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Two small ncRNAs jointly govern virulence and transmission in *Legionella pneumophila*

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Summary

To transit from intra- to extracellular environments, *L. pneumophila* differentiates from a replicative/non-virulent to a transmissive/virulent form using the two-component system LetA/LetS and the global repressor protein CsrA. While investigating how both regulators act coordinately we characterized two ncRNAs, RsmY and RsmZ that link the LetA/LetS and CsrA regulatory networks. We demonstrate that LetA directly regulates their expression and show that RsmY and RsmZ are functional in *E. coli* and are able to bind CsrA *in vitro*. Single mutants have no ($\Delta rsmY$) or a little ($\Delta rsmZ$) impact on virulence, but the $\Delta rsmYZ$ strain shows a drastic defect in intracellular growth in *Acanthamoeba castellanii* and THP-1 monocyte-derived macrophages. Analysis of the transcriptional programs of the $\Delta letA$, $\Delta letS$ and $\Delta rsmYZ$ strains revealed that the switch to the transmissive phase is partially blocked. One major difference between the $\Delta letA$, $\Delta letS$ and $\Delta rsmYZ$ strains was that the latter synthesizes flagella. Taken together, LetA activates transcription of RsmY and RsmZ, which sequester CsrA and abolish its post-transcriptional repressive activity. However, the RsmYZ-CsrA pathway appears not to be the main or only regulatory circuit governing flagella synthesis. We suggest that rather RpoS and LetA, by influencing LetE and probably cyclic-di-GMP levels, regulate motility in *L. pneumophila*.

Keywords

Legionella pneumophila; RsmY; RsmZ; non-coding RNA; CsrA; transcriptome; pathogenicity; *Acanthamoeba castellanii*

Introduction

Legionella pneumophila is the causative agent of the pneumonia-like Legionnaires' disease. The bacterium's survival and spread depends on its ability to replicate inside eukaryotic phagocytic cells like *Acanthamoeba castellanii*, *Hartmannella* sp. or *Naegleria* sp. It is thought that the interaction of *L. pneumophila* with aquatic protozoa has generated a pool of virulence traits, which also allow *Legionella* to infect human cells and to cause disease. Once internalized, *L. pneumophila* is able to survive and replicate by manipulating host cell functions to reprogram the endosomal-lysosomal degradation pathway of the phagocytic cell

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(for reviews see (Albert-Weissenberger *et al.*, 2007; Steinert *et al.*, 2002; Swanson and Hammer, 2000; Vogel and Isberg, 1999)).

For successful infection of host cells, *L. pneumophila* needs to ensure a precise timing of its life cycle. Signal transduction pathways, such as two-component systems (TCS) are known to be involved in these regulatory processes. TCS comprise a sensor histidine kinase that senses specific signals leading to autophosphorylation of the sensor and activation of the response regulators, which then activate or repress the transcription of target genes. In *L. pneumophila* strain Paris, 13 histidine kinases and 14 response regulators were identified (Cazalet *et al.*, 2004); however, only a few of these systems have been studied to date. Examples are CpxA/CpxR that regulate *icm* and *dot* virulence genes (Altman and Segal, 2008; Gal-Mor and Segal, 2003) or the PmrA/PmrS TCS that was shown to be a global regulator implicated in intracellular growth of *L. pneumophila* and in regulation of the Dot/Icm type IV secretion system (Al-Khodori *et al.*, 2008; Zusman *et al.*, 2007). An important TCS in *L. pneumophila*, described previously, is LetA/LetS (*Legionella* transmission activator and sensor, respectively) that induces traits necessary for efficient host transmission and survival in the environment (Hammer *et al.*, 2002). *letA* and *letS* mutants are non-motile, non-cytotoxic, sodium sensitive, and less proficient in infecting macrophages; however, *letA* mutants still multiply in macrophage host cells (Gal-Mor and Segal 2003; Hammer *et al.*, 2002). Furthermore, *letA* mutants are more sensitive to oxidative and acid stress than the wild type (Lynch *et al.*, 2003) and infectivity of *A. castellanii* is reduced (Hammer *et al.*, 2002; Lynch *et al.*, 2003; Molofsky and Swanson, 2004). Recently it was shown that the LetA/LetS TCS belongs to a family of signal-transducing proteins that employ a four-step phosphorelay to regulate gene expression. Histidine 307 of the LetS protein is the primary site of phosphorylation required to activate LetA (Edwards *et al.*, 2009). Additionally, a threonine substitution at position 311 of LetS generated a *L. pneumophila* mutant with an intermediate phenotype (Edwards *et al.*, 2009), in which gene expression of the flagellar regulon and numerous other loci was delayed when compared to wild type bacteria. Thus, histidine 307 and threonine 311 are necessary for the complete function of LetS (Edwards *et al.*, 2009).

There are orthologous systems to LetA/LetS in many other Gram-negative bacteria, such as *Salmonella enterica* (BarA/SirA), *Erwinia carotovora* (ExpA/ExpS), *Vibrio cholerae* (VarA/VarS), *Pseudomonas spp* (GacA/GacS), *Photobacterium luminescens* (UvrY/BarA) and *E. coli* (UvrY/BarA). These TCS are part of global regulatory circuits that control a variety of functions such as carbon metabolism, biosynthesis of secondary metabolites, cell motility, biofilm formation, quorum sensing, and, in pathogenic bacteria, virulence traits (Babitzke and Romeo, 2007; Cui *et al.*, 2001; Kay *et al.*, 2005; Krin *et al.*, 2008; Lenz *et al.*, 2005; Suzuki *et al.*, 2002). These regulatory networks comprise small non-coding RNAs (ncRNAs) that bind proteins of the CsrA (carbon storage regulator)/RsmA (repressor of secondary metabolites) family and antagonize their activity by sequestering CsrA (Babitzke and Romeo, 2007; Lapouge *et al.*, 2008; Liu *et al.*, 1997; Romeo, 1998). CsrA-mediated repression involves the binding of CsrA to the ribosome-binding site of target transcripts, thereby blocking ribosome access to the mRNA; activation by CsrA seems to be due to mRNA stabilization (Babitzke and Romeo, 2007; Pernestig *et al.*, 2003). The best-studied example is the BarA/UvrY system of *E. coli*, in which a few CsrA targets have been identified and studied so far. These are *glgC* (glycogen synthesis), *cstA* (peptide transport) and *pgaA* (synthesis of biofilm adhesin). CsrA of *E. coli* also binds to the untranslated region of the *flhDC* transcript (regulator of flagella biosynthesis) (Wei *et al.*, 2001) and has recently been shown to control cyclic di-GMP metabolism by directly binding to the mRNA leaders of different GGDEF/EAL protein encoding genes (Jonas *et al.*, 2008).

Legionella encodes a CsrA protein homologous to CsrA of *E. coli* (Fettes *et al.*, 2001; Molofsky and Swanson, 2003) that is able to complement an *E. coli* *csrA* mutant (Fettes *et al.*, 2001) as well as three CsrA homologues (Cazalet *et al.*, 2004). CsrA of *L. pneumophila* was shown to repress the production of virulence traits during the exponential (E) growth phase (replicative /non-virulent bacteria) (Bachman and Swanson, 2004b; Molofsky and Swanson, 2003). In the post exponential (PE) growth phase (transmissive/virulent bacteria), it appears that the LetA/LetS system is necessary to overcome post-transcriptional repression of transmissive traits by CsrA (Molofsky and Swanson, 2003, , 2004). However, the link between LetA/LetS regulation and the CsrA-system was missing; it was unknown whether the regulatory process in *Legionella* involved small ncRNAs like in *E. coli* and *Pseudomonas* sp.

In order to understand the regulation of the switch from replicative/non-virulent to transmissive/virulent *Legionella* and to elucidate the link between the LetA/LetS system and the CsrA regulatory network we took a genomic-level approach to analyze LetA/LetS regulation and to assess the role of two putative ncRNAs that were predicted in *L. pneumophila* by a bioinformatics approach (Kulkarni *et al.*, 2006). Here we present evidence that these ncRNAs, RsmY and RsmZ of *L. pneumophila*, are the missing link between LetA/LetS signaling and CsrA regulation. The genome-wide transcriptional profiles of *letS*, *letA* and *rsmYZ* double mutants showed the global effect of these different regulators on gene expression and their role in regulating virulence traits and transmission.

Results

The LetA/LetS regulatory system comprises two small ncRNAs - RsmY and RsmZ

A recent bioinformatics study searching for putative CsrB/C or RsmY/Z-like RNAs in a variety of bacterial genomes predicted two ncRNAs in the genome of *L. pneumophila*. One was designated RsmY and is present in the intergenic region between *gyrB* (*lpp0004*) and *lpp0005* (predicted 5' end at nucleotide position 6,714), while the other is located in the intergenic region between *lpp1662* and *lpp1663* (position 1,870,666) and was designated RsmZ (Kulkarni *et al.*, 2006) (Figure 1A). Repeated GGA motifs in the loop regions of the secondary structure are a crucial element in small RNAs that regulate CsrA and its homologs (Dubey *et al.*, 2005). Analysis of the putative structure of RsmY and RsmZ using the program MFOLD (Zucker, 2003) indeed predicted loops in which conserved GGA motifs were located (Figure 1B). To verify whether these two regions were transcribed, real-time PCR was performed. This showed that RsmY and RsmZ are expressed in a growth phase-dependent manner, with an mRNA transcript increase of 3.1 and 6.8-fold for *rsmY* and *rsmZ* in the PE (OD 4.3) compared to the E growth phase (OD 2.5), respectively. The predicted size of the *rsmY* transcript is 110 nts, and that of *rsmZ* 132 nts; which was confirmed by Northern blot analysis (data not shown). The LetA/LetS TCS is predicted to regulate expression of RsmY and RsmZ. To gain further insight into this regulatory cascade, we also constructed a *letA* and a *letS* deletion mutant in *L. pneumophila* strain Paris and compared their roles in gene regulation with that of the two ncRNAs RsmY and RsmZ. In contrast to the wt, in the *letA* and the *letS* mutants no up-regulation of *rsmY* or *rsmZ* in the PE phase was observed. Thus, the LetA/S TCS is essential for the growth phase dependent expression of *rsmY* and *rsmZ*.

To determine if LetA directly regulates *rsmY* and *rsmZ* expression by binding to their promoter regions, we searched for a putative LetA-binding site upstream *rsmY* and *rsmZ*. Indeed, 62 nts and 95 nts upstream these genes, respectively, a single, conserved motif similar to the *E. coli* UvrY binding site was identified (Figure 2A). A consensus motif of the likely LetA-binding site is the palindromic sequence TNAGAAATTTCTNA. To confirm this site we performed mobility shift assays with purified, recombinant LetA and a 35bp

fragment containing this predicted site. A band shift was observed for both *rsmY* and *rsmZ* upstream regions (Figure 2B), demonstrating that LetA binds specifically upstream of these loci. A stringent pattern search (no mismatch with respect to the consensus sequence) in the genome sequence of the *L. pneumophila* strain Paris genome did not identify additional LetA-binding sites, suggesting that *rsmY* and *rsmZ* might be the only direct targets of LetA. Thus LetA binds to the upstream regions of *rsmY* and *rsmZ* and regulates their expression.

RsmY* and *RsmZ* of *L. pneumophila* induce premature glycogen synthesis and biofilm formation in *E. coli

Known targets of CsrA in *E. coli* are mRNAs of genes involved in glycogen (*glgCAP*) and polysaccharide synthesis (*pgaABCD*). Inhibition of translation of these genes is effected by CsrA binding to these mRNAs during exponential growth (Wang *et al.*, 2005; Yang *et al.*, 1996). As it was shown that a functional similarity between the CsrA system of *E. coli* and *L. pneumophila* exists, and that CsrA of *L. pneumophila* can repress glycogen accumulation in *E. coli* (Fettes *et al.*, 2001), we hypothesized that RsmY and RsmZ of *L. pneumophila* might also be capable of interacting with CsrA of *E. coli*. In order to test our assumption and to verify the *in vivo* functionality of both small ncRNAs of *L. pneumophila*, we cloned *rsmY* and *rsmZ* into the IPTG-inducible vector pMMB207C (Chen *et al.*, 2004) generating the plasmid constructs *prsmY* and *prsmZ*, respectively. Overexpression of *rsmY* and *rsmZ* in *E. coli* led to drastic changes in two reporter phenotypes known to be controlled by CsrA/CsrB in *E. coli*, namely glycogen storage and biofilm formation. After induction with IPTG of exponentially growing *E. coli* cells, glycogen quickly accumulated (<2 hours) and reached a plateau 10-times higher than that of the control strain transformed with the empty vector pTS10 (Figure 3A, B). Similarly, biofilm formation was enhanced by a factor of 100 (Figure 3B) showing that RsmY and RsmZ of *L. pneumophila* can mimic the action of CsrB and CsrC in *E. coli*.

***CsrA* binds *RsmY* and *RsmZ* in vitro**

To determine whether RsmY and RsmZ are able to interact with CsrA of *L. pneumophila*, we prepared both ncRNA by *in vitro* transcription and performed mobility shift assays with Ni-NTA purified His-tagged *L. pneumophila* -CsrA, heterologously expressed in *E. coli* BL21 cells. At RNA concentrations of 100nM, a shift of RsmZ could be observed in the presence of 500nM of CsrA (Figure 4A). This additional band reached maximal intensity at 1μM CsrA; above this level (up to 5μM CsrA) no additional increase in the intensity of the shifted band was detectable. For RsmY, protein concentrations of 1μM CsrA were necessary to see a retarded band on the gel and the intensity of the band seemed to increase with higher CsrA concentrations up to 5μM (Figure 4B). The affinity for CsrA thus seems higher for RsmZ than for RsmY, suggesting that RsmZ is the major suppressor of CsrA in the PE phase. As controls, similar amounts of RNA of comparable size but lacking putative CsrA-binding structures did not lead to a mobility shift, indicating that CsrA bound specifically to RsmY and RsmZ *in vitro*. Thus, RsmY and RsmZ are two small ncRNAs that are able to bind CsrA. Altogether, our data suggest that LetA-dependent formation of small ncRNAs is required to dissociate RNA-binding proteins like CsrA from recognition sequences present in the target RNAs.

The *rsmYZ* double mutant is dramatically affected in infection and intracellular replication and is impaired in sodium sensitivity and pigment production

In order to gain further insight into the physiological role of these two small ncRNAs in *L. pneumophila*, we constructed $\Delta rsmY$ and $\Delta rsmZ$ single mutants. Their *in vitro* growth (either liquid or solid BYE) was not notably affected except for an elongated lag-phase (data not shown). The single knockout mutants have no ($\Delta rsmY$) or little ($\Delta rsmZ$) impact on virulence in the infection of *A. castellanii* (Figure 5A). In addition, the transcriptome of the

two mutants showed no significant differences as compared to that of the wild type (data not shown). These observations are consistent with the fact that overproduction of both RsmY and RsmZ in *E. coli* and *L. pneumophila* led to similar phenotypic changes, indicating that their expression probably triggers an identical response and that they can functionally replace the other one. In contrast, a double mutant strain should have a detectable phenotype.

To test this hypothesis we constructed a $\Delta rsmYZ$ double mutant. The double mutant infected *A. castellanii* less effectively than the wild type, and was not able to replicate efficiently. Moreover, after 24h of infection, when bacteria normally transit to the next host cell, the bacterial count of the $\Delta rsmYZ$ mutant declined, indicating that it was either not able to infect a new host or was degraded inside the amoeba (Figure 5A). Complementation of the $\Delta rsmZ$ mutant with plasmid *prsmZ* *in trans* rescued it in amoeba infection (Figure 5B). Deletion of *rsmY* and *rsmZ* as compared to the deletion of *letA*, had a more pronounced impact on intracellular growth (Figure 5A). In infection of THP-1 monocyte-derived macrophages the $\Delta rsmYZ$ double mutant was also strongly impaired in intracellular growth and infection of a new host (Figure 5C).

Wild type *L. pneumophila* are sodium sensitive as they grow poorly on agar containing 100mM NaCl (Catrenich and Johnson, 1989) and accumulate substantial pigment during growth (Wiater *et al.*, 1994). However, $\Delta letA$ or $\Delta letS$ mutants are sodium resistant and accumulate little pigment during the PE growth (Hammer *et al.*, 2002). Here we show that the double ($\Delta rsmYZ$) and the single knockout mutants ($\Delta rsmY$, $\Delta rsmZ$) were sodium resistant (Figure 6A) and accumulate little pigment (Figure 6B) comparable to a $\Delta letA$ mutant. This suggests that RsmY and RsmZ, through the CsrA system, are essential for intracellular replication of *L. pneumophila* and are involved in regulating pigment production and sodium sensitivity.

The over expression of *rsmY* or *rsmZ* in *L. pneumophila* wt induces expression of transmissive genes during exponential growth

Provided that RsmY and RsmZ interact with and sequester CsrA protein in the PE phase to allow the transition of wild-type cells to the transmissive phase, early expression (i.e. in the E phase) of these RNAs in *L. pneumophila* should abolish the post-transcriptional repression of transmissive traits by CsrA in the E phase. To verify this prediction *prsmZ*, was transformed into *L. pneumophila* Paris and cells were grown to OD 1.0. After 90 min of induction of *rsmZ* transcription cells were harvested and microarray analyses were performed to determine gene expression differences between the recombinant strain and wild-type strain harboring an empty plasmid pMMB207C. Differences in mRNA transcript levels could indeed be observed. Eighty-three genes were down- and 380 genes were significantly up-regulated. Among the down-regulated genes were typical replicative genes, while among the up-regulated genes were genes characteristic of the transmissive stage, as defined previously (Brüggemann *et al.*, 2006b) (the complete dataset is available at <http://genoscript.pasteur.fr>). For example, the strongest down-regulated gene was *lpp0706* (3.4-fold repressed) and the adjacent *lpp0707*, both typical replicative genes expected to be up-regulated at OD 1 (Brüggemann *et al.*, 2006b). These genes, together with a third repressed gene, *lpp1860*, code for proteins that are members of a subfamily of the major facilitator superfamily, previously described as phagosomal transporters (Pht) of *L. pneumophila* that may be involved in the transport of amino acids (Chen *et al.*, 2008). It was shown that one mutant, *phtA*, prematurely differentiates to the transmissive phase (Sauer *et al.*, 2005). Other down-regulated genes were *lpp0153* (glyceraldehyde 3-phosphate dehydrogenase) and *lpp0529* (acetyl-CoA biotin carboxyl carrier protein), *lpp2756* and *lpp2757* similar to stringent starvation protein A and B, respectively, or genes related to

ATP synthesis like *lpp3052* and *lpp3053*. These are again classically seen as replicative genes.

Several typical transmissive genes were already found to be up-regulated at OD 1. Those are genes encoding for proteins containing a GGDEF/EAL domain (*lpp1170*) or an EAL motif (*lpp0351*), respectively, and the superoxide-dismutase precursor (*lpp2297*) possibly related to stress response as well as several *Legionella* specific proteins with yet unknown function (e.g. *lpp1112* or *lpp1340*). Other strongly up-regulated genes in the RsmZ overexpressing strain with known or predicted functions were *lpp2788*, encoding the response regulator LqsR, a protein involved in phagocytosis and cytotoxicity (Tiaden *et al.*, 2007), *lpp1324*, coding a protein similar to a DNA-binding protein Fis, and *lpp2675* which exhibits similarity to Papain-like cysteine peptidases. Weak induction of *fleQ* (*lpp0915*), coding for the regulator of flagella synthesis FleQ, *lpp0969*, coding for the anti-sigma28 factor, a negative regulator of flagellin synthesis, and *lpp0541*, coding a putative sigma-54 modulation protein as well as two flagellar genes *fliE* (*lpp1725*) and *flgA* (*lpp0970*) was observed.

We also overexpressed *rsmY* by transforming plasmid *prsmY* into *L. pneumophila* strain JR32, since transformation of strain Paris was not successful. Comparison of microarray analyses at OD 0.5 and OD 1 showed similar results for RsmY on *L. pneumophila* gene expression as described above for RsmZ (the complete dataset is available at <http://genoscript.pasteur.fr>).

The LetA/LetS two-component system regulates *rsmY* and *rsmZ* expression and many genes involved in virulence formation of *L. pneumophila*

To compare the role of the LetA/LetS TCS in gene regulation with that of the two ncRNAs RsmY/RsmZ, we sought to identify all genes controlled by these systems through global gene expression profiling. Microarray analyses were performed with cells harvested in E and PE growth phases. No significant differences in gene expression between mutant and wild type strains were observed during the E phase (ODs 1 and 2.5), underlining the previous assumption that LetA/LetS senses a signal generated at the onset of the PE phase (Hammer *et al.*, 2002; Molofsky and Swanson, 2004). In contrast, drastic changes in gene expression of $\Delta letA$ and $\Delta letS$ were observed in the PE phase (OD 4.3), with 425 significantly down- and 314 significantly up-regulated genes (fold-change >1.5) as compared to the wild type (Supplementary material Table S1 and Table S2). The expression of *rsmY* and *rsmZ* was reduced three and two fold, respectively. However, expression was not completely abolished, suggesting that additional factors might regulate the expression of these small ncRNAs or that a basal level of RsmY and RsmZ is constitutively expressed. Direct comparison of the gene expression pattern of $\Delta letA$ and $\Delta letS$ identified no significant differences (data not shown), indicating that LetS has no additional function besides phosphorylating and activating LetA, and that no cross talk of LetA with other sensor kinases exists in these conditions.

Analyses of the transcriptome of the mutants revealed that up-regulated genes in the PE phase are those previously shown to be typical replicative traits (Brüggemann *et al.*, 2006b), such as protein biosynthesis (e.g. ribosomal structure and biogenesis), energy transfer (e.g. NADH dehydrogenase - *lpp2830-lpp2835*; NADH-quinone oxidoreductase - *lpp2827*, *lpp2828*; ATP synthetase - *lpp3052-lpp3059*), or proteins involved in carbohydrate metabolism (e.g. pyruvate dehydrogenase - *lpp1517*; phosphoenolpyruvate synthase - *lpp0867*) (Table 1). Furthermore, in contrast to the wild type, in *letA* and *letS* mutants the biosynthesis of the lipopolysaccharide (LPS) capsule and of legioaminic acid appears to continue also in the PE phase as several genes encoding proteins involved in LPS-biosynthesis (*lpp0814*, *hisF/lpp0815*, *hisH/lpp0816*, *neuA/lpp0817*, *neuB/lpp0818*, *neuC/*

lpp0819, *hisF*, *hisH*, *lpp2026*) are induced (Table 1 and the complete dataset is available at <http://genoscript.pasteur.fr>).

Conversely, down-regulated genes of both single mutants in the PE phase as compared to the wild type comprise functions associated with transmissive traits such as motility, virulence, and factors involved in host-cell manipulation (Table 2). Several substrates of the Dot/Icm type IV secretion system (T4SS), which are necessary at the onset of infection, were down regulated, such as RalF (*lpp1932*), PatA/VipD (*lpp2888*), LidA (*lpp1002*), the SidE homologs SdeA, SdeC (*lpp2096*, *lpp2092*), SidF (*lpp2637*) or SidC (*lpp2579*). T4SS-independent genes were also down-regulated, such as the structural toxin protein RtxA (*lpp0699*), the global stress protein GspA (*lpp2141*), the enhanced entry protein EnhB (*lpp2693*), EnhA homologs (*lpp0972*, *lpp1290*), and EnhC homologs (*lpp1310*, *lpp2174*). The highest repressed gene cluster in both $\Delta letS$ and $\Delta letA$ mutants is *lpp0726–0730*. This operon is of unknown function but *lpp0726* is predicted to encode a patatin-like esterase and *lpp0727*, *lpp0728* and *lpp0730* encode proteins with characteristics of a membrane-bound NADH dehydrogenase, an acetoacetate decarboxylase and an adenylate cyclase, respectively (Table 2 and the complete dataset is available at <http://genoscript.pasteur.fr>). The expression of ten selected genes (*lpp0952*, *flaA*, *mip*, *ralF*, *rpoS*, *rsmZ*, *lpp1170*, *lpp0351*, *fis2*, *csrA*) was also analyzed by qRT-PCR. These results were in perfect agreement with the microarray data (supplementary material, Figure S2). Thus, the LetA/LetS TCS is clearly implicated in regulating genes important for the switch from the replicative to transmissive phase.

LetA/LetS governs flagella biosynthesis largely independently of RsmY and RsmZ

Consistent with the lack of a functional flagellum in *letA* and *letS* mutants (Figure 7), the flagellin-encoding gene (*flaA*, *lpp1294*) was one of the strongest down-regulated genes in the dataset. Levels of *flaA* mRNA were 13-fold lower in $\Delta letA$ as compared to the wild-type (Table 3). Western blot analysis with anti-FlaA antibodies confirmed that no flagellin was present in $\Delta letA$ strains (data not shown). Other flagellum encoding genes were significantly repressed as well, such as *flgEF* (*lpp1226–1228*), *flgKL* (*lpp1231–1234*), *flgMNO* (*lpp1754–1756*), the *flaA*-associated operon containing *flaG*, *fliDS* (*lpp1291–93*), and the gene encoding the alternative sigma factor 28 (*fliA*, *lpp1746*) and its antagonist (*flgM*, *lpp0969*). Thus, LetA/LetS regulation seems to be hierarchically upstream of RpoN/FleQ, the master regulator of flagella biosynthesis genes (Albert-Weissenberger *et al.*, in preparation), since most flagellar genes are down-regulated in $\Delta letS$ and $\Delta letA$ strains.

Analysis of the transcription results of the $\Delta letA$ strain and the $\Delta rsmYZ$ double mutant as well as direct comparison between the two mutants in the PE phase showed, as expected, only slight differences in the expression patterns and transcript levels of most of the genes (the complete dataset is available at <http://genoscript.pasteur.fr>). Nevertheless, microarray analyses highlighted some genes and gene clusters which were expressed in $\Delta rsmYZ$ but strongly down-regulated in $\Delta letA$ (Table 2). Most surprisingly, these comprised the genes necessary for flagellar biosynthesis as they were expressed or even induced in the $\Delta rsmYZ$ but not in $\Delta letA$ as compared to the wild type strain (e.g. *flgB-L*, *fleN*, *flgM*, *flaA*) (Table 3). To further analyze these unexpected results we undertook western blot experiments with anti-FlaA antibodies. These revealed that FlaA protein was indeed expressed in wt and $\Delta rsmYZ$, but not in $\Delta letA$ (data not shown) indicating that, in contrast to $\Delta letA$ strains, $\Delta rsmYZ$ synthesizes a flagellum. We observed the $\Delta letA$, $\Delta rsmZ$ and $\Delta rsmYZ$ strains under the electron microscope; this confirmed that the different *rsm* mutants did indeed synthesize flagella (Figure 7).

Furthermore, *rpoS* (*lpp1247*), *lqsR* (*lpp2788*) and 6 GGDEF/EAL protein-coding genes (*lpp0351*, *lpp0352*, *lpp0809*, *lpp0952*, *lpp1170* and *lpp2355*) showed significantly lower

expression in $\Delta letA$ as compared to a $\Delta rsmYZ$ strain, with all of these genes encoding proteins putatively implicated in regulating *L. pneumophila* motility. Another protein previously implicated in influencing flagella expression, *letE*, encoding the *Legionella* transmission enhancer protein LetE (Bachman and Swanson, 2004b; Hammer *et al.*, 2002), seems also to play an important role as *letE* expression was induced nearly 3 fold in the $\Delta letA$ strain suggesting that LetA directly or indirectly represses *letE* expression, and that the repression of flagellum synthesis in a $\Delta letA$ strain may be linked to repression of *letE*.

Discussion

Bacterial regulatory RNAs participate in a broad range of cellular processes, including carbon storage and utilization, response to oxidative stress, quorum sensing, transition to stationary phase and virulence. Two major classes of ncRNAs are described. They can interact with 5' leader regions of target mRNAs by base pairing, facilitated by the RNA chaperone Hfq or they have high affinity to RNA binding proteins of the RsmA/CsrA family (for a review see (Repoila and Darfeuille, 2009)). Small RNAs that bind RsmA/CsrA are typically produced under the positive control of TCS like GacA/GacS of *P. aeruginosa*. In this way, the small RNAs expand the scope of genetic control exerted by the TCS.

In *L. pneumophila* no ncRNAs were characterized to date. However, *L. pneumophila* encodes a CsrA homologue of *E. coli* and a TCS homologous to GacA/GacS of *Pseudomonas* or BarA/UvrY of *E. coli*, whose regulatory activity is linked through ncRNAs in these bacteria. The present study was thus initiated to decipher the regulatory cascade under the control of the LetA/LetS two-component system (TCS) and the CsrA signaling pathway, to learn whether *L. pneumophila* also employs ncRNAs to link the two systems, and to identify the functions controlled by the *L. pneumophila* CsrA-pathway. Although many Gram-negative bacteria possess the Gac/Rsm signaling pathway, the target genes that are translationally regulated by this system vary considerable. In *L. pneumophila*, both systems had previously been shown to be implicated in regulating differentiation from a replicative/non-virulent form to a transmissive/virulent form (Forsbach-Birk *et al.*, 2004; Hammer *et al.*, 2002; Lynch *et al.*, 2003; Molofsky and Swanson, 2003, 2004). LetA/LetS was shown to influence the formation of the virulent phenotype of *L. pneumophila*, since it was reported to control the progression into the transmissive phase (Gal-Mor and Segal 2003; Hammer *et al.*, 2002; Lynch *et al.*, 2003). In contrast, CsrA activates replication and represses transmissive traits, as it was reported to repress motility, pigmentation, sodium resistance, cytotoxicity and efficient macrophage infection (Fettes *et al.*, 2001; Molofsky and Swanson, 2003).

Here we showed that *L. pneumophila* indeed employs ncRNAs, RsmY and RsmZ, two small RNAs predicted by a bioinformatics approach (Kulkarni *et al.*, 2006) that link this signaling pathway. At the onset of the transmissive phase, LetA induces the expression of *rsmY* and *rsmZ* by binding to an upstream LetA binding site with the proposed consensus sequence TNAGAAATTTCTNA, as shown by *in vitro* binding assays of purified LetA to a DNA fragment containing the predicted binding site (Figure 2). Subsequently RsmY and RsmZ bind CsrA, as evidenced by the *in vitro* binding of these two small ncRNAs to purified CsrA of *L. pneumophila*. The small ncRNAs of *L. pneumophila* are able to functionally complement the CsrA-regulating small RNAs of *E. coli*. Indeed, our study revealed that over-expression of the *L. pneumophila* *rsmY* and *rsmZ* gene in *E. coli* induces premature glycogen synthesis and increased biofilm formation, two known target traits of CsrA in *E. coli* (Wang *et al.*, 2005; Yang *et al.*, 1996). This suggests that *L. pneumophila* RsmY and RsmZ are able to interact with *E. coli* CsrA. However, the *E. coli* and *L. pneumophila* ncRNAs belong to two different classes of CsrA interacting RNAs. The ones similar to CsrB binding to *E. coli* CsrA are around 300 bp and those similar to RsmY, RsmZ, RsmX binding

to RsmA of *e.g. P. fluorescence* are about 100 bp (Babitzke and Romeo, 2007). They differ also in the number of GGA motifs present mainly in the loops. It has been shown that mutation of the GGA motifs leads to substantial reduction in CsrA binding affinity (Dubey *et al.*, 2005). There are 22 GGA motifs present in CsrB of *E. coli* but only 6 and 5 in RsmY and RsmZ of *L. pneumophila*, respectively. Still, RsmY and RsmZ of *L. pneumophila* are able to mimic CsrB or CsrC activity in *E. coli*, showing that the Csr-like systems in the different bacteria function by very similar mechanisms.

To further understand the impact of RsmY and RsmZ on virulence and its influence on the progression of *L. pneumophila* to the transmissive phase, single and double knockout mutants were constructed and analyzed. The double mutant was drastically affected in virulence and appeared unable to transit to a new host (Figure 5A). Interestingly, the intracellular replication defect was much stronger in the $\Delta rsmYZ$ double mutant than in the $\Delta letA$ mutant strain (Figure 5A). This might be due to the fact that a basal level of RsmY and RsmZ expression still remained in a $\Delta letA$ strain as judged from transcriptome analysis, but these two small RNAs were completely missing in the $\Delta rsmYZ$ double mutant. Thus, RsmY and RsmZ are crucial regulators of the transmissive/virulent phenotype of *L. pneumophila* and are indispensable for virulence of *L. pneumophila*.

Direct regulation by CsrA is post-transcriptional and occurs by CsrA binding to the mRNA of target genes (Babitzke and Romeo, 2007). Thus the target genes for which CsrA affects translation but not transcript levels will not be detected in standard microarray experiments. We therefore undertook a bioinformatics search for the occurrence of multiple GGA motifs in the vicinity of the Shine-Dalgarno sequence, a known feature of CsrA targets in other bacteria combined with a structural analysis of the identified sequences. By this approach 42 putative targets of CsrA were predicted (supplementary material Figure S1). Among those are LqsR, a recently identified pleiotropic regulator whose expression was reported to be influenced by RpoS and, to a lesser extent, also by LetA (Tiaden *et al.*, 2007; Spirig *et al.*, 2008), but also several flagella biosynthesis genes like *fleQ*, *fliL*, *fliH*, *flgE*, *fliQ* and *fliK* indicating that some influence of CsrA on flagella biosynthesis might exist. The bioinformatics approach did not predict *lly*, coding legiolysin, responsible for the pigmentation phenotype, as a target. However, when overexpressing RsmZ in the $\Delta letA$ mutant strain, pigmentation was restored, suggesting that *lly* may be a target of CsrA (Figure 8). Another target of CsrA might be Hfq, as shown for *E. coli* and suggested previously for *L. pneumophila* (McNealy *et al.*, 2005).

Comparison of the effect of the mutations of *rsmYZ*, *letA* and *letS* as compared to wt *L. pneumophila* by whole genome transcriptional profiling in different growth phases, revealed many similar but also different traits. The transcriptional changes induced by the LetA/LetS TCS were in line with phenotypes previously reported (Hammer *et al.*, 2002) and also observed in this study, like lack of flagella (Figure 7), reduced pigmentation and sodium sensitivity (Figure 6), or reduced infectivity and less efficient intracellular replication (Figure 5). In particular genes defined previously as typical replicative traits (Brüggemann *et al.*, 2006b) were expressed in all mutants in the PE (transmissive) phase, whereas several genes coding proteins characteristic of the transmissive/virulent phenotype were down-regulated. The genes affected by these changes have been shown to play central roles in the virulence of *L. pneumophila*. For example, SidF contributes to apoptosis resistance in *L. pneumophila*-infected cells by specifically interacting and neutralizing the effects of two pro-apoptotic members of the Bcl2 protein family (Banga *et al.*, 2007). SidC is a phosphatidylinositol-4 phosphate-binding type IV substrate that recruits endoplasmic reticulum vesicles to a replication-permissive vacuole (Weber *et al.*, 2006b; Ragaz *et al.*, 2008). The patatin-like protein PatA/VipD was reported to assist in the establishment of a vacuole suitable for bacterial replication by interfering with multi-vesicular body formation

at the late endosome and ER to Golgi transport (Shohdy *et al.*, 2005; VanRheenen *et al.*, 2006). SdeA is secreted early during macrophage infection, suggesting that it is important in the initial formation of the replicative vacuole (Bardill *et al.*, 2005). Taken together, our results from gene expression profiling of regulatory mutants identify the genes implicated in the observed phenotype of *letA* and *letS* mutants and confirm as suggested previously that the TCS LetA/LetS regulates the switch from replicative/non-virulent to transmissive/virulent *L. pneumophila* (Hammer *et al.*, 2002). Furthermore, we show here for the first time, that this regulation is dependent on two small ncRNAs. Thus, LetA/LetS is part of a Csr-like regulatory system in *Legionella* as previously proposed (Molofsky and Swanson, 2003).

If the LetA/LetS system only regulates RsmY and RsmZ, as suggested by a genome wide stringent search for additional LetA binding sites, a *rsmYZ* double mutant and a *letA* or *letS* mutant should show the same transcriptional profile. Indeed, in the transmissive phase the *rsmYZ* double mutant had a strong global impact on gene expression, very similar to the changes seen for a *letA* or a *letS* mutant. However, we observed one significant difference. The expression of the genes necessary for flagella biosynthesis were repressed in the *letA* mutant but induced or unchanged in the *rsmYZ* double mutant with respect to the wild type (Table 3). This result was unexpected, as it had been postulated that flagella biosynthesis in *L. pneumophila* was regulated through the LetA/LetS–CsrA signaling pathway (Fettes *et al.*, 2001; Heuner and Steinert, 2003; Molofsky and Swanson, 2003, 2004). However, we demonstrate here that although *letA* mutants do not synthesize flagella, *rsmY*, *rsmZ* and *rsmYZ* mutants synthesize flagella as judged by electron microscopy (Figure 7) and transcriptional profiling and flagellin is produced as assessed by western blot analysis with anti-FlaA antibodies (data not shown). Thus our results suggest that flagella expression in *L. pneumophila* is largely independent of the RsmYZ–CsrA pathway but is clearly dependent on the LetA/LetS TCS. This is different from what is seen in *E. coli* where CsrA positively regulates motility (Wei *et al.*, 2001) but similar to *Photobacterium luminescens*, where flagella synthesis is reported to be independent of the CsrA pathway but dependent on BarA/UvrY, a homologous system to LetA/LetS (Krin *et al.*, 2008).

Our transcriptome data also showed that expression of *letE*, encoding the LetA/LetS enhancer protein previously reported to enhance flagella synthesis (Bachman and Swanson, 2004b; Hammer *et al.*, 2002) is 3-fold induced in a Δ *letA* strain but not in a Δ *rsmYZ* mutant strain. Previous reports show that *letE* is mainly expressed during the replicative phase (Bachman and Swanson, 2004b), a stage where indeed no flagellum is synthesized. This might suggest, that in a Δ *letA* strain the direct or indirect repression of *letE* expression by LetA is abolished when *Legionella* progresses into the transmissive phase. Thus, in a Δ *letA* mutant, LetE may be further produced and may continue to repress flagella biosynthesis genes like in replicative phase. In contrast, in the presence of LetA *letE* is repressed in the transmissive phase and flagella repression is released.

Furthermore, according to the transcriptional profile, LetA, but not RsmYZ, influences the expression of genes coding the pleiotropic regulator LqsR and several GGDEF/EAL proteins. GGDEF/EAL proteins have been shown to repress motility in various bacteria (Romling, 2005). In contrast, the transcript level of the gene coding the sigma factor RpoS is strongly induced only in the Δ *rsmYZ* mutant during the PE phase. Furthermore it was recently shown that RpoS also influences *rsmY* and *rsmZ* expression (Hovel-Mine *et al.*, 2009), suggesting that a feedback loop between the two systems exists. Thus RsmY and RsmZ seem to have some influence on RpoS expression, a regulator known to positively influence flagella expression. But not only RpoS also the other regulators discussed above are all known to influence motility and virulence in *Legionella* (Bachman and Swanson, 2004a; Hickman and Harwood, 2008; Tiaden *et al.*, 2007; Weber *et al.*, 2006a). Therefore,

our results suggest that *rsmYZ* and LetA may directly or indirectly regulate the transcription of other regulators or regulatory elements important for flagella regulation and, moreover, alter the cyclic-diGMP level, which may influence motility by controlling the amount of specific GGDEF/EAL proteins.

However, impact of the CsrA pathway on flagella biosynthesis cannot be completely ruled out as a constitutively expressing CsrA strain was reported to have reduced motility in PE growth phase and it shows only 30% flagellin expression as compared to a wt strain (Molofsky and Swanson, 2003; Fettes et al., 2001). Thus, another, yet undiscovered small ncRNA, e.g. like RsmX of *Pseudomonas fluorescence* (Kay et al., 2005) could be present also in *L. pneumophila*. In *P. fluorescence* it was shown, that only a triple *rsmX*, *rsmY*, *rsmZ* mutation caused the same phenotypic effect as those observed in a *gacA* or *gacS* mutant (Kay et al., 2005). This arrangement may allow a more efficient regulatory response via a gene dosages effect and may allow fine-tuning of the LetA/LetS system in response to different stimuli. Furthermore, in other systems where small RNAs play a role, like in the control of quorum sensing in *Vibrio harvey* it has been shown that the action of small RNAs can be additive (Tu and Bassler, 2007). Search for this putative third small ncRNA and further analysis of CsrA and the different regulators involved in the CsrA signaling pathway are under way to shed light on this issue.

On the basis of the data presented here and of previously published work (reviewed in Molofsky and Swanson, 2004) we propose a refined model of this signaling network (Figure 9). Upon entering the PE phase LetS is auto-phosphorylated after having received a yet unknown signal (presumably a starvation signal related to the formation of ppGpp (Edwards et al., 2009; Hammer and Swanson, 1999) and then, in turn, phosphorylates LetA. Subsequently, activated LetA binds to a LetA-binding site in close proximity to the promoter region of the regulatory sRNA genes *rsmY* and *rsmZ* and therewith induces their expression. The RNA transcripts form a distinctive secondary structure, which is able to bind to and to sequester CsrA. Thus, CsrA is released from mRNA transcripts of its targets, which then allows either their translation or their decay. In *L. pneumophila*, this results in the production of transmissive phase traits, and probably also in the decay of mRNAs of replicative traits, thus allowing the cell to prepare itself for the last stage of the infectious cycle, the evasion from the host cell and the infection of a new host. Flagella biosynthesis appears to be independent of the RsmYZ-CsrA signaling pathway and seems mainly to be regulated by LetA, LetE, RpoS and probably specific levels of cyclic-diGMP controlled through the regulation of specific GGDEF/EAL proteins.

However, it is also possible that the CsrA pathway has more influence on flagella biosynthesis as evidenced here, if other not yet identified ncRNAs are implicated in the LetA/CsrA signaling pathway. Thus, determination whether other ncRNAs are part of this regulatory signaling cascade or not, is an important question for the future. Equally important is the elucidation of direct targets of CsrA, which would greatly enhance our understanding of this regulatory network in *L. pneumophila*.

Materials and Methods

Strains, media, growth conditions and *A. castellanii* and THP-1 infection assay

L. pneumophila strains Paris and Philadelphia JR32 were cultured in *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES)-buffered yeast extract broth or on ACES-buffered charcoal-yeast (BCYE) extract agar at 37°C. *A. castellanii* ATCC50739 was cultured in PYG 712 medium (2% proteose peptone, 0.1% yeast extract, 0.1 M glucose, 4 mM MgSO₄, 0.4 M CaCl₂, 0.1% sodium citrate dihydrate, 0.05 mM Fe(NH₄)₂(SO₄)₂ x 6H₂O, 2.5 mM NaH₂PO₃, 2.5 mM K₂HPO₃) at 20°C. THP-1 human monocytes were grown in RPMI 1640

GlutaMAX medium (GIBCO) supplemented with 10% foetal bovine serum at 37°C, 5% CO₂.

For *in vivo* growth of *L. pneumophila* Paris and its derivatives in *A. castellanii* we followed a protocol previously described (Brüggemann *et al.*, 2006b). In brief, three days old cultures of *A. castellanii* were washed in infection buffer (PYG 712 medium without proteose peptone, glucose, and yeast extract) and adjusted to 10⁵–10⁶ cells per ml. Stationary phase *Legionella* grown on BCYE agar, diluted in water were mixed with *A. castellanii* at a MOI of 0.01. After allowing invasion for 1 hour at 37°C the *A. castellanii* layer was washed twice with infection buffer (start point of time-course experiment). Intracellular multiplication was monitored using a 300µl sample, which was centrifuged (14500 rpm) and vortexed to break up amoeba. 72 hours before the infection cells were seeded into 12 well tissue culture trays (Falcon) at a density of 3 × 10⁵ cells/well and pretreated with 10⁻⁸ M phorbol 12-myristate 13-acetate (SIGMA) to induce differentiation into macrophage-like cells. Stationary phase *Legionella* were resuspended in RPMI 1640 serum free medium and added to THP-1 cell monolayers at a MOI of 10. After 1 hour of incubation cells were treated with 100 µg ml⁻¹ gentamycin for 1 hour to kill extracellular bacteria. Infected cells were then washed with PBS before incubation with serum-free medium. At 24, 48 and 72 THP-1 were lysed with 0.1% tritonX-100. The number of colony forming units (CFU) of *Legionellae* was determined by plating on BCYE agar. Each infection was carried out in duplicates.

Plasmids and *L. pneumophila* mutant construction

Mutant strains of *L. pneumophila* were constructed as described previously (Brüggemann *et al.*, 2006b; Heuner *et al.*, 2002). In brief, the gene of interest was inactivated by introduction of a kanamycin resistance (kan^R) cassette into the chromosomal gene. To construct the *letA* mutant strain, the chromosomal region containing the *letA* gene was PCR-amplified with the primers LetA_for TTGATGTGGATCCGGAAT and LetA_rev TAGTACGGTGCCTTCCTCGT. The product was cloned into the pGEM vector (Promega), the resulting plasmid was digested with *Xho*I and ligated to the kanamycin resistance cassette, which was amplified via PCR from the plasmid pGEM-Kan^R, using primers containing *Xho*I restriction sites at the ends (Kan_*Xho*I_for AGCTCGAGAAAGCAGGTAGCTTGCAG, Kan_*Xho*I_rev AGCTCGAGCCGCTGCTGGTTTCCTGG). A second *letA* mutant strain (in strain Philadelphia, kindly provided by M. Swanson, (Hammer *et al.*, 2002)) was also used in this study. For the *letS* mutant, the chromosomal region for *letS* was amplified using the primers LetS_for CCGAGTTTACCATGAACGCA and LetS_rev GGATGACACCACAAGCTGAT and the resulting 2.9 kb PCR fragment was cloned into the pGEM T easy vector (Promega). The resulting plasmid was digested with *Hind*III and the *Hind*III-kanamycin resistance cassette was inserted as described before.

To construct the *rsmY* mutant, the corresponding intergenic region between *lpp0004* and *lpp0005* plus ca. 400 bp up- and downstream sequence of *rsmY* was PCR-amplified with the primers rsmY_for AGGAAGATCCTAACTACCATCGCTT and rsmY_rev ATAGAGGAAGGCGCTATATTCAAC and cloned into pGEM T easy vector (Promega). On this template, an inverse PCR was performed using the primers rsmY_inv_*Hind*III_for AGAGAAGCTTATTCTCTAATTTACATTAGTATACC and rsmY_inv_*Hind*III_rev AGAGAAGCTTAGTTGACTTCCTGTGACACATAT. These primers contained a *Hind*III restriction site, and amplified the pGEM backbone and the 400 bp flanking regions of *rsmY*. The resulting PCR product was *Hind*III-digested and ligated to the kan^R cassette (amplified via PCR from the plasmid pGEM-Kan^R (Kanamycin resistance cassette (kindly provided by Klaus Heuner) inserted into a pGEM vector), using primers containing *Hind*III restriction sites at the ends (Kan_*Hind*III_for CTAAGCTTGCAGTGGGCTTACATG, Kan_*Hind*III_rev CTAAGCTTCGGGGGCATGGATGTGCGGATA). For chromosomal

recombination, the construct (i.e. PCR fragment containing the kan^R cassette with flanking regions of the gene of interest) was introduced into *L. pneumophila* by transformation.

For complementation experiments, pRsmZ was introduced into the *rsmZ* single mutant and *A. castellanii* infection assay were performed at presence of 0.25mM IPTG. As control *L. pneumophila* Paris wild type and the *rsmZ* mutant containing the empty plasmid pTS-10 were used, respectively. All mutants and plasmids used in this study are listed in Table 4.

RsmY and RsmZ overexpression in *L. pneumophila*

For overexpression of RsmZ in *L. pneumophila* we used the plasmid pMMB207C-RBS-lcsC (derived from pTS-10; kindly provided by H. Hilbi, see (Weber *et al.*, 2006b)). The *lcsS* gene was removed by cutting the plasmid with EcoRI and BamHI, and replaced with the *rsmZ* gene, which was amplified from chromosomal DNA with primers containing EcoRI and BamHI restriction sites (rsmZ_EcoRI_for GGCGAATTCGACTTATATGGATATGAGTC, rsmZ_BamHI_rev GCGGGATCCAAAAGTAAGCCAGGGACTC), respectively. The resulting plasmid (pRsmZ) and the control plasmid pTS-10 were introduced via electroporation into *L. pneumophila* strain Paris. For overexpression of RsmY in *L. pneumophila* the same plasmid and cloning strategy was used. For amplification of the *rsmY* genes primers rsmY_EcoRI_for GGCGAATTCTGACATGGATATGTCTGACAGGA and rsmY_BamHI_rev GCGGGATCCATACTAATGTAAATTAGAGAATAAGTGC, respectively were used. The resulting plasmid (pRsmY) and the control plasmid pTS-10 were introduced via electroporation into *L. pneumophila* strain JR32. For overexpression IPTG (100μM) was added to the plasmid-carrying strains at ODs of 0.5 and OD 1. Samples for RNA extraction were taken after 90 min of induction.

RsmY/RsmZ overexpression in *E. coli*

RsmY and RsmZ were amplified and cloned into pGEM (Promega), by using the following primers: rsmY_for AGGAAGATCCTAACTACCATCGCTT, rsmY_rev ATAGAGGAAGGCGCCTATATTCAAC and rsmZ_for CCAGCTATAGCACAAACC, rsmZ_rev GTTACCCAGACAAACACATG. The resulting plasmids (pGEM-rsmY, pGEM-rsmZ) were introduced into *E. coli* BL21 by electroporation.

Determination of *E. coli* intracellular glycogen content

Cell samples (about $2-5 \times 10^8$ cells) were collected by centrifugation (4000g, 4 min, 4°C), washed once with 1 ml cold water and the pellet stored at -20°C until further use. Cells were resuspended in 250 μL 0.25 M Na₂CO₃ and heated at 95°C for 2 h with occasional stirring. The suspension was adjusted to pH 5.2 with 150 μL of 1 M acetic acid and 600 μL of 0.2 M sodium acetate (pH 5.2). This mixture was incubated overnight at 57 °C with continuous shaking in the presence of 100 μg of α-amylglucosidase (Boehringer, 208464), freshly prepared as a 10 mg mL⁻¹ stock solution made in 0.2 M sodium acetate (pH 5.2). The released glucose was determined with the glucose oxidase reagent (Sigma, kit 510-A) in 96-well microplates and glycogen content was expressed in μg equivalent glucose per unit of OD₆₀₀.

Qualitative assessment of the *E. coli* glycogen content was carried out using the iodine-staining method (Govons *et al.*, 1969). Briefly, cells were grown overnight in Luria-Bertani (LB) liquid medium. An LB agar plate containing 0.5 mM isopropyl-β-d-thiogalactopyranoside (IPTG) was then streaked with large amounts of bacteria, which were grown for 7 hours at 37°C. Then a iodine crystal was positioned in the lid of the Petri dish positioned upside down. In 10 min, iodine vapor stained the cells according to the amount of glycogen they contained.

Biofilm formation assay

The initial steps of biofilm formation were assayed by determining the ability of cells to adhere to the wells of 96-well polyvinyl chloride (PVC) microtiter dishes (Falcon) as described previously (Roux *et al.*, 2005). The medium (LB for *E. coli*, BYE for *L. pneumophila*, 100 µl/well) was inoculated with a 1:100 dilution from an overnight culture. After inoculation, the plates were incubated at 37°C for 24 h (72 h in the case of *L. pneumophila*) and then rinsed and 125 µl of a 1% solution of crystal violet was added to each well. The plates were incubated at room temperature for 15 min and rinsed. For analyzing biofilm formation, the crystal violet was solubilized by addition of 200 µL of ethanol-acetone (80:20), and the OD₅₇₀ was determined. The results are averages of four replicate wells from three independent experiments.

Pigmentation and sodium sensitivity

For quantifying pigment accumulation, 1 ml samples - obtained from 5 day old broth cultures grown at 37°C - were centrifuged at 16,000 × g for 10 min and supernatants measured at OD_{550nm}. Sodium sensitivity was tested by plating dilutions of PE broth cultures on BYE agar containing or lacking 100 mM NaCl. CFUs were counted and percentage of sodium sensitive bacteria were calculated.

LetA overexpression, purification and Electrophoretic Mobility Shift Assay (EMSA)

To detect binding of LetA (Lpp2699) protein to the upstream regions of the *rsmY* and *rsmZ* genes we performed gel shift assays using the LightShift Chemiluminescent EMSA kit (Pierce, Rockford). For purification of the LetA protein, the *letA* gene was cloned into the vector pMMB207C using the primers letA_for TCTAGAATGATTAAAGTATTAATTGTTGATGAC and letA_Strep_rev CTGCAGTTACTTCTCAAATTGAGGATGTGACCAATCATCTTGTGACAAATCATTG G. The resulting plasmid (pletA_Strep) was sequenced and introduced into *E. coli* BL21, and the LetA Strep-tag fusion protein was over expressed and purified with a Strep-Tactin sepharose column according to the instructions of the manufacturer (IBA, Göttingen).

A set of complementary pairs of 35mers HPLC-purified *rsmY* and *rsmZ* oligonucleotides were used as substrates containing the predicted LetA binding site and a biotin tag at the 5' end: *rsmY*_upstream_for CTCTTCTTAATTGAGAAATTTCTTACATATTACTGC, *rsmY*_upstream_rev GCAGTAATATGTAAGAAATTTCTCAATTAAGAAGAG; *rsmZ*_upstream_for GTAATTGATGTAAGAAATTTCTCAATTCCATAGA, *rsmZ*_upstream_rev TCTATGGAATTGAGAAATTTCTTACATCAATTAC. As control peptide a random sequence containing motifs of the *rsmY* and *rsmZ* oligonucleotides, but without LetA binding site was used (control_for GTAATTGCAATTCCATAGATATTACTGCCATTTCAGA, control_rev TCTGAATGGCAGTAATATCTATGGAATTGCAATTAC). Binding assays of purified LetA protein with biotin-labeled DNA probes were performed at room temperature for 30 min. Binding reactions contained: 0.2 µM biotin end-labeled target DNA, LetA (0–6 µM), 1 x binding buffer, 5% glycerol, 5 mM MgCl₂. As nonspecific competitor 50 ng/µl poly(dI-dC) was used; as specific competitor unlabeled target DNA was added (4pmol). Subsequently, the binding reaction was loaded on an 8% native polyacrylamide gel, followed by electrophoretic transfer to a Nylon membrane. Cross-linking was performed under UV-light at 120 mJ/cm² for 60 sec. The biotin-labeled DNA probe was visualized on the nylon membrane by chemiluminescence using a streptavidin-horseradish peroxidase conjugate and the chemiluminescence substrate of the manufacturer.

CsrA overexpression and purification

Full-length cDNAs encoding CsrA (Lpp0845) were amplified by PCR using primer CsrA-for ATACCATGGTGATTTTGACTCGGCGTATAG and CsrA-rev CCCCCGGGTACTGCTTGCGAATCAGATTC. Fragments were cloned in frame into the expression vector pIVEX2.3d (Roche Diagnostics), which adds a hexa-histidine tag to the C-terminus of the protein; positive clones in *E. coli* DH5 α were selected on 50 μ g/ml ampicillin and sequences were verified. *E. coli* BL21 (Invitrogen) cells were used for protein overexpression. The cells were grown in M9 minimal medium, containing 1 mM MgSO $_4$, 0.1 mM CaCl $_2$, 0.2% (w/v) glucose, 50 μ g ml $^{-1}$ ampicillin, at 16°C. Protein production was induced by adding 0.5 mM IPTG at A $_{600}$ ~0.5, and cells were harvested late exponential phase by centrifugation at 4°C. Cells from one liter of culture were resuspended in 1 ml buffer A – PBS buffer (120 mM NaCl, 4 mM KH $_2$ PO $_4$, 16 mM Na $_2$ HPO $_4$, pH 7.8), 2 mM MgCl $_2$, 5 mM DTT, 5% (v/v) glycerol and a cocktail of protease inhibitors (Sigma) at the concentration recommended by the manufacturer. Cells were disrupted by sonication, centrifuged (18,000 $\times$ g, 30 min, 4°C) and the supernatant was applied to a Ni-NTA affinity column (GE Healthcare) equilibrated with buffer A including 40 mM imidazole. The column was washed with the same buffer containing 100 mM imidazole then the enzyme was eluted with 500 mM imidazole in buffer A. Fractions were dialyzed against buffer A and concentrated by centrifugation (Microcon, 3 kDa cut-off, Millipore) to a final concentration of 0.1 mg protein/ml. Proteins were quantified according to Bradford using BSA as standard. Aliquots of 20 μ l were frozen in liquid N $_2$ and kept at –80°C until further use. The quality of the purification was determined after SDS-PAGE analysis (4% stacking gel and 12% running gel) and staining with Brilliant Blue G - Colloidal Concentrate (Sigma).

In vitro transcription of RsmY/RsmZ-RNA and EMSA gel mobility assay

RsmY and RsmZ was amplified from bacterial DNA with the primer pairs RsmY-for TGACATGGATATGTCTGAC/RsmY-rev AAAGGTATACTAATGTAAATTAG and RsmZ-for GACTTATATGGATATGAGTC/RsmZ-rev AAAAGTAAGCCAGGGACTC, respectively, and cloned under the control of a T7 promoter into pGEMT-easy vector. Insertion was verified by sequencing and correct inserts were used in MEGascript T7 Kit (Ambion) to produce *in vitro* RNA; 2 μ M of Biotin-16-UTP was added into the reaction mix for later detection. As negative control, empty pGEMT-easy vector cut with NdeI was used as template to produce *in vitro* RNA without CsrA binding motif. The reaction was incubated at 37°C for 12h and RNA was purified by Phenol/Chloroform extraction according to the supplier's protocol. RNA concentration was estimated by UV absorption at 260nm. For 10 μ l interaction assays, 100nM of RNA was combined with varying concentrations of purified CsrA-His (0–5 μ M) and incubated in buffer A in presence of 10ng tRNA yeast (Invitrogen) and 1U Rnase Out (Invitrogen) for 30min. Subsequently, samples were fractionated under non-denaturing conditions on Blue-Native PAGE (Schägger and von Jagow, 1991, 1991) and blotted to Immobilon-P transfer membranes (Millipore). Membranes were blocked in PBS buffer containing 0.1% Tween-20 and 1% BSA for 1h at RT and, afterwards, incubated for 1h in the same buffer including streptavidin-horseradish peroxidase conjugate (Invitrogen). RNA-bands were visualized with ECL Plus Western blotting solutions (GE Healthcare) and detected with Chemi-Smart 5000 (Vilber Lourmet).

RNA Isolation, labeling and microarray hybridization

Total RNA was extracted as previously described (Milohanic *et al.*, 2003). Paris wild type and mutant strains were grown in AYE medium, and harvested for RNA isolation at the exponential (OD1 or OD 2.5) and post-exponential growth phase (OD. 4.3). RNA was prepared in triplicates (three independent cultures) and each RNA sample was hybridized twice to the microarrays (dye swap). RNA was reverse-transcribed with Superscript indirect

cDNA kit (Invitrogen) and labeled with Cy5 or Cy3 (Amersham Biosciences) according to the supplier's instructions. The design of microarrays containing gene-specific 70mer oligonucleotides based on all predicted genes of the genome of *L. pneumophila* strain Paris (CR628336) and its plasmid (CR628338) was previously described (Brüggemann *et al.*, 2006a). Hybridization was performed following the manufacturers' recommendations (Corning) using 250 pmol of Cy3 and Cy5 labeled cDNA. Slides were scanned on a GenePix 4000A scanner (Axon Instruments). Laser power and/or PMT were adjusted to balance the two channels and the resulting files were analyzed using Genepix Pro 4.0 software. Spots were excluded from analysis in case of high local background fluorescence, slide abnormalities, or weak intensity.

Data analysis and statistics

Data normalization and differential analysis were conducted using the R software (<http://www.R-project.org>). No background subtraction was performed, but a careful graphical examination of all the slides was conducted to ensure a homogeneous, low-level background in both channels. A loess normalization (Yang *et al.*, 2002) 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 (Delmar *et al.*, 2005)), together with the Benjamini and Yekutieli (Reiner *et al.*, 2003) p-value adjustment method. If not stated otherwise, only differently expressed genes with 2-fold-changes were taken into consideration. Empty and flagged spots were excluded from the data set, and only genes with no missing values for the comparison of interest were analyzed. The complete dataset is available at <http://genoscript.pasteur.fr> in a MIAME compliance public database maintained at the Institut Pasteur and was submitted to the ArrayExpress database maintained at <http://www.ebi.ac.uk/microarray-as/ae/> under the Acc. No A-MEXP-1539.

Real-time PCR

Real-time PCR was performed as described previously (Brüggemann *et al.*, 2006b) at cDNA concentrations from 100 pg to 0.1pg. Two biological repetitions were carried out and each transcript was quantified twice. The genes tested were *lpp0952*, *ralF*, *rpoS*, *rsmZ*, *lpp1170*, *lpp0351*, *fis2*, *csrA* (for *letA* mutant *flaA* and *mip* in addition). Primers used are listed in supplementary material, Table S2. As control *lpp1040* and *csrA* were used. Both were confirmed by microarray data to be stably expressed housekeeping genes under these conditions. Values of relative induction were calculated according to (Pfaffl, 2001), by group-wise comparison of induced samples versus control samples.

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Figure 1. Two small ncRNAs, named RsmY and RsmZ are located in intergenic regions
 A) Sequence of the small RNAs and the flanking regions; Blue letters, sequence of the small RNA. Red letters, LetA binding site; yellow boxes, GGA motifs representing the CsrA binding sites; arrows indicate flanking genes in the *L. pneumophila* strains Paris genome. B) Predicted structure of the RsmY and RsmZ of *Legionella pneumophila* predicted by the program Mfold (<http://mfold.burnet.edu.au/>). The typical GGA (arrows) motifs are located in the loop regions

Figure 2. LetA binds specifically upstream of *rsmY* and *rsmZ*

Electrophoretic Mobility Shift Assay with Strep-tag purified LetA protein and 35 bp fragment of HPLC-purified *rsmY* and *rsmZ* oligonucleotides containing the predicted LetA binding site and a 35 bp control DNA fragment without LetA binding site each with a biotin tag at the 5'ends A) Identified LetA-binding site and the *L. pneumophila* consensus sequence as compared to the *E. coli* consensus binding site for UvrY. B) Lane 1–4: 50ng control DNA; Lane 5–8: 50ng 35bp DNA fragment upstream *rsmY* containing the predicted LetA binding site, incubated with 0, 2, 4, 6 μ M LetA; Lane 9–12: 50ng 35bp DNA fragment upstream *rsmZ* containing the predicted LetA binding site incubated with 0, 2, 4, 6 μ M LetA, respectively.

Figure 3. RsmY and RsmZ of *L. pneumophila* induce glycogen accumulation and biofilm formation in *E. coli*

A) *Escherichia coli* wild-type (BL21) containing the empty plasmid pTS10 and *E. coli* producing *L. pneumophila* RsmY (*prsmY*) or RsmZ (*prsmZ*), respectively, were streaked onto Kornberg medium containing 1% glucose and 100 mg.ml⁻¹ Ampicillin, then stained with iodine vapour to visualize glycogen accumulation. B) Quantification of glycogen accumulation expressed in µg equivalent glucose per unit of OD₆₀₀ measures and biofilm formation of *E. coli* carrying RsmY and RsmZ of *L. pneumophila* as judged from OD₅₇₀ measures after solubilization of crystal violet.

Figure 4. RsmY and RsmZ bind CsrA *in vitro*

Electromobility shift assay with 100nM of RNA combined with varying concentrations of purified CsrA-His. A) RsmZ RNA and recombinant CsrA in 6% Blue Native Bis-Tris/Tricine Gel. Lane 1: no CsrA, 0.1 μ M control RNA; lane 2: 2.0 μ M CsrA + 0.1 μ M control RNA, lane 3: 5.0 μ M CsrA + 0.1 μ M control RNA, lane 4: no CsrA, 0.1 μ M RsmZ, lane 5: 0.5 μ M CsrA + 0.1 μ M RsmZ; lane 6: 1.0 μ M CsrA + 0.1 μ M RsmZ; lane 7: 2.0 μ M CsrA + 0.1 μ M RsmZ; lane 8: 5.0 μ M CsrA + 0.1 μ M RsmZ; lane 9: 1 μ g CsrA (silver-stained). B) RsmY RNA and recombinant CsrA in 6% Blue Native Bis-Tris/Tricine Gel. Lane 1: no CsrA, 0.1 μ M control RNA; lane 2: 2.0 μ M CsrA + 0.1 μ M control RNA, lane 3: 5.0 μ M CsrA + 0.1 μ M control RNA, lane 4: no CsrA, 0.1 μ M RsmY, lane 5: 0.5 μ M CsrA + 0.1 μ M RsmY; lane 6: 1.0 μ M CsrA + 0.1 μ M RsmY; lane 7: 2.0 μ M CsrA + 0.1 μ M RsmY; lane 8: 5.0 μ M CsrA + 0.1 μ M RsmY; lane 9: 1 μ g CsrA (silver-stained)

Figure 5. Intracellular replication of *L. pneumophila* in *Acanthamoeba castellanii* and THP-1 macrophages is dependent on functional RsmY and RsmZ

A) *A. castellanii* infection; Black, wild type *L. pneumophila* strain Paris; light blue, $\Delta rsmY$ *L. pneumophila* strain Paris; blue, $\Delta rsmZ$ *L. pneumophila* strain Paris; dark blue, $\Delta rsmYZ$ *L. pneumophila* strain Paris; green $\Delta letA$ *L. pneumophila* strain Paris. B) *A. castellanii* infection; Black, wild type *L. pneumophila* strain Paris; light blue, $\Delta rsmZ$ *L. pneumophila* strain Paris carrying the empty plasmid pMMB; red, $\Delta rsmZ$ *L. pneumophila* strain Paris carrying plasmid *prsmZ* with an IPTG inducible promoter; For the complementation experiment all strains were grown in presence of IPTG. C) Infection of THP-1 human macrophages with *Legionella pneumophila* Paris wild type and $\Delta rsmYZ$ *L. pneumophila* strain Paris. The number of viable bacteria within amoebae was evaluated by the standard plate count assay. Results are expressed as $\log_{10}$ ratio CFU Tn/T0. Each time point represents the mean of $\pm$ SD of two independent experiments. Infections were performed at 37°C.

Figure 6. Sodium sensitivity is repressed and pigment production is activated by LetA, RsmY and RsmZ

A) PE phase $\Delta rsmY$, $\Delta rsmZ$, $\Delta rsmYZ$ and $\Delta letA$ mutants of strain Paris are not sensitive to NaCl as compared to PE phase wild type strain Paris. B) RsmZ, RsmY, RsmYZ and LetA activate pigment production in *Legionella*

Figure 7. Electron microscopy of PE phase *Legionella pneumophila* wild type, $\Delta letA$, $\Delta rsmZ$ and $\Delta rsmYZ$

In contrast to the *letA* mutant, mutants in the small ncRNAs synthesize flagella in PE-growth phase. A) wild type, B) $\Delta rsmZ$, C) $\Delta rsmYZ$, D) $\Delta letA$.

Figure 8. RsmZ restores pigment production in a *letA* mutant strain

Wild type *Legionella pneumophila* and a *letA* mutant strain carrying *prsmZ* activate pigment production in PE growth phase, in contrast to a *letA* mutant strain.

Figure 9. Model of the regulatory network governing differentiation of *L. pneumophila* from replicative/non virulent to transmissive/virulent

During the transmissive phase, amino acid starvation triggers the ppGpp synthetase RelA and fatty acid starvation triggers the ppGpp synthetase/hydrolase SpoT to produce the alarmone (p)ppGpp. (p)ppGpp may be sensed by the sensor kinase LetS which then phosphorylates LetA. Phosphorylated LetA binds upstream of the small ncRNAs RsmY and RsmZ and activates their transcription. CsrA that is bound near the ribosomal binding site of its mRNAs targets (e.g. probably LqsR, FleQ and Hfq) inhibits their translation. The presence of RsmY and RsmZ titrate CsrA away from its targets, which then enables translation of the mRNAs, leading to expression of transmissive phenotypes. In contrast flagella biosynthesis does not seem to be dependent on the RsmYZ-CsrA-pathway but is controlled by the LetA/LetS TCS, probably via LetE repression. Furthermore, LetA/LetS influences directly or indirectly c-di-GMP levels which also may influence motility.

Table 1Selection of PE phase induced genes in the *letA* and *rsmYZ* mutants

Gene name	Annotation	$\Delta letA/wt$	$\Delta rsmYZ/wt$
<i>lpp3059</i>	AtpB (H ⁺ -transporting ATP synthase chain a)	2.45	-
<i>lpp3058</i>	AtpE (H ⁺ -transporting ATP synthase chain c)	1.57	-
<i>lpp3057</i>	AtpF (H ⁺ -transporting ATP synthase chain b)	1.55	-
<i>lpp3056</i>	AtpH (H ⁺ -transporting ATP synthase chain delta)	1.78	-
<i>lpp3055</i>	AtpA (H ⁺ -transporting ATP synthase chain alpha)	1.21	-
<i>lpp3054</i>	AtpG (H ⁺ -transporting ATP synthase chain gamma)	2.01	-
<i>lpp3053</i>	AtpD (H ⁺ -transporting ATP synthase beta chain)	2.47	-
<i>lpp3052</i>	AtpC (H ⁺ -transporting ATP synthase epsilon chain)	2.47	-
<i>lpp3039</i>	SodB (Superoxide dismutase)	-	3.11
<i>lpp3038</i>	GlrA (Glutaredoxin-like protein)	2.46	4.34
<i>lpp3037</i>	AhpC (Alkyl hydroperoxide reductase)	2.61	3.21
<i>lpp3033</i>	Major outer membrane protein precursor	3.41	7.74
<i>lpp3032</i>	Major outer membrane protein	4.22	7.52
<i>lpp2835</i>	NADH dehydrogenase I chain B	3.84	2.37
<i>lpp2831</i>	NADH dehydrogenase I chain F	2.35	1.69
<i>lpp2830</i>	NADH dehydrogenase I chain G	3.14	-
<i>lpp2828</i>	NADH-quinone oxidoreductase chain I	1.99	-
<i>lpp2827</i>	NADH-quinone oxidoreductase chain J	2.42	-
<i>lpp2768</i>	50S ribosomal protein L35	3.06	2.88
<i>lpp2689</i>	30S ribosomal subunit protein S2	3.33	1.78
<i>lpp2026</i>	Peptidoglycan-associated lipoprotein precursor	1.79	4.52
<i>lpp1958</i>	Major outer membrane protein	2.16	6.57
<i>lpp1773</i>	Similar to long-chain fatty acid transport protein	3.07	3.16
<i>lpp1731</i>	Thioredoxin reductase	2.77	2.63
<i>lpp1726</i>	FleR	-	3.01
<i>lpp1673</i>	AstB (Succinylarginine dihydrolase)	2.08	1.86
<i>lpp1672</i>	AstD (Succinylglutamic semialdehyde dehydrogenase)	2.46	1.96
<i>lpp1671</i>	AstA (Arginine N-succinyltransferase- beta chain)	2.16	2.21
<i>lpp1517</i>	Pyruvate dehydrogenase E2 component	3.74	2.09
<i>lpp1516</i>	Pyruvate dehydrogenase- (E1 beta subunit)	2.23	1.67
<i>lpp1346</i>	50S ribosomal subunit protein L32	2.39	2.46
<i>lpp1247</i>	RpoS (RNA polymerase sigma factor)	-	3.11
<i>lpp1223</i>	Oxygen-dependent coproporphyrinogen III oxidase	3.14	2.58
<i>lpp1204</i>	CydA (Cytochrome d ubiquinol oxidase subunit I)	3.43	2.69
<i>lpp1205</i>	CydB (Cytochrome d ubiquinol oxidase subunit II)	1.74	1.71
<i>lpp1202</i>	HisG	3.40	-
<i>lpp1201</i>	HisD	3.13	-

Gene name	Annotation	$\Delta\text{letA}/\text{wt}$	$\Delta\text{rsmYZ}/\text{wt}$
<i>lpp1200</i>	HisC	8.50	2.05
<i>lpp1199</i>	HisB	8.65	1.85
<i>lpp1198</i>	HisH	4.74	1.70
<i>lpp1197</i>	HisA	7.79	1.68
<i>lpp1196</i>	HisF	4.00	-
<i>lpp1195</i>	HisI	2.76	1.55
<i>lpp1117</i>	ChiA	4.88	2.23
<i>lpp0867</i>	PpsA (phosphoenolpyruvate synthase)	2.93	1.89
<i>lpp0855</i>	Mip (Macrophage infectivity potentiator)	2.51	3.91
<i>lpp0819</i>	NeuC (N-acetylglucosamine 2-epimerase)	1.65	-
<i>lpp0818</i>	NeuB (N-acetylneuraminic acid conden. enzyme)	2.01	-
<i>lpp0817</i>	NeuA (CMP-N-acetylneuraminic acid synthetase)	2.34	-
<i>lpp0816</i>	HisH (Imidazole glycerol phosphate synthase)	2.91	-
<i>lpp0815</i>	HisF (Imidazole glycerol phosphate synthase)	3.55	2.00
<i>lpp0814</i>	Similar to LPS biosynthesis protein	2.56	2.29
<i>lpp0602</i>	LetE (Transmission trait enhancer protein)	2.71	1.60
<i>lpp0570</i>	OmpH (Outer membrane protein)	3.08	3.54
<i>lpp0532</i>	ProA (Zinc metalloproteinase precursor)	3.01	3.07
<i>lpp0493</i>	similar to Cold shock-like protein CspD	2.84	5.88
<i>lpp0413</i>	50S ribosomal subunit protein L15	2.41	-
<i>lpp0384</i>	50S ribosomal protein L1	2.53	-
<i>lpp0383</i>	50S ribosomal protein L11	1.98	-
<i>lpp0845</i>	Global regulator CsrA	1.66	-

Table 2Selection of PE phase genes down-regulated in the *letA* and *rsmYZ* mutants

Gene name	Known or predicted product	$\Delta letA/wt$	$\Delta rsmYZ/wt$
<i>lpp0728</i>	Similar to acetoacetate decarboxylase	0.04	0.67
<i>lpp0972</i>	Similar to enhanced entry protein EnhA	0.05	0.15
<i>lpp0729</i>	Similar to protein	0.07	0.64
<i>lpp1294</i>	FlaA	0.08	-
<i>lpp1293</i>	FlaG	0.08	-
<i>lpp2096</i>	SdeA	0.10	0.18
<i>lpp0727</i>	NADH-ubiquinone oxidoreductase	0.11	-
<i>lpp1900</i>	Unknown	0.11	0.41
<i>lpp1942</i>	Unknown	0.11	0.34
<i>lpp0197</i>	Similar to adenine specific DNA methylase	0.13	-
<i>lpp2327</i>	<i>Legionella</i> specific protein	0.13	-
<i>lpp0730</i>	Similar to adenylate cyclase	0.14	-
<i>lpp0075</i>	Similar to <i>L. pneumophila</i> LvrA protein	0.14	-
<i>lpp1162</i>	<i>Legionella</i> specific protein	0.14	0.16
<i>lpp1291</i>	FliS	0.14	-
<i>lpp1292</i>	FliD	0.14	-
<i>lpp1453</i>	SidE homolog	0.14	0.22
<i>lpp1641</i>	Putative alpha amylase	0.14	0.23
<i>lpp2200</i>	Unknown	0.15	0.23
<i>lpp1233</i>	FlgK	0.16	1.80
<i>lpp1615</i>	SidE homolog	0.16	0.19
<i>lpp2357</i>	unknown	0.16	-
<i>lpp1341</i>	Some similarity with EnhA protein	0.17	0.17
<i>lpp2174</i>	Similar to EnhC	0.17	0.47
<i>lpp2578</i>	SdcA	0.17	0.26
<i>lpp1002</i>	LidA	0.18	-
<i>lpp1932</i>	RalF	0.18	0.26
<i>lpp2092</i>	SdeC	0.18	-
<i>lpp1290</i>	Similar to EnhA	0.19	0.40
<i>lpp1479</i>	PilB	0.19	0.29
<i>lpp2485</i>	Some similarities with eukaryotic proteins	0.19	0.42
<i>lpp2579</i>	SidC	0.20	0.31
<i>lpp0952</i>	Regulatory protein (GGDEF and EAL)	0.21	-
<i>lpp0969</i>	FlgM (Anti-sigma-28 factor)	0.23	-
<i>lpp1234</i>	FlgL	0.23	1.61
<i>lpp0686</i>	FimU	0.26	0.22
<i>lpp0683</i>	PilX	0.27	0.30

Gene name	Known or predicted product	$\Delta\text{letA}/\text{wt}$	$\Delta\text{rsmYZ}/\text{wt}$
<i>lpp2267</i>	MotB2	0.27	-
<i>lpp0726</i>	Similar to patatin-like esterase	0.28	0.67
<i>lpp1480</i>	PilC	0.28	0.26
<i>lpp1227</i>	FlgE	0.29	3.13
<i>lpp2487</i>	Some similarity with eukaryotic proteins	0.29	0.35
<i>lpp1228</i>	FlgF	0.30	4.02
<i>lpp1925</i>	PatE	0.30	0.34
<i>lpp2161</i>	Unknown	0.30	0.47
<i>lpp2265</i>	PatB	0.30	0.53
<i>lpp2362</i>	Chemiosmotic efflux system B protein B	0.30	-
<i>lpp2363</i>	Chemiosmotic efflux system B protein C	0.30	-
<i>lpp1324</i>	Similar to DNA-binding protein Fis	0.31	6.75
<i>lpp2266</i>	MotA2	0.31	0.62
<i>lpp2888</i>	PatA/VipD	0.31	0.23
<i>lpp0351</i>	Regulatory protein (EAL domain)	0.34	-
<i>lpp0352</i>	Regulatory protein (GGDEF domain)	0.34	0.42
<i>lpp1745</i>	MotA	0.34	-
<i>lpp1723</i>	FliG	0.35	2.01
<i>lpp1657</i>	FliK	0.36	-
<i>rsmY</i>	small ncRNA rsmY	0.36	0.05
<i>lpp2141</i>	Global stress protein GspA	0.37	2.67
<i>lpp1170</i>	Regulatory protein (GGDEF and EAL)	0.37	-
<i>lpp0809</i>	Regulatory protein (GGDEF domain)	0.38	-
<i>lpp2071</i>	Regulatory protein (GGDEF domain)	0.38	0.38
<i>lpp2355</i>	Regulatory protein (GGDEF domain)	0.38	-
<i>lpp0220</i>	Regulatory protein (EAL domain)	0.39	0.39
<i>lpp1744</i>	MotB	0.40	-
<i>lpp0440</i>	Regulatory protein (GGDEF domain)	0.41	0.32
<i>lpp2477</i>	Regulatory protein (GGDEF domain)	0.41	0.40
<i>lpp1754</i>	FliO	0.43	2.53
<i>lpp0029</i>	Regulatory protein (GGDEF and EAL)	0.44	0.36
<i>lpp1310</i>	EnhC homolog	0.45	-
<i>lpp1747</i>	FleN	0.45	2.92
<i>lpp2635</i>	FlhB	0.46	-
<i>lpp0166</i>	LvrA	0.47	-
<i>lpp1755</i>	FliN	0.47	2.76
<i>lpp2637</i>	SidF	0.48	0.44
<i>lpp2082</i>	Ankyrin repeat and a F-box	0.49	0.61
<i>lpp0699</i>	RtxA	0.50	0.37

Gene name	Known or predicted product	$\Delta\text{letA}/\text{wt}$	$\Delta\text{rsmYZ}/\text{wt}$
<i>lpp0150</i>	SdhB	0.51	0.34
<i>lpp1475</i>	Regulatory protein (GGDEF and EAL)	0.51	0.53
<i>lpp1748</i>	FlhF	0.51	2.54
<i>lpp2693</i>	EnhB	0.51	1.74
<i>lpp0304</i>	SidE	0.52	0.36
<i>lpp1746</i>	FliA (Sigma factor 28)	0.52	-
<i>lpp2788</i>	LqsR (Response regulator)	0.52	1.74
<i>lpp1722</i>	FliH	0.53	1.98
<i>rsmZ</i>	small ncRNA rsmZ	0.54	0.01
<i>lpp2095</i>	SdeB	0.55	0.47
<i>lpp1232</i>	FlgJ	0.57	2.16
<i>lpp0542</i>	RpoN (sigma-54 factor)	0.58	-
<i>lpp2546</i>	SdbB	0.58	0.38
<i>lpp1231</i>	FlgI	0.60	2.47
<i>lpp1226</i>	FlgD	0.61	3.66
<i>lpp0009</i>	Similar to host factor-1 protein Hfq	0.65	1.67
<i>lpp2692</i>	EnhC	0.68	-

Table 3

Flagella gene expression of *letA* and *rsmYZ* mutants as compared to wild type *L. pneumophila* strain Paris

Gene name	<i>Lpp</i>	Known or predicted product		$\Delta letA/ wt$	$\Delta rsmYZ/ wt$	$\Delta letA/\Delta rsmYZ$
<i>rpoN</i>	<i>lpp0542</i>	RNA polymerase sigma-54 factor	I	0.58	-	-
<i>fleQ</i>	<i>lpp0915</i>	Transcriptional regulator FleQ	I	-	-	-
<i>flgN</i>	<i>lpp0968</i>	Unknown	III	0.35	-	-
<i>flgM</i>	<i>lpp0969</i>	Anti-sigma-28 factor	III	0.23	-	7.16
<i>flgA</i>	<i>lpp0970</i>	Flagellar basal body P-ring biosynthesis	IIb	0.66	-	-
<i>flgB</i>	<i>lpp1224</i>	Flagellar basal-body rod protein FlgB	IIb	-	4.17	-
<i>flgC</i>	<i>lpp1225</i>	Flagellar basal-body rod protein FlgC	IIb	-	3.23	-
<i>flgD</i>	<i>lpp1226</i>	Flagellar basal-body rod modification	IIb	0.61	3.66	3.75
<i>flgE</i>	<i>lpp1227</i>	Flagellar hook protein FlgE	IIb	0.29	3.13	19.7
<i>flgF</i>	<i>lpp1228</i>	Flagellar biosynthesis protein FlgF	IIb	0.30	4.02	14.8
<i>flgG</i>	<i>lpp1229</i>	Flagellar biosynthesis protein FlgG	IIb	-	5.20	-
<i>flgH</i>	<i>lpp1230</i>	Flagellar L-ring protein precursor FlgH	IIb	-	4.62	5.50
<i>flgI</i>	<i>lpp1231</i>	Flagellar P-ring protein precursor FlgI	IIb	0.60	2.47	4.59
<i>flgJ</i>	<i>lpp1232</i>	Flagellar biosynthesis protein FlgJ	IIb	0.57	2.16	21.6
<i>flgK</i>	<i>lpp1233</i>	Flagellar hook-associated protein 1	IIb	0.16	1.80	16.4
<i>flgL</i>	<i>lpp1234</i>	Flagellar hook-associated protein FlgL	IIb	0.23	1.61	5.50
<i>flhS</i>	<i>lpp1291</i>	Flagellar protein FlhS	IV	0.14	-	11.3
<i>flhD</i>	<i>lpp1292</i>	Flagellar hook-associated protein 2	IV	0.14	-	13.0
<i>flaG</i>	<i>lpp1293</i>	Unknown	IV	0.08	-	20.6
<i>flaA</i>	<i>lpp1294</i>	Flagelline	IV	0.08	-	18.8
<i>flhK</i>	<i>lpp1657</i>	Flagellar hook-length control protein FlhK	IIb	0.36	-	2.54
<i>flhJ</i>	<i>lpp1720</i>	Flagellar protein FlhJ	IIa	-	-	-
<i>flhI</i>	<i>lpp1721</i>	Flagellum-specific ATP synthase FlhI	IIa	-	-	-
<i>flhH</i>	<i>lpp1722</i>	Polar flagellar assembly protein FlhH	IIa	0.53	1.98	-
<i>flhG</i>	<i>lpp1723</i>	Flagellar motor switch protein	IIa	0.35	2.01	2.50
<i>flhF</i>	<i>lpp1724</i>	Flagellar M-ring protein	IIa	0.42	2.23	2.99
<i>flhE</i>	<i>lpp1725</i>	Flagellar hook-basal body complex protein	IIa	-	2.58	-

Gene name	<i>Lpp</i>	Known or predicted product		$\Delta\text{letA}/\text{wt}$	$\Delta\text{rsmYZ}/\text{wt}$	$\Delta\text{letA}/\text{rsmYZ}$
<i>fleR</i>	<i>lpp1726</i>	Two-component response regulator	IIa	-	3.00	-
<i>fleS</i>	<i>lpp1727</i>	Sensor histidine kinase	IIa	-	-	-
<i>motB</i>	<i>lpp1744</i>	Chemotaxis MotB protein	III	0.40	-	2.78
<i>motA</i>	<i>lpp1745</i>	Flagellar motor protein MotA	III	0.34	-	4.32
<i>flaA</i>	<i>lpp1746</i>	Sigma factor 28	III	0.52	-	-
<i>fleN</i>	<i>lpp1747</i>	Flagellar synthesis regulator	IIb	0.45	2.92	3.61
<i>flhF</i>	<i>lpp1748</i>	Flagellar biosynthesis protein FlhF	IIb	0.51	2.54	-
<i>flhA</i>	<i>lpp1749</i>	Flagellar biosynthesis protein FlhA	IIa	-	-	-
<i>flhB</i>	<i>lpp1750</i>	Flagellar biosynthetic protein FlhB	IIa	0.46	-	-
<i>fliR</i>	<i>lpp1751</i>	Flagellar biosynthetic protein FliR	IIa	-	-	-
<i>fliQ</i>	<i>lpp1752</i>	Flagellar biosynthetic protein FliQ	IIa	-	-	-
<i>fliP</i>	<i>lpp1753</i>	Flagellar biosynthetic protein FliP	IIa	-	1.52	-
<i>fliO</i>	<i>lpp1754</i>	Flagellar protein FliO	IIa	0.43	2.53	-
<i>fliN</i>	<i>lpp1755</i>	Flagellar motor switch protein FliN	IIa	0.47	2.76	3.53
<i>fliM</i>	<i>lpp1756</i>	Flagellar motor switch protein FliM	IIa	0.47	2.37	3.30
<i>motA2</i>	<i>lpp2266</i>	Proton conductor component of motor,	III	0.31	-	3.14
<i>motB2</i>	<i>lpp2267</i>	Flagellar motor protein	III	0.27	-	5.86
<i>flhB</i>	<i>lpp2635</i>	Putative part of flagellar apparatus	III	0.46	-	-
<i>motY</i>	<i>lpp3034</i>	Sodium-type flagellar protein MotY	IV	-	-	-

Table 4

Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant properties ¹	Reference
<i>E. coli</i>		
DH5α		Invitrogen
BL21		Invitrogen
<i>L. pneumophila</i>		
CIP 107629	Virulent <i>L. pneumophila</i> serogroup 1, strain Paris	[5]
JR32	Virulent <i>L. pneumophila</i> serogroup 1, strain Philadelphia	[65]
Δ <i>letA</i>	Paris <i>letA</i> ::Km	This study
Δ <i>letS</i>	Paris <i>letS</i> ::Km	This study
Δ <i>rsmY</i>	Paris <i>rsmY</i> ::Cm	This study
Δ <i>rsmZ</i>	Paris <i>rsmZ</i> ::Km	This study
Δ <i>rsmYZ</i>	Paris <i>rsmYZ</i> ::Km::Cm	This study
Plasmids		
pGEM-T Easy	Cloning of PCR products, Ap	Promega
pMMB207C	<i>Legionella</i> expression vector, Δ <i>mobA</i>	[35]
pMMB207C–RBS–lcsC	<i>Legionella</i> expression vector for LcsC (with ribosome binding site)	[55]
pTS-10	pMMB207C–RBS	[40]
<i>prsmY</i>	pMMB207C, <i>rsmY</i>	This study
<i>prsmZ</i>	pMMB207C, <i>rsmZ</i>	This study
<i>pletA</i> _Strep	pMMB207C, <i>letA</i> with C-terminal Strep-Tag	This study
pIVEX2.3d	Expression of C-terminal hexa-histidine tag fusions	Roche Diagnostics
pIVEX2.3d– <i>csrA</i>	pIVEX2.3d, <i>csrA</i>	This study

¹ Abbreviations: Ap, ampicillin resistance; Cm, chloramphenicol resistance; Km, kanamycin resistance; Sm, Streptomycin resistance.