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Cloning and Genetic Characterization of the Flagellum Subunit Gene (*flaA*) of *Legionella pneumophila* Serogroup 1

KLAUS HEUNER,[1] LARISA BENDER-BECK,[1] BETTINA C. BRAND,[1] P. CHRISTIAN LÜCK,[2] KARL-HEINZ MANN,[3] REINHARD MARRE,[4] MANFRED OTT,[1] AND JÖRG HACKER[1]*

Institut für Molekulare Infektionsbiologie der Universität Würzburg, D-97070 Würzburg,[1] Institut für Medizinische Mikrobiologie, Medizinische Akademie Dresden, D-01309 Dresden,[2] Max-Planck-Institut für Biochemie, D-82152 Martinsried,[3] and Institut für Mikrobiologie und Immunologie der Universität Ulm, D-89081 Ulm,[4] Germany

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The gene *flaA*, encoding the flagellum subunit protein of *Legionella pneumophila* serogroup 1, has been isolated from an expression library of *L. pneumophila* isolate Corby in *Escherichia coli* K-12 by using an antiflagellin specific polyclonal antiserum. DNA sequence analysis of the *flaA* gene revealed the presence of a 1,428-bp open reading frame encoding a protein of 475 amino acids with an apparent molecular mass of 48 kDa that is expressed independently of an *E. coli* vector promoter. Peptide sequencing of the N terminus of the isolated flagellum subunit protein confirmed that this open reading frame encodes the flagellin. By comparing the FlaA amino acid sequence with those of flagellins of various other bacteria, high degrees of homology in the N-terminal and C-terminal amino acids could be observed. The *flaA*-specific mRNA was determined to be 1.6 kb in size, the expected size of a monocistronic mRNA. Temperature-dependent expression of flagellin was found to be regulated at the transcriptional level. Sequence analysis and primer extension experiments indicated that the transcription of the gene *flaA* is directed by a σ^{28}-like RpoF-FliA factor. By using *fliA* and *fliA*$^+$ *E. coli* K-12 mutants, it was shown that *flaA* expression in *E. coli* required the σ^{28} factor. A *flaA*-specific DNA probe hybridizes with genomic DNA isolated from *L. pneumophila* and with most of the genomic DNAs from non-*L. pneumophila Legionella* strains. Two *L. pneumophila* strains and isolates of *Legionella bozemanii* and *Legionella feeleii* (serogroup 1) carry *flaA*-specific sequences but were not able to produce flagella.

Legionella pneumophila, the causative agent of Legionnaires' disease, is a ubiquitous microorganism inhabiting freshwater biotopes. Inhalation of aerosolized legionellae leads to an infection of the human respiratory tract, in which *L. pneumophila* multiplies very efficiently in alveolar macrophages. In the natural environment, intracellular replication occurs in amoebae and other protozoa (59). To date, only a few well-defined virulence factors have been identified (16). The Mip-factor, a membrane-associated protein of 24 kDa showing peptidylprolyl *cis-trans* isomerase activity, contributes to the survival of *L. pneumophila* in macrophage-like cells and in protozoan organisms (13, 22, 25). More recently, two factors encoded by the *dot* and *icm* loci were identified and characterized as virulence factors (6, 7). Another potential virulence factor is the outer membrane protein MOMP, encoded by the *ompS* gene. This porin is necessary for the interaction between legionellae and host cells (29).

The influence of bacterial motility on the pathway of infection or on the survival of legionellae in aquatic habitats is not well understood. Shortly after the first report about *L. pneumophila*, it was demonstrated that legionellae are flagellated (9, 46). *L. pneumophila* cells produce a single, monopolar flagellum. Expression of flagella has been found in alveolar spaces of the human lung (10), and it has been demonstrated that the production of flagella is obviously not required for the intraperitoneal route of infection in guinea pigs (19). However, another report indicated that motility might be a virulence factor in the infection pathway of *L. pneumophila* in *Acanthamoeba castellanii* trophozoites (47).

Recently, it was shown that the expression of flagella in *L. pneumophila* is temperature regulated, since the expression of the monopolar flagellum is repressed at temperatures higher than 37°C (41). Temperature-dependent expression of flagellin was also demonstrated for *Listeria monocytogenes*, *Serratia marcescens*, and other organisms (15, 28, 42). The flagellin is the major subunit of the flagella of *L. pneumophila* Philadelphia I and was detected by Western blot (immunoblot) analysis, with an approximate molecular mass of 47 kDa (18, 41). Furthermore, it was demonstrated that various *L. pneumophila* strains and isolates of species other than *L. pneumophila* were able to produce flagella similar to the flagella of Philadelphia I (41). In this study, we have cloned and sequenced the flagellin gene *flaA* of *L. pneumophila* isolate Corby. It is shown that the N- and C-terminal regions of the flagellin exhibit significant homology to the corresponding regions of other flagellins. Furthermore, temperature-dependent expression of flagellin is regulated at the transcriptional level.

MATERIALS AND METHODS

Bacterial strains and plasmids. *L. pneumophila* Corby (serogroup 1) (31) was used for cloning of the *flaA* coding region. *L. pneumophila* strains are described in Table 1. *Escherichia coli* DH5α was used for propagation of recombinant plasmid DNA. Plasmid pUC 18 (Pharmacia LKB, Freiburg, Germany) was used for the construction of the expression library in *E. coli*. *E. coli* K-12 YK 410 (*fliA*$^+$) and YK 4104 (*fliA*) were described previously (11).

Media and chemicals. *E. coli* was cultivated in Luria-Bertani (LB) broth. *Legionella* strains were grown either on buffered charcoal-yeast extract (BCYE) agar plates supplemented with 0.025% ferric PP_i and 0.04% cysteine (Oxoid, Wesel, Germany) for 48 h before harvesting or in supplemented liquid GC-FC (containing 1.5% proteose peptone, 1% yeast extract, 0.4% K_2HPO_4, and 0.1% KH_2PO_4) unless otherwise stated. Enzymes were purchased from Pharmacia LKB, Boehringer GmbH (Mannheim, Germany), and GIBCO BRL (Eggenstein,

* Corresponding author. Mailing address: Institut für Molekulare Infektionsbiologie, Universität Würzburg, Röntgenring 11, D-97070 Würzburg, Germany. Phone: 49-931-312575. Fax: 49-931-571954.

TABLE 1. Characteristics of *Legionella* strains

Strain[a]	Reference or source[b]	Reaction with anti-FlaA antibodies[c]	Hybridization with *flaA* under[d]		Flagellation[e]
			High stringency	Low stringency	
L. pneumophila					
Corby	31	+	+	+	+
Philadelphia I (S1) (virulent)	ATCC 33152	+	+	+	+[f]
Philadelphia I XXXV (S1) (avirulent)	5	+	+	+	+[f]
U1 (S1) (environmental isolate)	5	+	+	+	+[f]
Msp19 (S1) (environmental isolate)	5	+/−	+	+	+
U22 (S3) (environmental isolate)	5	+	+	+	+
U21 (S6) (environmental isolate)	5	+	+	+	+[f]
685 (S1) (patient isolate)	5	+	+	+	+
667 (S4) (patient isolate)	5	−	+	+	−
640 (S5) (patient isolate)	5	+	+	+	+
664 (S6) (patient isolate)	5	+	+	+	+
Bloomington (S3)	ATCC 33155	+	+	+	+
Los Angeles (S4)	ATCC 33156	−	+	+	−
Chicago -2 (S6)	ATCC 33215	+	+	+	+
L. bozemanii	ATCC 33217	−	+	+	−
L. dumoffii	ATCC 33279	+/−	−	+	+
L. feeleii					
S1	ATCC 35072	−	+	+	−
S2	ATCC 35849	+	+	+	+
L. gormanii	ATCC 33297	+	+	+	+
L. hackeliae					
S1	ATCC 33250	+	+	+	+[f]
S2	ATCC 35999	+	+	+	+[f]
L. israeliensis	ATCC 43119	−	−	−	−
L. jordanis	ATCC 33623	+	+	+	+
L. longbeachae					
S1	ATCC 33462	−	−	−	−
S2	ATCC 33484	+	+	+	+
L. micdadei	ATCC 33218	+	+	+	+[f]
L. oakridgensis	ATCC 33761	−	−	−	−

[a] S1 through S6, serogroups 1 through 6.
[b] ATCC, American Type Culture Collection, Rockville, Md.
[c] Determined by Western blot analysis (56) of whole-cell extracts of the bacteria, using antiflagellin antibodies.
[d] For the specific conditions of low and high stringency, see Materials and Methods.
[e] Determined by electron microscopy.
[f] See reference 41.

Germany). Radiochemicals were supplied by Amersham (Braunschweig, Germany). Chemicals and oligonucleotides were purchased from Merck (Darmstadt, Germany), Oxoid, and Roth (Karlsruhe, Germany).

Preparation of polyclonal monospecific antibody against *L. pneumophila* Corby flagellin. In order to obtain an antibody against the flagellum subunit of *L. pneumophila* Corby, flagella were isolated and rabbits were immunized with the flagellum subunit as described previously (41). In order to reduce cross-reactivity of the resulting antibody, the polyclonal monospecific antibody was partially purified by binding the antibody-containing solution to total cell extracts of *E. coli* DH5α harboring plasmid pUC 18. The nonbinding fraction containing the purified antibody was then used for Western blot analysis.

SDS-PAGE and Western blot. Total cell extracts of *L. pneumophila* and *E. coli* strains were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. SDS-PAGE was carried out as described by Laemmli (35). *Legionella* cells were grown on BCYE agar plates for 48 h at 30°C, harvested in 1 ml of H_2O, and pelleted by centrifugation. The cells were then suspended in 50 μl of loading buffer and loaded onto an SDS–13% polyacrylamide gel. *E. coli* YK 410 (*fliA*$^+$) and YK 4104 (*fliA*) and recombinant *E. coli* K-12 strains expressing *L. pneumophila* flagellin were grown overnight in 5 ml of LB medium supplemented with ampicillin (100 μg/ml) at 30°C. Samples (0.5 ml) of the overnight cultures were transferred to 50 ml of fresh medium supplemented with ampicillin (100 μg/ml) and grown to late logarithmic phase at 30°C. A total of 10^9 cells were pelleted by centrifugation, suspended in 50 μl of loading buffer, and electrophoresed in an SDS–13% polyacrylamide gel. Western blots were carried out as described elsewhere (56).

N-terminal amino acid sequencing of *L. pneumophila* flagellin. A purified flagellin preparation was blotted onto polyvinylidene difluoride membranes (12) (Immobilontransfer; Millipore, Eschborn, Germany) to determine the N-terminal amino acid residues by Edman degradation using an Applied Biosystems 470 gas phase sequencer.

Electron microscopy. Bacteria grown on BCYE agar plates at 30°C were carefully suspended in distilled water, and a drop of the suspension was directly applied to Formvar-coated copper grids. After sedimentation of the bacteria and removal of remaining fluid, the samples were shadowed with platinum-palladium and examined with a Zeiss 10A transmission electron microscope.

DNA techniques. Preparation of genomic DNA and plasmid DNA and DNA cloning procedures were performed according to standard protocols (48). To construct the expression library of *L. pneumophila* Corby (serogroup 1), chromosomal DNA was partially digested with *Sau*3A and the fragments were electrophoresed in a 1.0% agarose gel. Fragments ranging from 1.0 to 5.0 kb were isolated and eluted by the freeze squeeze technique (54). The fragments were ligated into the *Bam*HI site of vector pUC 18 and transformed into *E. coli* DH5α.

Identification of recombinant *E. coli* K-12 expressing the flagellin of *L. pneumophila* Corby. Recombinant *E. coli* K-12 clones were grown in LB broth (ampicillin, 100 μg/ml) overnight at 37°C in 96-well microtiter dishes. The microtiter dishes were stored at −70°C after glycerol was added to a final concentration of 25%. Replicates of the clones were generated on nitrocellulose filters, which were placed on LB agar plates (ampicillin, 100 μg/ml) and incubated. The immuno-colony-dot assay (57) was then carried out with the antibodies raised against the flagellum subunit protein (41).

DNA sequencing. The nucleotide sequence of the 1,747-bp *L. pneumophila Sau*3A insert of pFLA 1 (see Fig. 1) was determined by the chain termination method of Sanger et al. (49) with α-^{32}P-labelled dATP (Amersham). Both strands were sequenced, and the sequenced region was analyzed with the Genetics Computer Group package (14). Synthetic oligonucleotide primers (5′-GTAATCAACACTAATGTGGC-3′, 5′-GCTGCCAACTCGACCAATAAC-3′, 5′-GGTGTCAGTAACCAAACTGG-3′, 5′-CGTATCAGACAACTTATCAGC-3′, 5′-GTTGCAGAATTTGGTTTTTGGTC-3′, and 5′-TGATGTCTGCATCATGTTGCC-3′) were obtained from Roth.

Southern hybridization. Chromosomal DNAs from various *Legionella* strains were digested with *Hin*dIII and electrophoresed in a 1% agarose gel. The DNA fragments were then transferred to nylon membranes (Pall, Dreieich, Germany) as described by Southern (51). The 987-bp *Hin*dIII-*Sac*II fragment of pFLA 1 was used as a flagellin-specific probe. The DNA probe was labelled and detected

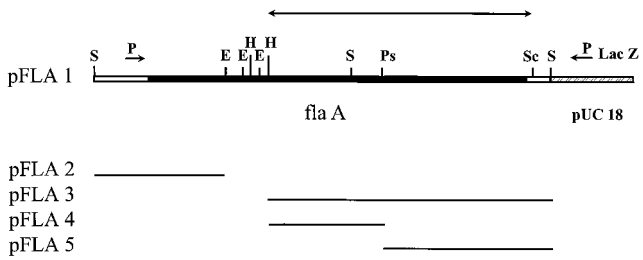


FIG. 1. Restriction maps of the *flaA* region of plasmids pFLA 1 through 5. The 987-bp *Hin*dIII-*Sac*II fragment used for Southern hybridization (arrow), the *flaA* coding region (black box), the flanking region (white box), and pUC 18 sequences (striped box) are indicated. Promoter regions (P) are also shown. Restriction sites: E, *Eco*RI; H, *Hin*dIII; Ps, *Pst*I; Sc, *Sac*II; S, *Sau*3A.

by using the nonradioactive enhanced chemiluminescence detection kit (ECL; Amersham). Hybridization was performed under high-stringency (6 M urea, 0.5 M NaCl; washing buffer, 0.4% SDS–0.5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]; 50°C) and low-stringency (6 M urea, 2 M NaCl; washing buffer, 0.1% SDS–2× SSC; 45°C) conditions at 42°C.

RNA isolation and Northern (RNA) blot analysis. Preparation of total RNA from *L. pneumophila* Corby was performed as described previously (53). A 20-ml sample of an *L. pneumophila* overnight culture (optical density at 600 nm, 0.6) grown at 30 or 37°C was pelleted by centrifugation and suspended in 10 ml of protoplasting buffer (15 mM Tris [pH 8], 0.45 M sucrose, 8 mM EDTA; pH 8). The bacteria were then incubated with 100 μl of lysozyme (100 mg/ml) for 20 min on ice, and the protoplasts were collected by centrifugation. The pellet was suspended in 0.5 ml of gram-negative lysing buffer (10 mM Tris [pH 8], 10 mM NaCl, 1 mM sodium citrate, 1.5% SDS). A 15-μl volume of diethyl pyrocarbonate was added, and the mixture was incubated for 5 min at 37°C. After the mixture had cooled on ice, 250 μl of saturated NaCl was added, and the mixture was blended by inversion and incubated for 10 min. Total RNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1), precipitated with 100% ethanol, and stored in H_2O at −80°C. A 60-μg sample of total RNA was suspended in formaldehyde loading buffer and electrophoresed in a 1% agarose-formaldehyde gel. The RNA was then transferred to a nylon membrane by vacuum blotting. The radiolabelled 987-bp *Hin*dIII-*Sac*II fragment (20) of pFLA 1 was used as a flagellin-specific probe. The hybridization procedure was carried out as described elsewhere (3). The hybridization was done at 37°C.

Primer extension. Primer extension analysis was performed as described elsewhere (3). Briefly, 60 to 90 μg of total cellular RNA of *L. pneumophila* was hybridized with a synthetic oligonucleotide primer (5′-CACATTAGTGTTGAT TACTTGAGCC-3′). The primer is complementary to a region 96 bp downstream of the proposed promoter. DNA products were separated by electrophoresis through a 7% polyacrylamide sequencing gel. The reference nucleotide sequence was generated from double-stranded pFLA 1 DNA with the same primer.

Nucleotide sequence accession number. The nucleotide sequence of the *flaA* gene has been submitted to the EMBL, GenBank, and DDBJ Nucleotide Sequence Database under accession no. X83232.

RESULTS

Cloning of the *L. pneumophila* flagellin gene. An expression library of *L. pneumophila* Corby was constructed in the vector pUC 18 and introduced into *E. coli* K-12. By using a polyclonal antiserum to *L. pneumophila* flagellin, a total of 8,000 *E. coli* K-12 clones were screened for flagellin production. One clone exhibited a strong reaction with the antiflagellin antibodies. Whole-cell extracts of this clone were subjected to SDS-PAGE and Western blot analysis using the antiflagellin antibodies, and a major band at 48 kDa was detected. The plasmid DNA isolated from this clone, designated pFLA 1, was analyzed. Figure 1 shows a restriction map of the insert DNA in pFLA 1 as well as subclones derived from pFLA 1. The origin of *L. pneumophila* DNA was confirmed by Southern blot analysis hybridizing of the radiolabelled *Hin*dIII-*Sac*II fragment of pFLA 1 to genomic DNA of the *L. pneumophila* Corby strain (Table 1). Results of additional digestions with *Pst*I, *Eco*RI, and *Bam*HI followed by Southern hybridization (data not shown) are consistent with the existence of only one copy of the flagellin gene in the chromosome of *L. pneumophila*.

Nucleotide sequence analysis of the *L. pneumophila flaA* gene. Both strands of the 1,747-bp *L. pneumophila* insert of pFLA 1 were sequenced and analyzed to identify the determinants responsible for the expression of flagellin. One open reading frame of 1,428 nucleotides encoding a protein of 475 amino acids was identified. The predicted molecular mass of 48 kDa is in good agreement with the size of the flagellum protein determined by SDS-PAGE analysis (41). Therefore, the corresponding gene was termed *flaA*. As shown in Fig. 2, the *flaA* locus starts at bp 193 (ATG) and ends at bp 1620 (TAG) and is preceded by a potential Shine-Dalgarno consensus sequence (AGGAGA) at position 178. At a position 94 nucleotides upstream of the initiation codon, a potential promoter sequence, −35 TAAA-N_{15}-TCCGATAA −10, which is almost identical to the consensus sequence of a σ^{28} promoter, was identified. This promoter was originally found in *Bacillus subtilis* (23) and is recognized by the *E. coli* RpoF-FliA σ^{28}-like factor (11, 26, 27). Downstream of *flaA*, the sequence shows features of a rho-independent transcriptional termination signal (bp 1629 to 1641). The free energy (7) of the putative hairpin loop is calculated to be −22 kJ. The GC content of the coding region is 45.6%.

Amino acid sequence analysis of the N-terminal part of FlaA. The FlaA protein was isolated and the N-terminal sequence of the protein was determined (Fig. 2). Among the 22 amino acids analyzed, neither the first residue (alanine) nor the 21st residue (glycine) could be determined by Edman degradation. Furthermore, methionine was not detected as a starting amino acid residue of the flagellum protein. This is consistent with various reports about flagella of other bacteria which demonstrated that the methionine is removed posttranslationally (24, 32, 36, 40). Moreover, the determination of the N-terminal amino acid sequence of the mature FlaA protein reveals that, like other flagellins, it does not contain a signal sequence characteristic of secreted proteins (Fig. 3). It is noteworthy that the entire amino acid sequence lacks the amino acid residues histidine, cysteine, and tryptophan. A similar situation has also been reported for other flagellins (15, 27, 34, 55). When the amino acid sequence of FlaA was compared with those of flagellins of other bacteria (Fig. 3), extended homologies in the N- and C-terminal parts of the proteins were detected. The overall similarity of the amino acid residues was calculated to be 50 to 70%.

Transcriptional analysis. As shown in Fig. 2, a putative σ^{28}-like promoter region was identified upstream of the *flaA* gene of *L. pneumophila*. In order to determine if this sequence acts as a *flaA* promoter in *L. pneumophila*, we mapped the transcriptional start site of the chromosomal *flaA* gene of *L. pneumophila* by primer extension experiments. Primer extension experiments determined the transcriptional start to be at the guanine residue at nucleotide 106, 8 nucleotides downstream of the −10 promoter region. This experiment indicates the function of this sequence as a *flaA* promoter in *L. pneumophila* Corby. The *flaA* transcription starts at a position identical to that of the conserved −10 region of σ^{28} promoters in the *Pseudomonas aeruginosa* PAK flagellin gene (Fig. 4B), the *Helicobacter pylori flaA* gene, and the *E. coli tar* gene (36).

The size of the *flaA* transcript was determined by Northern blot analysis with total RNA prepared from *L. pneumophila* cells grown at 30°C or at 37°C. By using a 987-bp *Hin*dIII-*Sac*II probe that corresponds to the *flaA* coding region, a specific transcript of approximately 1.6 kb was detected following hybridization with RNA prepared from cells grown at 30°C (Fig. 5, lane 1). The length of the 1.6-kb transcript corresponds to the coding region of *flaA*, indicating that *flaA* is transcribed as a monocistronic unit. Hybridization of RNA from *L. pneumo-*

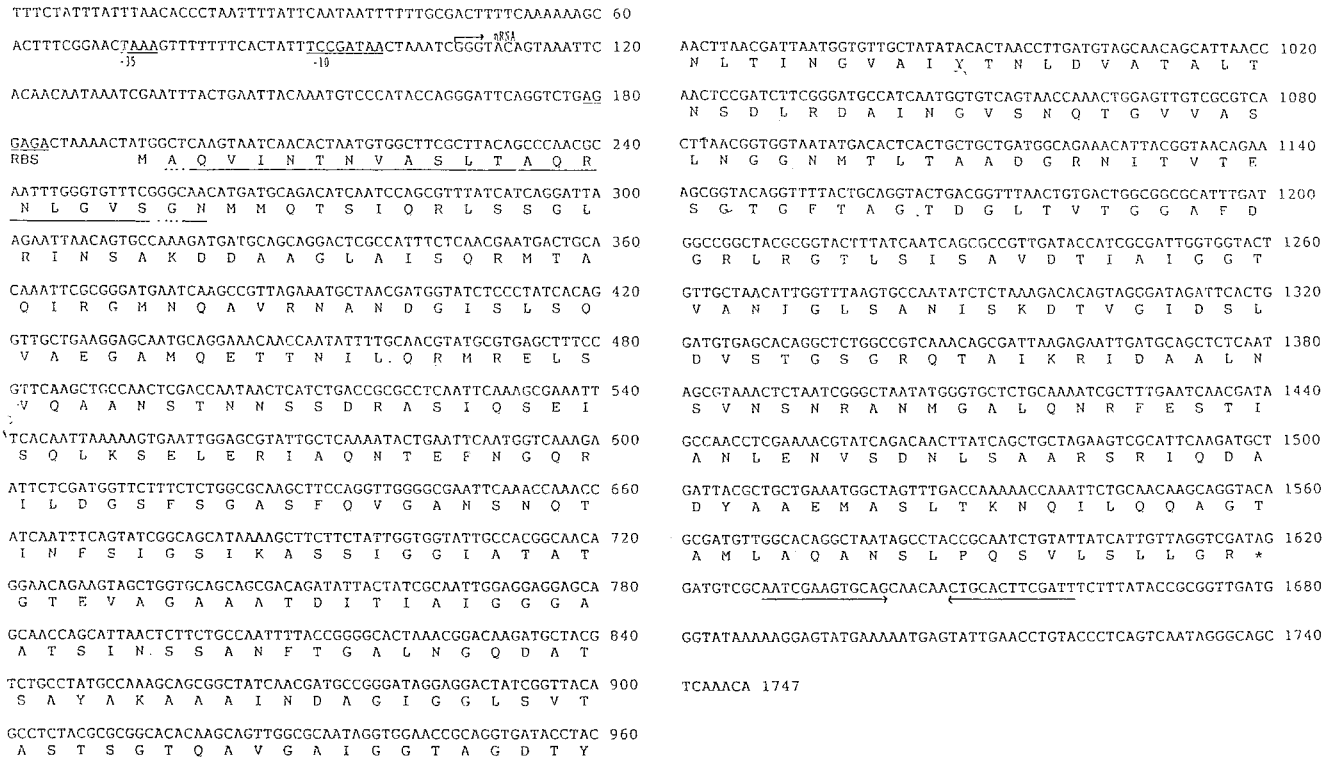


FIG. 2. Nucleotide sequence of the *flaA* gene. The deduced amino acid sequence of the flagellum subunit protein FlaA is shown below the nucleotide sequence. The −10 and −35 regions of the predicted *flaA* promoter, recognized by a FliA-RpoF σ^{28}-like factor, and the ribosome binding site (RBS) are underlined. The transcriptional start (arrow preceding "mRNA"), the N-terminal amino acid residues that have been determined by peptide sequencing (underlined), specific residues that could not have been determined by Edman degradation (dotted line), and the putative rho-independent transcriptional termination sequence (arrows) are indicated.

phila grown at 37°C with the *Hin*dIII-*Sac*II probe revealed only a minor *flaA* transcript of 0.8 kb (Fig. 5, lane 2), which presumably represents a degradation product of the 1.6-kb transcript. Neither the 1.6-kb transcript nor the 0.8-kb transcript could be detected during prolonged incubation at 37°C (Fig. 5, lane 3). These data suggest that the temperature-dependent expression of *flaA* is regulated at the level of transcription.

Expression of *L. pneumophila* flagellin in an *E. coli fliA* mutant. In order to determine whether the expression of the *L. pneumophila* FlaA protein in *E. coli* K-12 depends on σ^{28} plasmid pFLA 1 was introduced into *E. coli* strains with either a *fliA*$^+$ (YK 410) or an isogenic *fliA* (YK 4104) background. The *fliA* gene encodes the alternative σ^{28} factor. Total cell extracts of the recombinant *E. coli* strains grown at 30°C were prepared and subjected to SDS-PAGE. Western blot analysis was performed with the antiflagellin antiserum to determine the expression of the FlaA protein. Figure 6 shows that in the *fliA*$^+$ background (Fig. 6, lane 1), a clear band occurs at the position (48 kDa) of the wild-type flagellin protein of *L. pneumophila* Corby (Fig. 6, lane 5). In contrast, *E. coli fliA* carrying pFLA 1 (Fig. 6, lane 2) did not exhibit a significant reaction with the antiflagellin antibodies. Neither *E. coli fliA*$^+$ nor *E. coli fliA* carrying the vector pUC 18 (Fig. 6, lanes 3 and 4) showed any specific reaction with the antiserum. This suggests that the transcription of *flaA* is controlled by the FliA-RpoF σ^{28}-like factor in *E. coli.*

Distribution and expression of *flaA* in legionellae. To investigate the distribution of *flaA* sequences in the genus *Legionella* and especially in *L. pneumophila* strains, as well as in some other bacterial species, the *flaA*-specific 986-bp *Hin*dIII-*Sac*II fragment (Fig. 1) was used as a *flaA*-specific probe in Southern hybridization of chromosomal DNA of the strains described in Table 1. Hybridization was performed under high- and low-stringency conditions (see Materials and Methods). Furthermore, the antiflagellin antibodies were used in Western blot analysis of total cell extracts to detect proteins related to FlaA. In addition, expression of flagella was examined by electron microscopy (Fig. 7), which gave results consistent with the Western blot analysis. In fact, once we could observe flagella by electron microscopy, we also found a positive reaction with the FlaA-specific antibody (Table 1).

DNAs of all *L. pneumophila* isolates hybridized with the *flaA*-specific probe. Most of them produced proteins which were similar in size to the FlaA protein shown by Western blot analysis (Table 1). Two *L. pneumophila* isolates (strains 667 and Los Angeles [serogroup 4]) which hybridized with the *flaA* gene probe did not show any reaction in Western blot analysis. Moreover, these strains were not flagellated, as shown by electron microscopy. It is likely that strains 667 and Los Angeles possess sequences which are homologous to the *flaA* gene of *L. pneumophila* Corby but do not produce any flagella when grown on BCYE agar plates at 30°C. The majority of the non-*L. pneumophila Legionella* strains produced flagella similar to the flagella of strain *L. pneumophila* Corby. Genomic DNAs isolated from strains of *Legionella israelensis*, *Legionella longbeachae* (serogroup 1), *Legionella dumoffii*, and *Legionella oakridgensis*, however, did not hybridize with the *flaA* probe under high-stringency conditions. Similar results were observed in hybridization experiments under low-stringency conditions, except for *L. dumoffii*. Neither Western blot analysis nor electron microscopy provided evidence for the synthesis of flagella in these strains, suggesting that these isolates do not

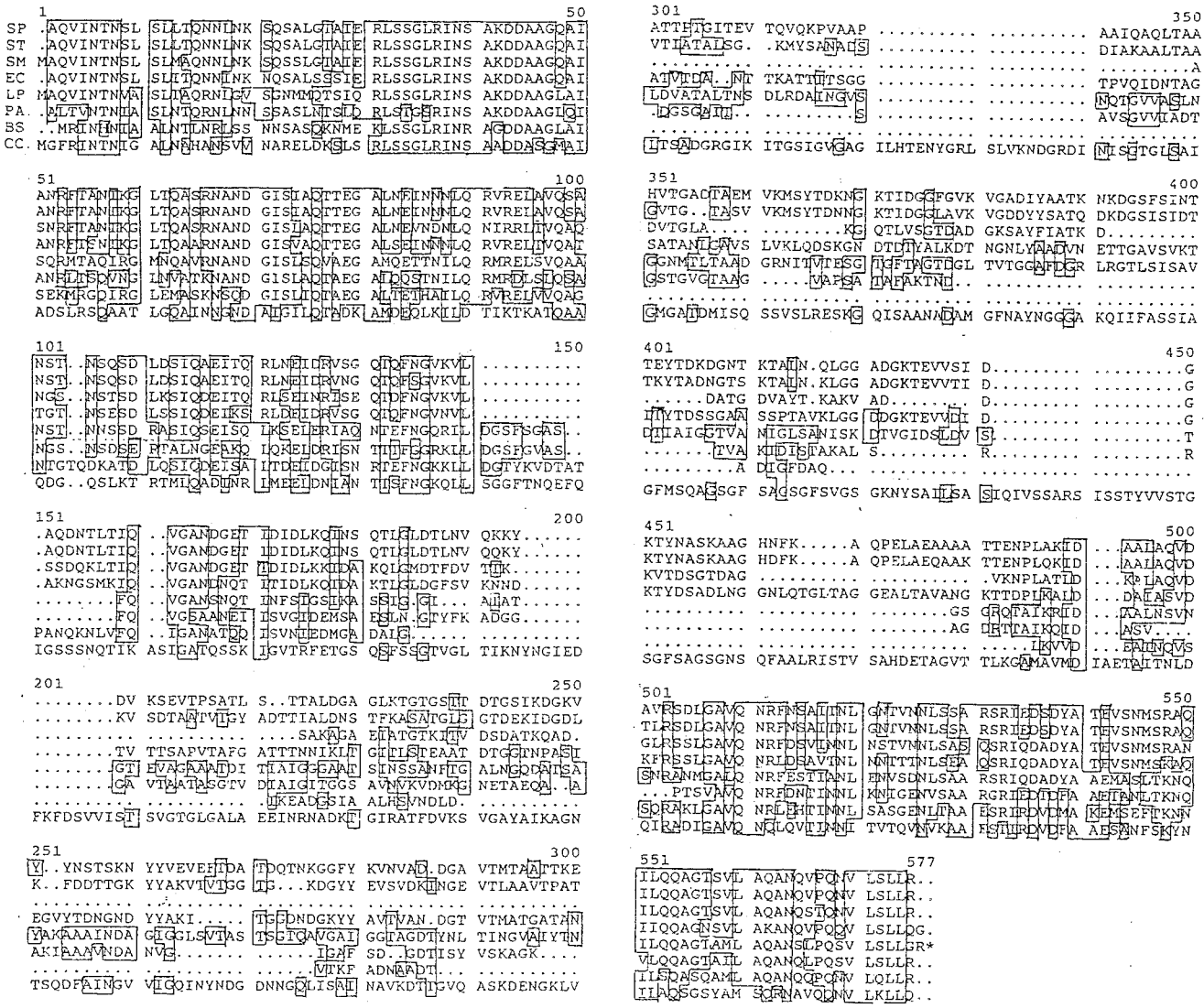
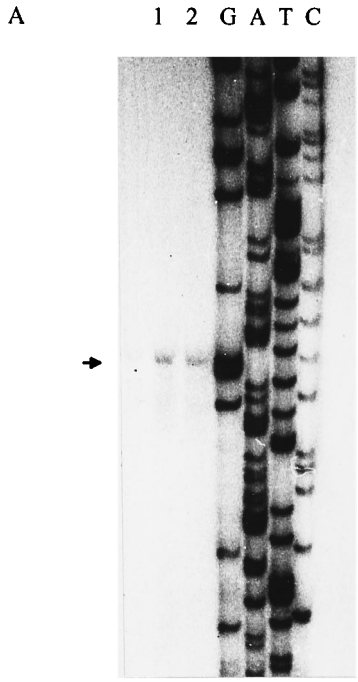


FIG. 3. Comparison of the amino acid sequence of the flagellum subunit protein FlaA of *L. pneumophila* (LP) with the amino acid sequences of flagellin proteins of *Salmonella paratyphi* (SP), *S. typhimurium* (ST), *Serratia marcescens* (SM), *E. coli* (EC), *P. aeruginosa* (PA), *B. subtilis* (BS), and *C. coli* (CC). Amino acid residues conserved between the *L. pneumophila* Corby protein and one or more other flagellin proteins are boxed.

possess flagellum-specific genes. In contrast, genomic DNAs isolated from strains of *Legionella bozemanii* and *Legionella feeleii* (serogroup 1) strongly hybridized with the *flaA*-specific probe of *L. pneumophila*. However, those isolates were not able to express flagellum proteins, as demonstrated by Western blot analysis and by electron microscopy. Genomic DNA isolated from enterobacterial strains (*E. coli* 536, *Salmonella typhimurium*, *Serratia marcescens*, *Serratia liquefaciens*, and *Yersinia enterocolitica*) as well as from *P. aeruginosa*, *Aeromonas sobria*, *Bordetella pertussis*, and *L. monocytogenes* (5, 8) did not hybridize with the *L. pneumophila flaA*-specific gene probe. Protein extracts of these strains did not give specific signals with the antiflagellin-specific antiserum in Western blot analysis (data not shown).

DISCUSSION

In this study, we have cloned and characterized the *L. pneumophila flaA* gene encoding flagellin. The *flaA* gene was identified by screening an expression library of *L. pneumophila* in *E. coli* with a polyclonal monospecific antiflagellin antibody. The nucleotide sequence of the *flaA* region showed one open reading frame of 1,428 nucleotides encoding a protein with a predicted molecular mass of 48 kDa. This is in good agreement with the molecular mass of 47 kDa determined by Ott et al. (41) using SDS-PAGE. The comparison of the sequenced N-terminal amino acids with the FlaA predicted amino acid sequence indicated that the start methionine is posttranslationally removed and that there is no signal sequence. It is noteworthy that the FlaA protein of *L. pneumophila* does not contain the amino acids cysteine, histidine, and tryptophan. Similarly, flagellins of various other species always lack cysteine and frequently lack histidine and tryptophan. Comparison of amino acid sequences in the N- and C-terminal regions shows the highest degree of similarity to *P. aeruginosa* flagellin. As demonstrated for the *E. coli* and the *S. typhimurium* flagellins, the conserved N- and C-terminal regions reflect the function of these domains in export and polymerization of flagellin



B

L. pneumophila Corby *fla A*

AACTAAA-15-TCCGATAACTAAATCGGGT

-35 -10

P. aeruginosa PAK flagellin

ACCTAAA-15-GCCGATAAAGATCACGAAT

-35 -10

FIG. 4. Map of the transcriptional start site of *L. pneumophila flaA*. The primer extension product is indicated (arrow) (lane 1, 90 μg of total RNA; lane 2, 60 μg of total RNA). The adjacent DNA sequence was determined by using pFLA 1 as the template (lanes G, A, T, and C). (B) Comparison of the sequence upstream of the transcriptional start for the *L. pneumophila flaA* gene with a similar sequence for the *P. aeruginosa* PAK flagellin gene. The consensus sequences recognized by the alternative σ^{28} factor (underlined) and the transcriptional start sites (arrows) are indicated.

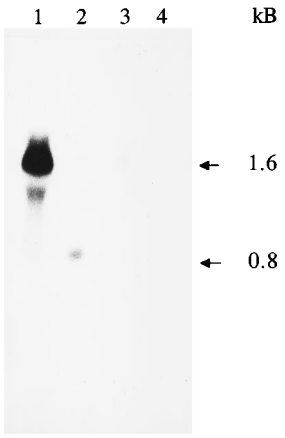


FIG. 5. Northern blot analysis of *flaA* gene transcription. RNA was extracted from *L. pneumophila* Corby grown for 18 h at 30°C (lane 1), for 18 h at 37°C (lane 2), or for 40 h at 37°C (lane 3) and from *E. coli* DH5α harboring plasmid pUC 18 grown for 18 h at 30°C (lane 4). A *Hin*dIII-*Sac*II fragment corresponding to the *flaA* coding region was used as a probe. The transcripts are indicated by arrows.

biogenesis (30, 32, 33). The internal region shows the highest degree of divergency. It can be deleted to construct a minimal-size functional flagellin (33). In *S. typhimurium*, for example, these highly variable regions are responsible for the antigenic diversity of *S. typhimurium* flagellin (32).

Southern hybridization using a *flaA*-specific gene probe was performed with genomic DNAs isolated from *L. pneumophila* isolates of various sources. Interestingly, the entire set of strains hybridized with the gene probe, even under high-stringency conditions, indicating that the *flaA* gene represents a well-conserved sequence in the *L. pneumophila* genome (Table 1). In contrast to strains of other species, such as *H. pylori*, *Campylobacter coli*, *Campylobacter jejuni*, *S. typhimurium*, or *Proteus mirabilis*, which carry two flagellin genes in their genomes (4, 24, 40, 58), only one copy of *flaA* was detected in the genome of *L. pneumophila*. Two of 14 *L. pneumophila* strains (isolates 667 [serogroup 4] and Los Angeles [serogroup 4]) were not able to produce flagella demonstrated by Western blot analysis and electron microscopy. It will be interesting to analyze whether mutations in the *flaA* locus or its flanking sequences or defects in the regulation of flagellin expression are responsible for the negative phenotypes. In addition to *L. pneumophila*, 10 of 13 isolates of non-*L. pneumophila Legionella* strains exhibited specific hybridization with the *flaA* probe of *L. pneumophila*, indicating that the *flaA* genes are widely distributed and conserved among most *Legionella* species. In *L. israelensis*, *L. longbeachae* (serogroup 1), and *L. oakridgensis*, no *flaA* homologous sequences could be detected by Southern hybridization and, consistently, no flagella could be visualized by electron microscopy, arguing for the absence of *flaA*-specific sequences in the genomes of these species. In contrast, strains of *L. bozemanii* and *L. feeleii* (serogroup 1) did not show expression of flagellin, although they strongly hybridized with the *flaA* probe of *L. pneumophila*. They might be defective in

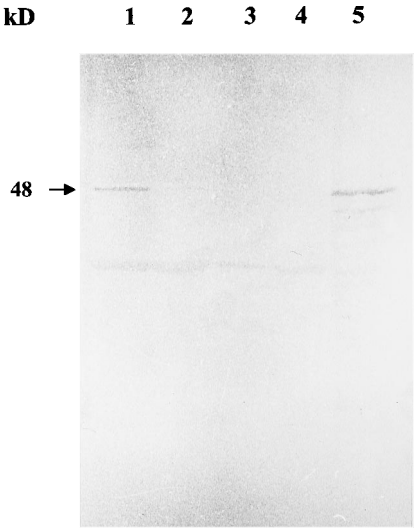


FIG. 6. Western blot analysis of total bacterial cell extracts using antiflagellin antiserum. Recombinant *E. coli* strains and *L. pneumophila* strains were grown overnight at 30°C under 5% CO_2–95% air. Equal amounts of extracts of *E. coli* K-12 strains YK 410 (*fliA*$^+$) harboring plasmid pFLA 1 (lane 1), YK 4104 (*fliA*) harboring plasmid pFLA 1 (lane 2), YK 410 harboring vector pUC 18 (lane 3), and YK 4104 harboring vector pUC 18 (lane 4) and *L. pneumophila* Corby (lane 5) were applied to each lane.

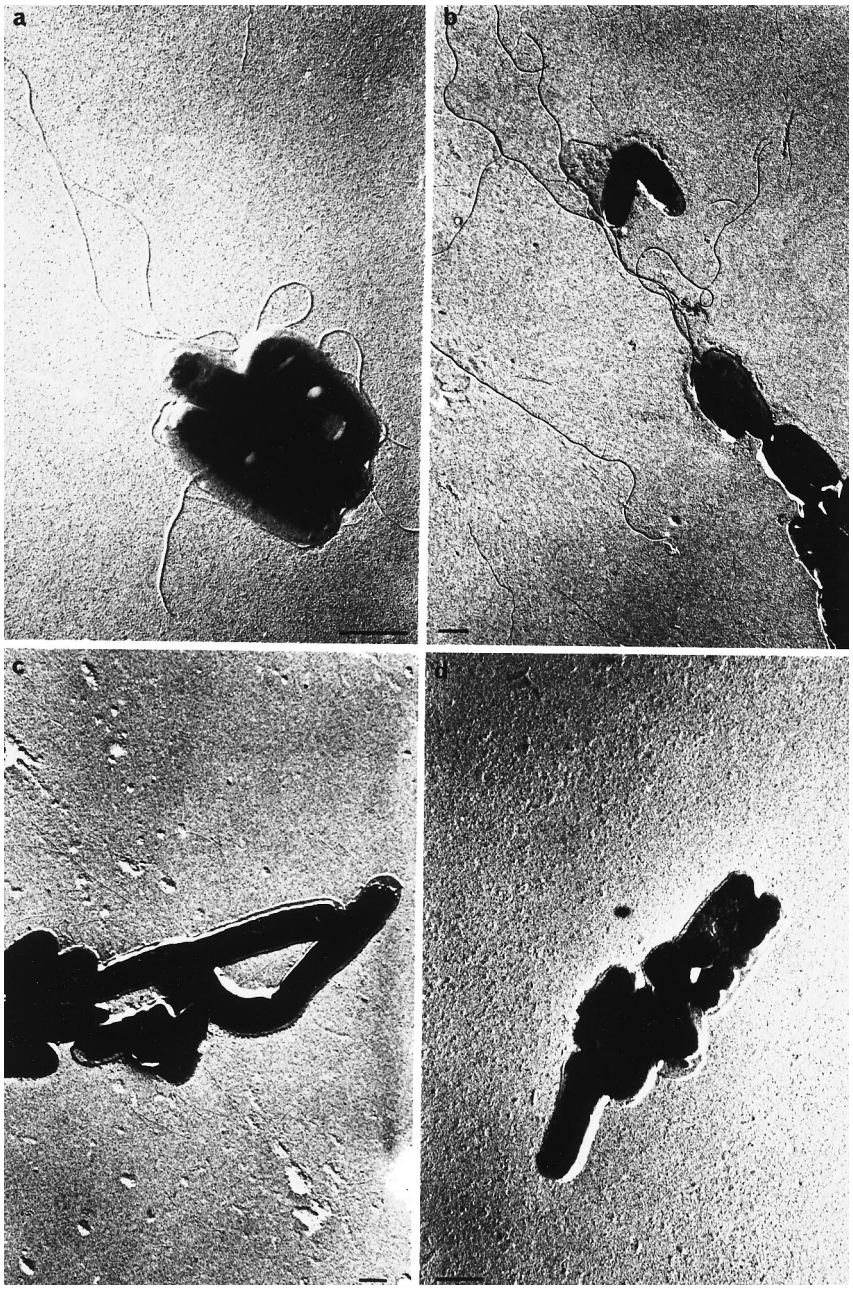


FIG. 7. Electron micrographs showing the flagellated strains *L. pneumophila* Corby (a) and *L. pneumophila* U22 (serogroup 3) (b) and the nonflagellated strains *L. longbeachae* (serogroup 1) (c) and *L. oakridgensis* (d) grown at 30°C. The samples were shadowed with platinum-palladium. Bars, 0.5 μm.

one step or another at the level of transcription or translation of the *flaA* gene.

Northern blot analysis showed a 1.6-kb transcript of the *flaA* locus, indicating that *flaA* is transcribed as a monocistronic mRNA. The size of the transcript corresponds to the distance between the transcriptional start site and the putative transcriptional termination signal. Furthermore, it has also been demonstrated that the temperature-dependent regulation of *flaA* in *L. pneumophila* occurs at the transcriptional level. The temperature-dependent regulation of flagellin is a common feature among different species; however, the molecular basis of this phenomenon is still unknown. The transcriptional start site was mapped to nucleotide 106 by primer extension. The promoter sequence 5′-TAAA-N_{15}-TCCGATAA-3′ is located 8 nucleotides upstream of the transcriptional start site. The primer extension experiment shows that this consensus sequence is a functional promoter in *L. pneumophila*. The −35/−10 promoter region is almost identical to the σ^{28} consensus sequence recognized by the alternative σ^{28} factor containing RNA polymerase which is required for the transcription of flagellin genes of *E. coli*, *B. subtilis*, and several other bacteria (26, 27, 39, 52). Unlike the wild-type *E. coli* strain YK 410, the *E. coli* YK 4104 (*fliA*) mutant containing the plasmid pFLA 1 shows no significant expression of *flaA*. Therefore, it is very likely that the transcription of the *flaA* gene of *L. pneumophila* is σ^{28} factor-dependently transcribed in *E. coli*, suggesting that

σ^{28} might also contribute to the regulation of flagella in *L. pneumophila.*

In contrast to the case for *L. pneumophila*, flagellin genes of *Caulobacter crescentus* (43) and one of the two flagellin-specific genes of *C. coli*, *C. jejuni*, and *H. pylori* are regulated by σ^{54} (24, 40, 58), which was found to be involved in the regulation of nitrogen assimilation, fimbrial expression, and glutamine biosynthesis in various species. For expression of flagella in *P. aeruginosa*, a cooperative effect of σ^{28} and σ^{54} has been described (55). Presently, we have no evidence whether σ^{54} in addition to σ^{28} is also involved in the expression of flagella in *L. pneumophila.*

A correlation of virulence and motility has been described for various bacterial pathogens (17, 21, 36–38, 43, 50). For *C. jejuni*, *P. aeruginosa*, *S. typhimurium*, and *Proteus mirabilis*, bacterial motility has been documented as a factor enhancing bacterial virulence in vivo. Furthermore, for *Proteus mirabilis*, *E. coli*, and *P. aeruginosa*, it has been demonstrated that flagellin genes are part of regulons which comprise other loci coding for virulence factors, such as urease production, hemolysin, metalloprotease, or fimbriae (45). In *Proteus mirabilis* and *C. jejuni*, flagella contribute to the invasion of eucaryotic host cells (1, 2, 60) and seem to be responsible for the attachment to the host cells, as shown for *Vibrio cholerae* (44). Furthermore, motility might be important for the survival of legionellae in aquatic habitats, because the interaction with and the intracellular growth within protozoa seem dependent on the active finding of the host cell by legionellae (47). However, nothing is known about chemotactic substances of protozoa that might attract legionellae. We are attempting to generate defined nonflagellated mutants of *L. pneumophila* to address the issue of the role of flagella in the ecology and pathogenesis of *L. pneumophila.*

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