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Minireview

Legionella pneumophila adaptation to intracellular life and the host response: Clues from genomics and transcriptomics

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Abstract ***Legionella pneumophila*** **is the causative agent of the pneumonia-like Legionnaires' disease. The bacterium's survival and spread depend on the ability to replicate inside eukaryotic phagocytic cells. A particular feature of *Legionella* is its dual host system allowing the intracellular growth in protozoa like *Acanthamoeba castellanii*, and during infection in human alveolar macrophages. Genome analysis and comparisons as well as expression profiling of the pathogen and the host helped to identify regulatory circuits mediating adaptation of the *L. pneumophila* transcriptome to the intracellular environment and gave clues for the metabolic needs of intracellular *Legionella*. This review will summarize what is currently known about intracellular gene expression of *L. pneumophila*, the transcriptional host response of the model host *Dictyostelium discoideum* and will present hypotheses drawn from these data with respect to subversion of host cell functions and virulence of *L. pneumophila*.**
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1. Introduction

Intracellular pathogens have developed outstanding abilities to survive their encounter with eukaryotic cells. Nearly all enter a membrane-bound vacuole as part of their invasion process, but their subsequent fates vary. Certain bacteria thrive within vacuoles that fuse with lysosomes, others have developed mechanisms to prevent fusion of the pathogen-containing vacuole with lysosomes, thereby maintaining a protected niche inside the host cell. Still others lyse the vacuole and survive and replicate within the cytoplasm.

Legionella pneumophila, the causative agent of the pneumonia-like Legionnaires' disease [1], is an intracellular pathogen that can exploit eukaryotic cells like aquatic protozoa or alveolar macrophages as sites of replication. After internalization by protozoa like *Hartmanella* sp. or *Acanthamoeba castellanii* or macrophages, *L. pneumophila* evades transport to the lysosome and establishes a unique endoplasmic reticulum-derived organelle (reviewed in [2]). To remodel its compartment, *L. pneumophila* uses a type IVB secretion apparatus, encoded by over 20 different *dot* and *icm* genes [3,4] that allows the translocation of effectors into host-cell cytosol [5–9]. Within this protected vacuole *L. pneumophila* replicates, becomes acid tolerant and downregulates virulence factors. As a consequence, the *Legionella* containing vacuole fuses with lysozomes where *L. pneumophila* replicates untill nutrients become limited. Nutrient limitation then leads to the transition to transmissive phase bacteria that express many virulence-associated traits allowing the release and transmission to new host cells [10,11].

A successful transition of *L. pneumophila* from the extracellular to the intracellular environment and from replicative to transmissive phase requires a precise adaptation response to conditions encountered in the host milieu. Although many steps in the intracellular lifestyle of *L. pneumophila* begin to be elucidated, our knowledge about the factors required for intracellular proliferation and the adaptation to the different environments is still rather limited.

The recent determination of the genome sequence of three *L. pneumophila* isolates [12,13] as well as that of *Dictyostelium discoideum* [14], an amoeba used as model host for *L. pneumophila*, opens the way for comparative and functional genomics to study host–pathogen interactions. Once the genome sequence available, microarrays provide a powerful tool to quantify all mRNAs encoded by the genome of a bacterial pathogen and their eukaryotic host cell. Such expression profiles serve as a monitor of the host cell environment as well as an indicator of the bacterial adaptation to its intracellular niche. Microarrays were recently used to study the interaction of *L. pneumophila* with its natural host *Acanthamoeba castellani* [15] as well as to study the response of the amoeba *D. discoideum* to the infection with *Legionella* [16].

In this review, we focus on recent analyses of the genome sequences, the transcriptional response of *L. pneumophila* to growth in *A. castellani* and the response of the model amoeba *D. discoideum* to infection with *Legionella* . These studies illustrate how microarray technology has expanded our understanding of the dialogue between the host and the pathogen.

2. The *L. pneumophila* genome sequence – a prerequisite for transcriptome studies

Determining and understanding the genetic basis of an organism is the challenging goal of genome analyses. The knowledge of the complete genome sequences of four distinct

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L. pneumophila isolates allow us now a better comprehension of the bacterium's lifestyle and host adaptation. The sequenced strains are Philadelphia 1, isolate from the first recognized outbreak of the disease in 1976 [13], and the endemic strain Paris and, the epidemic strain Lens, responsible for two major outbreaks in France in 2001 and 2004 [12]. Recently, the genome of a fourth isolate, that of *L. pneumophila* strain Corby, a virulent strain isolated from a human legionellosis case was sequenced [17]. Analysis of these sequences revealed several specific features of *L. pneumophila*, some of which are, undoubtedly related to its intracellular life.

2.1. High genome plasticity

Comparison of all four *L. pneumophila* genomes shows a very similar GC content of 38% and only a slight difference in size (3.35 Mbp for *L. pneumophila* Lens to 3.6 Mbp for *L. pneumophila* Corby). The sequence and the annotation of the *L. pneumophila* Paris, lens and Philadelphia-1 genomes are accessible at http://genolist.pasteur.fr/LegioList. All are predicted to contain about 3000 protein-coding genes. Subspecies Lens and Paris each contain additionally plasmids of different sizes (Paris 132 kb, Lens 60 kb). Though exhibiting a very similar genome organization, the genomes showed high plasticity and diversity [12,18]. Genome comparison identified a conserved backbone of 2400 genes and about 10% strain-specific genes in each isolate [12,13,17]. Although most of the strain-specific genes code for unknown functions, they also include several GGDEF/EAL regulators, different ankyrin proteins and several restriction modification systems that might be implicated in the adaptation to different environmental niches. In addition, all four strains contain chromosomal regions, which can be excised and maintained as plasmids [12,13,19,20]. These elements contain a type IVA secretion system: the Lvh T4SS in strains Paris, Lens and Philadelphia-1 and two new type IVA secretion systems (*trb*/*tra*) in strain Corby [20]. The Lvh type IVA secretion system was reported to be involved in virulence-related phenotypes under conditions mimicking the spread of Legionnaires' disease from environmental niches [21], thus diversity in this system as well as differences in copy number due to excision which occurs in a growth phase-dependent manner [22], might be of importance for survival and spread of *L. pneumophila* in the environment. Interestingly, although the *lvh* region is highly conserved in the three sequenced genomes the size and sequence of the flanking DNA regions probably acquired by horizontal gene transfer together with the *lvh* region are specific to each strain. The *lvh* region as well as the new type IVA secretion system of strain Corby are inserted in the same tmRNA in strains Paris and Lens but the *lvh* region is inserted in an Arg tRNA in strain Philadelphia 1. Preliminary comparative analysis of *L. pneumophila* with *L. longbeachae* (Cazalet C. et al., unpublished data) supports the high diversity not only of the species *L. pneumophila* but also the genus *Legionella*. One major challenge of microarray-based studies is now the identification of the conserved regulatory networks of the common backbone and the investigation of the impact diversity has in the regulation of environmental adaptation and virulence differences among strains.

2.2. Eukaryotic-like proteins

A key finding of the genome analysis was the identification of a large number of genes encoding eukaryotic-like proteins that are predicted to modulate host cell functions to the pathogen's advantage [12]. Examples for the *L. pneumophila* eukaryotic-like proteins are: two ecto-nucleoside triphosphate diphosphohydrolases (ecto-NTPDases) implicated in entry and replication [23], a sphingosine-1-phosphate lyase probably involved in the modulation of the host cell cycle signaling, a glucoamylase putatively implicated in glycogen degradation or three serine threonine protein kinases thought to be implicated in modulating eukaryotic signal transduction mechanisms and modifying host cell trafficking pathways (Table 1). Furthermore, many proteins with protein domains mainly found in eukaryotes are present in the *L. pneumophila* genomes. Among those are F-box and U-box containing proteins that are probably able to modulate the eukaryotic ubiquitination machinery or a large family of proteins carrying ankyrin domains, one of the most common modular protein–protein interaction motif of eukaryotes mediating many protein–protein interactions [24]. Additional Sel-1 domain proteins like EnhC and LidL that were previously shown to be implicated in interaction with the host cells or in the early signalling events that regulate *L. pneumophila* trafficking in macrophages [25,26] were identified. The high number and wide variety of eukaryotic-like genes reflect the evolution of *L. pneumophila* in association with eukaryotic cells like aquatic protozoa. Comparison of the distribution of eukaryotic-like genes in the published *L. pneumophila* genomes and hybridization results obtained from over 100 *L. pneumophila* strains reveal a strong conservation of these genes in the species *L. pneumophila* [12,18,27,28]. These findings underline the importance of eukaryotic-like proteins for the lifestyle of *Legionella* and raise the question at what stage of the infection the eukaryotic-like proteins act?

3. *Legionella pneumophila* gene expression during infection of *Acanthamoeba castellanii*

In the last years, microarray technology has emerged as the method of choice for large-scale gene expression studies. It provides an efficient and rapid method to investigate the entire transcriptome of a cell. *In vivo* time course transcriptome analysis of bacterial pathogens upon infection of their hosts has been studied for e.g. uropathogenic *Escherichia coli* [29], *Listeria monocytogenes* [30,31], *Mycobacterium tuberculosis* [32] and *Salmonella enterica* sv. Typhimurium [33]. The obtained data brought new insights into the sequential activation of virulence traits and allowed a better characterization of the infectious processes. In order to investigate the intracellular gene expression program of different *L. pneumophila* isolates and to learn about the impact genetic diversity may play, multiple-genome microarrays representing every gene predicted in three sequenced *L. pneumophila* genomes were used to study and compare gene expression of three strains during infection of *A. castellani*, the natural host of *L. pneumophila* [15].

3.1. The intracellular gene expression profile reflects the biphasic life cycle of L. pneumophila

In vitro studies in broth indicated that the life cycle of *L. pneumophila* consists of at least two phases, i.e. a replicative phase (RP) and a transmissive phase (TP). Bacteria in RP are avirulent, sodium resistant and not flagellated; in contrast TP bacteria are virulent, flagellated and highly motile [11].

Table 1
Expression of *Legionella pneumophila* eukaryotic-like protein encoding genes during growth in *Acanthamoeba castellanii*

Functions	Fold changes (FC) during infection *A. castellanii*					
	Paris	FC	Lens	FC	Phila	FC
Zinc metalloproteinase	*lpp3071*	−5,2	*lpl2927*	n.d.	*lpg2999*	−2,1
NuoE NADH dehydrogenase I chain E	*lpp2832*	−3,3	*lpl2701*	−2,3	*lpg2785*	−3,1
Hypothetical protein	*lpp0634*	−2,6	*lpl0618*	−2,4	*lpg0584*	−1,9
RNA-binding protein precursor	*lpp0321*	−2.5	–	–	*lpg0251*	−2.4
Glucoamylase	*lpp0489*	−1,9	*lpl0465*	−4,1	*lpg0422*	−2,4
Sphinogosine-1-phosphate lyase	*lpp2128*	n.d.	*lpl2102*	−4,2	*lpg2176*	−2,2
Uridine kinase	*lpp1167*	∼	*lpl1173*	−2,4	*lpg1165*	∼
Hypothetical protein	*lpp0358*	∼	*lpl0334*	∼	*lpg0282*	∼
Hypothetical protein	*lpp0379*	∼	*lpl0354*	∼	*lpg0301*	∼
Chromosome condensation 1-like	*lpp1959*	∼	*lpl1953*	∼	*lpg1976*	–
Nuclear membrane binding protein	*lpp1824*	∼	–	–	–	–
Hypersensitive response protein	*plpp0050*	∼	–	–	–	∼
Phytanoyl coA dioxygenase	*lpp0578*	∼	*lpl0554*	∼	*lpg0515*	∼
ExoA exodeosyribonuclease III	*lpp0702*	∼	*lpl0684*	∼	*lpg0648*	∼
DegP protease	*lpp0965*	∼	*lpl0935*	∼	*lpg0903*	∼
Apyrase	*lpp1033*	∼	*lpl1000*	∼	*lpg0971*	∼
Serine threonine protein kinase	*lpp1439*	∼	*lpl1545*	∼	*lpg1483*	∼
Thiamine biosynthesis protein NMT-1	*lpp1522*	∼	*lpl1461*	∼	*lpg1565*	∼
PurC	*lpp1647*	∼	*lpl1640*	∼	*lpg1675*	∼
Uracyl DNA glycosylase	*lpp1665*	∼	*lpl1659*	∼	*lpg1700*	∼
Apyrase	*lpp1880*	∼	*lpl1869*	∼	*lpg1905*	∼
Cytochrome *P*450	*lpp2468*	∼	*lpl2326*	∼	*lpg2403*	∼
Ser/Thr protein kinase domain	*lpp2626*	∼	*lpl2481*	∼	*lpg2556*	n.d.
Phytanoyl-coA dioxygenase	*lpp2748*	∼	*lpl2621*	∼	*lpg2694*	∼
6-Pyruvoyl-tetrahydropterin synthase	*lpp2923*	∼	*lpl2777*	∼	*lpg2865*	∼
SAM-dependent methyltransferase	*lpp2134*	∼	*lpl2109*	*2,1*	*lpg2182*	∼
Ca2+-transporting ATPase	*lpp1127*	*2,0*	*lpl1131*	*1,7*	*lpg1126*	*1,6*
Cytokinin oxidase	*lpp0955*	*2,5*	*lpl0925*	*7,7*	*lpg0894*	*3,7*
Pyruvate decarbosylase	*lpp1157*	*3,4*	*lpl1162*	*11,8*	*lpg1155*	*5,8*
SAM dependent methyltransferase	*lpp2747*	*4,4*	*lpl2620*	*4,1*	*lpg2693*	n.d.

Expression ratios are listed as negative fold changes (FC) when upregulated in exponential growth phase, and as positive FC when upregulated in post-exponential growth phase. The symbols "∼" and "–" indicate that genes were not differentially regulated or absent from the strain, respectively. n.d.: not determined. *lpp*, *lpl*, *lpg* indicate predicted coding sequences of *L. pneumophila* strain Paris, Lens and Philadelphia, respectively.

Transcriptional analyses of *L. pneumophila* during infection of *A. castellanii* revealed that these two phases also exist *in vivo* [15]. The. global expression profiles of all three sequenced *L. pneumophila* strains showed similar if not identical results, with sets of RP genes (405) upregulated at the earlier time points, and TP genes (393) upregulated at the late time points. The RP genes comprised DNA and RNA polymerase complexes, transcription and translation elongation factors, ribosomal proteins, protein secretion and translocation systems (*secAB*, *secEF*, *secGY*, etc.), genes coding proteins involved in purine and pyrimidine metabolism and other genes encoding metabolic processes. Among the identified TP genes several regulators (e.g. *fliA*, *cpxR*, *rpoE*), virulence factors (e.g. *ralF*, *dotA*, *letE*, *enhA*, *sdeA*, *sdcA*) and flagella biosynthesis genes (e.g. *fliS*, *fliD*, *fliN*) were overexpressed [15]. Thus the transcriptome during RP shows that cell division progresses as opposed to the TP, which prepares *L. pneumophila* to evade the host cell and to invade a new one.

3.2. Intracellular metabolism of L. pneumophila as deduced from gene expression

Although *L. pneumophila* growing in minimal medium obtains carbon and energy from amino acids, and not sugars [34,35], it is an open question whether the bacterium only scavenges amino acids or also carbohydrates from the host when growing inside the replicative vacuole. Supporting the amino acid diet, a *L. pneumophila* threonine transporter PhtA (Phagosomal transporter) was recently identified as essential for differentiation and proliferation in macrophages [36]. Likewise, our transcriptional analyses showed that the catabolism and uptake systems of amino acid encoding genes (>30) are upregulated in early and late RP, in particular those of serine, threonine (*phtA*), alanine, glycine, tyrosine and histidine [15]. Moreover, several aminopeptidase and protease encoding genes were upregulated during RP, which further suggest the acquisition of host amino acids (Fig. 1).

Listeria monocytogenes can use alternative carbon sources like phosphorylated glucose and glycerol during replication in epithelial cells [31]. Similarly, *L. pneumophila* may use carbohydrate-derivatives as indicated by the upregulation of the genes coding for the Entner–Doudoroff pathway (*lpp0483*, *lpp0487*), a putative glucokinase (*lpp0486*), a sugar transporter (*lpp0488*) and an eukaryotic-like glucoamylase (*lpp0489*, Fig. 1, [15]). The NADPH producing Entner–Doudoroff pathway is commonly used by bacteria for sugar and/or gluconate assimilation [37] and was also found to be upregulated during intracellular growth of *Salmonella enterica* [33]. Moreover, the *L. pneumophila* eukaryotic-like glucoamylase is upregulated *in vivo* and not *in vitro*, which suggests the presence of an *in vivo*-dependent signal that may trigger the use of the host glycogen.

3.3. The shift from replicative to transmissive phases seems to parallel oxygen limitation

Transcriptome analyses suggest that in the late replicative phase of infection of *A. castellani* oxygen becomes limited most

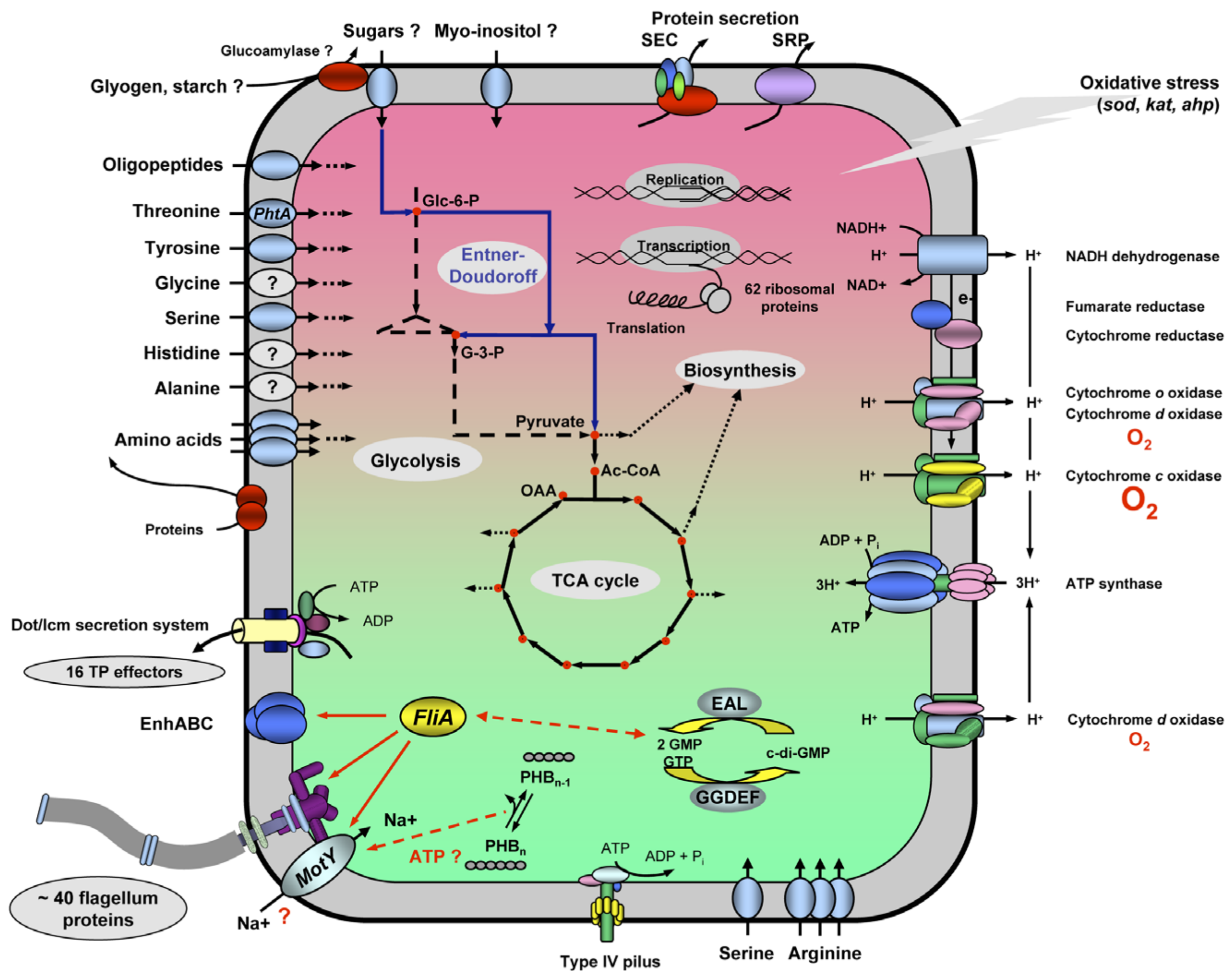

Fig. 1. Overview of major characteristics of the biphasic life cycle of *L. pneumophila* as deduced from transcriptional analyses (reproduced with permission from [15]). The color gradation of the background represents transcriptional changes from the replicative phase (RP, pink) to the transmissive phase (TP, cyan). Main replicative traits are: amino acid and oligopeptide transporters, proteases, sugar transporters, the glycolysis, and the Entner–Doudoroff pathways, Sec- and SRP (signal recognition protein)-dependent protein secretion systems, oxidative stress response proteins, and the replication, transcription and translation machinery. Endoxidases of the energy-producing respiratory chain are cytochrome o, d and c oxidases, upregulated during the RP, while in TP an alternative cytochrome d oxidase is overexpressed. Important transmissive traits are: GGDEF/EAL proteins, serine and arginine transporters, type IV pilus biosynthesis, synthesis and degradation of polyhydoxybutyrate (PHB), flagellum formation, invasion-associated traits (e.g. EnhABC) and host cell-modulators secreted by the Dot/Icm secretion system.

probably due to the high concentration of *L. pneumophila* cells inside the phagosome. When reaching the post-exponential growth phase, the ATP synthase (*atp* genes), c-type cytochrome (*ccmEFGH*), c-type cytochrome oxidase (*coxCAB*, *ctaG*), c-type cytochrome reductase (*petBA*) and NADH deshydrogenase (*nuo* genes) encoding genes are downregulated (Fig. 1). In contrast the d-type cytochrome oxidase (*qxtAB*), which has a high affinity for O2, was clearly upregulated [15]. Concomitantly, the genes putativly coding for polyhydroxybutyrate synthesis (*lpp0650*, *lpp2038*, *lpp2214*, *lpp2323*) are upregulated and the expression of genes implicated in virulence and flagellum synthesis is initiated.

However, does the lack of oxygen trigger the expression changes in *L. pneumophila?* In *Escherichia coli*, the two-component system Arc (Aerobic respiration control) coordinates, together with Fnr, the response to anaerobiosis. The transmembrane histidine kinase ArcB is activated during the transition from aerobic to microanaerobic growth and activates the upregulated cytosolic response regulator ArcA. It was recently shown that the phospho-histidine phosphatase SixA modulates the ArcB phosphorelay signal transduction [38], and that the Arc system, together with RssB, coordinates transcription and proteolysis of RpoS [39], which in turn induces the general stress response. Intriguingly, *L. pneumophila* upregulates the gene coding the SixA homologue (*lpp1968*) when reaching the transition phase. We thus searched whether in the *L. pneumophila* genomes a system similar to the *E. coli* Arc two-component system was present. By domain searches, we identified two genes, *lpp2132* and *lpp2133*, as probable *arcAB* of *L. pneumophila.* The *arcA* gene encoding the putative response regulator was 30-fold upregulated in TP and both *rpoS* and *arcB* were overexpressed. To date, no such regulatory pathway has been investigated in *L. pneumophila*, however genome analysis and transcriptome results suggest that the SixA and ArcAB homologues might participate in the control of the biphasic life cycle.

3.4. Expression of L. pneumophila type IV secretion systems during intracellular growth

The genomes of *L. pneumophila* encode each several type IV secretion systems (T4SS). The type-IVB secretion system similar to the *Agrobacterium tumefaciens* Vir system is the Dot (defective organelle trafficking) [40] /Icm (intracellular multiplication) [41] 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. The Dot/Icm T4BSS is of critical importance for the intracellular fate of *L. pneumophila*, as it is required for its ability to replicate and to cause disease (for reviews see [42,43]). We thus analyzed the intracellular expression pattern of the genes coding for this important secretion system in detail and identified three groups of *dot*/*icm* genes differentially expressed during intracellular growth. Group I comprises 8 genes (*dotJIHG*/*icmMLKE*, *dotK*/*icmN*, *dotCD*, *icmF*) that are overexpressed in late replicative phase with respect to transmissive phase, group II comprises two genes (*dotU*/*icmH*, *dotV*) whose expression does not change during intracellular growth, and group III contains 16 genes (*dotFEP*/*icmGCD*, *icmQ*, *dotNO*/*icmJB*, *icmTS*, *icmR*, *icmWX*, *icmV-dotA*, *dotB*, *dotML*/*icmPO*) whose expression increases continuously during intracellular growth (Fig. 2). The *dot*/*icm* genes are thought to be organized in at least 11 transcriptional units (*icmTS*, *icmR*, *icmQ*, *dotML*/*icmPO*, *dotK*/*icmN*, *dotJIHGFEP*/*icmMLKEGCD*, *dotNO*/*icmJB*, *dotU*/*icmHF-tphA*, *icmWX*, *icmV-dotA*, and *dotDCB*) [44]. According to *in vivo* microarray data, the proposed *icmMLKEGCD* transcriptional unit seems to contain two subunits represented by *icmMLKE* whose expression decreases during infection, and *icmGCD*, whose expression increases at post-exponential and stationary growth phases (Fig. 2). However, how these genes are regulated is mainly unknown. One regulator reported to be implicated in *dot*/*icm* gene expression is the CpxR response regulator. It was shown to act as a 10-fold activator of the *L. pneumophila icmR* gene expression and to a lesser extent also of that of two other transcriptional units (*icmV-dotA* and *icmWX*) [45]. This report is consistent with our *in vivo* expression data, showing that CpxR is upregulated in transmissive phase as are *icmV-dotA*, *icmWX* and *icmR* (Fig. 2). Interestingly, the *cpxR* gene was not differentially regulated during *in vitro* growth, as were some of the reported targets of CpxR like *icmR* and *icmW*.

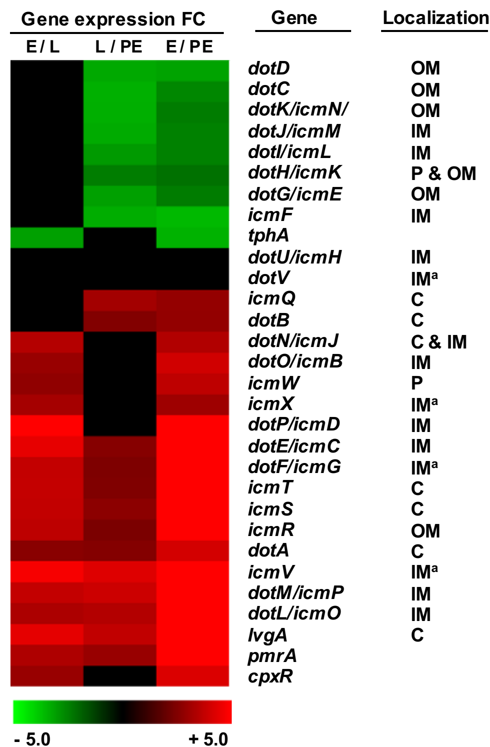

Fig. 2. Differential expression of the Dot/Icm T4BSS encoding genes of *L. pneumophila* during growth in *A. castellanii*. E, early exponential growth phase; L, late exponential growth phase; PE post-exponential growth phase. Black, not differentially regulated genes; green, genes upregulated in the early time points; red, genes upregulated in the later time points. *dot*/*icm*, gene names. Localization of gene products; C, cytoplasm; IM, inner membrane; IM[a], *in silico* predicted inner membrane; OM, outer membrane; P, periplasm according to [69].

In contrast to the Dot/Icm T4BSS, the Lvh T4ASS (11 genes) was not differentially regulated during infection of *A. castellanii* at 37 °C [15]. However, this is expected, as the Lvh system was reported to be dispensable for intracellular growth in both amoebae and macrophages [46]. This is consistent with its probable temperature-dependent regulation, as illustrated by the enhancement of *L. pneumophila* entry mediated by the *lvhB2* coding pilin at 30 °C but not at 37 °C [47]. Furthermore, the Lvh T4ASS was reported to be involved in virulence-related phenotypes under conditions mimicking the spread of Legionnaires' disease from environmental niches [21].

3.5. Dot/Icm substrates are mainly upregulated in transmissive phase

Many substrates of the Dot/Icm T4BSS have been identified. They inhibit phagosome maturation, alter its trafficking or are implicated in the egress from amoeba [5–9,26,48,49]. Though not all substrates of the Dot/Icm system showed significant gene expression changes during the intracellular life cycle, many are strongly upregulated in the TP: *ralF*, *sidB*-paralog *sdbB*; *sidC* and paralog *sdcA*; *sidG*; *sidE*-paralogs *sdeA*, *sdeB*, *sdeC*, *lpp1615* and *lpp1453*; *sidH*-paralogs *sdhB* and *lpp2886*; *vipD* and *vipE*; or more moderately upregulated *lidA* (*lpp1002*), *sidF* (*lpp2637*); *sidE* (*lpp0304*) and *ceg10* (*lpp0360*) [15]. Interestingly, most of these substrates are implicated in early steps of the intracellular life, thus indicating that late in TP, *L. pneumophila* primes itself for the next invasion. The late response regulator PmrA (Fig. 2) was recently shown to control the expression of 13 substrates of the Dot/Icm T4BSS [50]. However, no transcriptional correlation between these 13 targets during the cell cycle progression was observed. Thus additional, yet unknown transcriptional regulators might be implicated in their regulation. Importantly, many of the established and putative *in vivo* upregulated substrates of the Dot/Icm T4BSS (LidA, RalF, SdeA/LaiA, SdeB/LaiB, SidE/LaiD, SdhB and SdcA) did not show any differential regulation in *in vitro* growing cells [15]. The specific *in vivo* regulation of the expression of RalF, which interferes with vesicle trafficking [5], and LidA, which is implicated in the establishment of the replicative niche [51], suggest that "*in vivo* -dependent" signals trigger adaptation of *L. pneumophila* to its environment.

3.6. Expression of eukaryotic-like proteins during growth in A. castellanii

The *L. pneumophila* genome analysis identified various homologs of eukaryotic genes [12,27,28,52]. As shown in Table

1, 10 out of the 30 identified eukaryotic-like genes show significant expression changes related to growth phase. Four genes are significantly upregulated in TP in all three strains studied: a SAM-dependent methyltransferase (*lpp2747*) and three coding for proteins having probably metabolic functions (*lpp0955*, *lpp1127*, *lpp1157*). Interestingly, *lpp0955* encodes a putative cytokinin oxidase. Cytokinin oxidases/dehydrogenases catalyze the irreversible degradation of cytokinins by cleavage and release of an adenine moiety. It is noteworthy that cytokinin oxidases are among prokaryotes only found in the symbiotic cyanobacteria, like *Anabaena* sp., and plant pathogens such as *Streptomyces* sp. and *Rhodococcus fascians*. The cytokinin oxidase encoding gene in *Streptomyces* sp. is located on a pathogenicity island, most probably acquired from the *Rhodococcus fascians fas* operon, which is required in both bacteria for pathogenicity [53,54]. Cytokinins are phytohormones that play various roles in the development of plants by activating two-component phosphorelay pathways [55]. Interestingly, *D. discoideum* produces a cytokinin (discadenine) [56], which acts as an inhibitor of spore germination through its probable interaction with the histidine kinase DhkB, but it might also play a role in the initiation of sporulation [57]. Thus, although we cannot exclude a function of this *Legionella* enzyme in metabolism, it is tempting to assume that it might modulate the development of the host amoeba. In line, the *L. pneumophila* cytokinin oxidase encoding gene was upregulated in TP (Table 1) [15]. Furthermore, infection of *D. discoideum* with *L. pneumophila* leads to a strong inhibition (85%) of *Dictyostelium* differentiation into the multicellular fruiting body stage [58]. Perhaps the *L. pneumophila* cytokinin oxidase may trigger the arrest of the host cell differentiation, as a result of the degradation of discadenine.

Five (strain Paris), and six (strains Lens and Philadelphia-1) eukaryotic-like genes were upregulated during the exponential phase of growth (Table 1). Among those were a putative sphingosine-1-phosphate lyase (*lpp2128*) and a putative glycoamylase (*lpp0489*). This is in agreement with their suggested function as these enzymes might be implicated in induction or retardation of autophagy/apoptosis in RP and in scavenging carbohydrates during replication. Some of these factors are probably implicated in the subversion of host metabolic functions to provide carbon and energy to the growing bacterium.

3.7. Strain-specific genes induced during in vivo growth

As previously highlighted, a strong correlation of the transcriptional response of the core genome of the three-strains was observed during infection. When analysing the approximately 300 strain-specific genes of each isolate with respect to gene expression, it appeared that most of them are not differentially regulated during growth in *A. castellani* [15]. However, certain strain-specific genes were differentially expressed, indicating heterogeneity in the gene pool required for intracellular growth as well as specific adaptations to different intracellular environments. In RP only few strain-specific genes of strains Lens and Philadelphia were upregulated. These comprise putative transposases (*lpl2032*, *lpl2869*, *lpl0184*, *lpg2114*) and outer membrane proteins (*lpl2148*, *lpl1942*). However, strain Paris showed more diversity with the upregulation of a o-type cytochrome ubiquinol oxidase (*cyoCD*/*lpp0296-7*, *cyoA*/*lpp0295*) and a putative autotransporter or type V secretion system (*lpp0779*). The first one might improve environmental adaptation and survival of the bacterium whereas the autotransporter may be implicated either in better biofilm formation in the environment or confer an advantage in adhesion and invasion during infection.

In contrast, during TP several strain-specific genes probably important for virulence were highly upregulated. In strains Paris and Lens in particular specific eukaryotic-like and eukaryotic domain containing proteins (*lpp2168*, *lpl1158*, *lpl2330*, *lpl0063*, *lpl2477*, *lpp1100*, *lpp2344*, *lpl1681*) and GGDEF/EAL regulators (*lpp2477*, *lpl2826*) were overexpressed. In addition, in each of the strains upregulation of strain specific genes putatively involved in signal transduction (*lpp0300*, *lpl2476*) or transcriptional regulation (*lpl1048*, *lpl1926*, *lpg2524*, *lpl2105*, *lpl2107*) was observed. Strain Philadelphia-1 contains a specific pathogenicty island [59]. Interestingly, genes encoded on this island showed a strong TP upregulation (from 5-fold to 10-fold). An example is the *msrA* locus (*lpg2098*/*msrA3*, *lpg2099*/*msrA2*, *lpg2111*/*msrA1*) encoding putative methionine sulfoxide reductases and the gene encoding the 24 kDa macrophage- induced major protein (*lpg2112*, [60]). The methionine sulfoxide reductase (Msr) is an antioxidant repair enzyme that catalyzes the reduction of methionine sulfoxide [61] residues in proteins to methionine. It was shown to be involved in the adherence mechanism of *Mycoplasma genitalium* to erythrocytes, possibly by protecting mycoplasma protein structures from oxidative damage or through alternate virulence-related pathways [61]. Altogether, these data suggest that each strain has acquired genes allowing specific adaptation to different preferential hosts and/or to increase its fitness in the environment.

4. Trancriptional host response to infection with *Legionella pneumophila*

Intracellular replication of *L. pneumophila* is the result of an intimate relationship between the pathogen and its host. Understanding this complex cross-talk is essential to improve our understanding of the infection by *L. pneumophila*. One way to gain knowledge on host–pathogen interactions is the study of the host's transcriptional response to the infection with *L. pneumophila*. Recently the social amoeba *D. discoideum*, a model organism for the study of basic aspects of differentiation, signal transduction, phagocytosis, cytokinesis and cell motility has been described as infection model for *L. pneumophila* [58,62]. It is a particularly useful model system because a large number of mutants are available allowing the study of the role of specific host proteins during infection. Moreover, the genome of *D. discoideum* has been sequenced recently, allowing now the design of microarrays [14].

4.1. The transcriptional host response of D. discoideum to L. pneumophila infection

Using cDNA microarrays covering about half of the *Dictyostelium* genome, Farbrother and colleagues [16] studied the response of *D. discoideum* cells infected with *L. pneumophila* with respect to uninfected cells in a 48 h time course experiment [16].

Functional annotation of the differentially regulated genes revealed that apart from triggering a stress response, *Legionella* not only interferes with intracellular vesicle fusion but also

profoundly influences the metabolism of its host. The most pronounced response was observed at 24 h after infection, as more than 500 genes were differentially regulated. However, already 1 h after initial contact between the bacterium and the host, 18 genes were upregulated and one downregulated in the infected amoeba cells with respect to the uninfected ones, illustrating the fast response that might be triggered by the release of *L. pneumophila* virulence factors into the host cell.

As a result of the infection process, *D. discoideum* induces many stress protein encoding genes, like the superoxide dismutase (*sodB*) or the alternative oxidase (*aoxA*). As expected, *Legionella* seems to interfere with host signalling pathways. As an example, the *D. discoideum rtoA* gene is upregulated after infection with *L. pneumophila*. Interestingly, RtoA was shown to be required for maturation of the *L. pneumophila* replicative vacuole [16,63]. In addition to RtoA, the expression levels of *D. discoideum* ARF1 and CopB encoding genes are modulated during the infection process, supporting interference of the pathogen with vesicle transport [16]. Several other putative signaling pathways were hijacked, in particular the phosphoinositide metabolism (*pipA* and other putative PIP-6 kinase encoding genes), which is controlled by *L. pneumophila* to direct its entry into eukaryotic cells [64].

Exploitation of the host is also a likely explanation for the upregulation of about 10 amino-acyl tRNA synthetases and enzymes involved in nucleotide metabolism. Farbrother and colleagues [16] proposed that the bacterium induces such activities in order to take up products with energetically high added value to support the bacterial metabolism. Finally, several genes encoding calcium-binding proteins were downregulated in *D. discoideum* after infection with *L. pneumophila*. This is further consistent with the importance of cytoplasmic calcium levels for the intracellular growth of *L. pneumophila* [65] and the late upregulation of a eukaryotic-like calcium-transporting ATPase of *L. pneumophila* (Table 1).

4.2. The transcriptional host response of D. discoideum to infection with L. pneumophila ΔdotA or with L. hackaeliae

L. hackeliae is a *Legionella* species with reduced pathogenicity in macrophages and it is degraded by *D. discoideum* [58]. *L. pneumophila dotA* is a strain that lacks the protein DotA, an essential component of the type IV secretion system needed for infection. *L. pneumophila* Δ*dotA* is digested by *D. discoideum* and in macrophages almost all Δ*dotA*-mutants reside in phagolysosomes 15 min after uptake [66].

Analyses of the *D. discoideum* transcriptome upon infection with the *dotA* mutant as compared with that of the wild type is of particular interest in the course of functional genomics because it reflects effects that the secreted type IV effectors have on the host cell. Most interestingly, genes belonging to the *D. discoideum* ubiquitination machinery (*ubqG*, *ubqA*, *ubqF*, *ubcB*, *sonA*) were strongly upregulated at 24 h post infection with *L. pneumophila* wild type as compared to the *dotA* mutant. This points to the putative role of the Dot/Icm T4BSS in secreting factors interfering with the eukaryotic ubiquitination machinery. Good candidates for such substrates are the F- and U-box proteins encoded by *L. pneumophila*. Moreover, in contrast to wt *L. pneumophila*, the *dotA* mutant fails to induce the growth cessation of the amoeba as illustrated by the downregulation of more than 20 ribosomal genes. As another hypothesis, the putative cytokinin oxidase discussed before, might be a type IV secreted substrate implicated in this process. Interestingly, infection with the strongly attenuated *L. pneumophila dotA* mutant or with *L. hackaeliae*, triggered more changes in the transcriptional host response after 24 h of infection, than the wild-type *L. pneumophila* strain [16]. It may indicate that *L. pneumophila* controls through the T4BSS the host response in order to lower the host defense capacities.

5. Transcriptomics to explore the regulatory networks governing the *L. pneumophila* life cycle

L. pneumophila cycles between an infectious, non-replicating form thought to promote transmission to a new host, and an intracellular, replicative form, which does not express transmission traits. This cellular differentiation of *L. pneumophila* is governed by a complex regulatory system. Regulators shown or thought to be involved are specific sigma factors (RpoN, RpoD, RpoS, FliA), two-component systems (LetA/LetS), a mRNA-binding protein (CrsA), as well as probably small regulatory RNAs [11,67]. Thus, DNA microarray technology offers an ideal tool for genome-wide analysis of the regulatory circuits that mediate this adaptive response.

5.1. The L. pneumophila flagellum and the FliA (σ^{28}) sigma factor

Transcriptomic analyses of *L. pneumophila* wild type showed that in *in vitro* and *in vivo* conditions, the flagellin encoding gene *flaA* is upregulated up to 100 times in TP cells as compared to RP cells. Similarly, several genes encoding proteins implicated in flagellum biosynthesis (e.g. *fliS*, *fliD*, *fliN*, *flgBCDEFGHIJKL*, *fhF*, *fleN*) and the *fliA* gene, encoding the sigma factor FliA (σ^{28}) that regulates *flaA* gene expression, are strongly upregulated in the late phases of growth (Fig. 1). Analysis of the transcriptional profile of a *fliA* mutant as compared to the wt identified further FliA targets implicated in flagellum biosynthesis or movement (*flaA*, *fliD*, *fliS*, *motY*). Upstream of these genes a FliA binding consensus sequence is present suggesting direct regulation by FliA. From the expression profiling results, it appears that FliA controls only few targets. However, FliA also controls the expression of genes that were predicted to affect the first steps of cell invasion such as EnhA homologs (*lpp0972*, *lpp1290*) or a GGDEF/EAL regulator (*lpp0952*), which may explain lower invasiveness and cytotoxicity of *fliA* mutants and points to an implication of this sigma factor in the infection process [15].

5.2. The LetA/LetS two component system

Regulation of the transition from the replicative to the transmissive phase in *L . pneumophila* requires complex regulatory networks, which are only partially understood (Fig. 3). However, by analogy with the BarA/UvrY two-component system of *E. coli*, and genetic studies of regulatory interactions, a regulatory cascade for the transition from replicative to transmissive phase bacteria was proposed [11]. Under conditions of nutrient starvation RelA synthetizes the alarmone molecule (p)ppGpp, which in turn probably stimulates the LetA/LetS two-component system (Fig. 3). However, this might not be the only signal governing the biphasic life cycle. When stimulated, LetA/LetS induces the expression of transmissive traits by relieving CsrA repression [11]. The missing link between

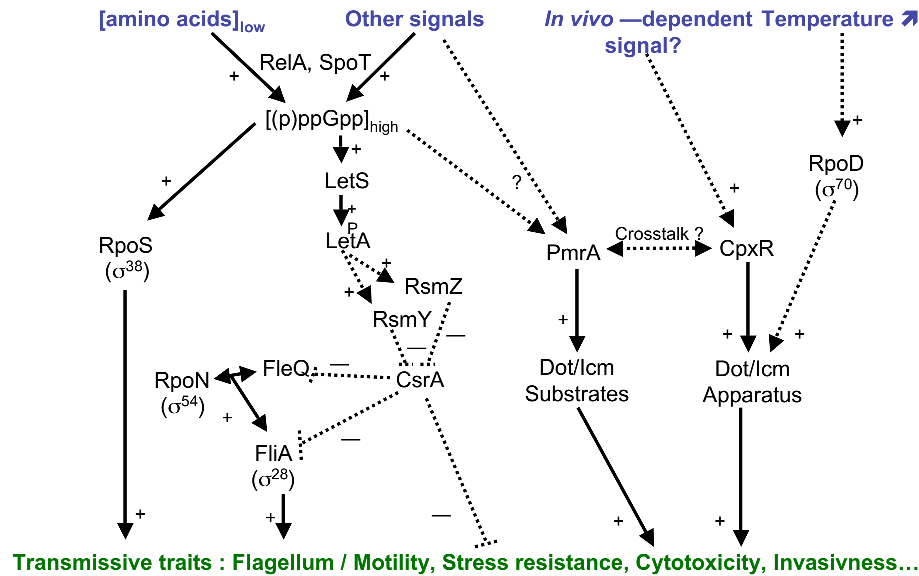

Fig. 3. Proposed regulatory cascade governing the life cycle switch of *L. pneumophila*. Blue, suggested external signals; green, transmissive traits. Known regulations are depicted with full traits, putative regulations are depicted by dotted lines.

the LetA activator and the CsrA RNA-binding protein is predicted to be a small regulatory RNA such as CsrB in *E. coli*. Although this CsrA-regulating sRNA has not yet been identified, two putative targets were proposed, namely RsmY and RsmZ [68]. Given the critical role played by CsrA in several bacteria, we started to characterize the interplay between each protagonist at both molecular and transcriptional levels (Fig. 3). First transcriptional analyses of both *letA* and *letS* mutants further confirmed the role of this two-component system in the control of the *L. pneumophila* biphasic life cycle (Brüggemann et al., unpublished).

6. Conclusions

Global approaches like DNA sequencing and transcriptome analysis have led to the identification of novel genes involved in host–bacterial interactions and allowed to decipher transcriptional networks. In *Legionella* research, the analysis of the genome sequence was an essential step forward, as it identified many new candidate genes for host–pathogen interactions. The investigation of the transcriptional adaptation of *L. pneumophila* to its host showed that the bacterium exhibits a biphasic life cycle also *in vivo* conditions and elucidated several pathways used for intracellular replication. The challenge to face now is to characterize these genes and their functions. With the availability of post genomic tools for functional characterization we move towards the understanding of the entire system. However, this will require the integration of large data sets into a genome-scale dynamic model, which will merge metabolic data and regulatory networks into a comprehensive system in order to understand the complex interactions between *Legionella* and its host.

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