JOURNAL OF BACTERIOLOGY, May 2004, p. 2540–2547 Vol. 186, No. 9
0021-9193/04/$08.00+0 DOI: 10.1128/JB.186.9.2540–2547.2004


# Characterization of the Alternative Sigma Factor $\sigma^{54}$ and the Transcriptional Regulator FleQ of *Legionella pneumophila*, Which Are Both Involved in the Regulation Cascade of Flagellar Gene Expression

Sebastian Jacobi,[1] Rüdiger Schade,[2] and Klaus Heuner[1]*

*Institut für Molekulare Infektionsbiologie, Julius-Maximilians Universität Würzburg, D-97070 Würzburg,[1] and Institut für Pharmakologie und Toxikologie, Universitätsklinikum Charité, Humboldt-Universität zu Berlin, 10117 Berlin,[2] Germany*

Received 14 November 2003/Accepted 13 January 2004

**We cloned and analyzed *Legionella pneumophila* Corby homologs of *rpoN* (encoding $\sigma^{54}$) and *fleQ* (encoding $\sigma^{54}$ activator protein). Two other genes (*fleR* and *pilR*) whose products have a $\sigma^{54}$ interaction domain were identified in the genome sequence of *L. pneumophila*. An *rpoN* mutant strain was nonflagellated and expressed very small amounts of the FlaA (flagellin) protein. Like the *rpoN* mutant, the *fleQ* mutant strain of *L. pneumophila* was also nonflagellated and expressed only small amounts of FlaA protein compared to the amounts expressed by the wild type. In this paper we show that the $\sigma^{54}$ factor and the FleQ protein are involved in regulation of flagellar gene operons in *L. pneumophila*. RpoN and FleQ positively regulate the transcription of FliM and FleN, both of which have a $\sigma^{54}$-dependent promoter consensus sequence. However, they seemed to be dispensable for transcription of *flaA*, *fliA*, or *icmR*. Our results confirmed a recently described model of the flagellar gene regulation cascade in *L. pneumophila* (K. Heuner and M. Steinert, Int. J. Med. Microbiol. 293:133-145, 2003). Flagellar gene regulation was found to be different from that of *Enterobacteriaceae* but seems to be comparable to that described for *Pseudomonas* or *Vibrio* spp.**

*Legionella pneumophila*, the causative agent of Legionnaires' disease, is a ubiquitous microorganism inhabiting man-made water systems and freshwater biotopes. *Legionella* infection occurs after inhalation of aerosolized bacteria. The organism invades and proliferates in alveolar macrophages of the human lung. In the environment, *Legionella* replicates intracellularly in amoebae and other protozoans (13).

Previous studies have demonstrated that the flagellum positively affects the establishment of infection but is not required for intracellular replication (9, 28). On the other hand, it has been shown that the flagellar system is needed for full fitness of *L. pneumophila* (20). Furthermore, it is known that the complex flagellar regulon is coordinately regulated with the expression of other virulence-associated factors (4, 6, 20, 32). Studies in our laboratory have demonstrated that the *flaA* gene (encoding the major subunit flagellin) is positively regulated by the alternative $\sigma^{28}$ factor (FliA) and seems to be negatively regulated by the transcriptional regulator FlaR (16, 18, 20). Furthermore, *flaA* expression is modulated by various environmental factors (17); for a review see reference 21).

Genome analysis revealed the presence of putative $\sigma^{54}$ promoter sites upstream of most of the flagellar operons, and we hypothesized that RpoN and FleQ may regulate these operons (21). For *Pseudomonas aeruginosa* it has been shown that $\sigma^{54}$ and a factor containing a $\sigma^{54}$ interaction domain are at the top of the cascade of flagellar gene regulation (2, 7, 8, 25). In order to obtain support for our hypothesis and to further characterize the cascade of flagellar gene expression, we screened the genome sequence of *L. pneumophila* for a homolog of *rpoN* and for factors having a $\sigma^{54}$ interaction domain. In this paper we describe identification of the $\sigma^{54}$ factor and the transcriptional regulator FleQ and the role of this factor and this regulator in flagellar gene regulation in *L. pneumophila*.

* Corresponding author. Mailing address: Institut für Molekulare Infektionsbiologie, Julius-Maximilians Universität Würzburg, Röntgenring 11, D-97070 Würzburg, Germany. Phone: 49-931312151. Fax: 49-931312578. E-mail: klaus.heuner@mail.uni-wuerzburg.de.

## MATERIALS AND METHODS

*L. pneumophila* Corby serogroup 1 (S1) (22) was used to clone the *rpoN* and *fleQ* genes. The legionellae used in a Southern blot analysis were *L. pneumophila* Philadelphia I (= ATCC 33152) (S1), *L. pneumophila* Msp19 (S1) (3), *L. pneumophila* 685 (S1) (3), *L. pneumophila* U22 (S3) (3), *L. pneumophila* U21 (S6) (3), *L. pneumophila* 664 (S6) (3), *L. pneumophila* type strains (S7, S10, S12, and S13) (P. C. Lück, Dresden, Germany), *L. pneumophila* Bloomington (= ATCC 33155) (S3), *L. pneumophila* Los Angeles (= ATCC 33156) (S4), *L. pneumophila* Chicago-2 (= ATCC 33215) (S6), *Legionella anisa* (12), *Legionella bozemanii* ATCC 33217, *Legionella dumoffii* ATCC 33279, *Legionella feelei* ATCC 35072 (S1), *Legionella gormanii* ATCC 33297, *Legionella hackeliae* ATCC 33250 and ATCC 35999 (S1 and S2), *Legionella israeliensis* ATCC 43119, *Legionella jordanis* ATCC 33623, *Legionella longbeachae* ATCC 33462 and ATCC 33484 (S1 and S2), *Legionella micdadei* ATCC 33218, *Legionella oakridgensis* ATCC 33761, *Legionella erythra* (34), and *Sarcobium* (*Legionella*) *lyticum* PCM 2298 (Polish Culture of Microorganisms). *Escherichia coli* DH5α was used for cloning of recombinant plasmid DNA. Plasmid pUC18 or pUC19 (Pharmacia LKB, Freiburg, Germany) was used for subcloning of DNA fragments, and the vector pBC KS (Stratagene) was used to construct plasmids for complementation of *L. pneumophila* mutants.

**Media and chemicals.** *E. coli* was cultivated in Luria-Bertani medium. The antibiotics used in *E. coli* cultures were chloramphenicol (20 mg $ml^{-1}$) and ampicillin (100 mg $ml^{-1}$). *L. pneumophila* was grown in YEB medium, which contained 1% yeast extract and was supplemented with 1% *N*-(2-acetamido)-2-aminoethanesulfonic acid, 0.025% ferric $PP_i$, and 0.04% L-cysteine, or on buffered charcoal yeast extract (BCYE) agar (10). Enzymes were purchased from MBI Fermentas (Vilnius, Lithuania), Amersham, Boehringer GmbH (Mannheim, Germany), and Invitrogen GmbH (Karlsruhe, Germany). AmpliTaq poly-

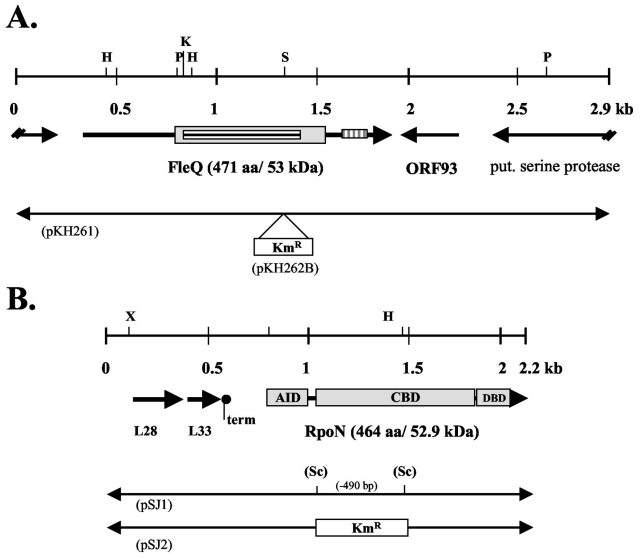


FIG. 1. Genetic maps of the FleQ-encoding region (A) and the RpoN-encoding region (B) of *L. pneumophila* Corby. (A) The gene was inactivated by inserting a kanamycin resistance cassette into the SnaBI site of pKH261 and was subcloned into vector pBCKS, resulting in plasmid pKH262B. For a description of the method used for integration of the *fleQ*::Km$^r$ resistance cassette into the chromosome, see Materials and Methods. Functional domains encoded by *fleQ* are indicated. The deduced protein contains a $\sigma^{54}$ activation domain (grey box), a putative ATP binding site (AAA) (box with horizontal lines), and a C-terminal HTH_8 motif (box with vertical lines). (B) The *rpoN* gene was inactivated by inserting a kanamycin resistance cassette into the SacII site of pSJ1, resulting in pSJ2. For a description of the methods used for integration of the *rpoN*::Km$^r$ resistance cassette into the chromosome, see Materials and Methods Typical $\sigma^{54}$ functional domains that are encoded by *rpoN* are indicated and included the AID, CBD, and DBD. Genes are indicated by arrows. L28, region encoding 50S ribosomal protein L28 homolog (78 amino acids); L33, region encoding 50S ribosomal protein L33 homolog (54 amino acids); term, putative rho-independent termination site; aa, amino acids; put., putative. Restriction endonuclease sites: H, HindIII; K, KpnI; P, PstI; S, SnaBI; Sc, SacII.

merase was purchased from Invitrogen GmbH. Chemicals and oligonucleotides were obtained from MWG-Biotech (Ebersberg, Germany).

**DNA techniques and nucleotide sequencing analysis.** Preparation of chromosomal or plasmid DNA, DNA manipulation, and Southern hybridization were performed by using standard protocols (33). PCR was carried out by using a TRIO-Thermoblock thermocycler (Biometra, Göttingen, Germany) and AmpliTaq polymerase (Invitrogen GmbH). Introduction of foreign DNA into bacterial strains by electroporation was performed by using a Bio-Rad gene pulser (Bio-Rad, Munich, Germany) according to the manufacturer's specifications. Electroporation of *E. coli* strains was carried out by using 1.8 kV, 200 Ω, and 25 mF, and electroporation of *Legionella* strains was carried out by using 2.3 kV, 100 Ω, and 25 mF.

Both strands of plasmid DNA were sequenced with infrared-dye-labeled primers by using an automated DNA sequencer (LI-COR-DNA 4000; MWG-Biotech). Sequences were analyzed by using the Genetics Computer Group package, Pendant (http://pendant.gfs.de), and SMART (http://smart.embl-heidelberg.de) programs, as well as data available on the website of the *Legionella* genome project (http://genome3.cpmc.columbia.edu/~legion).

**Generation of $\sigma^{54}$ (*rpoN*) and *fleQ* mutant strains of *L. pneumophila* Corby.** Mutant strains were generated as described recently (9). In brief, the gene of interest was inactivated by introduction of a kanamycin resistance cassette into the chromosomal gene by using an SnaBI restriction site (*fleQ*) or a PCR-introduced SacII restriction site (*rpoN*) (Fig. 1). Mutants were generated by using the natural competence of *L. pneumophila* (35). Correct insertion of the resistance gene cassette into the chromosome was verified by PCR and Southern blot analysis (data not shown). For complementation studies, the complete gene was cloned in the vector pBCKS (*fleQ*, pKH262C; *rpoN*, pKH268) and introduced into the mutant strain by electroporation.

**Southern hybridization.** Chromosomal DNA from various *Legionella* strains were digested with SacII and HindIII and electrophoresed, and fragments were transferred to nylon membranes (Pall, Dreieich, Germany) by capillary blotting. DNA probes containing the complete *L. pneumophila fleQ* or *rpoN* gene were used as *fleQ*- or *rpoN*-specific probes. DNA probes were labeled and detected by using a nonradioactive enhanced chemiluminescence detection kit (ECL; Amersham). Hybridization was performed under low-stringency conditions as described previously (15).

**RT-PCR analysis and primer extension.** Total RNA was extracted by using a High Pure RNA isolation kit (Roche, Mannheim, Germany) as described by the manufacturer. Additionally, purified RNA was incubated with 300 U of DNase I (Roche) per ml at 37°C for 60 min and then repurified by using an RNeasy Mini kit (Qiagen, Hilden, Germany). Reverse transcription (RT)-PCRs were performed with a OneStep RT-PCR kit (Qiagen) used according to the instructions of the manufacturer with gene-specific primers (Table 1). The RT reaction was performed at 50°C for 30 min with 100 ng of total RNA. PCR amplification was performed in the same tube after an initial activation step at 95°C for 15 min with each primer at a concentration of 0.6 μM, each deoxynucleoside triphosphate at a concentration of 400 μM, 5× OneStep RT-PCR buffer containing 12.5 mM $MgCl_2$, and 2 μl of OneStep RT-PCR enzyme mixture in a 50-μl (total volume) reaction mixture. Initial denaturation was performed at 95°C for 15 min (activation step), and final extension was performed at 72°C for 10 min. The cycling conditions were 94°C for 1 min, 50 to 52°C for 1 min (Table 1), and 72°C for 1 min for 30 cycles with a Biometra T3 thermocycler (Biometra). The purified RNA was analyzed for genomic DNA contamination by performing PCR with primers specific for the *flaA* gene as described above, except for the RT step (30 min at 50°C). In control experiments, the amounts of RT-PCR products were analyzed each third round of amplification by electrophoresis, starting with cycle 18 of 36 cycles, and the results showed that the yield of amplification products depended on the quantity of RNA present in the sample.

Primer extension analysis was carried out with IRD-labeled primer fleQPE (5′-AAATAGTCCGAAGCTTGTCAC-3′) by using an automated DNA sequencer (LI-COR-DNA 4000; MWG-Biotech). The primer (4 pmol) was annealed to 20 μg of total RNA, and the RT reaction was performed as described

TABLE 1. Primers used for RT-PCR

| Gene | Forward primer | Reverse primer | Temp for PCR (°C)[a] | Length (bp)[b] |
|---|---|---|---|---|
| *flaA* | CATGATGCAAACATCGATCGA | CTGCTACTTCTGTTCCTGTTG | 52 | 480 |
| *fliA* | AACGCATTGCACATCATCTGC | ATAAGACATCATCGGTTACTC | 50 | 400 |
| *fleN* | TAGCCATAGCGTTATCTCAAC | GTAATCTAACTGCACATCCAG | 52 | 540 |
| *fleQ* | GTGACAAGCTTCGGACTATTT | GAAAGAGAATGTATATTGCGA | 50 | 490 |
| *rpoN* | TGGTCAACATCTCACGTTAAC | CCTAGCAACTCAATGTCTTCA | 51 | 600 |
| *fliM* | GAGATCGATGCATTACTGGAT | TAATAATCGACCAAGTCATACACA | 51 | 450 |
| *icmR* | ATACTGATGACAGTGCACGAA | GATGATAATTTGAAACCACGTTC | 50 | 350 |
| *flesR* | AAGTATATCGATATGGAGTTT | AACTATTAAAACATGACTCAT | 45 | 480 |

[a] Temperature used for the amplification step in the RT-PCR.
[b] Length of amplified template.

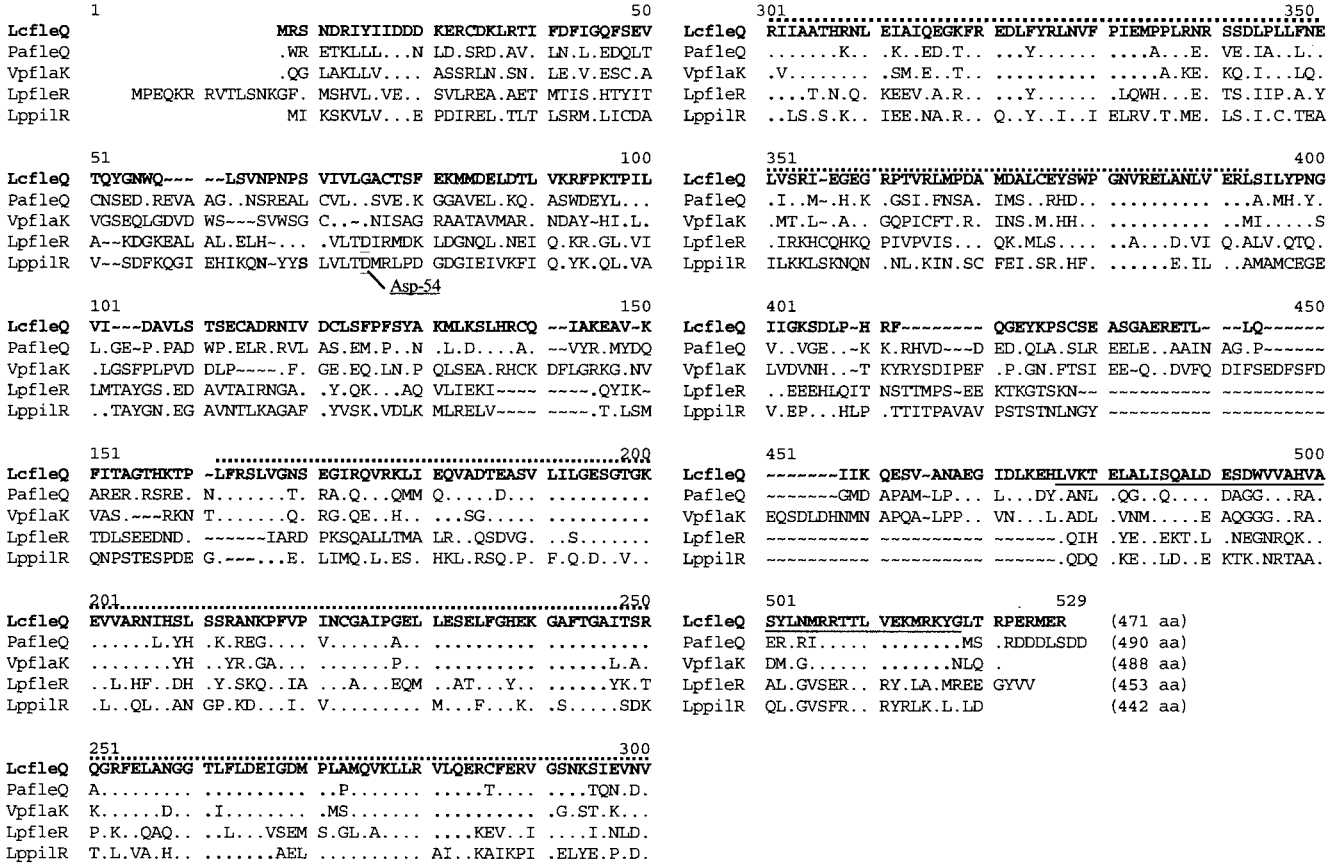


FIG. 2. Comparison of the amino acid sequences of *L. pneumophila* Corby FleQ (LcfleQ), FleQ of *P. aeruginosa* (PafleQ), FlaK of *V. parahaemolyticus* (VpflaK), FleR of *L. pneumophila* Philadelphia (LpfleR), and PilR of *L. pneumophila* Philadelphia (LppilR). The $\sigma^{54}$ interaction domain is indicated by a dotted line, and the C-terminal HTH_8 domain is underlined. Amino acids identical to the amino acids in *L. pneumophila* Corby FleQ are indicated by periods. The putative phosphor acceptor site of FleR and PilR at amino acid position 54 is indicated (Asp-54). ~, gaps; aa, amino acids.

recently (19). The sequencing reaction was performed by using the primer that was used for the primer extension analysis.

**SDS-PAGE and immunoblotting.** Total cell extracts of *L. pneumophila* strains were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. SDS-PAGE was performed as described by Laemmli (27). *Legionella* was grown on BCYE agar plates for 24 to 72 h at 30°C unless indicated otherwise, harvested, and suspended in distilled water, and the optical density at 600 nm was adjusted to 1. Three hundred microliters was centrifuged, and the cells were then suspended in 50 μl of Laemmli buffer and loaded onto an SDS–13% polyacrylamide gel. Western blotting was carried out by using polyclonal antibodies specific for *L. pneumophila* Corby flagellin and *P. aeruginosa* FleQ protein. The anti-FlaA antiserum was generated as described recently (19) by using purified flagella of *L. pneumophila* Corby.

**Intracellular replication assays.** U937 cells were cultured in RPMI 1640 (PAA) containing 2 mM L-glutamine and 10% fetal calf serum at 37°C with 5% $CO_2$. U937 cells ($10^6$ cells per well) were differentiated in 24-well plates with 10 ng of phorbol 12-myristate 13-acetate (Sigma) per ml for 48 h before use. Adherent cells were washed with RPMI 1640 prior to infection.

The ability of *L. pneumophila* strains to grow in macrophage-like U937 cells was determined in coculture assays. Bacterial strains were cultivated on BCYE agar plates for 3 days at 37°C. Differentiated U937 cells were infected with a bacterial suspension in RPMI 1640 (multiplicity of infection, 0.01), and the plates were centrifuged at 800 × *g* for 3 min and incubated at 37°C up to 4 days. The initial time was defined as 2 h postinfection. Due to the low multiplicity of infection, no washing or gentamicin treatment was performed. Macrophages were lysed daily with cold $H_2O$ and combined with the culture supernatant. Serial dilutions were spread on BCYE agar plates to determine the number of CFU. All assays were performed independently in triplicate.

**Electron microscopy.** Bacteria were grown for 4 days on BCYE agar plates at 30°C. Then bacteria were suspended in distilled water, and 1 drop of the suspension was applied to Pioloform (Merck)-coated copper grids. After sedimentation of the bacteria and removal of the remaining fluid, the samples were each stained with 1 drop of 1% phosphotungstic acid (Sigma) (pH 6.5) or shadowed with platinum-palladium and examined with a transmission electron microscope (EM10; Zeiss) at 60 kV.

**Nucleotide sequence accession number.** The sequences reported here have been deposited in the GenBank database under accession numbers AJ566390 (*fleQ*) and AJ580316 (*rpoN*).

## RESULTS

**Cloning of the *rpoN* and *fleQ* genes of *L. pneumophila* Corby.** Recently, we identified putative $\sigma^{54}$ promoter elements upstream of most of the major flagellar operons (21). Therefore, we searched for the presence of an RpoN homolog and for the presence of proteins with a $\sigma^{54}$ interaction domain in the deduced protein sequences of the genome of *L. pneumophila* (http://genome3.cpmc.columbia.edu/~legion). A homolog of RpoN and homologs of FleQ, FleR, and PilR proteins were identified. Primers specific for the putative *rpoN* (Rpon-F [5′-ATCTTACGTTGCATCACAATAACT-3′] and RpoN-R [5′-CAGTGAATGCTCTTAGTGCAGGAG-3′]) and *fleQ* (FleQ-F [5′-CCGTTATAATGATTACCGAGTGGA-3′ ] and FleQ-R

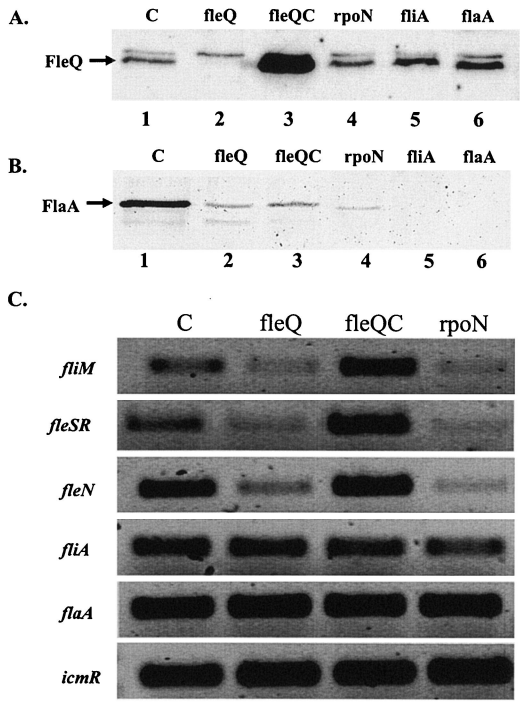


FIG. 3. Western blot and RT-PCR analyses of *L. pneumophila rpoN* and *fleQ* mutant strains. (A and B) Western blot analysis performed with strains grown on BCYE agar at 30°C for 4 days and with an anti-FleQ antibody (kindly provided by Reuben Ramphal, Gainesville, Fla.) (A) or an anti-FlaA antibody (B). Equal amounts of whole-cell lysates were loaded onto the polyacrylamide gel. (C) RT-PCR performed with whole-cell RNA isolated from strains grown on BCYE agar plates at 30°C for 3 days. Abbreviations: C, *L. pneumophila* Corby (wild type); flaA, *flaA* mutant strain; fleQ, *fleQ* mutant strain; fleQC, complemented *fleQ* mutant strain; fliA, *fliA* mutant strain; rpoN, *rpoN* mutant strain.

[5′-TCCCAGTTACAGCGAATCCGTGAT-3′]) homologs were generated, and the corresponding genes of *L. pneumophila* Corby were amplified, cloned, and analyzed further. The genetic maps of the cloned *fleQ* and *rpoN* regions are shown in Fig. 1.

**Nucleotide and protein sequence analysis of *fleQ* and FleQ.** The putative *fleQ* gene encompasses 1,413 bp and encodes a protein with a calculated molecular mass of 53 kDa (Fig. 2). A *P. aeruginosa*-specific anti-FleQ antibody cross-reacted in Western blot analysis with the FleQ protein of *L. pneumophila*, confirming the presence of a FleQ-like protein and that the molecular mass was approximately 53 kDa (Fig. 3A, lanes 1 and 3). Computer analysis revealed the presence of a Pfam-$\sigma^{54}$ interaction domain, an AAA domain (ATP binding site), and a C-terminal Pfam-HTH_8 domain (DNA binding site). The FleQ protein of *L. pneumophila* Corby is 99, 55, 54, and 54% identical to FleQ of *L. pneumophila* Philadelphia, FleQ of *P. aeruginosa*, the $\sigma^{54}$-dependent transcriptional activator of *Vibrio cholerae*, and FlaK of *Vibrio parahaemolyticus*, respectively. Downstream of *fleQ*, we identified two putative open reading frames encoding a putative serine protease and a hypothetical 93-amino-acid protein (ORF93) that exhibited no significant homology to any protein described so far (Fig. 1A).

As mentioned above, two other proteins containing a $\sigma^{54}$ interaction domain were identified in *L. pneumophila*. These proteins exhibtied 38% (FleR) and 37% (PilR) identity to FleQ. The corresponding genes were downstream of genes that coded for the putative sensor kinase FleS or PilS. Upstream of *fleS* and *pilS* we identified typical putative $\sigma^{54}$ promoters (Table 2). Furthermore, FleR and PilR also had a C-terminal HTH_8 domain and an Asp-54 residue that represented a putative phosphor acceptor site of an N-terminal sensor interaction domain (Fig. 2). An Asp-54 residue was not found in the FleQ proteins of *L. pneumophila* and *P. aeruginosa*. Furthermore, up- and downstream of *fleQ* no putative sensor kinase gene was identified. When the amino acid sequences of these five proteins were compared, it was obvious that the $\sigma^{54}$ interaction domain is the most conserved region (Fig. 2).

Five nucleotides upstream of the start codon (ATG) of FleQ, there is a ribosome binding site (AGGATA). Two putative $\sigma^{70}$-like promoter elements were identified 46 and 51 bp upstream of the start codon by primer extension analysis (Fig. 4 and Table 2). However, another transcription initiation site of *fleQ* was found, but upstream of the transcriptional start site $t_3$ no putative promoter element was identified (Fig. 4). Furthermore, a putative Vfr (homolog of the *E. coli* cyclic AMP

TABLE 2. Putative promoter sequences of various flagellar genes

| Region or gene | Sequence | Reference |
|---|---|---|
| $\sigma^{70}$ consensus | TTGACA--$N_{17}$--TATAAT[a] | |
| *fleQ* $t_1$ | GTCACA--$N_{17}$--CATTAT--$N_{46}$--ATG[a] | This study |
| *fleQ* $t_2$ | ATAAAT--$N_{17}$--AGTTAT--$N_{51}$--ATG[a] | This study |
| $\sigma^{28}$ consensus | TAAA---$N_{15}$--GCCGATAA[a] | |
| *flaA* | TAAA---$N_{15}$--TCCGATAA--$N_{94}$--ATG[a] | 15 |
| *fliD* | TATA---$N_{15}$--TCCGATAA--$N_{153}$-ATG[a] | 21 |
| $\sigma^{54}$ consensus | TGGCAC--$N_5$--TTGCA[b] | |
| *fliM* | TGGCAC--$N_5$--TTGCA---$N_{113}$---ATG[b] | 21 |
| *fleS* | TGGCCT--$N_5$--TTGCT---$N_{35}$---ATG[b] | 21 |
| *fleN* | TGGATG--$N_6$--TTGCA---$N_{88}$---ATG[b] | 21 |
| *pilS* | TGGTTC--$N_5$--ATGCG---$N_{137}$---ATG[b] | This study |

[a] The region before $N_{17}$ or $N_{15}$ is the −35 region, and the region after $N_{17}$ or $N_{15}$ is the −10 region.
[b] The region before $N_5$ or $N_6$ is the −24 region, and the region after $N_5$ or $N_6$ is the −12 region.

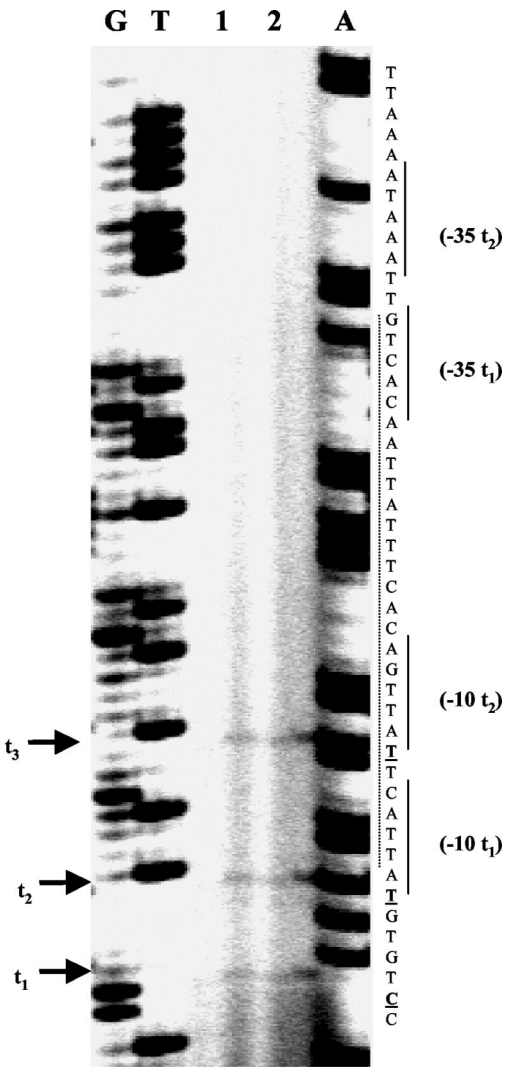


FIG. 4. Primer extension experiments to map the transcriptional start site of the *L. pneumophila fleQ* gene. Total RNA was isolated from *L. pneumophila* cultures grown on BCYE agar plates at 30°C for 3 days (see Materials and Methods). The results of two independent experiments are shown (lanes 1 and 2). Transcriptional start sites are indicated by arrows ($t_1$ to $t_3$). Lanes G, T, and A contained DNA sequencing ladders. The positions of putative promoter elements (−10, −35) are indicated, and the position of a putative Vfr (putative *E. coli* cyclic AMP receptor protein homolog) binding site is indicated by the dotted line.

receptor protein) binding site, containing an upstream activation sequence-like element (TGT-$N_{12}$-ACA), was observed overlapping the *fleQ* promoter element (Fig. 4).

**Nucleotide and protein sequence analysis of *rpoN* and RpoN.** The *L. pneumophila* RpoN homolog with a theoretical molecular mass of 52.9 kDa is encoded by a 1,392-bp open reading frame (Fig. 1B). Computer analysis revealed the presence of a $\sigma^{54}$ family signature and the Pfam_$\sigma^{54}$-activator interaction domain (AID), Pfam_$\sigma^{54}$-core binding domain (CBD), and Pfam_$\sigma^{54}$-DNA binding domain (DBD) commonly found in $\sigma^{54}$ factors. RpoN of *L. pneumophila* is 53.9 and 49.2% identical to RpoN of *V. cholerae* and *P. aeruginosa*, respectively. The genetic map is shown in Fig. 1B. Five nucleotides upstream of the start codon, there is a conserved ribosome binding site (AGAGGA), but no typical promoter sequences were identified. However, a putative $\sigma^{70}$-like −10 sequence (GATAAT) is present. Two genes encoding the putative 50S ribosomal proteins L28 and L33 are located upstream of *rpoN* (Fig. 1B). L28 and L33 of *L. pneumophila* Corby are 71.4 and 60.4% identical to L28 of *P. aeruginosa* and L33 of *Yersinia pestis*, respectively. In the genome of *L. pneumophila* Philadelphia genes for a putative $\sigma^{54}$ modulation protein and a putative phosphocarrier (HPr) were identified downstream of *rpoN* (data not shown). Identical arrangements of these three genes have been described for *E. coli*, *P. aeruginosa*, and *V. cholerae* (23, 24, 26).

**Analysis of the *rpoN* and *fleQ* mutant strains of *L. pneumophila* Corby.** After growth for 4 days on BCYE agar plates at 30°C, the *fleQ* mutant expressed smaller amounts of FlaA protein than the wild type expressed, as determined by Western blot analysis with an anti-FlaA antiserum (Fig. 3B, lanes 1 and 2). As expected, the *fleQ* mutant also did not exhibit any detectable FleQ protein (Fig. 3A, lane 2). The complemented strain did not express the flagellin as well as the wild-type strain expressed it (Fig. 3B, lane 3), but this was probably due to the overexpression of *fleQ* (Fig. 3A, lane 3). After three more days of incubation, no FlaA protein was detected in the *fleQ* mutant, whereas large amounts of FlaA protein were still detectable in the wild-type strain (data not shown). A similar behavior was observed for bacterial strains grown in supplemented YEB medium (data not shown). Agar-grown bacteria were examined by electron microscopy for the presence of flagella. The *fleQ* strain was nonflagellated at any time tested (Fig. 5B), whereas the wild type was flagellated after 4 days of incubation on agar plates (Fig. 5A). Electron microscopy of the complemented strain revealed the presence of flagella, but again the flagellation was not fully comparable to the wild-type flagellation (Fig. 5D). From these data we concluded that FleQ is required for full expression of *flaA* and for assembly of the flagellum in *L. pneumophila*.

The *rpoN* mutant was tested accordingly. Similar to the results obtained with the *fleQ* mutant, only very small amounts of the FlaA protein were detected in the *rpoN* mutant (Fig. 3B, lane 4), and expression of FlaA at the wild-type level could not be complemented by plasmid-encoded *rpoN* (data not shown). Furthermore, the *rpoN* mutant appeared to be nonflagellated in an electron microscopy analysis (Fig. 5C). As the *rpoN* mutant contained amounts of FleQ comparable to the amounts in the wild-type strain (Fig. 3A, lanes 1 and 4), we concluded that the phenotypes observed for the *rpoN* mutant were not based on reduced *fleQ* expression in this strain and that flagellar expression in *L. pneumophila* depends on the presence of an active $\sigma^{54}$ factor for direct expression of flagellar genes or for expression of an unknown additional factor.

To further analyze the abilities of the two mutants to express the flagellin but to be nonflagellated, we performed RT-PCR experiments (Fig. 3C) using RNA isolated from the *L. pneumophila* wild-type strain and *fleQ*, *rpoN*, and complemented *fleQ* mutant strains (see Materials and Methods) and gene-specific primer pairs (Table 1). Compared to the amounts in the wild-type strain, only small amounts of *fliM* transcripts were detectable in the *rpoN* and *fleQ* mutant strains, suggesting

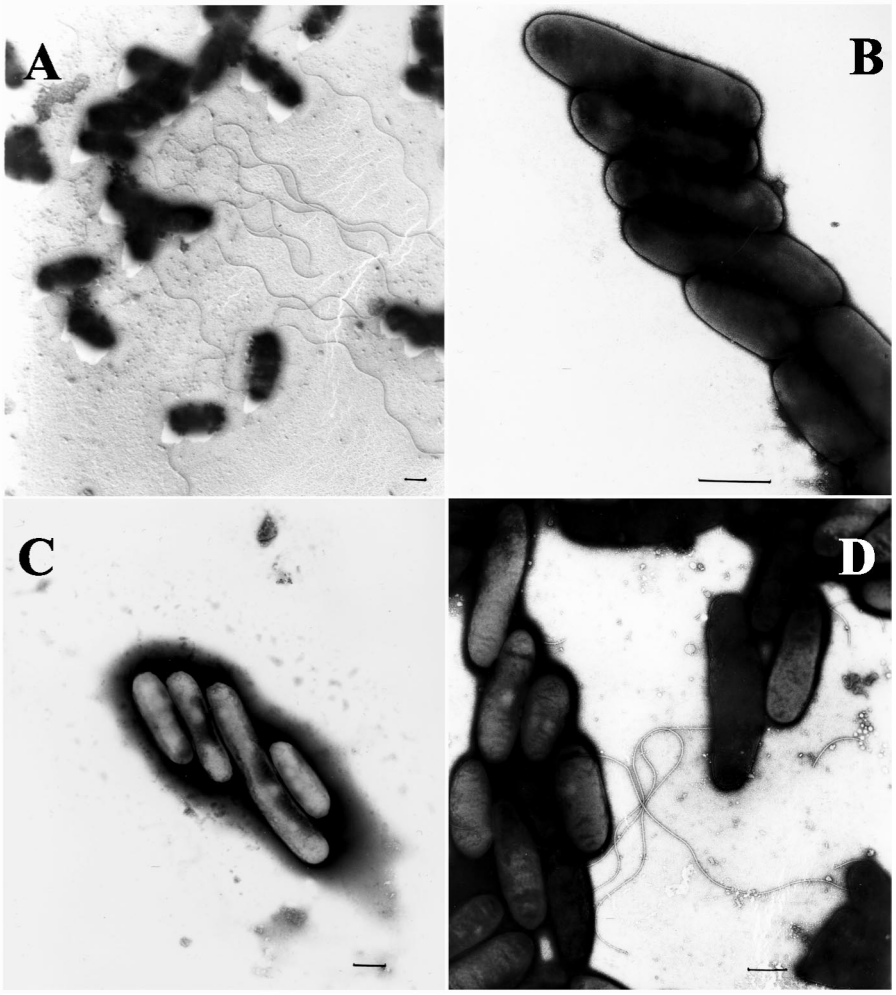


FIG. 5. Electron microscopy of *L. pneumophila* strains. Bacteria were grown on BCYE agar plates at 30°C for 4 days (see Materials and Methods). Bars = 0.5 μm. (A) *L. pneumophila* Corby (wild type); (B) *fleQ* mutant strain; (C) *rpoN* mutant strain; (D) complemented *fleQ* mutant strain.

that in both of these mutants this gene is positively regulated by RpoN and FleQ. The *fleN* gene, which had a putative $\sigma^{54}$-like promoter element, was also positively regulated by RpoN and FleQ (Fig. 3C). In the *fleQ* mutant these phenotypes were successfully complemented (Fig. 3C). It is likely that the identified $\sigma^{54}$ promoter elements of *fliM* and *fleN* are not recognized by the RNA polymerase when RpoN or FleQ is not present. The results of RT-PCR experiments suggest that transcription of the *fleSR* operon also is positively regulated by FleQ and RpoN (Fig. 3C). On the other hand, comparable amounts of *flaA* transcripts were identified in both mutant strains and the wild type (Fig. 3C). This confirmed the finding mentioned above obtained by Western blot analysis with the FlaA-specific antiserum that FlaA is produced in the mutants (Fig. 3B). Besides *flaA* transcripts, we detected in both mutants amounts of *fliA* transcripts that were comparable to the amounts in the wild type (Fig. 3C). The presence of FliA, a positive regulator of *flaA*, in the wild type and mutants may explain why the mutants are still able to express FlaA. However, as *fliA* seems to be expressed even though RpoN and FleQ are not present, it is not surprising that both mutants expressed the flagellin. RT-PCR also revealed that RpoN and FleQ seemed not to be involved in expression of *icmR* (Fig. 3C), which encodes a subunit of the type IV secretion system of *L. pneumophila*. This suggests that neither RpoN nor FleQ is involved in regulation of this virulence factor.

**Intracellular replication of the *rpoN* and *fleQ* mutants in host cells and distribution of *rpoN* and *fleQ* in legionellae.** The *rpoN* mutant and the *fleQ* mutant were tested for the ability to replicate intracellularly in the macrophage-like cell line U937. Compared to the replication of the wild-type strain, both mutants were still able to replicate (data not shown).

Southern blot analysis revealed that both *rpoN* and *fleQ* are conserved in *L. pneumophila* strains (data not shown). However, they seemed not to be as conserved in legionellae as the flagellin gene, because most of the non-*L. pneumophila* strains tested (see Materials and Methods) did not cross-hybridize with the *fleQ*-specific DNA probe (data not shown), whereas an *flaA*-specific probe was able to bind to the DNA of all flagellated strains tested (15). With the *rpoN*-specific probe,

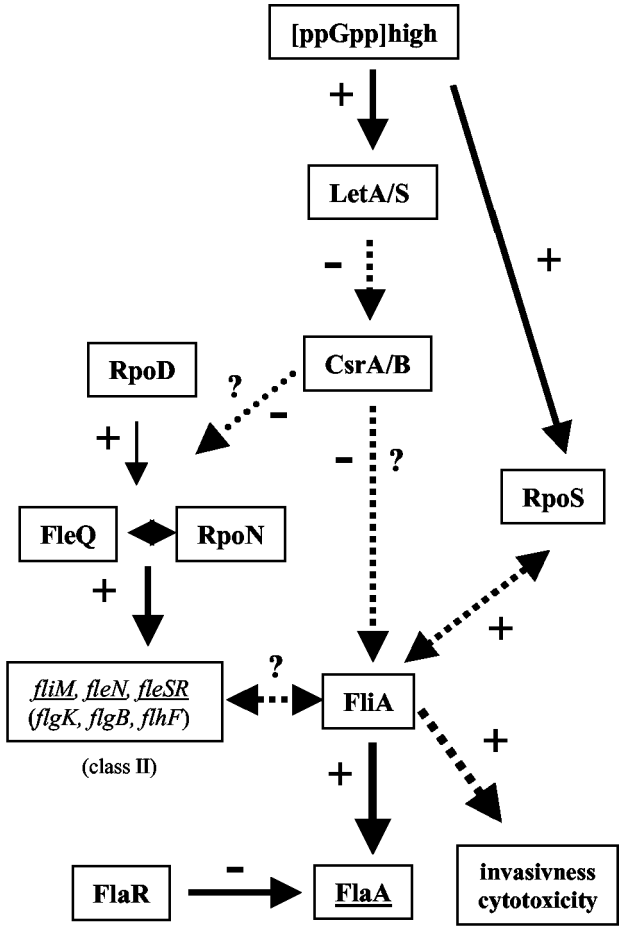


FIG. 6. Proposed cascade of *flaA* gene regulation in *L. pneumophila* Corby. The dotted arrows indicate unknown modes of regulation (direct or indirect). Putative class II genes of the regulation cascade are indicated. The role of FlaR is not known yet. +, positive regulation; −, negative regulation; ?, proposed link; CsrA/B, carbon storage regulator; FlaA, flagellin; FlaR, transcriptional regulator (LysR family); *fleSR*, putative two-component system; FleQ, transcriptional regulator; FliA, alternative $\sigma^{28}$ factor; LetA/S, two-component system; RpoN, alternative $\sigma^{54}$ factor; RpoD, $\sigma^{70}$ factor. (Modified from reference 21 with the permission of the publisher.)

weak hybridization signals were obtained only with *L. bozemanii*, *L. dumoffii*, *L. feelii* S1, *L. gormanii,* and *L. longbeachae* S1.

## DISCUSSION

Over 50 genes are required for assembly and functioning of the bacterial flagellum, and it has been shown that translational and posttranslational regulation also plays an important role in flagellar assembly. Furthermore, different promoter classes and regulators of various *fla* regulon hierarchies have been described (for a review see reference 1).

In this paper we describe cloning and characterization of *rpoN* and *fleQ* homologs of *L. pneumophila* Corby. The *rpoN* gene encodes an alternative $\sigma^{54}$ factor. $\sigma^{54}$ factors can be regarded as defective holoenzymes, because they initiate transcription only in concert with an activator protein (30). Most of these enhancer proteins are controlled by their own signal transducing pathways, which allows the bacteria to respond to a wide range of environmental signals through one sigma factor (5). We were able to identify three of these putative activators (FleQ, FleR, and PilR) in the genome sequence of *L. pneumophila* Philadelphia, all of which exhibited $\sigma^{54}$ interaction domains. DNA probes specific for the *rpoN* and *fleQ* genes hybridized with chromosomal DNA of all *L. pneumophila* strains but not with DNA of most of the non-*L. pneumophila* strains tested so far, suggesting that these factors are not very well conserved within the legionellae.

Analysis of the deduced amino acid sequence of *rpoN* revealed that RpoN has the $\sigma^{54}$ factor domains (AID, CBD, and DBD) commonly found in $\sigma^{54}$ factors (5). These domains are involved in activator interaction (AID), in interaction with the core RNA polymerase (CBD), and in DNA binding (DBD or RpoN box). The RpoN protein exhibited the highest identity (55%) to $\sigma^{54}$ of *V. cholerae*. Inactivation of *rpoN* or *fleQ* in *L. pneumophila* led to nonflagellated mutant strains. The flagellar operon genes (class II) were found to contain putative $\sigma^{54}$ promoter elements (Table 2) (21). Our results showed that flagellar expression depends on the presence of RpoN and its activator protein, FleQ. However, FlaA was still expressed at low levels in both mutants, but it was not assembled into a flagellum. It was shown recently that FliA directly regulates *flaA* expression (Fig. 3B, lane 5) (20). Here, we demonstrated by using RT-PCR that the *fliA* transcript is present in both mutant strains (Fig. 3C). This suggests that *flaA* is expressed in the *fleQ* and *rpoN* mutants, probably as a consequence of FliA expression. The flagellin may not be assembled into a flagellum because of the lack of expressed basal body genes. This hypothesis is supported by the reduced amounts of the transcript of *fliM* (an operon encoding several putative basal body genes) observed in the *fleQ* and *rpoN* mutants (Fig. 3C). These results also demonstrate that the *fliM*, *fleSR*, and *fleN* genes, all containing a putative $\sigma^{54}$-dependent promoter, are positively regulated by RpoN and FleQ. Now it has to be shown if this is also true for other class II genes containing putative $\sigma^{54}$-dependent promoters (Fig. 6) (21). We identified FleR of *L. pneumophila* as a putative $\sigma^{54}$ interaction protein. FleS has been cloned recently, but the role of FleSR in *flaA* expression in *L. pneumophila* has not been determined yet (31). We started to generate an FleR mutant to analyze the role of FleR in the cascade of flagellar gene regulation. RT-PCR results suggest that *fleSR* expression is positively regulated by FleQ and RpoN (Fig. 6). In *P. aeruginosa* the FleSR two-component system is also involved in flagellin expression, in addition to FleQ and RpoN. Furthermore, we also identified PilR as a putative $\sigma^{54}$ interaction protein, and experiments are under way to generate and analyze a *pilR* mutant strain of *L. pneumophila* Corby. It has to be determined if *pilR* is necessary for flagellation, for piliation, or for the virulence of *L. pneumophila*. A proposed cascade of flagellar regulation is shown in Fig. 6.

In this paper, we show that RpoN and FleQ are involved in flagellar gene expression in *L. pneumophila* Corby. RpoN and FleQ are necessary for flagellar expression and assembly. It is likely that FleQ expression is RpoD dependent, because we were able to identify $\sigma^{70}$-like promoter elements in front of the transcription initiation sites of *fleQ* by primer extension analysis (Fig. 4). We have to analyze if *fleQ* transcription is also Vfr dependent, as described for *P. aeruginosa* (7). A putative Vfr

binding site was identified overlapping the *fleQ* promoter element, and a homolog of the Vfr gene is present in the genome sequence of *L. pneumophila* Philadelphia. Furthermore, FleQ expression is not dependent on the presence of RpoN or FliA, as shown by Western blot analysis (Fig. 3A). RT-PCR results suggest that RpoN and FleQ are not involved in the regulation of *fliA* and *icmR* gene expression (Fig. 3C). The *icmR* gene encodes a protein of the type IV secretion system needed for intracellular replication of *L. pneumophila* in this host. Furthermore, both mutants were able to replicate intracellularly in U937 cells, suggesting that both genes are not required for intracellular replication of *L. pneumophila*. A putative cascade of flagellar gene expression has been determined, and this cascade is similar to those described for *Pseudomonas* and *Vibrio* (1, 8). Experiments are under way to characterize the expression of *fliA*, because FliA is known to be involved in the virulence of *Legionella* (14, 20). The CsrA protein seems to be involved in *fliA* expression, but the activator of *fliA* expression has not been identified (11, 29). Further analysis of this cascade of gene regulation should help us understand the role of FliA in the link between virulence and flagellar expression in *L. pneumophila*.

## ACKNOWLEDGMENTS

We thank Reuben Ramphal (Gainesville, Fla.) for providing the anti-FleQ antibody and Bianca Hochhut for careful reading of the manuscript.

This work was supported by grants GRK 587/1-01 and HE2845/2-1 from the Deutsche Forschungsgemeinschaft.

## REFERENCES

1. **Aldridge, P., and K. T. Hughes.** 2002. Regulation of flagellar assembly. Curr. Opin. Microbiol. **5:**160–165.
2. **Arora, S. K., B. W. Ritchings, E. C. Almira, S. Lory, and R. Ramphal.** 1997. A transcriptional activator, FleQ, regulates mucin adhesion and flagellar gene expression in *Pseudomonas aeruginosa* in a cascade manner. J. Bacteriol. **179:**5574–5581.
3. **Bender, L., M. Ott, A. Debes, U. Rdest, J. Heesemann, and J. Hacker.** 1991. Distribution, expression, and long-range mapping of legiolysin gene (*lly*)-specific DNA sequences in legionellae. Infect. Immun. **59:**3333–3336.
4. **Bosshardt, S. C., R. F. Benson, and B. S. Fields.** 1997. Flagella are a positive predictor for virulence in *Legionella*. Microb. Pathog. **23:**107–112.
5. **Buck, M., M.-T. Gallegos, D. J. Sudholme, Y. Guo, and J. D. Gralla.** 2000. The bacterial enhancer-dependent $\sigma^{54}$ ($\sigma^{N}$) transcription factor. J. Bacteriol. **182:**4129–4136.
6. **Byrne, B., and M. S. Swanson.** 1998. Expression of *Legionella pneumophila* virulence traits in response to growth conditions. Infect. Immun. **66:**3029–3034.
7. **Dasgupta, N., E. P. Ferrell, K. J. Kanack, S. E. West, and R. Ramphal.** 2002. *fleQ*, the gene encoding the major flagellar regulator of *Pseudomonas aeruginosa*, is $\sigma^{70}$ dependent and is downregulated by Vfr, a homolog of *Escherichia coli* cyclic AMP receptor protein. J. Bacteriol. **184:**5240–5250.
8. **Dasgupta, N., M. C. Wolfgang, A. L. Goodman, S. K. Arora, J. Jyot, S. Lory, and R. Ramphal.** 2003. A four-tiered transcriptional regulatory circuit controls flagellar biogenesis in *Pseudomonas aeruginosa*. Mol. Microbiol. **50:**809–824.
9. **Dietrich, C., K. Heuner, B. C. Brand, M. Steinert, and J. Hacker.** 2001. Flagellum of *Legionella pneumophila* positively affects the early phase of infection of eukaryotic host cells. Infect. Immun. **69:**2116–2122.
10. **Edelstein, P. H.** 1981. Improved semiselective medium for isolation of *Legionella pneumophila* from contaminated clinical and environmental specimens. J. Clin. Microbiol. **14:**298–303.
11. **Fettes, P. S., V. Forsbach-Birk, D. Lynch, and R. Marre.** 2001. Overexpression of a *Legionella pneumophila* homologue of the *E. coli* regulator *csrA* affects cell size, flagellation, and pigmentation. Int. J. Med. Microbiol. **291:**353–360.
12. **Fields, B. S., J. M. Barbaree, G. N. Sanden, and W. E. Morrill.** 1990. Virulence of a *Legionella anisa* strain associated with Pontiac fever: an evaluation using protozoan, cell culture, and guinea pig models. Infect. Immun. **58:**3139–3142.
13. **Fields, B. S.** 1996. The molecular ecology of legionellae. Trends Microbiol. **4:**286–290.
14. **Hammer, B. K., E. S. Tateda, and M. S. Swanson.** 2002. A two-component regulator induces the transmission phenotype of stationary-phase *Legionella pneumophila*. Mol. Microbiol. **44:**107–118.
15. **Heuner, K., L. Bender-Beck, B. C. Brand, P. C. Lück, K.-H. Mann, R. Marre, M. Ott, and J. Hacker.** 1995. Cloning and genetic characterization of the flagellum subunit gene (*flaA*) of *Legionella pneumophila* serogroup 1. Infect. Immun. **63:**2499–2507.
16. **Heuner, K., J. Hacker, and B. C. Brand.** 1997. The alternative sigma factor $\sigma^{28}$ of *Legionella pneumophila* restores flagellation and motility to an *Escherichia coli fliA* mutant. J. Bacteriol. **179:**17–23.
17. **Heuner, K., B. C. Brand, and J. Hacker.** 1999. The expression of the flagellum of *Legionella pneumophila* is modulated by different environmental factors. FEMS Microbiol. Lett. **175:**69–77.
18. **Heuner, K., C. Dietrich, M. Steinert, U. B. Göbel, and J. Hacker.** 2000. Cloning and characterization of a *Legionella pneumophila* specific gene encoding a member of the LysR family of transcriptional regulators. Mol. Gen. Genet. **264:**204–211.
19. **Heuner, K., U. Meltzer, B.-K. Choi, and U. B. Göbel.** 2001. Outer sheath associated proteins of the oral spirochete *T. maltophilum.* FEMS Microbiol. Lett. **197:**187–193.
20. **Heuner, K., C. Dietrich, C. Skriwan, M. Steinert, and J. Hacker.** 2002. Influence of the alternative $\sigma^{28}$ factor on virulence and flagellum expression of *L. pneumophila.* Infect. Immun. **70:**1604–1608.
21. **Heuner, K., and M. Steinert.** 2003. The flagellum of *Legionella pneumophila* and its link to the expression of the virulent phenotype. Int. J. Med. Microbiol. **293:**133–145.
22. **Jepras, R. I., R. B. Fitzgeorge, and A. Baskerville.** 1985. A comparison of virulence of two strains of *Legionella pneumophila* based on experimental aerosol infection of guinea-pigs. J. Hyg. **95:**29–38.
23. **Jin, S., K. Ishimoto, and S. Lory.** 1994. Nucleotide sequence of the *rpoN* gene and characterization of two downstream open reading frames in *Pseudomonas aeruginosa*. J. Bacteriol. **176:**1316–1322.
24. **Jones, D. H., F. C. Franklin, and C. M. Thomas.** 1994. Molecular analysis of the operon which encodes the RNA polymerase sigma factor sigma 54 of *Escherichia coli*. Microbiology **140:**1035–1043.
25. **Jyot, J., N. Dasgupta, and R. Ramphal.** 2002. FleQ, the major flagellar gene regulator in *Pseudomonas aeruginosa*, binds to enhancer sites located either upstream or atypically downstream of the RpoN binding site. J. Bacteriol. **184:**5251–5260.
26. **Klose, K. E., and J. J. Mekalanos.** 1998. Distinct roles of an alternative sigma factor during both free-swimming and colonizing phases of the *Vibrio cholerae* pathogenic cycle. Mol. Microbiol. **28:**501–520.
27. **Laemmli, U. K.** 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
28. **Merriam, J. J., R. Mathur, R. Maxfield-Boumil, and R. R. Isberg.** 1997. Analysis of the *Legionella pneumophila fliI* gene: intracellular growth of a defined mutant defective for flagellum biosynthesis. Infect. Immun. **65:**2497–2501.
29. **Molofsky, A. B., and M. S. Swanson.** 2003. *Legionella pneumophila* CsrA is a pivotal repressor of transmission traits and activator of replication. Mol. Microbiol. **50:**445–461.
30. **Morett, E., and L. Segovia.** 1993. The sigma 54 bacterial enhancer-binding protein family: mechanism of action and phylogenetic relationship of their functional domains. J. Bacteriol. **175:**6067–6074.
31. **Polesky, A. H., J. T. Ross, S. Falkow, and L. S. Tompkins.** 2001. Identification of *Legionella pneumophila* genes important for infection of amoebas by signature-tagged mutagenesis. Infect. Immun. **69:**977–987.
32. **Pruckler, J. M., R. F. Benson, M. Moyenuddin, W. T. Martin, and B. S. Fields.** 1995. Association of flagellum expression and intracellular growth of *Legionella pneumophila*. Infect. Immun. **63:**4928–4932.
33. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
34. **Saunders, N. A., N. Doshi, and T. G. Harrison.** 1992. A second serogroup of *Legionella erythra* serologically indistinguishable from *Legionella rubrilucens*. J. Appl. Bacteriol. **72:**262–265.
35. **Stone, B. J., and Y. Abu Kwaik.** 1999. Natural competence for DNA transformation by *Legionella pneumophila* and its association with the expression of type IV pili. J. Bacteriol. **181:**1395–1402.