# Control of Flagellar Gene Regulation in *Legionella pneumophila* and Its Relation to Growth Phase[▽][†]

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The bacterial pathogen *Legionella pneumophila* responds to environmental changes by differentiation. At least two forms are well described: replicative bacteria are avirulent; in contrast, transmissive bacteria express virulence traits and flagella. Phenotypic analysis, Western blotting, and electron microscopy of mutants of the regulatory genes encoding RpoN, FleQ, FleR, and FliA demonstrated that flagellin expression is strongly repressed and that the mutants are nonflagellated in the transmissive phase. Transcriptome analyses elucidated that RpoN, together with FleQ, enhances transcription of 14 out of 31 flagellar class II genes, which code for the basal body, hook, and regulatory proteins. Unexpectedly, FleQ independent of RpoN enhances the transcription of *fliA* encoding sigma 28. Expression analysis of a *fliA* mutant showed that FliA activates three out of the five remaining flagellar class III genes and the flagellar class IV genes. Surprisingly, FleR does not induce but inhibits expression of at least 14 flagellar class III genes on the transcriptional level. Thus, we propose that flagellar class II genes are controlled by FleQ and RpoN, whereas the transcription of the class III gene *fliA* is controlled in a FleQ-dependent but RpoN-independent manner. However, RpoN and FleR might influence flagellin synthesis on a posttranscriptional level. In contrast to the commonly accepted view that enhancer-binding proteins such as FleQ always interact with RpoN to fullfill their regulatory functions, our results strongly indicate that FleQ regulates gene expression that is RpoN dependent and RpoN independent. Finally, FliA induces expression of flagellar class III and IV genes leading to the complete synthesis of the flagellum.

Bacterial flagella are highly complex molecular machines. They are surface organelles assembled from over 40 different protein components that mediate bacterial motility. To ensure maximal efficiency and accuracy during flagellar biogenesis, bacteria use hierarchical regulatory networks involving transcriptional and posttranscriptional mechanisms to control the ordered expression of the individual components of the flagellar organelle. Although significant differences exist between the regulatory mechanisms used by different bacteria, a salient feature in all cases is that the flagellar genes can be classified based upon their temporal gene expression and on their dependence on various nested transcriptional regulators (for a recent review, see reference 33).

The bacterial pathogen *Legionella pneumophila* lives in natural and manmade water systems and replicates intracellularly within aquatic protozoa (41). When inhaled by humans, *L. pneumophila* is able to survive and replicate within alveolar macrophages (28). After entry into host cells, *L. pneumophila* inhibits phagolysosomal fusion (26, 27) and establishes a specialized *Legionella*-containing vacuole (LCV) surrounded by endoplasmic reticulum in which *L. pneumophila* represses transmissive traits and starts to replicate (15, 37, 43). During the bacterial late replicative phase, the LCV merges with lysosomes (44). Finally, induced by a nutrient decline the bacteria enter the transmissive phase, which is reflected by a major shift in gene expression (2, 8, 14, 19, 37, 51). In the transmissive phase, *L. pneumophila* expresses many virulence-associated traits promoting the release of the bacteria and infection of a new host (2, 3, 23, 36, 42, 45, 46, 51). One striking feature of transmissive *L. pneumophila* is the expression of a single monopolar flagellum composed of the flagellin subunit FlaA. The flagellum mediates invasivness of *L. pneumophila* for human macrophage-like cell lines and cytotoxicity to macrophages (13, 20). Furthermore, it was shown that flagellin sensed by nonpermissive mouse macrophages mediates cell death by activating the cytosolic Naip5 (Birc1e) receptor (35, 40). Expression of the flagellum is dependent on the regulatory circuit controlling phase transition (for a review, see reference 1) and different environmental factors (21, 22).

Several studies have been undertaken to understand the regulatory mechanisms governing this life cycle switch, including the regulation of flagellar gene expression. The two-component system LetA/LetS, a system homologous to BarA/UvrY of *Escherichia coli* and RsmA/RsmS of *Pseudomonas aeruginosa*, was shown to have an important role in the regulation of the life cycle switch and in flagellar gene expression (17, 20, 32, 36, 42). It is suggested that LetA/LetS responds to the alarmone molecule (p)ppGpp, synthesized by RelA and SpoT (8,

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19, 20, 51). Phosphorylated LetA then induces the expression of two small regulatory RNAs, RsmY and RsmZ, which in turn sequester CsrA, an RNA binding protein present in many bacteria. In consequence CsrA is released from its target mRNAs, allowing for the expression of transmissive traits (37, 42). Flagellar gene expression is thought to be regulated by this CsrA-dependent pathway; however, recently it was shown that the RsmYZ-CsrA pathway may not be the main or only regulatory circuit governing flagellar synthesis and that RpoS, LetA, LetE, and probably cyclic-di-GMP (c-di-GMP) levels have important regulatory influence on motility in *L. pneumophila* (42). In addition, the response regulator LqsR was shown to be involved in expression of several flagellar genes, including the flagellin gene (*flaA*). However, an *L. pneumophila lqsR* mutant strain had no obvious structural defect of the flagellum (47).

Based on the presence of homologs of the regulatory proteins FleQ, FleR, RpoN, and FliA of *Pseudomonas aeruginosa* in the *L. pneumophila* genomes, it was suggested that the flagellar gene regulation cascade in *L. pneumophila* is similar to that described in *P. aeruginosa* (2, 24, 25, 29). FleQ and RpoN of *L. pneumophila* are indeed involved in flagellar gene regulation by enhancing the expression of the flagellar class II genes *fliM*, *fleN*, and *fleSR*, and FliA controls the expression of the flagellar class IV genes *flaAG*, *fliDS*, and *motY* (2, 29). However, the exact regulatory circuit governing flagellar synthesis remains to be determined.

Here we investigated the role of FleQ, FleR, RpoN, and FliA in the regulation of the expression of transmissive traits in *L. pneumophila* strain Paris, particularly its effect on flagellation. Mutants with mutations in the regulatory genes coding FleQ, RpoN, FleR, and FliA are not flagellated. Expression profiling of these mutants showed that FleQ—but not FleR—enhances flagellar class II gene transcription together with RpoN and expression of the flagellar class III gene *fliA* encoding the $\sigma^{28}$ factor FliA. Surprisingly, FleQ regulates *fliA* independently of RpoN. FliA controls the expression of flagellar class III and IV genes, completing the flagellar assembly. Based on these results, we established a refined model of the complex regulatory cascade governing flagellum biosynthesis.

## MATERIALS AND METHODS

**Bacterial strains, media, and mutant construction.** *L. pneumophila* was cultured in *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES)-buffered yeast extract broth or on ACES-buffered charcoal-yeast extract (BCYE) agar at 37°C. The *rpoN* and *fleQ L. pneumophila* strain Paris mutants were constructed using the corresponding mutants of *L. pneumophila* strain Corby (29) as a template for amplifying the PCR product carrying a kanamycin cassette. The PCR product was then transformed into strain Paris as previously described (13). To construct the *L. pneumophila fleR* mutant in strain Paris, the *fleR* gene was amplified by PCR using primers fleR-F (5′-ACAAAAGCACAAGGTACCG GC-3′) and fleR-R (5′-AGCGGGTCTTTAAACTATCTGCTG-3′). The PCR fragment was purified (Qiaquick PCR purification kit; Qiagen) and ligated into pGEM-T Easy (Promega). By inverted PCR, using primers carrying XbaI restriction sites (fleR-XbaI-F, 5′-TTCTAGAATAGTCTCAGCC AACGCTTC-3′; and fleR-XbaI-R, 5′-TTCTAGAAACAACCAATAGTTCC AGTCA-3′), the sequence encoding the $\sigma^{54}$ interaction domain of *fleR* was deleted, and after self-ligation, the kanamycin cassette was inserted into the XbaI restriction site. The resulting plasmid, pCha11, was used as template for amplifying the PCR product, which was transformed into strain Paris as previously described (13). For complementation of the *fleQ* mutant strain, the *fleQ* gene with its native promoter was amplified from genomic DNA using primers fleQ-F (5′-CCGTTATAATGATTACCGAGTG GA-3′) and fleQ-R

| Strain or plasmid | Relevant characteristic(s)[a] | Source or reference |
|---|---|---|
| **Strains** | | |
|   *E. coli* DH5α | | Invitrogen |
|   *L. pneumophila* | | |
|     CIP 107629 | Virulent *L. pneumophila* serogroup 1, strain Paris | 4 |
|     Corby | Virulent *L. pneumophila* serogroup 1, strain Corby | 30 |
|     Δ*fleQ* mutant | Paris *fleQ*::Km$^r$ | This study |
|     Δ*rpoN* mutant | Paris *rpoN*::Km$^r$ | This study |
|     Δ*fleR* mutant | Paris *fleR*::Km$^r$ | This study |
|     Δ*fliA* mutant | Paris *fliA*::Km$^r$ | 2 |
| **Plasmids** | | |
|   pGEM-T Easy | Cloning of PCR products; Ap$^r$ | Promega |
|   pBC SK | Complementation vector; Cm$^r$ | Stratagene |

$^a$ Abbreviations: Km$^r$, kanamycin resistance; Ap$^r$, ampicillin resistance; Cm$^r$, chloramphenicol resistance.

(5′-TCCCAGTTAAGCGAATCCGTGAT-3′), cloned into pGEM-T Easy (Promega), excised as an EcoRI fragment, and cloned into the vector pBC SK (Stratagene) to obtain pChA15. The strains and plasmids used are listed in Table 1.

**Culture and infection of MH-S cells.** The MH-S murine alveolar macrophage cells were maintained in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum (FCS). Prior to infection, the cells were adhered to 12-well tissue culture plates at a concentration of $5 \times 10^5$ cells/ml for 2 h in 5% $CO_2$ at 37°C. The resulting monolayers were infected with *L. pneumophila* (infectivity ratio, 10 bacteria per cell) for 60 min, washed to remove nonphagocytized 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 (CFU) in cell lysates was determined by standard plate counts on BCYE agar.

**Infectivity assay.** Cells were seeded in 24-well tissue culture dishes at $5 \times 10^5$ cells per well 24 h prior to the experiments. To test the contributions of FleQ, FleR, and RpoN to the infectivity in MH-S cells, cell monolayers were incubated for 2 h after a mild centrifugation with each strain at a multiplicity of infection (MOI) of 0.3. After being washed three times with phosphate-buffered saline (PBS) and lysed by incubation for 10 min in 1 ml sterile water, dilutions were plated on BCYE agar to determine CFU. Cell-associated viable microbes were enumerated as CFU and expressed as the mean percentage of the initial microbial inoculum recovered ± standard deviation (SD), as calculated from three independent experiments.

**Electron microscopy.** Bacteria were grown for 3 days on BCYE agar. Then bacteria were suspended in distilled water and applied to Pioloform (Merck)-coated copper grids. After sedimentation of the bacteria and removal of the remaining fluid, the samples were shadowed with platinum-palladium and examined with a transmission electron microscope (EM10; Zeiss) at 60 kV.

**Immunoblotting (Western blotting) procedures.** Bacteria were grown in liquid broth to an optical density (OD) of 4.2. Cells were resuspended in lysis buffer (100 mM Tris-HCl [pH 8.0], 200 mM NaCl, 2 mM dithiothreitol [DTT]), 5% glycerol, and a cocktail of protease inhibitors (Sigma) and disrupted by sonication. After centrifugation (14,000 rpm, 30 min, 4°C), soluble protein in supernatant was collected and the protein concentration was determined according to the method of Bradford (1a) using BSA as a standard. Fifty micrograms total protein of each sample was mixed with Laemmli buffer, heated at 100°C for 5 min, and loaded on a 13% polyacrylamide-SDS gel. SDS-PAGE was performed as described by Laemmli (31). Then the proteins were transferred to nitrocellulose by electroblotting. Flagellin (FlaA) and FleQ were detected by an indirect immunostaining procedure with a rabbit polyclonal antiserum specific for *L. pneumophila* flagellin (29) or rabbit polyclonal antiserum specific for *L. pneumophila* FleQ and goat anti-rabbit immunoglobulin G coupled to horseradish peroxidase. Staining of immunoreactive bands was performed in the presence of hydrogen peroxide and 4-chloro-1-naphthol.

**RNA isolation and labeling for array hybridization.** For array hybridization and real-time PCR, total RNA was extracted as described previously (34). For *in*

TABLE 2. Primers used for real-time PCR

| Gene name | Sequence of: | |
| --- | --- | --- |
| | Forward primer | Reverse primer |
| csrA | 5′-TTTGACTCGGCGTATAGGTG-3′ | 5′-AGCGAACTTGATTGCCTTTT-3′ |
| flaA | 5′-CGGCAACAGGAACAGAAGTA-3′ | 5′-TTTGGCATAGGCAGACGTAG-3′ |
| fleN | 5′-GCATTTCCACATTCTGGCTA-3′ | 5′-ACTGTCCTGAGACCCGAAAC-3′ |
| flgC | 5′-GTCAGTCCAGGAGAATGCAA-3′ | 5′-AGTTTCACGCCAGCCTTAAT-3′ |
| fliA | 5′-GGTAAAACGCATTGCACATC-3′ | 5′-TCATAATGCCTTGCTGCTTC-3′ |
| fliM | 5′-CTTACGAGAAATCCCAATCTCTATGTTA-3′ | 5′-CGAAAAACTCCAAGATCAAGCAAAAATG-3′ |
| gyrA | 5′-TATTCCTGGCCCTGATTTTC-3′ | 5′-GCCTGAACTTTCATCCGTTT-3′ |
| letA | 5′-TAGATGGGTGGGAAGTGACA-3′ | 5′-AGGGAGAGGATCTGAGCAAA-3′ |
| lpp0952 | 5′-AGCGTGTGGGATCACAAATA-3′ | 5′-ACCATGTGTACTGGCCTCAA-3′ |
| motA | 5′-TTGGCCTTCTCTCCCTAGAA-3′ | 5′-TCTGCTTCCAAAACTTGTCG-3′ |

vitro experiments, the *L. pneumophila* strains were grown in broth and harvested for RNA isolation during the late replicative phase (OD at 600 nm [$OD_{600}$] = 3.3, equivalent to late exponential growth phase) and the transmissive phase ($OD_{600}$ = 4.0, equivalent to postexponential growth phase) (see Fig. S1 in the supplemental material). RNA was reverse transcribed and indirectly labeled with Cy5 or Cy3 as described by the manufacturer (Amersham Biosciences).

**Array hybridization.** For transcriptome analysis, the previously described *L. pneumophila* multiple genome microarrays were used (2). Array hybridization was performed following the manufacturers' recommendations (Corning) using 250 pmol of Cy3- and Cy5-labeled cDNA. Two biological replicates as well as a dye swap were carried out for each condition. Slides were scanned on a GenePix 4000A scanner (Axon Instruments). Laser power and/or a photomultiplier tube were adjusted to balance the two channels. The resulting files were analyzed using Genepix Pro 4.0 software. Spots with high local background fluorescence, slide abnormalities, or weak intensity were excluded.

**Data analysis and statistics.** For normalization and differential analysis, the R software (http://www.R-project.org/) was used. A loess normalization (50) 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 [12]), together with the Benjamini and Yekutieli (39) P value adjustment method. If not stated otherwise, only differentially expressed genes with a 2-fold change, meeting a P value of ≤0.001, were taken into consideration. Empty and flagged spots were excluded, and only genes with no missing values were analyzed.

**Accession numbers.** 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). The corresponding genes of strain Corby (*lpc*) are available via GenBank (accession no. CP000675). The complete data set 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 accession no. A-MEXP-1744.

**Real-time PCR.** Real-time PCR was conducted for confirming the transcriptome results on the total cellular RNAs in the same conditions. The primers used are listed in Table 2. These real-time experiments were performed as described previously (2). Briefly, 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 were 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, and 40 cycles at 95°C for 15 s and 60°C for 1 min. Each real-time PCR assay was performed twice using three different cDNA dilutions (150 ng to 1.5 ng). The quantity of cDNA for each target gene was normalized to the quantity of *csrA* cDNA in each sample. *csrA* is considered (and has been 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 (threshold cycle [$\Delta\Delta C_T$]). To check if contaminating chromosomal DNA was present, each sample was tested in control reactions that did not contain reverse transcriptase.

**RNA isolation and primer extension.** For primer extension experiments, total RNA was extracted by using a High Pure RNA isolation kit (Roche, Mannheim, Germany). Additionally, purified RNA was incubated with 300 U of DNase I (Roche) per ml at room temperature for 10 min and repurified using an RNeasy minikit (Qiagen). The 5′-IRD800-labeled primers flgB-PE (5′-CGGTGTATTA ACATTGGCTATGT-3′) and fleS-PE (5′-GGTTTGACTGCACAAGTTTGAT A-3′) (MWG-Biotech) were complementary to downstream regions of the *fleS* and *flgB* promoters, respectively. Extension reactions were carried out by using the Superscript II reverse transcriptase kit (Invitrogen). The gene-specific primer (4-pmol final concentration) was annealed with 5 to 10 μg total RNA in a volume of 20 μl containing 0.8 mM deoxynucleoside triphosphate (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 reverse transcriptase 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 $MgCl_2$, and 2.5 mM dithiothreitol) and incubated at 42°C for 90 min. Reactions were stopped by incubation at 70°C for 15 min, and the remaining RNA was removed at 37°C for 20 min with RNase H (Invitrogen). Nucleic acids were cautiously concentrated by precipitation and dissolved in 2 μl $H_2O$ and 2 μl formamide loading dye (Amersham Bioscience). Aliquots of the samples were applied to 4.3% polyacrylamide–urea Long Ranger (FMC Bioproducts, Rockland, ME) 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 data were processed by using the software supplied (MWG-Biotech). Reference sequencing reactions (A, C, G, and T) of cloned promoters, performed with the Thermo Sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Biosciences), were initiated using the same primers.

## RESULTS

***L. pneumophila* fleQ, fleR, and rpoN mutant strains are not flagellated.** FleQ, FleR, and RpoN of *L. pneumophila* are predicted to participate in the regulation of flagellar biosynthesis, similar to their homologs in *P. aeruginosa*. To unveil the regulation of flagellar genes by FleQ, FleR, and RpoN in *L. pneumophila*, we constructed *fleQ*, *fleR*, and *rpoN* mutants by inserting a kanamycin cassette into each of these genes. Comparison of growth properties of the wild-type (WT) and the mutant strains grown in broth or during infection of the macrophage cell line MH-S revealed no significant differences (data not shown). However, when infectivity was measured by enumerating cell-associated CFU after a 2-h incubation, the nonmotile *fleQ*, *fleR*, and *rpoN* mutant strains did not infect as efficiently as the WT, similar to the nonmotile *fliA* mutant (Fig. 1). Furthermore, we observed the effects of the gene deletions on motility and flagellation under both the light and electron microscopes. When compared to the *L. pneumophila* wild-type strain, it became evident that the three mutants were nonmotile, as judged by light microscopy, and nonflagellated, as evidenced by electron microscopy observation (see Fig. S2 in the supplemental material). Western blot analysis demonstrated that expression of flagellin (FlaA), the major flagellar subunit,
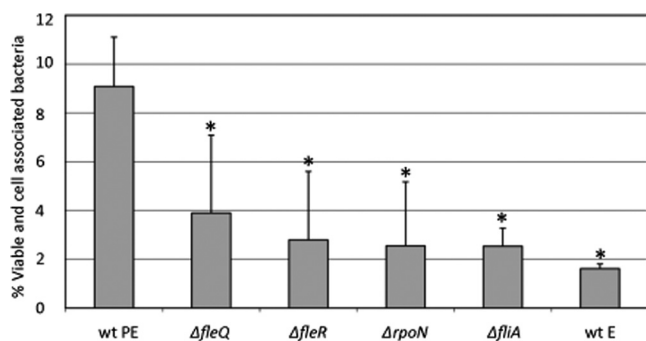
FIG. 1. The infectivity of the *L. pneumophila* Δ*fleQ*, Δ*fleR*, Δ*rpoN*, and Δ*fliA* mutant strains is reduced in MH-S macrophages. To test the contributions of the flagellar regulon to the infectivity in macrophages, MH-S monolayers were incubated for 2 h after a mild centrifugation, with each strain at an MOI of 0.3. Cell-associated viable *L. pneumophila* bacteria were enumerated as CFU and expressed as the mean percentage of the initial microbial inoculum recovered ± SD, calculated from six to nine independent experiments. The bars represent percentages of viable and cell-associated *L. pneumophila* (infectivity). PE, postexponetial growth phase; E, exponential growth phase. Asterisks indicate statistically significant differences (*, $P < 0.001$ by a two-tailed Student's *t* test) in comparison to WT PE-phase *L. pneumophila*.
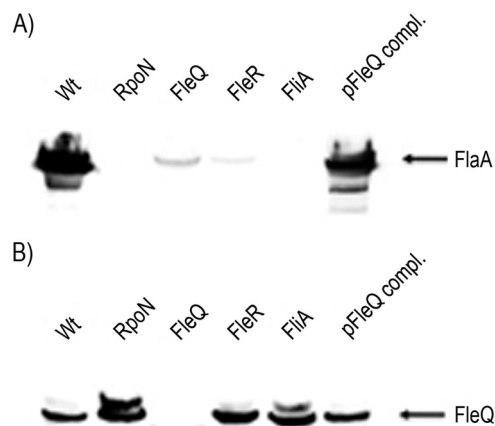


FIG. 2. Expression of flagellin and FleQ of *L. pneumophila* WT strain Paris; the *rpoN*, *fleQ*, *fleR*, and *fliA* mutant strains; and the complemented *fleQ* mutant strain. FleQ and FlaA were visualized by Western blot analysis of whole-cell lysates from liquid cultures grown to an OD of 4.2 (PE phase) using anti-FlaA (A) and anti-FleQ (B) antisera. Lane 1, wild-type (Wt) strain Paris; lane 2, *L. pneumophila* strain Paris Δ*rpoN* mutant; lane 3, *L. pneumophila* strain Paris Δ*fleQ* mutant; lane 4, *L. pneumophila* strain Paris Δ*fleR* mutant; lane 5, *L. pneumophila* strain Paris Δ*fliA* mutant; and lane 6, *L. pneumophila* strain Paris Δ*fleQ* mutant complemented (compl.) with native FleQ.

is strongly repressed in all mutant strains. Complementation of the *fleQ* mutant with the native *fleQ* gene restored flagellin and FleQ expression (Fig. 2). Thus, in contrast to the *L. pneumophila* wild-type strain, the *fleQ*, *fleR*, and *rpoN* mutants do not synthesize flagella in the transmissive phase.

**FleQ, FleR, and RpoN have a global effect on *L. pneumophila* gene expression.** Flagellar gene expression starts in the replicative phase and is turned off after completion of the flagellum in the transmissive phase. It was previously shown that FliA controls the final step of the flagellar biosynthesis (2, 21); however, other regulatory proteins involved in flagellar biosynthesis were not analyzed yet. By *in silico* analysis of the four *L. pneumophila* genomes sequenced (4, 5, 18), 46 genes organized in 10 genomic regions were predicted to participate in the flagellar biosynthesis or its regulation. Their expression starts in replicative phase and stops with the completion of the flagellum in the transmissive phase (2, 21, 22, 24). Here we selected for further analysis four regulatory proteins predicted to be implicated in flagellar gene regulation. We determined the impact of FleQ, FleR, and RpoN on gene expression by comparing the gene expression profiles of Δ*fleQ*, Δ*fleR*, and Δ*rpoN* strains to those of their isogenic wild-type strain by using whole-genome microarrays in the replicative and transmissive phases. Furthermore, the regulatory role of FliA in the transmissive phase was established.

Analysis of the global gene expression program of each of the three mutants as compared to the wild-type strain showed that during the replicative phase transcription of 34, 64, and 22 genes and during the transmissive phase transcription of 144, 269, and 261 genes were significantly differentially regulated in the *fleQ*, *fleR*, and *rpoN* mutants, respectively (Table 3). During the replicative phase, the expression of 1 to 2% of the 3,077 predicted *L. pneumophila* strain Paris genes is altered in each of the mutant strains, and during the transmissive phase, the expression of 4 to 9% of these genes is altered. Hence, our

results substantiate that FleQ, FleR, and RpoN have a great influence on the global gene expression in *L. pneumophila*. In contrast, only 43 genes (~1% of the predicted *L. pneumophila* genes) show an altered expression level during the transmissive phase in the *fliA* mutant. Real-time PCR analysis of selected genes validated the microarray results (see Fig. S3 in the supplemental material). For a complete list of significantly down- and upregulated genes, see Tables S1 to S7 in the supplemental material. The complete data set is available at http://genoscript.pasteur.fr.

**Transcriptional analyses of *fleQ*, *fleR*, *rpoN*, and *fliA* mutants identified FleQ as the master regulator of flagellar gene expression.** The σ⁵⁴ factor RpoN is known to initiate transcription of genes with σ⁵⁴ promoters in a concerted action with enhancer-binding proteins (for a review, see reference 48). Based on sequence similarity, the proteins FleQ and FleR of *L. pneumophila* are predicted to be enhancer-binding proteins that function together with RpoN (29). When analyzing the global transcriptional changes observed in the *rpoN*, *fleQ*, and *fleR* mutants compared to those in the wild-type strain, we identified, as expected, a set of RpoN-regulated genes that overlapped with those regulated by FleQ and FleR (Table 4). Genes coordinately regulated by FleQ and RpoN were nearly exclusively flagellar genes. Surprisingly, numerous genes regulated by FleQ were independent of RpoN, e.g., *fliA*. Furthermore, among the genes regulated by FleR and RpoN together, only a few were associated with flagellation. Instead, FleR and RpoN specifically controlled transcription of genes associated with protein biosynthesis. For instance, out of the 71 coordinately downregulated genes during the transmissive phase in the *fleR* and the *rpoN* mutants, 11 genes encoded ribosomal proteins.

In contrast to what was observed for FleQ, FleR, and RpoN,

TABLE 3. Number of genes showing altered expression in the wild-type strain compared to the mutant strains in the replicative and transmissive growth phases

| Growth phase | Mode of regulation | No. of genes showing altered expression in mutant[a] | | | |
| --- | --- | --- | --- | --- | --- |
| | | Δ*fleQ* | Δ*fleR* | Δ*rpoN* | Δ*fliA* |
| Replicative | Induced | 4 | 37 (3) | 3 | Not determined |
| | Repressed | 30 (22) | 26 (1) | 27 (16) | Not determined |
| Transmissive | Induced | 51 | 93 (13) | 101 (3) | 0 |
| | Repressed | 92 (15) | 178 | 159 (5) | 29 (9) |

[a] Numbers in parentheses represent flagellar genes.

only a very limited set of genes was affected by FliA, as in the *fliA* mutant expression of only 43 genes was altered. Among those, 10 had been previously described as belonging to the FliA regulon, as judged from analysis of gene expression of the *fliA* mutant during infection of *Acanthamoeba castellani* (2). Due to the FleQ-dependent *fliA* expression, the set of FliA- and FleQ-regulated genes shows a strong overlap: expression of the four flagellar operons *flaAG*, *flgMN*, *fliS*, and *flgL* and of the five nonflagellar genes *lpp1290* (encoding a protein similar to the enhanced entry protein EnhA), *lpp0952* (encoding a regulatory protein with GGDEF and EAL domains), *lpp0197*, and *lpp2282* was coordinately repressed in the *fleQ* and *fliA* mutants.

Investigation of the transcription level of genes located downstream of the kanamycin cassette insertion into *fleQ*, *fleR*, and *rpoN*, respectively, showed that interruption of *fleQ* did not led to missing transcription of the downstream genes, but kanamycin insertion into the *rpoN* mutant is probably responsible for missing transcription of *lpp0541* (encoding a σ⁵⁴ modulation protein). It was hypothesized previously that *fleR*, *fleS*, and *fliEFGHIJ* are expressed as an operon (24). However, the kanamycin cassette insertion into *fleR* led to missing transcription of the downstream gene *fliE* (encoding a flagellar basal body protein) but did not change the expression of the more distal genes, *fliFGHIJ*. Thus, either the insertion had a polar effect or FleR is required for *fliE* expression. The transcriptional level of mutated genes and the putative polar effects were not taken into consideration for data analyses.

**Transcriptional regulation of *L. pneumophila* motility genes is mainly governed by FleQ, RpoN and FliA.** The influence of FleQ (encoded by the flagellar class I gene *fleQ*) on flagellar gene transcription is important, as 27 out of the 46 flagellar genes were repressed in the *fleQ* mutant, in the replicative and/or transmissive phase. Transcription of 14 flagellar genes

TABLE 4. Numbers of genes coordinately regulated in *L. pneumophila* Δ*rpoN* and Δ*fleQ* or Δ*rpoN* and Δ*fleR* strains

| Growth phase | Mode of regulation (expression in the mutant) | No. of genes[a]: | |
| --- | --- | --- | --- |
| | | RpoN-FleQ dependent | RpoN-FleR dependent |
| Replicative | Induced | 0 | 2 |
| | Repressed | 15 (14) | 2 |
| Transmissive | Induced | 0 | 35 (1) |
| | Repressed | 10 (4) | 71 |

[a] Numbers in parentheses represent flagellar genes.

(*flgBDGIJ*, *fleN*, *flhFA*, and *fliMNOPQR*) was repressed in the *rpoN* and in the *fleQ* mutant, indicating that both regulators are needed for their transcription. All of them belong to the flagellar class II genes (Table 5). Additionally six (two) flagellar class II genes were repressed in the *fleQ* or *rpoN* mutant background, respectively. Importantly, transcription of the flagellar class III gene *fliA* (encoding the σ²⁸ regulator FliA)—which may be cotranscribed with the flagellar class III *motAB* genes—was repressed in the *fleQ* mutant but not in the *rpoN* mutant (Table 5). Accordingly, the FliA-dependent flagellar class II gene *flgL*, the flagellar class III genes *flgMN* and the flagellar class IV genes *fliS* and *flaAG* were repressed in the *fleQ* mutant but not in the *rpoN* mutant. This result was further confirmed by measuring the expression level of *fliA* in the Δ*fleQ*, Δ*rpoN*, and Δ*fleR* strains in the transmissve phase by quantitative reverse transcription-PCR (qRT-PCR). As shown in Table 6, *fliA* gene expression in the postexponential (PE) growth phase is repressed in a *fleQ* mutant but not in an *rpoN* mutant strain. Thus, FleQ regulates both flagellar genes dependent on RpoN and those independent from RpoN.

Interestingly, slight differences in flagellar gene regulation might exist between strains, as previous results studying *flaA* and *fliA* gene expression in a *fleQ* mutant in a different strain (*L. pneumophila* Corby) did not detect reduced levels of transcripts for these genes (29). We thus compared the expression of the two genes in *L. pneumophila* strains Corby and Paris by real-time PCR. This showed that in *L. pneumophila* strain Paris, *fliA* and *flaA* transcription is repressed in the absence of *fleQ*, while in strain Corby, it is repressed only in the replicative phase (data not shown). Taken together, our results provide evidence that FleQ is the master regulator for the flagellar biosynthesis genes in *L. pneumophila*, regulating gene expression in an RpoN-dependent as well as an RpoN-independent manner.

**FleR has little impact on transcriptional regulation of the flagellar genes.** 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 they are thus defined as flagellar class II genes (2). *fleS* and *fleR* transcription was significantly but only slightly repressed in the *fleQ* mutant as well as in the *rpoN* mutant (Table 5), indicating that *fleR* and *fleS* gene expression is under the control of FleQ. In contrast, transcription of only one flagellar gene, namely, *flaA*, was slightly repressed in the *fleR* mutant (0.54-fold), whereas 12 flagellar class II genes, one flagellar class III gene, and one flagellar class IV gene were induced, suggesting that FleR is not enhancing flagel-

TABLE 5. Flagellar gene expression of *L. pneumophila* Δ*rpoN*, Δ*fleQ*, Δ*fleR*, and Δ*fliA* mutants in the late replicative and transmissive phases in BCYE

| Gene name | Gene no. | Class | Relative mutant/WT gene expression in[a]: | | | | | | |
| | | | Replicative phase | | | Transmissive phase | | | |
| | | | Δ*rpoN*/WT | Δ*fleQ*/WT | Δ*fleR*/WT | Δ*rpoN*/WT | Δ*fleQ*/WT | Δ*fleR*/WT | Δ*fliA*/WT |
|---|---|---|---|---|---|---|---|---|---|
| *rpoN* | *lpp0542* | I | **0.104** | — | — | **0.061** | — | — | — |
| *fleQ* | *lpp0915* | I | —[b] | **0.216** | — | **2.138** | **0.364** | — | 1.436 |
| *fliJ* | *lpp1720* | IIa | — | — | — | — | — | — | — |
| *fliI* | *lpp1721* | IIa | — | — | — | — | — | — | — |
| *fliH* | *lpp1722* | IIa | — | **0.403** | — | — | — | — | — |
| *fliG* | *lpp1723* | IIa | — | **0.384** | 0.542 | — | — | — | — |
| *fliF* | *lpp1724* | IIa | — | — | — | — | — | — | — |
| *fliE* | *lpp1725* | IIa | — | 0.532 | **0.303** | — | — | **0.357** | — |
| *fleR* | *lpp1726* | IIa | — | 0.544 | **0.102** | — | — | **0.150** | — |
| *fleS* | *lpp1727* | IIa | — | 0.549 | — | — | — | — | — |
| *flhA* | *lpp1749* | IIa | **0.299** | **0.330** | 1.672 | — | — | — | — |
| *flhB* | *lpp1750* | IIa | — | — | — | — | — | — | — |
| *fliR* | *lpp1751* | IIa | **0.295** | **0.438** | — | — | — | — | — |
| *fliQ* | *lpp1752* | IIa | **0.392** | **0.382** | — | — | — | — | — |
| *fliP* | *lpp1753* | IIa | **0.306** | **0.382** | 1.695 | — | — | — | — |
| *fliO* | *lpp1754* | IIa | **0.174** | **0.243** | — | — | — | — | — |
| *fliN* | *lpp1755* | IIa | **0.060** | **0.095** | **4.015** | **0.446** | **0.456** | 1.878 | — |
| *fliM* | *lpp1756* | IIa | **0.058** | **0.113** | **2.362** | **0.278** | **0.335** | **2.220** | — |
| *flgA* | *lpp0970* | IIb | 0.605 | — | — | — | — | — | — |
| *flgB* | *lpp1224* | IIb | **0.212** | **0.363** | — | **0.357** | — | — | — |
| *flgC* | *lpp1225* | IIb | **0.384** | 0.536 | — | — | — | — | — |
| *flgD* | *lpp1226* | IIb | **0.403** | **0.470** | 1.684 | — | — | **2.564** | — |
| *flgE* | *lpp1227* | IIb | — | **0.304** | — | — | **0.231** | **3.998** | — |
| *flgF* | *lpp1228* | IIb | — | **0.215** | **2.499** | — | **0.429** | **5.280** | — |
| *flgG* | *lpp1229* | IIb | **0.262** | **0.279** | 1.865 | — | — | **3.023** | — |
| *flgH* | *lpp1230* | IIb | **0.376** | 0.556 | 1.752 | — | 0.526 | **2.965** | — |
| *flgI* | *lpp1231* | IIb | **0.310** | **0.177** | 1.783 | — | — | **2.662** | — |
| *flgJ* | *lpp1232* | IIb | **0.403** | **0.285** | 1.963 | — | 0.534 | **2.436** | — |
| *flgK* | *lpp1233* | IIb | — | **0.275** | **2.301** | — | **0.339** | **3.915** | 0.532 |
| *flgL* | *lpp1234* | IIb | — | **0.441** | — | — | **0.486** | **2.219** | **0.379** |
| *fliK'* | *lpp1657* | IIb | — | — | — | — | — | — | — |
| *fleN* | *lpp1747* | IIb | **0.118** | **0.157** | 1.993 | **0.233** | **0.362** | **2.604** | — |
| *flhF* | *lpp1748* | IIb | **0.102** | **0.147** | 1.834 | **0.308** | **0.435** | — | — |
| *flgN* | *lpp0968* | III | — | — | — | — | **0.420** | — | **0.068** |
| *flgM* | *lpp0969* | III | — | — | — | — | **0.309** | — | **0.081** |
| *motB* | *lpp1744* | III | — | **0.497** | — | — | **0.459** | — | — |
| *motA* | *lpp1745* | III | — | 0.606 | — | — | — | 1.922 | **0.052** |
| *fliA* | *lpp1746* | III | — | **0.457** | — | — | **0.484** | **2.587** | **0.042** |
| *motA2* | *lpp2266* | III | 1.849 | 0.583 | — | **2.545** | 0.521 | — | — |
| *motB2* | *lpp2267* | III | — | — | — | 1.864 | — | 1.950 | — |
| *flhB'* | *lpp2635* | III | — | — | — | **2.827** | — | 1.593 | **0.295** |
| *fliS* | *lpp1291* | IV | — | 0.643 | — | — | **0.399** | — | **0.048** |
| *fliD* | *lpp1292* | IV | — | — | — | **2.548** | 0.555 | **2.135** | **0.045** |
| *flaG* | *lpp1293* | IV | — | — | 0.527 | — | **0.212** | — | **0.007** |
| *flaA* | *lpp1294* | IV | — | — | **0.189** | — | **0.176** | 0.536 | **0.003** |
| *motY* | *lpp3034* | IV | — | — | — | — | — | — | **0.116** |

[a] Values in boldface show statistically significant altered expression compared to that of the wild type, with a fold change of $\geq 2$ or $\leq 0.5$ at $P \leq 0.001$. Values not in boldface show statistically significant altered expression compared to that of the wild type at $P \geq 0.001$.
[b] —, show no statistically significant gene expression changes.

TABLE 6. Real-time PCR results comparing the relative change in gene expression of *fliA* in the Δ*rpoN*, Δ*fleQ*, Δ*fleR*, and Δ*fliA* mutant strains compared to the wild-type strain *L. pneumophila* Paris

| Strain | *fliA* expression in the transmissive phase (log$_2$ ratio ± SD)[a] |
|---|---|
| Δ*rpoN* mutant | NS |
| Δ*fleQ* mutant | 0.41 ± 0.19 |
| Δ*fleR* mutant | NS |
| Δ*fliA* mutant | 0.13 ± 0.01 |

[a] The results were derived from three independent experiments, each performed in duplicate. NS, no significant change between the WT and mutant strain.

lar gene expression. Our data strongly indicate that FleR does not enhance flagellar class III gene transcription.

In previous studies, it was proposed that the flagellar class II genes are RpoN dependent, as RpoN binding sites were predicted (24, 29). However, flagellar class II gene transcription was not strictly dependent on the presence of RpoN. To test if transcription initiation correlates with putative RpoN binding sites upstream of the flagellar class II genes *fleS* and *flgB*, we determined the transcription initiation sites for both genes by primer extension. The results depicted in Fig. 3 show that transcription initiated from the putative RpoN binding site

FIG. 3. Primer extension-mediated mapping of the transcriptional start site of the *fleS* and *flgB* genes. Shown are reference sequencing reactions (lanes T, G, C, and A) and primer extension of *L. pneumophila* WT RNA harvested in the postexponential growth phase (lane PE). The sequence of the coding strand, encompassing the 3′ end of the extension product (*), is shown to the right. (A) Sequence of the upstream region of *fleS* indicating the start sites of the mRNA, as determined in the gel to the left, the RpoN and sigma 70 binding sites, and the primer used for primer extension. (B) Sequence of the upstream region of *flgB* indicating the start sites of the mRNA, as determined in the gels to the left, and the RpoN and sigma 70 binding sites.

preceding the *fleS* and *flgB* genes. However, in both cases two transcripts were detected, suggesting also dependence on a sigma 70 promoter (Fig. 3A and B).

**The second messenger c-di-GMP might influence motility in *L. pneumophila*.** GGDEF/EAL proteins have been shown to have important impact on flagellar regulation through changing the messenger bis-(3′,5′)-cyclic diguanylic acid (c-di-GMP) levels in different bacteria (49). Strikingly, also five nonflagellar genes encoding GGDEF/EAL regulatory proteins were repressed in the *fleQ* mutant but not in the *rpoN* mutant (*lpp0351*, *lpp0809*, *lpp0942*, *lpp1170*, and *lpp0952*). Expression of one of those genes—namely *lpp0952*—is also dependent on

FliA, indicating that FleQ is not directly regulating it. It is not known yet whether different expression of GGDEF/EAL proteins influences the cellular or subcellular c-di-GMP level; however, the gene expression data together with the knowledge from other bacteria suggest that this second messenger might also be important for *L. pneumophila* flagellar regulation and thus motility.

## DISCUSSION

In *L. pneumophila*, flagellation is associated with the transmissive/virulent phenotype during infection of proto-

zoa or macrophages and with the postexponential growth phase *in vitro*. However, the exact regulatory network governing the expression of the flagellar biosynthesis genes is not known yet. Four regulatory proteins of *L. pneumophila*, FleQ, FleR, RpoN, and FliA, are thought to have important regulatory functions in the flagellar biosynthesis pathway and may also be implicated in the switch from the replicative to transmissive phase. The regulatory gene mutants constructed in this study were not flagellated anymore in the transmissive phase, confirming their impact on flagellar biosynthesis. To get insight into which genes are under the control of these four regulators, we undertook for the first time comparative microarray analyses of *rpoN*, *fleQ*, *fleR*, and *fliA* mutants and their isogenic wild-type strains in the late exponential (late replicative) as well as postexponential (transmissive) growth phases. This genomewide study on the relative transcript abundances showed that FleQ, FleR, and RpoN influence not only on flagellar gene expression but also the global gene expression pattern. In each of the mutant strains, 1 to 2% of the genes show significantly altered expression patterns during the replicative phase and 4 to 9% show them during the transmissive phase. However, few genes regulated by FleQ, FleR, or RpoN seem to be associated with virulence. This result is in line with the results obtained from the infection of the murine alveolar macrophage cell line MH-S as the mutants had no defect in intracellular growth.

The $\sigma^{54}$ factor RpoN is known to initiate gene transcription in a concerted action with enhancer-binding proteins (48). *L. pneumophila* carries genes that encode three putative enhancer-binding proteins, FleQ, FleR, and PilR (29). Here we show that *L. pneumophila* genes coordinately regulated by FleQ and RpoN are nearly exclusively flagellar genes. This is similar to what is reported for *P. aeruginosa* (11). In contrast, FleR and RpoN together influence only the expression of *flaA* with respect to flagellum biosynthesis genes. The role of *L. pneumophila* FleR thus differs from that of *P. aeruginosa* (11). Instead, upon transition to the transmissive phase, expression of numerous genes involved in protein biosynthesis is coordinately enhanced by FleR and RpoN.

**FleQ is the master regulator of flagellar gene expression.** FleQ and RpoN together control the transcription of nearly all flagellar class II genes (Table 5), similar to what was reported for FleQ of *P. aeruginosa* (9–11). Transcription of *fliM*, *fleN*, *fleS*, *fleR*, *flgB*, *flgD*, *flgG*, *flgI*, *flgJ*, *flhF*, *flhA*, *fliR*, *fliQ*, *fliP*, *fliO*, and *fliN* is enhanced by FleQ and RpoN, underlining their major role in the timely regulation of flagellar gene expression (Table 5). Surprisingly, late flagellar genes (class III and IV) were transcribed dependent on FleQ but independent of the enhancer-binding protein RpoN or FleR. FleQ enhanced transcription of *fliA* independent of RpoN. Subsequently, transcription of the FliA-dependent flagellar class IV genes *fliS* and *flaAG* was induced. Further FliA-dependent flagellar genes, as deduced from the expression data, are *flgL*, *flhB'*, *flgMN* (reclassified as flagellar class IV genes), *motAB*, and the flagellar class IV gene *motY*. Thus, our results strongly suggest that FleQ is the master regulator for motility genes that controls flagellar gene expression both RpoN dependent as well as RpoN independent.

That FleQ clearly has an impact on gene transcription independent of RpoN is remarkable as it is a commonly accepted view that enhancer-binding proteins work in concert with a $\sigma^{54}$ factor like RpoN. However, it has been shown that the enhancer-binding protein NtrC from the aquatic photosynthetic bacterium *Rhodobacter capsulatus* may activate transcription of genes in an RpoN-independent fashion together with housekeeping RNA polymerase (RNAP)/$\sigma^{70}$ holoenzyme, representing an unusual class of enhancer-binding proteins (6, 7, 16). Thus, the same mechanism might apply to FleQ from *L. pneumophila*. Furthermore, it has been shown recently that FleQ of *P. aeruginosa* may influence gene transcription also independent of RpoN through binding of c-di-GMP (25). This leads to derepression of the transcription of certain genes, probably by influencing the FleQ DNA-binding properties (25).

Influence on flagellar gene expression and perhaps on FleQ of *L. pneumophila* by c-di-GMP is very likely. The *L. pneumophila* strain Paris genome encodes 24 proteins containing GGDEF and/or EAL domains putatively controlling the concentration of c-di-GMP. Expression of five of those (*lpp0351*, *lpp0809*, *lpp0942*, *lpp1170*, and *lpp0952*) is enhanced by FleQ; the latter indirectly via FliA as previously shown (2). These results strongly indicate that the c-di-GMP level is also involved in flagellar regulation in *L. pneumophila*. Interestingly, RpoN does not contribute to the FleQ regulation of proteins with GGDEF and/or EAL domains. However, sigma factor competition or ppGpp concentration might also contribute to the observed gene expression profiles. For example, the amount of ppGpp may have an influence on whether RpoN activates or instead represses a particular subset of flagellar regulon genes as it has been shown for *Escherichia coli* RpoN (38). Future studies investigating the mechanisms by which FleQ influences gene transcription independent of RpoN in *L. pneumophila* are challenging and might involve elucidation of new regulatory mechanisms.

**Regulation of flagellar gene transcription in *L. pneumophila* is distinct from that in *P. aeruginosa*.** In *L. pneumophila*, flagellar genes are expressed temporally during the transition to the transmissive phase. The results obtained here together with previous reports (3, 4, 15, 23, 26, 32, 44) suggest a model for the regulation of flagellar biosynthesis in *L. pneumophila* that is partly different from that described in *P. aeruginosa* (Fig. 4). In *L. pneumophila*, the enhancer-binding protein FleQ is the master regulator of the flagellar regulon, whose expression is probably transcriptionally controlled by the $\sigma^{70}$ factor. Together with the $\sigma^{54}$ factor RpoN, FleQ then enhances flagellar class II gene transcription. Surprisingly, FleQ enhances flagellar class III and IV gene transcription that is RpoN independent. FleR and RpoN might be responsible for a negative feedback loop on flagellar genes. This negative control may be an important mechanism employed by the cell to turn off flagellar gene expression once the gene products are no longer needed. The final step, leading to the completion of the flagellum, is controlled by the $\sigma^{28}$ factor FliA, encoded by *fliA*. Transcription of *fliA*—probably cotranscribed with *motAB*—is enhanced by FleQ independent of RpoN through a yet unknown regulatory mechanism. FliA regulates the expression of the flagellar class III genes *flgMN* and *flhB'* and the class IV genes *flaAG*, *fliDS*, and *motY*.

In conclusion, FleQ is the master regulator of the flagellar regulation cascade, controlling gene transcription in an RpoN-
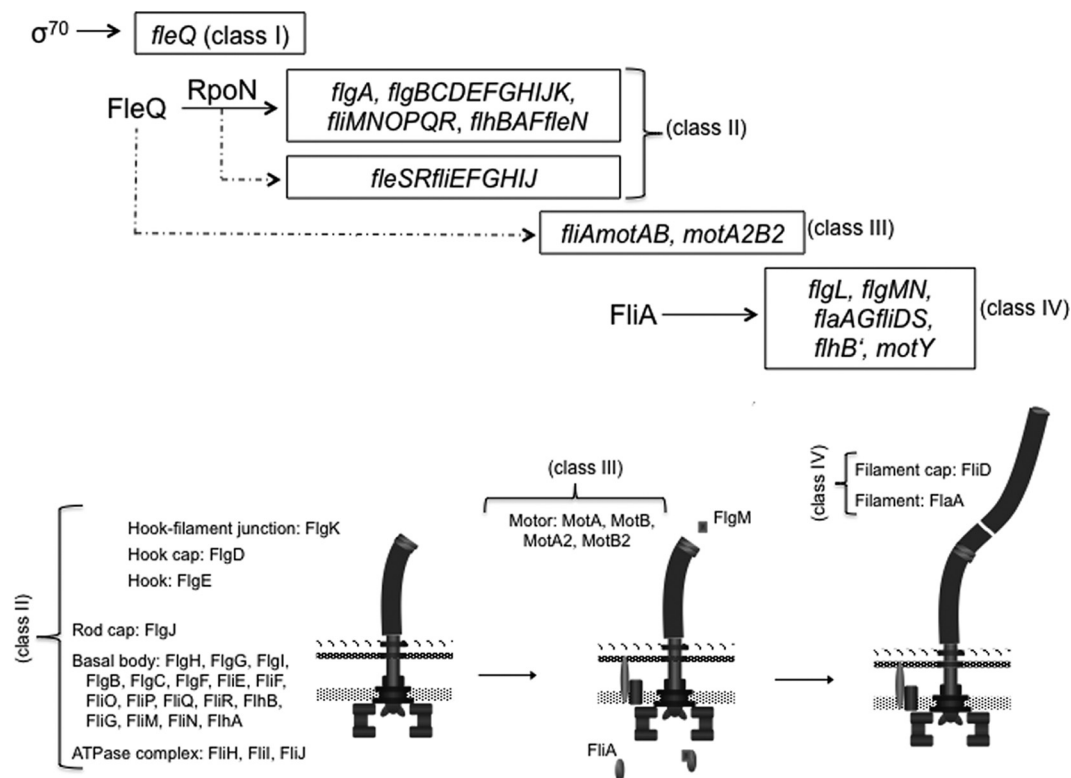
FIG. 4. Model for transcriptional regulation of the various flagellar genes (class I to IV) in *L. pneumophila*. "?" denotes an unknown factor or factors. FleQ is probably controlled by the $\sigma^{70}$ factor. Together with RpoN, FleQ then enhances flagellar class II gene transcription. FleQ independent from RpoN enhances flagellar class III and IV gene transcription, including that of *fliA*, encoding the $\sigma^{28}$ factor. FliA then regulates the expression of the flagellar class III genes *flgMN* and *flhB'* and the class IV genes *flaAG*, *fliDS*, and *motY* to complete the flagellum. FleR and RpoN seem to be responsible for a negative feedback loop on flagellar genes, possibly involving *letE*.

dependent as well as RpoN-independent manner. To understand this regulatory mechanism by which FleQ governs flagellar biosynthesis is now a challenging question for the future.

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