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A Transcriptional Activator, FleQ, Regulates Mucin Adhesion and Flagellar Gene Expression in *Pseudomonas aeruginosa* in a Cascade Manner

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Previous work has demonstrated that *fleR*, the gene for a transcriptional activator belonging to the NtrC subfamily of response regulators, is involved in the regulation of mucin adhesion and flagellar expression by *Pseudomonas aeruginosa*. This report describes the identification and characterization of *fleQ*, the gene for another transcriptional regulator which also regulates mucin adhesion and motility in this organism. The complete nucleotide sequence of the *fleQ* gene was determined on both DNA strands, and an open reading frame (ORF) consisting of 1,493 nucleotides was identified. This ORF coded for a gene product of predicted molecular weight, as confirmed by the overexpression of the *fleQ* gene as a fusion protein under an inducible promoter. The *fleQ* gene is flanked by a flagellar operon, *fliDSorf126*, at the 5′ end and the *fleSR* operon on the 3′ end. FleQ also had striking homology to a number of proteins belonging to the NtrC subfamily of response regulators, which work in concert with the alternate sigma factor RpoN (σ^{54}) to activate transcription. However, FleQ lacks the residues corresponding to Asp-54 and Lys-104 of the NtrC protein which are conserved in most of the members belonging to this subfamily of regulators. In addition, unlike some of the other transcriptional activators of this group, FleQ does not appear to have a cognate sensor kinase. A chromosomal insertional mutation in the *fleQ* gene abolished mucin adhesion and motility of *P. aeruginosa* PAK and PAK-NP. Both of these functions were regained by providing the complete *fleQ* gene on a multicopy plasmid. The location of *fleQ* immediately upstream of the *fleSR* operon, which is also necessary for the same process, suggested that these regulators may interact in some way. We therefore examined the regulation of the *fleSR* operon by *fleQ* and vice versa. Promoter fusion experiments showed that the *fleSR* operon was regulated by RpoN and FleQ. On the other hand, the *fleQ* promoter was independent of RpoN and FleR. FleQ, thus, adds another level of regulation to motility and adhesion in *P. aeruginosa*, above that of *fleSR*. We therefore propose the existence of a regulatory cascade which consists of at least two transcriptional regulators, FleQ and FleR, in the control of motility and adhesion in *P. aeruginosa*.

Pseudomonas aeruginosa is an opportunistic pathogen that colonizes the airways of individuals with cystic fibrosis and leads to the lung injury that is characteristic of most cases of this disease. The mechanisms by which this organism colonizes the human airways are not well understood, but clinical and laboratory studies have established that colonization involves the binding of *P. aeruginosa* to human respiratory mucus and mucins (31). However, *P. aeruginosa* has also been shown to bind to respiratory epithelial cells (20). Previous studies from our laboratories have demonstrated that the expression of mucin adhesin(s) by *P. aeruginosa* is linked to the expression of some of the genes of the flagellar regulon as detailed below (22). Mucin and cell adhesion as well as flagellar gene expression in *P. aeruginosa* are controlled by the alternative sigma factor RpoN (18, 29). Moreover, a mutation in a gene coding for part of the flagellar export apparatus, *fliO* (23), or the gene coding for the MS ring, *fliF* (2), the foundation for the flagellum, results in the concomitant loss of adhesion as well as motility. Based on these studies, our current model for explaining the relationship between adherence and motility is that (i) flagellar components are the actual bacterial adhesin(s) or that (ii) the adhesin(s) and flagellar proteins are distinct but are cotranscribed and share a common secretion/assembly machinery.

Consistent with these hypotheses, we found a pair of genes, *fleS* and *fleR*, which regulate both mucin adhesion and motility (19). Products of *fleS* and *fleR* are homologous to members of the subclass of two-component systems involved in transcriptional regulation of a number of genes from σ^{54} (RpoN) promoters. Since the promoter region of the putative *fleSR* operon contains the invariant nucleotides of the consensus RpoN-dependent promoters, we anticipated in analogy with all σ^{54}-dependent genes that additional regulatory elements control expression of motility and mucin adhesion by regulating the expression of *fleSR*. While sequencing the region upstream of *fleSR*, we have found a candidate gene for such a regulator.

In this report we describe a new gene, *fleQ*, which, based on the sequence homology data, belongs to the NtrC subfamily of transcriptional activators that work in concert with RpoN. In contrast to *fleR*, we have not found a sensor gene linked to *fleQ*. Insertional inactivation of the *fleQ* gene in *P. aeruginosa* resulted in a mutant which was nonmotile and nonadhesive, and both of these defects were complemented by providing the *fleQ* gene on a plasmid. To understand the possible role of RpoN and FleQ in regulation of the *fleSR* promoter, β-galactosidase assays were performed. The results from these assays

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TABLE 1. Bacterial strains, plasmids, and cosmid used

Strain, plasmid, or cosmid	Relevant genotype	Source or reference
E. coli DH5α	*hsdR recA lacZYA* ϕ80 *lacZΔM15*	GIBCO-BRL
P. aeruginosa		
PAK	Wild-type clinical isolate	D. Bradley
PAK-NP	PAK *pilA*::Tc^r	20
PAK-N1G	PAK *rpoN*::Gm^r	9
PAK-Q	PAK *fleQ*::Gm^r	This study
PAK-NPQ	PAK *pilA*::Tet^r *fleQ*::Gm^r	This study
PAK-RG	PAK *fleR*::Gm^r	19
ADD1976	Tc^r Cb^s mini-D180 T7 polymerase gene on the chromosome	4
Plasmids		
pUC18	*E. coli* cloning vector, Amp^r	GIBCO-BRL
pGEM3Z	Sequencing vector, Amp^r, LacZα peptide	Promega, Madison, Wis.
pBluescript KS (+)	*E. coli* cloning vector, Amp^r	Stratagene
pBSVI	pBluescript KS (+) with the *Pst*I site deleted from the polylinker	This study
pVIK	pBSVI having a 4.5-kb *Kpn*I fragment inserted at the *Kpn*I site	This study
pVIKG	pVIK with a 1.7-kb DNA fragment containing the gentamicin resistance gene inserted in the unique *Pst*I site of the *fleQ* gene	This study
pPZ375	*oriV* in pGEM3Z	27
pPZ375Q	pPZ375 with complete *fleQ* gene	This study
pDN19lacΩ	Promoterless *lacZ oriV oriT* Tet^r Str^r Ω fragment	28
placΩQ	pDN19lacΩ with a 600-bp *Eco*RI/*Bam*HI fragment containing the *fleQ* promoter region	This study
placΩS	pDN19lacΩ with 355-bp *Eco*RI/*Bam*HI fragment containing the *fleSR* promoter region	This study
pET15B	Expression vector, T7 promoter, His tag coding sequence, Amp^r, pBR322 origin	Novagen
pET15BVP	*oriV* cloned as a *Pst*I fragment into the *Pst*I site in the Amp^r gene of pET15B	This study
pET15BVPQ	*fleQ* gene inserted as a PCR product into the *Nde*I/*Bam*HI sites of pET15BVP	This study
Cosmid		
pRR194	pVK102 with 20-kb *P. aeruginosa* DNA insert containing the *fleQ* gene	19

suggested that the *fleSR* promoter was regulated by RpoN and FleQ. Thus, FleQ and FleR appear to work together in a cascade to control motility and mucin adhesion in *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. All bacterial strains, plasmid vectors, and their derivatives are described in Table 1. All cultures were grown in liquid Luria broth (12), in Terrific broth (21), or on agar plates (1.7% agar) with or without antibiotics. The antibiotic concentrations used were as follows: for *Escherichia coli*, ampicillin at 200 μg/ml and gentamicin at 10 μg/ml; for *P. aeruginosa*, tetracycline at 200 μg/ml, carbenicillin at 150 μg/ml, gentamicin at 50 μg/ml, and streptomycin at 300 μg/ml.

Enzymes and chemicals. All restriction enzymes, T4 DNA ligase, and *Taq* polymerase were purchased from GIBCO-BRL Inc., Gaithersburg, Md. *Pfu* DNA polymerase was purchased from Stratagene, La Jolla, Calif. The Isotherm sequencing kit was purchased from Epicentre Technologies Inc., Madison, Wis. The chemicals were purchased either from Sigma Chemical Co., St. Louis, Mo., or from Amresco, Inc., Solon, Ohio.

Electroporations. Electroporations were performed by using a modification of the protocol of Smith and Iglewski (24). The DNA used for the electroporations was prepared by the alkaline lysis procedure (3). For gene replacement experiments involving chromosomal recombinations, the plasmid DNA was linearized by a restriction enzyme and gel purified. About 1 μg of linear DNA fragment was electroporated into the electrocompetent *P. aeruginosa* cells. For complementation experiments, 50 to 100 ng of supercoiled, covalently closed circular plasmid DNA was electroporated into the target strains.

PCR amplification. PCRs were performed in a DNA Thermal Cycler 480 (Perkin-Elmer Cetus). The reactions were performed in 100-μl volumes containing *Pfu* polymerase or *Taq* polymerase. Each reaction mixture contained a final concentration of 50 ng of DNA template, 2.5 U of *Pfu* polymerase or *Taq* polymerase, 2.0 mM $MgCl_2$, 0.1 mM deoxynucleoside triphosphate mix, 10% dimethyl sulfoxide, and 0.2 μM primers. Thirty cycles were performed for each reaction when *Taq* polymerase was used. Each cycle consisted of incubations for 1 min at 94°C, 1 min at 52°C, and 3 min at 72°C. When *Pfu* polymerase was used, 40 cycles, each consisting of incubations for 2 min at 95°C, 1 min at 46°C, and 6 min at 72°C, were run. The annealing temperature was kept low due to the low ionic strength of the *Pfu* reaction buffer, and the extension time was increased to 6 min to accommodate the low proofreading capacity of the *Pfu* polymerase.

The primers used for PCRs were purchased from GIBCO-BRL. Restriction sites were added at the ends of primers (shown below in boldface) to facilitate subsequent cloning of the PCR products. Additional nucleotides were added 5′ to the restriction sites to ensure efficient cleavage. The following primers were used in the PCRs. RER30 and RER31 were used for the PCR amplification of the *fleQ* promoter. RER30 (5′CCCAAA**GAATTC**CCGGTTGGGATGCGATT G3′) was located 328 nucleotides upstream of nucleotide 1; an *Eco*RI site was added to this primer. RER31 (5′CCCAAA**GGATCC**CGCCGAGGAAGTTGA GAA3′) was located between nucleotides 349 and 366; a *Bam*HI site was added to this primer. RER17 and RER18 (Fig. 1) were used for the PCR amplification of the *fleSR* promoter. To RER17 (5′CCCAAA**GGATCC**CGTTGAGGGCTG GTTGC3′), a *Bam*HI site was added; to RER18 (5′CCC**GAATTC**GGCTGCCG GGAATGGAC3′), an *Eco*RI site was added. 15CNTG615 (5′TCCGCCAGCT CCTCCAT3′) [Fig. 1]) was used in the primer extension experiments. NDE-FLEQ (5′AGGCAGCTGATC**CATATG**TGGCGCGAAACC3′) was used as a 5′ primer to clone the complete *fleQ* gene into pET15BVP; an *Nde*I site was added to this primer. Q3PBAM (5′CCCAAA**GGATCC**TCAATCATCCGACAG GTC3′) was used as the 3′ primer to clone the complete *fleQ* gene into the vector pET15BVP; a *Bam*HI site was added to this primer.

Plasmid constructions. A plasmid vector called pBSVI was first constructed by digesting the vector pBluescript KS (+) (Stratagene) with two blunt-end-cutting enzymes, *Eco*RV and *Sma*I, in the polylinker and religating the vector. Thus, the small DNA fragment between the *Eco*RV and the *Sma*I sites was removed and the *Pst*I site was lost. A 4.5-kb *Kpn*I fragment containing the complete *fleQ* gene was cloned into the *Kpn*I site of the polylinker in pBSVI, resulting in the construction of pVIK, which contained a unique *Pst*I site. A 1.7-kb gentamicin resistance cassette with *Pst*I ends was then inserted into this unique *Pst*I site located in the *fleQ* gene (Fig. 1). This plasmid, called pVIKG, was used for insertional inactivation of the *fleQ* gene. To construct a plasmid which could be used for complementation of the *fleQ* mutant, a 2.0-kb *Cla*I-*Spe*I (Fig. 2) fragment containing the complete *fleQ* gene was inserted into the *Cla*I/*Spe*I-cut vector pBluescript KS (+). This fragment was then excised as a 2.0-kb *Hin*dIII/*Sst*I fragment and cloned into the *Hin*dIII/*Sst*I sites of pPZ375 (27), which has the *oriV* fragment that allows this plasmid to replicate in *P. aeruginosa.*

The *lacZ* fusion plasmid placΩQ was constructed by inserting a 600-bp DNA fragment containing the region upstream of the *fleQ* gene into the promoter probe vector pDN19lacΩ (28). This 600-bp DNA fragment was generated by

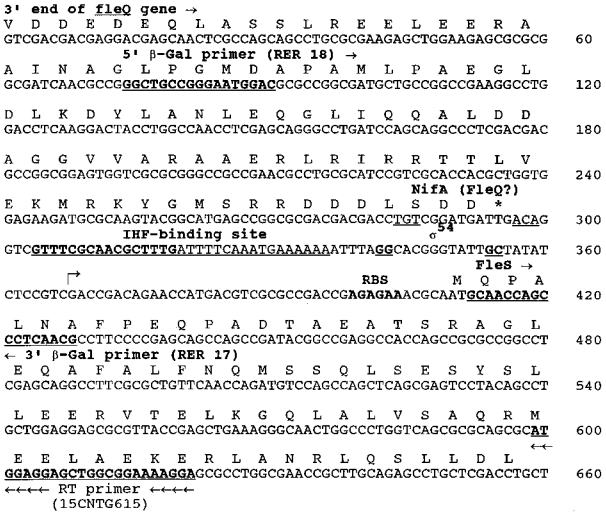


FIG. 1. Promoter region of the *fleSR* operon. The promoter region of *fleSR* used for β-galactosidase (β-Gal) assays included the 3′ end of the *fleQ* gene and extended into the coding region of *fleS*. The primers used for both primer extension and β-galactosidase experiments are shown. In the upstream sequence, the putative RpoN binding site (GG-N_{10}-GC), NifA binding site (TGT-N_{10}-ACA), and the IHF binding site (nucleotide 304 to 336) are indicated. The transcription start site as determined by primer extension is shown at bp 367. RBS, ribosome binding site.

PCR using primers RER30 and RER31, with *Eco*RI and *Bam*HI sites added at the 5′ and the 3′ ends, respectively. This *Eco*RI/*Bam*HI fragment was then cloned into the *Eco*RI and *Bam*HI sites of pDN19lacΩ to generate placΩQ. Plasmids constructs for β-galactosidase assays testing the promoter activity of the *fleSR* operon were made by cloning the putative *fleSR* promoter region into the *Eco*RI/*Bam*HI sites upstream of the promoterless *lacZ* gene in the previously described lacΩ plasmid (28). The promoter region of *fleSR* was cloned with primers RER18 and RER17 (Fig. 1).

Plasmid pET15BVP was constructed by inserting the *oriV* fragment from pPZ375 (27) with *Pst*I ends into the unique *Pst*I site in the β-lactamase gene of pET15B (Novagen Inc., Madison, Wis.). Plasmid pET15BVP can replicate in both *P. aeruginosa* and *E. coli* hosts and retains ampicillin and carbenicillin resistance (15). Plasmid pET15BVPQ was derived from pET15BVP by cloning an approximately 2.0-kb PCR product containing the complete *fleQ* gene into *Nde*I/*Bam*HI sites.

DNA sequencing. DNA sequencing was performed by using Taq DyeDeoxy terminator and dye primer cycle sequencing protocols developed by Applied Biosystems (Perkin-Elmer Corp., Foster City, Calif.). Fluorescence-labeled dideoxynucleotides and primers were used. The labeled extension products were analyzed on an Applied Biosystems model 373A DNA Sequencer. Double-stranded sequences were aligned and assembled by using programs in the Sequencer software package (Gene Codes Corp., Ann Arbor, Mich.).

β-Galactosidase assay. Expression of the *lacZ* gene under the control of the putative *fleQ* promoter region or the *fleSR* promoter was measured by β-galactosidase assays as described by Miller (12), with minor modifications. The cells were grown to late log phase (A_{600} of 0.7 to 1.0), which usually took about 4 to 4.5 h. At this point, the cells were harvested and assayed for β-galactosidase activity. The bacteria containing the lacΩ plasmids were grown in L broth with streptomycin.

Motility assay. Bacterial strains were grown overnight at 37°C on fresh agar plates with or without antibiotics. The cells were then transferred with a sterile toothpick to 0.3% agar plates with or without antibiotics. These plates were incubated at 37°C for 16 h, and motility was assessed qualitatively by examining the circular swarm formed by the growing bacterial cells.

Adhesion assay. Human tracheobronchial mucins were prepared from sputum of a patient with chronic bronchitis by ultracentrifugation as described previously (33). The bacterial strains were grown in Trypticase soy broth (BBL Microbiology Systems) overnight at 37°C, and the inoculum was adjusted by spectrophotometer to between 10^7 and 2×10^7 CFU/ml. Strains containing plasmids which coded for antibiotic resistance were grown in broth containing carbenicillin (150 μg/ml). Microtiter plates were coated with mucins at a concentration of 50 μg/ml (31). Bacteria were added to the wells, and the plates were incubated at 37°C. The plates were washed 15 times in a manually operated microtiter plate washer, and the bacteria bound to the wells were desorbed with Triton X-100 and plated for enumeration. Each strain was tested a minimum of three times. The results are mean values derived from these experiments.

SDS-polyacrylamide gel electrophoresis and immunoblotting. Whole bacterial cells were denatured by boiling in 2% sodium dodecyl sulfate (SDS)–1% β-mercaptoethanol–50 mM Tris HCl (pH 7.5). These samples were separated on 15% polyacrylamide gels (11), and the proteins were electrophoretically transferred to nitrocellulose (30). The filters were treated with 2% nonfat dry milk in Tris-buffered saline, incubated with antisera, washed, and probed with horseradish peroxidase-labeled anti-mouse immunoglobulins G and M (Kirkegaard & Perry, Gaithersburg, Md.). Monoclonal antiflagellin was kindly provided by A. Siadak, Oncogen, Seattle, Wash.

Transcriptional start site determination. To establish the transcriptional start site of the *fleSR* operon, primer 15CNTG615 (Fig. 1) complementary to the noncoding strand near the 5′ end of the *fleS* gene was end labeled with [γ-^{32}P]ATP by using polynucleotide kinase as instructed by the manufacturer (Bethesda Research Laboratories). The labeled primer was gel purified on a 20% nondenaturing polyacrylamide gel. After purification from the gel by extraction in elution buffer (21) and ethanol precipitation, the resulting labeled primer gave 3×10^6 cpm/ml by scintillation counting. This primer was then used for both sequencing and reverse transcription (RT) reactions. Sequencing was done with an Isotherm sequencing kit (Epicentre Technologies) and was chosen because it gave superior performance in A/T-rich regions, one such region being present in our sequence adjacent (3′) to the putative integration host factor (IHF) binding site (Fig. 1). The DNA template for the sequencing reaction was a 4.5-kb *Kpn*I fragment spanning the start region which had been cloned into pUC19. The total RNA template for the primer extension reaction was prepared by a modification of the method of Deretic et al. (6). The only change to the protocol was the addition of 50 mM EDTA to the lysis buffer. The absence of DNA contamination in the RNA preparation was confirmed by comparing the results of PCR and RT-PCR, using the RT primer and an upstream primer. Only the latter reaction gave a product (data not shown). Finally, the products of the sequencing and RT reactions were run on a standard 8% sequencing gel and visualized by autoradiography.

Expression and purification of FleQ. The complete *fleQ* coding sequence was inserted as a PCR product into the *Nde*I/*Bam*HI sites of plasmid pET15BVP. The resulting plasmid, called pET15BVPQ, and the vector control plasmid pET15BVP were electroporated into *P. aeruginosa* ADD1976 (4), which has the T7 polymerase gene inserted into the chromosome. Bacterial cultures were grown to an A_{550} of 0.4 to 0.5, and the T7 promoter was induced by the addition of 1.0 mM isopropylthiogalactopyranoside (final concentration) (IPTG). The cultures were grown for an additional 3 h and then harvested. The induced cultures of *P. aeruginosa* ADD1976 containing pET15BVPQ were lysed by the addition of lysozyme and Triton X-100 and then spun for 25 min at 13,200 rpm. The pellets containing the insoluble His-FleQ as inclusion bodies were saved for purification of His-FleQ. These pellets were resuspended in 1× binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl [pH 7.9]) with 6 M urea. A small

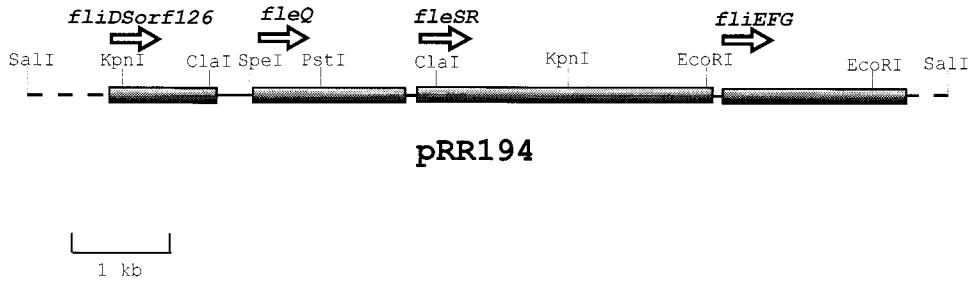


FIG. 2. Map of the relevant region of cosmid pRR194 showing the location of the *fleQ* gene. The map is drawn approximately to scale. The solid rectangles show the coding regions. The arrows beside the solid rectangles indicate the direction of transcription.

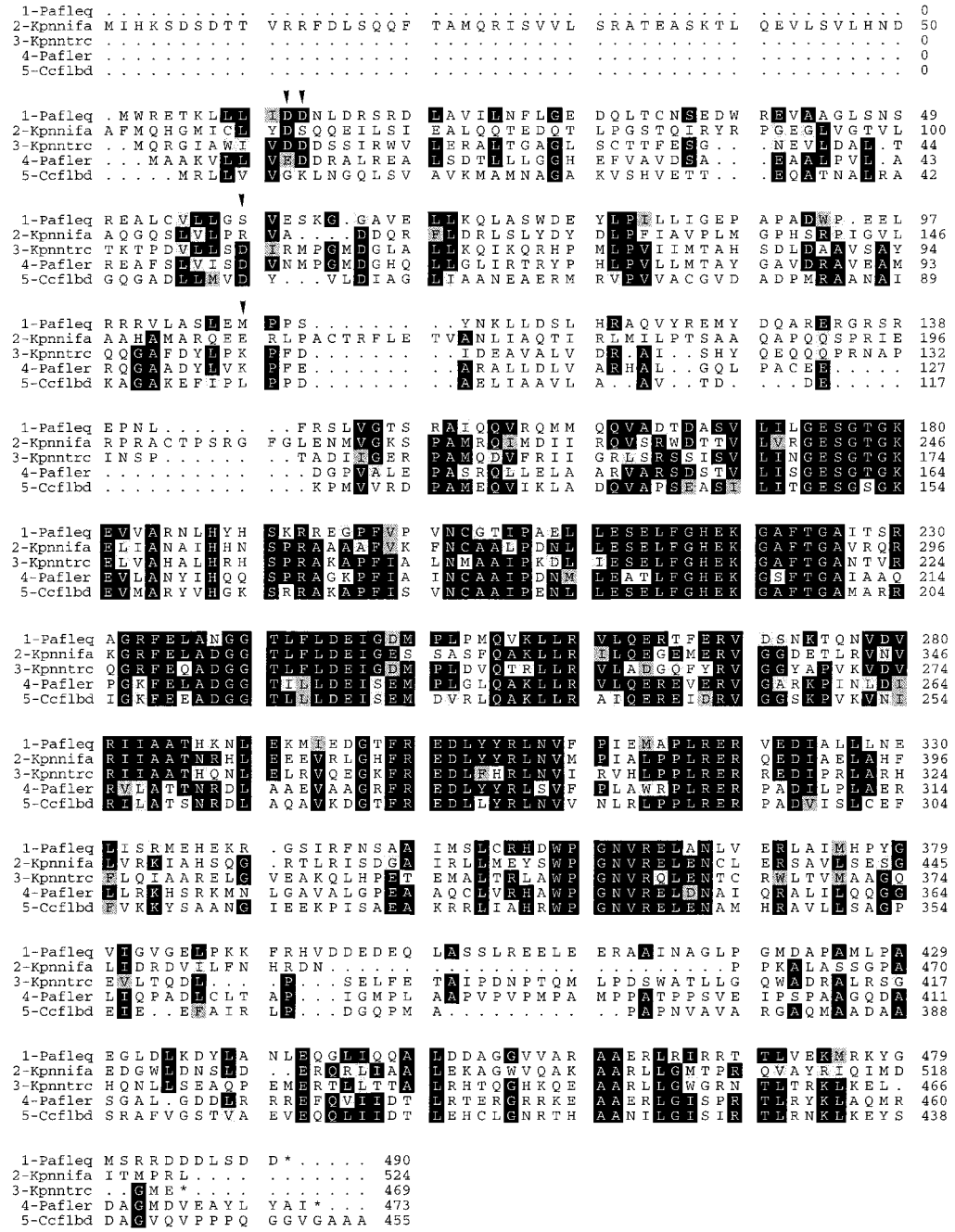


FIG. 3. Computer-generated alignment (Prettybox program, developed by Richard Westerman, Purdue University) of FleQ of *P. aeruginosa* with homologous transcriptional regulators of other organisms. Dark shading shows identity of amino acids, while two shades of gray show degrees of similarity (based on the GCG comparison table). The arrowheads indicate the conserved amino acids which are believed to constitute the acid pocket and are involved in the phosphorylation of these proteins.

disposable column containing 2.5 ml of Chelating Sepharose Fast Flow resin (Pharmacia Biotech Inc., Piscataway, N.J.) was packed. The column was charged with 50 mM $NiSO_4$ according to the pET instruction manual provided by Novagen. Further steps in the purification of His-FleQ were performed according to the pET instruction manual. All buffers contained 6 M urea in order to keep the His-FleQ protein solubilized. The His-FleQ protein was finally eluted with 1× elution buffer (1 M imidazole, 0.4 M NaCl, 20 mM Tris-HCl [pH 7.9]) containing 6 M urea. The protein was dialyzed against 1-liter volumes of buffer containing 50 mM Tris (pH 7.4), 50 mM KCl, and 6 mM $MgCl_2 \cdot 6H_2O$ with stepwise decreases in urea concentration (4 M→2 M→1 M→0.5 M→no urea).

Nucleotide sequence accession number. The 1,816-nucleotide sequence containing the complete sequence of the *fleQ* gene was submitted to GenBank (accession no. L49378).

RESULTS

DNA sequence analysis. By sequencing the region upstream of the *fleSR* operon, we have identified a new gene, which we have named *fleQ* because of its effect on flagellar expression. The location of the *fleQ* gene on cosmid pRR194 relative to *fleSR* and other flagellar genes is shown in Fig. 2. The nucleotide sequence of a 1.8-kb segment of *P. aeruginosa* DNA containing the complete *fleQ* gene was determined on both DNA strands. An open reading frame (ORF) consisting of 1,473 nucleotides which is predicted to code for a polypeptide contain-

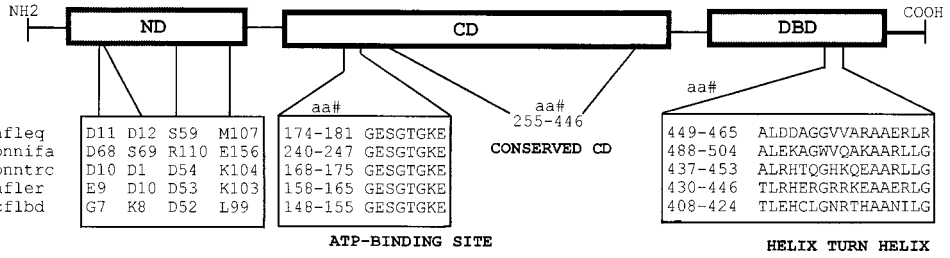


FIG. 4. Schematic diagram showing the comparison of the structural and functional domains (determined or proposed) of the transcriptional regulators FleQ of *P. aeruginosa* (Pafleq), NifA and NtrC of *K. pneumoniae* (Kpnnifa and Kpnntrc), FleR of *P. aeruginosa* (Pafler), and FlbD of *C. crescentus* (Ccflbd). Shown are the amino acids conserved among the family of transcriptional regulators which work in association with RpoN. The amino acid sequences were aligned by using the GCG multiple sequence analysis program PILEUP. Common to all proteins are the N-terminal domain of low sequence similarity (ND), the highly conserved central domain (CD), and the C-terminal DNA binding domain (DBD). The conserved amino acid residues and the amino acid sequences of characteristic motifs are shown in boxes.

ing 491 amino acids (M_r, 54,000) was identified on this DNA. A potential translational initiation codon is located at nucleotide 282 and is preceded by a potential ribosome binding site. The ORF terminates with a TGA codon at nucleotide 1752. The *fleQ* stop codon is separated from the *fleS* ORF by 112 bp. Codon usage in the *fleQ* gene was characteristic of *P. aeruginosa* genes, as judged by the CODONPREFERENCE and CODONFREQUENCY programs of the Genetics Computer Group (GCG). The codon frequency table for *P. aeruginosa* was created by Temple (26a).

The deduced amino acid sequence of the *P. aeruginosa fleQ* gene was compared to known protein sequences in the GenBank, PI, and SWISS-PROT databases, using the BLAST program (1). These searches revealed significant homology of the *P. aeruginosa* FleQ protein to a number of response regulators which belong to the NtrC subfamily of regulators that work in concert with σ^{54}. The computer-generated alignment of the FleQ protein of *P. aeruginosa* with NifA and NtrC of *Klebsiella pneumoniae*, FleR of *P. aeruginosa*, and FlbD of *Caulobacter crescentus* is shown in Fig. 3. As is noted with regulators belonging to the NtrC subfamily, FleQ had poor homology in the N-terminal region. A domain structure of FleQ is shown in Fig. 4, and the conserved amino acids are shown underneath. Interestingly, of the four residues conserved in the N-terminal domains of other members of this family (arrowheads in Fig. 3), FleQ contained the residues corresponding to Asp-10 and Asp-11 of NtrC but lacked the residues corresponding to Asp-54 and Lys-104 (Fig. 4). In the response regulators belonging to this subfamily, Asp-11, Asp-12, and Asp-54 constitute the acid pocket which accommodates Mg^{2+}, while Asp-54 also acts as the phospho-accepting aspartate from the cognate sensory kinase (26). It has been postulated that the conserved Asp-57 in CheY, which corresponds to Asp-54 in NtrC, forms a salt bridge with Lys-109 (8) (Lys-104 in NtrC) and that phosphorylation of Asp-57 breaks this interaction and induces the subsequent conformational change leading to regulator activation (25, 32). The absence of the corresponding aspartic acid and lysine residues in FleQ suggests that FleQ is probably not phosphorylated by a cognate kinase, which is in agreement with our observation that no gene encoding a homolog of the sensor kinases of the two-component regulatory family can be identified upstream of the *fleQ* gene. However, in place of the aspartic acid, there was a serine residue which is a potential site of phosphorylation.

Central domains c1 to c7, involved in ATP binding and activation of σ^{54} (13), were conserved in FleQ (Fig. 3 and 4). The carboxy terminus of FleQ contained a sequence similar to the helix-turn-helix present in many DNA binding proteins (16). FleQ contained all of the conserved amino acids in this region except for residue 465, which is an arginine in FleQ but a conserved glycine residue in the other members of this group (Fig. 4).

Analysis of the hydropathic characteristics of the FleQ protein by the method of Kyte and Doolittle (10) suggested that FleQ is relatively hydrophilic, lacking any long hydrophobic stretches characteristic of transmembrane segments (data not shown), and therefore is probably a soluble cytoplasmic protein.

Construction and complementation of a *fleQ* mutant. To determine the possible function of FleQ, a chromosomal *fleQ* mutant was constructed in *P. aeruginosa* PAK-NP by gene replacement. The *P. aeruginosa fleQ* gene located on a 4.5-kb *Kpn*I fragment was inactivated by inserting a gentamicin resistance gene cassette into a unique *Pst*I site in the *fleQ* gene (Fig. 2). The insertionally inactivated *fleQ* gene on the plasmid was electroporated into PAK-NP, where it replaced the corresponding chromosomal copy of the *fleQ* gene by double-reciprocal recombination, giving rise to a *fleQ* mutant strain, PAK-NPQ. The insertional inactivation of *fleQ* in PAK-NPQ was confirmed by Southern blot analysis (data not shown). Another *fleQ* mutant, PAK-Q, was constructed in *P. aeruginosa* PAK by using the same strategy (data not shown), to test whether the same phenotype would be obtained. Since this mutant is sensitive to tetracycline, it was used in the promoter fusion experiments which required the use of a plasmid carrying tetracycline resistance.

Since the *fleQ* gene was located close to the pair of genes in the *fleSR* operon, which have been shown to be involved in motility and mucin adhesion in *P. aeruginosa* (19), we tested this mutant in motility and mucin adhesion assays. These results showed that the *fleQ* mutant, PAK-NPQ, was nonmotile (data not shown) and nonadhesive (Fig. 5). Western blots of crude extracts of this strain, using a monoclonal antiflagellin antibody (29), indicated that it lacked flagellin (Fig. 6). To confirm that the nonmotile and nonadhesive phenotype of PAK-NPQ was indeed due to inactivation of the *fleQ* gene, this gene was cloned as a 2.0-kb *Spe*I-*Cla*I fragment (Fig. 2) on a multicopy plasmid (pPZ375Q), which was then introduced into PAK-NPQ. Motility (data not shown), flagellin synthesis, and mucin adhesion functions were restored in PAK-NPQ by the *fleQ* gene provided on a plasmid, while the vector alone did not complement the *fleQ* mutation (Fig. 5 and 6). The *fleQ* mutant of strain PAK exhibited the same phenotype.

Analysis of the *fleSR* and *fleQ* promoters. Since *fleQ* and *fleSR* genes are adjacent to each other (Fig. 2) and *fleR* and *fleQ* mutants had the same phenotype, it was possible that *fleQSR* formed one operon. To resolve this issue, the transcriptional start site of the *fleSR* operon was localized by primer

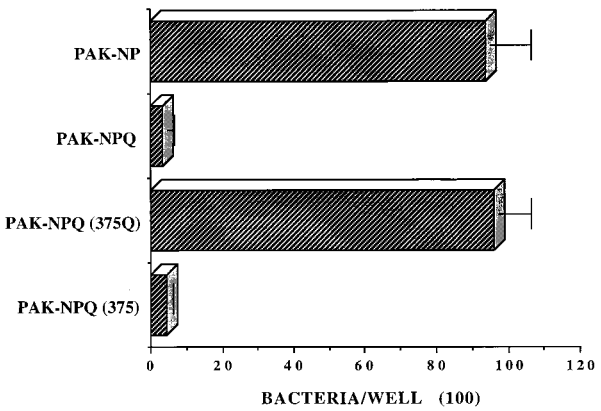


FIG. 5. Adhesion of *pilA* and *fleQ* mutants of *P. aeruginosa* PAK to mucin. PAK-NP, *pilA* mutant of PAK; PAK-NPQ, *pilA fleQ* mutant of PAK; PAK-NPQ (375Q), PAK-NPQ complemented with the complete *fleQ* gene on multicopy plasmid vector pPZ375; PAK-NPQ (375), PAK-NPQ with the vector pPZ375.

extension. Figure 7 shows the sequencing reaction and the primer extension (RT) reactions using primer 15CNTG615 (Fig. 1). A single band in the RT lane corresponds to a G in the sequence. Since the RT reaction extends the strand complementary to the RNA, the *fleSR* transcriptional start site is located at the C complementary to the G in the sequence (bp 367 in Fig. 1). Exactly 12 bp upstream of this start site, a σ^{54} binding sequence (GG-N_{10}-GC) was identified (Fig. 1). In addition, a palindromic sequence (shown in boldface in Fig. 1) which overlaps with a putative IHF DNA binding site (19) was recognized. Finally, two potential recognition sites for the nitrogen fixation transcriptional regulator NifA (TGT-N_{10}-ACA) (5) were located upstream of the *fleSR* start site. One of the NifA binding sites was an exact match with the consensus NifA binding site (Fig. 1), while the second putative NifA binding site (TGT-N_{10}-CCA) had one mismatch with the consensus NifA binding site and was located about 440 bp upstream of the first NifA binding site, within the *fleQ* gene.

To understand the effect of RpoN and FleQ on the *fleSR* promoter region, the *fleSR* promoter was fused with the promoterless *lacZ* gene and the activity of the *fleSR* promoter was measured in a number of *P. aeruginosa* strains. Table 2 shows the results of these β-galactosidase assays. Both *rpoN* (PAK-N1G) and *fleQ* (PAK-Q) mutants had greatly reduced activity of the *fleSR* promoter, showing that transcription from the *fleSR* promoter requires σ^{54} (product of the *rpoN* gene) and the transcriptional activator FleQ. In addition, the *fleSR* promoter retained wild-type levels of β-galactosidase activities in the *fleR* mutant strain (PAK-RG), thus suggesting that *fleSR* is not autoregulated.

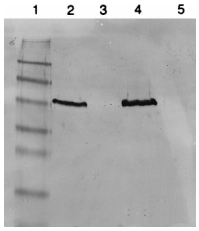
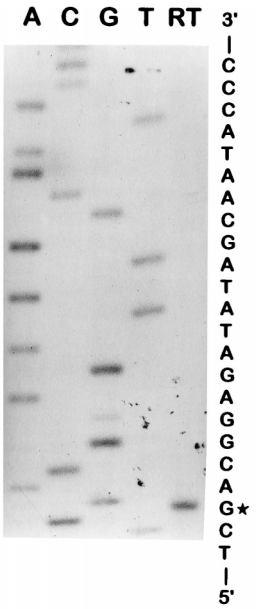


FIG. 6. Western immunoblots of PAK-NP and PAK-NPQ probed with a monoclonal antibody raised against purified flagellin. Lane 1, protein molecular weight markers (64, 50, 36, 30, 16, and 6 kDa); lane 2, PAK-NP (wild-type strain); lane 3, PAK-NPQ (*fleQ* mutant); lane 4, complemented strain PAK-NPQ (375Q), *fleQ*$^+$; lane 5, vector control, PAK-NPQ (375), *fleQ* mutant. Bands in lanes 2 and 4 run slightly above the 36-kDa marker, corresponding to the expected size (45 kDa) of the *P. aeruginosa* flagellin protein.



Portion of *fleSR* promoter (underlined part shown in sequence above):

5'- CAGGGTATTGCTATATCTCCGTCGACCGACAG -3'
3'- GTCCCATAACGATATAGAGGCAGCTGGCTGTC -5'

FIG. 7. Start site determination of *fleSR* by primer extension. The autoradiograph shows a sequencing gel with RT product run alongside. Both reactions used the same primer, 15CNTG615 (Fig. 1). A single band in the RT lane (marked with star) corresponds to the G in the sequencing reaction. A portion of the *fleSR* promoter is shown at the bottom; the arrow marks the transcriptional start site.

The promoter region of the *fleQ* gene was visually examined for the presence of specific motifs for NtrC, NifA, σ^{70}, σ^{28}, or σ^{54} binding. We were unable to identify either the NifA binding site (TGT-N_{10}-ACA) (7) or NtrC binding site (TGCACY-N_5-GGTGCA) (7) in this region. Neither σ^{70} nor σ^{28} recognition sites could be identified in this upstream region. However, two potential σ^{54} binding sites (GG-N_{10}-GC) (14) were present between nucleotides 103 and 117 and between nucleotides 189 and 205. To determine the role of σ^{54} in the regulation of the *fleQ* gene, we fused a 600-bp sequence upstream of the *fleQ* gene to a promoterless *lacZ* reporter gene, and the levels of β-galactosidase activities were compared by introducing this construct into PAK (wild-type strain) and PAK-N1G (*rpoN* mutant). As shown in Table 3, the *rpoN* mutant strain PAK-

TABLE 2. Control of the *fleSR* promoter

Host strain	Genetic background	Mean β-galactosidase activity (Miller units) ± SD	
		Vector alone	*fleSR* promoter
PAK	Wild type	75 ± 70	14,453 ± 551
PAK-N1G	*rpoN* mutant	135 ± 12	1,106 ± 136
PAK-Q	*fleQ* mutant	115 ± 2	438 ± 8
PAK-RG	*fleR* mutant	99 ± 171	21,023 ± 206

TABLE 3. Control of the *fleQ* promoter

Host strain	Genetic background	Mean β-galactosidase activity (Miller units) ± SD	
		Vector alone	*fleQ* promoter
PAK	Wild type	75 ± 70	3,062 ± 298
PAK-RG	*fleR* mutant	99 ± 171	2,493 ± 358
PAK-N1G	*rpoN* mutant	62 ± 54	4,320 ± 194
PAK-Q	*fleQ* mutant	123 ± 116	1,904 ± 325

N1G did not exhibit a reduction in β-galactosidase activity compared with the wild type, suggesting that RpoN was probably not necessary for transcription of the *fleQ* gene. Additionally, a kinetic analysis of β-galactosidase expression was performed on strains PAK and PAK-N1G at time points ranging from 2 to 18 h of growth after inoculation (data not shown). No significant difference in β-galactosidase activity was observed between the two strains at different phases of growth.

The activity of the *fleQ* promoter was also tested in the *fleR* mutant (PAK-RG) and *fleQ* mutant (PAK-Q) strains to test whether FleR or FleQ was involved in regulation of the *fleQ* promoter. As shown in Table 3, the activity of the *fleQ* promoter was unaffected by either the *fleR* or the *fleQ* mutation. These results suggest that *fleQ* transcription is independent of FleQ and FleR.

Overexpression and purification of the FleQ fusion protein. The *fleQ* gene was overexpressed under the control of an inducible T7 promoter on a plasmid in *P. aeruginosa*. The complete *fleQ* coding sequence was inserted as a PCR product into the *Nde*I/*Bam*HI sites of plasmid pET15BVP. This insertion created a fusion of six histidine residues in frame with the FleQ ORF. The resulting plasmid pET15BVPQ and the vector control plasmid pET15BVP were electroporated into *P. aeruginosa* ADD1976, which has the T7 polymerase gene inserted into the chromosome (4). Bacterial cultures were grown and induced as explained in Materials and Methods. The induced and uninduced whole-cell extracts of *P. aeruginosa* ADD1976 containing pET15BVP (vector) or pET15BVPQ (vector plus FleQ) were analyzed on SDS–10% polyacrylamide gels (Fig. 8). A new band representing the FleQ fusion protein (His-FleQ) was observed at the expected location (Fig. 8, lane 3). To test whether the His-FleQ protein was functional in vivo, we introduced the FleQ expression plasmid (pET15BVPQ) into the *fleQ* null strains PAK-Q and PAK-NPQ. The motility in these *fleQ* mutants was restored, suggesting that the His-FleQ protein was functionally active (data not shown).

The His-FleQ protein was purified from the cell lysates of *P. aeruginosa* ADD1976 carrying pET15BVPQ as described in Materials and Methods. Since the overexpressed His-FleQ protein was localized in the insoluble fraction, 6 M urea was used to solubilize it. The purified His-FleQ was renatured by the stepwise removal of urea. A small aliquot of the purified His-FleQ was analyzed on an SDS–10% polyacrylamide gel (Fig. 8, lane 5). We observed a single band which migrated at the same location as the induced *fleQ* gene product in the whole-cell extracts of *P. aeruginosa* ADD1976 carrying pET15BVPQ. This protein preparation was found to be 90% pure, as determined by a laser scan (not shown) using a Zeineh SOFT LASER scanning densitometer (Biomed Instruments Inc., Fullerton, Calif.).

DISCUSSION

This report describes the cloning, sequencing, and characterization of *fleQ*, the gene for a transcriptional regulator in *P. aeruginosa*. Analysis of the deduced amino acid sequence of the *fleQ* gene revealed that FleQ belongs to a subclass of transcriptional regulators which have been shown to control the expression of genes transcribed by RNA polymerase containing the alternative sigma factor RpoN. However, unlike some of the other transcriptional activators of this subclass, FleQ does not appear to have a cognate sensor kinase. Insertional inactivation of the *fleQ* gene resulted in the concomitant loss of motility, mucin adhesion, and the ability to synthesize flagellin. It was possible that this insertional mutation caused a polar effect leading to the inactivation of the downstream operon *fleSR*. However, complementation of the mutant strain with a plasmid clone encoding FleQ restored all of these functions and thus confirmed that the mutant phenotype was due to the disruption of the *fleQ* gene. The significance of two sequences in the promoter region of the *fleQ* gene, resembling RpoN-dependent promoters, was addressed by fusion of this region to a promoterless *lacZ* gene and examining promoter activity in various mutant backgrounds. Results of β-galactosidase expression studies indicated that the transcription of *fleQ* is independent of RpoN and is not subject to regulation by FleR or FleQ. The structure of this new gene and the possible role of this gene in the regulation of motility and mucin adhesion in *P. aeruginosa* are discussed.

The translated product of the *P. aeruginosa fleQ* gene has many similarities with the transcriptional regulator FlbD of *C. crescentus*, which also appears to lack a sensor kinase (17). It is also notable that FlbD lacks the residues corresponding to Asp-10 and Asp-11 of NtrC, which are parts of the acid pocket that is the site of phosphorylation by the sensor kinases which phosphorylate homologous regulators (17). FleQ may be even more aberrant than FlbD in that it lacks the Asp-54 residue that is the phosphate acceptor site of transcriptional regulators of this group and carries a serine residue instead. It is therefore possible that FleQ is either not phosphorylated or phosphorylated at the serine residue. This raises several possibilities: (i) this protein acts constitutively, i.e., without the need for phosphorylation; (ii) its activity is regulated by phosphorylation at the serine residue; and (iii) its activity is controlled by novel signal-transducing mechanisms. The other similarity between FleQ and FlbD is that FlbD is a regulator of the flagellar genes of *C. crescentus*, a function which FleQ very likely performs in *P. aeruginosa.*

The discovery of the regulatory network controlling mucin adhesion and motility in *P. aeruginosa* was based on our initial observation that both functions were regulated by RpoN (18). We subsequently discovered two additional regulatory genes, *fleS* and *fleR*, which regulate adhesion and motility. Since FleS

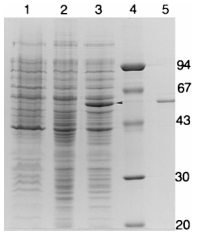


FIG. 8. Overexpression and purification of FleQ. FleQ was overexpressed in *P. aeruginosa* host ADD1976 (4) by using a derivative of the pET15B vector (Novagen). Lane 1, ADD1976(pET15BVP), vector control, induced with 1 mM IPTG for 3 h at 37°C; lane 2, ADD1976(pET15BVPQ), vector with FleQ insert, uninduced; lane 3, ADD1976(pET15BVPQ), vector with FleQ insert, induced with 1 mM IPTG for 3 h at 37°C; lane 4, Pharmacia low-molecular-weight markers; lane 5, approximately 400 ng of purified His-FleQ protein. Sizes are indicated in kilodaltons.

and FleR were highly homologous to members of the subclass of two-component systems which work in concert with RpoN, we proposed that FleR was the response regulator working with RpoN to control adhesion and motility (19). We suggested that FleR regulates some of the *P. aeruginosa* flagellar genes and the mucin adhesin in concert with RpoN. However, with the discovery of *fleQ* and the preliminary knowledge of its functions, it appears that there are other possible models for regulation of mucin adhesion and motility. Since RpoN recognition sequences were identified in the promoter region of the putative *fleSR* operon (19), it was anticipated that FleQ might regulate the putative *fleSR* operon. Our results from β-galactosidase assays suggest that this is the case.

The presence of two σ^{54}-driven transcriptional activators, FleQ and FleR, acting in a series, creates a cascade of transcriptional control over the expression of both the flagellar assembly pathway and the genes which control mucin adhesion of *P. aeruginosa*. It is possible that the purpose of this cascade is to allow rapid up- and down-regulation of the structural genes controlling these functions in response to environmental signals yet unidentified. One possible scenario in *P. aeruginosa* could be that FleQ regulates *fleSR* and the adhesin(s) and that FleSR regulates flagellar operons which may be responsible for the export and localization of the adhesin. It is already known that the export system for flagellar proteins is required for adhesion (23), but the mechanism of regulation of the flagellar export apparatus of *P. aeruginosa* has not been elucidated. It may be possible to identify the adhesin more directly by characterization of additional genes that are regulated by FleQ and FleR, perhaps by identifying DNA targets for FleQ and FleR binding. In summary, we have discovered a new transcriptional regulator of adhesion and motility in *P. aeruginosa* which has an interesting structure. It lacks the Asp-54 phosphorylation site found in homologous regulators and is not found in an operon with a potential sensor kinase. To fully understand its role in regulation of bacterial virulence factors, it is essential to determine the range of genes that are regulated by the motility/adhesion regulatory cascade, as well as identify the signals that initiate the signal transduction sequence.

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