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The Alternative Sigma Factor σ^{28} of *Legionella pneumophila* Restores Flagellation and Motility to an *Escherichia coli fliA* Mutant

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Gene expression in *Legionella pneumophila*, the etiological agent of Legionnaires' disease, can be controlled by alternative forms of RNA polymerase programmed by distinct σ factors. To understand the regulation of *L. pneumophila* flagellin expression, we cloned the σ factor (FliA) of RNA polymerase responsible for the transcription of the flagellin gene, *flaA*. FliA is a member of the σ^{28} class of alternative σ factors identified in several bacterial genera. The gene *fliA* has been isolated from an expression library of *L. pneumophila* isolate Corby in *Escherichia coli* K-12. This library was transformed into a *fliA* mutant of *E. coli* K-12 containing a plasmid carrying the *L. pneumophila*-specific *flaA* promoter fused to the reporter gene *luxAB*. Screening the obtained transformants for luciferase activity, we isolated the major part of the *fliA* gene on a 1.64-kb fragment. This fragment was sequenced and used for reverse PCR in order to recover the complete *fliA* gene. The resulting 1.03-kb fragment was shown to contain the entire *fliA* gene. *L. pneumophila* FliA has 55 and 43% amino acid identity with the homologous sequences of *Pseudomonas aeruginosa* and *E. coli*. Furthermore, the *L. pneumophila fliA* gene was able to restore the flagellation and the motility defect of an *E. coli fliA* mutant. This result suggests that the *L. pneumophila* σ^{28} protein can bind to the *E. coli* core RNA polymerase to direct transcription initiation from the *flaA*-specific promoter.

Legionella pneumophila, a ubiquitous microorganism inhabiting freshwater biotopes and man-made water systems, is also a pathogen of humans causing severe pneumonia termed Legionnaires' disease. In the environment, the bacteria are able to replicate intracellularly in amoebae and other protozoa (14, 39, 46). *Legionella* infection occurs after inhalation of aerosolized bacteria. The bacteria enter the human lungs, where they are capable of invading and proliferating in alveolar macrophages and blood monocytes.

A growing number of virulence factors elaborated by *L. pneumophila* contribute to the pathogenicity of the organism. These include a number of genetic loci that have been implicated in being important for intracellular growth (for a review, see reference 6). The *icm* locus is essential for the intracellular multiplication of *L. pneumophila* within human macrophages and is also required for virulence in guinea pigs (5, 30). Adjacent to *icm* is *dot*, a gene essential for intracellular replication and proper trafficking of host organelles during macrophage infection (3–5). Several surface-associated cellular components have been implicated in virulence. The Mip (macrophage infectivity potentiator) protein contributes to intracellular survival of *L. pneumophila*, and invasion experiments using a null mutation in *mip* revealed that Mip is required for early survival processes of *L. pneumophila* in eukaryotic cells (9, 10, 47). Convincing evidence suggests that the major outer membrane protein (encoded by *ompS*) is involved in the uptake of bacteria by the host cell (22). The major outer membrane protein is a porin (15) that binds to complement components which in turn mediate binding to monocyte receptors involved in phagocytosis (36).

Flagellar motility, via a single monopolar flagellum, may be a potential virulence factor in the infection pathway of *L. pneumophila* in *Acanthamoeba castellanii* trophozoites (40), in *Hartmannella vermiformis*, and to a limited degree in human U937 cells (38). In contrast, the production of flagella seems not to be required for the intraperitoneal route of infection in guinea pigs (13). The flagellin is the major subunit of the flagella of *L. pneumophila*, and it has recently been shown that various *L. pneumophila* strains and isolates of species other than *L. pneumophila* are able to produce flagella (21).

Previous reports from this laboratory have shown that the temperature-dependent expression of the gene *flaA*, encoding the flagellum subunit, is regulated at the transcriptional level (21). Sequence analysis and primer extension experiments indicated that transcription of the *flaA* gene is directed by an alternative σ factor of RNA polymerase, FliA (21). Genes of the σ^{28} family (designated *sigD*, *fliA*, and *rpoF*) are required for expression of motility and chemotaxis genes in several organisms, including *Bacillus subtilis*, *Salmonella typhimurium*, *Escherichia coli*, and *Pseudomonas aeruginosa* (2, 19, 32, 35, 45). These genes are expressed in a complex transcriptional cascade. This regulatory hierarchy acts to ensure that the highly expressed filament structural protein, flagellin, is synthesized only after a prerequisite set of structural proteins has been expressed and properly assembled.

As a first step in understanding the regulation of flagellin synthesis in *L. pneumophila*, we identified and cloned the *fliA* gene. The sequence of the *fliA* gene predicts a protein with 43% amino acid identity with the *E. coli fliA* protein. The close correspondence between these two alternative σ factors in their genetic functions and promoter specificities encouraged us to attempt to complement an *E. coli fliA* mutant with the corresponding cloned *fliA* gene from *L. pneumophila*. Remarkably, the expression of σ^{28} protein restores motility to a *fliA* mutant of *E. coli*, implying that the σ^{28} factor can interact productively with the RNA polymerase core enzyme.

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MATERIALS AND METHODS

Strains, plasmids, and oligonucleotides. *L. pneumophila* Corby (serogroup 1) (23) was used to construct an expression library in *E. coli* K-12 as recently described (21). *E. coli* DH5α (48) was used for propagation of recombinant

TABLE 1. Plasmids and oligodeoxyribonucleotides

Plasmid or oligonucleotide	Characteristics or sequence	Reference or source
Plasmids		
Mini Tn5 LuxAB	pUT 3,200-bp *Sal*I-*Bam*HI fragment containing *luxAB*	27
pKH20	pUC18 3,200-bp *Sal*I-*Bam*HI fragment containing *luxAB*	This study
pKH21	pUC18 360-bp *Sph*I-*Sal*I fragment containing p*flaA*	This study
pKH22	pUC18 3,560-bp *Sph*I-*Bam*HI fragment containing the p*flA-luxAB* fusion	This study
pKH23	pMMB207 3,560-bp *Hin*dIII-*Kpn*I fragment containing the p*flaA-luxAB* fusion from pKH22	This study
pKH24	pMMB207 3,200-bp *Hin*dIII-*Kpn*I fragment from pKH20	This study
pKH25	pUC18 1,636-bp *Hin*dIII-*Sma*I fragment containing part of *fliA*	This study
pKH26	pUC18 412-bp reverse PCR product containing part of *fliA*	This study
pKH27	pUC18 1,028-bp PCR product containing *fliA* and part of *ylxH*	This study
Oligonucleotides		
luxU	5′-CATGTCTATATTGGGCATGCATTTC-3′	This study
luxR	5′-CCATAGTTTTAGTCTCCGTCGACCTG-3′	This study
fliU1	5′-TAGCAGCCATTTATATGGT-3′	This study
fliU2	5′-GGTCCGCCTGGCGTCAGGG-3′	This study
fliU3	5′-TTAGCTGTACTCTGTTTG-3′	This study
fliU4	5′-GGGAAATACTGGAGGTTAGTG-3′	This study
fliU5	5′-GGATGTGCAGTTAGATTAC-3′	This study
fliR1	5′-GATATCATCAATTACTCC-3′	This study
fliR2	5′-CCTTGGATGAATCATAATG-3′	This study
fliR4	5′-CCGCTGAAACAGCAATCACTT-3′	This study
fliR5	5′-TTTATTCCGGTAATCTTGATC-3′	This study

plasmid DNA. *E. coli* K-12 YK410 (*fliA*$^+$) and YK4104 (*fliA*) were described previously (24) and served as hosts for the expression of the *fliA* gene of *L. pneumophila*. Plasmid pUC18 (Pharmacia LKB, Freiburg, Germany) was used for the construction of pKH25, pKH26, and pKH27. Vector pMMB207 (33) was used to construct plasmids pKH23 and pKH24. Plasmids and oligonucleotides used for PCR and DNA sequencing are listed in Table 1.

Media, chemicals, and swarm assay. *E. coli* was cultivated in Luria-Bertani (LB) medium. Antibiotics used for selection of markers in *E. coli* were streptomycin (12 μg ml^{-1}), chloramphenicol (20 μg ml^{-1}), and ampicillin (50 μg ml^{-1}). Swarm assays were performed by stabbing fresh bacteria onto semisolid agar (10 g of Bacto Tryptone, 5 g of Bacto Yeast Extract, and 2 g of Bacto Agar per liter) and incubating the plates at 30°C overnight. Restriction enzymes were purchased from Pharmacia LKB, Boehringer GmbH (Mannheim, Germany), and Gibco BRL (Eggenstein, Germany). The thermal polymerase Goldstar DNA polymerase was purchased from Eurogentec (Seraing, Belgium). Radiochemicals were supplied by Amersham (Braunschweig, Germany). Chemicals and oligonucleotides were purchased from Sigma and Roth (Karlsruhe, Germany).

DNA techniques. Preparation of genomic DNA and plasmid DNA, DNA cloning, DNA manipulations, and Southern hybridization procedures were performed according to standard protocols (42). PCR was carried out by the method of Saiki et al. (41), using a Thermocycler 60 apparatus from Biomed, Theres, Germany, and GoldStar DNA polymerase (Eurogentec). Introduction of foreign DNA into bacterial strains by electroporation was performed by using a Bio-Rad gene pulser according to the manufacturer's specifications. Electroporation of *E. coli* strains was carried out at 2.3 kV, 200 Ω, and 25 μF.

Construction of a plasmid containing a p*flaA-luxAB* fusion. Plasmid pKH20 is a pUC18 derivative containing a *Sma*I-*Bam*HI fragment encoding the promoterless *luxAB* gene. In order to fuse the *flaA*-specific promoter (p*flaA*) to the *luxAB* gene, the promoter was amplified by PCR with primers luxU and luxR. The resulting 360-bp fragment was cloned into the *Sph*I-*Sal*I site of pUC18, resulting in pKH21. The p*flaA* sequence was confirmed by DNA sequencing. Fusion of the promoter to the *luxAB* gene was accomplished as follows. Plasmid pKH20 was digested with *Sal*I and *Bam*HI to precisely excise the *luxAB* gene. This fragment was ligated into pKH21 after digestion with *Sal*I and *Bam*HI, generating a p*flaA-luxAB* fusion and resulting in pKH22. The fusion point was verified by DNA sequencing. pKH22 was digested with *Hin*dIII and *Kpn*I in order to subclone the p*flaA-luxAB* fusion into pMMB207. This fusion plasmid was designated pKH23. The control plasmid pKH24 was constructed by excising the promoterless *luxAB* gene from pKH20, using *Hin*dIII and *Kpn*I, followed by ligation into pMMB207.

Cloning of the *fliA* gene from *L. pneumophila*. We pooled 2,000 clones of our *L. pneumophila* Corby genomic library in pUC18 in groups of 1,000 and extracted plasmid DNA. Plasmids were electroporated into *E. coli* YK4104(pKH23) containing the plasmid-encoded p*flaA-luxAB* fusion and the chromosomal *fliA* mutation. Transformants were subsequently screened for luciferase (*luxAB*) activity. Luciferase activity was determined by adding the substrate decanal to the lid of the petri dish followed by a brief incubation at room temperature and observation for light emission of individual clones. Plasmid pKH25, a clone of the expression library, produced luciferase activity in YK4104(pKH23). Subsequently it was shown by sequence analysis that the entire *fliA* gene with the exception of four C-terminal amino acids was present on this plasmid. To isolate the complete *fliA* gene directly from the chromosome of *L. pneumophila*, genomic DNA was digested with various restriction enzymes known not to cut within the *fliA* gene. Fragments were separated by gel electrophoresis, transferred to membranes, and hybridized with a probe generated by PCR using primers fliU2 and fliR1. This probe corresponded to internal parts of pKH25 in order to identify fragments with a size of approximately 2 kb which were expected to contain the complete *fliA* gene. On the basis of these results, genomic DNA was digested with *Hin*dIII and the fragments were religated and used for reverse PCR with primers fliU4 and fliR4. The resulting fragment was cloned into pUC18, sequenced with pUC18-specific primers, and designated pKH26. On the basis of sequence data obtained from pKH25 and pKH26, we designed a synthetic primer set (fliU5-fliR5) in order to amplify the entire chromosomal *fliA* gene by PCR. The resulting fragment was cloned into pUC18, giving pKH27. Nucleotide sequence analysis indicated that the entire *fliA* gene is present on this fragment.

The reaction conditions for reverse PCR with religated *Hin*dIII genomic DNA fragments as templates were the following. After an initial denaturation step of 2 min at 94°C, 30 cycles, each at 94°C for 60 s, 57°C for 75 s, and 72°C for 90 s, were completed, with a final elongation step at 72°C for 3 min. Amplification of the chromosomal *fliA* gene was performed under the same conditions but with each cycle at 94°C for 60 s, 45°C for 90 s, and 72°C for 90 s.

Nucleotide sequencing analysis. DNA sequencing of supercoiled plasmid DNA was determined by the chain termination method of Sanger et al. (43) with [α-^{32}P]dATP (Amersham). Both strands of each DNA fragment to be analyzed were sequenced. Sequence data for pKH25 and pKH27 were edited, analyzed, and displayed by using the University of Wisconsin Genetics Computer Group (GCG) sequence analysis software package (11). Homology searches were conducted against the GenBank, EMBL, and Swiss-Prot databases, using the FASTA program (37). Comparisons of two sequences were conducted by using the Gap program of the GCG package; multiple alignments were accomplished by using the Pileup program of the GCG package. Sequence similarities and homologies reported are those calculated by Gap.

Luciferase activity assay. *E. coli* strains grown to the late logarithmic growth phase were adjusted to an optical density at 600 nm of 1 and kept on ice. Four hundred microliters of reaction buffer (50 mM sodium phosphate buffer [pH 7.0], 50 mM β-mercaptoethanol, 2% bovine serum albumin was added to 10 μl of bacterial suspension in a luminometer cuvette. The cuvette was placed into a luminometer (Lumat LB 9501 [automated]; Berthold, Bad Wildbad, Germany), and 300 μl of a decanal (*n*-decyl-aldehyde; Sigma)-reaction buffer (1:2,000) solution was injected before measurement of the light output. The reaction time was limited to 5 s, and luciferase activity was expressed in relative light units (RLU).

Electron microscopy. Bacteria were grown in LB broth until late logarithmic phase, and a drop of suspension was directly applied to Formvar-coated copper grids. After sedimentation of the bacteria and removal of the remaining fluid, the

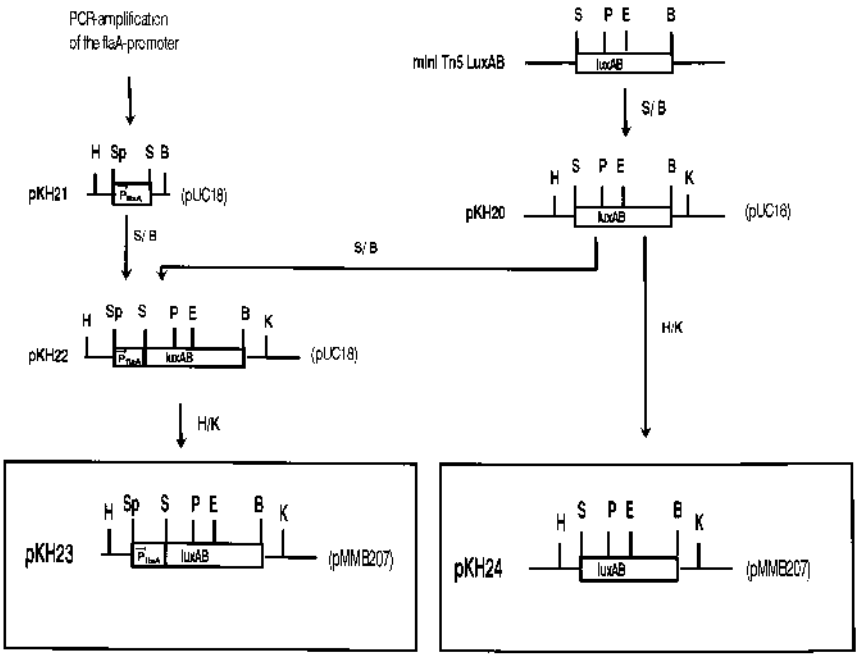

FIG. 1. Construction of an *L. pneumophila pflA* fusion to the *luxAB* reporter gene using vectors pUC18 and pMMB207. Mini Tn5 LuxAB was used solely to subclone the *luxAB* gene into pUC18, followed by fusion to the *flaA*-specific promoter sequence and subcloning into pMMB207. Restriction endonuclease sites: S, *Sal*I; P, *Pst*I; E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; Sp, *Sph*I; K, *Kpn*I.

samples were shadowed with platinum-palladium and examined with Zeiss 10A transmission electron microscope.

Nucleotide sequence accession number. The sequence reported here has been entered in GenBank under accession number X98892.

RESULTS

Construction and characterization of a *flaA* promoter fusion to *luxAB*. To identify the σ^{28}-encoding gene of *L. pneumophila*, we developed a screening system based on the fact that transcription of the flagellar genes of *L. pneumophila* and *E. coli* depends on the activity of the alternative σ^{28} factor. We constructed plasmid pKH23, containing the *flaA* promoter fused to the luciferase-encoding *luxAB* gene, and pKH24, containing the promoterless *luxAB* gene (Fig. 1). Plasmid pKH23 was transformed into *E. coli* YK410 and YK4104, which is a *fliA* derivative of wild-type strain YK410. The resulting transformants were grown to late logarithmic growth phase and tested for luciferase activity. As shown in Fig. 2, the luciferase activity of YK4104(pKH23) (5,023 RLU) was 13-fold less than that of wild-type YK410(pKH23) (66,794 RLU). This result indicates that the *E. coli* RNA holoenzyme is able to accurately initiate transcription from the *flaA*-specific promoter of *L. pneumophila*. YK410 containing plasmid pKH24 with the promoterless *luxAB* gene exhibited a basal luciferase activity of 390 RLU, corresponding to the transcription of the promoterless *luxAB* gene. The difference in the luciferase activities of YK410 (pKH24) and YK4104(pKH23) is probably due to nucleotide sequences of p*flaA* that can also be recognized by σ^{70} of *E. coli*, therefore allowing a low level of transcription.

Isolation and identification of the *fliA* gene from *L. pneumophila*. To isolate the *fliA* gene, we screened plasmids from our genomic library of *L. pneumophila* Corby (21) for the ability to direct transcription of a p*flA-luxAB* fusion (pKH23) in a *fliA* mutant (YK4104) of *E. coli* K-12. Plasmid pKH25 exhibited luciferase activity, but sequence analysis revealed that the 3′ end of the *fliA* gene was not present on pKH25 (Fig. 3). To obtain the complete *fliA* gene from chromosomal DNA fragments of *L. pneumophila*, we designed internal *fliA*-specific probes to identify fragments of approximate 2 kb. Following digestion of chromosomal DNA with *Hin*dIII, we used *fliA* internal primers for reverse PCR in order to amplify the 3′-end adjacent sequence to obtain the entire *fliA* gene. The resulting fragment, pKH26 (Fig. 3), was sequenced, and the data were

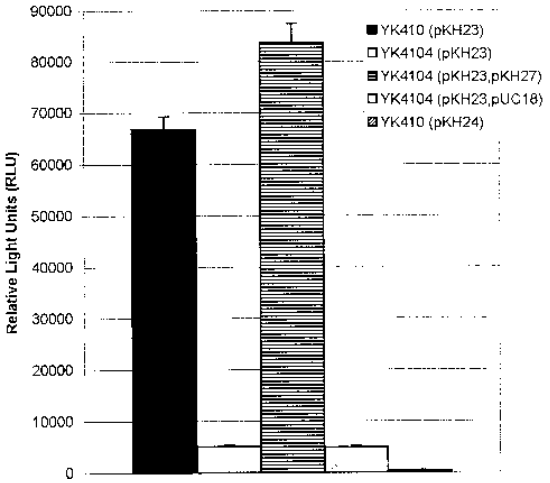

FIG. 2. Expression of luciferase from a flagellin promoter fusion to *luxAB*. Shown are results for *E. coli* wild-type strain YK410 and the *fliA* mutant strain YK4104 transformed with pKH23, YK4104(pKH23) containing vector pUC18, and YK4104(pKH23) complemented with pKH27. Expression from YK4104 (pKH23) or YK4104(pKH23, pUC18) yielded <6,000 RLU, and expression from YK4104(pKH24) yielded <500 RLU. Error bars indicate the standard error of the mean determined from six samples taken from one experiment. The absence of error bars indicated that the standard error of the mean was smaller than the capacity of the graphing program. Very similar results were obtained in three independent experiments.

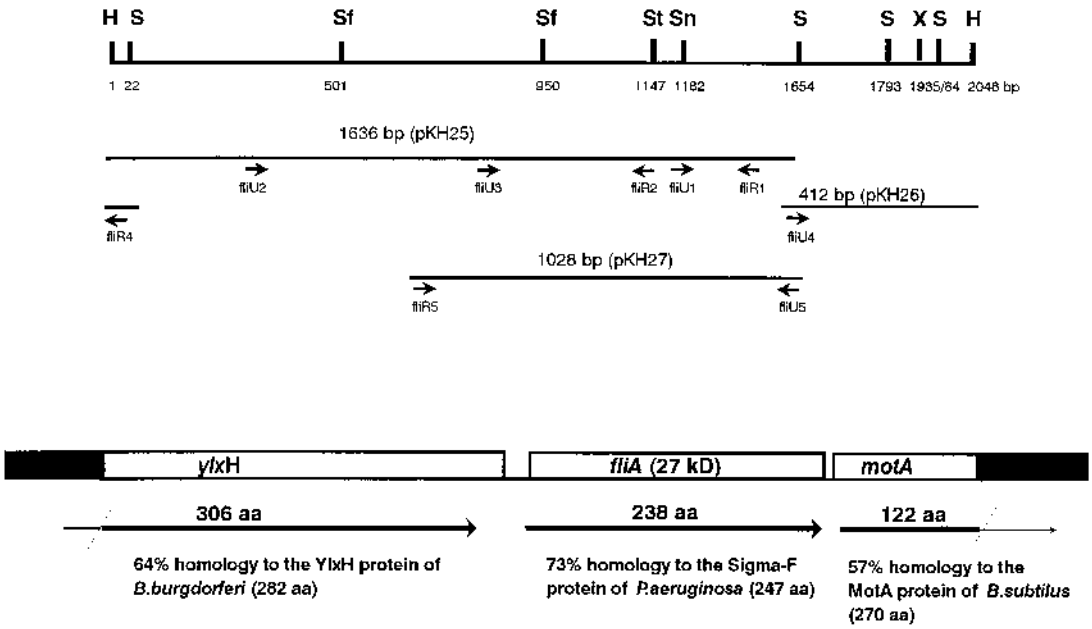

FIG. 3. Restriction map of the *L. pneumophila fliA* region. Coding regions corresponding to *fliA*, *motA*, and *ylxH* are indicated by arrows. Small arrows represent the primers used for PCR. Restriction endonuclease sites: H, *Hin*dIII; S, *Sma*I; Sf, *Sfi*I; St, *Sty*I; Sn, *Sna*BI; X, *Xba*I. aa, amino acids.

then used to design a new set of primers for the final amplification of the chromosomal *fliA*. The PCR fragment containing the entire *fliA* gene of *L. pneumophila* was cloned and designated pKH27 (Fig. 3).

Sequence analysis of the DNA region containing *fliA* locus. The nucleotide sequence of the entire 1,023-bp fragment of pKH27 and of pKH25 was determined (Fig. 4). The *fliA* gene is preceded by a Shine-Dalgarno sequence (AGGAGA) located 11 bp upstream from the GTG start codon (Fig. 4). It

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1    TTAGATTTACCAAATTACTAATAACTACAATCTGCTGTTATTGTAAGGAG
       L  D  L  P  N  Y  *  (ylxH)                  SD
51   AGTATCGTGGATGCTTTGGCTGCATACAGCAAAGTAAATCAACAAACCCA
       (fliA) M  D  A  L  A  A  Y  S  K  V  N  Q  Q  T  Q
101  GGAAGCTCTGGTAAAAACACATGCCACATTGGTAAAACGCATTCGACATC
         E  A  L  V  K  T  H  A  T  L  V  K  R  I  R  H
151  ATCTGCTCGGGCGATTACCGCAGAGTGTACAACTGGATGACTTGATACAA
       H  L  L  G  R  L  P  Q  S  V  Q  L  D  D  L  I  Q
201  TCAGGGATGCTCGGTCTGCTTGAAGCAGCAAGACATTATGATTCATCCAA
         S  G  M  L  G  L  L  E  A  A  R  H  Y  D  S  S  K
251  GGGGGCATCCTTTGAAACTTATGCAGCAATACGTATCAGGGCACATATGC
         G  A  S  F  E  T  Y  A  G  I  R  I  R  G  H  M
301  TTGATGAAGTAAGACGTAATGATTGGGTGCCGCGTTCTGTTTATCGCAAT
       L  D  E  V  R  R  N  D  W  V  P  R  S  V  Y  R  N
351  TCCAGAATGATTTCTGATGCTGTGCGCATATTGGAGCATAAACTTGGGCG
         S  R  M  I  S  D  A  V  R  I  L  E  H  K  L  G  R
401  TGAGGCTAAGGATAATGAAATTGCTGAAGAGTTGGGTGTGTCATTAAATG
         E  A  K  D  N  E  I  A  E  E  L  G  V  S  L  N
451  ATTATCATAGTATGTTGCAAGATTCCATTAGCAGCCATTTATATGGTTTT
       D  Y  H  S  M  L  Q  D  S  I  S  C  G  N  I  H  N
501  GATGACTTGGGAGTAACCGATGATATCTTATTTGATGATGAAGGGAATGG
       Q  H  S  P  L  L  R  H  Y  R  I  Q  H  H  G  N  G
551  CTCAACTGAACCGCACAGGAATGTTATGCGAAGTGATATGATGAATCGAT
         S  T  E  P  H  R  N  V  M  R  S  D  M  M  N  R
601  TAACCCAAGTGATTGATAGTCTCCCTCGTAAAGAGAGGCTGGTGCTTTCT
       L  T  Q  V  I  D  S  L  P  R  K  E  R  L  V  L  S
651  TTGTATTATGAGCAGGATTTGAATTTGAAGGAAATCGGGGAAATACTGGA
       L  Y  Y  E  Q  D  L  N  L  K  E  I  G  E  I  L  E
701  GGTTAGTGAGTCACGAATTTCACAGATTCTAAGTCAAGCAACGCATCGCA
         V  S  E  S  R  I  S  Q  I  L  S  Q  A  T  H  R
751  TTAGATCAAGATTACCGGAATAAAAAATAA
       I  R  S  R  L  P  F  *
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FIG. 4. Nucleotide sequence of the *fliA* gene. The deduced amino acid sequence of the FliA protein is shown below the nucleotide sequence. The ribosome binding site (SD) and the start codon are underlined.

encodes a protein of 238 amino acid residues with a predicted molecular mass of 27.2 kDa. The deduced amino acid sequence had a high degree of homology to members of the σ^{28} class of sigma factors. Among the most homologous (Table 2) were the proteins encoded by *P. aeruginosa fliA* (73% similarity and 55% identity), *E. coli fliA* (65% similarity and 43% identity), *Streptomyces coelicolor whiG* (57% similarity and 33% identity), and *B. subtilis sigD* (58% similarity and 33% identity). In addition, we analyzed conserved regions 2, 3, and 4 of the *L. pneumophila* σ^{28} factor in an alignment between the *L. pneumophila fliA* and *P. aeruginosa sigF* genes. As expected, these regions contained the most highly conserved residues indicated in Fig. 5. Downstream of *fliA*, we identified the beginning of a second open reading frame designated *motA* (Fig. 3). The first 122 N-terminal amino acids exhibited high homology (57% similarity on the amino acid level) to *motA* of *B. subtilis* (data not shown). Part of a third open reading frame (*ylxH*), corresponding to a protein of approximately 306 amino acids but lacking the N terminus, was found upstream of *fliA*. This gene was designated *ylxH*, and the gene product seemed to be related to the Ylxh protein of *Borrelia burgdorferi* (64% similarity on the amino acid level), exhibiting putative ATP-binding properties. Interestingly, this gene is located in an operon which also comprises numerous flagellum-associated genes.

Complementation of an *E. coli fliA* mutant. To determine if the cloned *fliA* homolog encoded a functional σ^{28}, we attempted to use the cloned *L. pneumophila fliA* gene to com-

TABLE 2. Amino acid sequence comparison of the *L. pneumophila* FliA protein and other members of the σ^{28} class of sigma factors

Protein	Species	% Identity	% Similarity
FliA	*P. aeruginosa*	55	73
FliA	*E. coli*	43	65
WhiG	*S. coelicolor*	33	57
SigD	*B. subtilis*	33	58

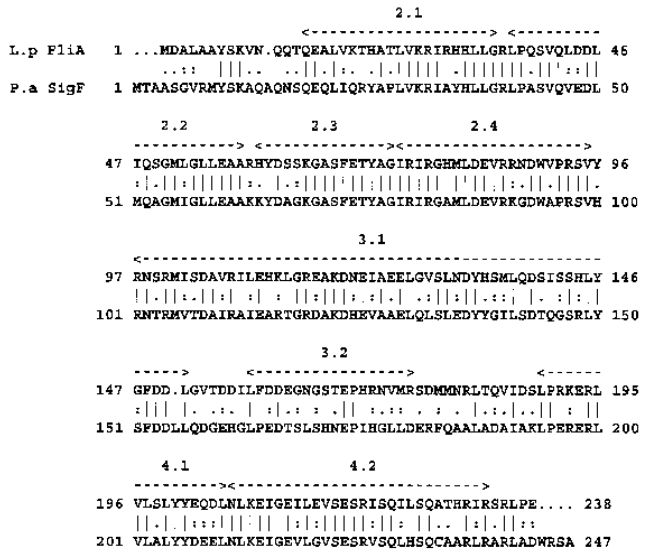

FIG. 5. Amino acid sequence comparison of the proteins encoded by the *L. pneumophila fliA* and *P. aeruginosa sigF* genes. The arrows define regions 2, 3, and 4 and the corresponding subregions conserved among sigma factors. Sequence comparison was generated by using the Gap program in the GCG package (29).

plement the defect in directing transcription of the *flaA* promoter in an *E. coli fliA* mutant (YK4101). We first determined the level of transcription by measuring the luciferase activity in mutant strains YK4104(pKH23) and YK4104(pKH23) containing vector pUC18 (Fig. 2). Both strains exhibited a 13-fold decrease in enzyme activity compared to the wild-type strain YK410 transformed with pKH23. In contrast, the complemented mutant strain YK4104(pKH23, pKH27) was shown to exhibit a level of enzyme activity similar to that of strain YK410 (pKH23), indicating that the *L. pneumophila fliA* gene product is functional in *E. coli.*

Furthermore, strain YK410(pKH23) and strain YK4104 (pKH23, pKH27) were motile when inoculated into motility medium, while the cloning vector (pUC18) in the same background was not (Fig. 6). Although YK4104(pKH23, pKH27) was motile, complementation of the *E. coli fliA* defect with the *L. pneumophila* gene on a multicopy plasmid did not restore motility to the level seen in the wild-type carrying pKH23. In addition, expression of flagella was examined by electron microscopy (data not shown), which gave results consistent with the swarm plate assay. In fact, once we could observe motility, we also found morphologically normal flagellated bacteria at least in a population subset.

DISCUSSION

This report describes the identification and cloning of the *L. pneumophila fliA* gene by using a *flaA* promoter fusion to *luxAB*. This specific reporter gene system represents an excellent tool for identifying other σ^{28}-regulated genes and, moreover, may be used to identify master regulators involved in modulating the activity or expression of σ^{28}. The *fliA* gene product is a member of the σ^{28} class of sigma factors used in the transcription of flagellar and chemotaxis genes in various species. The cloned fragment contains an open reading frame homologous to those for other sigma factors of the σ^{28} class. In addition, there is preliminary evidence that the *fliA* gene is located upstream of a gene (*motA*) whose product may be involved in motility (Fig. 3).

Genes involved in flagellum expression are tightly regulated and organized in a complex hierarchy. In *E. coli* and *S. typhimurium*, the first level of this cascade includes the *flhDC* operon, coding for master regulators which control class II genes. Class II genes encode proteins forming the basal body hook structure and also the FliA protein, which is required for transcription of class III genes. This last level of hierarchy includes flagellin genes as well as genes involved in motility such as *motA* and chemotaxis genes (for a review, see reference 18). Besides the necessity of FliA for the transcription of class III genes, FliA may also act as an activator in the transcription of class II genes (25). The activity of FliA in *S. typhimurium* is negatively regulated by FlgM, which acts as an anti-sigma factor, binding to FliA and preventing its association with the RNA polymerase core (26).

When the cloned *L. pneumophila fliA* gene was introduced into an *E. coli* strain with a mutation in *fliA*, it was able to complement the defect, restoring flagellin production and motility. However, complementation analysis using the cloned *L. pneumophila fliA* gene did not enable the *E. coli* mutant to spread through motility medium as efficiently as the wild-type *E. coli*. This could be a result of poor expression of the cloned gene in *E. coli*, but at least in *B. subtilis*, as few as 20 molecules of σ^{D} allow near normal motility (29). Therefore, the defect in the *fliA* mutant strain is probably not primarily a quantitative deficit that can be overcome by producing more protein. On the other hand, overexpression of FliA may also interfere with the regulation of genes downstream of *fliA.*

Furthermore, differences in the gene product of *Legionella fliA* and *E. coli fliA* may not allow complete complementation of the *E. coli* defect. It is conceivable that on one hand, the *L. pneumophila* FliA recognizes the *flaA*-specific promoter and therefore directs transcription of the p*flA-luxAB* fusion as shown in Fig. 2. On the other hand, FliA may not be able to bind to σ^{28}-specific promoter sequences of other genes whose gene products may be required for the correct assembly of the flagella, thus resulting in a reduced motility. Alternatively, the

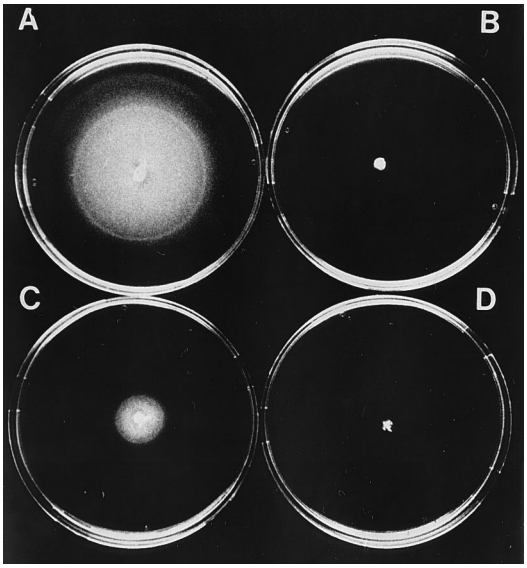

FIG. 6. Complementation of a *fliA* mutation in *E. coli* by using the cloned *L. pneumophila fliA* gene. Strains were inoculated into motility agar and incubated overnight at 30°C. (A) *E. coli* wild-type strain YK410(pKH23); (B) the isogenic *fliA* derivative [YK4104(pKH23)]; (C) YK4104(pKH23) with the cloned *L. pneumophila fliA* gene (pKH27); (D) YK4104(pKH23) containing vector pUC18.

nature of the mutation in the *fliA* mutant strain is not well defined (8), and the reduced ability of *Legionella* FliA to complement may be the result of reduced expression of genes downstream of *fliA*, whose products may be directly or indirectly involved in motility. There is evidence that the *E. coli fliA* gene is located in an operon with *fliZ* and *fliY*. These gene products are not required for motility but are required for the regulation of σ^F activity (34).

The amino acid sequences of many sigma factors have been separated into regions of highest homology, and each region has been studied for possible functions (for a review, see reference 20). The amino acid sequence of the cloned *L. pneumophila fliA* gene has homology to sequences of other sigma factors in regions 2, 3, and 4, as is typical of members of the σ^{28} family. Region 2 contains domains which have been implicated in binding of the sigma factor to the core RNA polymerase. Region 2 may also be involved in the recognition of the −10 sequence of bacterial promoters and in catalyzing the open-promoter complex formation. It has been suggested that region 3 may be important for the structural integrity of some σ factors. The sequence of region 4 predicts a helix-turn-helix structure possibly involved in the recognition of the −35 region of bacterial promoter sequences. The regions of the *L. pneumophila fliA* gene corresponding to conserved regions 2, 3, and 4 are shown in Fig. 5.

The interplay of various regulatory elements, including alternative sigma factors, may also be linked to the cell cycle of a bacterium. Because *L. pneumophila* has only a single polar flagellum, it is necessary for the bacterium to produce flagellin only for a short time during every cell division. Flagellin synthesis may be temporally controlled by FliA, like the control of sporulation in *S. coelicolor* by σ^{28} (7, 31). There also may be a feedback mechanism mediated by FliA connecting flagellin synthesis to accurate flagellum assembly. Such a mechanism is proposed for *S. typhimurium*, where the product of the *flgR* gene exerts negative control over *fliA* expression if the assembly process is defective (17).

For *Campylobacter jejuni*, *P. aeruginosa*, *Salmonella typhi*, and *Proteus mirabilis*, bacterial motility has been documented as a factor enhancing bacterial virulence in vivo (1, 12, 27, 49). In addition, there is evidence that in *Vibrio cholerae*, motility is tightly coupled to the expression of multiple ToxR-regulated and non-ToxR-regulated virulence determinants (16). One attractive model suggests that FliA, which positively regulates several flagellar genes, might negatively regulate virulence gene expression (44). Finally, motility might be important for the survival of legionellae in aquatic habitats, because the interaction with the intracellular growth within protozoa seem dependent on finding the host cell (40).

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