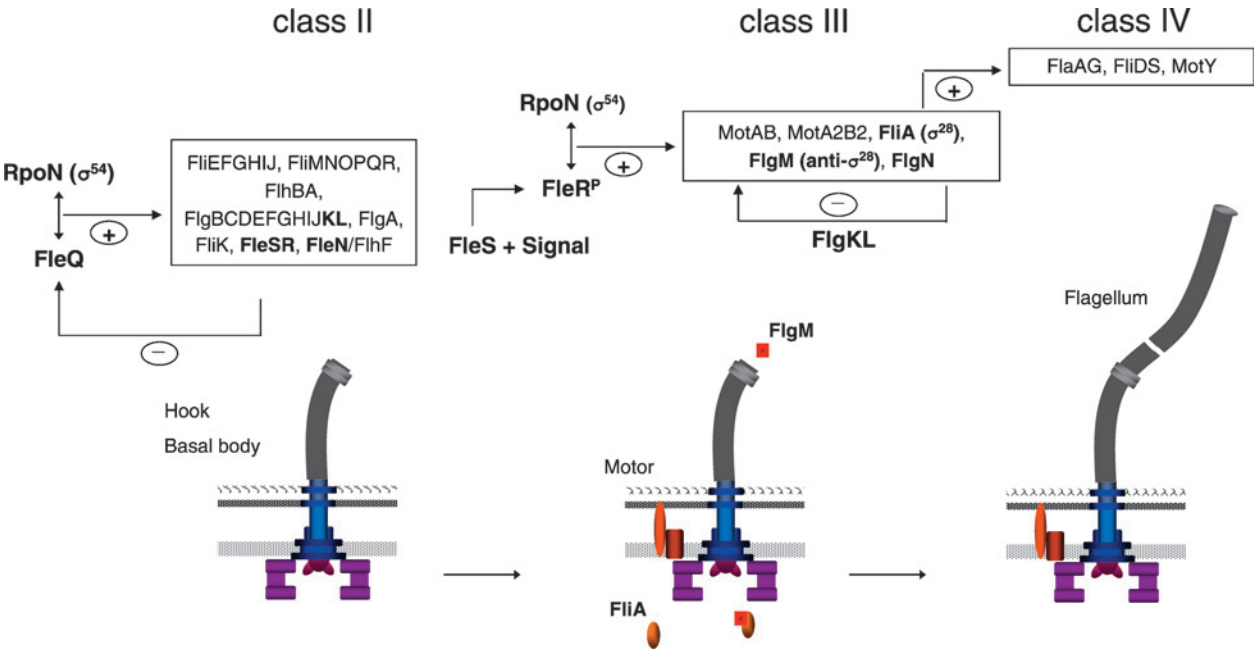

Figure 2. Model of the hierarchy in the transcriptional regulation of the flagellar. RpoN (σ_{54}) and FleQ control class II gene expression. The class II gene *fleN* encodes a putative anti-activator of FleQ and *fleSR* encodes a two-component system. The sensor FleS could be activated by an external signal, and subsequently activates FleR via phosphorylation. $FleR^P$ probably triggers together with RpoN the expression of class III genes as *fliA* (encoding the alternative σ^{28} factor FliA) and *flgM*. The FlgM, an anti-σ^{28} factor binds to FliA and thus antagonizes activation of FliA-dependent gene expression. FlgM might be controlled by another class III protein, FlgN, a chaperone for the hook-associated proteins FlgK and FlgL, involved in a sensing mechanism to determine the stage of flagellar. When the flagellum hook-basal body structure is built, the FlgM is exported and repression of FliA is removed.

~57% are located on the leading strand. No function could be predicted for 42.1% of the *L. pneumophila* Paris, 44.1% of the *L. pneumophila* Lens and for 28% of the *L. pneumophila* Philadelphia 1 genes, a proportion similar to that found in most other sequenced bacterial genomes. Many of the predicted genes (21% for strain Paris, 20.4% for strain Lens, 17% for strain Philadelphia 1) are unique to the genus *Legionella*.

Co-evolution with protozoa is reflected in the *L. pneumophila* genome sequence

Freshwater protozoa are essential for the growth of *Legionella*. The intracellular growth of *L. pneumophila* within aquatic protozoa seems to have generated a pool of virulence traits during evolution, which allow *Legionella* to also infect human cells. This is reflected in the genome sequence of this pathogen, as it codes an unexpected high number and variety of eukaryotic-like proteins, which may be able to interfere in all different stages of the intracellular cycle by mimicking functions of eukaryotic proteins [21, 56].

Proteins that we defined as eukaryotic-like show the strongest similarity to eukaryotic proteins or contain motifs known to be implicated in protein-protein interactions mostly or uniquely present in eukaryotes [21]. A first screen for such proteins identified 113 and 109 candidates in strain Paris and Lens, respectively. Those proteins that showed at least 20% amino acid identity over more than a third of the length of the most similar protein were defined as eukaryotic-like (Table 1). A search against Pfam and Prosite databases identified 38 additional genes containing motifs that are present mostly in eukaryotes (ankyrin, sel-1, sec7, F-box, U-box) (Table 2) [21]. Recently, de Felipe and colleagues [57] published a similar search. However, the number of proteins reported in this study differs, due to different criteria used for the definition of eukaryotic-like genes. According to de Felipe and colleagues [57], 50 eukaryotic-like proteins are present in the *L. pneumophila* genomes, 28 of which are common with those reported by Cazalet and colleagues [21]. The 22 additional proteins show a similarity to eukaryotic proteins that is lower than the above-mentioned cut-off and contain essentially leucine-rich repeats and coiled-coil domains, domains commonly found in several bacterial species. Thus, the comparison and discussion in this review will concentrate on the eukaryotic-like genes reported previously [21, 56]. As nearly all eukaryotic-like proteins are conserved in the three sequenced *L. pneumophila* genomes, it is evident that most of these proteins should fulfill

Table 1. Proteins with the highest similarity score to eukaryotic proteins and their distribution in the three sequenced strains

Predicted product	*L.p.* Paris	G-C	*L.p.*Lens	G-C	*L.p.*Phila	G-C	Best hit BLASTp
RNA binding protein precursor	*lpp0321*	34%	–	–	*lpg0251*	37%	(AAL07519 *Solanum tubeosum*)
Hypothetical protein	*lpp0358*	38%	*lpl0334*	38%	*lpg0282*	39%	(EAA20288.1 *Plasmodium yoelii yoelii*)
Hypothetical protein	*lpp0379*	39%	*lpl0354*	40%	*lpg0301*	40%	(CAD21525.1 *Taenia solium*)
Glucoamylase	*lpp0489*	39%	*lpl0465*	39%	*lpg0422*	39%	(P42042 *Arxula adeninivorans*)
Phytanoyl coA dioxygenase	*lpp0578*	36%	*lpl0554*	37%	*lpg0515*	37%	(EAA70100.1 *Gibberella zeae*)
Hypothetical protein	*lpp0634*	39%	*lpl0618*	39%	*lpg0584*	39%	(XP_306643.1 *Anopheles gambiae*)
ExoA exodeoxyribonuclease III	*lpp0702*	39%	*lpl0684*	39%	*lpg0648*	39%	(EAA20230.1 *Plasmodium yoelii yoelii*)
Cytokinin oxidase	*lpp0955*	39%	*lpl0925*	39%	*lpg0894*	40%	(NP_484368.1 *Nostoc sp.*)
DegP protease	*lpp0965*	39%	*lpl0935*	40%	*lpg0903*	40%	(NP_189431.2 *Arabidopsis thaliana*)
Ecto nucleoside triphosphate diphosphohydrolase (apyrase)	*lpp1033*	40%	*lpl1000*	39%	*lpg0971*	40%	Nucleoside phosphatase signature conserved (Q9MYU4 *Sus scrofa*)
Ca^{2+}-transporting ATPase	*lpp1127*	37%	*lpl1131*	37%	*lpg1126*	38%	(AAB81284.1 *Paramecium tetraurelia*)
Pyruvate decarboxylase	*lpp1157*	39%	*lpl1162*	39%	*lpg1155*	40%	(AAB16855.1 *Arabidopsis thaliana)*
Uridine kinase	*lpp1167*	33%	*lpl1173*	34%	*lpg1165*	34%	(AAM09314.2 *Mus musculus*)
Serine threonine protein kinase	*lpp1439*	36%	*lpl1545*	35%	*lpg1483*	36%	Conserved domain
Thiamine biosynthesis protein NMT-1	*lpp1522*	38%	*lpl1461*	39%	*lpg1565*	40%	(AAC64375.1 *Botryotinia fuckeliana*)
PurC	*lpp1647*	38%	*lpl1640*	39%	*lpg1675*	40%	(AAR06292.1 *Nicotania tabacum*)
Uracyl DNA glycosylase	*lpp1665*	36%	*lpl1659*	36%	*lpg1700*	37%	(EAA36774.1 *Giardia lamblia*)
Nuclear membrane binding protein	*lpp1824*	34%	–	–	–	–	(NP_082559.1 *Mus musculus*)
Ectonucleoside triphosphate diphosphohydrolase (apyrase)	*lpp1880*	39%	*lpl1869*	39%	*lpg1905*	40%	(CAE70887.1 *Caenorhabditis briggsae*)
Chromosome condensation 1-like	*lpp1959*	41%	*lpl1953*	38%	*lpg1976*	43%	Pattern of chromosome condensation regulator conserved
Sphingosine-1-phosphate lyase	*lpp2128*	41%	*lpl2102*	41%	*lpg2176*	40%	(NP_775139.1 *Rattus norvegicus*)
SAM dependent methyltransferase	*lpp2134*	35%	*lpl2109*	36%	*lpg2182*	36%	(BAC98835.1 *Bombyx mori*)
Cytochrome P450	*lpp2468*	39%	*lpl2326*	39%	*lpg2403*	38%	(NP_487786.1 *Nostoc sp.*)
Ser/Thr protein kinase domain	*lpp2626*	32%	*lpl2481*	32%	*lpg2556*	32%	conserved domain
SAM dependent methyltransferase	*lpp2747*	35%	*lpl2620*	35%	*lpg2693*	36%	(EAA20288.1 *Plasmodium yoelii yoelii*)
Phytanoyl-coA dioxygenase	*lpp2748*	36%	*lpl2621*	36%	*lpg2694*	36%	(XP_372144.1 *Homo sapiens*)
NuoE NADH dehydrogenase I chain E	*lpp2832*	38%	*lpl2701*	38%	*lpg2785*	37%	(BAA25988.1 *Homo sapiens*)
6-pyruvoyl-tetrahydropterin synthase	*lpp2923*	34%	*lpl2777*	35%	*lpg2865*	35%	(NP_703938.1 *Plasmodium falciparum*)
Zinc metalloproteinase	*lpp3071*	38%	*lpl2927*	38%	*lpg2999*	38%	(AAF56122.1 *Drosophila melanogaster*)
Hypersensitive induced response protein	*plpp0050*	36%	–	–	–	–	(AAN17462.1 *Hordeum vulgare subsp. Vulgare*)

lpp, *lpl*, *lpg* indicate predicted coding sequences of *L. pneumophila* strain Paris, Lens and Philadelphia, respectively; gray lines indicate proteins that are also mentioned in Table 2 because of their conserved eukaryotic domains; protein accession numbers are given in parentheses. Eukaryotic-like was defined as a protein having the best BLASTp hit(s) to an eukaryotic protein and that shared more than 20% amino acid identity over more than a third of the length with this most similar protein.

important functions (Tables 1 and 2). Recently, analysis of the gene content of 180 strains by DNA-DNA hybridization showed that the eukaryotic-like genes identified during sequence analysis are, with minor exceptions, conserved in all the strains tested [58].

What could the function of these eukaryotic-like proteins be? They may have a role in different regulatory pathways or may be secreted into the eukaryotic cell and then be implicated in different stages of the intracellular life cycle of *Legionella* such as invasion, trafficking into host cell, modulation of host cell functions and evasion.

As an example, the *L. pneumophila* genome contains F-box- and U-box-domain-containing proteins. These domain-containing proteins may allow *L. pneumophila* strains to interfere with the ubiquitin machinery of eukaryotic cells. Three F-box proteins, one of which is specific for each strain, are present in strains Paris and Philadelphia 1, strain Lens contains two F-box proteins (Table 2). Strain Philadelphia 1 and Paris encode the first U-box protein identified in a pro-

Table 2. *L. pneumophila* proteins encoding domains preferentially found within eukaryotic proteins and their distribution in the three sequenced strains.

L.p. Paris	*L. p.*Lens	*L.p.*Philadelphia	*Motif identified*
EnhC (Lpp2692)	EnhC (Lpl2564)	EnhC (Lpg2639)	21 sel-1 domains
LidL(Lpp1174)- EnhC paralog	LidL (Lpl1180) – EnhC paralog	LidL (Lpg1172) – EnhC paralog	6 sel-1 domains
Lpp1310- EnhC paralog	Lpl1307 – EnhC paralog	Lpg1356- EnhC paralog	4 sel-1 domains
Lpp2174- EnhC paralog	Lpl1303 – EnhC paralog	Lpg2222 – EnhC paralog	3 sel-1 domains
–	Lpl1059 – EnhC paralog	Lpg1062 – EnhC paralog	7 sel-1 domains
RalF (Lpp1932)	RalF Lpl1919	RalF (Lpp1950)	sec7 domain
Lpp0267	Lpl0262	Lpg0208	Ser/Thr kinase domain
Lpp2626	Lpl2481	Lpg2556	Ser/Thr kinase domain
Lpp1439	Lpl1545	Lpg1483	Ser/Thr kinase domain
Lpp2065	Lpl2055	Lpg2214	Ankyrin repeat
Lpp0037	Lpl0038	Lpg0038	Ankyrin repeat
pLpp0098	–	–	Ankyrin repeat
Lpp2058	Lpl2048	–	Ankyrin repeat
Lpp0750	Lpl0732	Lpg0695	Ankyrin repeat
Lpp2061	Lpl2051	–	Ankyrin repeat
Lpp2270	Lpl2242	Lpg2322	Ankyrin repeat
Lpp0503	Lpl0479	Lpg0436	Ankyrin repeat
Lpp1905	–	–	Ankyrin repeat
Lpp1683	Lpl1682	Lpg1718	Ankyrin repeat + SET
Lpp2248	Lpl2219	Lpg2300	Ankyrin repeat
Lpp0202	–	–	Ankyrin repeat
Lpp0469	Lpl0445	Lpg0403	Ankyrin repeat
Lpp2517	Lpl2370	Lpg2452	Ankyrin repeat
Lpp1100	–	–	Ankyrin repeat
Lpp0126	Lpl0111	Lpg0112	Ankyrin repeat
Lpp0356	–	–	Ankyrin repeat
Lpp2522	Lpl2375	Lpg2456	Ankyrin repeat
Lpp0547	Lpl0523	Lpg0483	Ankyrin repeat
–	Lpl1681	–	Ankyrin repeat
–	Lpl2344	–	Ankyrin repeat
–	Lpl2058	Lpg2128	Ankyrin repeat
–	–	Lpg0402	Ankyrin repeat
–	–	Lpg2131	ankyrin repeat
Lpp2082	Lpl2072	Lpg2144	F-box domain + ankyrin repeat
Lpp2486	–	–	F-box domain + coiled-coil
–	–	Lpg2224	F-box domain
Lpp0233	Lpl0234	Lpg0171	F-box domain
Lpp2887	–	Lpg2830	2 U-box domains

Lpp, Lpl, Lpg indicate predicted proteins of *L. pneumophila* strain Paris, Lens and Philadelphia, respectively.

karyotic genome. Ubiquitination is a protein modification generally used by cells to tag proteins that are destined for proteasomal degradation. For example, F-box proteins assembled into Skp1–Cul1–F-box protein (SCF)-ubiquitin ligase complexes determine which substrate will be targeted for ubiquitination and subsequent proteolysis by the proteasome. It is tempting to assume that *L. pneumophila* is able to modulate the eukaryotic ubiquitination machinery by tagging host proteins for ubiquitination, which are in turn degraded by the proteasome.

Another interesting example is the high number of ankyrin repeat proteins present in the *L. pneumophila* genomes. This protein family is the most prominent and heterogeneous protein family of the eukaryotic-like proteins, as only 12 ankyrin repeat proteins are shared by all three isolates. Strain Paris, Lens and Philadelphia 1 encode each 6, 4 or 2 specific ankyrin proteins, respectively (Table 2). Large families of ankyrin repeat proteins are so far only identified in *C. burnetii* [59], *Wolbachia pipientis* [60] and *Rickettsia felis* [61], all intracellular bacteria. Eukaryotic ankyrin proteins are multifunctional and involved in many cellular pathways such as interaction with the cytoskeleton [62], or targeting proteins to the plasma membrane or to the ER [63].

The presence of three additional proteins with homologies to eukaryotic proteins: a sphingosine-1-phosphate lyase and two secreted apyrases, which are conserved in all three *L. pneumophila* genomes is an exciting finding (Table 1). In eukaryotes, sphingosine-1-phosphate lyase is part of the sphingomyelin degradation pathway. Sphingosine kinase phosphorylates the catabolite of ceramide sphingosine into sphingosine-1-phosphate, which is cleaved irreversibly by sphingosine-1-phosphate lyase. As the balance of both ceramide and sphingosine-1-phosphate is important for inducing authophagy or apoptosis [64], the presence of this protein in the *Legionella* genomes suggests it may influence the autophagy machinery, which recognizes *Legionella* phagosomes for lysosome delivery [20, 65]. To affect the eukaryotic cell, *L. pneumophila* must translocate this protein into the host. However, de Felipe and colleagues [57] were unable to show translocation of the *L. pneumophila* homolog of sphingosine-1-phosphate lyase (Spl) into the host cell.

The two apyrases identified in *Legionella* are not found in any other prokaryote to date (Table 1). These enzymes have been isolated in authophagy vacuoles [66] and may thus influence the *L. pneumophila* phagosome by decreasing the concentration of NTPs and NDPs. A recent study reports that one of the two apyrases (Lpp1880/Lpl1869/Lpg1905) appears to be involved in infection of the host [67].

Ser/Thr protein kinases (STPKs) are known to be essential virulence factors in other pathogens. In *Yersinia pseudotuberculosis*, the STPK named YpkA was shown to be translocated into the host cells, where it counteracts the host defense by interfering with eukaryotic signal transduction pathways [68, 69], and PknG of *Mycobacterium tuberculosis* is secreted into macrophage phagosomes, inhibiting phagosome-lysosome fusion and promoting intracellular survival [70]. The *L. pneumophila* strains encode three eukaryote-like STPKs (Table 2). Within the group of eukaryotic STPKs, two of the STPKs of *L. pneumophila* (Lpp2626/Lpl2481/Lpg2556 and Lpp1439/Lpl1545/Lpg1483) are closest to STPKs from *Entamoeba histolytica* [21]. This suggests that the *L. pneumophila* STPKs may also modulate eukaryotic signal transduction mechanisms and are involved in modulation of host cell trafficking pathways.

Eukaryotic-like proteins have also been identified in other bacterial pathogens such as *S. typhimurium* and *P. aeruginosa* [71, 72]; however, a comparative analysis of *L. pneumophila* and 12 other bacterial pathogens, as well as *E. coli* K12 as reference genome, shows that *L. pneumophila* ranks as one of the pathogens that encodes the most and the widest variety of eukaryotic-like proteins or proteins with eukaryotic domains (Table 3). Thus, the genome sequence reflects the co-evolution of *Legionella* with fresh-water amoebae and suggests that the different eukaryotic-like proteins are important for the intracellular life cycle of *L. pneumophila*.

To act in the eukaryotic cell these proteins should be secreted. This could be achieved by many different secretion systems present in *L. pneumophila*.

Secretion machineries of *L. pneumophila* – central to its life and to pathogenesis

The importance of protein secretion for *L. pneumophila* is highlighted by the presence of a wide variety of secretion systems in the three sequenced strains. Associated with the virulence of *L. pneumophila* are mainly the Dot/Icm type-IV secretion system and the Lsp type II secretion system. However, *L. pneumophila* possesses more secretion systems including a second type-IV system, Lvh, a type I secretion system, Lss, a twin arginine translocation (TAT) pathway and several Tra-like systems. In addition to these conserved secretory pathways, strain Paris encodes a type-V secretion system specific to this strain.

Secretion across the cytoplasmic membrane

All three *L. pneumophila* strains Philadelphia 1, Lens and Paris possess two systems for protein transport across the cytoplasmic membrane. The general secretory pathway (Sec) translocates proteins in an unfolded state. All proteins of the Sec apparatus are encoded by the *L. pneumophila* genome with the exception of SecG. In addition, *L. pneumophila* encodes a TAT pathway for translocation of folded proteins. Contrary to the usual organization of *tatA*, *tatB* and *tatC* in an operon, only the *tatA* and *tatB* genes are co-transcribed and *tatC* is in a different

Table 3. Distribution of eukaryotic-like domains in the genome of the three *L. pneumophila* genomes and among 12 other bacterial genomes.

	F-box proteins	Ser/Thr protein kinases	Sec7 domain	Ankyrin domain	SET domain	U-box proteins
L. pneumophila Paris	3	3	1	20	1	1
L. pneumophila Lens	2	3	1	17	1	0
L. pneumophila Philadelphia 1	2	3	1	13	1	1
Coxiella burnetii RSA 493	0	1	0	16[a]	0	0
Chlamydia pneumoniae AR39	0	2	0	0	1	0
Pseudomonas aeruginosa PAO1	0	2	0	3	1	0
Escherichia coli K12	0	0	0	0	0	0
Listeria monocytogenes EGDe	0	3	0	0	0	0
Salmonella typhimurium LT2	0	0	0	0	0	0
Shigella flexneri 301	0	0	0	1	0	0
Mycobacterium tuberculosis H37Rv	0	8[b]	0	0	0	0
Bartonella henselae Houston-1	0	0	0	0	0	0
Wolbachia pipientis wMel	0	0	0	23[c]	0	0
Rickettsia prowazekii Madrid E	0	0	1	3	0	0
Ricketsia felis URRWXCal2	0	0	1	25	0	0

[a] 13 reported in [59] [b] 11 described in [137]
[c] 23 reported in [60]

location of the *L. pneumophila* chromosome. The TAT secretion system is implicated in biofilm formation, growth under low iron conditions and growth within macrophages and *A. castellanii* [73, 74]. Most TAT substrates are characterized by a double-arginine motif in their signal peptide. Based on this criterion, 20 proteins were identified as putative TAT substrates from analysis of the genome sequence, including 12 transport/binding proteins and lipoproteins and 3 proteins involved in respiration [74]. Secretion of a subunit of cytochrome c oxidase, phospholipase C and LvrE in a TAT-dependent has been demonstrated [73, 74].

A putative type-I secretion system in *Legionella*

L. pneumophila contains a putative type-I secretion system encoded by the *lssXYZABD* genes. This locus encodes a protein of the ATP binding cassette (ABC) protein family, LssB and a protein of the membrane fusion protein (MFP) family LssD. *L. pneumophila* also encodes for TolC-like proteins, the third component of type-I secretion systems. This type-I secretion system might, like in other bacterial pathogens, mediate transport of toxins, degradative enzymes or other virulence factors across both bacterial membranes. It seems to be specific to *L. pneumophila* strains [75]. The *lssXYZABD* locus is highly conserved between the three sequenced strains as well as in *L. pneumophila* strain Corby (94–98% DNA identity; 95–99% protein identity). The downstream *lssE* gene, encoding a putative signaling protein, is more variable (75–96% DNA identity; 70–94% protein identity). In most type-I secretion systems the target protein-encoding genes are located upstream of the secretion machinery itself, but no putative substrate was identified in *L. pneumophila* to date.

A type-II secretion system in *Legionella*

Until now, *L. pneumophila* is the only intracellular pathogen known to encode a type-II secretion system. This secretion system is dependent on the *pilBCD* locus that is involved in the biogenesis of type IV pili and on the *lsp* (*Legionella* secretion pathway) *FGHIJK* locus. The prepilin peptidase PilD, which processes the pseudopilin (LspG,H,I,J,K) assembly into a functional type-II secretion apparatus, the outer membrane secretin LspD, the ATPase LspE and the inner membrane protein LspF are involved in *L. pneumophila* type-II protein secretion. The secretion system is highly conserved in the three sequenced genomes (protein identities of 96–100%) and seems also to be conserved throughout the genus *Legionella* [76]. The Lsp secretion system promotes the ability of *L. pneumophila* to infect both protozoan and macrophage hosts, to grow in the mammalian lung [76] and

to grow at low temperatures [77]. It is involved in secretion of various enzymes (see below: Virulence factors).

Type-IV secretion systems in *Legionella*

Many bacterial pathogens encode type-IV secretion systems. *L. pneumophila* encodes a type-IVB secretion system similar to the Tra/Trb system of IncI plasmids and also type-IVA systems similar to the *Agrobacterium tumefaciens* Vir system. The type-IVB secretion system in *Legionella* is the Icm (intracellular multiplication) [78] and/or Dot (defective organelle trafficking) [79] system. The type-IVA secretion systems are the Lvh (*Legionella vir* homologue) system and several Tra systems, which are homologous to Tra proteins of the *E. coli* F plasmid. Both the Lvh and the Dot/Icm type IV secretion system mediate conjugal DNA transfer [80–83].

The Dot/Icm type-IVB secretion system. The Dot/Icm type-IV secretion system of *L. pneumophila* is probably involved in many different stages of the intracellular life cycle. This macromolecular complex is encoded by 25 genes located on two genomic regions: region I contains seven genes (*icmV*, *W*, *X*; *dotA*, *B*, *C*, *D*), and region II is composed of 18 genes (*icmT*, *S*, *R*, *Q*, *P*, *O*, *N*, *M*, *L*, *K*, *E*, *G*, *C*, *D*, *J*, *B*, *F*, *H*). This system is conserved and present in the same chromosomal location in strains Paris, Lens and Philadelphia 1. Comparison of 18 *L. pneumophila* and 17 other *Legionella* species by sequence and hybridization analysis showed that the *dot/icm* regions are present in all strains sequenced and that their organization is conserved [84]. In the Dot/Icm system of *L. longbeachae* and *L. micdadei*, the global organization is also conserved, but interestingly, in region II in *L. micdadei* the gene *icmR* is replaced by two genes, *migA* and *migB,* which do not show any homology to *icmR*. The same gene is replaced in *L. longbeachae* by a gene called *ligB* [85]. The *icmR* gene encodes the chaperone of IcmQ, a protein that forms pores in lipid membranes, and thus regulates the activity of IcmQ. IcmQ and IcmR are essential components of the Dot/Icm system [86]. A recent analysis of the *icmR-icmQ* region in 29 additional *Legionella* species identified a large hypervariable gene family named functional homologs of *icmR* (*fir*). Despite the highly variable sequence of the *fir* genes, they are located next to *icmQ* in all investigated species, and encode a functionally similar protein that interacts with IcmQ. Thus, co-evolution between the IcmQ and its relevant FIR protein seemed to have occurred, possibly allowing adaptation to various protozoan hosts [87].

The type-IV secretion system encoded by the *dot*/*icm* loci is required for replication of *Legionella* in both host systems, protozoa and human macrophages [88–90] The *icm* gene products participate in establishing a replicative niche in alveolar macrophages after invasion, and it is proposed that this secretion system exports effector molecules into the eukaryotic host cell during infection [86, 91]. However, the Dot/Icm secretion system also mediates an anti-apoptotic signaling cascade in human macrophages [92]. At present, several effectors have been identified (see below: Virulence factors). The first characterized substrate was RalF [93]. Most identified Dot/Icm substrates possess a hydrophobic residue or a proline residue at the third or fourth C-terminal positions, supporting the idea that these residues are critical for secretion by the type-IV system [94].

The Lvh type-IVA secretion system. The second *Legionella* type-IV secretion system, Lvh (*Legionella vir* homologues), is dispensable for intracellular growth in both macrophages and amoebae [95], but it is implicated in host cell infection by *L. pneumophila* at 30°C [96]. This secretion system is encoded by 11 genes, which are located on a plasmid-like element that can exist either integrated in the chromosome or excised as a multicopy plasmid in strains Paris and Philadelphia 1. This region appeared to be stably integrated in strain Lens. However, a 28-bp perfect repeat present in the flanking regions in strain Lens, suggested that it is also able to excise, a fact that was recently confirmed [97]. The *lvh*-element is probably of plasmid origin as it has a higher GC content than the rest of the chromosome and it carries some plasmid related genes. The elucidation of the biological implication of these different forms is another challenging question to answer.

A putative type-V secretion pathway – autotransporter – identified in strain Paris

When comparing the three sequenced *L. pneumophila* isolates, only strain Paris contains a gene (*lpp0779*) that is predicted to encode an autotransporter protein. It shows the typical structure of a type-V secretion protein and accordingly contains three major domains: an N-terminal leader peptide for secretion across the inner membrane, a C-terminal domain that forms a pore in the outer membrane and processes the third domain, a passenger domain to the cell surface [98]. The passenger domain exposed to the cell surface might confer to the protein a function in cell-cell aggregation. Consistent with this, the passenger domain of the autotransporter protein is com-

posed of hemagglutinin repeats, similar to those of the *E. coli* autotransporters AIDA-I and Ag43 [99, 100]. The *L. pneumophila* autotransporter protein may thus mediate adherence to mammalian cells and/or autoaggregation during biofilm formation. However, the RGD interaction motif present in AIDA-I and Ag43 is absent in the *L. pneumophila* autotransporter. The presence of many remnants of insertion sequences upstream of this autotransporter, as well as its GC content of 41%, which is higher than the genome average GC of 38%, suggest acquisition through horizontal gene transfer.

Virulence factors

Although various properties contribute to the virulence of *L. pneumophila*, in this review we mainly focus on secreted factors and surface-associated virulence properties. Putative virulence factors as predicted from the genome sequence of *L. pneumophila* of strains Paris, Lens and Philadelphia 1 are discussed.

Surface proteins of *L. pneumophila*

The ability to attach to and invade the host cell is central to *L. pneumophila*'s ability to cause Legionnaires' disease. A *Legionella* surface protein, major outer membrane protein (MOMP), was shown to be implicated in this process. MOMP binds to the complement component CR3 of human monocytes, and the subsequent uptake of the bacteria occurs by attachment to the complement receptors CR1 and CR3. The MOMP, a porin, is encoded by the *ompS* gene [101, 102]. However, complement-independent binding to macrophages also seems to occur, probably via a bacterial surface protein structure with lectin-like properties that appears intimately associated with carbohydrate or lipid structures located on the outer membrane of the bacteria [103]. Attachment to mammalian and amoebal host cells are also mediated by type-IV pili [104] and the 60-kDa heat shock protein Hsp60 [105]. Another surface protein implicated in adhesion and invasion is the 16-kDa *L. pneumophila*-specific outer membrane protein encoded by the *ligA* gene, which appears to contribute to the initial uptake of *L. pneumophila*. The most prominent effect of the *L. pneumophila ligA* mutant is a nearly completely reduced replication within the natural host *A. castellanii*. Furthermore, deletion of the *ligA* gene results in sodium resistance, decreased cytotoxicity to human monocytes, and decreased hemolytic activity to red blood cells [106].

A very interesting *Legionella* protein exposed on the cell surface is the macrophage infectivity potentiator (Mip). Mip belongs to the enzyme family of FK-506 binding proteins that exhibit peptidyl prolyl cis/trans isomerase (PPIase) activity. The homodimeric protein is involved in the infection of eukaryotic host cells and promotes a *p*-nitrophenol phosphorylcholine (*p*-NPPC) hydrolase activity in culture supernatants [107–112]. Furthermore, Mip contributes to the bacterial dissemination within the lung tissue and the spread of *Legionella* to the spleen, as determined in the guinea pig infection model [113]. Mip binds to the extracellular matrix protein collagen (type I, II, III, IV, V VI) and enables *L. pneumophila* to transmigrate across the lung epithelial barrier. A particular feature of Mip is that it functions in a concerted action with a serine protease, a novel mechanism for bacterial penetration of the lung epithelial barrier [113].

Secreted factors of *L. pneumophila*

Once inside a host cell, *L. pneumophila* avoids the intracellular killing mechanisms that normally follow uptake. Indispensable for establishing a replication vacuole is the Dot/Icm type-IVB secretion system. This secretion system exports virulence factors that inhibit phagolysosomal fusion and reprograms the *Legionella*-containing vacuole. Given the central role of the Dot/Icm system in *Legionella* pathogenesis, many recent studies aimed at identifying and characterizing its substrates. The first characterized substrate was RalF, required for localization of the host GTPase protein ARF-1, a key regulator of vesicle trafficking from the ER, to the phagosomes [93]. RalF is conserved in all three sequenced strains, like LidA, another substrate involved in recruitment of vesicles during vacuole biogenesis and in maintaining integrity of the Dot/Icm complex [114], and LepA and LepB, which are involved in non-lytic egress of *Legionella* from amoeba [115]. A number of candidate effector proteins named SidA-H, were identified in the Philadelphia 1 strain by a two-hybrid screen using IcmG/DotF as bait followed by a screen of proteins transferred inter-bacterially with a Cre/loxP-based protein translocation assay [116]. SidA, SidB, SidC, SidE and SidF are conserved in strains Paris and Lens; in contrast, SidG is missing in strain Lens, SidD is missing in strains Paris and Lens, and SidH is interrupted by two IS elements in strain Paris and missing in strain Lens. SidA, SidB, SidC, SidE and SidF proteins contain a coil-coiled domain, a motif known to be involved in protein-protein interactions.

Recently, it was shown in two independent studies that SidM/DrrA, a guanosine nucleotide exchange factor, is a Dot/Icm substrate protein that is translocated into host cells. Enhanced by LidA, SidM/DrrA recruits Rab1 (a small host GTPase regulating ER-to-Golgi traffic) to *Legionella*-containing vacuoles [117, 118]. Interestingly, the gene encoding SidM/DrrA is absent from strain Paris, again showing that each strain has a slightly different array of effector proteins, perhaps indicating subtle differences in host adaptation. Weber and colleagues [119] proposed that *L. pneumophila* modulates host cell phosphoinositide metabolism and exploits the Golgi lipid second messenger phosphatidylinositol(4) phosphate [PI(4)P] to anchor secreted proteins to the *Legionella*-containing vacuole [119]. Implicated are SidC and SdcA that have been shown to specifically bind PI(4)P. Also secreted via the Dot/Icm secretion system are VipA, VipD and VipF. VipA is a coiled-coil protein and VipF contains an acetyltransferase domain. Both are highly conserved in strains Philadelphia 1, Paris and Lens, while VipD, a protein containing a patatin domain, is absent in strain Lens. These vacuole protein sorting inhibitor proteins (Vip) inhibit lysosomal protein trafficking by different mechanisms [120]. Furthermore, WipA, WipB, YlfA (yeast lethal factor A), YlfB and six additional proteins containing leucine-rich repeats and/or coiled-coil domains were shown to be translocated via the Dot/Icm secretion system [57, 121, 122].

The Dot/Icm type-IVB secretion system plays an indisputable decisive role in allowing *L. pneumophila* to inhibit phagolysosome fusion. However, recently it was shown that LPS-rich outer membrane vesicles are also sufficient to inhibit phagosome-lysosome fusion by a mechanism that is correlated with developmentally regulated modifications of the pathogen's surface, but independent of type-IV secretion [123].

Interestingly, in addition to the Dot/Icm type-IVB secretion system, the type-II secretion system of *L. pneumophila* secretes virulence factors. This is interesting as to date *L. pneumophila* is the only intracellular pathogen known to encode a type-II secretion system. In *L. pneumophila*, protein export via the type-II secretion system includes many virulence-associated proteins. It is known to transport a tartrate-resistant and tartrate-sensitive acid phosphatase, an RNase, the zinc metalloprotease Msp, mono-, di- and triacylglycerol lipases, phospholipases A and C, the lysophospholipase A PlaA, the lysophospholipase A-homolog PlaC and a p-nitrophenyl phosphorylcholine hydrolase [76, 124–128].

Putative virulence factors as deduced from genome analysis

In addition to the eukaryotic-like proteins (see above: Genomics of *Legionella*), proteins with homology to virulence factors of other organisms were identified through sequence analysis. The three sequenced *L. pneumophila* strains encode a protein homologous to the enteropathogenic *E. coli* (EPEC) virulence regulator BipA. These three *Legionella* proteins (encoded by *lpp2875, lpl2737, lpg2822*) are identical and show 68% similarity to the *E. coli* BipA protein. The GC content of the *Legionella bipA* is significantly higher (43%) than that of the rest of the chromosome (38%), indicating horizontal gene transfer. The BipA protein is a member of the ribosome binding GTPase superfamily that is widely distributed in bacteria and plants. In EPEC, BipA is thought to be involved in diverse regulatory cascades. It was shown to regulate the *espC* pathogenicity island, a type-III secretion system and flagella-mediated cell motility [129, 130]. In *L. pneumophila* no homologue of the EspC toxin nor a type-III system is present; however, BipA may be involved in regulation of flagellum expression. In a global gene expression analysis of *L. pneumophila* in its natural host *A. castellanii*, we showed that *bipA* gene expression is growth phase dependent, as its expression is decreased between two- and three-fold in the stationary phase with respect to exponential growth [23]. As flagella expression is also growth phase dependent, the hypothesis that BipA is involved in this complex regulation is possible.

A gene encoding a putative inner membrane efflux protein similar to the virulence factor MviN is present in the three sequenced *L. pneumophila* strains, while strain Philadelphia 1 encodes two orthologs (*lpg1087, lpg2635*). Absence of this protein in *S. typhimurium* results in reduced virulence in a mouse typhoid disease model and a homolog seems to be indispensable in *Sinorhizobium meliloti*, suggesting that MviN of *L. pneumophila* may also be involved in virulence or may be essential [131, 132].

Interestingly, three homologs of the factor for inversion stimulation, Fis, are present in *L. pneumophila*. This exists in only one copy in other genomes including *C. burnetii*, *E. coli* and *Salmonella* species. This DNA-binding protein is involved in many different processes including modulation of transcription. Recently, this protein has been implicated in the control of virulence gene expression in *E. coli* and *S. typhimurium* [133–135] and in biofilm formation in enteroaggregative *E. coli* [136]. As the concentration of the Fis protein varies tremendously between exponential and stationary phases due to the stringent response, these proteins could be implicated in differ-

ential expression of virulence genes during the *Legionella* cell cycle.

Conclusions and perspectives

Over evolutionary time the interactions between aquatic protozoa and *L. pneumophila* selected a pool of virulence traits, which preadapted *L. pneumophila* as human pathogen. Although much has been learned about *L. pneumophila* pathogenesis, more work is needed to elucidate the mode of its interactions with host cells, and further genomic, proteomic and functional analyses will promote our knowledge in this field. Currently, the impact of the metabolic properties of *L. pneumophila* on its pathogenic life cycle is underestimated. *L. pneumophila* replicates intracellularly in protozoa and human macrophages, and therefore it may have evolved mechanisms that counteract the metabolic constraints imposed by phagocytic cells. The ongoing reconstruction of the metabolic capacities of *L. pneumophila* strains Paris, Lens and Philadelphia 1 based on the genome sequences will shed light on the metabolic properties and nutrition requirements of this intracellular pathogen.

Little is also known about the degree of genetic diversity, on the genomic level, among different isolates of *L. pneumophila* of different serogroups or among different *Legionella* species. In particular, the relationship between a specific gene content and virulence is unknown. However, this knowledge is a prerequisite for understanding differences in pathogenicity and the ecology of this organism. It is also the basis for the development of rational and accurate diagnostic tools. In this context, additional sequence information will be very helpful and the ongoing sequencing projects including a fourth *L. pneumophila* isolate, strain Corby by the Institute for Molecular Biotechnology (IMB, Jena, Germany) and the genome sequences of *L. hackeliae* and *L. micdadei* by the IMB and that of an isolate of *L. longbeachae* by the Institut Pasteur (Paris, France) will give enormous new insight. The availability of these different genome sequences of *Legionella* will pave the way for in depth comparative genomics and the identification of currently unknown virulence determinants.

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