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

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We cloned and analyzed *Legionella pneumophila* Corby homologs of *rpoN* (encoding σ^{54}) and *fleQ* (encoding σ^{54} activator protein). Two other genes (*fleR* and *piIR*) whose products have a σ^{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 σ^{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 σ^{54}-dependent promoter consensus sequence. However, they seemed to be dispensable for transcription of *fliA*, *fliC*, 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.

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Taq polymerase (Invitrogen GmbH). Restriction endonuclease sites: H, HindIII; K, KpnI; P, PstI; S, SnaBI; Sc, SacII. putative rho-independent termination site; aa, amino acids; put., putative encoding 50S ribosomal protein L33 homolog (54 amino acids); term, terminator.

Materials and Methods. Functional domains encoded by the FleQ-encoding region (B) of L. pneumophila were inferred from a database searching and sequence analysis. The FleQ gene is encoded by the FleQ operon, which contains the FleQ protein and the FleQ-encoding region. The FleQ protein is predicted to be a membrane-bound protein with two transmembrane domains.

Restriction endonuclease sites: H, HindIII; K, KpnI; P, PstI; S, SnaBI; Sc, SacII.

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 resistance cassette into the chromosome, see Materials and Methods. Functional domains encoded by fleQ are indicated. The deduced protein contains a domain (grey box), a putative ATP binding site (AA) (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 pS31, resulting in pS32. For a description of the methods used for integration of the rpoN::Km resistance cassette into the chromosome, see Materials and Methods. Typical rpoN functional domains that are encoded by rpoN are indicated and include the AID, CBD, and DBD. Genes are indicated by arrows. L28, region encoding 50S ribosomal protein L28 homolog (76 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.

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). 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 μF, and 25 mF, and electroporation of Legionella strains was carried out by using 2.3 kV, 100 μF, 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, 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 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 by using chromogenic detection kits (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 RNeasy Mini columns (Qiagen) according to the manufacturer’s instructions. cDNA synthesis was 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 MgCl2, 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 plasmid DNA strain. Primer extension analysis was carried out with IRD-labeled primer PCR (5'-AAATAGTCCGAGAGTTCGAC-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 previously (15).

TABLE 1. Primers used for RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Temp for PCR (°C)</th>
<th>Length (bp)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>flaA</td>
<td>CATGATGCAACACATCGATCGA</td>
<td>CTGCTACTTCTGTCCTGTG</td>
<td>52</td>
<td>480</td>
</tr>
<tr>
<td>flaB</td>
<td>AACGCATTGCACTATCTGC</td>
<td>ATAGACATCCTCAGTTCTACT</td>
<td>50</td>
<td>400</td>
</tr>
<tr>
<td>flaC</td>
<td>TGGACGATGCTGATGACAGTACGACGAA</td>
<td>GTAATCTAATCTGACATCCAG</td>
<td>52</td>
<td>540</td>
</tr>
<tr>
<td>flaD</td>
<td>GTGCAAGAATCTGGCAGTAATTT</td>
<td>GAAAGAGAATAATCATCCGCG</td>
<td>50</td>
<td>490</td>
</tr>
<tr>
<td>rpoN</td>
<td>TGTGTCACATCTCAGTTAAC</td>
<td>CCTAGCAACTCAATGTCCTCA</td>
<td>51</td>
<td>600</td>
</tr>
<tr>
<td>flaM</td>
<td>GAGATCGATGCTGACTGATT</td>
<td>TAATAATCGACCAATGCTACACA</td>
<td>51</td>
<td>450</td>
</tr>
<tr>
<td>icmR</td>
<td>ATACCTGATCAGTCAGCAGAAA</td>
<td>GATGATAAATTGGAAAACACGTCG</td>
<td>50</td>
<td>350</td>
</tr>
<tr>
<td>fleR</td>
<td>AAGTATGATGATGATGGTTT</td>
<td>AACTTAAAACATGACTCAT</td>
<td>45</td>
<td>480</td>
</tr>
</tbody>
</table>

* Temperature used for the amplification step in the RT-PCR.

* Length of amplified template.
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% CO2. 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 H2O 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 37°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 (fl eQ) and AJ580316 (rpoN).

RESULTS

Cloning of the rpoN and fleQ genes of L. pneumophila Corby. Recently, we identified putative ρ54 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 ρ54 interaction domain in the deduced protein sequences of the genome of L. pneumophila (htp://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'-ATCTTACGTTCATACAAATACT-3'] and Rpon-R [5'-CAGTGAATGCTCTTAGTGCAGGAG-3']) and fleQ (FleQ-F [5'-CGTTTAATGATTACCGAGTGGA-3']) and FleQ-R were designed.
Nucleotide and protein sequence analysis of \textit{fleQ} and FleQ. The putative \textit{fleQ} gene encompasses 1,413 bp and encodes a protein with a calculated molecular mass of 53 kDa (Fig. 2). A \textit{P. aeruginosa}-specific anti-FleQ antibody cross-reacted in Western blot analysis with the FleQ protein of \textit{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 \textit{L. pneumophila} Corby is 99, 55, 54, and 54\% identical to FleQ of \textit{L. pneumophila} Philadelphia, FleQ of \textit{P. aeruginosa}, the \(\sigma^{54}\)-dependent transcriptional activator of \textit{Vibrio cholerae}, and FlaK of \textit{Vibrio parahaemolyticus}, respectively. Downstream of \textit{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 \textit{L. pneumophila}. These proteins exhibited 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 \textit{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 transcriptional activator of \textit{V. cholerae}, and FlaK of \textit{V. parahaemolyticus}, respectively. Downstream of \textit{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).

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Five nucleotides upstream of the start codon (ATG) of \textit{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 \textit{fleQ} was found, but upstream of the transcriptional start site \(\tau_3\) no putative promoter element was identified (Fig. 4). Furthermore, a putative Vfr (homolog of the \textit{E. coli} cyclic AMP activator) interaction domain was the most conserved region (Fig. 2).

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A, B, and C (See Table 2).}

<table>
<thead>
<tr>
<th>Region or gene</th>
<th>Sequence</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>(\sigma^{70}) consensus</td>
<td>TTGACA(\rightarrow)N1(\rightarrow)TATAAT(^a)</td>
<td>This study</td>
</tr>
<tr>
<td>\textit{fleQ} (\tau_1)</td>
<td>GTCTCA(\rightarrow)N1(\rightarrow)CATTAT(\rightarrow)N1(\rightarrow)AGT(^a)</td>
<td>This study</td>
</tr>
<tr>
<td>\textit{fleQ} (\tau_2)</td>
<td>ATAAAT(\rightarrow)N1(\rightarrow)AGTTAT(\rightarrow)N1(\rightarrow)AGT(^a)</td>
<td>This study</td>
</tr>
<tr>
<td>(\sigma^{28}) consensus</td>
<td>TAAA(\rightarrow)N1(\rightarrow)GCGGATA(^a)</td>
<td>15</td>
</tr>
<tr>
<td>\textit{flaA}</td>
<td>TAAA(\rightarrow)N1(\rightarrow)TCCGATA(\rightarrow)N3(\rightarrow)AGT(^a)</td>
<td>21</td>
</tr>
<tr>
<td>\textit{fliD}</td>
<td>TATA(\rightarrow)N1(\rightarrow)TCCGATA(\rightarrow)N1(\rightarrow)AGT(^a)</td>
<td>21</td>
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<tr>
<td>(\sigma^{54}) consensus</td>
<td>TGGCAC(\rightarrow)N3(\rightarrow)TTGCA(^b)</td>
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<tr>
<td>\textit{fliM}</td>
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<td>21</td>
</tr>
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<td>21</td>
</tr>
<tr>
<td>\textit{pilS}</td>
<td>TGGTCA(\rightarrow)N2(\rightarrow)AGCG(\rightarrow)N1(\rightarrow)AGT(^b)</td>
<td>This study</td>
</tr>
</tbody>
</table>

\(^a\) The region before N1 or N2 is the \(-35\) region, and the region after N1 or N2 is the \(-10\) region.

\(^b\) The region before N3 or N5 is the \(-24\) region, and the region after N9 or N3 is the \(-12\) region.

\[5'.\text{TTCCAGTACAGCGAATCCGTGAT-3'}\] homologs were generated, and the corresponding genes of \textit{L. pneumophila} Corby were amplified, cloned, and analyzed further. The genetic maps of the cloned \textit{fleQ} and \textit{rpoN} regions are shown in Fig. 1.

\[VOL. 186, 2004\] RpoN AND FleQ HOMOLOGS OF \textit{L. PNEUMOPHILA} 2543
The presence of an active 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 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 contained amounts of FleQ comparable to the wild-type strain (Fig. 3C), and gene-specific primer pairs (Table 1) were detectable in the *rpoN* mutant strains, suggesting

![Image](https://example.com/image.png)

**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 (t1 to t5). 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-N12-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 σ54 family signature and the Pfam_σ54-activator interaction domain (AID), Pfam_σ54-core binding domain (CBD), and Pfam_σ54-DNA binding domain (DBD) commonly found in σ54 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 σ70-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 σ54 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*.
that in both of these mutants this gene is positively regulated by RpoN and FleQ. The \textit{fleN} gene, which had a putative $\sigma^{54}$-like promoter element, was also positively regulated by RpoN and FleQ (Fig. 3C). In the \textit{fleQ} mutant these phenotypes were successfully complemented (Fig. 3C). It is likely that the identified $\sigma^{54}$-promoter elements of \textit{fliM} and \textit{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 \textit{fleSR} operon also is positively regulated by FleQ and RpoN (Fig. 3C). On the other hand, comparable amounts of \textit{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 \textit{flaA} transcripts, we detected in both mutants amounts of \textit{fliA} transcripts that were comparable to the amounts in the wild type (Fig. 3C). The presence of FliA, a positive regulator of \textit{flaA}, in the wild type and mutants may explain why the mutants are still able to express FlaA. However, as \textit{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 \textit{icmR} (Fig. 3C), which encodes a subunit of the type IV secretion system of \textit{L. pneumophila}. This suggests that neither RpoN nor FleQ is involved in regulation of this virulence factor.

**Intracellular replication of the \textit{rpoN} and \textit{fleQ} mutants in host cells and distribution of \textit{rpoN} and \textit{fleQ} in legionellae.** The \textit{rpoN} mutant and the \textit{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 \textit{rpoN} and \textit{fleQ} are conserved in \textit{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-\textit{L. pneumophila} strains tested (see Materials and Methods) did not cross-hybridize with the \textit{fleQ}-specific DNA probe (data not shown), whereas an \textit{flaA}-specific probe was able to bind to the DNA of all flagellated strains tested (15). With the \textit{rpoN}-specific probe,
weak hybridization signals were obtained only with L. bozemanii, L. dumoffii, L. felii S1, L. gormanii, and L. longbeachae S1.

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; FleA, flagellin; FleR, transcriptional regulator (LasR family); fleSR, putative two-component system; FleQ, transcriptional regulator; FliA, alternative ς54 factor; LetA/S, two-component system; RpoN, alternative ς54 factor; RpoD, ς70 factor. (Modified from reference 21 with the permission of the publisher.)

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 ς54 factor. ς54 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 ς54 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 ς54 factor domains (AID, CBD, and DBD) commonly found in ς54 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 ς24 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 ς54 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 FleA directly regulates flaA expression (Fig. 3B, lane 5) (20). Here, we demonstrated by using RT-PCR that the flaA 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 FleA 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 ς54-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 ς54-dependent promoters (Fig. 6) (21). We identified FleR of L. pneumophila as a putative ς54 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 FleE 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 ς54 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 ς52-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*.

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