Die Regulation der Flagellenbiogenese in *Legionella pneumophila*

Regulation of the Flagellar Biogenesis in *Legionella pneumophila*

Dissertation zur Erlangung des

naturwissenschaftlichen Doktorgrades

der Bayerischen Julius-Maximilians-Universität Würzburg

vorgelegt von

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Würzburg 2008

Eingereicht am:

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Tag des Promotionskolloquiums:

Doktorurkunde ausgehändigt am:

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Danksagung

Mein Dank gilt in ganz besonderem Maße meinen drei Betreuern, die sich gegenseitig wunderbar ergänzten und so maßgeblich zum Gelingen dieser Arbeit beigetragen haben:

Prof. Dr. Dr. h. c. mult. Jörg Hacker danke ich insbesondere für sein Vertrauen in mich und seine Unterstützung, die diese Arbeit erst ermöglicht hat. Seine wertvollen Anregungen prägten den Fortgang dieser Arbeit.

Dr. Carmen Buchrieser danke ich für ihre exzellente wissenschaftliche Anleitung und Betreuung. Sie hat es immer geschafft mich zu motivieren und für die Forschung zu begeistern.

Dr. Klaus Heuner danke ich für die hervorragende Betreuung und Diskussionsbereitschaft während der gesamten Doktorarbeit. Besonders hilfreich war seine große praktische Erfahrung für den Laboralltag.

Ich danke allen Mitgliedern der ehemaligen Unit GMP unter Leitung von Dr. Frank Kunst des Institut Pasteurs für die abwechslungsreiche, von Herzlichkeit geprägte Arbeitsatmosphäre und die vielen wissenschaftlichen als auch nicht-wissenschaftlichen Gespräche. Dr. Holger Brüggemann und der Platform 2 der Pasteur Genopole Île-de-France danke ich für die kompetente Einführung in die Welt der Microarrays.

Den Mitgliedern des Instituts für Molekulare Infektionsbiologie und des Zentrums für Infektionsforschung danke ich für ihre stete Hilfsbereitschaft und die ausgezeichnete Arbeitsatmosphäre. Das gilt insbesondere für die beiden *Legionellen*-Arbeitsgruppen, welche inzwischen an das Robert Koch-Institut in Berlin und die Technische Universität in Braunschweig gezogen sind.

Von ganzem Herzen danke ich Marco, meinen Eltern und Geschwistern für ihre große Unterstützung und rege Anteilnahme.

Ich danke der Bayerischen Forschungsförderung, die meine Forschung von Dezember 2005 bis November 2008 unterstützt hat.

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SUMMARY

The bacterial pathogen *Legionella pneumophila* replicates intracellularly in protozoa, but can also cause severe pneumonia, called Legionnaires' disease. The bacteria invade and proliferate in the alveolar macrophages of the human lung. *L. pneumophila* bacteria exhibit a biphasic life cycle: replicative bacteria are avirulent; in contrast, transmissive bacteria express virulence traits and flagella.

Primarily aim of this thesis was to evaluate the impact of the regulatory proteins FleQ, FleR, and RpoN in flagellar gene regulation. Phenotypic analysis, Western blot and electron microscopy of regulatory mutants in the genes coding for FleQ, RpoN and FleR demonstrated that flagellin expression is strongly repressed and that these mutants are non-flagellated in transmissive phase. Transcriptomic studies of these putative flagellar gene expression regulators demonstrated that *fleQ* controls the expression of numerous flagellar biosynthetic genes. Together with RpoN, FleQ controls transcription of 14 out of 31 flagellar class II genes, coding for the basal body, hook, and regulatory proteins. Unexpectedly, 7 out of 15 late flagellar genes (class III and IV) are expressed dependent on FleQ but independent of RpoN. Thus, in contrast to the commonly accepted view that enhancer binding proteins as FleQ always interact with RpoN to initiate transcription, our results strongly indicate that FleQ of *L. pneumophila* regulates gene expression RpoN-dependent as well as RpoN-independent. Moreover, transcriptome analysis of a *fleR* mutant strain elucidated that FleR does not regulate the flagellar class III genes as previously suggested. Instead FleR regulates together with RpoN numerous protein biosynthesis and metabolic genes. Based on these experimental results our modified model for the transcriptional regulation of flagellar genes in *L. pneumophila* is that flagellar class II genes are controlled by FleQ and RpoN, while flagellar class III and IV genes are controlled in a *fleQ*-dependent but *rpoN*-independent manner.

Although all *L. pneumophila* strains share the same complex life style, various pathotypes have evolved. This is reflected by the genomes, which contain e.g. genomic islands. The genomic island Trb-1 of *L. pneumophila* Corby, carries all genes necessary for a type-IV conjugation system, an integrase gene and a putative oriT site.

The second aim of this thesis was to investigate the implication of this genomic island in conjugative DNA transfer. Using conjugation assays we showed that the oriT site located on Trb-1 is functional and contributes to conjugation between different *L.*

pneumophila strains. As this is the first oriT site of *L. pneumophila* known to be functional our results provide evidence that conjugation is a major mechanism for the evolution of new pathotypes in *L. pneumophila.*

ZUSAMMENFASSUNG

Das pathogene Bakterium *Legionella pneumophila* repliziert sich in der Natur intrazellulär in Protozoen. Beim Menschen kann das Bakterium eine schwere Pneumonie, die sogenannte Legionärskrankheit auslösen. Hierbei vermehren sich die Bakterien in Alveolarmakrophagen der Lunge. Der Lebenszyklus von *L. pneumophila* Bakterien ist gekennzeichnet durch zwei Phase: replikative Bakterien sind avirulent; im Gegensatz dazu sind transmissive Bakterien virulent und flagelliert.

Hauptziel dieser Arbeit war es die Beteiligung der regulatorischen Proteins FleQ, FleR, and RpoN an der Flagellengenregulation zu ermitteln. Mutanten für die Gene welche für FleQ, FleR oder RpoN codieren exprimieren in der transmissiven Phase im Genesatz zum Wildtyp nur wenig Flagellin und sind nicht flagelliert. Nachgewiesen wurde dies durch eine phänotypische Analyse, Western blot und Ektronenmikroskopie. Studien des Transkripoms dieser Mutanten zeigten, daß FleQ die Expression zahlreicher Flagellenbiosynthesegenen kontrolliert. Gemeinsam mit RpoN kontrolliert FleQ die Transkription von 14 der 31 Klasse II Flagellengene, welche für Basalkörper, Haken und regulatorische Proteine codieren. Überraschenderweise sind 7 der 15 späten Flagellengenen (Klasse III und IV) abhängig von FleQ, aber unabhängig von RpoN exprimiert. Daher und entgegen der allgemeinen Auffassung dass sogenannte ‚enhancer binding' Proteine wie FleQ zur Transkriptionsinitiation immer mit RpoN interagieren, deuten unsere Ergebnisse darauf hin, dass FleQ von *L. pneumophila* Genexpression sowohl RpoN-abhängig, als auch RpoN-unabhängig reguliert. Ebenso anders als zuvor vorgeschlagen, verdeutlichen Studien des Transkriptoms einer *fleR* Mutante, dass FleR nicht die Expression der Klasse III Flagellengene induziert. Statt dessen reguliert FleR gemeinsam mit RpoN zahlreiche Gene der Proteinbiosynthese und des Metabolismus. Basierend auf diesen experimentellen Ergebnissen sind in unserem modifizierten Modell für die transkriptionelle Regulation der *L. pneumophila* Flagellengene die Flagellengene der Klasse II von FleQ und RpoN kontrolliert, während die Flagellengene der Klasse III und IV in einer *fleQ*-abhängigen aber *rpoN*-unabhängigen Weise kontrolliert sind.

Obwohl alle *L. pneumophila* Stämme den zweiphasigen Lebenszyklus aufweisen haben sich unterschiedliche Pathotypen evolviert. Das ist auch in den Genomen sichtbar, die z. B. genomische Inseln enthalten. Die genomische Insel Trb-1 von *L.*

pneumophila Corby trägt alle Gene eines Typ-IV Konjugationssystem, ein Integrase-Gen und einen putative oriT-Bereich.

Das zweite Ziel dieser Arbeit war es also zu untersuchen, inwieweit Trb-1 an konjugativem DNA-Transfer beteiligt ist. Mit Hilfe von Konjugationsexperimenten, zeigten wir, dass der oriT-Bereich von Trb-1 funktional ist und zur Konjugation zwischen verschiedenen *L. pneumophila* Stämmen beiträgt. Dies ist der erste oriT-Bereich von *L. pneumophila*, dessen Funktionalität nachgewiesen wurde. Damit bekräftigen unsere Ergebnisse, dass Konjugation eine treibende Kraft für die Evolution neuer Pathotypen in *L. pneumophila* ist.

1 INTRODUCTION

1.1 *Legionella* and Legionnaires' disease

During the 58th state convention of the American Legion of Pennsylvania July 21-24, 1976 in Philadelphia occurred an epidemic of pneumonia. The total number of cases was 182, whereof 29 cases were lethal. The disease was named Legionnaires' disease and as etiological agent an at that time unknown bacterium was isolated and named *Legionella pneumophila* (45, 128, 232, 257). By retrospective analyses, several earlier cases of pneumonia could be attributed to *Legionella* ssp. (103). This section provides an overview of the genus and ecology of *Legionella* and the Legionnaires' disease, which is caused by *Legionella* ssp..

1.1.1 The genus *Legionella*

Members of the genus *Legionella* are ubiquitous, usually harmless and facultative intracellular Gram-negative bacteria, belonging to the class of γ-proteobacteria (102, 259). Depending on the growth phase, they are 0.5–0.7 µm wide and 2–20 µm long (35). Major reservoirs are natural freshwater environments (121), but *Legionella* are also widespread in man-made water systems (115). The genus *Legionella* comprises 50 species with 73 serogroups (242) and the number of newly recognized species continues to increase (105). The ability of *Legionella* to multiply intracellularly in freshwater protozoa as well as in human macrophages (325) has resulted in a new precept in microbiology: bacteria can parasitize protozoa and can then utilize a variation of that mechanisms to infect humans (114, 116, 141).

1.1.2 Ecology of *Legionella*

1.1.2.1 Legionella*-protozoa interaction*

That *Legionella* ssp. bacteria are ubiquitous present in natural and artificial water environments and are associated with protozoa was known soon after identification of the genus *Legionella* (121, 325). Free-living protozoa are parasitized by *Legionellae* and provide the intracellular environment required for its replication. 14 species of protozoa, including *Acanthamoeba, Naegleria* and *Hartmanella* ssp., the ciliates *Tetrahymena pyriformis*, *Tetrahymena vorax* and one species of slime mold

are known to allow multiplication of *Legionellae*. The presence of *Legionellae* appears to be depend also on the spectrum of host-protozoa in the environment (17, 115-117, 274, 324, 325, 363, 372). Beside, *Legionellae* can survive unfavorable environmental conditions in encysted amoebal cells (12, 221, 348).

1.1.2.2 Viable but nonculturable form

In low-nutrient environments *L. pneumophila* is able to enter a non-replicative viable but nonculturable state, which can be reactivated when nutrition's are available (346). This might be a strategy to survive extended periods in low-nutrition environments without protozoa. However, very little is known about the mechanism by which *L. pneumophila* develops the viable but nonculturable form.

1.1.2.3 Colonization of man-made habitats

Legionellae are found worldwide in various natural but also artificial aquatic environments, such as cooling towers, water systems in hotels and hospitals, and swimming pools. Partly due to human alterations of the environment, Legionnaires' disease has emerged in the second half of the 20th century. Through inhaling aerosols containing *Legionellae*, the bacteria enter the human lung. On the epithelial surface of lung alveoli, *Legionellae* infect alveolar macrophages and thus cause Legionnaires' disease. For the prevention of Legionnaires' disease, many countries have developed guidelines for the control of *Legionellae* in water systems. However, humans are dead-end hosts since transmission of *Legionellae* among humans has never been observed so far.

Besides aquatic environments as source for transmission of *Legionellae* to humans, a number of cases are documented where the infection is soil-derived (216, 344, 345).

1.1.3 Legionnaire's disease

1.1.3.1 Clinical features

Legionnaires' disease is a severe pneumonia, often lacking specific symptoms. However, several symptoms are associated with Legionnaires' disease rather than with other causes of pneumonia. The average incubation period is 2-10 days. The most common symptoms are weakness, high fever, headache, nonproductive and

dry cough, chills, muscle pain, chest pain, diarrhea (25–50% of cases), vomiting and nausea (10–30% of cases), central nervous system manifestations such as confusion and delirium (50% of cases), renal failure, hyponatraemia, high lactate dehydrogenase levels (5, 269, 350, 384, 392). If untreated, Legionnaires' disease usually worsens during the first week and can be fatal.

Not everyone exposed to the organism will develop symptoms of Legionnaires' disease; most susceptible are elderly, male, smokers and immuno-compromised persons (154, 159, 245, 319, 339, 391). Besides, *Legionellae* can also cause Pontiac fever, an acute, self-limiting, influenza-like illness (150) and extrapulmonary syndromes. The ladder illness is caused when *L. pneumophila* spreads from the respiratory system to the body, e.g. spleen (241).

1.1.3.2 Diagnostic and treatment

The currently available methods for diagnosis of Legionnaires' disease are culture, urinary antigen testing, direct fluorescent antibody testing, detection of nucleic acids, and detection of specific antibodies in serum samples (242).

Important for epidemiological investigations are culture and detection of antibodies in patient's serum (242). The epidemiological subtyping is important for identifying the sources of infection and broaden our knowledge that is fundamental to scientifically proven risk assessment (242). Several methods have been described, including monoclonal antibody typing (205), analysis of total genomic DNA (e.g., amplified fragment length polymorphism typing (130-132)), and sequence-based typing (3, 135, 136, 308). Of these various approaches, the multi-locus sequence typing including the *mip* sequence is probably the best method (105). Sequences of the *mip* genes and other genes from all validly described Legionella ssp. are available in the web database (http://www.ewgli.org/) established by members of the European Working Group for *Legionella* Infections (105).

Appropriate antimicrobial agents for treatment of Legionnaires' disease includes macrolides, azalides, ketolides, tetracyclines, and antibacterial quinolones. The most active drugs are azithromycin and levofloxacin and are used for severely ill or immunocompromised persons (103).

1.1.3.3 Epidemiology

Approximately one-third of the 50 *Legionella* species have been associated with human disease, foremost *L. pneumophila* (168, 242). About 90% of Legionnaires�' disease cases are caused by *L. pneumophila* and only about 10% are caused by non-*pneumophila Legionella* species. This does not correspond to the environmental distribution of *Legionella* strains (Figure 1) (242). However, the distribution of *Legionella* species as cause for Legionnaires' disease may differ geographically. In Australia as an example, approximately 45 % of Legionnaires' disease cases are caused by *L. pneumophila* and *L. longbeachae*, each, and 10% by other *Legionella* species (28, 284).

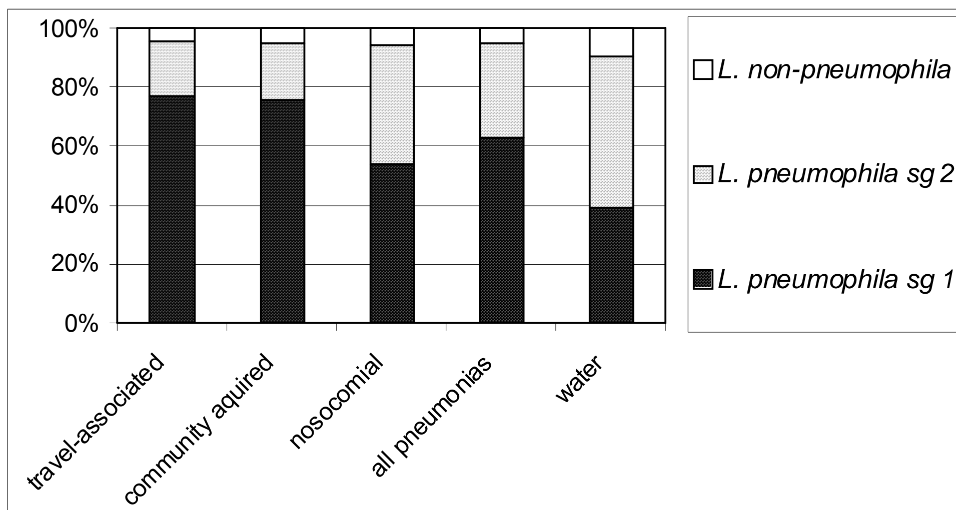


Figure 1: Prevalence of *Legionella* species and serogroups among clinical and environmental *Legionella* isolates 1986–2006, Dresden, Germany. Data from: (242).

1.2 Diversity within *L. pneumophila* genomes

The epidemiological data support that *L. pneumophila* is more virulent than other *Legionella* strains. For that reason *L. pneumophila* is of particular interest. To date, four *L. pneumophila* genomes are sequenced: these are strain Philadelphia I, strain Paris, strain Lens, and strain Corby (60, 64, 151). The *L. pneumophila* genomes comprise 3,503,610 base pairs (bp) (Paris), 3,345,687 bp (Lens) 3,397,754 bp (Philadelphia-1) and 3,576,461 bp (Corby) with an average GC content of 38% and ~3000 predicted protein-coding genes in each genome (60, 64, 151). About 2,400 genes have an orthologous gene in all *L. pneumophila* genomes, constituting the common backbone of this species (59). However, many differences in gene content are also present, including genes involved in the ability of *L. pneumophila* to replicate intracellularly within protozoa and to cause disease in humans (59). In *L. pneumophila*, gene gain as a result of horizontal gene transfer is presumably the most important source of genome variation. This is reflected by the genomes, which contain plasmids, genomic and pathogenic islands, IS elements and ‘islets‘ (transposases small DNA pieces) (59). This section focuses on the distribution and variability of known *L. pneumophila* genomic islands and plasmids.

1.2.1 Genomic and pathogenicity islands in *L. pneumophila*

Pathogenicity islands are a class of mobile elements. The term 'pathogenicity island' originated from the study of uropathogenic *E. coli* but has subsequently been widely applied to bacterial pathogens (162). Typical features of pathogenicity island are a GC content that often differs from that of the rest of the genome, the presence of direct repeats at their ends, the association with tRNA genes, the presence of integrase determinants and other mobility loci, and their genetic instability (162). Genomic islands have the same characteristics as pathogenicity islands with the exception that they apparently do not contribute to virulence. The *L. pneumophila* genomes carry several genomic and pathogenicity islands which differ from strain to strain in size and in the encoded proteins. These genetic elements are preferable inserted in plasticity zones of the *L. pneumophila* genomes (59).

One example for a pathogenicity island is the region encoding the Lvh type-IV secretion system. The *lvh* cluster itself is highly conserved in the genomes of the

strains Paris, Lens, and Philadelphia 1 while the flanking DNA regions are strain specific in size as well as in the genetic content: in strain Paris the *lvh* region is flanked by 11 kb and 22 kb, in strain Lens by 5.8 kb and 30 kb and in strain Philadelphia 1 by 14 kb and 30 kb. This island has the capacity to exist in an integrated and an excised form as multi copy plasmid in strain Paris and strain Philadelphia 1, and due to the presence of flanking direct repeats perhaps also in strain Lens. In strain Philadelphia 1 this island is inserted in an Arg tRNA and in strains Paris and strain Lens the same tmRNA (57, 330).

In *L. pneumophila* Corby two similar large genomic islands are present called Trb-1 and Trb-2 (151). Both islands encode all genes necessary for a functional type-IV secretion system with a *trb*/*tra* gene organization similar to the *tra*/*trb* region of plasmid R751 (IncP) of *Enterobacter aerogenes* (358) and contain an oriT-like site. Beside the *trb*/*tra* genes, the gene content of the islands is specific. Trb-1 is integrated in a Pro tRNA gene while Trb-2 is inserted in a tmRNA. The ladder integration site is identical to that of the pathogenicity island containing the *lvh* region in strain Paris and strain Lens. Both, the Trb-1 and Trb-2 can exist as an integrated and an excised form (151, 347).

A 65 kb pathogenicity island (LpPI-1), present in strain Philadelphia 1 is absent from strains Paris, Lens and Corby. This island is inserted in a Val tRNA gene, encodes putative virulence factors and a cluster of genes encoding homologues of Tra proteins, and contains mobile elements. The Tra region shows in average 55% similarity to the Tra proteins associated with the F plasmid of *E. coli*. In addition, the LpPI-1 *tra* gene homologues are arranged identically to those of the F plasmid, excepting *traM*, *traY*, and *traX*, which are missing in *L.pneumophila* (44, 57).

1.2.2 *L. pneumophila* plasmids

L. pneumophila strain Lens and strain Paris contain plasmids of different sizes (Paris 132 kb, Lens 60 kb). Both plasmids, of strain Paris and strain Lens show a heterogeneous distribution and can either be intact or truncated. These plasmids contain several mobile elements and a gene cluster encoding homologues of Tra proteins. The plasmid identified in strain Paris carries many genes coding proteins probably conferring antibiotic resistances (59, 347).

1.3 The intracellular life cycle of *L. pneumophila*

This section aims to provide a brief overview of the *L. pneumophila* infection cycle in host cells. Furthermore, the life cycle of *L. pneumophila* and the underlying regulatory circuit are discussed.

1.3.1 Infection cycle in host cells

L. pneumophila has a similar infection cycle within protozoa and human macrophages. however, dependent on the host cell, different mechanisms might be used to enter and exit from the respective host cell types (115, 141, 167). Once inside the host cell, *L. pneumophila* remains in the phagosome which does not enter the endolysosomal pathway. *L. pneumophila* inhibits phagolysosomal fusion and acidification of the phagosome (196, 197). It establishes phagosomes, which are completely isolated from the endosomal pathway but are surrounded by endoplasmic reticulum. Within this vacuole, *L. pneumophila* starts to replicate. During the late replicative phase, the vacuole merges with lysosomes, providing a nutrient-rich replication niche (351). Finally, a nutrient decline leads to the transition of *L. pneumophila* to the transmissive phase (53), expressing many virulence-associated traits promoting the release of the bacteria and infection of a new host cell (53, 265, 353, 354) (Figure 2).

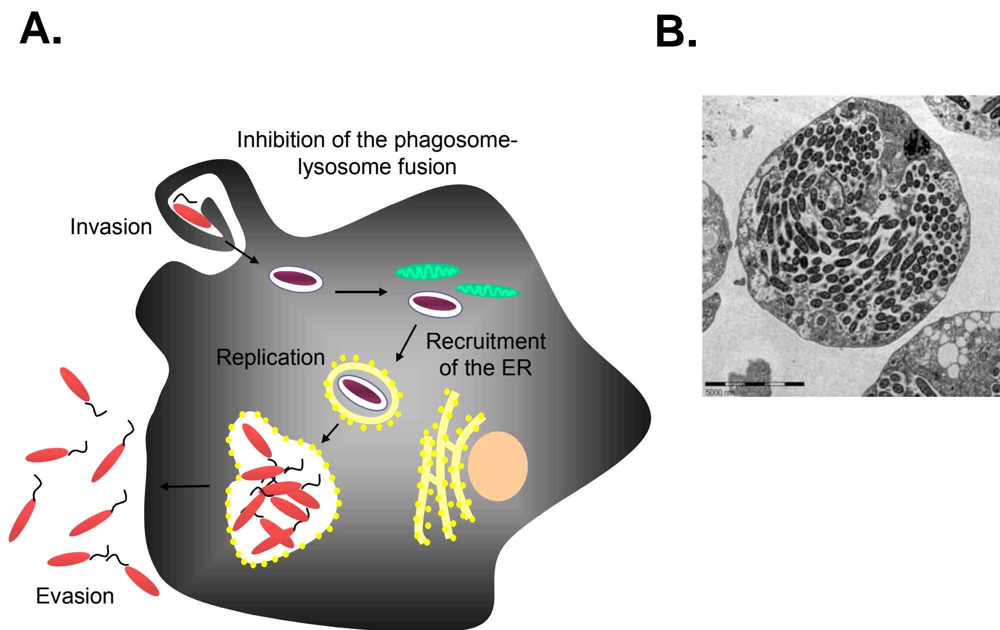


Figure 2: The *L. pneumophila* infection cycle. **A.** The infection cycle of *L. pneumophila* begins and ends with free, flagellated bacteria. **B.** *Hartmannella vermiformis* amoeba filled with *L. pneumophila*. Photo: Holland/Özel, Robert Koch-Institut.

1.3.2 Cellular differentiation – a key feature for *L. pneumophila* pathogenesis

1.3.2.1 Characterization of replicative and transmissive phase bacteria

The infection cycle is reflected by a major shift in gene expression from replicative to transmissive phase in *L. pneumophila* (49). As a consequence of this shift in gene expression, the life cycle of *L. pneumophila* consists of two distinguishable phenotypes: replicative bacteria and transmissive bacteria.

Replicative bacteria are found in the replicative vacuole, are sodium resistant and unflagellated (19-21, 53, 112, 166, 179, 181, 200, 244, 266, 324, 325, 354, 394). In contrast, transmissive phase bacteria are highly motile and express numerous virulence traits (Table 1) (9, 19-21, 53, 109, 112, 155, 164, 166, 179, 181, 200, 211, 244, 266, 324, 325, 351, 394). They are able to evade from the host cell and to infect a new host cell. After infection of a new host cell, *L. pneumophila* reverts again to the replicative form. This biphasic life cycle can be mimicked in broth culture with exponential and post-exponential grown bacteria.

Table 1: Reciprocal expression of various traits in replicative and transmissive phase of *L. pneumophila*.

Traits	Replicative phase	Transmissive phase
Replication	+	–
Motility / flagellation	–	+
Contact-dependent cytotoxity	–	+
Stress resistance	–	+
Sodium sensitivity	–	+
Pigment production	–	+
β-hydroxybutyrat storage granules	–	+
Small coccoid cell shape	–	+
Lysosome evasion	–	+

– indicates repression; + indicates activation.

1.3.2.2 Regulatory control of the L. pneumophila *life cycle*

Transition from the replicative to the transmissive phase in *L. pneumophila* is governed by complex regulatory networks, which are up to now only partially understood. In *Escherichia coli*, the small RNA-binding protein Carbon Storage Regulator A (CsrA) (320) and the regulatory RNA's *csrB* and *csrC* function as a global post-transcriptional regulatory system. CsrA regulates translation initiation of several genes by binding to the mRNA and the two regulatory RNA's sequester CsrA and compensate its regulatory activity (248, 320). A two-component signal transduction system named BarA/UvrY regulates expression of *csrB*. (172, 248, 320). CsrD, presumably part of an autoregulatory loop, is required for the decay of *csrB* and *csrC* through an RNase E-mediated pathway (352). Carbon storage regulatory (Csr) systems like in *E. coli*, control gene expression post-transcriptionally and are found in several bacteria (248, 320). For *L. pneumophila*, it is supposed that a CsrA homologue of *E. coli* represses the expression of transmission traits in replicative phase (112, 266). Accordingly, for transition to the transmissive phase the CsrA repression must be relieved (112, 266).

The two-component system LetA/LetS of *L. pneumophila*, homologues to BarA/UvrY or GacA/GacS in *Pseudomonas aeruginosa* (166, 244, 248) was studied in more detail. During nutritional deprivation, LetA/LetS induces the expression of transmissive traits by relieving CsrA repression (266). Accordingly, *L. pneumophila* LetA/LetS is predicted to induce the expression of one or more regulatory RNA's which alleviate binding of CsrA to mRNA's. The nucleotide sequence of these regulatory RNA's has not been conserved in evolution while the structure and function has. Much attempt has been undertaken to identify regulatory RNA's in different bacteria. Kulkarni and colleagues (222) have developed a computer program (CSRNA_FIND) designed to locate potential CsrA-regulating small RNA's in bacteria and they predicted two putative CsrA-regulating small RNA genes for *L. pneumophila*, named *rsmY* and *rsmZ*. Also involved in differentiation caused by LetA/S is LetE, a small novel protein (20, 166). *L. pneumophila* possess also a homologue of CsrD of *E. coli* (*lpp0891*), which accordingly might be required for the decay of the regulatory RNA's sequestering CsrA.

The LetA/LetS two-component system of *L. pneumophila* probably responds to ppGpp (20, 166, 266). In accordance, the entry in the transmissive phase is initiated

by a mechanism called ‘stringent response’ (165). Under conditions of nutrient starvation, signaled probably by low amino acid levels (201), RelA synthesizes the alarmone molecule ppGpp (165, 166, 394). Another factor involved in regulation of the biphasic life cycle in *L. pneumophila* is the alternative sigma factor RpoS. However, its implication and exact role is not understood yet, as contradictory results are reported (2, 19, 21, 138, 164, 258, 265, 266). By analogy to *E. coli*, it has been speculated that ppGpp increases the amount of RpoS. But recent reports did not find a link between ppGpp and RpoS expression in *L. pneumophila* (2). It was speculated that there might exist a link between LetA/LetS and RpoS (19, 46, 138, 244). This link is perhaps identified by the finding that both LetA and RpoS promote the expression of the response regulator LqsR. LqsR promotes pathogen-host cell interaction and suppresses the transition from transmissive to replicative phase (359).

1.4 Expression of a flagellum in *L. pneumophila*

Below the implication of the flagellum in virulence as well as its regulation will be discussed.

1.4.1 The flagellar genes

In *L. pneumophila*, 46 genes organized in 10 genomic regions were predicted to participate in flagella biosynthesis or its regulation (49, 183). Based on their temporal expression seen in intracellular transcriptome study they were classified in flagellar class I-IV genes (Table 2) (49). Similar to the flagellated bacterium *Aquifex aeolicus* (95), chemotaxis genes seem to be absent in the sequenced *L. pneumophila* strains.

1.4.2 Regulatory control of flagellation

Flagellation of *L. pneumophila* is dependent on the regulatory control of the life-cycle described in subsection 1.3.2.2 (19-21, 112, 123, 165, 166, 244, 266) and different environmental factors (179). For the subsequent flagellar gene expression varying models were proposed (7, 49, 177, 184, 200). Based on the presence of *P. aeruginosa* homologous of FleQ, FleSR, FliA, and RpoN, it is believed that the flagellar gene regulation in *L. pneumophila* is similar to that in *P. aeruginosa* (see subsection 1.7.4.). That FleQ and RpoN are involved in flagellar gene regulation and FliA controls flagellar class IV gene expression was verified recently (49, 199).

1.4.3 Implication of the flagellar regulon in virulence

Expression of a single monopolar flagellum correlates with transition to transmissive phase and thus it was speculated that its expression is regulatory linked to other transmissive phase traits (39, 53, 139, 165, 180, 184, 301, 324). This hypothesis is supported by Pruckler et al., as they examined the role of insertional mutants with mini-Tn10 in the infection of amoebae and human monocyte-like cells and observed that most flagellin-deficient mutant strains were attenuated in infectivity but non of the flagellin-positive mutant strains (301). Though this study does not

distuigish if the flagellum itself or regulators involved in regulation of flagellar biosynthesis are implicated in infectivity.

To my knowledge, the first evidence that the flagellum is directly implicated in virulence was reported by Dietrich et al. (97). Hereby it was proved that the invasion capacity of a *L. pneumophila flaA* (*flaA* encodes flagellin, the major subunit of the flagellum) mutant strain was attenuated in eukaryotic cells while intracellular replication was not significantly affected. However, due to the experimental protocol it is possible that motility rather than the pure presence of the flagellum promotes contact with host cells. This hypothesis is supported by experiments performed with a *L. pneumophila motAB* mutant strain. Motility of this strain is highly reduced and bacteria are less flagellated compared to the wild type. This mutant was attenuated in establishing the first contact to host cells but not in intracellular replication, like the *flaA* mutant (264). Presumably the flagellum-mediated motility facilitates to reach and infect new host cells and this contributes to fitness of *L. pneumophila*

There is accumulating evidence that the σ^{28} factor FliA is implicated in virulence. It was reported that FliA enhances infectivity and intracellular replication (166, 180, 264), biofilm formation (249), and hemolytic contact-dependent activity (183). Contact-dependent hemolysis is also enhanced by FleQ and RpoN (183). Moreover, flagellar biosynthesis is regulatory coupled to other transmissive phase traits *via* the regulatory control governing transition to transmissive phase.

1.4.4 Flagellin restricts replication in macrophages

The importance of flagella, and flagellin in particular, was recently shown by different groups. Flagellin is sensed by the innate immunity of a mammalian host through the Toll-like receptor (TLR5). But recent studies indicated that flagellin is also sensed TLR5-independent (10, 125, 233, 260).

In mice, different alleles of the Nod-like receptor gene NAIP5/Birc1e determine whether macrophages restrict or support intracellular replication of *L. pneumophila* and whether a mouse is resistant or susceptible to *Legionella* infection. *L. pneumophila* has been shown to replicate more readily in macrophages that are derived from the A/J strain of mice, compared with those that are derived from the C57BL/6 strain of mice (387) due to mutations affecting the NAIP5/Birc1e locus (98,

385). Also caspase-1-deficient macrophages show an increased susceptibility to *L. pneumophila* intracellular replication (393).

Restriction of *L. pneumophila* replication in mice macrophages is a consequence of caspase-1 activation dependent on the inflammosome (a cytosolic multiprotein complex) containing NAIP5/Birc1e and Ipaf that responds to cytosolic flagellin (11, 233, 313, 355, 369). While in most mice strains powerful innate immune mechanisms mediate resistance to *Legionella* infection, humans can develop a severe pneumonie. Nevertheless, a recent study demonstrated that NAIP and Ipaf also restrict to a certain extend *L. pneumophila* replication in human cells by recognizing flagellin (369).

Table 2: Flagellar genes in *L. pneumophila* Paris (*Lpp*).

Gene name	Synonym in *Lpp*	Known or predicted product	Class
rpoN	*lpp0542*	RNA polymerase sigma-54 factor	I
fleQ	*lpp0915*	Transcriptional regulator FleQ	I
flgN	*lpp0968*	Unknown	III
flgM	*lpp0969*	Negative regulator of flagellin synthesis (Anti-sigma-28 factor)	III
flgA	*lpp0970*	Flagellar basal body P-ring biosynthesis protein FlgA	IIb
flgB	*lpp1224*	Flagellar basal-body rod protein FlgB	IIb
flgC	*lpp1225*	Flagellar basal-body rod protein FlgC	IIb
flgD	*lpp1226*	Flagellar basal-body rod modification protein FlgD	IIb
flgE	*lpp1227*	Flagellar hook protein FlgE	IIb
flgF	*lpp1228*	Flagellar biosynthesis protein FlgF	IIb
flgG	*lpp1229*	Flagellar biosynthesis protein FlgG	IIb
flgH	*lpp1230*	Flagellar L-ring protein precursor FlgH	IIb
flgI	*lpp1231*	Flagellar P-ring protein precursor FlgI	IIb
flgJ	*lpp1232*	Flagellar biosynthesis protein FlgJ	IIb
flgK	*lpp1233*	Flagellar hook-associated protein 1	IIb
flgL	*lpp1234*	Flagellar hook-associated protein FlgL	IIb
fliS	*lpp1291*	Flagellar protein FliS	IV
fliD	*lpp1292*	Flagellar hook-associated protein 2 (flagellar capping protein)	IV
flaG	*lpp1293*	Unknown	IV
flaA	*lpp1294*	Flagellin	IV
fliK'	*lpp1657*	Flagellar hook-length control protein FliK	IIb
fliJ	*lpp1720*	Flagellar protein FliJ	IIa
fliI	*lpp1721*	Flagellum-specific ATP synthase FliI	IIa

Table 2 - continued

fliH	*lpp1722*	Polar flagellar assembly protein FliH	IIa
fliG	*lpp1723*	Flagellar motor switch protein	IIa
fliF	*lpp1724*	Flagellar M-ring protein	IIa
fliE	*lpp1725*	Flagellar hook-basal body complex protein	IIa
fleR	*lpp1726*	Two-component response regulator	IIa
fleS	*lpp1727*	Sensor histidine kinase	IIa
motB	*lpp1744*	Chemotaxis MotB protein	III
motA	*lpp1745*	Flagellar motor protein MotA	III
fliA	*lpp1746*	Sigma factor 28	III
fleN	*lpp1747*	Flagellar synthesis regulator	IIb
flhF	*lpp1748*	Flagellar biosynthesis protein FlhF	IIb
flhA	*lpp1749*	Flagellar biosynthesis protein FlhA	IIa
flhB	*lpp1750*	Flagellar biosynthetic protein FlhB	IIa
fliR	*lpp1751*	Flagellar biosynthetic protein FliR	IIa
fliQ	*lpp1752*	Flagellar biosynthetic protein FliQ	IIa
fliP	*lpp1753*	Flagellar biosynthetic protein FliP	IIa
fliO	*lpp1754*	Flagellar protein FliO	IIa
fliN	*lpp1755*	Flagellar motor switch protein FliN	IIa
fliM	*lpp1756*	Flagellar motor switch protein FliM	IIa
motA2	*lpp2266*	Proton conductor component of motor, chemotaxis and motility protein	III
motB2	*lpp2267*	Flagellar motor protein	III
flhB‘	*lpp2635*	Putative part of export apparatus for flagellar proteins	III
motY	*lpp3034*	Sodium-type flagellar protein MotY	IV

1.5 Protein secretion systems – central to pathogenesis of *L. pneumophila*

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 protein secretion systems, which are also shortly described in this section.

1.5.1 Secretion across the cytoplasmic membrane

L. pneumophila possesses two systems for protein transport across the cytoplasmic membrane: the Sec pathway which translocates proteins in an unfolded state and the Tat pathway for translocation of folded proteins. The Tat pathway is involved in biofilm formation, growth under low iron conditions and growth within macrophages and *A. castellanii*. To date, several proteins were predicted to be Tat substrates but until now only the 3',5'-cyclic nucleotide phosphodiesterase and the iron-sulfur subunit of the ubiquinol-cytochrome c reductase were confirmed to be Tat substrates (86-89, 321).

1.5.2 A putative type-I secretion system

The *lssXYZABD* locus of *L. pneumophila* encodes a protein of the ATP binding cassette protein family LssB and a protein of the membrane fusion protein family LssD. Beside, the *L. pneumophila* genome encodes TolC-like proteins, the third component of type-I secretion systems. This putative type-I secretion system is called Lss for *Legionella* secretion system (199). Two putative substrates have been identified. However, for one of those it has also been shown to be secreted dependent on the type-II secretion system (6, 94).

1.5.3 A type-II secretion system

The type-II secretion system of *L. pneumophila* is dependent on the *pilBCD* locus that is also involved in the biogenesis of type IV pili and on the *lsp* (*Legionella*

secretion pathway) *FGHIJK* locus. In addition to the prepilin peptidase PilD, the outer membrane secretin LspD, the ATPase LspE and the inner membrane protein LspF play a role in *L. pneumophila* type-II protein secretion (322). The Lsp secretion system promotes the ability of *L. pneumophila* to infect protozoan and macrophage hosts, to grow in the mammalian lung (322) and at low temperatures (337). It is involved in secretion of various enzymes (94) (see also subsection 1.6.2).

1.5.4 Type-IV secretion systems

Type-IV secretion systems can mediate different cellular functions: they can transfer DNA substrates and/or proteins between bacteria or between bacteria and eukaryotic cells. Type-IV secretion systems whose function is predominantly protein secretion often transfer proteins directly into the host cell cytosol and thus contribute to successful infection of hosts by pathogenic bacteria (65, 67, 311). In *L.pneumophila* two type-IV secretion systems known to be implicated in virulence are encoded: a type-IVB secretion system similar to the Tra/Trb system of IncI plasmids and a type-IVA system similar to the *Agrobacterium tumefaciens* Vir system.

The Dot/Icm type-IVB secretion system (34, 251) of *L. pneumophila* 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 type-IV secretion system is required for replication of *Legionella* in both host systems, protozoa and human macrophages (13, 104, 331). It translocates effector molecules into the eukaryotic host cell during infection, which contribute to a successful infection cycle during all stages of infection (see subsection 1.6.2).

The Lvh type-IVA secretion system is encoded by 11 genes. It is dispensable for intracellular growth in both macrophages and amoebae (330), but is implicated in host cell infection by *L. pneumophila* at 30°C (315). Recently, it was suggested that this system can replace a defective Dot/Icm system under certain conditions (23). This system is absent from *L. pneumophila* Corby (151).

1.5.5 A putative type-V secretion pathway - autotransporter

Strain Paris, but not the other three sequenced *L. pneumophila* strains possess a gene (*lpp0779*) that is predicted to encode an autotransporter protein. It shows the typical structure of a type-V secretion protein and it was speculated that it might mediate adherence to mammalian cells and/or autoaggregation during biofilm formation (7). However, involvement in virulence was not shown yet.

1.5.6 'Vesicle-mediated secretion'

Besides the secretion of individual proteins, many gram-negative bacteria, including *L. pneumophila*, shed vesicles derived from the outer membrane (4, 111, 137). Enterotoxigenic *E. coli* vesicles as an example serve as specifically targeted transport vehicles that mediate entry of active enterotoxin and other bacterial envelope components into host cells (213). Although the *L. pneumophila* vesicles might be associated with virulence (111, 137), they must not essentially serve as specifically targeted transport vehicle.

1.6 Virulence factors of *L. pneumophila*

The interaction of *L. pneumophila* with protozoa seems to have generated a pool of virulence traits during evolution. The same virulence traits allowing to infect a broad host range of protozoa, enables *L. pneumophila* to infect also human cells. Though various properties contribute to the virulence of *L. pneumophila*, this section focuses on secreted and surface associated virulence properties.

1.6.1 Surface proteins

Attachment to and invasion of the host cell is central to *L. pneumophila*'s ability to cause Legionnaires' disease. The *Legionella* surface protein MOMP, is implicated in this process by binding to the complement component CR3 of human monocytes. The subsequent uptake of the bacteria occur by attachment to the complement receptors CR1 and CR3. The MOMP protein, a porin, is encoded by the *ompS* gene (*lpp1958*) (31, 133, 134, 190, 296). However, also complement-independent binding to macrophages 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 bacterial outer membrane (148). Attachment to mammalian and amoebal hosts are also mediated by type-IV pili (349) and the 60 kDa heat shock protein Hsp60 (349). Another surface protein, the 16 kDa *L. pneumophila*-specific outer membrane protein encoded by the *ligA* gene is a putative adhesion that probably contributes to the initial uptake of *L. pneumophila*. Deletion of the *ligA* gene resulted in sodium resistance, decreased cytotoxicity, decreased hemolytic activity and avirulence in *A. castellanii* (113).

A very interesting *Legionella* protein that is exposed on the cell surface is Mip. Mip belongs to the enzyme family of FK-506 binding proteins that exhibit PPIase activity. It promotes the presence of a p-nitrophenyl phosphorylcholine hydrolase activity in culture supernatants, the infection of eukaryotic host cells, and perhaps dissemination in the human body (69-71, 93, 107, 118, 174, 175, 215, 327, 373, 381). The surface protein Lpa, might promote dissemination in the human body. Lpa is a homologue of the plasminogen activator protein (Pla) of *Yersinia pestis* and has the capacity to convert plasminogen into plasmin (371).

Probably membrane-associated is also the major cell-associated phospholipase A PlaB. PlaB contributes to the cytotoxicity of *L. pneumopila* (120) and plays a role for successful dissemination of *L. pneumophila* in tissues (Heuner and Flieger, unpublished).

1.6.2 Secreted factors

The Dot/Icm type-IV secretion system is indispensable for a successful infection of host cells by *L. pneumophila*, as it translocates a number of effector proteins which subvert host cell functions (239). Accordingly, many studies aimed at identifying and characterizing its substrates and the number of effectors translocated by the Dot/Icm system permanently grows.

The first characterized effector was RalF. This effector is required for localization of the host GTPase protein ARF-1, a key regulator of vesicle trafficking from the endoplasmic reticulum to the phagosomes (273). The substrate LidA is involved in recruitment of vesicles during vacuole biogenesis and in maintaining integrity of the Dot/Icm complex (75). The substrates LepA and LepB are involved in egress of *Legionella* from protozoan hosts but not mammalian hosts (62, 63). Numerous candidate effector proteins named SidA-H, were identified in the Philadelphia 1 strain by a two hybrid screen with IcmG/DotF as bait followed by a screen of proteins transferred inter bacterially with a Cre/loxP based protein translocation assay (243). SidA, SidB, SidC, SidE and SidF proteins contain a coil-coiled domain, a protein motif involved in protein-protein interactions. SidC anchors to phosphatidylinositol-4 phosphate on *Legionella* containing vesicles and recruits ER vesicles to a replication-permissive vacuole (78). It was suggested that SidF and SdhA, a paraloug of SidH are involved in the inhibition the host cell death (26, 225). SidF interacts with the proteins BNIP3 and Bcl-rambo, both members of the Bcl2 family (26). Two independent studies reported that SidM/DrrA, a guanosine nucleotide exchange factor is a Dot/Icm substrate translocated into host cells. SidM/DrrA - enhanced by LidA - recruits Rab1 (a small host GTPase regulating ER-to-Golgi traffic) to *Legionella*-containing vacuoles (90, 246, 270). Further substrates of the Dot/Icm secretion system are the proteins VipA, VipD and VipF. These vacuole protein sorting inhibitor proteins (Vip) inhibit lysosomal protein trafficking by different mechanisms (332). Also translocated *via* the Dot/Icm secretion system are WipA,

WipB, YlfA (yeast lethal factor A), YlfB and six additional proteins containing Leu-rich repeats and/or coiled-coil domains (54, 91, 281). A recent approach using a systematic screening technique identified further 19 novel putative Dot/Icm substrate proteins. One of those substrates, named LubX belongs to the eukaryotic-like proteins of *L. pneumophila* as it contains two domains that have a remarkable similarity to the U-box, a domain found in eukaryotic E3 ubiquitin ligases and was studied in more detail. LubX has ubiquitin ligase activity in conjunction with UbcH5a or UbcH5c E2 enzymes and mediates polyubiquitination of host Clk1. One U-box domain is critical to the ubiquitin ligation, and the other U-box domain mediates interaction with Clk1 (219). The eukaryotic-like ankyrin proteins AnkB and AnkX are translocated into the host cell dependent on the Dot/Icm secretion system (8, 286). AnkB is required for intracellular proliferation within human macrophages and protozoa (8) AnkX prevents microtubule-dependent vesicular transport to interfere with fusion of the *L. pneumophila*-containing vacuole with late endosomes after infection of macrophages (286).

Eliminating one or several effector proteins often has little impact on intracellular multiplication of *L. pneumophila* (91, 239, 246, 270, 273, 368). This led to the speculation that effectors might have redundant functions (280). Redundant functions of the effectors might also explain that each strain has a slightly different array of effector proteins (7). However, this might also reflect subtly differences in host adaptation and thus host-specificity of different *L. pneumophila* strains (7).

Beside the Dot/Icm type-IVB secretion system also the Lsp type-II secretion system of *L. pneumophila* is involved in the secretion of virulence factors. Remarkably, *L. pneumophila* is to my knowledge the only intracellular pathogen known to encode a type-II secretion system which is implicated in virulence so far. This secretion system secretes a tartrate-resistant and tartrate-sensitive acid phosphatase, a RNase, the zinc metalloprotease ProA (MspA), mono-, di- and triacylglycerol lipases, phospholipases A and C, the lysophospholipase A PlaA, the lysophospholipase A-homologue PlaC, a p-nitrophenyl phosphorylcholine hydrolase, and two distinct aminopeptidases encoded by *lapA* and *lapB*, among others (14, 15, 24, 25, 119, 163, 322, 323).

For various newly identified and presumably secreted virulence factors the secretion pathway is still unknown. As an example, for the Rtx toxin (encoded by

rtxA), which is involved in entry and replication in protozoa and human macrophages (72-74) no secretion pathway was identified so far. Rtx proteins are a large family of pore-forming cytolysins present in a number of different bacterial pathogens that are characterized by a nonapeptide Gly-reach repeat motif (377). In contrast to the extracellular bacteria *Vibrio cholerae*, in which the *rtx* gene is highly conserved, the gene region coding for the Rtx toxin in *L. pneumophila* is highly variable concerning the number and sequence of these repeats. Changes in the repeatscould play a role in pathogenicity of *L. pneumophila*. The interplay of the Rtx toxin with host membranes might lead to the evolution of new variants that are able to escape host cell defenses (58, 85). LpnE is present in the culture supernatants, however, secretion is independent of both the Lsp type-II secretion system and the Dot/Icm type-IV secretion system. The Sel1 repeat protein LpnE influences vacuolar trafficking and can interact with eukaryotic proteins containing immunoglobulin-like folds (275, 276). Another protein family presumably secreted are the glycosyltransferases termed Lgt1-3 which modify the eukaryotic elongation factor eEF1A. This modification results in inhibition of protein synthesis and causes death of the host cells (32, 33). The eukaryotic-like ankyrin proteins AnkH and AnkJ play a role in intracellular replication, probably in modulation of the phagosome biogenesis by *L. pneumophila* (161). To fulfill those functions these proteins presumably must be transferred to the host cell.

1.7 Bacterial flagella

Although the basic flagellar structure is well conserved across bacteria, dependent on the speciec they vary with respect to the number of flagella per cell, the location of flagella on the cell surface, and the overall number of flagellar genes (27, 297). Relatively little is known about the evolutionary origins of the flagella so far. This section summarizes the current knowledge of the evolutionary origin of bacterial flagella, points out how a lack of scientific knowledge is exploited for dissemination of religious doctrines, and gives an overview of flagella structure and regulatory concepts.

1.7.1 The origin of bacterial flagella

1.7.1.1 Evolution of bacterial flagella

Several of the flagellar proteins share common ancestry with components from different other biological systems (285). Due to extensive similarity between some flagellar genes and genes dedicated to protein secretion systems, it was speculated that the flagellum arose from a secretion system that was later adapted to bacterial motility (37, 56, 271, 277).

Besides, all bacterial flagella share a conserved core set of about 20 proteins (285) and Lui and Ochman (236) suggested that the genes encoding those proteins have evolved through successive duplications of one or a few genes whereby horizontal gene transfer played a minor role. This suggestion, however, was harshly criticized as perhaps too simple by Doolittle and Zhaxybayeva (99) who argue for a model involving horizontal gene transfer, cobbling together parts with separate origins and multiple other original functions.

The evolution of peritrichous flagellar systems was investigated in another recent study of Liu and Ochsman (235). They proposed that lateral flagellar systems originated twice: once in α-proteobacteria and again in the common ancestor of the β- and γ-proteobacteria. The genetic basis of flagella has been subject to many modifications which is reflected by the order and organization of flagellar genes. In the ancestors the flagellar gene operons existed as small units and have expanded through the recruitment of new genes and fusion of gene units. In contrast to the evolutionary trend towards larger flagellar gene operons, operon structures in ε- and

α-proteobacteria have been highly disrupted through gene disassociation and rearrangements.

1.7.1.2 The myth of the flagella to be irreducible complex

Out of the traditional creationist movement in the United States, the intelligent design creationist movement arose. This movement promotes the intelligent design theory - standing for the religious belief in a supernatural creator - as a purported scientific alternative to evolutionary theory (122). A main argument made by proponents of intelligent design is the irreducible complexity of certain biological systems, which they claimed as too complex to have evolved through natural selection (1, 99, 271, 285).

One of the examples given for irreducible complex systems by the intelligent design creationists, first and foremost by the scientist Behe are bacterial flagella (29). This example was also used in the much noticed trial Kitzmiller *versus* Dover Area School District in 2005 concerning the dissemination of the intelligent design theory in a United States public school. But the judge adjudicated that teaching of creationism in any form in United States public schools is unconstitutional and stated that the bacterial flagellum is in fact not irreducible complex (1). Accordingly, numerous scientific arguments support the view that bacterial flagella have evolved, rather than designed (1, 271, 285). Thus the irreducible complexity of bacterial flagella disseminated by the intelligent design creationists is a religious belief rather than a scientific theory, although the intelligent design creationists attempt to manufacture a ‘scientific‘ controversy (122).

1.7.2 Bacterial flagella fulfill various functions

Movement driven by flagella is the most common strategy for motility in bacteria and represents an important advantage in moving towards favorable conditions or in avoiding unfavorable conditions, e.g. by chemotaxis (110). In addition to motility, bacterial flagella are involved in adhesion, biofilm formation and colonization (68, 79, 101, 140, 300, 343), and in pathogenic bacteria they are often considered as virulence factor (210, 283).

As an example most *E. coli* strains are motile by peritrichous (lateral) flagella. It was proposed that in *E. coli* motility promotes initial cell-to-surface contact and thus

flagella are critical for normal biofilm formation (300, 383). However, as *E. coli* strains are genotypic and accordingly phenotypic highly diverse this is not appropriate for all *E. coli* strains. Several recent publications revealed the importance of flagella in colonizing the urinary tract caused by uropathogenic *E. coli* (UPEC). Thereby flagella and type 1 fimbriae mediate opposite actions. Flagella enable the bacteria to disseminate through urine and along mucus layers, while type 1 fimbriae allow bacteria to adhere to specific receptors present on uroepithelial cells (333). Flagellum-mediated motility and chemotaxis may not be absolutely required for virulence; nevertheless these traits contribute to the fitness of UPEC and therefore significantly enhance the pathogenesis of urinary tract infections caused by UPEC (226, 227, 386).

In *Campylobacter jejuni* infections, the flagella is an important virulence factor. *C. jejuni* is a commensal of animals and an important cause of human bacterial diarrhea. The motility imparted by the polar flagella is required for colonization of animals and humans (268, 272, 295, 356, 374). Virulence factors and flagella are probably co-regulated (55, 144) and, in addition, recent studies showed that secretion of several virulence factors - the Cia proteins, the FlaC protein (homologue to flagellin proteins but apparently not part of the flagellar filament in *C. jejuni*), and the FspA protein - requires a minimal flagella structure (152, 217, 218, 299, 317, 338). The flagellins of *C. jejuni* are heavily glycosylated (326, 357) and changes in glycan composition affect autoagglutination and microcolony formation on intestinal epithelial cells (157). Altogether, the role of flagella in the virulence of *C. jejuni* (and also other *Campylobacter* species) is complex and manifold (reviewed in Ref. (156)).

Nevertheless, flagella usually show strong antigenic properties in animal and plant hosts (80, 170, 171, 208, 334).

1.7.3 Structure of bacterial flagella

Bacterial flagella are complex and fascinating organelles whose assembly is dependent on multiple cooperating components. The main structural aspects of flagella are common to all bacterial flagella (Figure 3) (reviewed in (106) and (247)).

The flagellar motor is proton- or Na^+-driven and composed of a rotor and a stator. The switch proteins FliM, FliN, and FliG (building the C ring) are responsible for the

state of motor rotation. Herein FliM connects the flagellar system with the chemotaxis system as the N-terminus binds to the signaling protein CheY. The torque, generated by the motor, is transmitted *via* the basal body, which is composed of a central rod surrounded by an MS ring, a P ring, and an L ring to the hook. The hook is believed to serve as flexible joint that converts the torque into a force. This force then is transmitted to the filament. The filament is a highly rigid helical structure mainly built from flagellin proteins which finally confers motility to the cell (18, 30, 36, 47, 92, 126, 127, 186-188, 191-195, 206, 207, 212, 220, 223, 230, 250, 253, 254, 293, 294, 341, 360, 361, 365-367). Flagella of *E. coli* can rotate clockwise, counterclockwise, and can also pause (229). Other bacterial species may have similarly three functional states of the motor or have two. In the latter case, the flagella rotate in one direction and pause (e.g. *Rhodobacter sphaeroides*) or rotate in two directions without pausing (e.g. in *Pseudomonas* ssp.) (169, 267, 336).

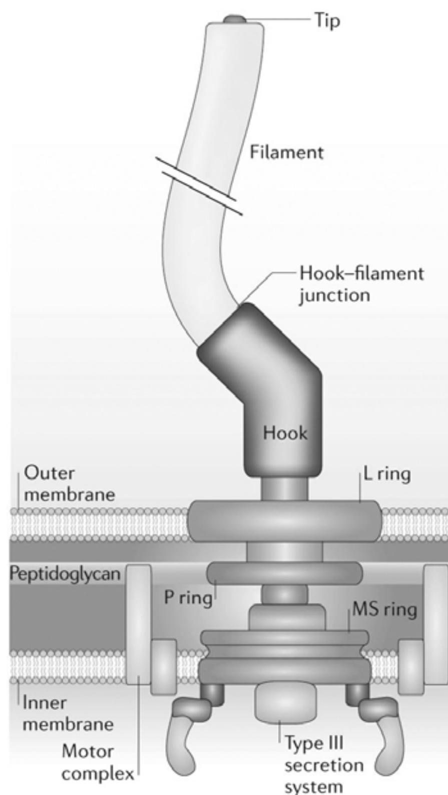


Figure 3: Bacterial flagella structure. From: (285)

1.7.4 Regulatory cascades of bacterial flagella

The flagellar biosynthesis and flagellar rotation is very energy-consumptive. Consequently, the flagellar biosynthesis is highly regulated by various environmental factors. To ensure maximal efficiency and accuracy during flagellar biogenesis, bacteria utilize hierarchical regulatory networks involving transcriptional and post-transcriptional mechanisms to control the ordered expression of the individual components of the flagellar organelle. Whereas significant differences exist between the regulatory programs used by the different bacteria, a salient feature is that the flagellar genes can be classified based on their temporal expression and on their dependence on various nested transcriptional regulators. Involved in the regulatory cascade of the polar flagellar system are enhancer binding proteins, which generally activate transcription of target genes together with the RNA polymerase in complex with a σ^{54} factor. In the regulatory cascade of the peritrichous flagellar systems FlhD and FlhC are the master regulators. While generally the regulatory cascade of the polar flagellar systems is four-tired, the regulatory cascade of the peritrichous flagellar systems is three-tired (340). The master regulators of both types of regulatory cascades are often involved also in regulation of non-flagellar genes. Thus the master regulator of the flagellar cascade in *P. aeruginosa* plays also a role in biofilm formation (16, 84, 185) and FlhD/FlhC is involved in many cellular processes as anaerobic respiration (302-305, 342).

This subsection provides examples of regulatory cascades controlling the flagellar biosynthesis in different gram-negative bacteria (reviewed in (256) and (340)). The current knowledge of the regulatory control of the flagellar biosynthesis in *L. pneumophila* is summarized in subsection 1.4.2.

In the γ-proteobacterium *P. aeruginosa*, the enhancer binding protein FleQ (a class I gene product) is the master regulator of the flagellar regulon. Expression of FleQ is σ^{70}-dependent and repressed by Vfr (82). It directly or indirectly regulates the expression of the majority of flagellar genes with the exception of *fliA*. The transcription of *fliA*, encoding the σ^{28} factor FliA appears to be constitutive and not dependent on flagellar regulators. Class II gene expression is directly induced by FleQ and the σ^{54} factor RpoN (82, 84, 316). The activity of FleQ is post-transcriptionally controlled in a feedback-loop by FleN which interacts with FleQ (81, 83). After phosphorylation-dependent activation of FleR by its cognate sensor kinase

FleS (both class II gene products), FleR and RpoN induce class III gene expression (84, 316). Finally, expression of FliA induces expression of flagellar class IV genes coding e.g. for flagellin which leads to the completion of the flagellum (50, 84, 129) (Figure 4).

The cascade of the ε-proteobacterium *Helicobacter pylori* is presumably similar to that of *P. aeruginosa*. However, in *H. pylori*, no master flagellar regulator has been identified; expression of many early flagellar genes seem to be constitutive. Similarly to *P. aeruginosa*, the enhancer binding protein FlgR and its cognate sensory partner FlgS control together with the σ^{54} factor the expression of class II genes. A σ^{28} factor transcribes late flagellar genes (class IV) and also participates in directing the expression of the intermediate class genes (class III) (43, 209 , 278, 279, 298, 390).

In the peritrichous flagellated *E. coli*, expression of the flagellar master regulators FlhD and FlhC is controlled by numerous regulators, including the RNA-binding protein CsrA (376). FlhD and FlhC induce flagellar gene transcription at two different types of promoters. First they activate transcription of flagellar genes dependent on the σ^{70} factor, including the *fliA* gene, encoding the σ^{28} factor FliA (238). In the following FlhD and FlhC also induce transcription of a subset of flagellar promoters that are depend upon FliA. Additionally, FliA induces transcription of a set of flagellar genes without direct influence of FlhD and FlhC (237, 282).

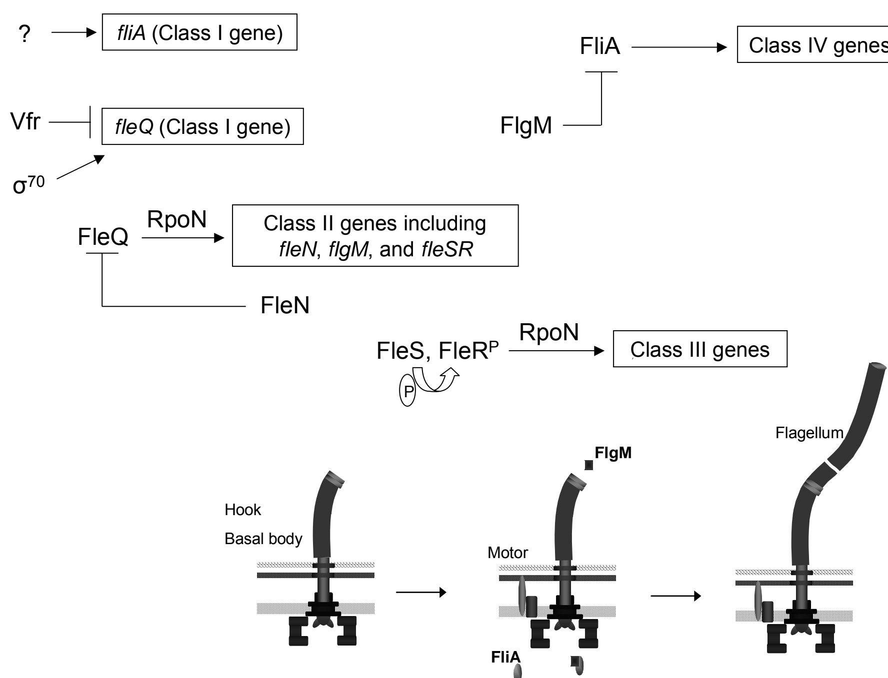


Figure 4: Model for flagellar regulation cascade in *P. aeruginosa*.

1.8 Conjugation plays a major role in bacterial evolution

Type-IV secretion systems are macromolecular transporters of gram negative bacteria that mediate intercellular transfer of DNA and/or proteins between bacteria or between bacteria and eukaryotic cells (173, 375). Transfer of DNA substrates is called conjugation and plays a crucial role for bacterial genome evolution, e.g. the *L. pneumophila* genome. Through conjugation newly developed enhancements can spread quickly to other bacterial species in communities.

1.8.1 Conjugation – a mechanisms for the transfer of genetic information

The transfer machinery required for conjugation, usually is encoded by self-transmissible and mobilizable plasmids termed conjugative plasmids, integrative conjugative elements forming high frequency recombinant strains, and conjugative transposons. The transfer functions are subdivided into mating pair functions (Mpf) and DNA transfer and replication functions (Dtr). The Mpf system is required for establishing the donor-recipient contact while the Dtr system is involved in DNA processing during conjugation (228). Based on the interrelationship between the regulatory systems controlling plasmid replication, the conjugative plasmids are classified in incompatibility groups IncF (e.g. F), IncP (e.g. RP4, R751), IncI (e.g. R144) and IncW (e.g. R388) (228).

1.8.2 The type-IV secretion system encoded by pRP4

The plasmid RP4 was isolated 1969 in Birmingham from *Pseudomonas* strains and carries antibiotic resistance genes (240). It is the best investigated plasmid of the incompatibility group IncP and has a broad host range (gram-negative and gram-positive bacteria as well as eukaryotic cells (149, 158, 173, 255, 362, 375)). The transfer genes of RP4 are functionally highly clustered: the Tra2 region encodes proteins necessary for mating pair formation and surface extension (287); the Tra1 region encodes all essential DNA processing functions and the oriT site is located within an intergenic region of Tra1 (Figure 5A) (228).

Bacteria carrying RP4 have short, rigid pili (Figure 5C). Efficient transfer of RP4 is adapted to solid surfaces or biofilms, what is in accordance with the hypothesis that IncP plasmid originated in soil bacteria (240, 380). The process of bacterial conjugation is not completely elucidated. Conjugal transfer of RP4 begins with the Tra2 (Figure 5A and 5B) encoded pilus, which extends from the donor, attaching to the recipient. The pilus then retracts until intimate contact is made between the donor and the recipient and the mating pore is formed. For DNA processing, TraG-M assemble to a nucleoprotein structure called relaxome at the oriT site (287). The relaxase TraI initiates the DNA transfer by cleaving the DNA at the *nic-site* in the oriT and the coupling protein TraG probably couples the DNA substrate to the Dtr-system (22, 231, 328). The ssDNA transferred to the recipient through a cytoplasmic conjugation bridge is probably synthesized by *rolling circle* replication (288, 289) and presumably protected from the action of proteases by proteins. Conjugation is finished perhaps by the action of the relaxase TraI (228).

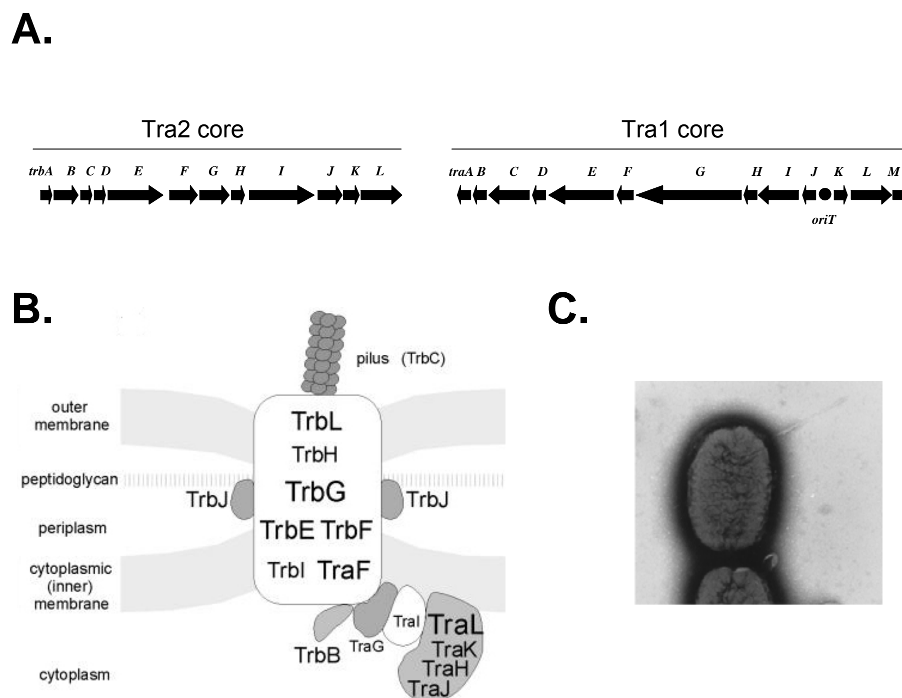


Figure 5: The conjugative plasmid RP4. **A.** Organization of the Tra regions. Adapted from: (228). **B.** Model of the DNA transfer apparatus. From: (153). **C.** Negative-staining electron microscopy of *E. coli* K12 JE2571 cells containing the conjugative plasmid RP4. Photo: (160).

1.8.3 Type-IV secretion systems are adapted conjugation systems

Many pathogens use type-IV secretion systems to secrete virulence factors into hosts as proteins or nucleoprotein complexes. Those systems are ancestrally related to type-IV secretion systems mediating conjugation (65, 67, 311). As an example, the soil bacterium *A. tumefaciens* causes a plant disease called `crown gall´. Essential for pathogenesis is the *vir* encoded type-IV secretion system which transports T-DNA from the bacterial Ti (tumor-inducing) plasmid associated with virulence proteins into plant cells. Integration of the T-DNA in the host cell genome and expression of the introduced genes follows. This results in neoplastic cell growth and synthesis of nutritive compounds that provide a selective advantage for *A. tumefaciens* (66, 108, 147, 364). Another example is the Dot/Icm type-IV secretion system of *L. pneumophila*, which secretes various effectors in the host cell and is essential for successful infection (see subsections 1.5.4 and 1.6.2).

1.9 Aims of the thesis

The complex life style of *L. pneumophila* requires that different traits are expressed at the appropriate time, e.g. flagellation in transmissive phase. Thus, complex regulatory mechanisms that confer temporal regulation of specific traits have evolved. The present thesis aims to investigate two issues:

- Flagellar regulation in *L. pneumophila*

It is evident from the introduction that flagellation in *L. pneumophila* is basically dependent on the regulatory control governing transition to transmissive phase. Though little is known about the subsequent regulatory cascade governing flagellar gene expression, but it is believed to resemble that of the well studied flagellar system of *P. aeruginosa* (7, 49, 177, 184, 200). However, to what extend the *P. aeruginosa* homologues FleQ, FleR and RpoN influence flagellar gene expression in *L. pneumophila* was not investigated so far. Therefore, the primarily aim of this thesis is to characterize the implication of FleQ, FleR, and RpoN in flagellar gene transcription and to propose a model for the regulatory cascade controlling the flagellar system in *L. pneumophila*.

Although all *L. pneumophila* strains share the same complex life style, they differ in virulence (203). Reflected is this also in the genome sequences of the four sequenced *L. pneumophila* isolates (60, 64, 151). One of the driving forces for the observed genome variability between different isolates is presumably horizontal gene transfer, e.g. conjugation.

- Conjugation in *L. pneumophila*

Although conjugation of DNA between *L. pneumophila* strains has been observed and the existence of oriT sites has been proposed (329, 330, 347, 370), to my knowledge, functionality of none of those oriT sites was verified. The genomic island Trb-1 of the *L. pneumophila* Corby carries genes involved in conjugation and, moreover, an oriT-like site (151, 347). Thus our second aim is to investigate its implication in conjugation.

2 MATERIAL AND METHODS

2.1 Material

2.1.1 *Legionella* strains

The strains used in this study are *L. pneumophila* strain Paris CIP107629, strain Corby (203), and strain Philadelphia JR-32 (252) and the mutant strains *L. pneumophila fliA* strain Paris (49), the *L. pneumophila fleQ* strain Corby, *L. pneumophila rpoN* strain Corby, *L. pneumophila traG1* strain Corby (151, 200), and *L. pneumophila dotA* strain Philadelphia JR-32.

L. pneumophila bacteria were grown in ACES-buffered Yeast Extract broth (BYE) (1% Yeast extract, 1% ACES [pH:6.9 with KOH], 0.04% Cys-HCl and 0.025% ferric pyrophosphate). Bacteria from frozen vials (20% glycerin at -80°C) were streaked onto ACES-buffered Charcoal Yeast Extract (BCYE) agar plates (1% Yeast extract, 1% ACES [pH:6.9 with KOH], 2.5 g/l charcoal, 15 g/l agar, 0.4 g/l Cys-HCl and 0.25 g/l iron pyrophosphate). Recombinant bacteria were selected using antibiotics at the following concentrations: 12.5 µg/ml of kanamycin and 15 µg/ml (8 µg/ml in broth) of chloramphenicol. For selection of the streptomycin-resistant strain Philadelphia-1, 20 µg/ml of streptomycin was used.

2.1.2 *E. coli* strains

The *E. coli* strain used is *E. coli* DH5α [supE44 ΔlacU169 (ϕ80lacZ ΔM15) hsdR17 recA endA1 gyrA96 thi-1 relA1] (Invitrogen).

E. coli bacteria were grown on LB medium. For selection of recombinant bacteria, antibiotics were used at the following concentrations: 40 µg/ml of chloramphenicol, 100 µg/ml of ampicillin, and 50 µg/ml of kanamycin.

2.1.3 Amoebae

Axenic cultures of the amoeba *Acanthamoeba castellanii* ATCC 3023 were prepared in PYG 712 medium (2% proteose peptone, 0.1% yeast extract, 0.1 M

glucose, 4 mM $MgSO_4$, 0.4 M $CaCl_2$, 0.1% sodium citrate dihydrate, 0.05 mM $Fe(NH_4)_2(SO_4)_2 * 6H_2O$, 2.5 mM NaH_2PO_3, 2.5 mM K_2HPO_3) at room temperature.

2.1.4 Eukaryotic cell lines

The MH-S cell line ATCC CRL-2019, used in this study, was derived by SV40 transformation of an adherent cell enriched population of mouse alveolar macrophages. Cells grow adherent as well as in suspension.

The cells were cultivated in RPMI 1640, 0.05 mM 2-mercaptoethanol, 10% fetal bovine serum at 37°C and 5% CO_2. The medium was renewed every 2 to 3 days. For subcultivation (when cells are confluent) the floating cells were transferred to a centrifuge tube and the adherent cells were rinsed with 0.25% trypsin, 0.53 mM EDTA solution. After removing of the solution and adding an additional 1 to 2 ml of trypsin-EDTA solution, the flask were kept at 37°C until the cells detach and consequently added to the floating cells collected above and centrifuge the cell suspension. The pellet was resuspend in fresh medium with a ratio 1:6 to the original volume.

For cryopreservation approximately $1x10^7$ cells per ml were resuspended in cold RPMI 1640, 20% FCS and aliquots of 0.5 ml transferred to cold cryotubes. After that 0.5 ml cold RPMI 1640, 20% DMSO was added to each of the cryotubes, mixed, and immediately frozen overnight at -80°C using Mr. Frosty (Nalgene), which provides a constant cooling rate of -1°C/minute. Until the recovery, the kryotubes were stored in liquid nitrogen. For recovery, one vial of cells was quickly thawn in a 37°C water bath. Upon thawing, the cells were immediately diluted into constant volumes of pre-warmed growth media supplemented with 20% glucose and seed in a 24-well plate.

2.1.5 Oligonucleotides

All oligsonucleotides used in this study were purchased from Eurofins MWG Operon or Sigma-Aldrich and are listed in Table 3.

Table 3: Oligonucleotides used in this study.

Name	Sequence (5'→3')	Application(s) and relevant properties
pUC-F pUC-R	TTTCCCAGTCACGACGTTGTA GTGGAATTGTGAGCGGATAAC	Sequencing of DNA fragments inserted into cloning vectors
SP6 T7	CATACGATTTAGGTGACACTA ATACGACTCACTATAGGGCGAATTG	Sequencing of DNA fragments inserted into cloning vectors
km-F km-R	TGAATGTCAGCTCCGCGGCTATC TGCCGCGGCACTCCTGGAGTT	Amplification of kanamycin resistance cassette
flreSP-F fleSPr-R	AAATTGGTATAACCCGGTGCAA ATTACCAAGGCGGTAGGCAAA	Amplification of promoter region of *fleS* for cloning into pChA 7
flgBPr-F flgBPr-R	CAAATTGTATTTTGGAGTTGGAGC CGGTGTATTAACATTGGCTATGT	Amplification of promoter region of *flgB* for cloning into pChA 8
rpoN-F rpoN-R	ATCTTACGTTGCATCACAATAACT CAGTGAATGCTCTTAGTGCAGGAG	Amplification of disrupted *rpoN* of pSJ 1 for natural transformation
fleQ-F fleQ-R	CCGTTATAATGATTACCGAGTGGA TCCCAGTTACAGCGAATCCGTGAT	Amplification of disrupted *fleQ* of pKH 262B for natural transformation
fleR-F fleR-R	ACAAAAGCACAAGGTACCGGC AGCGGGTCTTTAAACTATCTGCTG	Amplification of *fleR* for cloning into pChA 10 Amplification of disrupted *fleR* of pChA 11 for natural transformation
fleRIn-F fleRIn-R	T**TCTAGA**ATAGTCTCAGCCAACGCTTCT T**TCTAGA**AACAACCAATAGTTCCAGTCA	Used for inverse PCR of pChA 10 to disrupt *fleR* Xba I restriction sites
Km-F Km-R	TGAATGTCAGCT**TCTAGA**CTATCTGGACAAG GCGGCCATCGTG**TCTAGA**CACTCCTGGAGT	Amplification of the kanamycin resistance cassette for cloning in pChA 12 Xba I restriction sites
csrA-RT-F csrA-RT-R	TTTGACTCGGCGTATAGGTG AGCGAACTTGATTGCCTTTT	Real-time PCR
flaA-RT-F flaA-RT-R	CGGCAACAGGAACAGAAGTA TTTGGCATAGGCAGACGTAG	Real-time PCR
fleN-RT-F fleN-RT-R	GCATTTCCACATTCTGGCTA ACTGTCCTGAGACCCGAAAC	Real-time PCR
flgC-RT-F flgC-RT-F	GTCAGTCCAGGAGAATGCAA AGTTTCACGCCAGCCTTAAT	Real-time PCR
fliA-RT-F fliA-RT-R	GGTAAAACGCATTGCACATC TCATAATGCCTTGCTGCTTC	Real-time PCR
fliM-RT-F fliM-RT-R	CTTACGAGAAATCCCAATCTCTATGTTA CGAAAAACTCCAAGATCAAGCAAAAATG	Real-time PCR
gyrA-RT-F gyrA-RT-R	TATTCCTGGCCCTGATTTTC GCCTGAACTTTCATCCGTTT	Real-time PCR

Table 3 - continued

letA-RT-F letA-RT-R	TAGATGGGTGGGAAGTGACA AGGGAGAGGATCTGAGCAAA	Real-time PCR
letE-RT-F letE-RT-R	ATGAATGTGCATTGGGAGAA TATTGGCAACTTCCGTCAGA	Real-time PCR
lpp0952-RT-F lpp0952-RT-R	AGCGTGTGGGATCACAAATA ACCATGTGTACTGGCCTCAA	Real-time PCR
lpp1452-RT-F lpp1452-RT-R	ATGACGGCTACCCTTTGATG TATCGCACCACCAAT	Real-time PCR
motA-RT-F motA-RT-R	TTGGCCTTCTCTCCCTAGAA TCTGCTTCCAAAACTTGTCG	Real-time PCR
fleSPE	GGTTTGACTGCACAAGTTTGATA	5'IRD$_{800}$-labeled oligo for primer extension
flgBPE	CGGTGTATTAACATTGGCTATGT	5'IRD$_{800}$-labeled oligo for primer extension
trbI-F (2aF)	GATGCTTTTGGCATCAGCCTG	Reverse transcription PCR
trbI-R (2bR)	GCTCTGAGTTCATATCGGGTG	Reverse transcription PCR
trbHI-F (2bF)	CTGTGTTTGTGGTGTTGATAG	Reverse transcription PCR
traG-F (4aF)	TGCTGATGCCATTCATCAGCG	Reverse transcription PCR
traG-R (4R)	CATCTAATGGATTCCATCGGG	Reverse transcription PCR
trbL-traG-F (4bF)	CATCAGTATGCAGGTTGGCAC	Reverse transcription PCR
traH-MutX-F (5F)	CCGGCTTGAATTGAGCAATCC	Reverse transcription PCR
traH-MutX-R (5aR)	CCAACGCTGACAGCATTAGAC	Reverse transcription PCR
parR-R (5bR)	GTTGTTCCATATCATCGGCAAGAT	Reverse transcription PCR
traJ-F (6F)	AAACACGGTCGCCATCTTCGT	Reverse transcription PCR
traJ-R (6R)	GCTAGCCAATGCAGCATT	Reverse transcription PCR

2.1.6 Plasmids

All plasmids used and constructed in this study are listed in Table 4.

Table 4: Vectors and plasmids used in this study.

Plasmid	Relevant properties	Reference
pGEM-T Easy	cloning vector, carries ampicillin resistance, allows blue/white screening of recombinants	Promega
pBC SK	cloning vector, MCS, carries chloramphenicol resistance, allows blue/white screening of recombinants	Stratagene
pBC KS	cloning vector, MCS, carries chloramphenicol resistance, allows blue/white screening of recombinants	Stratagene

Table 4 - continued

pKH 542	vector pGEM-T Easy, ,carries *traHJJKLM* genes and putative oriT site (PCR fragment with Primer F AATCCGCGGACTTCCTAATTATTCCCT and Primer R TTTTGGTCTAGATGGATGCATGAGTTA)	Erik Weinmann
pChA 1	vector pBC SK, *traHJJKLM* genes and putative oriT site from pKH 542, used for conjugation assays	this study
pChA 7	vector pGEM-T Easy, promoter region of *fleS*, used for primer extension	this study
pChA 8	vector pGEM-T Easy, promoter region of *flgB*, used for primer extension	this study
pKH 262B	vector pBOC, *fleQ* gene disrupted by insertion of a kanamycin cassette, used for mutagenesis	Klaus Heuner
pSJ 1	vector pUC18, *rpoN* gene disrupted by inverse PCR of pKH 266 (Primer F C**CCGCGG**TTTTGGCTACTGCCAAGAAAG and Primer R A**CCGCGG**TGGATAATTGCAGCAGTCTTA), religation and insertion of a kanamycin cassette in Sac II restriction site, used for mutagenesis	Sebastian Jacobi
pChA 10	vector pGEM-T Easy, *fleR* gene, used for construction of pChA 11	this study
pChA 13	vector pGEM-T Easy, *fleR* gene with deletion, resulted from inverse PCR of pChA 10 with primer fleRIn-F and fleRIn-R, used for construction of pChA 11	this study
pChA 11	vector pGEM-T Easy, *fleR* gene disrupted by insertion of a kanamycin cassette, used for mutagenesis	this study
pChA 12	vector pGEM-T Easy, kanamycin resistance cassette, used for mutagenesis	this study

2.1.7 Chemicals, enzymes, and antibodies

All chemicals and enzymes used in this study were purchased from the following companies: New England Biolabs, Invitrogen, MBI Fermentas, Roche Diagnostics, Gibco BRL, Dianova, Difco, Merck, Oxoid, GE Healthcare/Amersham Biosciences, Roth, Serva, Sigma-Aldrich, and Applied Biosystems.

The following commercial kits were used:

Atlas PowerScript Fluorescent Labeling Kit, Clontech

Agilent RNA 6000 Nano Kit, Agilent Technologies

High Pure RNA Isolation Kit, Roche

Plasmid Mini Kit, Qiagen

PCR Purification Kit, Qiagen

Gel Extraction Kit, Qiagen

RNeasy Kit, Qiagen

One Step RT PCR, Qiagen

DNeasy Tissue Kit, Qiagen

Superscript III Reverse Transcriptase, Invitrogen

pGEM-Teasy Vector System, Promega

ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit, Applied Biosystems

ThermoSequenase Fluorescent Labelled Primer Cycle Sequencing Kit with 7-deaza-dGTP, Amersham Biosciences

Atlas PowerScript Fluorescent Labeling Kit, Clonech

2.1.8 DNA and Protein markers

To determine the size of DNA fragments in agarose gels, the GenerulerTM 1-kb DNA ladder, purchased from MBI Fermentas, was used and to determine the molecular weight of protein fractions separated by polyacrylamide gel electrophoresis, Rainbowmarker (RPN800) purchased from Amersham Biosciences or PageRuler Prestained Protein Ladder purchased from Fermentas was used.

2.1.9 Technical equipment

Autoclaves	Integra Bioscience, H+P Varoclav
Balances	IL-180, Chyo Balance Corp
	MP-3000, Chyo Electronic Balance
Centrifuges (cooled)	Beckmann J2-HC . JA10 and JA20 rotors
	Heraeus Sepatech Megafuge1.OR
	Heraeus Sepatech Biofuge 13R
Centrifuges (table top)	Heraeus Biofuge pico
Clean bench	Nunc Microflow 50726
Developer	Agfa Curix 60
DNA chip reader	Axon Instruments GenePix 4000A scanner
Documentation	BioRad GelDoc2000
Electrophoresis systems	BioRad
Electroporator Gene Pulser	BioRad GenePulser Xcel
Fast Prep System	LabCentral Bio101
Hybridization oven	HybAid Mini 10

Incubators	Heraeus B5050E (30°C, 37°C)
Magnetic stirrer	Heidolf MR3001K
Micropipettes	Eppendorf Reference 0.5-10 µl
	Eppendorf Research 2-20 µl, 20-200 µl, 100-1000 µl
	RAININ AutoRep E
	RAININ L12-20, L12-200
Microscopes	Zeiss LSM 510 confocal laser scanning microscope
	Zeiss Transmission electron microscope EM10
Microwave	AEG Micromat
PCR-Thermocycler	Biometra T3
pH-meter	Methrom-Herisau E512
Photometer	Amersham Bioscience Ultrospec 3100 Pro
	Eppendorf BioPhotometer Plus
	ThermoScientific NanoDrop 1000
Power supplies	BioRad Power Pac 300
Real-time PCR Instruments	ABI Prism 7700 sequence detection system, PE Applied Biosystems
	BioRad MyiQ Single-Color Real-Time PCR Detection System
RNA/DNA Analyzer	Agilent 2100 Bioanalyzer
Sequencer	ABI PRISM 310 Genetic Analyzer
	LI-COR DNA sequencer model 4000
Shaker	B. Braun Biotech International Certomat BS-1 (37°C, 150 rpm)
Sonicator	Branso Sonifier B12
Thermoblocks	Eppendorf Thermostat 5320
Vortexer	Vortex-Genie 2TM Scientific Industries
Waterbath	GFL 1083, Memmert

2.2 Methods

2.2.1 Nucleic acid methods

2.2.1.1 Isolation of chromosomal DNA

The culture was inoculated with a single colony into 80 ml medium and incubated overnight at 37°C with vigorous agitation. When the culture reached the mid-exponential growth phase, the culture was centrifuged at 1,500 g for 15 min at 4°C. The supernatant was removed and the pellet was resuspended in 4 ml of lysis buffer (0.1 M Tris HCl pH:8; 0.1 M EDTA; 0.15 M NaCl; 2 mg/ml lysozyme). Incubation was performed at 37°C for 30 min and thereafter inactivation of lysozyme was done by incubation at 65°C for 3 min. 200 μg/ml of RNase was added and the suspension was incubated at 50°C for 15 min. Then, 500 μl of 10% SDS and 3.35 mg/ml of Proteinase K were added and incubated at 60°C for about 2 h. After incubation, 0.5 ml of phenol:chloroform:isoamylalcohol (25:24:1) was added, the suspension was mixed vigorously and allowed to stand for several minutes. Separation of the phases was achieved by centrifugation at 4,500 g for 30 min. The aqueous phase was taken by Pasteur pipette, transferred to a fresh tube, and precipitated by 2.5 volumes of ice-cold ethanol. DNA was enrolled on the Pasteur pipette, dried on air for 5-15 min, and resuspended in 300 μl TE buffer (10 mM Tris pH 7.5, 1 mM EDTA).

Alternatively, the DNeasy Tissue Kit (Qiagen) was used following the recommendation of the manufacturer.

2.2.1.2 Small scale plasmid DNA extractions

Minipreparations of plasmid DNA were obtained from 2 ml cultures with the QIAprep Spin miniprep Kit (Qiagen) following the recommendations of the manufacturer.

2.2.1.3 PCR reactions

For standard PCR reactions, two different protocols were used. Using the Taq DNA polymerase kit from Qiagen and the dNTP Mix from Sigma-Aldrich, usually, the reaction was performed in a final volume of 50 µl with a final concentration of 1x PCR Buffer, 1x Q-Solution, 200 µM of each dNTP, 0.5 µM of each of the two primers, 1.25

units Taq DNA polymerase, and a variable amount of template DNA. For the REDTaq ReadyMix PCR Reaction Mix from Sigma-Aldrich a final concentration of 1x REDTaq ReadyMix, 0.5 µM of each of the two primers, and a variable amount of template DNA was used.

DAp GoldStar DNA polymerase with 3'→5' proofreading activity from Eurogentec was used to amplify long DNA fragments. The PCR was usually performed in 50 µl reaction mixture containing a final concentration of 1x Opti buffer, 2-8 mM $MgCl_2$, 1-2 mM dNTP Mix, 0.5 µM of each of the two primers, 4 units DAp GoldStar DNA polymerase, and a variable amount of template DNA.

The following protocol was used for the thermal cycler:

Initial denaturation	3 min	94°C
3-step cycling with 29 cycles		
1 min		94°C
1 min		approx. 5°C below Tm of primers
approx. 1 min/kb PCR product		72°C
Final extension	10 min	72°C

2.2.1.4 Enzymatic digest of DNA with restriction endonucleases

Approximately 10 µg plasmid DNA, dissolved in dH_2O was mixed with 5 µl of the appropriate 10x NEBuffer and 10 U of restriction enzyme (New England Biolabs), so that the final volume of the sample was 50 µl. Following the specific requirements of the enzyme indicated on the product sheets, the plasmid DNA was digested for one to 2 h, for most of the restriction enzymes incubated at 37°C. Afterwards, inactivation of the restriction enzyme was carried out by heating the samples for 20 min at 65°C.

2.2.1.5 Horizontal gel electrophoresis

For routine analytical and preparative separation of DNA fragments, horizontal gel electrophoresis under non-denaturing conditions was performed using agarose gels with 1% (w/v) agarose in running buffer (TAE).

To prevent diffusion of the DNA and to have a visible running front, 0.2 volumes loading dye was added to the samples before loading. The electrophoresis was carried out at a voltage between 100-160 V, afterwards stained in an ethidium

bromide solution (10 mg/ml), washed with water and photographed under a UV-transilluminator.

50x TAE buffer: 2 M Tris
6% (v/v) acetic acid (99.7%)
50 mM EDTA (pH 8.0)
ad 1 l dH_2O

6x loading dye: 0.25% bromphenol blue
0.25% xylenecyanol FF
15% Ficoll (Type 400, Pharmacia)
30% glycerol
ad 50 ml dH_2O

2.2.1.6 Isolation of DNA fragments from agarose gels

Agarose pieces containing the DNA fragment of interest were cut out of the gel and DNA purified from agarose gels using the QIAquick Gel Extraction Kit (Qiagen). The piece of DNA containing agarose was subsequently melted for 10 min at 50°C in QG buffer (supplied by the manufacturer). By applying the mixture to QIAquick spin columns followed by centrifugation for 1 min, the DNA was separated from the rest of the solution. The columns were then washed with 750 µl PE buffer (supplied by the manufacturer, supplemented with ethanol) and residual PE buffer was removed by centrifugation (2x 1 min). Finally, the DNA was by eluted from the column with 20-50 µl dH_2O.

2.2.1.7 Ligation of DNA fragments

Linearized vector and insert DNA after restriction digest can be ligated either due to the presence of sticky ends or by blunt-end ligation. The modifying enzyme for ligation process was a T4-DNA ligase (New England Biolabs). Best efficiencies were obtained using a insert/vector ratio of 3/1. Reactions were performed over night at 16°C in a final volume of 40 µl containing 4 µl 10x ligation buffer and 100 units T4 ligase.

Prior to electroporation, ligation mixtures were precipitated with 0.1 volume 3 M NaAc, pH 4.8 and 2 volumes of cold ethanol. After centrifugation 10 min with 15,000 g, the DNA pellet was solubilized in 10 µl dH_2O.

2.2.1.8 DNA Sequencing

The nucleotide sequences of various mutagenized chromosomal genes or plasmid constructs was determined using fluorescent dye terminators (ABI prism BigDye terminator kit, Applied Biosystems).

The sequencing-PCR mix for one sample was:

30 ng PCR product (or: 0.5 µg plasmid DNA)
1.5 µl 10 pM primer
2 µl 5x buffer (kit component)
2 µl premix (kit component)
ad 10 µl ABI-H_2O

The thermal profile for the PCR reaction was: 40 cycles of denaturation at 96°C for 30 sec, annealing at ≤ 60 C for 15 sec, and extension at 60°C for 4 min, followed by final extension at 60 °C for 2 min. Sequencing products were purified by ethanol precipitation and analyzed in a ABI prism sequencer (Perkin Elmer).

2.2.1.9 Transformation of bacterial cells

2.2.1.9.1 Preparation of electrocompetent cells

For electrocompetent *E. coli*, 50 ml LB medium were inoculated with 500 µl of an over night culture and grown OD_{600} of 0.6-0.8. For electrocompetent *L. pneumophila*, the cells were grown 3 days at 37°C on BCYE agar, then transferred to dH_2O. The cells were collected by centrifugation for 10 min at 1,500 × g at 4°C and the pellet was washed with 50 ml ice-cold 10% (v/v) glycerol. After a second centrifugation step at the same conditions, the pellet was resuspended in 25 ml 10% (v/v) glycerol, centrifuged again and finally resuspended in 500 µl 10% glycerol.

2.2.1.9.2 Electroporation of *E. coli* electrocompetent cells

For electroporation, one vial of 80 µl electrocompetent cells was thawed on ice and mixed with ~ 1 µg DNA. Electroporation was performed in a GenePulser Xcel (Biorad) electroporator, with settings of 1.7 kV, 25 µF, and 200 Ω in 1-mm-wide GenePulser electroporation cuvettes. After electroporation, cells were resuspended in 1 ml of LB medium, allowed to recover for 1 h at 37°C, and then plated on LB agar

containing chloramphenicol (40 μg/ml) or ampicillin (40 μg/ml) , X-gal (40 μg/ml), and IPTG (100 μM). The plates were incubated overnight at 37°C.

2.2.1.9.3 Electroporation of *L. pneumophila* electrocompetent cells

For electroporation, one aliquot of 80 μl electrocompetent cells was thawed on ice and mixed with ~ 1 µg DNA and incubated for 10 min on ice. Electroporation was performed in a GenePulser Xcel (Biorad) electroporator, with settings of 2.5 kV, 25 μF, and 100 Ω in 1-mm-wide GenePulser electroporation cuvettes. After electroporation, cells were resuspended in 1 ml of LB medium, allowed to recover overnight at 37°C, and then plated on BCYE agar containing chloramphenicol (12 μg/ml). The plates were incubated 4 days at 37°C.

2.2.1.9.4 Transformation of *L. pneumophila* by natural competence

Legionella is naturally competent for transformation with linear DNA. For mutant construction, *L. pneumophila* bacteria were grown to early stationary phase in 3 ml of BCYE shaking at 37°C. Prior to addition of PCR product, 2 ml of BYE broth was removed from the culture without disturbing the bacteria settled at the bottom of the culture tube. Then PCR product was added to the bacterial culture, gently mixed, and incubated without shaking at 37°C or 30°C for additionally two days. Transformants were selected on BCYE supplemented by 12.5 µg/ml kanamycin.

2.2.1.9.5 Site-directed cassette mutagenesis in *L. pneumophila*

The *rpoN* and *fleQ* mutants in *L. pneumophila* strain Paris were constructed using the corresponding mutants of strain Corby (200) as template for amplifying the PCR product carrying a kanamycin cassette. The PCR product was then transformed into strain Paris. To construct a *L. pneumophila fleR* mutant in strain Paris, the fleR gene was amplified by a PCR using Primers fleR-F and fleR-R. This PCR fragment was purified (Qiaquick PCR purification kit; Qiagen) and ligated into pGEM-T Easy (Promega). By inverted PCR, using primers carrying Xba I restriction sites (fleRIn-F and fleRIn-R), the sequence encoding the σ^{54} interaction domain of FleR was deleted and after self-ligation, the kanamycin cassette was inserted via the XbaI restriction site. The resulting plasmid, pChA 11 was then used as template for amplifying the PCR product, which was then transformed into strain Paris.

2.2.1.10 Isolation of total RNA

Bacterial pellets obtained from broth culture or agar were flash frozen on dry ice-ethanol and stored at -80°C. For total RNA extraction, two different protocols were used.

Total RNA prepared for microarray experiments and/or quantitative real-time PCR cells were resuspended by vortexing in 400 µl of resuspension buffer (12.5 mM Tris, 5 mM EDTA and 10% glucose). Then, 500 µl of acid phenol (pH 4.6) and 0.4 g of glass beads (0.2-0.3 mm diameter; Sigma) were added. The cells were sheared mechanically using a Fastprep apparatus. After centrifugation at 15,000 g for 5 min, the supernatant was transferred to a fresh tube, and 1 ml of Trizol reagent (Gibco BRL) was added. The sample was incubated for 5 min at room temperature. Total RNA was extracted twice with chloroform–isoamyl alcohol (24:1, v/v) and precipitated in 0.7 volumes of isopropanol. After a washing step with 70% ethanol, the RNA pellet was dissolved in sterile DNase- and RNase-free water (ICN Biomedicals) and quantified by absorbance at 260 and 280 nm. Purity and integrity of RNA were controlled on agarose gels, and RNA was stored at -80°C until use.

Total RNA prepared for semi-quantitative reverse transcription PCR and primer extension experiments was extracted by using a High Pure RNA isolation kit (Roche) as described by the manufacturer. Additionally, purified RNA was incubated with 300 U of DNase I (Roche) per ml at 37°C for 10 min and then repurified by using an RNeasy Mini kit (Qiagen).

The quality of the RNA was analyzed using the Agilent RNA 6000 Nano Kit and Agilent Bioanalyzer 2100.

2.2.1.11 cDNA synthesis and reverse transcription PCR

Reverse transcription-PCRs were performed with a OneStep RT-PCR kit (Qiagen) used according to the instructions of the manufacturer with gene-specific primers (Table 1). Each reaction sample contained 100 ng of total RNA, each primer at a concentration of 0.6 µM, each dNTP at a concentration of 400 µM, 5x OneStep RT-PCR buffer containing 12.5 mM MgCl2, and 2 µl of OneStep RT-PCR enzyme.

The following protocol was used for the thermal cycler:

Reverse Transcription 30 min 50°C

Initial PCR activation step	15 min	95°C
3-step cycling with 25-35 cycles		
	1 min	94°C
	1 min	52°C
	30 sec	72°C
Final extension	10 min	72°C

2.2.1.12 cDNA synthesis and quantitative real-time PCR

Quantitative real-time PCR for confirmation of the transcriptome results was conducted on the same total RNA's as used for transcriptome experiments. Synthesis of cDNA from 5 µg RNA was performed using AMV reverse transcriptase (Roche) for 1 h at 42°C. Primers were designed using the Primer3 Software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) such that the amplified products were in the size range of 70-150 bp. Real-time PCR was performed in a 25 µl reaction volume containing cDNA, 12.5 µl SYBR PCR Master Mix (Applied Biosystems) and gene specific primers (300 nM) (Table 2). Amplification and detection of specific products was performed with an ABI Prism 7700 sequence detection system (PE Applied Biosystems) with the following cycle profile: 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, 40 cycles at 95°C for 15 s and 60°C for 1 min. Each real-time PCR assay was performed twice with three different cDNA dilutions (150 ng−1.5 ng). The quantity of cDNA for each target gene was normalized to the quantity of *csrA* (*lpp0845*) cDNA in each sample, because *csrA* was considered (and confirmed by microarray analysis) to represent a stable expressed housekeeping gene and is not differentially expressed in the tested mutant strains. The relative change in gene expression was recorded as the ratio of normalized target concentrations (ΔΔ ct) (307). To check if contaminating chromosomal DNA was present, each sample was tested in control reactions that did not contain reverse transcriptase.

For comparison of *fliA*, *letE*, and *lpp1452* transcription in *L .pneumophila fleR* strain Paris to its isogenic wild type and for the comparison of *L. pneumophila* strain Paris and strain Corby concerning the influence of *fleQ* on *fliA* and *flaA* transcription, a similar protocol was performed as described above. The following details were modified:

The real-time PCR was performed using the iQ SYBR Green supermix (BioRad) and for amplification and detection of specific products the MyiQ Single-Color Real-Time PCR Detection System. Instead of *csrA*, the stable expressed housekeeping gene *gyrA* (*lpp1372* and *lpc0833*) was used for data normalization.

2.2.1.13 Reverse transcription for cDNA synthesis and primer extension

The 5'-IRD_{800}-labeled primers flgBPE and fleSPE, were complementary to downstream regions of *fleS* and *flgB* promoters, respectively. Extension reactions were carried out by using the Superscript II Reverse Transcriptase Kit (Invitrogen). Therefore the gene-specific primer (4 pmol final concentration) was annealed with 5-10 µg total RNA in a volume of 20 µl containing 0.8 mM dNTP Mix by heating at 90°C for 2 min and subsequent cooling to 45°C within 20 min in a thermocycler. For extension of the primer, annealing reaction mixtures were combined with 200 U Superscript III RT and 40 U RNaseOUT (Invitrogen) in 40 µl reaction volumes (containing 31,25 mM Tris hydrochloride (pH 8.3), 46.9 mM KCl, 1.9 mM MgCl2 and 2.5 mM DTT) and incubated at 42°C for 90 min. Reactions were stopped by incubation at 70°C for 15 min and remaining RNA was removed at 37°C for 20 min with RNaseH (Invitrogen). Nucleic acids were cautiously concentrated by precipitation and dissolved in 2 µl dH_2O and 2 µl Formamide loading dye (Amersham Bioscience). Aliquots of the samples were applied to 4.3% polyacrylamide-urea Long Range (FMC Bioproducts, Rockland, USA) sequencing gels of 66 cm with a 64 well shark tooth comb. Gels were run under standard electrophoresis conditions in a LI-Cor-DNA4000 nucleotide sequence analyzer, and outcoming data were processed by using the software supplied with this apparatus. Reference sequencing reactions (A, C, G, T) of cloned promoters, performed with the Thermo Sequenase Fluorescent labelled Primer Cycle Sequencing Kit with 7-Deaza-dGTP (Amersham Biosciences), were initiated by the same primers.

2.2.2 Expression profiling using DNA arrays

2.2.2.1 cDNA synthesis and fluorescent labeling

To synthesize the probes for microarray hybridization, the Atlas PowerScript Fluorescent Labeling kit from Clontech was used following the manual provided by the manufacturer.

As first step, the cDNA was synthesized and purified. Then the cDNA probe was coupled with the fluorescent Cy3 or Cy5 Mono-Reactive Dye from GE Healthcare, respectively. After purification of labeled cDNA, the absorbance for Cy3 or respectively Cy5, was measured with NanoDrop 1000. If necessary, the probes were concentrated using Microcon Centrifugal Filter Units (Millipore).

2.2.2.2 Microarray hybridization's

L. pneumophila multiple genome microarrays containing also each gene of strain *L. pneumophila* Paris was constructed in collaboration with the microarray platform at the Institut Pasteur Genopole (48, 49).

Array hybridization's were performed following the manufacturers' recommendations (Corning). Each slide was prehybridized for 1 h at 42°C in 50 ml of 5x SSC (75 mM NaCl, 7.5 mM trisodium citrate pH 7), 1 mg/ml BSA, 0.1% SDS. Slides were washed two times in water for 1 min with shaking; the last wash was realized in isopropanol and slides were dried by centrifugation for 2 min at 400 g. In parallel, lifterslips were washed first in 0.1% SDS for 1 min and two times in water and wiped between each wash.

250 pmol of Cy3 and Cy5 labelled DNA were mixed and concentrated on Microcon Centrifugal Filter Units (Millipore). The hybridization solution was prepared in a final volume of 50 µL and is composed of 5X SSC, 0.1 mg/mL of salmon sperm DNA, 30% Formamide, 0.1% SDS and the concentrated labelled probes. The mixture was denatured for 3 min at 95°C and hybridization's were realized in Telechem hybridization chambers overnight at 42°C. A biological replicate as well as a dye-swap was carried out. Slides were scanned. Laser power and/or PMT were adjusted to balance the two channels.

2.2.2.3 Microarray analysis

The resulting files were analyzed using Genepix Pro 5.0 software. Spots with high local background fluorescence, slide abnormalities, or weak intensity were excluded. For normalization and differential analysis of the data the R software (http://www.R-project.org) was used. A loess normalization (389) was performed on a slide-by-slide basis (BioConductor package marray; http://www.bioconductor.org/packages/bioc/stable/src/contrib/html/marray.html). Differential analysis was carried out separately for each comparison between two time points, using the VM method (VarMixt package (96)), together with the Benjamini and Yekutieli (312) P value adjustment method. If not stated otherwise, only differentially expressed genes with twofold changes meeting a P value ≤ 0.001 were taken into consideration. Empty and flagged spots were excluded, and only genes with no missing values were analyzed. Gene names (*lpp*) refer to strain Paris. Corresponding genes of strain Lens (*lpl*) and strain Philadelphia (*lpg*) are available at the LegioList web server http://genolist.pasteur.fr/LegioList and at http://genome3.cpmc.columbia.edu/~legion/, respectively. The corresponding genes of strain Corby (*lpc*) are available via GenBank (accession number CP000675).

2.2.3 Phenotypic assays

2.2.3.1 Protein fractionation

Transmissive phase *L. pneumophila* bacteria grown on BCYE agar plates were harvested for fractionation. Following washing in 1 ml cold PBS-Tween (0.01%) the bacterial pellet was resuspended in cold 200 µl of 10 mM Tris (pH 8.0), 30 mM NaCl. After centrifugation (20,000 × g, 5 min, 4°C) the bacterial pellet was resuspended in cold 200 µl of 10 mM Tris (pH 8.0), 20% sucrose, 0.1 mM EDTA, incubated 10 min at room temperature and centrifuged (16,000 × g, 2 min, 4°C). The supernatants of those washing steps were collected, combined to the 'extracellular protein fraction' and TCA precipitated (see subsection 2.2.3.2). The bacterial pellet was resuspended in cold 300 µl 0.5 mM $MgCl_2$ and centrifuged. Proteins of the supernatant are 'periplasmic protein fraction` and were TCA precipitated. The pellet was resuspended in 500 µl dH_2O and sonicated with 4 bursts of 30 sec. Intact bacteria were removed via centrifugation (5 min, 10,000 × g at 4°C) and the supernatant was centrifuged 1 h

with 50,000 × g at 4°C. The pellet from this spin was deemed the 'outer membrane protein fraction' containing also outer membrane proteins as MOMP, which was resuspended in 30 µl 20 mM Tris (pH 8). The supernatant was TCA precipitated and the resulting protein precipitate was washed according to subsection 2.2.3.2, before being resuspended in 10 mM Tris, pH 7.0, with 0.5% N-laurylsarcosine to solubilize inner membrane proteins. This suspension was incubated for 30 min at room temperature and then centrifuged (1h, 50,000 × g at 4°C). The supernatant was TCA precipitated and constituted the 'inner membrane protein fraction'. The pellet, containing the 'cytoplasmic protein fraction', was resuspended in 30 µl 20 mM Tris (pH 8).

2.2.3.2 TCA precipitation

1 volume of 100% (w/v) TCA to 9 volumes of protein sample were added and incubated at least 30 min at 4°C. The precipitated proteins were pelleted by centrifugation (50,000 g, 15 min) and the supernatant removed. The protein pellet was washed 5 times with cold ethanol and one time with cold 70% ethanol. After that, the pellet was dried by placing the tube in 95°C heat block for 5-10 min to drive off ethanol. For SDS-PAGE, the proteins were solved in 20 mM Tris (pH 8).

2.2.3.3 SDS-PAGE and immunoblotting (Western blot)

L. pneumophila transmissive phase bacteria (grown on BCYE agar for 3 days), suspended in distilled water, and OD_{600} was adjusted to 2. Alternatively samples derived by protein fractionation (see 2.2.3.1). The samples were mixed with Laemmli buffer, heated at 95°C for 5 min and loaded onto a 13% SDS-PAGE was performed as described by Laemmli (224). Then the proteins were transferred to nitrocellulose by electroblotting. Flagellin (FlaA) was detected by an indirect immunostaining procedure with a rabbit polyclonal antisera specific for *L. pneumophila* flagellin (97) and goat antirabbit immunoglobulin G coupled to horseradish peroxidase (Dianova). Staining of immunoreactive bands was performed in the presence of hydrogen peroxide and 4-chloro-1-naphthol.

2.2.3.4 Electron microscopy

L. pneumophila transmissive phase bacteria (grown on BCYE agar for 3 days) were suspended in dH_2O, and applied to Pioloform (Merck)-coated copper grids. After sedimentation of the bacteria and removal of the remaining fluid, the samples were

each shadowed with platinum-palladium or stained with 1 drop of 1% phosphotungstic acid (Sigma) (pH 6.5) and examined with a transmission electron microscope (EM10; Zeiss) at 60 kV.

2.2.3.5 Fluorescence staining, immunofluorescence microscopy and imaging

A. castellanii cells were seeded on glass cover slips in 6-well plates in regular amoeba buffer. The next day the amoeba cells were infected the next day with an MOI of 0.2. After incubation of 8 and 14 h at 37°C, the cells were fixed for 20 min with 4% paraformaldehyd in PBS, washed 3 times with PBS and then blocked for 5 min in blocking buffer (PBS, 10% FCS, 0.2% saponin). To the fixed cells rabbit polyclonal antisera specific for *L. pneumophila* flagellin (97) diluted 1:100 in blocking buffer was added and they were incubated for 45 min at room temperature. After that the samples were washed twice with PBS, blocked for 5 min in blocking buffer, and incubated with in blocking buffer 1:260 diluted Alexa Flour 488-conjugated goat α-rabbit (Molecular Probes) for 45 min at room temperature. After three washes with PBS, the cover slips were mounted in embedding medium on glass slides, sealed with nail polish and viewed with an LSM 510 confocal laser scanning microscope (Zeiss) using a 40 ×, 1.3 NA Plan Neofluar oil-immersion objective. Fluorescence signals of double- and triple-labelled specimens were serially recorded with appropriate excitation and emission filters to avoid bleed-through. Images were digitally processed with LSM 5 Image Browser (Zeiss) and merged to yield pseudo-colored pictures.

2.2.3.6 Infection assay in MH-S cells

The MH-S murine alveolar macrophage cell line was described as a valuable model for *L. pneumophila* (388). The cells were maintained in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum (FCS) and prior to infection the cells were adhered to 12-well tissue culture plates at a concentration of 5×10^5 cells/ml for 2 h in 5% CO_2 at 37°C. The resulting monolayers were infected with an MOI of 0.1 for 1 h, washed three times with RPMI 1640 medium to remove non-phagocytized bacteria, and incubated in RPMI 1640 medium containing 10% FCS. The cultures were then incubated for up to 48 h in 5% CO_2 at 37°C. The number of viable bacteria in cell lysates was determined by standard plate counts on BCYE agar after 0, 24, and 48 h of incubation.

2.2.3.7 Contact-dependent hemolytic assay and statistical evaluation

In Eppendorf tubes, 200 µl transmissive phase *L. pneumophila* bacteria (grown on BCYE agar for 3 days) adjusted to OD_{600} 2 were incubated for 2 h with 800 µl sheep erythrocytes diluted 1:80 in PBS at 37°C for 2 h after pelleting with 850 g for 3 min. Afterwards Eppendorf tubes were carefully mixed and again centrifuged with 850 g for 3 min and lysis was quantified by the hemoglobin released, as determined by reading the absorbance at 415 nm of the supernatant. Each reaction was performed in duplicate. The percentage of lysis of the mutant strains was determined in relation to lysis of the wild type. The Student's two-paired t-test was used for statistical evaluation of the experiments.

2.2.3.8 Sodium sensitivity assay

To measure the sodium sensitivity of *L. pneumophila* in transmissive phase, 10 µl of 10-fold serial dilutions in dH_2O of transmissive phase cultures adjusted to OD_{600} 1 were dropped onto BCYE agar plates with and without 100 mM NaCl. The result was evaluated after a 4 day incubation at 37°C. The protocol is similar to (53).

2.2.3.9 Conjugation assay and statistical evaluation

As recipient the Streptomycin-resistant *L. pneumophila* Philadelphia JR-32 strain and as donor *L. pneumophila* Corby wild type or *traG* mutant each carrying pChA 1 (contains the oriT site of the genomic island Trb-1) was used. The recipient was grown in broth shaking at 37°C until the culture reaches stationary growth phase and the donor was grown in broth gently rolling at 37°C until the early exponential phase. The cell number of the donor culture was determined by standard plate counts. Of each, donor and recipient culture, 1.5 ml were taken, pelleted and carefully washed with dH_2O. The recipient cells were resuspended in 150 µl dH_2O, carefully mixed with the donor cells and transferred onto a solid conjugation plate (1% Yeast extract, 1% ACES [pH:6.9 with KOH], 2.5 g/l charcoal, 20 g/l agar). Afterwards this conjugation probe was incubated 24 h at 37°C. Then, the bacteria were rinsed with 10 ml dH_2O, pelleted, and resuspended in 1 ml dH_2O. The bacteria were directly plated on selection plates with 20 µl/ml chloramphenicol and 50 µl/ml streptomycin and the number of transconjugants was counted after 3-5 days. The transconjugation rate was determined by division of the donor cell number by the number of

transconjugants. As control, the same experiment was performed in parallel with *L. pneumophila* Corby wild type carrying the vector pBC KS.

The Wilcoxon signed-rank test with two paired samples (379) was used to calculate a p value for the conjugation assays. Herein the results of nine single conjugation experiments using *L. pneumophila* wild type carrying pChA 1 as donor were compared to the 9 matching control experiments with *L. pneumophila* carrying pBC KS as donor.

3 Results

3.1 Flagellar regulation

The primarily aim of the thesis was to characterize the implication of the enhancer binding proteins FleQ, FleR, and the σ^{54} factor RpoN on flagellar gene regulation in *L. pneumophila* Paris, in order to better understand the flagella biosynthesis.

3.1.1 Construction of *fleQ*, *fleR*, and *rpoN* mutants

fleQ, *fleR*, and *rpoN* mutants in *L. pneumophila* Paris were generated by exploiting the natural competence of *L. pneumophila*. Linear DNA carrying a kanamycin cassette flanked by regions of the target sequence for homologue recombination (for *fleQ* and *rpoN* mutants as described in (200) and for the *fleR* mutant as depicted in Figure 6) was constructed. After transformation of *L. pneumophila* by natural competence, recombination events were proofen by PCR amplification (Figure 7) and subsequent sequencing.

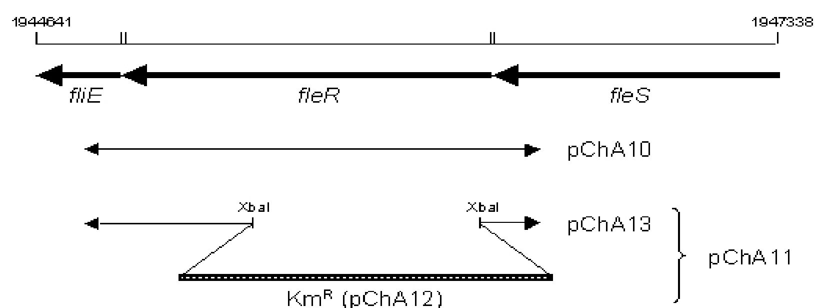
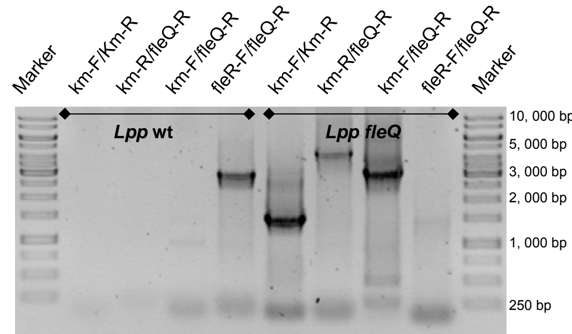
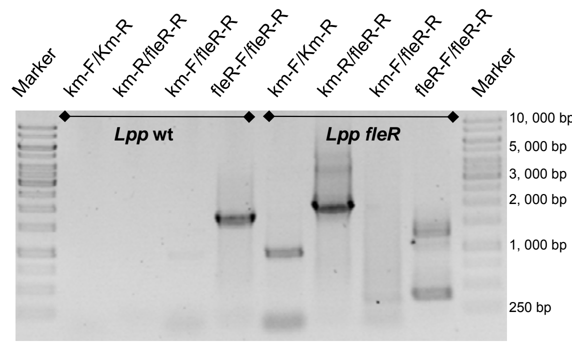


Figure 6: Construction of the *fleR* mutant in *L. pneumophila* Paris.

A.



B.



C.

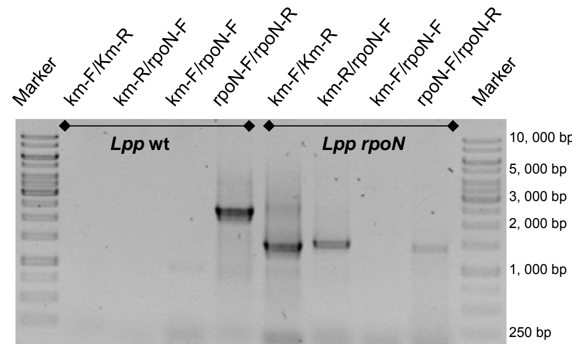


Figure 7: Verification of the *fleQ* (A.), *fleR* (B.), and *rpoN* (C.) mutations in *L. pneumophila* Paris by PCR using primer binding inside the kanamycin resistance cassette and primer binding outside the kanamycin resistance cassette. Thus no band is expected in the wild type but bands of 3 kb (km-F/fleQ-R), 1.8 kb (km-R/fleR-R), and 1.4 kb (km-R/rpoN-F) are expected in the *fleQ*, *fleR,* and *rpoN* mutant respectively. Marker (1 kb GeneRuler, Fermentas).

3.1.2 Phenotype of *fleQ*, *fleR*, and *rpoN* mutants

3.1.2.1 Impact on in vitro *and* in vivo *growth*

In different laboratories the maximum growth of *L. pneumophila* in BYE broth varied depending on unknown changes in experimental conditions; the bi-phasic life-cycle however, remains the same (142). Comparing the growth properties of the wild type and mutant strains grown in BYE broth (Figure 8) and MH-S cells (Figure 9) confirms that *fleQ*, *fleR*, and *rpoN* are dispensable for bacterial replication *in vitro* and *in vivo*.

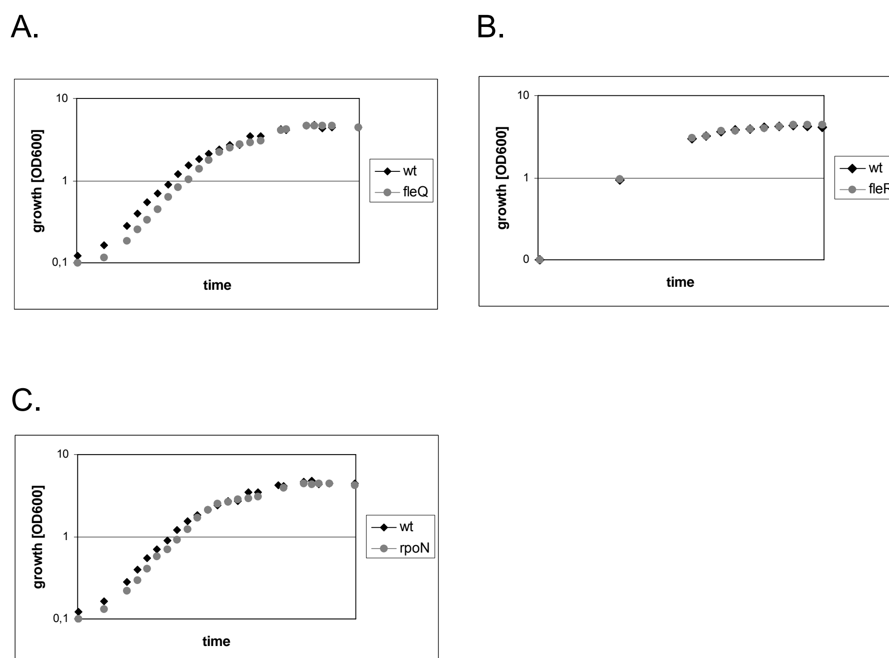


Figure 8: Growth curve of *L. pneumophila* Paris wild type and deletion mutant *fleQ* (A.), *fleR* (B.), and *rpoN* (C.) in BYE broth at 37°C. Experiments with the *fleQ*, and *rpoN* deletion mutant were performed twice, with the *fleR* deletion mutant three times. Shown are the average values.

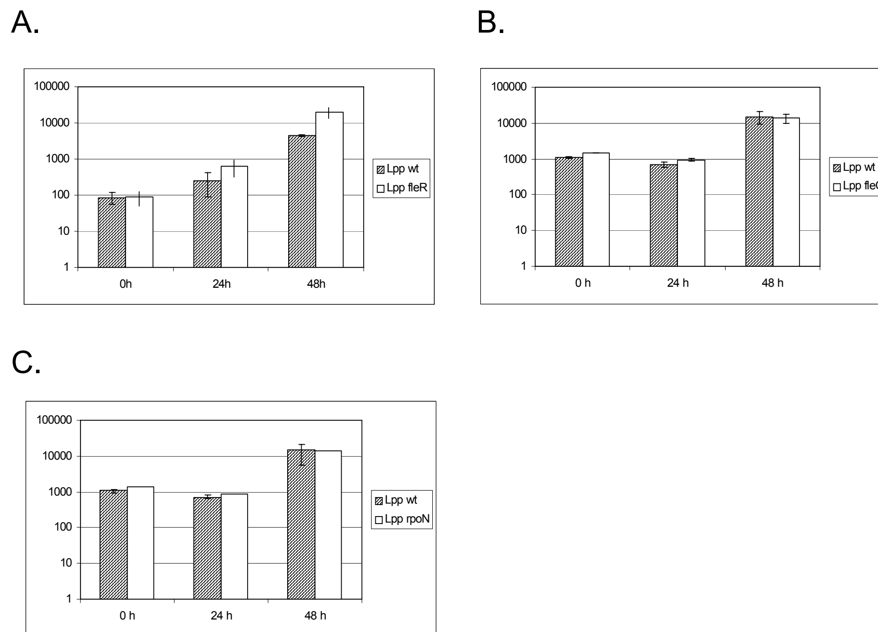


Figure 9: Infection of the macrophage cell line MH-S with *L. pneumophila* Paris wild type and deletion mutant *fleQ* (A.), *fleR* (B.), and *rpoN* (C.). Presented is the mean ± SD derived from two independent experiments, each performed in duplicate.

3.1.2.2 Impact on flagellation and cell shape

The effects of the *fleQ*, *fleR*, and *rpoN* gene deletions on motility, flagellation, and cell shape were observed under the light and electron microscope in transmissive phase. The three mutant strains are like the wild type strain small coccoid in transmissive phase with approximately 1-2 μm in length. In contrast to the wild type strain, the mutants were non-motile as judged by light microscopy and non-flagellated as evidenced by electron microscopy observation (Figure 10). In addition, flagellin (FlaA) was not detectable by Western blotting and thus flagellin expression is strongly reduced in the mutant strains (Figure 11). Taken together these results demonstrate that in contrast to the wild type, the *fleQ*, *fleR*, and *rpoN* mutants do not synthesize a flagella in transmissive phase.

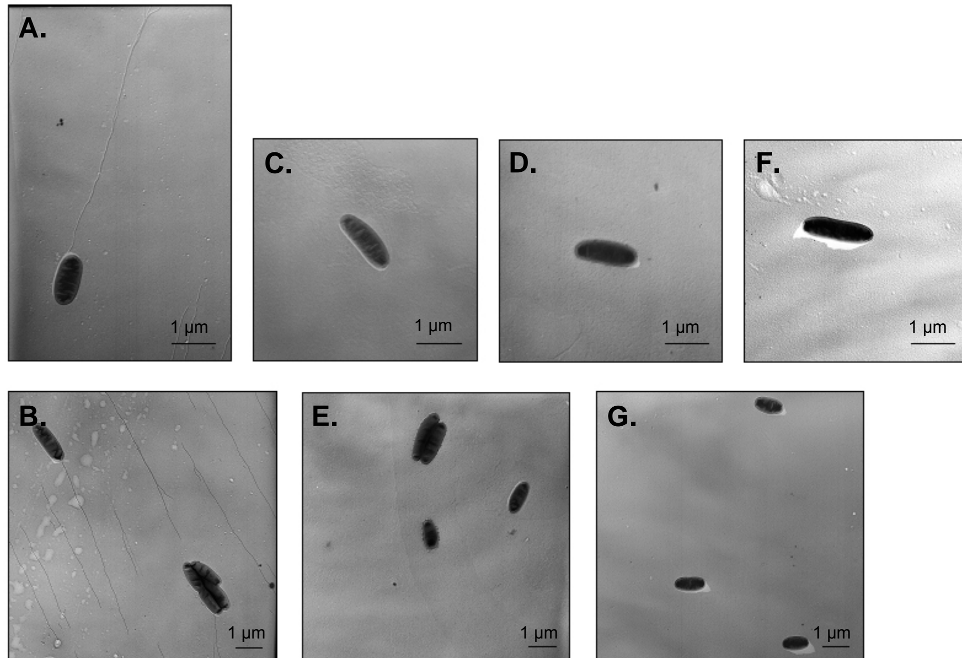


Figure 10: Transmission electron microscopy of *L. pneumophila* Paris cells showing that the wild type (A., B.) is flagellated while the *fleQ* (C.), the *fleR* (D., E.) and *rpoN* (F., G.) mutant cells are unflagellated in transmissive phase.

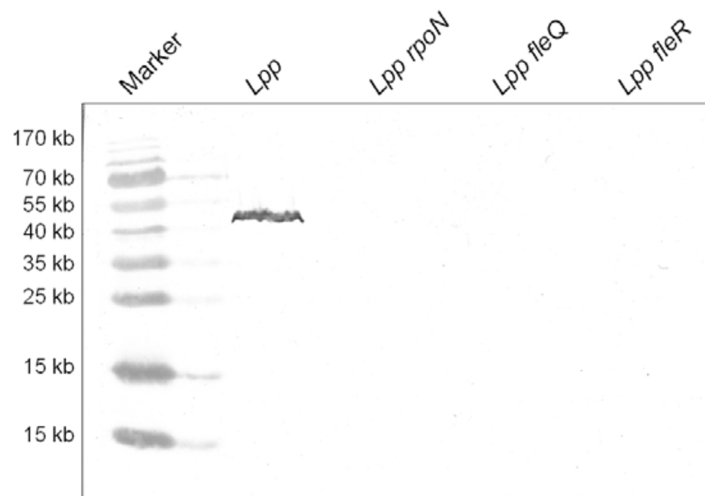


Figure 11: Expression of flagellin tested *via* Western blotting and an anti-FlaA antiserum using whole cell lysate of *L. pneumophila* strain Paris wild type and *rpoN*, *fleQ*, and *fleR* mutant strains. Marker: PageRuler Prestained Protein Ladder, Fermentas.

3.1.2.3 Impact on contact-dependent hemolysis

Beside flagellation, other traits, e.g. contact-dependent hemolytic activity on sheep erythrocytes are characteristic for the transmissive phase phenotype of *L. pneumophila* (214). Thus we tested the contact-dependent hemolytic activity on sheep erythrocytes for various flagellar mutant strains, showing that it was strongly reduced in the *fleQ*, *rpoN*, and *fliA* mutants of *L. pneumophila* Paris; the remaining contact-dependent hemolytic activity was 61%, 48%, and 66% respectively (Figure 12).

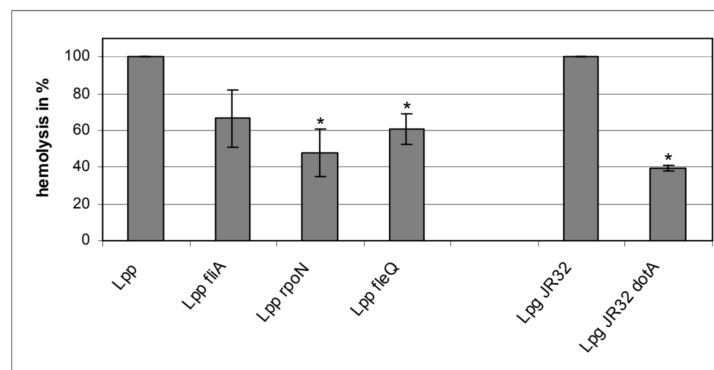


Figure 12: Comparison of contact-dependent hemolysis. Data are presented as mean percentage in relation to the wild type ± SD. Data derived from three independent experiments for *L. pneumophila* Paris strains and as control two independent experiments with the *L. pneumophila* Philadelphia JR32 wild type and *dotA* mutant strain (as negative control (214)). Each experiment was performed in duplicate. *, P value ≤ 0.02 (two-sided Student's t-test).

3.1.3 Transcriptional profile of *fleQ*, *fleR*, and *rpoN* mutants

3.1.3.1 Global impact

We examined the transcriptional impact of mutations in *fleQ*, *fleR*, and *rpoN* by expression profiling using a *L. pneumophila* whole genome microarray. In particular we focused our analysis on the expression of flagellar genes. As their expression starts in replicative phase and stops with the completion of the flagellum in transmissive phase (49, 178, 179, 184), we compared the relative transcript abundances during replicative and transmissive phase. To identify possible polar effects of the kanamycin cassette insertion into *fleQ*, *fleR*, and *rpoN* respectively, the transcription level of the downstream genes was investigated. Insertion of the

kanamycin cassette into *fleQ* did not have a polar effect on downstream gene transcription. In contrast, kanamycin cassette insertion into *rpoN* led to missing transcription of the downstream gene *lpp0541* (encoding a σ^{54} modulation protein). It was hypothesized that *fleR* and the genes *fleS*, *fliEFGHIJ* are expressed as one operon (184). However, kanamycin cassette insertion into *fleR* led to missing transcription of the downstream gene *fliE* (encoding a flagellar basal body protein) but expression of the more distal genes *fliFGHIJ* was comparable to the wild type. The influence of the missing *fliE* transcription might be extensive as a *fliE* mutant in *Salmonella* did not produce flagellin and did not secrete flagellin and other flagellum components, lacks flagella, and was nonmotile (261, 262, 310) or negligible as a *fliE* mutant in *E. coli* did not show any motility defect (306). The transcriptional level of mutated genes and the supposable polar effects were not taken into consideration for data analyses. Real-time PCR analysis of seven (for the *fleQ* and *rpoN* mutant) and eight (for the *fleR* mutant) selected genes validated the microarray results (Figure 13).

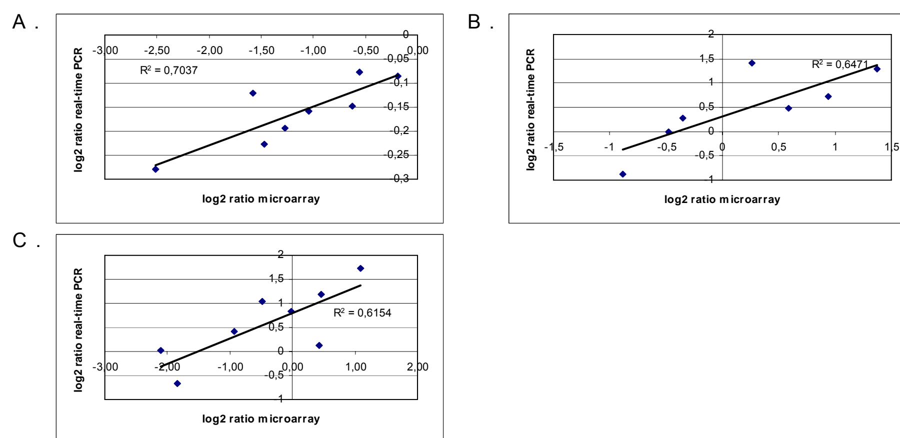


Figure 13: Correlation of microarray and real-time PCR data in transmissive phase for a *L. pneumophila fleQ* mutant (A.), *fleR* mutant (B.), and *rpoN* mutant (C.) compared to the wild type. The Pearson's correlation coefficient is given.

Global analysis of the transcriptome results showed that during replicative phase, transcription of 34, 63, and 30 genes and during transmissive phase, transcription of 143, 271, and 260 genes was significantly altered in the *fleQ*, *fleR*, and *rpoN* mutant as compared to the wild type strain, respectively (Figure 14 and 15). Thus, expression of 1-2% in replicative phase and 4-9% during transmissive phase of the

3077 predicted *L. pneumophila* Paris genes was altered in each of the mutant strains. Our results substantiate that *fleQ*, *fleR*, and *rpoN* have a great influence on the global gene expression in *L. pneumophila* Paris. For a complete list of significantly down- and upregulated genes in mutant strains see Tables S1-S6.

The σ^{54} factor RpoN is known to initiate gene transcription in a concerted action with enhancer binding proteins (38, 50, 51, 378). The three *L. pneumophila* proteins FleQ, FleR, and PilR are predicted to function together with RpoN. *pilR*, encoding PilR was not mutated in this study.

As expected, the set of *rpoN* regulated genes overlapped with those regulated by *fleQ* and *fleR* (Figure 16 and 17). Genes co-ordinately regulated by *fleQ* and *rpoN* were nearly exclusively flagellar genes. However, the set of genes regulated by *fleQ* seems partly to be independent of *rpoN* what was also reflected in flagellar gene expression and described in more detail in subsection 3.1.4.2. Surprisingly, flagellar genes were underrepresented in co-ordinately regulated genes by *fleR* and *rpoN*. Nevertheless, in transmissive phase 101 genes were co-ordinately regulated by *fleR* and *rpoN* (Table 5) (see subsections 3.1.4.3 - 3.1.4.5).

Although it was reported earlier that *fleR* expression is positively regulated by FleQ (200), the overlap of the genes regulated by *fleQ* and *fleR* was very small. Thus, the set of genes regulated by *fleQ* and *fleR* seem to be independent of each other.

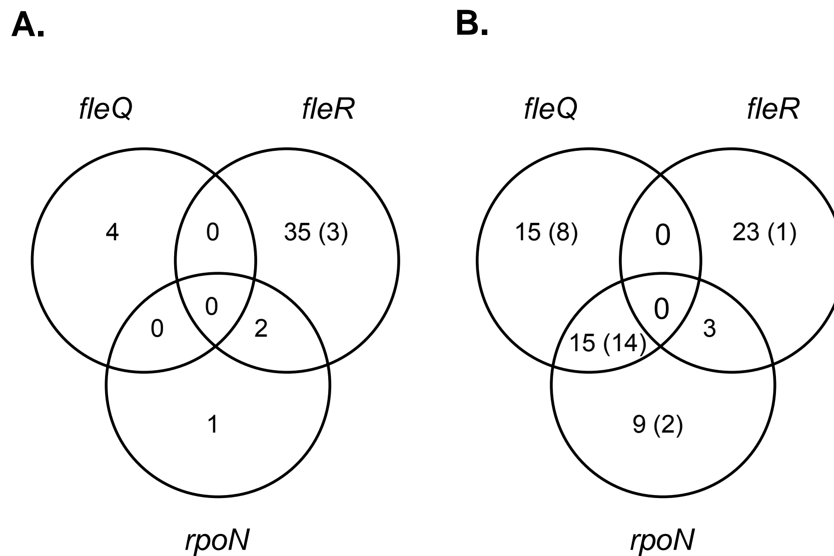


Figure 14: Venn diagrams, showing induced (A.) and repressed (B.) gene numbers (fold change ≥ 2.00 ∨ ≤ 0.50, P value ≤ 0.001) and the overlap of genes in the transcriptome of the mutant strains compared to the wild type *L. pneumophila* Paris in **replicative phase**. Numbers in parenthesis represent flagellar genes.

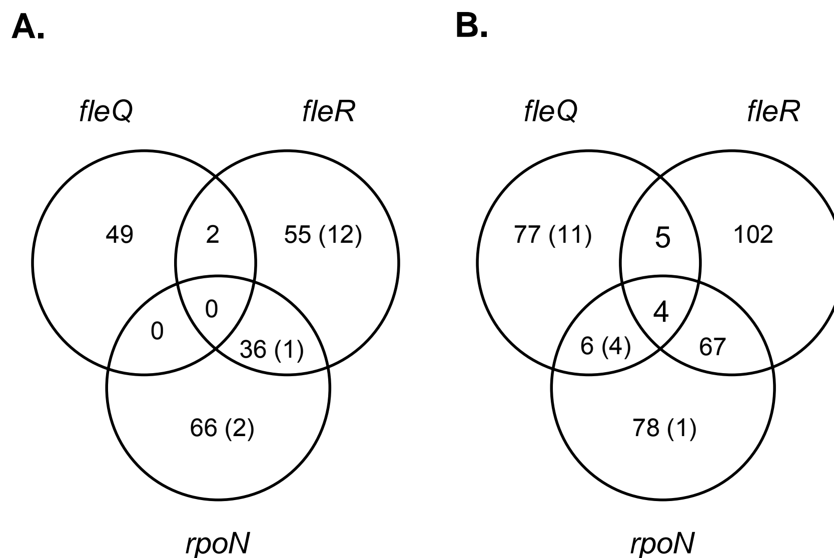


Figure 15: Venn diagrams, showing induced (A.) and repressed (B.) gene numbers (fold change ≥ 2.00 ∨ ≤ 0.50, P value ≤ 0.001) and the overlap of genes in the transcriptome of the mutant strains compared to the wild type *L. pneumophila* Paris in **transmissive phase**. Numbers in parenthesis represent flagellar genes.

Table 5: Co-ordinately down- and upregulated genes in the *fleR* and *rpoN* mutant during transmissive phase. Flagellar genes are excluded and can be found in Figure 16. Given are also the fold changes of the *fleQ* mutant. Significant values are in bold (fold change ≥ 2.00 ∨ ≤ 0.50, P value ≤ 0.001). RP, replicative phase; TP, transmissive phase.

Gene name	Function/Putative function	Fold change in *fleQ* mutant	Fold change in *fleR* mutant	Fold change in *rpoN* mutant	Predominantly expressed[1]
lpp2461	-	0.636	**7.576**	**4.870**	TP
lpp0962	-	**0.329**	**7.282**	**4.121**	RP
lpp0963	-	**0.365**	**6.899**	**4.697**	RP
lpp1638	-	0.587	**5.608**	**3.847**	RP
lpp0493, cspD	Similar to Cold shock-like protein CspD	0.863	**4.892**	**2.780**	RP
lpp1113	-	0.822	**4.406**	**3.956**	-
lpp0799	-	0.792	**4.309**	**3.867**	RP
lpp0936	-	0.922	**3.765**	**5.509**	-
lpp0012	-	0.727	**3.721**	**2.786**	-
lpp3049	Similar to protease	0.777	**3.585**	**5.381**	-
lpp1957	-	0.966	**3.166**	**2.615**	RP
lpp0964	-	0.628	**3.177**	**2.667**	-
lpp2322	Similar to acetoacetyl-CoA reductase	0.840	**2.976**	**4.329**	RP
lpp1639	-	1.078	**2.863**	**3.738**	-
lpp1743	-	0.934	**2.863**	**2.172**	-
lpp2290	-	0.822	**2.771**	**2.364**	-
lpp1738, rir1	Similar to ribonucleoside-diphosphate reductase-α subunit	0.995	**2.706**	**3.052**	RP
lpp1630	-	0.546	**2.593**	**2.096**	TP
lpp0621	Similar to acetoacetyl-CoA-reductase	0.685	**2.526**	**2.698**	RP

Table 5 - continued

lpp2258, murI	Similar to glutamate racemase	1.042	**2.459**	**2.638**	TP
lpp2198	-	0.947	**2.437**	**2.217**	-
lpp0009	Similar to host factor-1 protein	0.890	**2.389**	**2.539**	TP
lpp0045	Similar to sterol desaturase	0.539	**2.356**	**2.170**	TP
lpp1324	Similar to DNA-binding protein Fis	**0.440**	**2.315**	**3.144**	RP
lpp0359	Similar to NAD+-dependent formate dehydrogenase	0.869	**2.300**	**2.501**	RP
lpp0004, gyrB	DNA gyrase- subunit B (type II topoisomerase)	0.928	**2.228**	**2.443**	-
lpp1796	Similar to ABC transporter - ATP-binding protein	0.948	**2.207**	**2.312**	-
lpp2199	Similar to C4-dicarboxylate transport protein	0.890	**2.189**	**2.866**	-
lpp0026	Similar to amino acid permease	0.591	**2.172**	**2.186**	-
lpp2246	-	0.634	**2.145**	**2.567**	RP
lpp2320	-	1.017	**2.136**	**3.455**	-
lpp2077	Similar to transcriptional regulator - LysR family	0.571	**2.085**	**2.387**	TP
lpp1411	Similar to *L. pneumophila* putative phospholipase C	0.693	**2.053**	**2.784**	-
lpp1452	-	0.935	**0.082**	**0.144**	TP
lpp0788	-	**0.456**	**0.089**	**0.086**	RP
lpp0688	-	0.987	**0.132**	**0.147**	RP
lpp1818	-	0.712	**0.132**	**0.414**	RP
lpp2519	-	0.650	**0.151**	**0.180**	-
lpp1546	Some similarity with *Legionella* 33 kDa polypeptide	1.218	**0.156**	**0.268**	TP
lpp2943	-	0.559	**0.165**	**0.278**	RP
lpp2164	Similar to hemin binding protein Hbp	0.525	**0.175**	**0.226**	RP

Table 5 - continued

lpp1346, rpmF	50S ribosomal subunit protein L32	0.759	**0.192**	**0.225**	RP
lpp2594	-	0.945	**0.196**	**0.148**	-
lpp2275	-	0.900	**0.206**	**0.232**	-
lpp1438	-	0.949	**0.206**	**0.228**	RP
plpp0129	Some similarity with transcriptional regulator - Mer family	1.105	**0.219**	**0.242**	RP
lpp0602, letE	Transmission trait enhhancer protein LetE	0.466	**0.240**	**0.244**	-
lpp1805	Similar to outer membrane protein	0.713	**0.249**	**0.261**	RP
lpp1516	Similar to pyruvate dehydrogenase E1 (β subunit)	0.835	**0.297**	**0.265**	RP
lpp1305	Similar to aldehyde dehydrogenase	0.893	**0.299**	**0.324**	RP
lpp2438	-	0.960	**0.301**	**0.407**	RP
lpp0532, proA1	Zinc metalloproteinase precursor	0.488	**0.301**	**0.343**	RP
lpp2768, rpmI	50S ribosomal protein L35	0.728	**0.313**	**0.364**	RP
lpp1706	-	0.836	**0.319**	**0.293**	-
lpp0988	-	1.058	**0.328**	**0.175**	RP
lpp2866	Similar to aminopeptidase	**0.482**	**0.337**	**0.427**	RP
lpp2587	-	0.910	**0.338**	**0.388**	-
lpp0569	Similar to protective surface antigen	**0.343**	**0.343**	**0.337**	RP
lpp1419, secA	Preprotein translocase- secretion protein SecA subunit	0.934	**0.343**	**0.490**	RP
lpp0024, hbp	hemin binding protein	0.519	**0.342**	**0.347**	-
lpp0543, rpmG	50S ribosomal subunit protein L33	0.583	**0.348**	**0.417**	RP
lpp1711	Similar to putative tRNA/rRNA methyltransferase	0.892	**0.356**	**0.352**	RP
lpp2230	Similar to Leu dehydrogenase	0.577	**0.355**	**0.450**	RP

Table 5 - continued

lpp1662	-	0.635	**0.358**	**0.364**	-
lpp2263	-	0.654	**0.357**	**0.327**	TP
lpp1146	-	1.421	**0.361**	**0.436**	RP
lpp0755	Similar to L-isoaspartate carboxylmethyltransferase protein Pcm	**0.361**	**0.361**	**0.443**	RP
lpp1740	-	0.764	**0.372**	**0.450**	-
lpp1304	Similar to dehydrogenase	0.852	**0.374**	**0.372**	RP
lpp2690	-	1.102	**0.376**	**0.417**	-
lpp0250	-	0.904	**0.384**	**0.375**	RP
lpp0205	-	1.050	**0.386**	**0.374**	-
lpp3058, atpE	Highly similar to H+-transporting ATP chain c	0.732	**0.385**	**0.072**	RP
lpp1517	Pyruvate dehydrogenase E2 (dihydrolipoamide acetyltransferase)	0.828	**0.387**	**0.227**	RP
lpp0814	Similar to LPS biosynthesis protein	0.588	**0.388**	**0.309**	RP
plpp0128	-	0.836	**0.394**	**0.242**	-
lpp0385, rplJ	50S ribosomal subunit protein L1	0.569	**0.395**	**0.450**	RP
plpp0125	Similar to acetyltransferase	1.127	**0.406**	**0.479**	RP
lpp3017	-	0.644	**0.405**	**0.346**	RP
lpp0535	Similar to fructose-bisphosphate aldolase	0.808	**0.407**	**0.208**	RP
lpp2186	Similar to acyl-carrier protein	0.835	**0.412**	**0.420**	RP
lpp1550, rpsF	30S ribosomal protein S6	0.709	**0.415**	**0.379**	RP
lpp0417, rpsK	30S ribosomal protein S11	0.748	**0.422**	**0.492**	RP
lpp0619	-	0.519	**0.420**	**0.408**	RP
lpp0384, rplA	50S ribosomal protein L1	0.560	**0.422**	**0.398**	RP

Table 5 - continued

lpp0834	-	0.949	**0.435**	**0.320**	RP
lpp2094	-	1.256	**0.435**	**0.392**	TP
lpp1515	Similar to pyruvate dehydrogenase - (E1 α subunit)	0.737	**0.436**	**0.263**	RP
lpp2400	-	0.791	**0.437**	**0.362**	RP
lpp2689, rpsT	30S ribosomal subunit protein S2	0.584	**0.441**	**0.322**	RP
lpp1420, mutT	Mutator protein MutT	0.713	**0.459**	**0.421**	RP
lpp1882	-	0.793	**0.463**	**0.495**	-
lpp0544, rpmB	50S ribosomal protein L28	0.534	**0.464**	**0.254**	RP
lpp2009	Similar to DAPH synthase	0.718	**0.468**	**0.435**	-
lpp0407, rpsN	30S ribosomal protein S14	0.679	**0.470**	**0.354**	RP
lpp0873, mreB	Rod shape-determining protein MreB	0.735	**0.468**	**0.370**	RP
lpp3065	-	0.618	**0.475**	**0.490**	RP
lpp1809	Conserved lipoprotein	0.784	**0.480**	**0.466**	RP
lpp1830, tig	Peptidyl-prolyl cis-trans isomerase (trigger factor)	0.830	**0.484**	**0.393**	RP
lpp0408, rpsH	30S ribosomal protein S8	0.699	**0.492**	**0.462**	RP
lpp0984, etfB	Electron transfer flavoprotein β-subunit	0.747	**0.494**	**0.361**	RP
lpp0986, ald	Similar to Ala dehydrogenase	0.682	**0.495**	**0.357**	RP
lpp2882	-	1.014	**0.497**	**0.400**	RP
lpp1363	Similar to putative choline kinase	0.764	**0.500**	**0.300**	-

[1]According to (49)

3.1.3.2 Impact on genes associated with flagellation

The influence of *fleQ* on flagellar gene transcription is immense, as 27 out of 46 flagellar genes were repressed in the *fleQ* mutant (Figure 16): Transcription of 14 flagellar genes (*flgBDGIJ*, *fleN*, *flhFA*, *fliMNOPQR*) were concertedly repressed in the *rpoN* and *fleQ* mutant; all of them belong to the flagellar class II genes. Additionally six (two) flagellar class II genes were repressed in the *fleQ* (*rpoN*) mutant background, respectively. Importantly, transcription of 7 out of 15 late flagellar genes (class III and IV) were repressed solely in the *fleQ* mutant. These were the flagellar class III genes *motB*, *flgMN*, *fliA* (encoding the σ^{28} regulator FliA) and the FliA-dependent (49) flagellar class IV genes *fliS*, *flaAG*.

Strikingly, the non-flagellar genes *lpp2282*, *lpp0952* (coding for a GGDEF/EAL and PAS/PAC domain protein) and *lpp1290* (coding for a homologue of the enhanced entry proteins (EnhA)) were described earlier as belonging to the FliA regulon (49) and were also repressed solely in the *fleQ* mutant. Beside *lpp0952*, four more genes encoding GGDEF/EAL regulatory proteins were repressed in the *fleQ* mutant (Table 6). This is noteworthy as GGDEF/EAL proteins are often involved in flagellar regulation through changing the messenger bis-(3',5')-cyclic diguanylic acid (c-di-GMP) levels in the cell (382). Taken together our results prove that *fleQ* and *rpoN* enhance transcription of flagellar genes, whereby the role of *fleQ* is more pronounced.

Table 6: GGDEF/EAL proteins repressed in *fleQ* mutant during transmissive phase. Values stated in bold show statistically significant (fold change $\geq 2.00 \vee \leq 0.50$, P value ≤ 0.001) altered expression compared to the wild type *L. pneumophila* Paris.

Gene name	Fold change	Domains	Transmembrane helices
lpp0351	**0.467**	EAL	-
lpp0809	**0.472**	GGDEF	5
lpp0942	**0.452**	GGDEF	-
lpp0952	**0.414**	GGDEF, EAL, 2 PAS	-
lpp1170	**0.464**	GGDEF, EAL	9

According to the temporal order of flagellar gene transcription, *fleQ* belongs to the flagellar class I genes while transcription of *fleS* and *fleR* - encoding a two-

component system - takes place later and thus they were defined as flagellar class II genes (49). It was suggested that *fleSR* expression might be dependent on *fleQ* and *rpoN* (200). Nevertheless, *fleS* and *fleR* transcription was only slightly repressed in the *fleQ* as well as in the *rpoN* mutant (in replicative phase: 0.55-fold and 0.54-fold in the *fleQ* mutant, and 0.52fold and 0.70fold in the *rpoN* mutant respectively).

It was also suggested, that FleR and FleS, encoded by *fleR* and *fleS* respectively, regulate flagellar class III genes together with RpoN. However, as proved by microarray and real-time analyses, transcription of only one flagellar gene was repressed in the *fleR* mutant (Figure 16). Remarkably, also expression of *letE,* encoding a regulatory protein described earlier to influence flagellation was repressed in the *fleR* and *rpoN* mutant (Table 5).

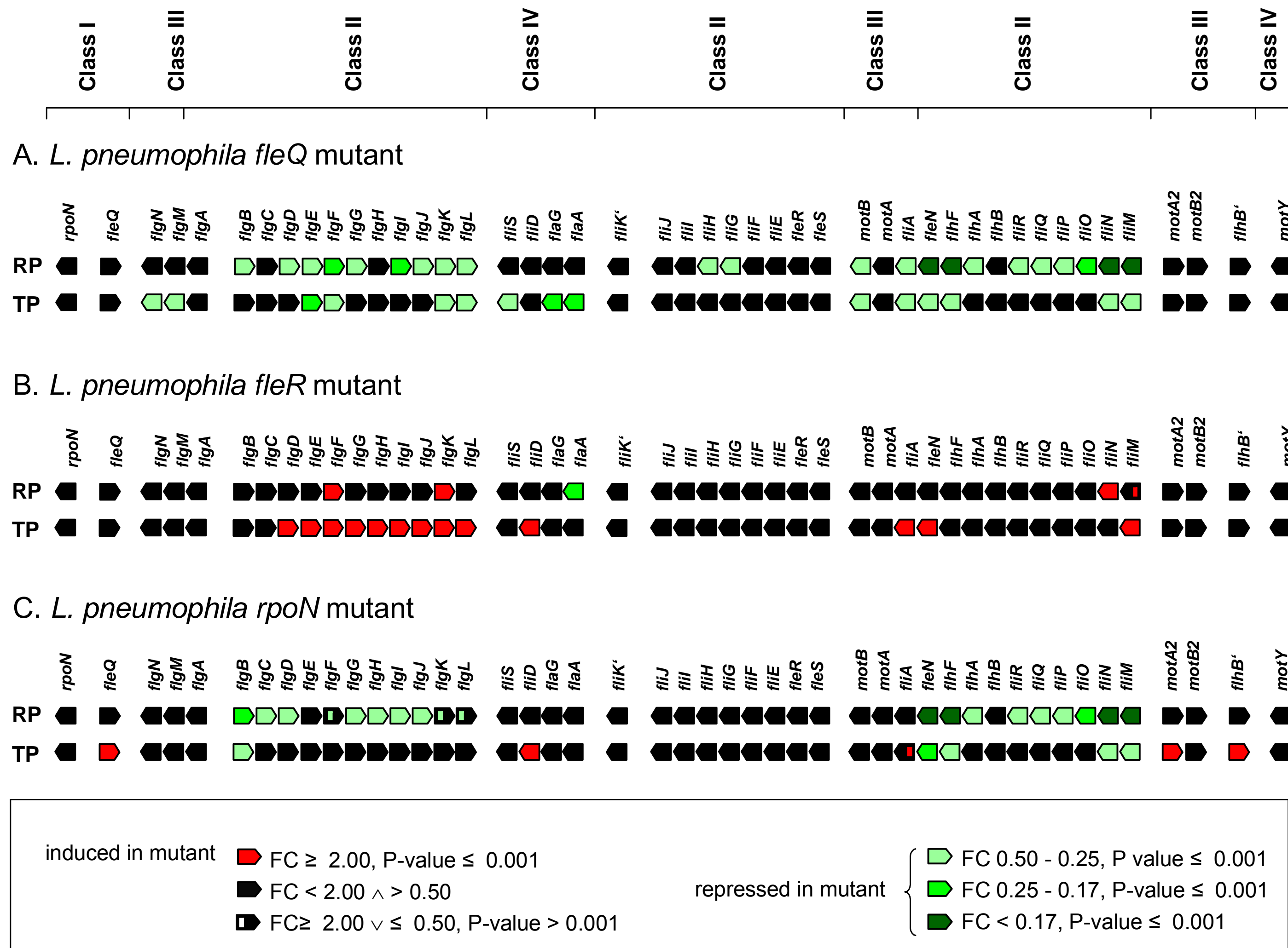


Figure 16: Expression of flagellar genes in the *fleQ* mutant (A.), *fleR* mutant (B.) and *rpoN* mutant (C.) relative to wild type *L. pneumophila* Paris in replicative (RP) and transmissive phase (TP). Different growth phases represent different stages of flagellum biosynthesis. Values derived from transcriptional profiling experiments and are defined in the key at the bottom. Because of hierarchical regulation these results do not necessarily indicate direct effects. FC, fold change

3.1.3.3 Impact on genes associated with protein biosynthesis

Out of the 71 co-ordinately repressed genes during transmissive phase in *fleR* and *rpoN* mutant, numerous genes were associated with protein biosynthesis, e.g. 11 genes encoding ribosomal proteins (Table 5), thus suggesting, that *fleR* and *rpoN* have an impact on protein biosynthesis.

3.1.3.4 Impact on genes associated with metabolism

Expression of *lpp0535*, encoding a putative fructose-bisphosphate aldolase, an important enzyme of the glycolysis and gluconeogenesis was repressed in the *fleR* and *rpoN* mutant. Output of glycolysis is pyruvate, which can be transformed to acetyl-CoA by the pyruvate dehydrogenase E1, the first component enzyme of the pyruvate dehydrogenase complex. The pyruvate dehydrogenase complex links the glycolysis to the citric acid cycle. Interestingly, genes encoding homologues of the α- and β-subunit of E1 and the unit E2 of the dehydrogenase complex were repressed in the *fleR* and *rpoN* mutant (Table 5). However, expression of the genes *aceE* and *aceF*, also encoding the units E1 and E2 of the dehydrogenase complex was neither repressed in the *fleR* nor the *rpoN* mutant.

Acetyl-CoA is used in the citric acid cycle or for polyhydroxybutyrate biosynthesis. In the ladder, acetoacetyl-CoA reductases are involved. Expression of two out of three encoded putative acetoacetyl-CoA reductases (*lpp0621* and *lpp2322*) was induced in both, the *fleR* and *rpoN* mutant (Table 5). Also expression of the third putative acetoacetyl-CoA reductase *lpp0620* was 2.15-fold induced in the *fleR* mutant and not significantly 2.17-fold induced in the *rpoN* mutant.

3.1.3.5 Impact on virulence genes

Expression of the virulence gene *proA1* (*mspA*) was repressed in the *fleR*, and *rpoN* mutant. Also in the *fleQ* mutant, *proA1* (*mspA*) expression seemed to be repressed although not significantly (Table 5). In addition, numerous other virulence genes, e.g. Dot/Icm substrates are differentially regulated in at least one of the mutant strains (Table S1-S6).

3.1.4 Comparison of the influence of *fleQ* in different strains

Differing from our microarray results, using reverse-transcription PCR a previous study could not detect reduced *flaA* and *fliA* mRNA levels in a *L. pneumophila fleQ* mutant strain Corby compared to the isogenic wild type (200). To test if *fleQ* enhances *fliA* and *flaA* transcription in *L. pneumophila* Corby, like in strain Paris, we compared *fleQ* mutants of both strains to the corresponding wild type by real-time PCR. Our results indicate that in *L. pneumophila* strain Paris *fliA* and *flaA* transcription is permanently repressed in absence of *fleQ* while in strain Corby only in replicative phase (Table 7). Thus, the method used previously might not have been sensitive enough to detect these differences and the influence of FleQ on *fliA* and *flaA* transcription.

Table 7: Real-time PCR results comparing the relative change in gene expression of the *fleQ* mutant to the corresponding wild type *L. pneumophila* strain Paris and strain Corby. The results derived from three independent experiments, each performed in duplicate.

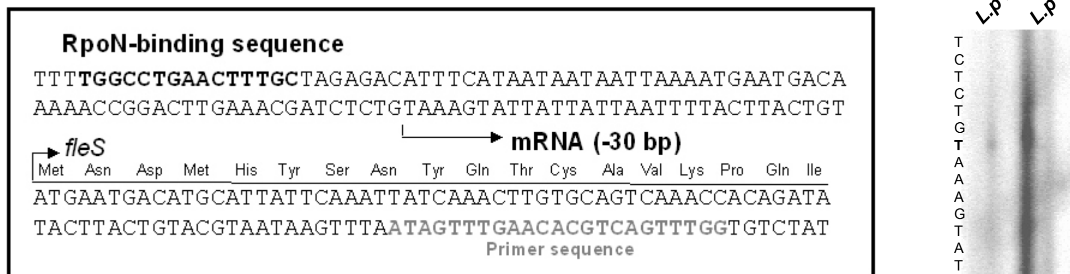
	strain Paris		strain Corby	
	replicative phase	transmissive phase	replicative phase	transmissive phase
Gene	log2 ratio ± SD	log2 ratio ± SD	log2 ratio ± SD	log2 ratio ± SD
fliA	0.32 ± 0.01	0.41 ± 0.19	0.32 ± 0.21	1.13 ± 0.01
flaA	0.18 ± 0.03	0.27 ± 0.06	0.55 ± 0.15	0.70 ± 0.05

3.1.5 Promoter mapping in regions of the flagellar regulon

According to our microarray analyses, transcription of most of the flagellar class II genes was enhanced by *rpoN*. However, in all cases transcription was not strictly dependent on the presence of *rpoN*. To test if the transcription initiation correlates with putative RpoN binding sites, predicted upstream of the flagellar class II genes *fleS* and *flgB*, we determined the transcription initiation sites by primer extension. The results, depicted in Figure 17, show that the putative operon transcribed from the promoter preceding the *fleS* gene initiated from the putative RpoN binding site. In contrast, upstream of *flgB* two transcription initiation sites were determined but none of them initiate from the putative RpoN binding site. Using the promoter prediction

software for identifying prokaryotic promoters (http://www.fruitfly.org/cgi-bin/seq_tools/promoter.pl), those promoter sites were predicted with a high probability (scores 0.85 and 0.93) and are similar to σ^{70} promoters. For the determined transcription initiation sites, reduced transcription initiation upstream of *fleS* and *flgB* was still observed in the *rpoN* mutant.

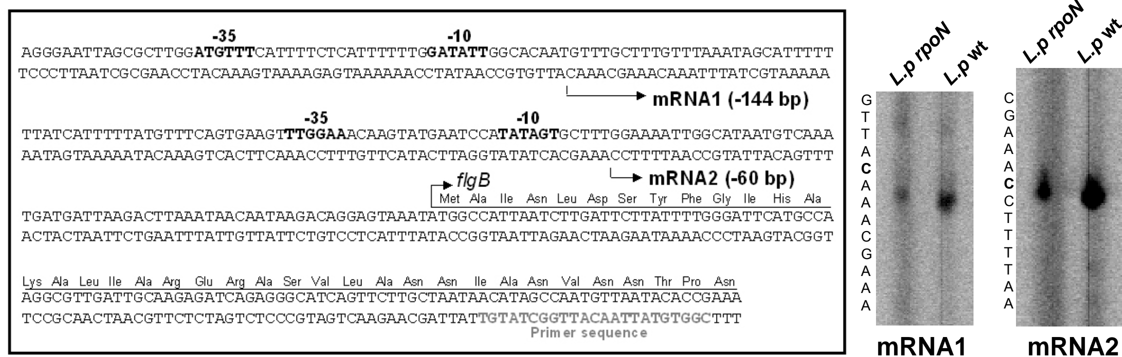
A. Start site upstream of *fleS*



L.p rpoN L.p wt

T C T C T G T A A A G T A T

B. Start sites upstream of *flgB*



L.p rpoN L.p wt

G T T A C A A A C G A A A

mRNA1

L.p rpoN L.p wt

C G A A A C C T T T T A A

mRNA2

Figure 17: Primer extension-mediated mapping of the transcriptional start site(s) of the *fleS* gene (A.) and *flgB* gene (B.). RNA's were isolated from the *L. pneumophila* Paris wild type and *rpoN* mutant. Transcriptional start sites are indicated by arrows and the promoter sequences are shown in bold.

3.1.6 Expression of flagella

To correlate our microarray analyses with expression of flagellin and thus probably biosynthesis of flagella, which takes place in late stages of *A. castellanii* infection after transition from replicative to transmissive phase (Figure 18), weexamined the flagellin (FlaA) localization in transmissive phase cultures, whole cell lysate and protein fractions, *via* Western blotting, for the presence of flagellin. We detected a reactive band of the predicted size (47.9 kDa), which was present in the whole cell lysate, the extracellular and periplasmic protein fractions (Figure 19).

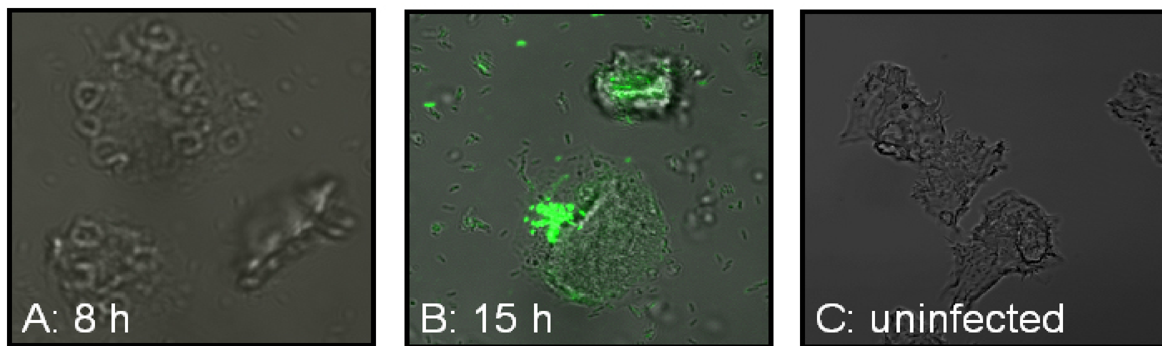


Figure 18: Flagellin expression during infection of *A. castellanii*. *A. castellanii* cells were infected with *L. pneumophila* Paris wild type and processed for immunofluorescence using an anti-FlaA antiserum.

A. No flagellin (FlaA) expression is visible after 8 h of infection.

B. Flagellin (FlaA) expression is visible after 14 h of infection inside *A. castellanii*.

C. Uninfected control.

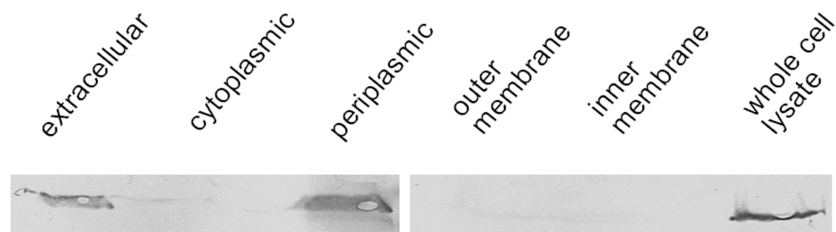


Figure 19: Localization of flagellin tested *via* Western blotting and an anti-FlaA antiserum by using transmissive phase *L. pneumophila* Paris whole cell lysate and protein fractions.

Usually flagellated bacteria are chemotactic what enables them to move towards favorable conditions or to avoid unfavorable conditions (110). Herein, the switch protein FliM connects the flagellar system with the chemotaxis system as the N-terminus binds to the signaling protein CheY. Moreover, FliM is supposed to be responsible for the state of motor rotation (292, 335, 360, 361). Consequently, different rotation properties of *E. coli*, *P. aeruginosa* and *R. sphaeroides* are reflected in the FliM protein sequence. A salient feature is the presence of a conserved CheY binding site. Interestingly, although a chemotaxis system is not found in *L. pneumophila* (60, 64, 151) and taxis was not observed so far (184), the CheY binding domain is also present in FliM of *L. pneumophila* (Figure 20).

CheY binding

```
Pa        1  mavqdllsqdeidallhgvddglveteveatpgs...............
Ec        1   mgdsilsqaeidallngdsevkdeptasvsgesd..............
Rs        1 maatprklsskevaalvgnlmeasestslenglevr.............
Lpp       1  mtekdvlsqeeidalldsvdesidgetndeelnssdqparkkpedttsq

Pa       35 ................vksydltsqdrivrgrmptleminerfarytris
Ec       35 ................irpydpntqrrvvrerlqaleiinerfarhfrmg
Rs       37 ..................pyafgenelnqlgdyhalriinerfcrtardv
Lpp      50 elnstfdsdikvaeegvktlnftgqerivkgqlpvldkiydravrlfaad

Pa       69 mfnllrrsadvavggvqvmkfgeyvhslyvptslnlvkmkplrgtalfil
Ec       69 lfnllrrspditvgairiqpyhefarnlpvptnlnlihlkplrgtglvvf
Rs       69 flpmlrlqprissfppevrsfddyrssqdnfvsitasrieelrgnqmivi
Lpp     100 iyhltardfeikqdpllitkhkefmkslpnpsligiykfkplrgkgiilf

Pa      119 daklvfklvdnffggdgrh.akiegreftptelrvvrmvleqafvdlkea
Ec      119 spslvfiavdnlfggdgrfptkvegrefthteqrvinrmlklalegysda
Rs      119 pppfislltdsyyggqirhvpttr.teftateervielvtdrlnvalqva
Lpp     150 dstfvydlvdyyfggnsqfgaqkdktdftatelrvmevvtkklvanliha

Pa      168 wqavlemnfeyvnsevnpamanivs.psevvvvstfhield.ggggdlhi
Ec      169 wkainpleveyvrsemqvkftnittspndivvntpfhveig.nltgefni
Rs      168 wrdlmaltftvvsreesmqfasfvd.gedmvvncsfmvqlpntepasfdi
Lpp     200 wepiiqlditkfndetnpqlvniae.peemllvarfvlnfg.ketgsfyf

Pa      216 tmpysmiepiremldagfqsdhddqderwikalredvldvqvplgatvvr
Ec      218 clpfsmieplrellvnpplensrnedqnwrdnlvrqvqhsqlelvanfad
Rs      217 lyplqtlkpissqlrsrmqsdfvdddrswreklerailsipltlsarlce
Lpp     248 ilpysmlepikqqlelgasrpddeidpnwinslkeelmdvelsvsasmae

Pa      266 rqlklrdilhmqpgdvipvempehmvmrangvpafkvklgahkgnlalqi
Ec      268 islrlsqilklnpgdvlpiekpdriiahvdgvpvltsqygtlngqyalri
Rs      267 pevplrqlmqmqpgdvlpvhltealsllvegqpifeaapgerggqaalnl
Lpp     298 tvstlgqvmswkvgdfvpleineevtldiegtpsftatlgstnekralki

Pa      316 leav.........ersr
Ec      318 ehlinpilnslneeqpk
Rs      317 trrhvrg
Lpp     348 ikiiry
```

Figure 20: ClustalW alignment of multiple FliM protein sequences. The ClustalW output was obtained from the FliM protein sequences of *L. pneumophila* Paris (Lpp), *R. sphaeroides* (Rs), *E. coli* K12 (Ec), and *P. aeruginosa* PAO1 (Pa). For the alignment, the Structure based Alignment Program (STRAP) was used (www.charité.de/bioinf/strap/).

3.2 Conjugation

The genomic island Trb-1 of the *L. pneumophila* Corby carries all genes necessary for conjugation and moreover, an oriT-like site (151, 347). Thus the second aim of this thesis was to investigate its implication in conjugation.

3.2.1 Presence of pili-like structures on the surface

The presence of putative pili structures on the surface of the *L. pneumophila* Corby when grown on BCYE agar was proven by electron microscopic examinations. To avoid a mixing up with flagella structures we used the flagellum-negative *flaA* mutant strain (Figure 21); but same structures were also seen in the isogenic wild type. In a *traG1* mutant strain no pili-like structures were observed.

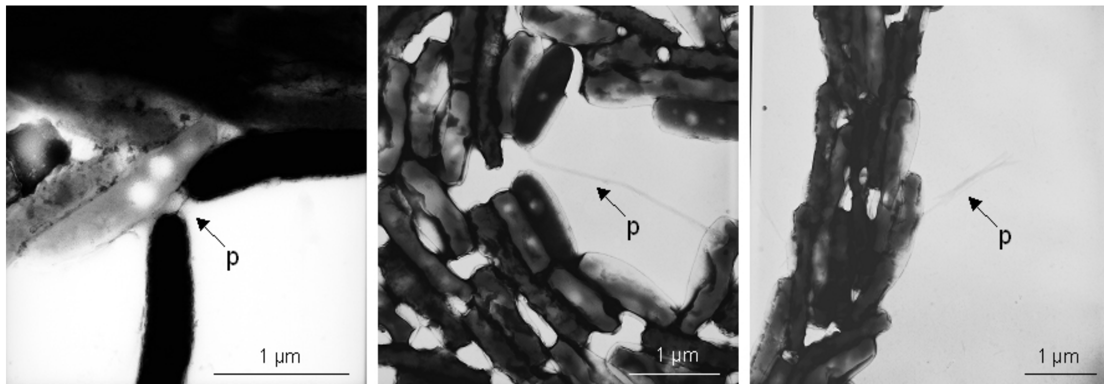


Figure 21: Pili-like structures observed by electron microscopy of the *L. pneumophila flaA* mutant strain Corby (unflagellated). Same structures were seen in wild type strain. Strains were grown on BCYE agar plates at 30 °C for 3 to 5 days.; p: putative pilus.

3.2.2 Functional analyses of the oriT site of the genomic island Trb-1

Mobilization of DNA during conjugation requires an oriT site. To test whether the putative oriT site of Trb-1 is functional we cloned the *traH1J1*-oriT-*traK1L1M1* region of Trb-1 into the non-mobile vector pBC KS, resulting in plasmid pChA 1. For conjugation assays we used as donor the *L. pneumophila* Corby wild type or *traG*

mutant strain, each carrying pChA 1, and as recipient the streptomycin-resistant *L. pneumophila* Philadelphia JR-32 strain. Control experiments were performed with the non-mobile vector pBC KS.

These experiments proved that this oriT site of Trb-1 is able to mobilize the non-mobile plasmid pBC KS with a conjugation rate of 0.0075 ± 0.01 from one *L. pneumophila* strain to another. The presence of DNase during conjugation had no significant influence on the conjugation rate indicating that DNA is transferred through a conjugation pore. The conjugation ability of a *traG*1 mutant strain was not significantly reduced as compared to the wild type (Figure 22).

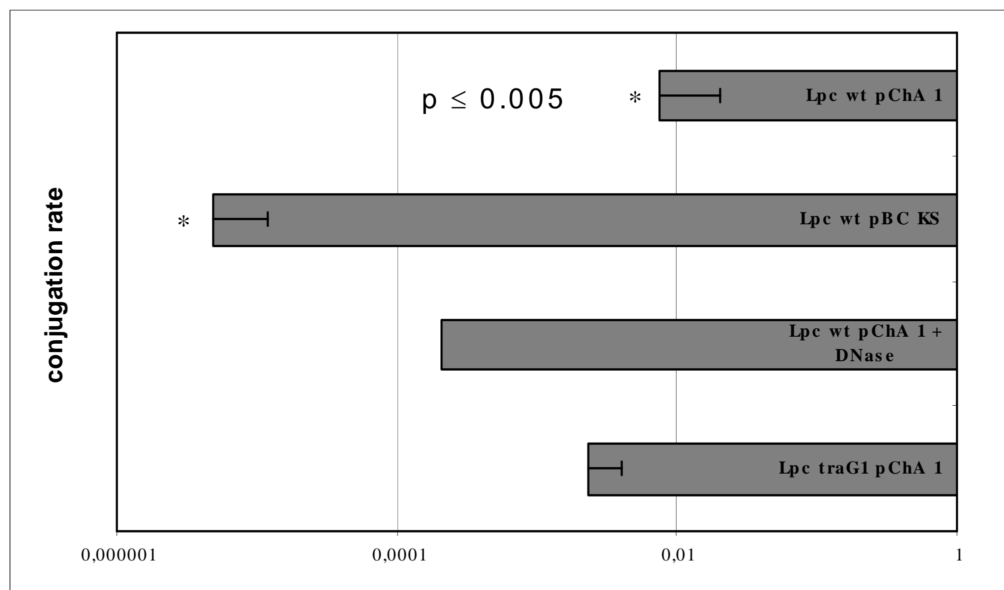


Figure 22: Conjugation experiments. Represented is the mean of nine independent experiments with *L. pneumophila* Corby containing pChA 1 or pBC KS as donor and three for the *traG1* mutant strain Corby as donor ± SD; a single conjugation experiment was performed with the presence of DNase. *, P ≤ 0.005 (Wilcoxon signed-rank test with two paired samples).

3.2.3 Expression of genes located on Trb-1

Reverse transcription PCR expression analysis indicate that the genes of the putative type-IV secretion system Trb1/Tra1 encoded by the genomic island Trb-1 are active in *L. pneumophila* Corby. Expression of *trbI1* (co-transcribed with *trbH1*), *traG1*, *traH1*, and *traJ1* was confirmed. Interestingly the *lpc2796* gene, which is

integrated in the locus encoding the putative type-IV secretion system is co-transcribed with *traH1* (Figure 23).

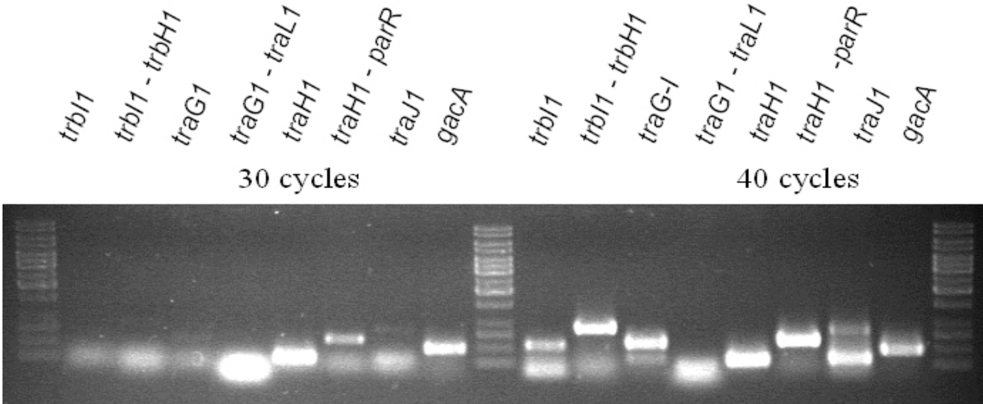


Figure 23: Reverse transcription experiments with selected genes of the genomic island Trb-1 *in L. pneumophila* Corby wild type.

4 Discussion

4.1 Regulatory impact of FleQ, FleR, and RpoN

The primarily aim of this thesis was to characterize the implication of FleQ, FleR, and RpoN in flagellar gene transcription and to propose a model for the regulatory cascade controlling the flagellar system in *L. pneumophila*.

4.1.1 FleQ, FleR, and RpoN have a great impact on global gene transcription

The data derived from microarray analyses in replicative phase as well as in transmissive phase substantiate that *fleQ*, *fleR*, and *rpoN* have an important influence not only on flagellar gene expression but also on the global gene expression pattern. In each of the mutant strains, expression of 1-2% genes is significantly altered during replicative phase and 4-9% genes during transmissive phase.

The σ^{54} factor RpoN is known to initiate gene transcription in a concerted action with enhancer binding proteins (38, 50, 51, 378) like the three *L. pneumophila* proteins FleQ, FleR, and PilR. *pilR*, encoding PilR was not mutated in this study. However, the set of genes regulated by *rpoN* might also comprise genes co-regulated by *pilR*. Based on homology to corresponding genes in *P. aeruginosa*, it is predicted that PilR regulates expression of *pilA* together with RpoN (42, 189, 198, 204). This seems to be different in *L. pneumophila* Paris, as expression of the *pilA*-homologous genes in the *rpoN* mutant were not altered.

As expected, the set of *rpoN* regulated genes overlaps with those regulated by *fleQ* and *fleR.* Genes co-ordinately regulated by *fleQ* and *rpoN* are nearly exclusively flagellar genes. Surprisingly, *fleQ* has in addition a great impact on gene transcription independent of *rpoN*. Both, *rpoN*-dependent and *rpoN*-independent regulation by *fleQ* is reflected in flagellar gene expression and described in more detail in subsection 4.1.2. In contrast and unexpectedly, flagellar genes are under-represented in co-ordinately regulated genes by *fleR* and *rpoN*, but many other gene groups are co-ordinately regulated by *fleR* and *rpoN* (see subsection 4.1.3).

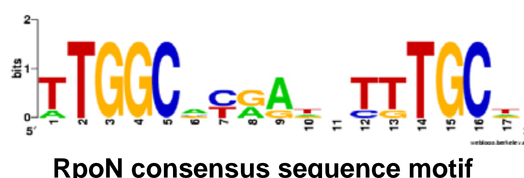
4.1.2 Flagellar gene transcription is mainly dependent on FleQ

Western blot analyses and microscopic examinations confirm that the *fleQ*, the *fleR*, and the *rpoN* mutant are all unflagellated in transmissive phase, indicating that those three regulators are implicated in flagella biosynthesis, similar to what was already reported for a *fleQ* and a *rpoN* mutant in *L. pneumophila* Corby (200).

Our microarray and real-time PCR analyses substantiate that FleQ regulates together with RpoN the flagellar class II gene transcription similar to FleQ of *P. aeruginosa* (82-84, 316). It was shown previously, that expression of the flagellar class II genes *fliM*, *fleN*, *fleS*, and *fleR* is enhanced by *fleQ* and *rpoN* (200). Our data now confirm these results and show that transcription of the flagellar class II genes *flgB*, *flgD*, *flgG*, *flgI*, *flgJ*, *flhF*, *flhA*, *fliR*, *fliQ*, *fliP*, *fliO*, and *fliN* is also enhanced by FleQ and RpoN. The flagellar genes *fleR* and *fleS* of *L. pneumophila* are predicted to encode a two-component system with FleS being the sensor protein and FleR the response regulator that enhance together with RpoN transcription of genes with σ^{54} promoters. In homology to the *P. aeruginosa* flagellar cascade we had proposed that *L. pneumophila* FleR activates together with RpoN the expression of flagellar class III genes (49, 177, 184). This proposal however, is not supported by our microarray and real-time PCR analyses data; in other words our results suggest that FleR and RpoN do not enhance flagellar class III gene transcription. Instead, transcription of flagellar class II genes seem to be repressed by FleR. Interestingly, FleR and RpoN enhance transcription of *letE*. As recently a putative RpoN binding sequence upstream of *letE* was identified (Table 8) (49), we suggest that *letE* is directly regulated by RpoN together with FleR. Strikingly, results of a very recent study indicate that *letE* encodes a flagellar gene repressor (Carmen Buchrieser, in preparation).

Table 8: Putative RpoN binding site upstream of *letE*. Letters in bold highlight the identified motif within the upstream sequence of the respective genes.

Gene name	Distance from start	Sequence
lpp0602, *letE*	241	GT**TGGC**C**TAA**TAC**TTGC**



RpoN consensus sequence motif

Transcription of late flagellar genes (class III and IV) is solely enhanced by FleQ. FleQ regulates the class III genes *motB*, *flgMN*, *fliA* (encoding the σ^{28} regulator FliA) and thereby also the FliA-dependent flagellar class IV genes *fliS*, *flaA* and *flaG*. Moreover, transcription of three genes (*lpp0952*, *lpp1290*, *lpp2282*) described previously as being part of the FliA-regulon (49) are solely enhanced by FleQ. While upstream of *lpp2282* and *lpp0952*, FliA consensus motifs are present, *lpp1290* is probably under control of the vicinal promoter of *fliD* (49, 184).

Thus our results strongly suggest that FleQ is the master regulator for motility that enhances flagellar class II gene transcription together with RpoN, but flagellar class III/IV genes RpoN-independent. The impact of FleQ on gene transcription independent of RpoN, is remarkable as it has been generally accepted that enhancer binding proteins function in concert with a σ^{54} factor like RpoN. However, it has been shown that the enhancer binding protein NtrC of *Rhodobacter capsulatus* activates transcription of genes in a RpoN-independent fashion together with the housekeeping RNAP/σ^{70} holoenzyme (40, 76, 77, 124, 314).

Also FleQ of *P. aeruginosa* influences gene transcription independent of RpoN. Beside activating the transcription of flagellar genes, FleQ in *P. aeruginosa* is also transcriptional repressor of certain non-flagellar genes. Hereby binding of c-di-GMP to FleQ leads to derepression, probably by influencing the FleQ DNA-binding properties (185). In several bacteria the second messenger bis-(3',5')-cyclic diguanylic acid (c-di-GMP) influences motility (382). The *L. pneumophila* Paris genome encodes 24 proteins containing GGDEAF and/or EAL domains putatively controlling the concentration of c-di-GMP of which interestingly transcription of five of those is enhanced by FleQ but not by RpoN. Thus, we hypothesize, that FleQ of *L. pneumophila* also may regulate gene transcription in response to c-di-GMP.

However, another reason for the observed RpoN-independent influence on gene transcription by FleQ in *L. pneumophila* might be that *fleQ* mRNA influences the level of free CsrA. Bioinformatic analyses identified potential CsrA binding sites in the *fleQ* mRNA (Carmen Buchrieser, in preparation), suggesting that *fleQ* mRNA translation is controlled by CsrA. This is compatible with our results showing that although *fleQ* is constitutively transcribed (49, 200), the FleQ protein in *L. pneumophila* Corby is detectable by Western blot only between mid-exponential and late-exponential growth phase (Sebastian Jacobi, unpublished). Assuming that CsrA

binds to *fleQ* mRNA, a *fleQ* mutation would lead to greater amounts of unbound CsrA and consequently to a phenotype similar to CsrA overexpression (Figure 24).

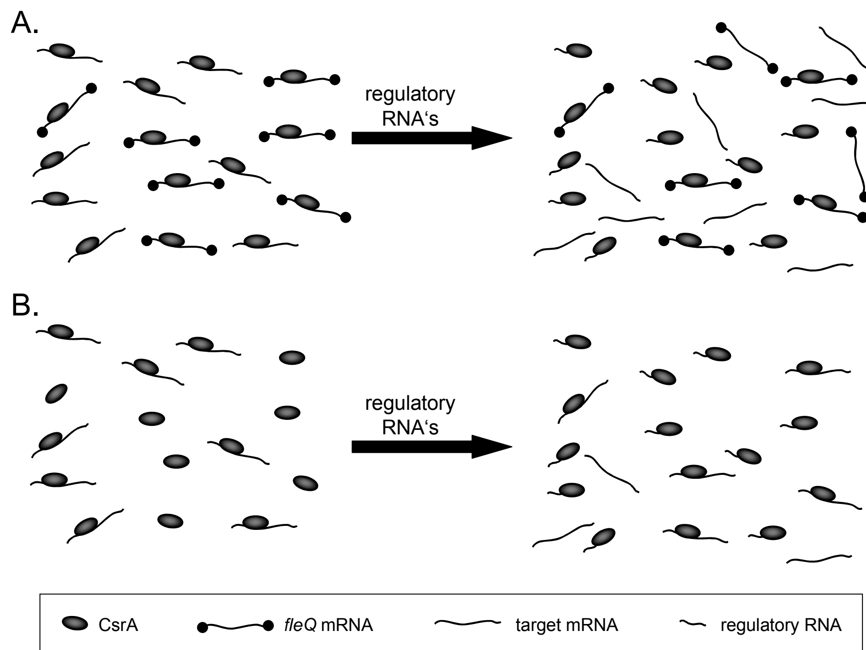


Figure 24: Model for balancing the level of CsrA by *fleQ* mRNA in the *L. pneumophila* wild type (A.) in comparison to a *fleQ* mutant (B.).

Using Northern blot analysis it was determined by the group of R. Marre that CsrA overexpression leads to reduced amounts of *flaA* and also *fliA* mRNA (Figure 25) (112). In accordance to these results, *fliA* and *flaA* transcription is repressed in the *fleQ* mutants of *L. pneumophila* strain Paris and strain Corby.

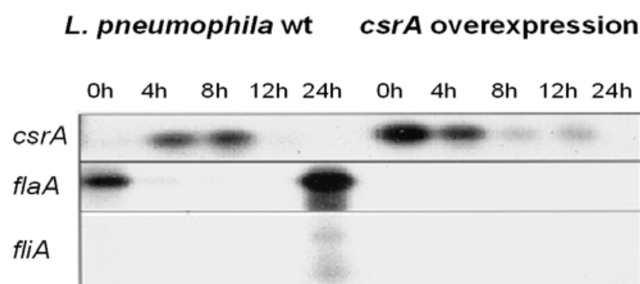


Figure 25: Northern blot of *fliA*, *flaA*, and *csrA* transcripts in *L. pneumophila* L1 wild type and a strain overproducing *csrA*. Total RNA was extracted from bacteria grown in BYE broth at 37°C for the indicated times and subjected to Northern blot analysis. From: (112).

Thus, FleQ might have an indirect influence on the expression of many genes, like *fliA* and *flaA via* balancing the level of free CsrA. Similarly, the CsrA in *E. coli* controls motility as activator of *flhDC* expression, encoding the *E. coli* master flagellar regulators (376). However, to date, *in vitro* binding assays of *fleQ* mRNA to purified CsrA have been unsuccessful and it is suggested that flagella biosynthesis might be largely independent of the CsrA system or need another, yet undiscovered factor (Carmen Buchrieser, in preparation).

Further details of flagellar gene regulation derived from the identification of promoter sites upstream of flagellar gene operons. The only flagellar promoter sites determined experimentally previously, were those of *fleQ* and *flaA* suggesting that their transcription is initiated by the σ^{70} factor (200) and the σ^{28} factor FliA (178, 182) respectively. Furthermore, putative FliA binding sequences are present upstream of the class IV operons *fliDS* and *motY* (49, 184) and putative RpoN binding sequences are present upstream of all putative flagellar class II gene operons (49, 184, 200). As flagellar class II gene transcription is indeed enhanced by *rpoN*, but not strictly dependent on *rpoN*, the question arose if those putative RpoN binding sequences are functional. Therefore we determined by primer extension the transcriptional start site(s) of the mRNA transcripts for the regulatory regions upstream of the flagellar class II genes *flgB* and *fleS.* While the transcription start site upstream of *fleS* corresponds to the putative RpoN binding sequence, transcription upstream of *flgB* initiates from two σ^{70}-like promoter sequences. Thus, *flgB* transcription seems to be regulated outside the typical hierarchy. This has also been observed for the *flk* gene in *Salmonella enterica* serovar Typhimurium (212), the operon containing the *flgBC* and *fliE* genes in *Caulobacter crescentus* (41), flagellar genes in several spirochetes (145, 146, 234, 291) and most recently the *flaG* gene and the *fliST* operon in *Pseudomonas fluorescens* (309). In conclusion we categorize *fleS* as direct and *flgB* as indirect target of RpoN.

Taken together, our data strongly suggest that FleQ is the master regulator of the *L. pneumophila* flagellar biosynthesis genes and substantiate that FleQ enhance flagellar class II genes together with RpoN and flagellar class III/IV genes RpoN-independent. Moreover we hypothesize that in the following, FleR and RpoN probably repress flagellar gene transcription *via letE*.

4.1.3 FleR and RpoN couple protein biosynthesis and metabolism to flagellar biosynthesis

Transcription of numerous genes involved in protein biosynthesis is enhanced by FleR and RpoN as deduced from microarray and real-time PCR analyses. Genes involved in gene transcription and mRNA translation generally show a decrease in mRNA levels upon transition to transmissive phase (49). Importantly, our data show that FleR and RpoN clearly counteract this tendency.

Besides, FleR and RpoN control expression of several metabolic genes. This suggests that they could control the overall rate of the glycolysis, pentose phosphate pathway, citric acid cycle, and polyhydroxybutyrate synthesis. Our results indicate that FleR and RpoN might increase the overall rate of glycolysis or gluconeogenesis by enhancing expression of *lpp0535*, encoding a putative fructose-bisphosphate aldolase. Output of the glycolysis is pyruvate, which can be transformed to acetyl-CoA by activity of the pyruvate dehydrogenase. Interestingly, expression of genes encoding homologous of the α- and β-subunit of E1 and the unit E2 of the dehydrogenase complex are enhanced by FleR and RpoN. Nevertheless, expression of the genes *aceE* and *aceF*, also encoding the units E1 and E2 of the dehydrogenase complex is not affected by *fleR* or *rpoN* . Acetyl-CoA then can be used in the citric acid cycle for amino acid and porphyrine biosynthesis. Beside, acetyl-CoA can be used for polyhydroxybutyrate biosynthesis whereby acetoacetyl-CoA reductases are involved. Polyhydroxybutyrate is employed by *L. pneumophila* as a form of energy storage molecule which is used under conditions of starvation (202) and inclusions of polyhydroxybutyrate in the cytoplasm are typically found in the transmissive phase (143). The overall rate of polyhydroxybutyrate biosynthesis seems to be repressed by FleR and RpoN, as they repress expression of all three genes putatively encoding acetoacetyl-CoA reductases (*lpp0621*, *lpp2322* and *lpp0620*).

For flagellar biosynthesis in *L. pneumophila*, thousands of flagellin subunits must be expressed to built up the flagella within a short time slot. Thus the capacity of protein biosynthesis must be important. Considering the regulatory influence of FleR and RpoN, we consequently hypothesize that they couple protein biosynthesis and metabolism to fulfill the requirements of flagellar biosynthesis.

4.1.4 FleQ, FleR, and RpoN are dispensable for phase transition

The most prominent transmissive phase trait is the monopolar flagellum which is absent in the *fleR*, the *fleQ*, and the *rpoN* mutant strains. Besides, also other transmissive phase traits, e.g. the contact-dependent hemolytic activity on sheep erythrocytes (214) might be affected by these three regulators. This kind of hemolytic activity is dependent on various genes, like the *dotA* (214) and the *rpoS* gene (2). A phenotypic assay revealed that the hemolytic contact-dependent activity is strongly enhanced by FleQ, RpoN, and also FliA in strain Paris. Same results were reported earlier for strain Corby (a *fleR* mutant strain was not tested so far) (183). Various enzymes might be responsible for this observed hemolysis activity. According the expression profile deduced from the microarray analyses, FleQ enhances the expression of gene *lpp2281*, encoding a protein similar to membrane-associated metalloprotease proteins and RpoN enhances the expresion of gene *lpp0549*, encoding the membrane protease subuntit HflC. Interestingly, expression of *proA1* (*msp*, *lpp0532*), encoding the zinc metalloprotease ProA (MspA), which is active on a variety of substrates (100) is enhanced by FleQ, FleR, and RpoN. Also further virulence genes are differentially expressed in the mutant strains.

However, the small coccoid shape in transmissive phase was not affected by FleQ, FleR, and RpoN. As proved by *in vitro* and *in vivo* growth experiments, the *fleQ*, the *fleR*, and the *rpoN* mutant are able to replicate and then to exit the replicative phase similar to the wild type, indicating that FleQ, FleR, and RpoN are not absolutely required for transition from replicative to transmissive phase.

4.1.5 Biological function of the flagella

In accordance to our results, prediction of flagellin localization performed with PSORTb and PA-SUB predicted that flagellin is localized extracellular and periplasmic respectively (137). That flagellin expression restricts bacterial replication in macrophages verify that *L. pneumophila* is adapted to survival in natural fresh water, replicating in protozoa rather than to cause Legionnaires‘ disease in humans, replicating in macrophages.

In its natural fresh water reservoir chemotaxis would enable *L. pneumophila* bacteria to move towards favorable conditions and to avoid unfavorable conditions.

Though *L. pneumophila* does not encode a typical chemotaxis system (60, 64, 151), and chemotaxis was not observed so far (184). In contrast to the thermopilic bacterium *A. aeolicus* which also lacks a chemotaxis system (95), *L. pneumophila* encodes the switch protein FliM including the CheY binding domain. Possibly *L. pneumophila* responds to a different set of factors than the most studied chemotaxis systems respond to (e.g. sugars and amino acids) and input for controlling taxis is mediated through an unidentified system. Also possibly, motility in *L. pneumophila* is undirected. As demonstrated during *A. castellanii* infection by confocal laser scanning microscopy with an anti-flagellin antibody and substantiated by many publications (19-21, 112, 123, 165, 166, 244, 266) it seems that phase transition induced by nutrient starvation regulates the production of flagella in *L. pneumophila* and undirected motility is perhaps the best strategy to disperse evenly in the environment after evasion from the host cell.

4.1.6 Modified model for transcriptional regulation of flagellar genes

In *L. pneumophila*, flagellar genes are expressed temporally during the transition to the transmissive phase and is repressed once the flagellum is completed. For the transcriptional regulation of flagellar genes, varying models have been proposed, which was thought to be similar to *P. aeruginosa* (7, 49, 177, 184, 200) (see subsection 1.7.4.).

Our data obtained during this study suggest a modified model for this regulatory cascade in *L. pneumophila* (Figure 25). In this model *fleQ*, encoding the enhancer binding protein FleQ is the master regulator of the flagellar regulon. Expression of FleQ is probably transcriptionally controlled by the σ^{70} factor and post-transcriptionally controlled by an unknown factor. Together with the σ^{54} factor RpoN, FleQ enhances flagellar class II gene transcription (200). Differing from *P. aeruginosa* (82-84, 316), at least some flagellar class II genes are not direct targets of RpoN and FleQ in *L. pneumophila*. The next step in *P. aeruginosa*, expression of flagellar class III genes is induced by phosphorylation-dependent activated FleR and RpoN (84). In contrast, FleR in *L. pneumophila* does not activate the expression of flagellar class III genes (84, 316). Instead FleR and RpoN seem to couple protein biosynthesis and metabolism to the requirements of flagellar biosynthesis. Moreover, they enhance

LetE expression, which now emerges as a repressor for flagellar gene expression (Carmen Buchrieser, in preparation). Transcription of flagellar class III genes in *L. pneumophila* is solely enhanced by FleQ. As last step in flagellar biosynthesis, expression of the σ^{28} factor FliA (encoded by *fliA*) induces expression of flagellar class IV genes coding e.g. for flagellin which leads to the completion of the flagellum in both, *P. aeruginosa* and *L. pneumophila* (49, 178, 180, 182, 184). FliA in *L. pneumophila* seems also to be responsible for a negative feedback loop on flagellar class II and III genes (Table S7) (49). This negative control, as a response to the completion of the flagellum, may be an important mechanism used by the cell to turn off flagellar gene expression once the gene products are no longer needed.

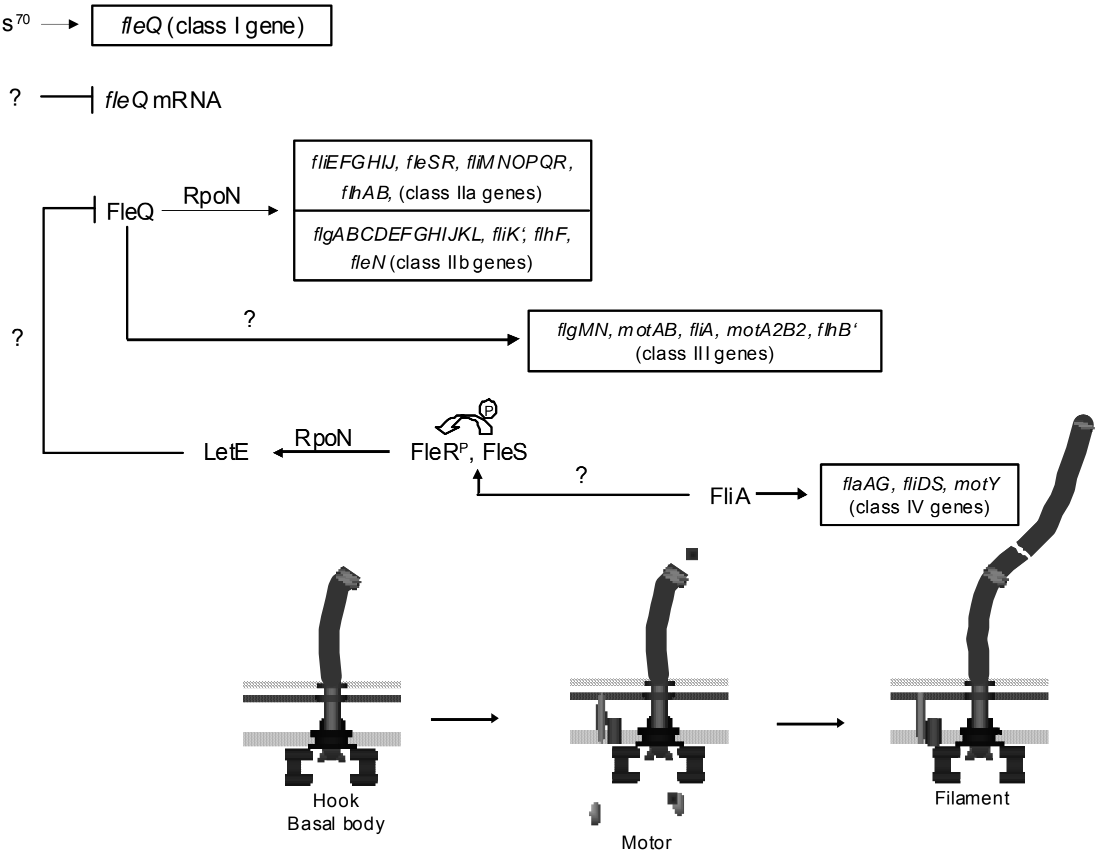


Figure 25: Model for transcriptional regulation of the various flagellar genes (Class I-IV) in *L. pneumophila*. ? denotes an unknown factor(s).

4.1.7 Concluding remarks and future directions

In conclusion, this study allowed to get deeper insight in the complex regulatory network controlling flagellar gene transcription in *L. pneumophila* that is different from that of *P. aeruginosa*. Our data show that a modified model for flagellar gene regulation needs to be established. Therein flagellar genes are controlled by FleQ and RpoN, while FleR has little impact on flagellar gene transcription.

Of particular interest is the unexpected finding that FleQ of *L. pneumophila* regulates gene expression RpoN-dependent as well as RpoN-independent. Future studies might investigate the mechanisms by which FleQ influences gene transcription independent of RpoN. This is a special challenge, as it might involve unknown regulatory mechanisms.

4.2 Impact of the genomic island Trb-1 on conjugative DNA transfer

One of the driving forces for the evolution of variation in virulence between different *L. pneumophila* strains (203) might have been horizontal gene transfer, e.g. conjugation. It was already experimentally shown that chromosomal virulence genes (*icm-dot* locus) can be horizontally transferred from one *L. pneumophila* strain to another (263), that *L. pneumophila* can conjugate RSF1010-related plasmids in a Dot/Icm type-IV secretion system-dependent manner and that also the Lvh type-IV secretion system contributes to the ability to mobilize a plasmid (329, 330, 370). In addition to a type-IV conjugation system, a cognate oriT site is essential for conjugative DNA transfer.

The *L. pneumophila* Corby genome possesses the genomic island Trb-1, which encodes an integrase and all proteins necessary for a type-IV conjugation system (Figure 26). Moreover, Trb-1 contains a putative oriT site where DNA processing might be initiated during conjugation (Figure 27) (151, 347). Thus the second aim of this thesis was to investigate the implication of Trb-1 in conjugative DNA transfer.

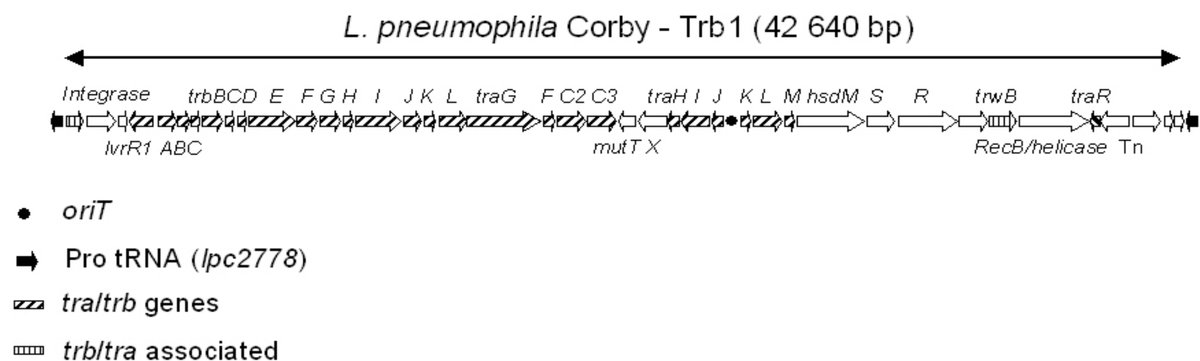


Figure 26: Genetic organization of the genomic island Trb-1 of *L. pneumophila* Corby. Adapted from: (151).

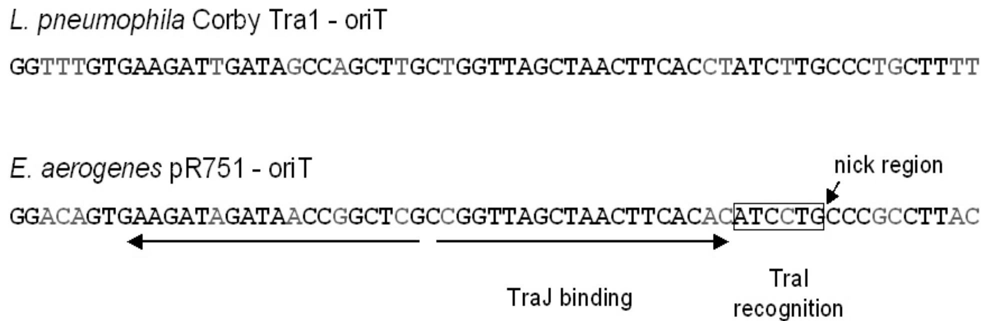


Figure 27: Comparison of the putative oriT site of Trb-1 and the oriT site of the conjugative plasmid R751 (290). The TraJ-binding site (large arrows), the TraI-binding site (rectangle) and the nick region (small arrow) of the pR751 oriT site are indicated. Adapted from: (151).

4.2.1 Presence of putative conjugation pili

Different pili or pili-like structures have been observed on the surface of various *L. pneumophila* strains (61, 318, 349), indicating that *L. pneumophila* might excrete multiple pili by different pathways. Only the long type-IV pili described by Stone and Abu Kwaik have been specifically identified and are dependent on the *pilEL* genes (349).

On the surface of *L. pneumophila* Corby we identified long pili structures by electron microscopic examination. As reverse-transcription PCR analyses confirmed that the genes encoding the type-IV conjugation system Trb1/Tra1 are active and, moreover, as those pili structures have not been observed in a *traG1* mutant, we suggest that the Trb1/Tra1 system might be responsible for those structures. Nevertheless, this suggestion remains to be confirmed by future experiments, e.g. immunogold labeling of the pilus with epitope-specific antibodies and subsequent electron microscopy.

4.2.2 The oriT site of the genomic island Trb-1 is functional

Although putative oriT sites are present in the four sequenced *L. pneumophila* genomes (347) their functionality was not proven so far. To prove the hypothesis that the putative oriT site located on Trb-1 plays a role in conjugation, we conducted

conjugation assays using a recombinant plasmid carrying the oriT region. The results clearly showed that this oriT site is functional.

To test if the Trb1/Tra1 type-IV conjugation system initiates DNA processing during conjugation at this oriT site, we conducted the same experiment using a *traG1* mutant strain. However, the conjugation ability of the *traG1* mutant strain was not significantly reduced. This indicates that either the Trb1/Tra1 type-IV conjugation system does initiate DNA transfer at this oriT site or that other type-IV secretion systems, e.g. the homolog type-IV secretion system encoded on by the genomic island Trb-2 also can initiate DNA transfer at this oriT site. In the ladder case, the conjugation assays might not have been sensitive enough to detect differences between wild type and *traG1* mutant.

4.2.3 Concluding remarks and future directions

Our results indicate that conjugation plays a powerful role for genome diversity and evolution of new pathotypes within *L. pneumophila*. Using conjugation assays, we showed, for the first time, that *L. pneumophila* Corby possesses a functional oriT site contributing to conjugation between different *L. pneumophila* strains.

This oriT site is located on the genomic island Trb-1. In addition to a functional oriT site, this island carries an integrase gene and all genes necessary for a type-IV conjugation system. Later on we verified that Trb-1 can be excised from the chromosome forming episomal circles, horizontally transferred to other *L. pneumophila* strains, and then integrated site-specific in the genome of the recipient (Figure 28) (151).

Thus, future studies may investigate if Trb-1 is self-transmissible. As Hentschel and Hacker defined a genomic island as never self-transmissible (176), it might be necessary to reclassify Trb-1 as an integrative conjugative element. These integrative conjugative elements have both, plasmid- and phage-like features: similar to plasmids, they transfer *via* conjugation and like many phages they integrate into and replicate with the host chromosome (52).

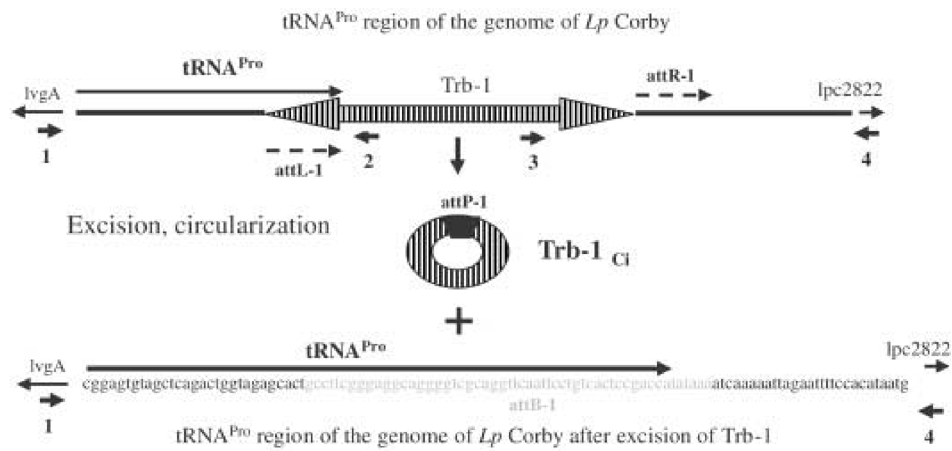


Figure 28: Mechanism of generating the episomal circular form of Trb-1 of *L. pneumophila* Corby. After excision and circularization of Trb-1 (Trb-1 Ci) as an episomal element, an intact Pro tRNA gene is left at the "core" genome. The genomic island Trb-1 is indicated by a vertical striped arrow and the sequence of the Pro tRNA gene (*lpc2778*) after excision of Trb-1 is given. From: (151).

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Supplementary material

Table S1: Coordinately down- and upregulated genes in the *fleQ* mutant during replicative phase (fold change ≥ 2.00 ∨ ≤ 0.50, P value ≤ 0.001).

Gene name	Annotation	Fold change	Functional group
lpp2943	Unknown	2.114	Unknown
lpp2275	Unknown	2.099	Unknown
lpp1740	Unknown	2.028	Unknown
lpp2594	Unknown	2.028	Unknown
lpp1755, fliN	Flagellar motor switch protein FliN	0.095	Cellular processes
lpp1756, fliM	Flagellar motor switch protein FliM	0.113	Cellular processes
lpp1748, flhF	Flagellar biosynthesis protein FlhF	0.147	Cellular processes
lpp1747, fleN	Similar to flagellar synthesis regulator	0.157	Cellular processes
lpp1231, flgI	Flagellar P-ring protein precursor FlgI	0.177	Cellular processes
lpp1228, flgF	Flagellar biosynthesis protein FlgF	0.215	Cellular processes
lpp0915, fleQ	Transcriptional regulator FleQ	0.216	Information pathways
lpp1754, fliO	Flagellar protein FliO	0.243	Cellular processes
lpp1233, flgK	Flagellar hook-associated protein 1	0.275	Cellular processes
lpp1229, flgG	Flagellar biosynthesis protein FlgG	0.279	Cellular processes
lpp1232, flgJ	Flagellar biosynthesis protein FlgJ	0.285	Cellular processes
lpp1227, flgE	Flagellar hook protein FlgE	0.304	Cellular processes
lpp1749, flhA	Flagellar biosynthesis protein FlhA	0.330	Cellular processes
lpp1224, flgB	Flagellar basal-body rod protein FlgB	0.363	Cellular processes
lpp0953	Similar to kynurenine 3-monooxygenase	0.374	Intermediary metabolism
lpp1753, fliP	Flagellar biosynthetic protein FliP	0.382	Cellular processes
lpp1752, fliQ	Flagellar biosynthetic protein FliQ	0.382	Cellular processes
lpp1723, fliG	Flagellar motor switch protein	0.384	Cellular processes
lpp1722, fliH	Polar flagellar assembly protein FliH	0.403	Cellular processes
lpp0725	Similar to hypothetical protein	0.409	Similar to unknown proteins
lpp2554	Hypothetical gene	0.418	Unknown
lpp1656	Unknown	0.435	Unknown
lpp1751, fliR	Flagellar biosynthetic protein FliR	0.438	Cellular processes
lpp1234, flgL	Flagellar hook-associated protein FlgL	0.441	Cellular processes
lpp1746, fliA	Sigma factor 28	0.457	Information pathways

Table S1 - continued

lpp2010, hemE	Uroporphyrinogen decarboxylase	0.460	Intermediary metabolism
lpp1226, flgD	Flagellar basal-body rod modification protein FlgD	0.470	Cellular processes
lpp1170	Regulatory protein (GGDEF and EAL domains)	0.476	Cellular processes
lpp2327	Unknown	0.490	Unknown
lpp0351	Regulatory protein (EAL domain)	0.493	Cellular processes
lpp1744, motB	Similar to chemotaxis MotB protein	0.497	Cellular processes

Table S2: Coordinately down- and upregulated genes in the *fleQ* mutant during transmissive phase (fold change ≥ 2.00 ∨ ≤ 0.50, P value ≤ 0.001).

Gene name	Annotation	Fold change	Functional group
lpp0661	Similar to major facilitator family transporter	4.759	Cellular processes
lpp0660	Similar to a domain of alanyl-tRNA synthetase	3.431	Information pathways
lpp0240	Similar to hypothetical protein	3.032	Similar to unknown proteins
lpp2117	Similar to bacteriophage protein	2.741	Other functions
lpp2584, smlA	Major facilitator superfamily transporter	2.620	Similar to unknown proteins
lpp2390	Similar to conserved hypothetical protein	2.612	Similar to unknown proteins
lpp2120	Similar to conserved hypothetical protein	2.552	Similar to unknown proteins
lpp2058	Ankyrin repeat protein	2.540	Similar to unknown proteins
lpp2141, gspA	Global stress protein GspA	2.520	Other functions
lpp1124	Similar to amino acid ABC transporter	2.500	Cellular processes
lpp1048	Weakly similar to integrase	2.446	Other functions
lpp0801	Similar to DNA/RNA helicases - superfamily II	2.417	Information pathways
lpp2105	Similar to transcriptional regulator - deoR family	2.375	Information pathways
lpp2682	Putative membrane protein	2.359	Similar to unknown proteins
lpp0237	Similar to pyoverdine biosynthesis protein PvcB	2.344	Intermediary metabolism
lpp1906	Similar to N-terminal part of putative transposase (IS91 family)	2.330	Other functions
lpp0865	Similar to acyl-CoA dehydrogenase	2.324	Intermediary metabolism
lpp1057	Putative membrane protein similar to conserved hypothetical protein	2.315	Similar to unknown proteins
lpp0357	Similar to amino acid transporter	2.288	Cellular processes
lpp2383	Similar to unknown protein	2.276	Similar to unknown proteins
lpp1972	Predicted membrane protein	2.273	Similar to unknown proteins
lpp0360	Unknown	2.268	Unknown
lpp0652	Similar to ABC transporter - permease component	2.268	Cellular processes
lpp2235	Unknown	2.253	Unknown
lpp1098	Similar to transposase (IS5 family)	2.235	Other functions
lpp1885	Similar to D-alanyl-D-alanine carboxypeptidase	2.206	Cellular processes
lpp0067	Similar to plasmidic transfer origin protein TraK	2.205	Other functions
lpp1087	Similar to putative transcriptional regulator	2.196	Information pathways
lpp2407	Similar to antirestriction protein	2.196	Information pathways
lpp2089	Unknown	2.164	Unknown
lpp0225	Similar to conserved hypothetical protein	2.163	Similar to unknown proteins
lpp1631	Unknown	2.163	Unknown

Table S2 - continued

lpp0212	Hypothetical gene	2.155	Unknown
lpp0658	Similar to putative lysyl-tRNA synthetase	2.152	Information pathways
lpp1769	Similar to hypothtical proteins	2.138	Similar to unknown proteins
lpp0911	Highly similar to ABC transporter - ATP-binding protein	2.134	Cellular processes
lpp2299	Similar to alkyl hydroperoxide reductase AhpC	2.127	Other functions
lpp0218	Unknown	2.126	Unknown
lpp0324	Unknown	2.119	Unknown
lpp0239	Some similarity with transporters	2.118	Cellular processes
lpp0301	Similar to cation transport ATPase	2.107	Cellular processes
lpp2056	Similar to transposase (IS5 family)	2.105	Other functions
lpp0647	Similar to 1-acyl-sn-glycerol-3-phosphate acyltransferase	2.102	Intermediary metabolism
lpp2517	Ankyrin repeat protein	2.102	Unknown
lpp0284	Similar to RND efflux membrane fusion proteins	2.099	Cellular processes
plpp0095	-	2.098	Unknown
lpp1888, pfp	Similar to PPi dependent phosphofructokinase	2.096	Intermediary metabolism
lpp2309, dnaG	DNA primase	2.088	Information pathways
lpp0017	Unknown	2.086	Unknown
lpp1714, clpB	Endopeptidase Clp ATP-binding chain B (ClpB)	2.079	Other functions
plpp0096	Similar to unknown protein	2.078	Unknown
lpp1200, hisC1	Histidinol-phosphate aminotransferase (Imidazole acetol-phosphate transaminase)	0.142	Intermediary metabolism
lpp1197, hisA	Phosphoribosylformimino-5-aminoimidazole carboxamide ribotide isomerase	0.173	Intermediary metabolism
lpp1294, flaA	Flagelline	0.176	Cellular processes
lpp0725	Similar to hypothetical protein	0.191	Similar to unknown proteins
lpp1198, hisH2	Imidazole glycerol phosphate synthase subunit HisH (IGP synthase glutamine amidotransferase subunit)	0.192	Intermediary metabolism
lpp1293, flaG	Unknown	0.212	Unknown
lpp1290	Similar to enhanced entry protein EnhA	0.224	Other functions
lpp1227, flgE	Flagellar hook protein FlgE	0.231	Cellular processes
lpp1177	Unknown	0.236	Unknown
lpp0623	Unknown	0.237	Unknown
lpp1890	Similar to type IV pilin PilA	0.265	Cellular processes
lpp2849	Similar to putative coproporphyrinogen oxidase A	0.277	Similar to unknown proteins
lpp2525	Similar to guanylate cyclase-related protein	0.289	Intermediary metabolism

Table S2 - continued

lpp1201, hisD	Histidinol dehydrogenase	0.304	Intermediary metabolism
lpp0969, flgM	Similar to negative regulator of flagellin synthesis (Anti-sigma-28 factor)	0.309	Information pathways
lpp0728	Similar to acetoacetate decarboxylase	0.310	Intermediary metabolism
lpp3061	Unknown	0.316	Unknown
lpp2265	Similar to conserved hypothetical protein	0.319	Similar to unknown proteins
lpp2245b	Similar to transposase (IS4 family)	0.325	Other functions
lpp0962	Unknown	0.329	Unknown
lpp1756, fliM	Flagellar motor switch protein FliM	0.335	Cellular processes
lpp1233, flgK	Flagellar hook-associated protein 1	0.339	Cellular processes
lpp0197	Similar to adenine specific DNA methylase (Mod-related)	0.344	Information pathways
lpp1202, hisG	ATP phosphoribosyltransferase	0.348	Intermediary metabolism
lpp1864	Unknown	0.348	Unknown
lpp2281	Similar to membrane-associated metalloprotease proteins	0.349	Intermediary metabolism
plpp0089	Weakly similar to stability protein StbE	0.350	Unknown
lpp1747, fleN	Similar to flagellar synthesis regulator	0.362	Cellular processes
lpp0915, fleQ	Transcriptional regulator FleQ	0.364	Information pathways
lpp1110	Hypothetical protein	0.364	Unknown
lpp0963	Unknown	0.365	Unknown
lpp2327	Unknown	0.366	Unknown
lpp0954	Similar to unknown proteins	0.367	Similar to unknown proteins
lpp1340	Unknown	0.381	Unknown
lpp0686	Similar to type-IV fimbrial pilin related protein	0.385	Cellular processes
lpp0727	Similar to NADH-ubiquinone oxidoreductase	0.390	Cellular processes
lpp1291, fliS	Similar to flagellar protein FliS	0.399	Cellular processes
lpp0910	Similar to conserved hypothetical protein	0.405	Similar to unknown proteins
lpp1029	Unknown	0.405	Unknown
lpp2209	Unknown	0.406	Unknown
lpp2559	Similar to small heat shock protein	0.408	Other functions
lpp2124	Unknown	0.410	Unknown
lpp0952	Regulatory protein (GGDEF and EAL domains)	0.414	Cellular processes
lpp2282	Unknown	0.416	Unknown
lpp2145	Similar to ornithine cyclodeaminase	0.418	Intermediary metabolism
lpp0968, flgN	Similar to unknown protein	0.420	Similar to unknown proteins
lpp1606	Similar to acetyltransferase- GNAT family	0.427	Intermediary metabolism
lpp2809	Unknown	0.427	Unknown

Table S2 - continued

lpp1015	Similar to long-chain-fatty-acid-CoA ligase	0.428	Intermediary metabolism
lpp1228, flgF	Flagellar biosynthesis protein FlgF	0.429	Cellular processes
lpp1748, flhF	Flagellar biosynthesis protein FlhF	0.435	Cellular processes
lpp0160	Similar to Wolinella succinogenes hypothetical protein	0.436	Similar to unknown proteins
lpp0907	Weakly similar to anti-anti-sigma factor	0.436	Information pathways
lpp0161	Similar to Wolinella succinogenes hypothetical protein	0.438	Similar to unknown proteins
lpp1324	Similar to DNA-binding protein Fis	0.440	Information pathways
lpp0353	Similar to two-component sensor histidine kinase	0.443	Cellular processes
lpp0070	Unknown	0.445	Unknown
lpp1396	Similar to phosphate starvation-inducible protein PhoH	0.445	Intermediary metabolism
lpp1085	Unknown	0.448	Unknown
plpp0090	Weakly similar to stability protein StbD	0.450	Unknown
lpp2958	Unknown	0.450	Unknown
lpp3028	Similar to protease	0.450	Intermediary metabolism
lpp0942	Regulatory protein (GGDEF domain)	0.452	Cellular processes
lpp1203	Weakly similar to E. coli Trp operon repressor	0.452	Information pathways
lpp1440	Weak similarity to myosin	0.454	Unknown
lpp0788	Unknown	0.456	Unknown
lpp1755, fliN	Flagellar motor switch protein FliN	0.456	Cellular processes
lpp2524, shkA	Similar to two-component sensor histidine kinase	0.456	Cellular processes
lpp2762, rplM	50S ribosomal subunit protein L13	0.458	Information pathways
lpp1744, motB	Similar to chemotaxis MotB protein	0.459	Cellular processes
lpp0394, rplC	50S ribosomal subunit protein L3	0.461	Information pathways
lpp0590, lvgA	Unknown virulence protein	0.462	Similar to unknown proteins
lpp0257	Similar to chitin-binding protein CbpD	0.464	Intermediary metabolism
lpp1170	Regulatory protein (GGDEF and EAL domains)	0.464	Cellular processes
lpp0351	Regulatory protein (EAL domain)	0.467	Cellular processes
lpp0855, mip	Macrophage infectivity potentiator	0.467	Information pathways
lpp2340	Predicted membrane protein	0.469	Unknown
lpp0724	Similar to conserved hypothetical protein	0.470	Similar to unknown proteins
lpp0959	Unknown	0.470	Unknown
lpp0052	Unknown	0.472	Unknown
lpp0809	Regulatory protein (GGDEF domain)	0.472	Cellular processes
lpp2962, coxB	Cytochrome c oxidase- subunit II	0.473	Cellular processes
lpp1823	Unknown	0.474	Unknown
lpp2368	Unknown	0.478	Unknown

Table S2 - continued

lpp1447	Some similarity with eukaryotic proteins	0.479	Similar to unknown proteins
lpp2866	Similar to aminopeptidase	0.482	Intermediary metabolism
lpp0506	Unknown	0.483	Unknown
lpp1199, hisB	Histidinol-phosphatase/imisazoleglycerol-phosphate dehydratase	0.483	Intermediary metabolism
lpp1618	Similar to conserved hypothetical proteins	0.483	Similar to unknown proteins
lpp1746, fliA	Sigma factor 28	0.484	Information pathways
lpp1234, flgL	Flagellar hook-associated protein FlgL	0.486	Cellular processes
lpp1009	Similar to 2-oxoglutarate ferredoxin oxidoreductase α subunit	0.494	Intermediary metabolism
lpp1900	Unknown	0.494	Unknown

Table S3: Coordinately down- and upregulated genes in the *fleR* mutant during replicative phase (fold change ≥ 2.00 ∨ ≤ 0.50, P value ≤ 0.001).

Gene name	Annotation	Fold change	Functional group
lpp1755, fliN	Flagellar motor switch protein FliN	4.015	Cellular processes
lpp0799	Unknown	3.544	No similarity
lpp2276	Unknown- N-terminal similar to Legionella 33 kDa polypeptide	3.423	Similar to unknown proteins
lpp2894	Similar to lysophospholipase A	3.175	Intermediary metabolism
lpp0934	Unknown	2.870	No similarity
lpp0620	Similar to acetoacetyl-CoA reductase	2.816	Intermediary metabolism
lpp2482	Unknown	2.700	No similarity
lpp0962	Unknown	2.643	No similarity
lpp2190	Similar to multidrug resistance ABC transporter ATP-binding protein	2.592	Cellular processes
lpp0640	Similar to competence protein comM	2.565	Similar to unknown proteins
lpp2322	Similar to acetoacetyl-CoA reductase	2.542	Intermediary metabolism
lpp1228, flgF	Flagellar biosynthesis protein FlgF	2.499	Cellular processes
lpp0963	Unknown	2.477	No similarity
lpp2675	Weakly similar to cysteine protease	2.438	Intermediary metabolism
lpp1638	Unknown	2.393	No similarity
lpp0829a	Similar to N-terminal part of *Legionella* hypothetical protein	2.331	Similar to unknown proteins
lpp1382	Similar to short-chain dehydrogenase	2.329	Similar to unknown proteins
lpp2569	Similar to carbonic anhydrase	2.305	Other functions
lpp1631	Unknown	2.304	No similarity
lpp1113	Similar to hypothetical proteins	2.302	Intermediary metabolism
lpp1233, flgK	Flagellar hook-associated protein 1	2.301	Cellular processes
lpp1936	Unknown	2.239	No similarity
lpp0866	Similar to hydrolase	2.235	Other functions
lpp0359	Similar to NAD+-dependent formate dehydrogenase	2.235	Cellular processes
lpp0976	Similar to cell division protein FtsL	2.234	Cellular processes
lpp1859	Signal peptide predicted	2.226	No similarity
lpp0012	Unknown	2.161	No similarity
lpp2332	Similar to ATP synthase C chain	2.125	Cellular processes
lpp0122	Unknown	2.124	No similarity
lpp1626	Similar to metalloprotease	2.122	Intermediary metabolism
lpp3071	Similar to eukaryotic zinc metalloproteinase	2.113	Intermediary metabolism
lpp0621	Similar to acetoacetyl-CoA reductase	2.111	Intermediary metabolism

Table S3 - continued

lpp2631	Unknown	2.105	No similarity
lpp2246	Unknown	2.095	No similarity
lpp1324	Similar to DNA-binding protein Fis	2.073	Information pathways
lpp2491	Unknown	2.039	No similarity
lpp2788	Putative response regulator	2.020	Information pathways
lpp1726, fleR	Similar to two-component response regulator	0.102	Information pathways
lpp1294, flaA	Flagelline	0.189	Cellular processes
lpp0788	Unknown	0.219	No similarity
lpp2476	Unknown	0.241	No similarity
lpp3023	Unknown	0.252	No similarity
lpp0561	Similar to carboxy-terminal protease family protein	0.259	Intermediary metabolism
lpp2164	Similar to hemin binding protein Hbp	0.290	Similar to unknown proteins
lpp1725, fliE	Flagellar hook-basal body complex protein	0.303	Cellular processes
lpp2433	Similar to transporters	0.307	Cellular processes
lpp1948	Unknown	0.309	No similarity
lpp1856	Similar to esterase/lipase	0.319	Information pathways
lpp0959	Unknown	0.358	No similarity
lpp1445	Unknown	0.359	No similarity
lpp0332	Unknown	0.378	No similarity
lpp1547, rplI	50S ribosomal protein L9	0.390	Information pathways
lpp0684	Similar to type IV pilus assembly protein PilW	0.407	Cellular processes
lpp1740	Unknown	0.409	No similarity
lpp0602, letE	Transmission trait enhancer protein LetE	0.423	Other functions
lpp0543, rpmG	50S ribosomal subunit protein L33	0.429	Information pathways
lpp1452	Unknown	0.435	No similarity
lpp1680	16 kD immunogenic protein	0.444	Similar to unknown proteins
lpp0009	Similar to host factor-1 protein	0.469	Other functions
lpp2943	Unknown	0.470	No similarity
lpp1409	Unknown	0.470	No similarity
lpp2594	Unknown	0.470	No similarity
lpp0385, rplJ	50S ribosomal subunit protein L1unknown	0.482	Information pathways
lpp2486	Some similarity with eukaryotic proteins- contains a F-box domain	0.495	No similarity
lpp0286	Unknown	0.499	No similarity

Table S4: Coordinately down- and upregulated genes in the *fleR* mutant during transmissive phase (fold change ≥ 2.00 ∨ ≤ 0.50, P value ≤ 0.001).

Gene name	Annotation	Fold change	Functional group
lpp2461	Unknown	7.576	No similarity
lpp0962	Unknown	7.282	No similarity
lpp0963	Unknown	6.899	No similarity
lpp0934	Unknown	5.867	No similarity
lpp1638	Unknown	5.608	No similarity
lpp1228, flgF	Flagellar biosynthesis protein FlgF	5.280	Cellular processes
lpp0493, cspD	Similar to Cold shock-like protein CspD	4.892	Other functions
lpp0865	Similar to acyl-CoA dehydrogenase	4.710	Intermediary metabolism
lpp1113	Similar to hypothetical proteins	4.406	Similar to unknown proteins
lpp0799	Unknown	4.309	No similarity
lpp1227, flgE	Flagellar hook protein FlgE	3.998	Cellular processes
lpp0866	Similar to hydrolase	3.919	Other functions
lpp1233, flgK	Flagellar hook-associated protein1	3.915	Cellular processes
lpp0936	Unknown	3.765	No similarity
lpp0012	Unknown	3.721	No similarity
lpp0298	Hypothetical gene	3.673	No similarity
lpp3049	Similar to protease	3.585	Intermediary metabolism
lpp2495	Similar to malonate decarboxylase - γ subunit	3.443	Intermediary metabolism
lpp1631	Unknown	3.286	No similarity
lpp1957	Unknown	3.166	Similar to unknown proteins
lpp0964	Similar to hypothetical protein	3.177	Similar to unknown proteins
lpp2482	Unknown	3.126	No similarity
lpp1229, flgG	Flagellar biosynthesis protein FlgG	3.023	Cellular processes
lpp2322	Similar to acetoacetyl-CoA reductase	2.976	Intermediary metabolism
lpp1230, flgH	Flagellar L-ring protein precursor FlgH	2.965	Cellular processes
lpp1639	Unknown	2.863	No similarity
lpp1743	Similar to hypothetical poteins	2.863	Similar to unknown proteins
lpp0309, gabT	Similar to 4-aminobutyrate aminotransferase	2.841	Intermediary metabolism
lpp2290	Unknown	2.771	No similarity
plpp0047	similar to putative anti restriction protein KlcA	2.736	Unknown
lpp2894	Similar to lysophospholipase A	2.728	Intermediary metabolism
lpp1738, rir1	Similar to ribonucleoside-diphosphate reductase - α subunit	2.706	Intermediary metabolism
lpp0095	Unknown	2.703	No similarity

Table S4 - continued

lpp2660	Similar to peptidase	2.703	Intermediary metabolism
lpp2245b	Similar to transposase (IS4 family)	2.663	Other functions
lpp0905	Similar to unknown protein	2.663	Similar to unknown proteins
lpp1231, flgI	Flagellar P-ring protein precursor FlgI	2.662	Cellular processes
lpp1080	Similar to very-short-patch-repair endonuclease Vsr	2.613	Information pathways
lpp1747, fleN	Similar to flagellar synthesis regulator	2.604	Cellular processes
lpp1630	Similar to hypothetical proteins	2.593	Similar to unknown proteins
lpp1746, fliA	Sigma factor 28	2.587	Information pathways
lpp2279	Similar to biotin synthesis protein	2.568	Intermediary metabolism
lpp1226, flgD	Flagellar basal-body rod modification protein FlgD	2.564	Cellular processes
lpp2669, murD	UDP-N-acetylmuramoylalanine-D-glutamate ligase	2.559	Cellular processes
lpp0621	Similar to acetoacetyl-CoA reductase	2.526	Intermediary metabolism
lpp0904	Similar to permease of ABC transporter	2.519	Cellular processes
plpp0013	Weakly similar to conserved hypothetical proteins	2.479	Unknown
lpp2258, murI	Similar to glutamate racemase	2.459	Intermediary metabolism
lpp1232, flgJ	Flagellar biosynthesis protein FlgJ	2.436	Cellular processes
lpp2198	Unknown	2.437	No similarity
lpp1482	Putative cAMP/cGMP binding protein	2.408	Other functions
lpp0308	Similar to betaine aldehyde dehydrogenase BetB	2.404	Other functions
lpp0009	Similar to host factor-1 protein	2.389	Other functions
lpp0045	Similar to sterol desaturase	2.356	Intermediary metabolism
lpp2401	Unknown	2.317	No similarity
lpp1324	Similar to DNA-binding protein Fis	2.315	Information pathways
lpp0993, pilQ	Type IV pilus assembly protein PilQ	2.311	Cellular processes
lpp0359	Similar to NAD+-dependent formate dehydrogenase	2.300	Cellular processes
lpp1156	Unknown	2.298	No similarity
lpp2666, murB	UDP-N-acetylenolpyruvoylglucosamine reductase	2.293	Cellular processes
lpp2261	Similar to conserved hypothetical protein	2.290	Similar to unknown proteins
lpp0809	Regulatory protein (GGDEF domain)	2.286	Cellular processes
lpp1974	Similar to putative polysaccharide deacetylase-related protein	2.285	Intermediary metabolism
lpp2332	Similar to ATP synthase C chain	2.278	Cellular processes
lpp0004, gyrB	DNA gyrase- subunit B (type II topoisomerase)	2.228	Information pathways
lpp1756, fliM	Flagellar motor switch protein FliM	2.220	Cellular processes
lpp1234, flgL	Flagellar hook-associated protein FlgL	2.219	Cellular processes
lpp1936	Unknown	2.214	No similarity
lpp1796	Similar to ABC transporter - ATP-binding protein	2.207	Cellular processes

Table S4 - continued

lpp2199	Similar to C4-dicarboxylate transport protein	2.189	Cellular processes
lpp0026	Similar to amino acid permease	2.172	Cellular processes
lpp2667, murC	UDP-N-acetylmuramate-L-alanine ligase	2.151	Cellular processes
lpp0620	Similar to acetoacetyl-CoA reductase	2.147	Intermediary metabolism
lpp2246	Unknown	2.145	No similarity
lpp2320	Unknown	2.136	No similarity
lpp1292, fliD	Similar to flagellar hook-associated protein 2 (flagellar capping protein)	2.135	Cellular processes
lpp0730	Similar to adenylate cyclase	2.109	Intermediary metabolism
lpp1219	Similar to thiocyanate hydrolase γ subunit	2.090	Other functions
lpp2736	Similar to hypothetical protein	2.094	Similar to unknown proteins
plpp0043	-	2.086	Unknown
lpp2077	Similar to transcriptional regulator - LysR family	2.085	Information pathways
lpp0992, pilP	Type IV pilus assembly protein PilP	2.071	Cellular processes
lpp2807	Unknown	2.068	No similarity
lpp1176, pilR	Similar to type-IV fimbriae expression regulatory protein PilR (two-component response regulator)	2.063	Information pathways
lpp2929	Similar to predicted permeases	2.056	Cellular processes
lpp1411	Similar to *L. pneumophila* putative phospholipase C	2.053	Intermediary metabolism
lpp2013, argS	Arginine tRNA synthetase	2.043	Information pathways
plpp0049	Similar to abortive infection bacteriophage resistance protein	2.038	Unknown
lpp0125	Similar to transposase (IS4 family)	2.035	Other functions
lpp1256	Similar to intracellular septation protein	2.031	Cellular processes
lpp2857	Similar to conserved hypothetical protein	2.013	Unknown
lpp0521, icmJ/dotN	Unknown	2.003	Cellular processes
lpp0171, lvhB4	*Legionella* vir homologue protein	2.002	Cellular processes
lpp0972	Similar to enhanced entry protein EnhA	0.062	Similar to unknown proteins
lpp1452	Unknown	0.082	No similarity
lpp0788	Unknown	0.089	No similarity
lpp2559	Similar to small heat shock protein	0.120	Other functions
lpp0688	Unknown	0.132	No similarity
lpp1818	Unknown	0.132	No similarity
lpp1726, fleR	Similar to two-component response regulator	0.150	Information pathways
lpp2519	Unknown	0.151	No similarity
lpp1546	Some similarity with Legionella 33 kDa polypeptide	0.156	Similar to unknown proteins

Table S4 - continued

lpp2943	Unknown	0.165	No similarity
lpp2164	Similar to hemin binding protein Hbp	0.175	Similar to unknown proteins
lpp1346, rpmF	50S ribosomal subunit protein L32	0.192	Information pathways
lpp2594	Unknown	0.196	No similarity
lpp2275	Unknown	0.206	No similarity
lpp1438	Unknown	0.206	No similarity
plpp0129	Some similarity with transcriptional regulator, MerR family	0.219	Unknown
lpp0602, letE	Transmission trait enhancer protein LetE	0.240	Other functions
lpp1805	Similar to outer membrane protein	0.249	Cellular processes
lpp1162	Unknown	0.255	No similarity
lpp2269, sdaC	Similar to serine transporter	0.289	Cellular processes
lpp1516	Similar to pyruvate dehydrogenase E1 (β subunit)	0.297	Intermediary metabolism
lpp1445	Unknown	0.298	No similarity
lpp1305	Similar to aldehyde dehydrogenase	0.299	Intermediary metabolism
lpp2438	Hypothetical gene	0.301	No similarity
lpp0532, proA1	Zinc metalloproteinase precursor	0.301	Intermediary metabolism
lpp2898	Similar to SsrA-binding protein	0.302	Other functions
lpp1893	Weakly similar to endoglucanase	0.304	Intermediary metabolism
lpp2703, rpmA	50S ribosomal protein L27	0.307	Information pathways
lpp2088	Unknown	0.310	No similarity
lpp0044	Unknown	0.313	No similarity
lpp2768, rpmI	50S ribosomal protein L35	0.313	Information pathways
lpp1112	Unknown	0.315	No similarity
lpp2947	Unknown	0.316	No similarity
lpp1409	Unknown	0.317	No similarity
lpp1547, rplI	50S ribosomal protein L9	0.318	Information pathways
lpp3077, rpmH	50S ribosomal protein L34	0.319	Information pathways
lpp1706	Unknown	0.319	No similarity
lpp0248	Similar to Zn metalloprotein	0.323	Intermediary metabolism
plpp0044	Unknown	0.324	Unknown
lpp2504	Unknown	0.326	No similarity
lpp0988	Unknown	0.328	No similarity
lpp1138	Unknown	0.332	No similarity
lpp1948	Unknown	0.334	No similarity
lpp1667	Unknown	0.335	No similarity

Table S4 - continued

lpp2866	Similar to aminopeptidase	0.337	Intermediary metabolism
lpp2587	Unknown	0.338	No similarity
lpp0469	Ankyrin repeat protein	0.340	No similarity
lpp1549, rpsR	30S ribosomal subunit protein S18	0.341	Information pathways
lpp0024, hbp	hemin binding protein	0.342	Other functions
lpp0569	Similar to protective surface antigen	0.343	Similar to unknown proteins
lpp1419, secA	Preprotein translocase- secretion protein SecA subunit	0.343	Cellular processes
lpp2232, lly	4-hydroxyphenylpyruvate dioxygenase (legiolysin)	0.345	Intermediary metabolism
lpp0543, rpmG	50S ribosomal subunit protein L33	0.348	Information pathways
lpp0581	Unknown	0.352	No similarity
lpp2230	Similar to leucine dehydrogenase	0.355	Intermediary metabolism
lpp0102	Similar to arginine-binding periplasmic protein	0.355	Cellular processes
lpp1711	Similar to putative tRNA/rRNA methyltransferase	0.356	Information pathways
lpp0410, rplR	50S ribosomal subunit protein L18	0.357	Information pathways
lpp2263	Unknown	0.357	No similarity
lpp1725, fliE	Flagellar hook-basal body complex protein	0.357	Cellular processes
lpp1662	Conserved hypothetical protein	0.358	Similar to unknown proteins
lpp1146	Unknown	0.361	No similarity
lpp1029	Unknown	0.361	No similarity
lpp0755	Similar to L-isoaspartate carboxylmethyltransferase protein Pcm	0.361	Information pathways
lpp0386, rplL	50S ribosomal subunit protein L7/L12	0.363	Information pathways
lpp1740	Unknown	0.372	No similarity
lpp0383, rplK	50S ribosomal protein L11	0.372	Information pathways
lpp1304	Similar to dehydrogenase	0.374	Other functions
lpp2690	Unknown	0.376	No similarity
lpp0805	Similar to surface antigens (17 kDa)	0.376	Similar to unknown proteins
lpp2480	Unknown	0.377	No similarity
lpp0413, rplO	50S ribosomal subunit protein L15	0.381	Information pathways
lpp2697	Similar to putative protein from Stx2 converting bacteriophage I	0.382	Other functions
lpp3061	Unknown	0.383	No similarity
lpp0250	Unknown	0.384	No similarity
lpp3058, atpE	Highly similar to H+-transporting ATP synthase chain c	0.385	Cellular processes
lpp0205	Unknown	0.386	No similarity
lpp1517	Pyruvate dehydrogenase E2 (dihydrolipoamide acetyltransferase)	0.387	Intermediary metabolism
lpp0814	Similar to LPS biosynthesis protein	0.388	Similar to unknown proteins

Table S4 - continued

lpp2026, pal	Peptidoglycan-associated lipoprotein precursor (19 kDa surface antigen)	0.389	Cellular processes
lpp1132	Unknown	0.394	No similarity
plpp0128	-	0.394	Unknown
lpp0385, rplJ	50S ribosomal subunit protein L1	0.395	Information pathways
lpp0412, rpmD	50S ribosomal subunit protein L3	0.397	Information pathways
lpp2583	Unknown	0.399	No similarity
lpp2976	Unknown	0.400	No similarity
lpp2803	Similar to hypothetical protein	0.402	Similar to unknown proteins
lpp3017	Unknown	0.405	No similarity
plpp0125	similar to acetyltransferase, GNAT family	0.406	Unknown
lpp0535	Similar to fructose-bisphosphate aldolase	0.407	Intermediary metabolism
lpp2186	Similar to acyl-carrier protein	0.412	Intermediary metabolism
lpp0483, zwf	Similar to Glucose-6-phosphate 1-dehydrogenase	0.413	Intermediary metabolism
lpp2933	Unknown	0.413	No similarity
lpp1004	Similar to GTPases	0.414	Other functions
lpp1550, rpsF	30S ribosomal protein S6	0.415	Information pathways
lpp0403, rpsQ	30S ribosomal protein S17	0.416	Information pathways
lpp0381, secE	Preprotein translocase SecE subunit	0.416	Cellular processes
lpp2762, rplM	50S ribosomal subunit protein L13	0.420	Information pathways
lpp0619	Unknown	0.420	No similarity
lpp2032	Unknown	0.421	No similarity
lpp3064	Similar to phosphoheptose isomerase	0.422	Intermediary metabolism
lpp2705	Similar to 50S ribosomal subunit protein L25 - RplY	0.422	Information pathways
lpp0384, rplA	50S ribosomal protein L1	0.422	Information pathways
lpp0417, rpsK	30S ribosomal protein S11	0.422	Information pathways
lpp2810	Unknown	0.423	No similarity
lpp0030	Unknown	0.423	No similarity
lpp0411, rpsE	30S ribosomal subunit protein S5	0.424	Information pathways
lpp1535, rpoE	Sigma factor RpoE (sigma 24)	0.426	Information pathways
lpp2745	Similar to cation transport ATPase	0.427	Cellular processes
lpp0008	Unknown	0.428	No similarity
lpp0140	Unknown	0.429	No similarity
lpp0402, rpmC	50S ribosomal subunit protein L29	0.430	Information pathways
lpp0820	Similar to acetyl transferase	0.431	Intermediary metabolism
lpp1938	Similar to chloromuconate cycloisomerase	0.431	Intermediary metabolism

Table S4 - continued

lpp2094	Unknown	0.435	No similarity
lpp0834	Unknown	0.435	Similar to unknown proteins
lpp3072	Unknown	0.435	No similarity
lpp2908	Similar to cold shock protein	0.436	No similarity
lpp1515	Similar to pyruvate dehydrogenase - (E1 α subunit)	0.436	Intermediary metabolism
lpp1898	Similar to ferredoxin	0.436	Cellular processes
lpp2400	Hypothetical gene	0.437	No similarity
lpp3003, trxA	Highly similar to thioredoxin	0.437	Cellular processes
lpp2476	Unknown	0.438	No similarity
plpp0126	Similar to conserved hypothetical protein	0.439	Unknown
lpp2938	Unknown	0.440	No similarity
lpp2133	Similar to response regulator	0.440	Information pathways
lpp1105	Unknown	0.440	No similarity
lpp2689, rpsT	30S ribosomal subunit protein S2	0.441	Information pathways
lpp0365, efp	Similar to elongation factor P	0.442	Information pathways
lpp0409, rplF	50S ribosomal subunit protein L6	0.442	Information pathways
lpp2948, gidA	Highly similar to glucose-inhibited division protein A GidA	0.442	Cellular processes
lpp1349, fabD	Malonyl CoA-acyl carrier protein transacylase	0.443	Intermediary metabolism
plpp0127	Similar to acetyltransferase (C-terminal part)	0.446	Unknown
lpp0709	Similar to tyrosine-specific transport protein	0.446	Cellular processes
lpp0397, rplB	50S ribosomal subunit protein L2	0.446	Information pathways
lpp2656	Unknown	0.447	No similarity
lpp2222	Putative membrane protein	0.449	No similarity
lpp1707	Similar to DNA-binding protein Fis	0.451	Information pathways
lpp0617	Hypothetical protein	0.452	No similarity
lpp1878	Similar to conserved hypothetical protein	0.452	Similar to unknown proteins
lpp0419, rpoA	DNA-directed RNA polymerase alpha chain	0.454	Information pathways
lpp0392, tufA2	Translation elongation factor Tu	0.454	Information pathways
lpp0406, rplE	50S ribosomal protein L5	0.456	Information pathways
lpp2511	Unknown	0.457	No similarity
lpp0959	Unknown	0.457	Similar to unknown proteins
lpp0418, rpsD	30S ribosomal subunit protein S4	0.459	Information pathways
lpp1420, mutT	Mutator protein MutT	0.459	Information pathways
lpp0538	Conserved hypothetical protein	0.463	Similar to unknown proteins
lpp1882	Unknown	0.463	No similarity

Table S4 - continued

lpp0544, rpmB	50S ribosomal protein L28	0.464	Information pathways
lpp2629	Unknown	0.465	No similarity
lpp0873, mreB	Rod shape-determining protein MreB	0.468	Cellular processes
lpp2009	Similar to DAHP synthase	0.468	Intermediary metabolism
lpp2236, asnS	Asparagine tRNA synthetase	0.469	Information pathways
lpp0407, rpsN	30S ribosomal protein S14	0.470	Information pathways
lpp0416, rpsM	30S ribosomal protein S13	0.475	Information pathways
lpp3065	Similar to conserved hypothetical protein	0.475	Similar to unknown proteins
lpp1809	Conserved lipoprotein	0.480	Similar to unknown proteins
lpp0751	Unknown	0.482	No similarity
lpp1164	Similar to other proteins	0.482	No similarity
lpp2726	Similar to zinc protease	0.483	Intermediary metabolism
lpp2159	Similar to oxidoreductase	0.484	Intermediary metabolism
lpp1830, tig	Peptidyl-prolyl cis-trans isomerase (trigger factor)	0.484	Information pathways
lpp1302, leuS	Leucyl-tRNA synthetase	0.483	Information pathways
lpp2591	Unknown	0.484	No similarity
lpp1821, fabI	Similar to Enoyl-[acyl-carrier-protein] reductase	0.488	Intermediary metabolism
lpp1620, iolE	Similar to myo-inositol catabolism protein iolE	0.492	Intermediary metabolism
lpp0408, rpsH	30S ribosomal protein S8	0.492	Information pathways
lpp0984, etfB	Electron transfer flavoprotein β subunit	0.494	Cellular processes
lpp1342	Unknown	0.492	No similarity
lpp0986, ald	Similar to alanine dehydrogenase	0.495	Intermediary metabolism
lpp1955	Unknown	0.494	No similarity
lpp1980	similar to pterin-4-alpha-carbinolamine dehydratase PhhB	0.495	Intermediary metabolism
lpp1223, hemF	Oxygen-dependent coproporphyrinogen III oxidase	0.497	Intermediary metabolism
lpp2882	Unknown	0.497	No similarity
lpp0684	Similar to type IV pilus assembly protein PilW	0.497	Cellular processes
lpp0472	Similar to conserved hypothetical proteins	0.498	Similar to unknown proteins
lpp1399, trpS	Tryptophanyl-tRNA synthetase TrpS	0.500	Information pathways
lpp0960, mutY	Similar to A/G-specific adenine glycosylase	0.500	Information pathways
lpp1363	Similar to putative choline kinase	0.500	Intermediary metabolism

Table S5: Coordinately down- and upregulated genes in the *rpoN* mutant during replicative phase (fold change ≥ 2.00 ∨ ≤ 0.50, P value ≤ 0.001).

Gene name	Annotation	Fold change	Functional group
lpp0962	Unknown	2.235	Unknown
lpp0963	Unknown	2.158	Unknown
lpp2849	Similar to putative coproporphyrinogen oxidase A	2.114	Similar to unknown proteins
lpp1756, fliM	Flagellar motor switch protein FliM	0.058	Cellular processes
lpp1755, fliN	Flagellar motor switch protein FliN	0.060	Cellular processes
lpp1748, flhF	Flagellar biosynthesis protein FlhF	0.102	Cellular processes
lpp0542, rpoN	RNA polymerase sigma-54 factor (sigma-L)	0.104	Information pathways
lpp1747, fleN	Similar to flagellar synthesis regulator	0.118	Cellular processes
lpp1754, fliO	Flagellar protein FliO	0.174	Cellular processes
lpp1224, flgB	Flagellar basal-body rod protein FlgB	0.212	Cellular processes
lpp0541	Similar to putative sigma-54 modulation protein	0.233	Information pathways
lpp2435	Similar to conserved hypothetical protein	0.243	Similar to unknown proteins
lpp1229, flgG	Flagellar biosynthesis protein FlgG	0.262	Cellular processes
lpp2434	Unknown	0.262	Unknown
lpp2068	Unknown	0.281	Unknown
lpp1751, fliR	Flagellar biosynthetic protein FliR	0.295	Cellular processes
lpp1749, flhA	Flagellar biosynthesis protein FlhA	0.299	Cellular processes
lpp1753, fliP	Flagellar biosynthetic protein FliP	0.306	Cellular processes
lpp1231, flgI	Flagellar P-ring protein precursor FlgI	0.310	Cellular processes
lpp3023	Unknown	0.310	Unknown
lpp2433	Similar to transporters	0.332	Cellular processes
lpp2432	Similar to hypothetical protein	0.342	Similar to unknown proteins
lpp0953	Similar to kynurenine 3-monooxygenase	0.363	Intermediary metabolism
lpp0561	Similar to carboxy-terminal protease family protein	0.366	Intermediary metabolism
lpp1230, flgH	Flagellar L-ring protein precursor FlgH	0.376	Cellular processes
lpp1225, flgC	Flagellar basal-body rod protein FlgC	0.384	Cellular processes
lpp2430	Unknown	0.384	Unknown
lpp1752, fliQ	Flagellar biosynthetic protein FliQ	0.392	Cellular processes
lpp1232, flgJ	Flagellar biosynthesis protein FlgJ	0.403	Cellular processes
lpp1226, flgD	Flagellar basal-body rod modification protein FlgD	0.403	Cellular processes
lpp1512	Similar to arginine 3rd transport system periplasmic binding protein	0.435	Cellular processes

Table S5 - continued

lpp0706	Similar to major facilitator family transporter	0.480	Cellular processes

Table S6: Coordinately down- and upregulated genes in the *rpoN* mutant during transmissive phase (fold change ≥ 2.00 ∨ ≤ 0.50, P value ≤ 0.001).

Gene name	Annotation	Fold change	Functional group
lpp0866	Similar to hydrolase	7.333	Other functions
lpp0936	Unknown	5.509	No similarity
lpp3049	Similar to protease	5.381	Intermediary metabolism
lpp1187	Unknown	5.333	No similarity
lpp2461	Unknown	4.870	No similarity
lpp1100	Ankyrin repeat protein	4.733	No similarity
lpp0963	Unknown	4.697	No similarity
lpp1642	Unknown	4.326	No similarity
lpp2322	Similar to acetoacetyl-CoA reductase	4.320	Intermediary metabolism
lpp0962	Unknown	4.121	No similarity
lpp1113	Similar to hypothetical proteins	3.956	Similar to unknown proteins
lpp0799	Unknown	3.867	No similarity
lpp1638	Unknown	3.847	No similarity
lpp0450	Unknown	3.841	No similarity
lpp1639	Unknown	3.738	No similarity
lpp0304, sidE	SidE protein- substrate of the Dot/Icm system	3.521	Similar to unknown proteins
lpp2320	Unknown	3.455	No similarity
lpp1799	Some similarity with eukaryotic protein	3.415	No similarity
lpp1223, hemF	Oxygen-dependent coproporphyrinogen III oxidase	3.372	Intermediary metabolism
lpp0034	Unknown	3.199	No similarity
lpp2592	Unknown	3.146	No similarity
lpp1324	Similar to DNA-binding protein Fis	3.144	Information pathways
lpp0934	Unknown	3.075	No similarity
lpp1738, rir1	Similar to ribonucleoside-diphosphate reductase - α subunit	3.052	Intermediary metabolism
lpp1965	Similar to hydantoin-racemase	3.052	Intermediary metabolism
lpp1643	Unknown	3.036	No similarity
lpp1177	Unknown	2.911	No similarity
lpp0987, mrcA	Similar to peptidoglycan synthetase; penicillin-binding protein 1A	2.905	Cellular processes
lpp2149	Unknown	2.891	No similarity
lpp2199	Similar to C4-dicarboxylate transport protein	2.866	Cellular processes
lpp2577, sdeD	SdeD protein (substrate of the Dot/Icm system)	2.850	Similar to unknown proteins
lpp1121	Unknown	2.838	No similarity
lpp0939, pntA	Pyridine nucleotide transhydrogenase - α subunit	2.828	Intermediary metabolism

Table S6 - continued

lpp2635, flhB'	Similar to FlhB protein- putative part of export apparatus for flagellar proteins	2.827	Cellular processes
lpp0314	Similar to oxydoreductase	2.822	Other functions
lpp0012	Unknown	2.786	No similarity
lpp1411	Similar to Legionella pneumophila putative phospholipase C	2.784	Intermediary metabolism
lpp0493, cspD	Similar to Cold shock-like protein CspD	2.780	Other functions
lpp2578, sdcA	SdcA protein- paralog of SidC (substrate of the Dot/Icm system)	2.767	Similar to unknown proteins
lpp1963	Some similarity with eukaryotic proteins	2.765	Similar to unknown proteins
lpp2158	Similar to unknown protein	2.736	Similar to unknown proteins
lpp0937, pntB	NAD(P) transhydrogenase subunit β (Pyridine nucleotide transhydrogenase subunit β)	2.714	Cellular processes
lpp0449	Similar to putative hyperosmotically inducible periplasmic proteins	2.702	Similar to unknown proteins
lpp0621	Similar to acetoacetyl-CoA reductase	2.698	Intermediary metabolism
lpp0964	Similar to hypothetical protein	2.667	Similar to unknown proteins
lpp2321	Similar to cold shock protein	2.665	Other functions
lpp1441	Similar to unknown protein	2.653	Similar to unknown proteins
lpp2525	Similar to guanylate cyclase-related protein	2.648	Intermediary metabolism
lpp2258, murI	Similar to glutamate racemase	2.638	Intermediary metabolism
lpp1031	Unknown	2.638	No similarity
lpp1957	Unknown	2.615	Similar to unknown proteins
lpp2246	Unknown	2.567	No similarity
lpp2809	Unknown	2.554	No similarity
lpp1292, fliD	Similar to flagellar hook-associated protein 2 (flagellar capping protein)	2.548	Cellular processes
lpp2266, motA2	Similar to proton conductor component of motor - chemotaxis and motility protein	2.545	Cellular processes
lpp0009	Similar to host factor-1 protein	2.539	Other functions
lpp2298	Similar to alkyl hydroperoxide reductase AhpD	2.539	Other functions
lpp0359	Similar to NAD+-dependent formate dehydrogenase	2.501	Cellular processes
lpp1136	Similar to transcriptional regulator - (TetR family?)	2.495	Information pathways
lpp0761	Similar to conserved hypothetical protein- predicted membrane protein	2.469	Similar to unknown proteins
lpp0036	Similar to arginine transport system periplasmic binding protein	2.454	No similarity
lpp0004, gyrB	DNA gyrase- subunit B (type II topoisomerase)	2.443	Information pathways
lpp1823	Unknown	2.434	No similarity
lpp1990, spoT	Guanosine-3 -5 -bis(diphosphate) 3 -pyrophosphohydrolase	2.432	Intermediary metabolism

Table S6 - continued

lpp1133, cfa	Cyclopropane fatty acyl phospholipid synthase (Cyclopropane fatty acid synthase)	2.424	Intermediary metabolism
lpp2694, enhA	Enhanced entry protein EnhA	2.388	Other functions
lpp2077	Similar to transcriptional regulator- LysR family	2.387	Information pathways
lpp2290	Unknown	2.364	No similarity
lpp2086	Unknown	2.356	No similarity
lpp2490	Unknown	2.351	No similarity
plpp0068	Weakly similar to *L. longbeachae* spectinomycin 3' adenylyltransferase	2.330	Unknown
lpp2035	Similar to 3-hydroxy-3-methylglutaryl-coenzyme A reductase	2.330	Intermediary metabolism
lpp2692, enhC	Enhanced entry protein EnhC	2.322	Other functions
plpp0047	Similar to putative anti restriction protein KlcA	2.313	Unknown
lpp1796	Similar to ABC transporter- ATP-binding protein	2.312	Cellular processes
plpp0066	Similar to ATPase components of ABC transporters	2.299	Unknown
lpp1925	Similar to conserved hypothetical protein	2.288	Similar to unknown proteins
lpp2202	Unknown	2.283	No similarity
lpp0215	Unknown	2.276	No similarity
lpp2637, sidF	Substrate of the Dot/Icm system	2.266	Similar to unknown proteins
lpp2572	Unknown	2.239	No similarity
lpp2198	Unknown	2.217	No similarity
lpp0859	Unknown	2.216	No similarity
lpp0900	Similar to conserved hypothetical protein	2.211	Cellular processes
lpp0946	Similar to glycosyl hydrolase	2.207	Cellular processes
lpp2877, recN	DNA repair protein RecN	2.198	Information pathways
lpp0026	Similar to amino acid permease	2.186	Cellular processes
lpp2039	Unknown	2.183	No similarity
lpp0045	Similar to sterol desaturase	2.170	Intermediary metabolism
lpp2788	Putative response regulator	2.165	Information pathways
lpp1030	Unknown	2.146	No similarity
lpp0915, fleQ	Transcriptional regulator FleQ	2.138	Information pathways
lpp1009	Similar to 2-oxoglutarate ferredoxin oxidoreductase α subunit	2.126	Intermediary metabolism
lpp1002, lidA	LidA protein- substrate of the Dot/Icm system	2.121	Similar to unknown proteins
lpp1186	Unknown	2.110	No similarity
lpp2096, sdeA	SdeA unknown	2.103	Similar to unknown proteins
lpp0965	Similar to protease	2.098	Intermediary metabolism
lpp1630	Similar to hypothetical proteins	2.096	Similar to unknown proteins

Table S6 - continued

lpp1688, guaB	Similar to IMP dehydrogenase/GMP reductase	2.081	Intermediary metabolism
lpp0443, sdhA1	SdhA- substrate of the Dot/Icm system	2.078	Similar to unknown proteins
lpp1641	Unknown	2.054	No similarity
lpp1255	Similar to two component response regulator	2.044	Information pathways
lpp0557, argH	Argininosuccinate lyase	0.019	Intermediary metabolism
lpp0556, argG	Argininosuccinate synthase	0.021	Intermediary metabolism
lpp0553	Similar to putative glutamine-binding periplasmic protein precursor	0.025	Intermediary metabolism
lpp0558, argF	Ornithine carbamoyltransferase	0.027	Intermediary metabolism
lpp0555	Similar to amino acid (glutamine) ABC transporter (ATP-binding protein)	0.038	Cellular processes
lpp0542, rpoN	RNA polymerase sigma-54 factor (sigma-L)	0.061	Information pathways
lpp0541	Similar to putative sigma-54 modulation protein	0.064	Information pathways
lpp0554	Similar to amino acid ABC transporter permease	0.069	Cellular processes
lpp0788	Unknown	0.086	No similarity
lpp1452	Unknown	0.144	No similarity
lpp0688	Unknown	0.147	No similarity
lpp2594	Unknown	0.148	No similarity
lpp0988	Unknown	0.175	No similarity
lpp2519	Unknown	0.180	No similarity
lpp0535	Similar to fructose-bisphosphate aldolase	0.208	Intermediary metabolism
lpp1346, rpmF	50S ribosomal subunit protein L32	0.225	Information pathways
lpp2164	Similar to hemin binding protein Hbp	0.226	Similar to unknown proteins
lpp1517	Pyruvate dehydrogenase E2 (dihydrolipoamide acetyltransferase)	0.227	Intermediary metabolism
lpp2516	Similar to N-hydroxyarylamine O-acetyltransferase	0.227	Intermediary metabolism
lpp1438	Unknown	0.228	No similarity
lpp2275	Unknown	0.232	No similarity
lpp0953	Similar to kynurenine 3-monooxygenase	0.232	Intermediary metabolism
lpp1747, fleN	Similar to flagellar synthesis regulator	0.233	Cellular processes
lpp0954	Similar to unknown proteins	0.234	Similar to unknown proteins
lpp2068	Unknown	0.235	No similarity
plpp0128	-	0.242	Unknown
plpp0129	Some similarity with transcriptional regulator, MerR family	0.242	Unknown
lpp0602, letE	Transmission trait enhancer protein LetE	0.244	Other functions
lpp0544, rpmB	50S ribosomal protein L28	0.254	Information pathways

Table S6 - continued

lpp2839, lepA	Effector protein A- substrate of the Dot/Icm secretion system	0.257	Similar to unknown proteins
lpp1805	Similar to outer membrane protein	0.261	Cellular processes
lpp1515	Similar to pyruvate dehydrogenase- (E1 α subunit)	0.263	Intermediary metabolism
lpp1546	Some similarity with Legionella 33 kDa polypeptide	0.264	Similar to unknown proteins
lpp1786	Similar to conserved hypothetical protein	0.264	Similar to unknown proteins
lpp1516	Similar to pyruvate dehydrogenase E1 (β subunit)	0.265	Intermediary metabolism
lpp1756, fliM	Flagellar motor switch protein FliM	0.278	Cellular processes
lpp2943	Unknown	0.278	No similarity
lpp1706	Unknown	0.293	No similarity
lpp2897	Similar to unknown protein	0.295	Similar to unknown proteins
lpp1363	Similar to putative choline kinase	0.300	Intermediary metabolism
lpp0833	Similar to sialic acid synthase	0.303	Cellular processes
lpp3058, atpE	Highly similar to H+-transporting ATP synthase chain c	0.307	Cellular processes
lpp0540	Similar to sugar transport PTS phosphocarrier protein Hpr	0.307	Cellular processes
lpp1748, flhF	Flagellar biosynthesis protein FlhF	0.308	Cellular processes
lpp0814	Similar to LPS biosynthesis protein	0.309	Similar to unknown proteins
lpp1663	Similar to activator of osmoprotectant transporter ProP (N-terminal part)	0.312	Similar to unknown proteins
lpp0552	Similar to transcriptional regulator of arginine metabolism	0.315	Information pathways
lpp0055	Similar to hypothetical protein	0.320	Similar to unknown proteins
lpp0834	Unknown	0.320	Similar to unknown proteins
lpp2689, rpsT	30S ribosomal subunit protein S2	0.322	Information pathways
lpp1305	Similar to aldehyde dehydrogenase	0.324	Intermediary metabolism
lpp2263	Unknown	0.327	No similarity
lpp3016, lpxA2	Similar to acyl-[acyl carrier protein]-UDP-N-acetylglucosamine O-acyltransferase	0.327	Cellular processes
lpp1681	Unknown	0.328	No similarity
lpp0798	Weakly similar to outer membrane protein	0.332	Cellular processes
lpp2313	Unknown	0.335	No similarity
lpp0532, proA1	Zinc metalloproteinase precursor	0.343	Intermediary metabolism
lpp3017	Unknown	0.346	No similarity
lpp0024, hbp	Hemin binding protein	0.347	Other functions
plpp0122	Similar to unknown protein	0.347	Unknown
lpp1199, hisB	Histidinol-phosphatase/imisazoleglycerol-phosphate dehydratase	0.350	Intermediary metabolism
lpp1705	Unknown	0.350	No similarity
lpp1711	Similar to putative tRNA/rRNA methyltransferase	0.352	Information pathways

Table S6 - continued

lpp0407, rpsN	30S ribosomal protein S14	0.354	Information pathways
lpp1224, flgB	Flagellar basal-body rod protein FlgB	0.357	Cellular processes
lpp2104	Unknown	0.357	No similarity
lpp0986, ald	Similar to alanine dehydrogenase	0.357	Intermediary metabolism
lpp0056	Unknown	0.360	No similarity
lpp0984, etfB	Electron transfer flavoprotein β subunit	0.361	Cellular processes
lpp1680	16 kD immunogenic protein	0.361	Similar to unknown proteins
lpp2400	Hypothetical gene	0.362	No similarity
lpp1182, ribA	Riboflavin biosynthesis protein RibA	0.362	Intermediary metabolism
lpp2768, rpmI	50S ribosomal protein L35	0.364	Information pathways
lpp1662	Conserved hypothetical protein	0.364	Similar to unknown proteins
lpp1348, fabH	3-oxoacyl-[acyl-carrier-protein] synthase III	0.364	Intermediary metabolism
lpp0503	Ankyrin repeat protein	0.369	Similar to unknown proteins
lpp0873, mreB	Rod shape-determining protein MreB	0.370	Cellular processes
lpp1304	Similar to dehydrogenase	0.372	Other functions
lpp0205	Unknown	0.374	No similarity
lpp0250	Unknown	0.375	No similarity
lpp1550, rpsF	30S ribosomal protein S6	0.379	Information pathways
lpp1454	Similar to aminopeptidase N	0.386	Intermediary metabolism
lpp0561	Similar to carboxy-terminal protease family protein	0.387	Intermediary metabolism
lpp2587	Unknown	0.388	No similarity
lpp2094	Unknown	0.392	No similarity
lpp0144	Weakly similar to conserved hypothetical protein	0.393	Similar to unknown proteins
lpp1830, tig	Peptidyl-prolyl cis-trans isomerase (trigger factor)	0.393	Information pathways
lpp3057, atpF	Highly similar to H+-transporting ATP synthase chain b	0.395	Cellular processes
lpp0606	Similar to DNA-binding proteins Fis	0.396	Information pathways
lpp2838, tpi	Triosephosphate isomerase	0.397	Intermediary metabolism
lpp0384, rplA	50S ribosomal protein L1	0.398	Information pathways
lpp2882	Unknown	0.400	No similarity
plpp0121	Weakly similar to acetyltransferase	0.401	Unknown
plpp0089	Weakly similar to stability protein StbE	0.404	Unknown
lpp2438	Hypothetical gene	0.407	No similarity
lpp0619	Unknown	0.408	No similarity
lpp0549, hflC	Membrane protease subunit HflC	0.412	Intermediary metabolism
lpp1818	Unknown	0.414	No similarity

Table S6 - continued

lpp0743, htpB	6unknown kDa chaperonin (Protein Cpn6unknown)(groEL protein)(Heat shock protein B).	0.415	Information pathways
lpp0543, rpmG	50S ribosomal subunit protein L33	0.417	Information pathways
lpp2690	Unknown	0.417	No similarity
plpp0091	Highly similar to spectinomycin 3' adenylyltransferase	0.418	Unknown
lpp2968	Unknown	0.418	No similarity
lpp2931	Similar to probable (di)nucleoside polyphosphate hydrolase NudH	0.419	Intermediary metabolism
lpp2008, grpE	Heat-shock protein GrpE (HSP-7unknown cofactor)	0.420	Other functions
lpp2186	Similar to acyl-carrier protein	0.420	Intermediary metabolism
lpp1169	Similar to conserved hypothetical protein	0.421	Similar to unknown proteins
lpp1420, mutT	Mutator protein MutT	0.421	Information pathways
lpp0423	Similar to 3-oxoacyl-[acyl-carrier protein] reductase	0.421	Intermediary metabolism
lpp2704, rplU	50S ribosomal protein L21	0.421	Information pathways
lpp0914	Unknown	0.421	No similarity
lpp2866	Similar to aminopeptidase	0.427	Intermediary metabolism
lpp2984	Weakly similar to conserved hypothetical protein	0.429	No similarity
lpp1994, rph	Ribonuclease PH (RNase PH) (tRNA nucleotidyltransferase)	0.433	Information pathways
lpp2009	Similar to DAHP synthase	0.435	Intermediary metabolism
lpp0399, rplV	50S ribosomal subunit protein L22	0.436	Information pathways
lpp1146	Unknown	0.436	No similarity
lpp1320	Similar to conserved hypothetical protein	0.437	Similar to unknown proteins
plpp0124	bifunctional protein, similar to acetyl transferase and to methyl transferase	0.441	Unknown
lpp2486	Some similarity with eukaryotic proteins- contains a F-box domain	0.441	No similarity
lpp2632, gcdH	Similar to glutaryl-CoA dehydrogenase	0.442	Cellular processes
lpp0755	Similar to L-isoaspartate carboxylmethyltransferase protein Pcm	0.443	Information pathways
lpp2521	Unknown	0.445	No similarity
lpp1755, fliN	Flagellar motor switch protein FliN	0.446	Cellular processes
lpp0487, eda	Similar to 2-deydro-3-deoxyphosphogluconate aldolase/4-hydroxy-2-oxoglutarate aldolase	0.446	Intermediary metabolism
lpp1740	Unknown	0.450	No similarity
lpp0385, rplJ	50S ribosomal subunit protein L1unknown	0.450	Information pathways
lpp2230	Similar to leucine dehydrogenase	0.450	Intermediary metabolism
lpp3053, atpD	Highly similar to H+-transporting ATP synthase β chain	0.455	Cellular processes
lpp1499	Conserved hypothetical protein	0.455	Similar to unknown proteins

Table S6 - continued

lpp1198, hisH2	Imidazole glycerol phosphate synthase subunit HisH (IGP synthase glutamine amidotransferase subunit)	0.456	Intermediary metabolism
lpp1181, ribE	Riboflavin synthase alpha chain	0.458	Intermediary metabolism
lpp0569	Similar to protective surface antigen	0.458	Similar to unknown proteins
lpp1386	Unknown	0.460	No similarity
lpp0408, rpsH	30S ribosomal protein S8	0.462	Information pathways
lpp1248, hmgA	Homogentisate 1-2-dioxygenase	0.464	Intermediary metabolism
lpp1809	Conserved lipoprotein	0.466	Similar to unknown proteins
lpp0830	Unknown	0.467	No similarity
lpp0277, purE	Phosphoribosylaminoimidazole carboxylase catalytic subunit	0.472	Intermediary metabolism
lpp0932	Similar to enoyl-CoA hydratase/carnithine racemase	0.474	Intermediary metabolism
lpp0134	Some similarity with *L. pneumophila* IcmL/DotI	0.475	Similar to unknown proteins
lpp0484, pgl	Similar to 6-phosphogluconolactonase	0.475	Intermediary metabolism
lpp0539, pssA	Similar to CDP-diacylglycerol-serine O-phosphatidyltransferase (Phosphatidylserine synthase)	0.476	Intermediary metabolism
lpp1795	Similar to unknown proteins	0.476	Similar to unknown proteins
lpp0528, accC	Biotin carboxylase (A subunit of acetyl-CoA carboxylase)	0.477	Intermediary metabolism
lpp1664, ubiG	3-demethylubiquinone-9 3-methyltransferase	0.478	Other functions
plpp0125	similar to acetyltransferase, GNAT family	0.479	Unknown
lpp2912	Hypothetical tetratricopeptide repeat protein	0.479	No similarity
lpp1704	Similar to Adenylate cyclase 1(ATP pyrophosphate-lyase 1; Adenylylcyclase 1)	0.482	Intermediary metabolism
lpp0950	Similar to acetyltransferase	0.484	Other functions
lpp0976	Similar to cell division protein FtsL	0.485	Cellular processes
lpp0480	Similar to hypothetical proteins	0.487	Similar to unknown proteins
lpp2208	Similar to conserved hypothetical protein	0.489	Similar to unknown proteins
lpp3065	Similar to conserved hypothetical protein	0.490	Similar to unknown proteins
lpp1419, secA	Preprotein translocase- secretion protein SecA subunit	0.490	Cellular processes
lpp0417, rpsK	30S ribosomal protein S11	0.492	Information pathways
lpp2437	Similar to hypothetical protein	0.493	Similar to unknown proteins
lpp0420, rplQ	50S ribosomal protein L17	0.494	Information pathways
plpp0088	Similar to transcriptional regulator	0.495	Unknown
lpp1882	Unknown	0.495	No similarity
lpp1684	Similar to methionine aminopeptidase - type I	0.496	Intermediary metabolism

Table S7: Gene expression data for *L. pneumophila* Paris and the *fliA* mutant at 8h *versus* 14h of growth *in vivo* in *A. castellani*. Data from: (49).

	wild type 8h-14h post-infection		***fliA* mutant 8h-14h post-infection**		
Gene name	Fold change	P value	Fold change	P value	Fold change *fliA* mutant *versus* wild type
flaA	29.930	0.0000	1.808	0.0000	-28.122
fliD	6.005	0.0000	2.142	0.0000	-3.863
fliS	5.150	0.0000	1.845	0.0001	-3.305
motB	4.366	0.0000	1.132	0.2972	-3.234
flaG	2.861	0.0000	0.424	0.0000	-2.436
motA	4.069	0.0000	1.695	0.0002	-2.373
flgE	0.676	0.0064	35.069	0.0000	34.393
flgF	0.906	0.0000	34.355	0.0000	33.448
flhF	0.681	0.0060	26.776	0.0000	26.095
fleN	0.755	0.0301	26.070	0.0000	25.315
flgI	0.819	0.1246	17.207	0.0000	16.388
fliN	0.291	0.0000	16.092	0.0000	15.801
fliM	0.419	0.0001	15.875	0.0000	15.456
flgG	0.343	0.0000	15.717	0.0000	15.374
fliO	0.397	0.0000	14.615	0.0000	14.218
flgC	0.282	0.0000	11.710	0.0000	11.429
flgJ	1.082	0.5403	11.811	0.0000	10.730
flgB	0.206	0.0000	10.433	0.0000	10.227
flgK	1.170	0.2209	10.749	0.0000	9.579
flgL	1.674	0.0015	10.786	0.0000	9.111
letE	1.535	0.0044	10.187	0.0000	8.652
flhA	0.921	0.5209	9.421	0.0000	8.500
fliK'	1.195	0.1694	9.300	0.0000	8.105
flgA	0.545	0.0010	8.481	0.0000	7.937
fliR	1.096	0.4728	9.011	0.0000	7.915
fliE	0.246	0.0000	7.042	0.0000	6.796
fliQ	0.774	0.0722	7.505	0.0000	6.730
flgD	0.610	0.0011	7.065	0.0000	6.455
flgH	0.347	0.0000	6.227	0.0000	5.881
fliP	0.927	0.5560	6.576	0.0000	5.649

Table S7 - continued

fleR	0.342	0.0000	4.545	0.0000	4.203
flgM	2.727	0.0000	6.696	0.0000	3.969
fleS	0.276	0.0000	4.181	0.0000	3.905
rpoS	0.366	0.0000	4.126	0.0000	3.760
fliH	0.559	0.0003	3.992	0.0000	3.433
fliG	0.229	0.0000	3.188	0.0000	2.959
flgN	2.769	0.0000	5.094	0.0000	2.325

Abbreviations

α	alpha
A	Ampere
A	adenosine
Ala	alanine
ABC	amino acid binding protein
APS	ammonium persulfate
Arg	arginine
Asn	asparagine
ATP	adenosine-5'-triphosphate
β	beta
b	base
BCYE	ACES-buffered Charcoal Yeast Extract
BLAST	Basic Local Alignment Search Tool
bp	base pairs
BSA	bovine serum albumin
BYE	ACES-buffered Yeast Extract broth
C	cytidine
cAMP	cyclic adenosine monophosphate
cDNA	copy DNA
CFU	colony forming unit
cGMP	cyclic guanosine monophosphate
Clk1	Cdc2-like kinase 1
CR	complement receptor
Cys	cysteine
Da	Dalton
DAHP	3-deoxy-D-arabino-heptulosonat-7-phosphate
dATP	desoxyadenosin-5'-triphosphate
dH_2O	destilled H_2O
Δ	Delta, difference operator
DMSO	dimethylsulfoxid
DNA	desoxyribonucleic acid
DNase	desoxyribonuclease
dNTP	2'-desoxyribonukleosid-5'-triphosphate
Dot/Icm	defective organelle trafficking/intracellular trafficking
Dtr	DNA transfer and replication functions
DTT	dithiothreitol
ε	epsilon
e.g.	exempli gratia
EDTA	ethylendiamintetraacetat
ER	endoplasmic reticulum
et al.	et altera
EtOH	ethanol

FC	fold change
FCS	fetal calf serum
γ	gamma
g	gram
G	guanosine
Gln	glutamine
Glu	glutamate
Gly	glycine
GMP	guanosine monophosphate
h	hour
His	histidine
Ile	isoleucine
IMP	inosine monophosphate
IncI	Incompatibility group I
IncP	Incompatibility group P
IS	insertion sequence element
Ipaf	ICE protease-activating factor
IPTG	Isopropyl-β-D-thiogalactopyranosid
k	kilo
kb	kilo bases
Km	kanamycin
l	liter
LB	lysogeny broth
Leu	leucine
LPS	lipopolysaccharide
Lsp	*Legionella* secretion pathway
Lss	*Legionella* secretion system
Lvh	*Legionella vir* homologues
Lys	lysine
µ	micro
m	milli
M	molar
min	minute
mip	macrophage infectivity potentiator
MOMP	major outer membrane protein
Mpf	mating pair functions
mRNA	messenger RNA
n	nano
NAD	nicotinamide adenine dinucleotide
NAIP	neuronal apoptosis inhibitory protein
OD_{600}	optical density at 600 nm
oriT	origin of transfer
PBS	phosphate buffered saline
PCR	polymerase chain reaction
ppGpp	guanosine tetraphosphate

PPi	$P_2O_7^{4-}$
PPIase	peptidyl prolyl cis/trans isomerase
Pro	proline
RNA	ribonucleic acid
RNAP	RNA polymerase
RNase	ribonuclease
RP	replicative phase
rpm	rounds per minute
rRNA	ribosomal RNA
RT	reverse transcription
σ	sigma
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
Sec	general secretory pathway
Ser	serine
Sg	serogroup
SSC	sodium salt citrate
ssDNA	single stranded DNA
ssp.	subspecies
T	thymidine
TAE	Tris-acetate-EDTA
Tat	twin arginine translocation pathway
TBS	Tris-buffered saline
TCA	trichloroacetic acid
TE	Tris-EDTA
TEMED	N,N,N`,N`-tetramethyldiamin
Thr	threonine
tmRNA	transfer messenger RNA
Tn	transposon
Tris	Trishydroxylmethylaminomethan
TLR	Toll-like receptor
tRNA	transfer RNA
Trp	tryptophane
Tyr	tyrosine
UPEC	uropathogenic *Escherichia coli*
V	Volt
Val	valine
wt	wild type

Publikationen

1. Sahr*, T., Brüggemann*, H., Jules*, M., **Albert-Weissenberger, C.**, Cazalet, C., and Buchrieser, C. Two small ncRNAs jointly govern virulence and transmission in *Legionella pneumophila*. (in Vorbereitung).

2. Newton, H., Sansom, F., Dao, J., Cazalet, C., Brüggemann, H., **Albert-Weissenberger, C**., Buchrieser, C., Cianciotto, N., and Hartland, E. (2008). A significant role for *ladC* in the initiation of *Legionella pneumophila* infection. Infect Immun. 76: 3075-3085.

3. Heuner, K., and **Albert-Weissenberger, C.** (2008). The Flagellar Regulon of *Legionella pneumophila* and the Expression of Virulence Traits. *Legionella*: Molecular Microbiology. Heuner, K., and Swanson, M., Editors. Portland: USA, Caister Academic Press. Chapter 6: 101-121.

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8. **Albert, C.**, Jacobi, S., De Buck, E., Lammertyn, E., and Heuner, K. (2006). Identification of target proteins of the Lss secretion system of *Legionella pneumophila* Corby. *Legionella*: State of the art 30 years after its recognition. Cianciotto, N., Abu Kwaik, Y., Edelstein, P., Fields, B., Geary, D., Harrison, T., Joseph, C., Ratcliff, R., Stout, J., and Swanson, M., Editors. Washington, D.C.: USA, ASM Press. Chapter 55: 221-223.

*** trugen gleichmäßig zu dieser Arbeit bei**

Tagungsbeiträge

2008 Genomes: Functional Genomics of Microorganisms. April 8-11, Paris, Frankreich
Poster

2007 Infectivity and Host Cells. 19.-21. November, Wittenberg, Deutschland
Vortrag

32nd FEBS Congress: Molecular Machines and their Dynamics in Fundamental Cellular Functions. 7.-12. Juli, Wien, Österreich
Poster

Young investigator meeting, International Symposium on New Aspects of Infectious Diseases: From Genomics to Vaccine Development. 1.-2. April, Würzburg, Deutschland
Poster

2006 4th STAPA Young Researcher´s Congress of the Institut Pasteur. 6. Juni, Paris, Frankreich
Poster

2005 Infectivity and Host Cells. 24.-26. Oktober, Wittenberg, Deutschland
Vortrag

6th International Conference on *Legionella*. 16.-20. Oktober, Chicago, USA
Poster

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Würzburg, November 2008