

## *fleN*, a Gene That Regulates Flagellar Number in *Pseudomonas aeruginosa*

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Received 29 June 1999/Accepted 11 October 1999

**The single polar flagellum of *Pseudomonas aeruginosa* plays an important role in the pathogenesis of infection by this organism. However, regulation of the assembly of this organelle has not been delineated. In analyzing the sequence available at the *Pseudomonas* genome database, an open reading frame (ORF), flanked by flagellar genes *flhF* and *flhA*, that coded for a protein (280 amino acids) with an ATP-binding motif at its N terminus was found. The ORF was inactivated by inserting a gentamicin cassette in *P. aeruginosa* PAK and PAO1. The resulting mutants were nonmotile on motility agar plates, but under a light microscope they exhibited random movement and tumbling behavior. Electron microscopic studies of the wild-type and mutant strains revealed that the mutants were multiflagellate, with three to six polar flagella per bacterium as rather than one as in the wild type, indicating that this ORF was involved in regulating the number of flagella and chemotactic motility in *P. aeruginosa*. The ORF was named *fleN*. An intact copy of *fleN* on a plasmid complemented the mutant by restoring motility and monoflagellate status. The  $\beta$ -galactosidase activities of eight flagellar operon or gene promoters in the wild-type and *fleN* mutant strains revealed a direct correlation between six promoters that were upregulated in the *fleN* mutant (*fljLMNOPQ*, *flgBCDE*, *fljEFG*, *fljDS orf126*, *fleSR*, and *fljC*) and positive regulation by FleQ, an NtrC-like transcriptional regulator for flagellar genes. Based on these results, we propose a model where FleN influences FleQ activity (directly or indirectly) in regulating flagellar number in *P. aeruginosa*.**

Flagella serve primarily as locomotory organelles in flagellated bacterial species. They have also been implicated in biofilm development in *Pseudomonas aeruginosa* and *Escherichia coli* (24, 26) and in the pathogenesis of infections by *P. aeruginosa*, *Campylobacter jejuni*, *Helicobacter pylori*, and *Vibrio cholerae* (23, 25). The bacterial flagellum is a complex structure requiring more than 40 genes for its assembly (18). Over the years, significant progress has been made in identifying various flagellar structural and regulatory genes, elucidating the composition of flagellar substructures, and understanding the mechanisms of its assembly in a number of bacterial species, including *E. coli*, *Salmonella enterica* serovar Typhimurium (1, 18, 22), and *Caulobacter crescentus* (28, 38). Work is in progress to elucidate the pathway of flagellar assembly in the pathogens *P. aeruginosa*, *V. cholerae*, and *H. pylori*.

Most flagellated bacterial species have extracellular flagella; exceptions are spirochetes such as pathogenic *Treponema pallidum* and *Borrelia burgdorferi*, which have flagella implanted in the periplasmic space. In some cases, extracellular flagella are covered by a sheath, as in *V. cholerae* and *H. pylori* (23). The distribution of flagella may be monotrichous polar as in *P. aeruginosa* (14) and *V. cholerae* (39) or peritrichous (5 to 10 flagella) as in *E. coli* and *Salmonella* serovar Typhimurium (18). Flagellar number, a characteristic feature of each species, is successfully maintained over the generations, but nothing is known about the genes and the mechanisms which contribute to its regulation. In a recent model proposed for *Salmonella*, the number of flagellar filaments has been linked to the cell cycle (2).

A major goal of our laboratory is to gain an insight into the

biogenesis pathway of the flagellum of *P. aeruginosa*. The published information from our laboratory and elsewhere (5, 6, 29, 33) indicates that the system deviates from the well-described enteric model (18). It has certain similarities to the *Caulobacter* (38), *Vibrio* (17), and *Helicobacter* (32) systems, which utilize the alternative sigma factor RpoN and an NtrC transcriptional regulator homologue at some stage(s) of flagellar biogenesis.

The availability of the partial genome sequence of *P. aeruginosa* from strain PAO1 at the *Pseudomonas* genome database website ([www.pseudomonas.com](http://www.pseudomonas.com)) has simplified our quest to understand the flagellar biogenesis pathway in this organism. In this paper, we report the identification of *fleN*, a gene that is involved in controlling the number of flagella and chemotactic motility in *P. aeruginosa*.

### MATERIALS AND METHODS

**Bacterial strains, plasmids, and media.** The bacterial strains and plasmids used in this study are listed in Table 1. They were grown in Luria-Bertani (LB) broth (30) at 37°C with shaking at 250 rpm or on LB agar plates unless stated otherwise. The following antibiotics were used to maintain the plasmid and chromosomal insertions in *P. aeruginosa*: gentamicin at 50  $\mu$ g/ml (100  $\mu$ g/ml for plates), carbenicillin at 150  $\mu$ g/ml (300  $\mu$ g/ml for plates), streptomycin at 300  $\mu$ g/ml, and tetracycline at 50  $\mu$ g/ml (100  $\mu$ g/ml for plates). In *E. coli*, the concentrations used were 200  $\mu$ g/ml for ampicillin, 10  $\mu$ g/ml for gentamicin, and 25  $\mu$ g/ml for tetracycline.

**Computer analysis.** The nucleotide sequences of earlier contigs and contig 53 from the *Pseudomonas* genome database (release date, March 15, 1999) were subjected to an open reading frame (ORF) search using the ORF Finder program at the National Center for Biotechnology Information (NCBI) website ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). Later, the deduced amino acid sequence of the uncharacterized ORF, *fleN*, was subjected to BLASTP (3) searches using the GenBank database entries. TBLASTN (3) was used to search for homologues of FleN in the preliminary sequence data of *V. cholerae* (obtained from The Institute of Genomic Research [TIGR] website at [www.tigr.org](http://www.tigr.org)) and *C. crescentus* ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). The deduced amino acid sequence of FleN was subjected to an online PROSITE database search ([www2.ebi.ac.uk](http://www2.ebi.ac.uk)).

**Transformation and electroporation.** Frozen competent *E. coli* DH5 $\alpha$  cells were prepared and transformed by essentially using the standard procedure (30). Electroporations in *P. aeruginosa* were performed by using a modification of the protocol of Smith and Iglewski (31). For gene replacement experiments involving

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TABLE 1. Bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Relevant information	Source or reference
<i>E. coli</i> DH5 $\alpha$	<i>hsdR recA lacZYA</i> $\phi$ 80 <i>lacZ</i> $\Delta$ M15	GIBCO-BRL
<i>P. aeruginosa</i>		
PAK	Wild-type clinical isolate	D. Bradley
PAO1	Wild type	M. Vasil
PAK-Q	PAK <i>fleQ</i> ::Gm <sup>r</sup>	5
PAK-N	PAK <i>fleN</i> ::Gm <sup>r</sup>	This study
PAO-N	PAO <i>fleN</i> ::Gm <sup>r</sup>	This study
Plasmids		
pGEM3Zf(+)	Cloning vector, Amp <sup>r</sup> , LacZ $\alpha$ peptide	Promega Corp., Madison, Wis.
pGEM- <i>fleN</i>	pGEM3Zf(+) with a 2.0-kb PCR product ( <i>fleN</i> locus) cloned into the <i>EcoRI</i> / <i>Bam</i> HI sites	This study
pGEM- <i>fleNG</i>	pGEM- <i>fleN</i> with a gentamicin resistance gene inserted in the unique <i>EcoRV</i> site of <i>fleN</i>	This study
pPZ375	<i>oriV</i> in pGEM3Zf(+)	35
pPZ375- <i>fleN</i>	pPZ375 containing <i>fleN</i> as a 1.0-kb <i>Hind</i> III/ <i>Sst</i> I PCR fragment	This study
pET15bVP	<i>oriV</i> cloned as a <i>Pst</i> I fragment in <i>bla</i> of pET15b	5
pET- <i>fleN</i>	<i>fleN</i> inserted as a PCR product into the <i>Nde</i> I/ <i>Bam</i> HI sites of pET15bVP	This study
pDN19lac $\Omega$	Promoterless <i>lacZ</i> <i>oriV</i> <i>oriT</i> Tet <sup>r</sup> Str <sup>r</sup> $\Omega$ fragment	37
plac $\Omega$ Q	pDN19lac $\Omega$ containing the <i>fleQ</i> promoter region	5
plac $\Omega$ S	pDN19lac $\Omega$ containing the <i>fleSR</i> promoter region	5
plac $\Omega$ E	pDN19lac $\Omega$ containing the <i>fliEFG</i> promoter region	4
plac $\Omega$ D	pDN19lac $\Omega$ containing the <i>fliDSorf126</i> promoter region	6
plac $\Omega$ flgE	pDN19lac $\Omega$ containing the <i>flgBCDE</i> promoter region	This study
plac $\Omega$ L	pDN19lac $\Omega$ containing the <i>fliLMNOPQ</i> promoter region	This study
pMS565	pDN19lac $\Omega$ containing the <i>fliA</i> promoter region	33
pPT269	pDN19lac $\Omega$ containing the <i>fliC</i> promoter region	37
pMSZ5	pDN19lac $\Omega$ containing the <i>pilA</i> promoter region	15
Primers <sup>a</sup>		
pPAO4	5' cccaaagaat <b>TCCCGGCCAGT</b> CGCTGAT 3', <i>EcoRI</i> site incorporated	
pPAO5	5' cccaaagg <b>ATCCGCCAGGGCCG</b> CCTGG 3', <i>Bam</i> HI site incorporated	
flnHind	5' cccaaaaagctt <b>GAGGACGTG</b> GGAAGAAC 3', <i>Hind</i> III site incorporated	
flnSst	5' cccaaagag <b>CTCCAGAGG</b> CCCGCTGTC 3', <i>Sst</i> I site incorporated	
flnPst	5' GTGAGCCT <b>GCAGGCACCG</b> GAAGAGCC 3', <i>Pst</i> I naturally present	
flnNde	5' GACAACACA <b>acATGAAGC</b> AGATGGG 3', <i>Nde</i> I site incorporated	
flnBam	5' CCTTGCTATAC <b>gggTCCAGAGG</b> CCCGCTG 3', <i>Bam</i> HI site incorporated	
5Pflilbgal	5' cccaaagaat <b>ctc</b> CGGGCGATGAGGAAC 3', <i>EcoRI</i> site incorporated	
3Pflilbgal	5' cccaaaggat <b>ctc</b> GCCTCTTCTTAGCC 3', <i>Bam</i> HI site incorporated	
RER 41	5' cccaaagaat <b>ctc</b> GGCTTGCCACCCTTGCC 3', <i>EcoRI</i> site incorporated	
RER 42	5' cccaaaggat <b>ctc</b> GTGGCCAGGTTGTTGG 3', <i>Bam</i> HI site incorporated	

<sup>a</sup> In primer sequences, lowercase denotes nucleotides added or modified to facilitate restriction digestion at the sites marked in bold.

chromosomal recombinations, about 1  $\mu$ g of linearized plasmid was used. For introducing replicative plasmids, 50 to 100 ng of plasmid DNA prepared by the alkaline lysis procedure (8) was electroporated into the strains.

**PCR.** PCR was performed in a DNA Thermal Cycler 480 (Perkin-Elmer Cetus, Norwalk, Conn.), using either *Taq* DNA polymerase or eLongase (GIBCO-BRL Inc., Gaithersburg, Md.) in 100- $\mu$ l reaction volumes. Briefly, the reaction mixture consisted of 100 ng of template DNA, 1.5 mM MgCl<sub>2</sub>, 1 $\times$  polymerase buffer, 0.2 mM deoxynucleoside triphosphates, 0.5  $\mu$ M concentrations of each primer (Table 1; custom synthesized at GIBCO-BRL), 2% dimethyl sulfoxide, 1 U of *Taq* DNA polymerase, or 2 U of eLongase. PCR was performed as follows: initial denaturation of 10 min at 94°C, followed by 35 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 55°C (pPAO4-pPAO5), 50°C (flnHind-flnSst and flnPst-flnSst), or 52°C (flnNde-flnBam), and an extension of 1 min/kb at 72°C with *Taq* or 2 min/kb at 68°C with eLongase. With primer pairs 5Pflilbgal-3Pflilbgal and RER41-RER42, *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.) was used according to the manufacturer's instructions, with 45°C as the annealing temperature. The template DNA used for PCR was either purified genomic DNA isolated by the cetyltrimethylammonium bromide procedure (7) or a plasmid preparation made by the alkaline lysis method. The PCR products were electrophoresed on a 1% SeaPlaque GTG agarose (FMC Bioproducts, Rockland, Maine) gel and stained with ethidium bromide, and the desired bands were electroeluted for further applications.

**Sequencing.** DNA sequencing was accomplished by using the *Taq* DyeDeoxy terminator and DyePrimer cycle sequencing protocols developed by Applied Biosystems (Perkin-Elmer Corp., Foster City, Calif.), using fluorescence-labeled dideoxynucleotides. The labeled extension products were analyzed on an Applied Biosystems model373A DNA sequencer.

**Generation of *fleN* chromosomal mutants.** The 2.0-kb PCR product generated by using amplifiers pPAO4 and pPAO5 (Fig. 1) from the PAO1 genome was restricted at the *EcoRI* and *Bam*HI unique sites incorporated in the primers and cloned into pGEM3Zf(+), yielding pGEM-*fleN*. The cloned insert contained the ~1.0-kb coding region of the new ORF, *fleN*, and its flanking regions. A 1.7-kb

gentamicin resistance cassette with blunt ends was inserted into the unique *EcoRV* site of the cloned ORF in pGEM-*fleN* (Fig. 1). The resulting plasmid, pGEM-*fleNG*, was linearized and electroporated into both PAK and PAO1 strains of *P. aeruginosa* for insertional inactivation of *fleN* by homologous recombination.

**Construction of plasmids for complementation and promoter activity studies.** *fleN* was amplified from pGEM-*fleN* by using primers flnHind and flnSst (Fig. 1).

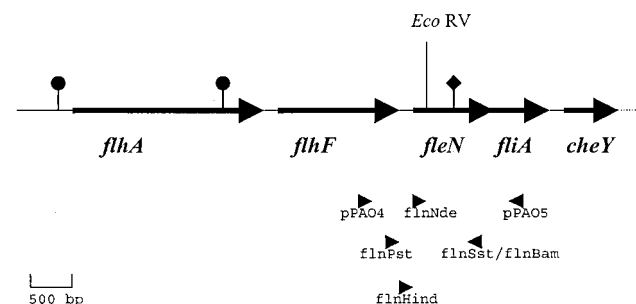


FIG. 1. Schematic representation of the *fleN* locus based on the *P. aeruginosa* PAO1 sequence of contig 53 from the *Pseudomonas* genome database. Bold arrows indicate locations of the ORFs (*flhA*, *flhF*, *fleN*, *fliA*, and *cheY*) in this region, and their respective orientations; ♦ indicates the presence of a  $\sigma^{54}$  consensus sequence; ♦ denotes the location of the *fliA* promoter. The position of the unique *EcoRV* site in *fleN*, used for inserting the gentamicin resistance gene, is shown. Arrowheads show positions and orientations of primers used in this study.

The 1.0-kb PCR product was digested with *Hind*III and *Sst*I and cloned into pPZ375, a high-copy-number plasmid, yielding pPZ375-*fleN*. pET-*fleN* was derived from pET15bVP by cloning an 880-bp PCR product obtained from PAK by using primers flnNde and flnBam (Fig. 1) sequentially digested with *Nde*I and *Bam*HI.

To clone the putative promoters of *fliLMNOPQ* and *flgBCDE* operons (S. K. Arora and R. Ramphal, unpublished data), 340- and 260-bp stretches mapping immediately upstream of the respective operons were amplified by using primer pairs 5Pflilbgal-3Pflilbgal and RER41-RER42 (Table 1), respectively. They were digested at the unique *Eco*RI/*Bam*HI sites incorporated in the primers and cloned into pDN19lac $\Omega$  in *E. coli*. The resulting plasmids were named plac $\Omega$ L and plac $\Omega$ flgE, respectively.

**Electron microscopy (EM).** Static cultures were grown overnight at 37°C with the appropriate antibiotics. A drop of the culture was allowed to adhere to a carbon-coated grid for 10 s and drained off; the grid was then rinsed in a drop of saline, and adherent cells were negatively stained with a 2% aqueous solution of phosphotungstic acid for 10 s. Samples were examined with a Hitachi H-7000 transmission electron microscope.

**Video light microscopy.** To test the motility of the various strains under examination by the hanging drop method, a 50- $\mu$ l drop of the culture was placed in the center of a glass coverslip edged with vacuum grease, and the coverslip was inverted carefully on a concave glass slide. The bacteria were examined with high-dry and oil immersion objectives of a Zeiss light microscope (Carl Zeiss Inc., Thornwood, N.Y.) equipped with a chilled charge-couple device camera (Hamamatsu Photonics K. K., Hamamatsu City, Japan).

**$\beta$ -Galactosidase assay.** The promoter regions of flagellar operons and genes were cloned upstream of a promoterless *lacZ* in pDN19lac $\Omega$  (Table 1). The constructs and vector pDN19lac $\Omega$  were electroporated into wild-type PAK and mutants PAK-N and PAK-Q and then tested for  $\beta$ -galactosidase activity (20). The strains were grown to late log phase ( $A_{600}$  of 0.7 to 1.0) in LB medium containing either streptomycin or tetracycline.

**Nucleotide sequence accession number.** The *P. aeruginosa* PAK *fleN* sequence reported in this paper has been submitted to the GenBank database and assigned accession no. AF133657.

## RESULTS

**Nucleotide sequence analysis of the *fleN* locus.** Analysis of the available sequence at the *Pseudomonas* genome project ([www.pseudomonas.com](http://www.pseudomonas.com); release date, March 15, 1999) revealed an ORF (Fig. 1), located on contig 53, encoding a protein of 280 amino acids. The ORF was named *fleN* for reasons described below. It mapped between *flhF*, encoding the putative homologue of a protein implicated in flagellin-specific export in *Bacillus subtilis* (11) and *fliA*, encoding the flagellar sigma factor  $\sigma^{28}$  in *P. aeruginosa* (33). *flhA*, coding for a flagellin export apparatus protein homologue of *Salmonella* serovar Typhimurium (21), mapped further upstream of *flhF*. The *che* locus consisting of genes involved in chemotaxis (13) mapped downstream of *fliA*. In *E. coli* and *Salmonella* serovar Typhimurium, the master regulator operon of the flagellar biogenesis pathway, *flhCD*, maps upstream of the *che* locus (18), but in *P. aeruginosa* we were unable to identify *flhCD* sequence homologues in the same or different loci of the genomic sequence. Thus, the location of the previously uncharacterized ORF *fleN*, upstream of the *che* locus, among flagellar genes tempted us to investigate its possible involvement in flagellar biogenesis.

Sequence analysis of *fleN* and the adjacent genes revealed stretches of 83 bp between the coding regions of *flhA* and *flhF* and 139 bp between those of *flhF* and *fleN*, which were devoid of alternative sigma factor consensus sequences for  $\sigma^{54}$  (CTG GYAYRN<sub>4</sub>TTGCA) and  $\sigma^{28}$  (TAAAN<sub>15</sub>GCCGATAA) that some flagellar genes are known to possess (29, 37). Search for a  $\sigma^{54}$  promoter consensus sequence further upstream of *fleN* revealed two candidate sites both having a 14/17 match. One of the sites lay centered 424 bp upstream of the *flhF* initiation codon, mapping within *flhA*, and the other was centered 94 bp upstream of the *flhA* initiation codon (Fig. 1). The *fleN* stop codon TGA and the initiation codon ATG for the subsequent gene, *fliA*, overlap, and the promoter of *fliA* (33) maps within the coding region of *fleN* (Fig. 1).

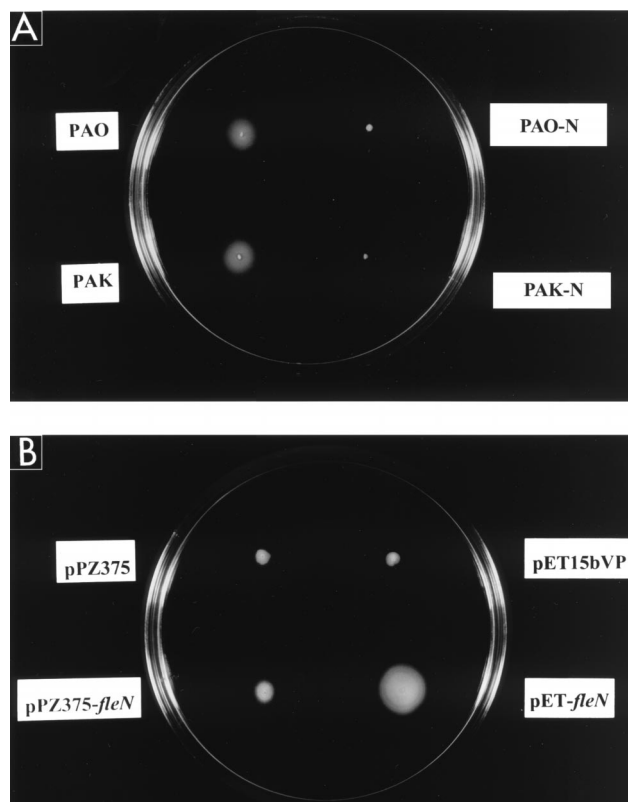


FIG. 2. (A) Motility phenotype of *P. aeruginosa* wild-type strains PAK and PAO (PAO1) and their *fleN* mutants, PAK-N and PAO-N. (B) Motility phenotype of PAK-N containing plasmid constructs pPZ375-*fleN*, pET-*fleN*, and their vector controls, pPZ375 and pET15bVP, respectively. The strains were freshly grown on LB plates with appropriate antibiotics; cells were transferred to 0.3% agar plates with a sterile toothpick and incubated at 37°C for 8 h (A) or 12 h (B).

***fleN* regulates the number of polar flagella and motility in *P. aeruginosa*.** Chromosomal *fleN* mutants of *P. aeruginosa* PAK and PAO1 were constructed as described in Materials and Methods. The insertional inactivation of *fleN* in the Gm<sup>r</sup> Carb<sup>s</sup> clones was confirmed by PCR using primers flnHind and flnSst, which yielded a ~2.7-kb product in the mutants, while the wild type displayed a 1.0-kb product as expected (data not shown). The mutants were tested for their motility phenotypes on motility plates. Both PAK and PAO1 mutants were non-motile (Fig. 2A). Nonmotility could be attributed to either the absence of flagellum or an impairment in its function when present. EM studies revealed that the nonmotile mutants of both PAK (Fig. 3) and PAO1 (data not shown) had a tuft of three to six polar flagella, as opposed to a single polar flagellum in the wild-type parents. This observation suggested that disruption of the ORF was associated with the loss of flagellar number control. The ORF was thus named *fleN*, and the PAK and PAO1 mutants were named PAK-N and PAO-N, respectively.

Direct sequencing of the *fleN* PCR product obtained from PAK by using primers flnPst and flnSst (Fig. 1) indicated that the sequence of *fleN* in PAK was identical to that of PAO1 available at the *Pseudomonas* genome database. Therefore, further studies were restricted to the PAK mutant, PAK-N.

Examination of the multiflagellated PAK-N strain by the hanging drop method (14) revealed that the flagella were not paralyzed, as the majority of the bacteria exhibited random movement, tumbling behavior, and polar spinning but lacked

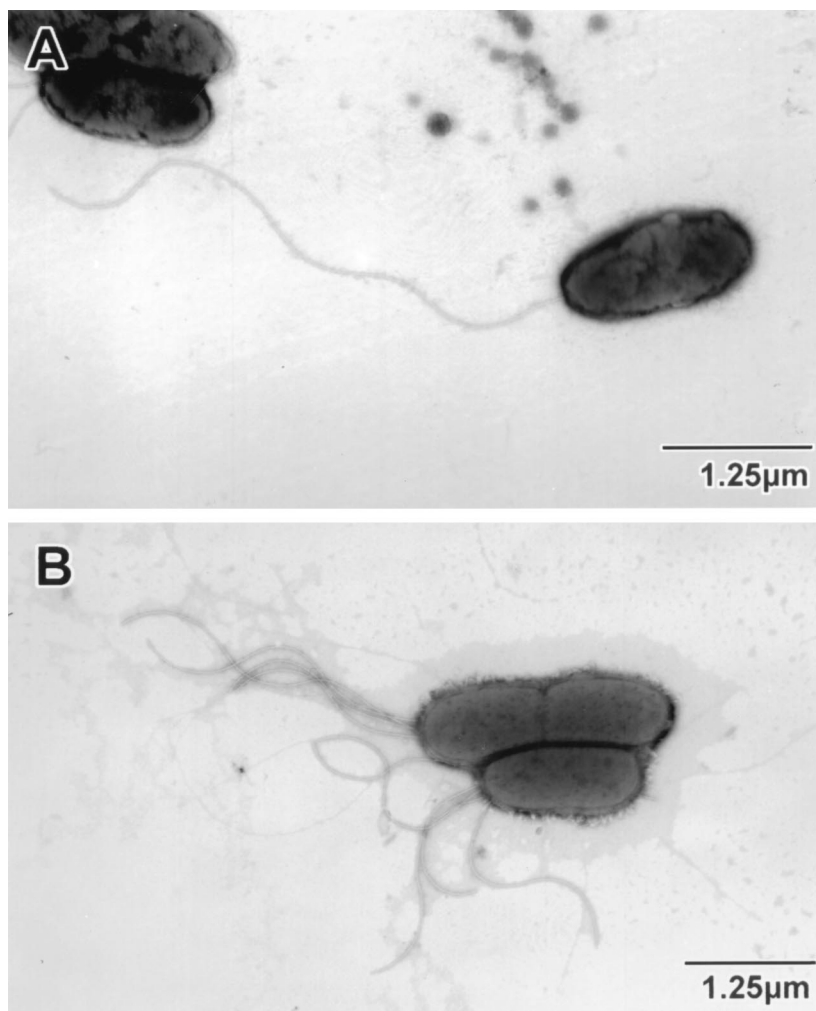


FIG. 3. Electron micrographs of wild-type PAK (A) and mutant PAK-N (B). The wild-type cell has a long single polar flagellum, as opposed to the polar tuft of shorter flagella present on the mutant bacteria visible in this field.

the directional swimming typical of the wild-type PAK strain. This could account for their nonmotile phenotype on motility plates, where chemotactic motility is essential for swarming behavior.

**Complementation of PAK-N.** Successful complementation of PAK-N to the wild-type phenotype with an intact copy of *fleN* on a plasmid was important to demonstrate that *fleN* was solely responsible for the nonmotility associated with the multiflagellate status. The *fleN* PCR product generated by using primers *flnHind* and *flnSst* was cloned into the broad-host-range plasmid pPZ375 (pPZ375-*fleN*), allowing the transcription of *fleN* from the unrepressed strong *lac* promoter of the vector. The sequence of the PCR product was identical to that of the wild-type parent PAO1 sequence. When examined for complementation, pPZ375-*fleN* was not able to confer motility to PAK-N (Fig. 2B). However, EM studies showed that the majority of these cells were now devoid of flagella, with few having a single flagellum (Fig. 4B). The mutant strain with pPZ375, the vector control, remained nonmotile (Fig. 2B) and multiflagellate (Fig. 4A). This suggested that FleN was probably being synthesized from pPZ375-*fleN*, but the excessive amount was inhibiting the formation of the flagellum in the majority of the bacteria. Subsequently, pET15bVP, an expression vector with low basal activity of the T7 promoter, was used

in a *P. aeruginosa* host lacking T7 RNA polymerase to clone *fleN* downstream of the T7 promoter (pET-*fleN*) and examined for its ability to complement the *fleN* mutant. It conferred wild-type motility phenotype to the nonmotile *fleN* mutant (Fig. 2B), and most of the cells displayed one to two flagella (Fig. 4D).

**Flagellar genes that are positively regulated by FleQ are upregulated in the *fleN* mutant.** The multiflagellate phenotype of the *fleN* mutant suggested that an excess of flagellar components was being synthesized for the assembly of multiple flagella. Therefore, we compared the  $\beta$ -galactosidase activities of eight flagellar gene promoter fusion constructs available in the laboratory, as well as that of a nonflagellar gene promoter of *pilA*, in the PAK and PAK-N strains (Table 2). The assay showed a 2- to 27-fold upregulation in the transcriptional activities of six flagellar promoters in PAK-N. These were the promoters of the *fliEFG*, *fliLMNOPQ*, *fliDS orf126*, *flgBCDE*, and *fleSR* operons and *fliC*. The promoter activities of *fleQ*, *fliA*, and *pilA* did not show any appreciable change.

Previous studies from our laboratory showed that FleQ, an NtrC-like transcriptional activator of *P. aeruginosa* that works in concert with RpoN ( $\sigma^{54}$ ), positively regulates two of the above six upregulated promoters, namely, *fliDSorf126* (6) and *fleSR* (29), either directly or indirectly. These results were

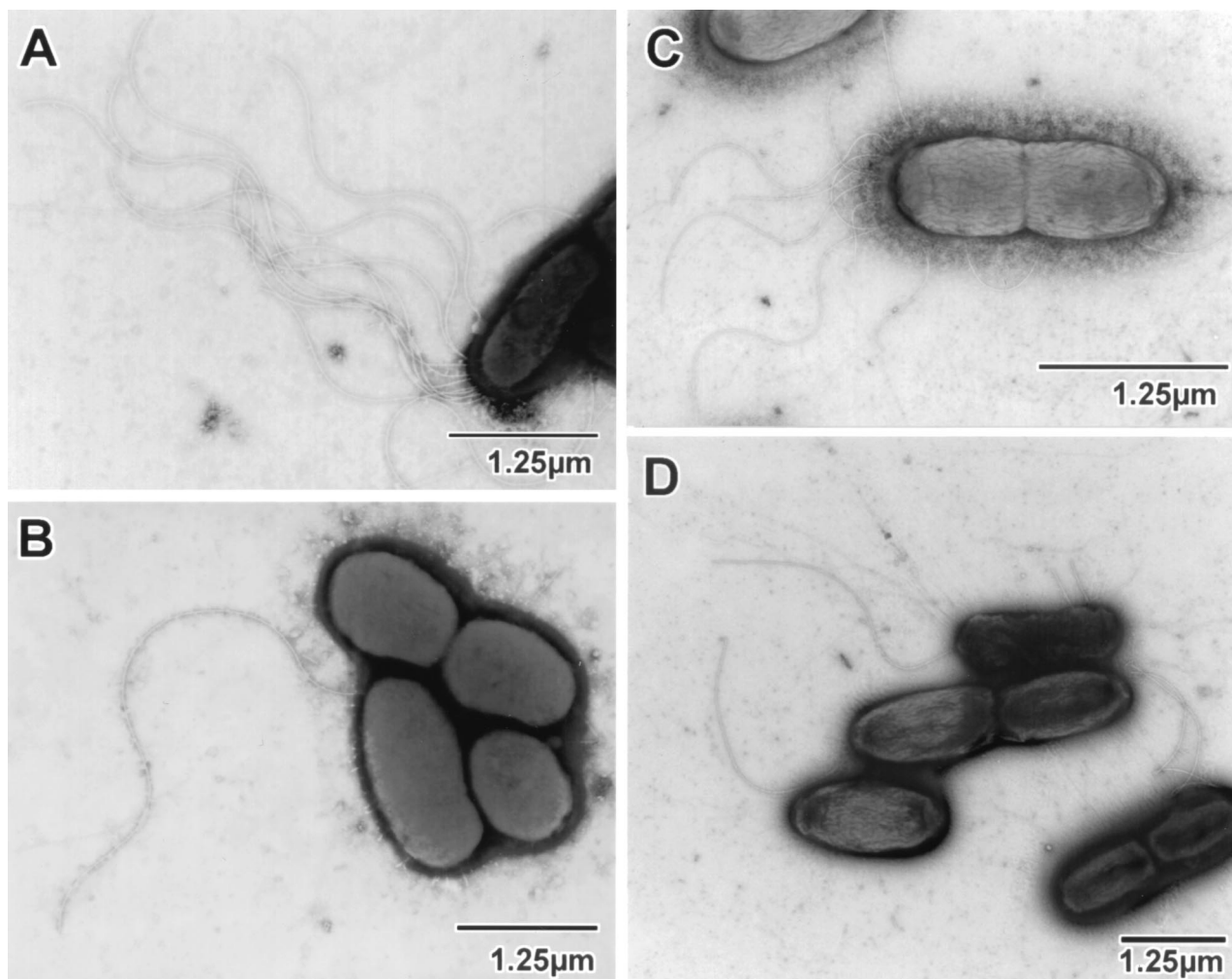


FIG. 4. Electron micrographs of mutant PAK-N containing plasmid constructs pPZ375 (A), pPZ375-*fleN* (B), pET15bVP (C), and pET-*fleN* (D). The vector controls (A and C) retain the multiple polar flagella of the mutant, whereas most of the cells in panel B, with pPZ375-*fleN*, are nonflagellated. pET-*fleN* (D) restores the wild-type monoflagellate status to the mutant, as most of the cells are seen to possess a single polar flagellum.

based on  $\beta$ -galactosidase assays performed with wild-type PAK, the *rpoN* mutant PAK-N1G, and the *fleQ* mutant PAK-Q. The *fleQ* promoter is not autoregulated by FleQ (5). To determine whether there was a direct correlation between upregulation of a promoter in the *fleN* mutant and FleQ dependence, the flagellar promoters of the genes and operons *fliC*, *fliEFG*, *flgBCDE*, *fliLMNOPQ*, and *fliA* were tested in an FleQ-deficient background, PAK-Q (Table 3). Reduced activity of the *fliC*, *fliEFG*, *flgBCDE*, and *fliLMNOPQ* promoters in PAK-Q implied that these genes and operons were positively regulated by FleQ, either directly or indirectly. The *fliA* promoter was not dependent on FleQ under these conditions, as it did not exhibit an appreciable decrease in its activity in PAK-Q.

In summary, among the eight flagellar gene promoters tested, the six promoters that were positively regulated by FleQ, namely, *fliEFG*, *fliLMNOPQ*, *fliDS orf126*, *flgBCDE*, *fliC*, and *fleSR*, were upregulated in the *fleN* mutant. This finding suggests that the absence of functional FleN in PAK-N enhances the transcriptional activator abilities of FleQ by an unknown mechanism.

**FleN homologues from other microbial genomes share an N-terminal ATP-binding motif.** Computer-based searches of

the various databases revealed that FleN homologues were also present in other microbial genomes (Fig. 5). The majority of the top scorers from the annotated entries were found to be positional homologues of FleN, as respective genes mapped

TABLE 2. Assessment of the transcriptional activities of flagellar gene promoters in strains PAK and PAK-N

Operon or gene (promoter construct)	$\beta$ -Galactosidase activity (Miller units; mean $\pm$ SD)		Fold upregulation of promoter activity in PAK-N
	PAK	PAK-N	
<i>flgBCDE</i> (plac $\Omega$ flgE)	1,000 $\pm$ 100	27,000 $\pm$ 2,000	27
<i>fleSR</i> (plac $\Omega$ S)	6,300 $\pm$ 80	80,000 $\pm$ 500	12.6
<i>fliDS orf126</i> (plac $\Omega$ D)	1,900 $\pm$ 100	18,000 $\pm$ 90	9.5
<i>fliLMNOPQ</i> (plac $\Omega$ L)	2,600 $\pm$ 40	14,800 $\pm$ 1,800	5.6
<i>fliEFG</i> (plac $\Omega$ E)	1,000 $\pm$ 30	5,000 $\pm$ 180	5
<i>fliC</i> (pPT269)	3,200 $\pm$ 200	7,400 $\pm$ 300	2.3
<i>pilA</i> (pMSZ5)	10,000 $\pm$ 700	12,000 $\pm$ 500	1.2
<i>fleQ</i> (plac $\Omega$ Q)	2,800 $\pm$ 100	2,800 $\pm$ 300	1
<i>fliA</i> (pMS565)	650 $\pm$ 10	640 $\pm$ 2	1
Promoterless <i>lacZ</i> (plac $\Omega$ )	289 $\pm$ 143	238 $\pm$ 187	0.8

downstream of the *flhF* counterparts, similar to that of *fleN*. They included OrfC of *P. putida* (90% identity) (13), Orf304 of *T. pallidum* (35% identity (J. M. Hardham, J. G. Frye, N. R. Young, and L. V. Stamm, GenBank accession no. U36839 [unpublished data]), YlxH of *B. burgdorferi* (34% identity) (J. J. Dunn, L. Butler-Loffredo, J. Kieleczawa, J. Medalle, and B. J. Luft, GenBank accession no. U43739 [unpublished data]), Orf298/YlxH of *Bacillus subtilis* (33% identity) (16), YlxH of *H. pylori* (31% identity) (36), and MinD-1 of *Aquifex aeolicus* (31% identity) (12). *ctrA* of *P. aeruginosa* W51D (accession no. AF052586) displayed 94 and 60% identities to *fleN* at the deduced amino acid and nucleic acid sequence levels, respectively. It maps upstream of *rhlG*, a gene involved in rhamnolipid synthesis (10). The function of *ctrA* has not been reported. Neither FleN nor CtrA of *P. aeruginosa* displayed sequence homology to the *C. crescentus* master regulator of flagellar biogenesis, also named CtrA (27).

Searches with the unannotated partial genome sequences of *V. cholerae*, a monoflagellate, revealed an ORF on contig asm913, coding for a protein of 313 amino acids, that displayed 60% deduced amino acid sequence identity with FleN (Fig. 5). We refer to it as Orf313 in this study. It mapped downstream of the *V. cholerae* putative *flhF* as does *fleN* in *P. aeruginosa*, classifying it as another positional homologue of FleN.

The striking similarity among all of the proteins described above was that they all have an ATP-binding motif {[AG]-X<sub>4</sub>-G-K-[ST]} (PROSITE PS000017) at their N termini (Fig. 5) except for OrfC of *P. putida*. A closer inspection of the nucleotide sequence of this region in *P. putida* (accession no. AF031898) (13) revealed that an unannotated ORF mapping upstream of *orfC* coded for a polypeptide with 76 amino acid residues that bore the missing ATP-binding motif. This finding indicated the possibility of either a sequence error or an inherent mutation(s) leading to a reading frame shift in this locus in *P. putida*. The available genomic sequences of *C. crescentus*, *E. coli*, and *Salmonella* serovar Typhimurium did not have a FleN homologue.

## DISCUSSION

Insertional inactivation of *fleN*, an ORF of *P. aeruginosa* flanked by the flagellar genes, *flhF* and *flhA*, conferred a multiflagellate, nonmotile phenotype to strains PAK and PAO1 (Fig. 2A). Though nonmotile in the motility plate assay, the *fleN* mutant displayed tumbling, spinning, and poor swimming abilities when examined under the light microscope. This defect in motility could not be solely attributed to the newly acquired multiflagellated phenotype of the *P. aeruginosa fleN* mutants, because a closely related species, *P. putida*, is characteristically known to possess a tuft of polar flagella (three or more) and simultaneously retain its motility (14). Moreover, the *fleN* mutant seemed distinct from a previously described (34) *P. aeruginosa* multiflagellate mutant which bore multiple polar flagella but possessed better swarming ability than its wild-type parent. The latter mutant was generated by chemical mutagenesis, and the mutation was mapped to a *fla* locus, but the exact gene was not identified. These observations suggest that the phenotypes of multiflagellation and nonmotility are not necessarily linked.

For a flagellated cell to chemotactically respond to an environmental stimulus, proper transmission of the message to the flagellar switch is necessary to determine the rotational direction of the flagella. In *E. coli* and *Salmonella* serovar Typhimurium, this transmission proceeds via a phosphorylation cascade involving CheA, CheW, CheY, CheZ, CheR, and CheB. Counterclockwise rotation forces the flagellar filaments into a

TABLE 3. Assessment of flagellar gene promoters for their dependence on FleQ, a transcriptional activator

Operon or gene (promoter construct)	β-Galactosidase activity (Miller units; mean ± SD)	
	PAK	PAK-Q
<i>flgBCDE</i> (placΩ <sub>flgE</sub> )	1,000 ± 120	290 ± 40
<i>fliLMNOPQ</i> (placΩ <sub>L</sub> )	2,500 ± 20	640 ± 20
<i>fliEFG</i> (placΩ <sub>E</sub> )	760 ± 10	250 ± 10
<i>fliC</i> (pPT269)	2,100 ± 200	330 ± 7
<i>fliA</i> (pMS565)	400 ± 20	360 ± 20
Promoterless <i>lacZ</i> (placΩ)	170 ± 50	190 ± 70

bundle that propels the cell smoothly forward. Reversal of flagellar rotation to clockwise causes the bundle to fly apart and the cell to tumble (19). Unusually high tumbling frequencies have been reported for strains carrying certain mutations in either CheZ or the flagellar switch proteins FliG, FliM, and FliN and in wild-type strains overproducing CheY (9). In the *P. aeruginosa* multiflagellate *fleN* mutant, a defect in motility associated with tumbling behavior could arise if the proteins involved in flagellar rotation and chemotaxis were unable to function normally due to abnormal signal transduction in the absence of FleN. Alternatively, the probable lack of an equivalent increment in the number of transducing elements involved in chemotaxis relative to that of the flagellar structural components could explain the associated lack of motility. Thus, FleN could have a dual role in maintaining the single polar flagellum and chemotactic motility in *P. aeruginosa*.

Efforts to complement the *fleN* mutant revealed that the flagellar assembly of *P. aeruginosa* was sensitive to the amounts of FleN being expressed. Two constructs, pPZ375-*fleN* and pET-*fleN*, had distinctly different effects on the phenotype of the *fleN* mutant, though they had essentially the same region of *fleN* cloned in them. Most of the pPZ375-*fleN* containing cells were nonflagellate and remained nonmotile, whereas pET-*fleN* complemented the mutant to its wild-type motility phenotype with one to two polar flagella. Such a phenomenon can be explained based on the amounts of FleN being expressed as a function of the promoter activities of these two constructs. Expression of FleN from pET-*fleN*, with a low basal activity of the T7 promoter, was probably closer to the normal physiological level, allowing assembly of the flagellum, whereas in pPZ375-*fleN*, with an unrepressed *lac* promoter, the amount of FleN in the majority of the cells was high enough to signal complete cessation of flagellar assembly. Such an effect of FleN on flagellar assembly suggested that the chromosomal *fleN* locus is tightly regulated to control the amount of FleN that is made. The ability to complement the defect in PAK-N with a copy of *fleN* alone proved that the disruption of the genomic *fleN* did not jeopardize the functioning of the downstream *fliA* and *che* genes.

Multiflagellation in the *fleN* mutant could be attributed to the upregulation of FleQ-dependent flagellar operons and genes (Tables 2 and 3), which code for homologues of both structural proteins known to be involved in the formation of the flagellar motor and switch (FliM, FliN, and FliG), the basal body (FliE and FliF), the basal body rod (FlgB and FlgC), the hook (FlgD and FlgE), the cap (FliD), and the filament (FliC), and regulatory proteins (FleS and FleR), which are all essential for flagellar assembly. The *fleQ* promoter is itself not upregulated in the *fleN* mutant, which rules out the possibility of FleQ overexpression leading to upregulation of its dependent promoters. Further, the observed promoter upregulation sug-



distribution is important for motile bacterial species. *fleN* of *P. aeruginosa* is the first gene to be partially characterized as having a dual role in controlling the number of flagellar filaments and motility in flagellated microbial species.

#### ACKNOWLEDGMENTS

We thank S. Lory for helpful discussions and acknowledge the electron microscopy and sequencing core laboratories of the Interdisciplinary Center for Biotechnology Research at the University of Florida for assistance with the EM and sequencing projects, respectively. W. Zeile's help with the video microscopy is thankfully acknowledged. B. W. Ritchings is acknowledged for technical assistance in constructing placcQL.

This work was supported by NIH grants HL33622 and AI45014 to R.R.

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