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1 # Control of flagellar gene regulation in *Legionella pneumophila* and its
2 # relation to growth phase

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Abstract

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 blot and electron microscopy of regulatory mutants in the genes encoding RpoN, FleQ, FleR and FliA demonstrated that flagellin expression is strongly repressed and that the mutants are non-flagellated in 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 five remaining flagellar class III genes and the flagellar class IV genes. Surprisingly, FleR does not induce but inhibit expression of at least 14 flagellar class III genes on 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 post-transcriptional level. In contrast to the commonly accepted view that enhancer binding proteins as FleQ always interact with RpoN to fullfill their regulatory functions, our results strongly indicate that FleQ regulates gene expression RpoN-dependent and RpoN-independent. Finaly, FliA induces expression of flagellar class III and IV genes leading to the complete synthesis of the flagellum.

INTRODUCTION

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 post-transcriptional 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 (33)).

The bacterial pathogen *Legionella pneumophila* lives in natural and man made 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 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 non-permissive 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 (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 homologues system to BarA/UvrY of *E. coli* and RsmA/RsmS of *Pseudomonas aeruginosa* was shown to have an important role in the regulation of the 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, 19, 20, 51). Phosphorylated LetA then induces the expression of two small regulatory RNAs, RsmY and RsmZ, that in turn sequester CsrA, a 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 flagella synthesis, and that RpoS, LetA, LetE and probably cyclic-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, a *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 on the regulation of the expression of transmissive traits in *L. pneumophila* strain Paris, particularly on flagellation. Mutants 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 σ^{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-aminoethanesulphonic acid (ACES)-buffered yeast extract broth or on ACES-buffered charcoal-yeast extract (BCYE) agar at 37°. The *rpoN* and *fleQ* mutants in *L. pneumophila* strain Paris were constructed using the corresponding mutants of *L. pneumophila* strain Corby (29) as 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'-ACAAAAGCACAAGGTACCGGC-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 *Xba*I restriction sites (fleR-XbaI-F 5'-TTCTAGAATAGTCTCAGCCAACGCTTC-3' and fleR-XbaI-R 5'-TTCTAGAAACAACCAATAGTTCCAGTCA-3'), the sequence encoding the σ^{54} interaction

domain of *fleR* was deleted and after self-ligation, the kanamycin cassette was inserted into the *Xba*I 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 (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. 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 5×10^5 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 non-phagocytized bacteria, and incubated in RPMI 1640 medium containing 10% FCS. The cultures were then incubated for up to 48 h in 5% CO_2 at 37°C. The number of viable bacteria (colony-forming unit [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 5×10^5 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 an MOI of 0.3. After washing 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 colony-forming units (CFU). Cell-associated viable microbes were enumerated as CFU and expressed as the mean percentage of the initial microbial inoculum recovered ± SD, calculated from three independent experiments.

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

6 **Immunoblotting (western blotting) procedures.** Bacteria were grown in liquid broth
7 to OD 4.2. Cells were resuspended in Lysis buffer (100mM Tris/HCl pH 8.0, 200mM NaCl,
8 2mM DTT), 5% Glycerol and a cocktail of protease inhibitors (Sigma) and disrupted by
9 sonication. After centrifugation (14,000rpm, 30 min, 4°C), soluble protein in supernatant was
10 collected and the protein concentration was determined according to Bradford using BSA as
11 standard 50 µg total protein of each sample were mixed with Laemmli buffer, heated at 100°C
12 for 5 min and loaded on a 13% polyacrylamide-SDS (sodium dodecyl sulfate) gel. SDS-
13 polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli
14 (31). Then the proteins were transferred to nitrocellulose by electroblotting. Flagellin (FlaA)
15 and FleQ were detected by an indirect immunostaining procedure with a rabbit polyclonal
16 antisera specific for *L. pneumophila* flagellin (29) or rabbit polyclonal antisera specific for *L.*
17 *pneumophila* FleQ and goat antirabbit immunoglobulin G coupled to horseradish peroxidase.
18 Staining of immunoreactive bands was performed in the presence of hydrogen peroxide and
19 4-chloro-1-naphthol.

20 **RNA isolation and labelling for array hybridization.** For array hybridization and
21 real-time PCR, total RNA was extracted as described previously (34). For *in vitro*
22 experiments, the *L. pneumophila* strains were grown in broth and harvested for RNA isolation
23 during late replicative phase (OD_{600} = 3.3, equivalent to late exponential growth phase) and
24 transmissive phase (OD_{600} = 4.0, equivalent to post-exponential growth phase) (**Figure S1**).
25 RNA was reverse-transcribed and indirectly labelled with Cy5 or Cy3 as described by the
26 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 PMT 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 twofold change, meeting a P-value ≤ 0.001, were taken into consideration. Empty and flagged spots were excluded, and only genes with no missing values were analyzed. Gene names (*lpp*) refer to strain Paris. Corresponding genes of strain Lens (*lpl*) and strain Philadelphia (*lpg*) are available at the LegioList web server http://genolist.pasteur.fr/LegioList. The corresponding genes of strain Corby (*lpc*) are available *via* GenBank (accession number CP000675). 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 pending.

Real-time PCR. Real-time PCR was conducted for confirming the transcriptome results on the total cellular RNAs in the same conditions. Primers used are listed in **Table 2**. These real-time experiments were performed as described previously (2). Briefly, Real-time

1 PCR was performed in a 25 µl reaction volume containing cDNA, 12.5 µl SYBR PCR Master
2 Mix (Applied Biosystems) and gene specific primers (300 nM) (Table 2). Amplification and
3 detection of specific products was performed with an ABI Prism 7700 sequence detection
4 system (PE Applied Biosystems) with the following cycle profile: 1 cycle at 50°C for 2 min, 1
5 cycle at 95°C for 10 min, 40 cycles at 95°C for 15 s and 60°C for 1 min. Each real-time PCR
6 assay was performed twice using three different cDNA dilutions (150 ng–1,5 ng). The
7 quantity of cDNA for each target gene was normalized to the quantity of *csrA* cDNA in each
8 sample. *csrA* is considered (and confirmed by microarray analysis) to represent a stable
9 expressed housekeeping gene and is not differentially expressed in the tested mutant strains.
10 The relative change in gene expression was recorded as the ratio of normalized target
11 concentrations (ΔΔ ct). To check if contaminating chromosomal DNA was present, each
12 sample was tested in control reactions that did not contain reverse transcriptase.
13 **RNA isolation and Primer Extension.** For primer extention experiments total RNA
14 was extracted by using a High Pure RNA isolation kit (Roche, Mannheim, Germany).
15 Additionally, purified RNA was incubated with 300 U of DNase I (Roche) per ml at room
16 temperature for 10 min and repurified using an RNeasy Mini Kit (Qiagen). The 5'-IRD800-
17 labeled primers flgB-PE 5'-CGGTGTATTAACATTGGCTATGT-3' and fleS-PE 5'-
18 GGTTTGACTGCACAAGTTTGATA-3' (MWG-Biotech), were complementary to
19 downstream regions of *fleS* and *flgB* promoters respectively. Extension reactions were carried
20 out by using the Superscript II Reverse Transcriptase Kit (Invitrogen). The gene-specific
21 primer (4 pmol final concentration) was annealed with 5-10 µg total RNA in a volume of 20
22 µl containing 0.8 mM dNTP Mix by heating at 90°C for 2 min and subsequent cooling to
23 45°C within 20 min in a thermocycler. For extension of the primer, annealing reaction
24 mixtures were combined with 200 U Superscript III RT and 40 U RNaseOUT (Invitrogen) in
25 40 µl reaction volumes (containing 31,25 mM Tris hydrochloride (pH 8.3), 46.9 mM KCl, 1.9
26 mM MgCl2 and 2.5 mM dithiothreitol) and incubated at 42°C for 90 min. Reactions were

1 stopped by incubation at 70°C for 15 min and remaining RNA was removed at 37°C for 20
2 min with RNaseH (Invitrogen). Nucleic acids were cautiously concentrated by precipitation
3 and dissolved in 2 µl H_2O and 2 µl formamide loading dye (Amersham Bioscience). Aliquots
4 of the samples were applied to 4.3 % polyacrylamide-urea Long Ranger™ (FMC
5 Bioproducts, Rockland, USA) sequencing gels of 66 cm with a 64 well shark tooth comb.
6 Gels were run under standard electrophoresis conditions in a LI-Cor-DNA4000 nucleotide
7 sequence analyzer and data were processed by using the software supplied (MWG-Biotech).
8 Reference sequencing reactions (A, C, G, T) of cloned promoters, performed with the Thermo
9 Sequenase Fluorescent labeled Primer Cycle Sequencing Kit with 7-Deaza-dGTP (Amersham
10 Biosciences), were initiated using the same primers.

11 **RESULTS**

12 ***L. pneumophila fleQ*, *fleR*, and *rpoN* mutant strains are not flagellated.** FleQ,
13 FleR, and RpoN of *L. pneumophila* are predicted to participate in the regulation of flagellar
14 biosynthesis similar to their homologs in *P. aeruginosa*. To unveil the regulation of flagellar
15 genes by FleQ, FleR, and RpoN in *L. pneumophila*, we constructed mutants in the genes *fleQ*,
16 *fleR*, and *rpoN* by inserting a kanamycin cassette into each of these genes. Comparison of
17 growth properties of the wild type and the mutant strains grown in broth or in infection of the
18 macrophage cell line MH-S, revealed no significant differences (data not shown). However,
19 when infectivity was measured by enumerating cell-associated CFU after a 2-h incubation, the
20 nonmotile *fleQ*, *fleR*, and *rpoN* mutant strains did not infect as efficiently as the WT, similar
21 to the non-motile *fliA* mutant (**Figure 1**). Furthermore, we observed the effects of the gene
22 deletions on motility and flagellation under the light and the electron microscope. When
23 compared to the *L. pneumophila* wild type strain it became evident that the three mutants
24 were non-motile as judged by light microscopy and non-flagellated as evidenced by electron
25 microscopy observation (**Figure S2**). Western blot analysis demonstrated that expression of

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

5 **FleQ, FleR, and RpoN have a global effect on *L. pneumophila* gene expression.**
6 Flagellar gene expression starts in replicative phase and is turned off after completion of the
7 flagellum in transmissive phase. It was previously shown, that FliA controls the final step of
8 the flagella biosynthesis (2, 21), however, other regulatory proteins involved in flagella
9 biosynthesis were not analysed yet. By *in silico* analysis of the four *L. pneumophila* genomes
10 sequenced (4, 5, 18), 46 genes organized in 10 genomic regions were predicted to participate
11 in the flagella biosynthesis or its regulation. Their expression starts in replicative phase and
12 stops with the completion of the flagellum in transmissive phase (2, 21, 22, 24). Here we
13 selected four regulatory proteins predicted to be implicated in flagella gene regulation for
14 further analysis. We determined the impact of FleQ, FleR, and RpoN on gene expression by
15 comparing the gene expression profiles of a *ΔfleQ*, *ΔfleR*, and *ΔrpoN* strain to their isogenic
16 wild type strain using whole genome microarrays in replicative and in transmissive phase.
17 Furthermore, the regulatory role of FliA in transmissive phase was established.

18 Analysis of the global gene expression program of each of the three mutants as
19 compared to the wild type strain showed that during replicative phase transcription of 34, 64,
20 and 22 genes and during transmissive phase transcription 144, 269, and 261 genes was
21 significantly differentially regulated in the *fleQ*, *fleR*, and *rpoN* mutant, respectively (**Table
22 3**). During replicative phase the expression of 1-2% of the 3077 predicted *L. pneumophila*
23 strain Paris genes and during transmissive phase 4-9% of these genes is altered in each of the
24 mutant strains. Hence, our results substantiate that FleQ, FleR, and RpoN have a great
25 influence on the global gene expression in *L. pneumophila*. In contrast, only 43 genes (~1% of
26 the predicted *L. pneumophila* genes) show an altered expression level during transmissive

1 phase in the *fliA* mutant. Real-time PCR analysis of selected genes validated the microarray
2 results (**Figure S3**). For a complete list of significantly down- and upregulated genes see
3 supplementary material **Tables S1-S7**. The complete dataset is available at
4 http://genoscript.pasteur.fr.

5 **Transcriptional analyses of *fleQ*, *fleR*, *rpoN*, and *fliA* mutants identified FleQ as**
6 **the master regulator of flagellar gene expression.** The σ^{54} factor RpoN is known to initiate
7 transcription of genes with σ^{54} promoters in a concerted action with enhancer binding proteins
8 (for a review see (48)). Based on sequence similarity, the proteins, FleQ and FleR of *L.*
9 *pneumophila* are predicted to be enhancer-binding proteins that function together with RpoN
10 (29). When analyzing the global transcriptional changes observed in the *rpoN*, *fleQ* and *fleR*
11 mutants as compared to the wild type strain, we identified, as expected, a set of RpoN
12 regulated genes that overlapped with those regulated by FleQ and FleR (**Table 4**). Genes co-
13 ordinately regulated by FleQ and RpoN were nearly exclusively flagellar genes. Surprisingly,
14 numerous genes regulated by FleQ were independent of RpoN, e.g. *fliA*. Furthermore, among
15 the genes regulated by FleR and RpoN together, only few were associated with flagellation.
16 Instead, FleR and RpoN specifically controlled transcription of genes associated with protein
17 biosynthesis. For instance, out of the 71 co-ordinately downregulated genes during
18 transmissive phase in the *fleR* and the *rpoN* mutant 11 genes encoded ribosomal proteins
19 (**Table S8**).

20 In contrast to what was observed for FleQ, FleR and RpoN, only a very limited set of
21 genes was affected by FliA as in the *fliA* mutant expression of only 43 genes was altered.
22 Among those ten had been previously described as belonging to the FliA regulon as judged
23 from analysis of gene expression of the *fliA* mutant during infection of *Acanthamoeba*
24 *castellani* (2). Due to the FleQ-dependent *fliA* expression, the set of FliA and FleQ regulated
25 genes shows a strong overlap: expression of the five flagellar operons *flaAG*, *flgMN*, *fliS*,
26 *flgL*, and of five non-flagellar genes *lpp1290* (encoding a protein similar to the enhanced

1 entry protein EnhA), *lpp0952* (encoding a regulatory protein with GGDEF and EAL
2 domains), *lpp0197*, and *lpp2282* was co-ordinately repressed in the *fleQ* and the *fliA* mutant.
3 Investigation of the transcription level of genes located downstream of the kanamycin
4 cassette insertion into *fleQ*, *fleR*, and *rpoN* respectively, showed that interruption of *fleQ* did
5 not led to missing transcription of the downstream genes, but kanamycin insertion into the
6 *rpoN* mutant is probably responsible for missing transcription of *lpp0541* (encoding a σ^{54}
7 modulation protein). It was hypothesized previously, that *fleR* and *fleS*, *fliEFGHIJ* are
8 expressed as an operon (24). However, the kanamycin cassette insertion into *fleR* led to
9 missing transcription of the downstream gene *fliE* (encoding a flagellar basal body protein)
10 but did not change the expression of the more distal genes *fliFGHIJ*. Thus either the insertion
11 had a polar effect of FleR is required for *fliE* expression. The transcriptional level of mutated
12 genes and the putative polar effects were not taken into consideration for data analyses.
13 **Transcriptional regulation of *L. pneumophila* motility genes is mainly governed**
14 **by FleQ, RpoN and FliA.** The influence of FleQ (encoded by the flagellar class I gene *fleQ*)
15 on flagellar gene transcription is important, as 27 out of the 46 flagellar genes were repressed
16 in the *fleQ* mutant, either in replicative and/or in transmissive phase. Transcription of 14
17 flagellar genes (*flgBDGIJ*, *fleN*, *flhFA*, *fliMNOPQR*) was repressed in the *rpoN* and in the
18 *fleQ* mutant indicating that both regulators are needed for their transciption. All of them
19 belong to the flagellar class II genes (**Table 5**). Additionally six (two) flagellar class II genes
20 were repressed in the *fleQ* or *rpoN* mutant background, respectively. Importantly,
21 transcription of the flagellar class III gene *fliA* (encoding the σ^{28} regulator FliA) - which may
22 be co-transcribed with the flagellar class III genes *motAB* - was repressed in the *fleQ* mutant
23 but not in the *rpoN* mutant (**Table 5**). Accordingly, the FliA-dependent flagellar class II gene
24 *flgL*, the flagellar class III genes *flgMN* and the flagellar class IV genes *fliS* and *flaAG* were
25 repressed in the *fleQ* mutant but not in the *rpoN* mutant. This result was further confirmed by
26 measuring the expression level of *fliA* in the Δ*fleQ*, Δ*rpoN*, and ΔfleR strains in transmissve

1 phase by qRT-PCR for. As shown in **Table 6**, *fliA* gene expression in PE phase is repressed in
2 a *fleQ* but not in an *rpoN* mutant strain. Thus, FleQ regulates flagella genes dependent but
3 also independet from RpoN.

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

13 **FleR has little impact on transcriptional regulation of the flagellar genes.**
14 According to the temporal order of flagellar gene transcription, *fleQ* belongs to the flagellar
15 class I genes while transcription of *fleS* and *fleR* - encoding a two-component system - takes
16 place later, and they are thus defined as flagellar class II genes (2). *fleS* and *fleR* transcription
17 was significantly but only slightly repressed in the *fleQ* as well as in the *rpoN* mutant (**Table**
18 **5**) indicating that *fleR* and *fleS* gene expression is under the control of FleQ. In contrast,
19 transcription of only one flagellar gene, namely *flaA* was slightly repressed in the *fleR* mutant
20 (0.54-fold) whereas 12 flagellar class II genes, one flagellar class III gene, and one flagellar
21 class IV gene were induced, suggesting that FleR is not enhancing flagella gene expression.
22 Our data strongly indicate that FleR does not enhance flagellar class III gene transcription.

23 In previous studies it was proposed that the flagellar class II genes are RpoN
24 dependent as RpoN binding sites were predicted (24, 29). However, flagellar class II gene
25 transcription was not strictly dependent on the presence of RpoN. To test if transcription
26 initiation correlates with putative RpoN binding sites upstream of the flagellar class II genes

1 *fleS* and *flgB*, we determined the transcription initiation sites for both genes by primer
2 extension. The results depicted in **Figure 3**, show that transcription initiated from the putative
3 RpoN binding site preceding the *fleS* and *flgB* genes. However, in both cases two transcripts
4 were detected, suggesting also dependence on a sigma 70 promoter (**Figure 3a and 3b**).
5 **The second messenger c-di-GMP might influence motility in *L. pneumophila*.**
6 GGDEF/EAL proteins have been shown to have important impact on flagellar regulation
7 through changing the messenger bis-(3',5')-cyclic diguanylic acid (c-di-GMP) levels in
8 different bacteria (49). Strikingly, also five non-flagellar genes encoding GGDEF/EAL
9 regulatory proteins were repressed in the *fleQ* mutant but not in the *rpoN* mutant (*lpp0351*,
10 *lpp0809*, *lpp0942*, *lpp1170*, and *lpp0952*). Expression of one of those - namely *lpp0952* - is
11 also dependent on FliA indicating that FleQ is not directly regulating it. It is not known yet,
12 whether different expression of GGDEF/EAL proteins influences the cellular or subcellular c-
13 di-GMP level, however the gene expression data together with the knowledge from other
14 bacteria suggest that this second messenger might also be important for *L. pneumophila*
15 flagella regulation and thus motility.

16 ## DISCUSSION

17 In *L. pneumophila*, flagellation is associated with the transmissive/virulent phenotype
18 during infection of protozoa or macrophages and with the post exponential growth phase *in*
19 *vitro*. However, the exact regulatory network governing the expression of the flagella
20 biosynthesis genes is not known yet. Four regulatory proteins, FleQ, FleR, RpoN, and FliA of
21 *L. pneumophila* are thought to have important regulatory functions in the flagella biosynthesis
22 pathway and may also be implicated in the switch from replicative to transmissive phase. The
23 here constructed mutants in these regulatory genes were not flagellated anymore in
24 transmissive phase, confirming their impact on flagella biosynthesis. To get insight, which
25 genes are under control of these four regulators, we undertook for the first time comparative

1 microarray analyses of *rpoN, fleQ, fleR*, and *fliA* mutants and their isogenic wild type strains
2 in late exponential (late replicative) as well as post exponential (transmissive) growth phase.
3 This genome-wide study on the relative transcript abundances showed that FleQ, FleR, and
4 RpoN influence not only on flagellar gene expression but also the global gene expression
5 pattern. In each of the mutant strains, 1-2% of the genes show significantly altered expression
6 patterns during RP and 4-9% during TP. However, few genes regulated by FleQ, FleR, or
7 RpoN seem to be associated with virulence. This result is in line with the results obtained
8 from the infection of the murine alveolare macrophage cell line MH-S as the mutants had no
9 defect in intracellular growth.

10 The σ^{54} factor RpoN is known to initiate gene transcription in a concerted action with
11 enhancer binding proteins (48). *L. pneumophila* encodes three putative enhancer binding
12 proteins, FleQ, FleR, and PilR (32). Here we show that *L. pneumophila* genes co-ordinately
13 regulated by FleQ and RpoN are nearly exclusively flagellar genes. This is similar to what is
14 reported for *P. aeruginosa* (11). In contrast, FleR and RpoN together influence only the
15 expression of *flaA* with respect to flagellum biosynthesis genes. The role of *L. pneumophila*
16 FleR thus differs from that of *P. aeruginosa* (11). Instead, upon transition to transmissive
17 phase expression of numerous genes involved in protein biosynthesis are co-ordinately
18 enhanced by FleR and RpoN.

19 **FleQ is the master regulator of flagellar gene expression.** FleQ and RpoN together
20 control the transcription of nearly all flagellar class II genes (**Table 5**) similar to what was
21 reported for FleQ of *P. aeruginosa* (9-11). Transcription of *fliM*, *fleN*, *fleS*, *fleR*, *flgB*, *flgD*,
22 *flgG*, *flgI*, *flgJ*, *flhF*, *flhA*, *fliR*, *fliQ*, *fliP*, *fliO*, and *fliN* is enhanced by FleQ and RpoN
23 underlining their major role in the timely regulation of flagellar gene expression (**Table 5**).
24 Surprisingly, late flagellar genes (class III and IV) were transcribed dependent on FleQ but
25 independent of the enhancer binding proteins RpoN or FleR. FleQ enhanced transcription of
26 *fliA* independent of RpoN. Subsequently transcription of the FliA-dependent flagellar class IV

1 genes *fliS* and *flaAG* were induced. Further FliA-dependent flagellar genes as deduced from
2 the expression data are *flgL, flhB', flgMN* (reclassified as flagella class IV genes), *motAB* and
3 the flagellar class IV gene *motY*. Thus our results strongly suggest that FleQ is the master
4 regulator for motility genes that controls flagellar gene expression RpoN-dependent but also
5 RpoN-independent.
6 That FleQ clearly has an impact on gene transcription independent of RpoN is
7 remarkable as it is a commonly accepted view that enhancer-binding proteins work in concert
8 with a σ^{54} factor like RpoN. However, it has been shown that the enhancer binding protein
9 NtrC from the aquatic photosynthetic bacterium *Rhodobacter capsulatus* may activate
10 transcription of genes in a RpoN-independent fashion together with housekeeping RNAP/σ^{70}
11 holoenzyme, representing an unusual class of enhancer-binding proteins (6, 7, 16). Thus the
12 same mechanism might apply for FleQ from *L. pneumophila.* Furthermore, it has been shown
13 recently, that FleQ of *P. aeruginosa* may influence gene transcription also independent of
14 RpoN through binding of c-di-GMP (25). This leads to derepression of the transcription of
15 certain genes, probably by influencing the FleQ DNA-binding properties (25).
16 Influence on flagellar gene expression and perhaps on FleQ of *L. pneumophila* by c-di-GMP
17 is very likely. The *L. pneumophila* strain Paris genome encodes 24 proteins containing
18 GGDEF and/or EAL domains putatively controlling the concentration of c-di-GMP.
19 Expression of five of those (*lpp0351*, *lpp0809*, *lpp0942*, *lpp1170*, and *lpp0952*) is enhanced
20 by FleQ; the latter indirectly *via* FliA as previously shown (2). These results strongly indicate
21 that the c-di-GMP level is also involved in flagella regulation in *L. pneumophila*.
22 Interestingly, RpoN does not contribute to the FleQ regulation of proteins with GGDEF
23 and/or EAL domains. However, sigma factor competition or ppGpp concentration might also
24 contribute to the observed gene expression profiles. For example, the amount of ppGpp may
25 have an influence whether RpoN activates or instead represses a particular subset of flagellar
26 regulon genes as it has been shown for *Escherichi 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 flagella biosynthesis in *L. pneumophila* that is partly different from that described in *P. aeruginosa* (**Figure 4**). In *L. pneumophila*, the enhancer binding protein FleQ is the master regulator of the flagella regulon whose expression is probably transcriptionally controlled by the σ^{70} factor. Together with the σ^{54} factor RpoN, FleQ then enhances flagellar class II gene transcription. Surprisingly, FleQ enhances flagellar class III and IV gene transcription 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 σ^{28} factor FliA, encoded by *fliA*. Transcription of *fliA* - probably co-transcribed 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*, *flhB'* and the class IV genes *flaAG*, *fliDS*, and *motY*.

In conclusion, FleQ is the master regulator of the flagellar regulation cascade, controling gene transcription in an RpoN-dependent but also RpoN-independent manner. To understand this regulatory mechanism by which FleQ governs flagella biosynthsis is now a challenging question for the future.

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4
5

Figure Legends

Figure 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* 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 percent viable and cell-associated *L. pneumophila* (infectivity). PE, post exponetial growth phase; E, exponential growth phase; Asterisk, indicates statistically significant differences (*, $P < 0.001$ by a two-tailed Student *t* test) in comparison to wt PE phase *L. pneumophila*.

Figure 2: Expression of flagellin and FleQ of *L. pneumophila* strain Paris wt, *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 OD 4.2 (PE phase) using anti-FlaA (A) and anti-FleQ (B) antiserum. lane 1, Size in kDa, lane 2 wild type strain Paris, lane 3 *L. pneumophila* strain Paris *ΔrpoN*, lane 4 *L. pneumophila* strain Paris *ΔfleQ*, lane 5 *L. pneumophila* strain Paris *ΔfleR,* lane 6 *L. pneumophila* strain Paris *ΔfliA,* lane 7 *L. pneumophila* strain Paris *ΔfleQ* complemented with native FleQ.

Figure 3: Primer extension-mediated mapping of the transcriptional start site of the *fleS* and *flgB* genes. Reference sequencing reactions (lanes T, G, C and A) and primer extension of *L. pneumophila* wt RNA harvested in post exponential 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

1 primer used for primer extension. B) Sequence of the upstream region of *flgB* indicating
2 the start sites of the mRNA as determined in the gels to the left, the RpoN and sigma 70
3 binding sites
4
5 **Figure 4: Model for transcriptional regulation of the various flagellar genes (Class I-IV)**
6 **in *L. pneumophila*.** ? denotes an unknown factor(s). FleQ is probably controlled by the σ^{70}
7 factor. Together with RpoN, FleQ then enhances flagellar class II gene transcription. FleQ
8 independet from RpoN enhances flagellar class III and IV gene transcription including fliA,
9 encoding the σ^{28} factor. FliA then regulates the expression of the flagellar class III genes
10 *flgMN*, *flhB'* and the class IV genes *flaAG*, *fliDS*, and *motY* to complte the flagellum. FleR
11 and RpoN seem to be responsible for a negative feedback loop on flagellar genes possibly
12 involving *letE*.
13

1 **Table 1.** Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant properties[1]	Reference
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*	Paris *fleQ*::Km	This study
Δ*rpoN*	Paris *rpoN*::Km	This study
Δ*fleR*	Paris *fleR*::Km	This study
Δ*fliA*	Paris *fliA*::Km	(2)
Plasmids		
pGEM-T Easy	Cloning of PCR products, Ap	Promega
pBC SK	Complementation vector, Cm	Stratagene

2 [1]Abbreviations: Km, kanamycin resistance; Ap, Ampicillin resistance; Cm, Chrloamphenicol
3 resistance.
4
5

1 **Table 2:** Primers used for real-time PCR

Gene name	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'

2

1 **Table 3:** Number of genes showing altered expression in the wild type strain as compared to

2 the mutant strains in replicative and transmissive growth phase.

Growth phase	Mode of regulation	*ΔfleQ*	*ΔfleR*	*ΔrpoN*	*ΔfliA*
RP	induced	4	37 (3)	3	not testified
	repressed	30 (22)	26 (1)	27 (16)	not testified
TP	induced	51	93 (13)	101 (3)	0
	repressed	92 (15)	178	159 (5)	29 (9)

3 Numbers in parenthesis represent flagellar genes. RP, replicative phase; TP, transmissive phase.

1 **Table 4:** Numbers of genes co-ordinately regulated in *L. pneumophila ΔrpoN* and *ΔfleQ* or

2 *ΔrpoN* and *ΔfleR* strains.

Growth phase	**Mode of regulation (expression in the mutant)**	**RpoN- FleQ-dependent**	**RpoN- FleR-dependent**
RP	induced	0	2
	repressed	15 (14)	2
TP	induced	0	35 (1)
	repressed	10 (4)	71

3 Numbers in parenthesis represent flagellar genes. RP, replicative phase; TP, transmissive phase.

1 **Table 5:** Flagellar gene expression of *L. pneumophila* mutants Δ*rpoN*, Δ*fleQ,* Δ*fleR,* Δ*fliA* in
2 late replicative (RP) and transmissive phase (TP) in BCYE

			Replicative Phase			**Transmissive phase**			
Gene name	**Gene N°**	**Class**	**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	-	**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**	-

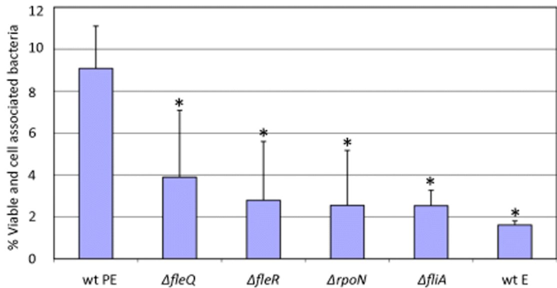
flgE	*lpp1227*	IIb	-	**0.304**	-	-	**0.231**	**3.998**	-
flgF	*lpp1228*	IIb	-	**0.215**	**2.499**	-	**0.429**	**5.280**	-
flgG	*lpp1229*	IIb	**0.262**	**0.279**	1.865	-	-	**3.023**	-
flgH	*lpp1230*	IIb	**0.376**	0.556	1.752	-	0.526	**2.965**	-
flgI	*lpp1231*	IIb	**0.310**	**0.177**	1.783	-	-	**2.662**	-
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**

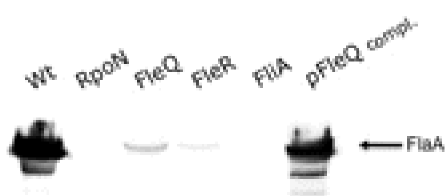
Values stated in bold show statistically significant altered expression compared to the wild type with a fold change of ≥ 2 or ≤ 0.5 meeting a p value of ≤ 0.001. Values stated not in bold show stratistically significant altered expression compared to the wild type meeting a p value ≥ 0.001.

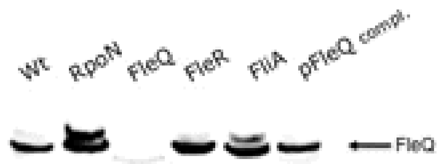
1 **Table 6:** Real-time PCR results comparing the relative change in gene expression of *fliA* in
2 the Δ*rpoN*, Δ*fleQ,* Δ*fleR* and Δ*fliA* mutant strains as compared to the wild type
3 *L. pneumophila* strain Paris.
4

Strain	***fliA* expression in transmissive phase**
	log2 ratio ± SD
Δ*rpoN*	n.s.
Δ*fleQ*	0.41 ± 0.19
Δ*fleR*	n.s.
Δ*fliA*	0.13 ± 0.01

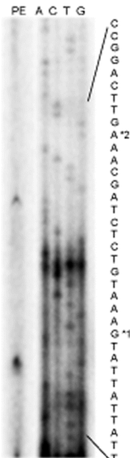
5 The results are derived from three independent experiments, each performed in duplicate. n.s.,
6 no significant change between wt and mutant strain
7
8

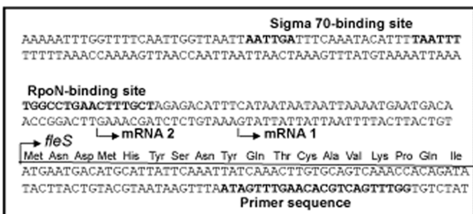

% Viable and cell associated bacteria
0
2
4
6
8
10
12
*
wt PE
ΔfleQ
ΔfleR
ΔrpoN
ΔfliA
wt E


A)
Wt
RpoN
FleQ
FleR
FliA
pFleQ compl.
FlaA

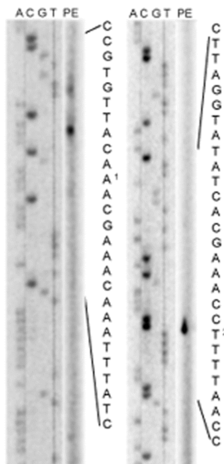

B)
Wt
RpoN
FleQ
FleR
FliA
pFleQ compl.
FleQ

A)





B)



σ^{70} → *fleQ* (class I)

FleQ —RpoN→ *flgA, flgBCDEFGHIJK, fliMNOPQR, flhBAFfleN* (class II)

fleSRfliEFGHIJ (class II)

fliAmotAB, motA2B2 (class III)

FliA → *flgL, flgMN, flaAGfliDS, flhB', motY* (class IV)

(class II)

Hook-filament junction: FlgK

Hook cap: FlgD

Hook: FlgE

Rod cap: FlgJ

Basal body: FlgH, FlgG, FlgI, FlgB, FlgC, FlgF, FliE, FliF, FliO, FliP, FliQ, FliR, FlhB, FliG, FliM, FliN, FlhA

ATPase complex: FliH, FliI, FliJ

(class III)

Motor: MotA, MotB, MotA2, MotB2

FlgM

FliA

(class IV)

Filament cap: FliD

Filament: FlaA