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

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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 flagellar, 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 σ54 (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 σ54-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...
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 restriction enzymes 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 *E. coli* strains, ampicillin at 200 μg/mL; for *P. aeruginosa* strains, ampicillin at 150 μg/mL, carbenicillin at 1 g/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 Promega, Madison, Wis.

**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, 50 to 100 ng of supercoiled, covalently closed circular plasmid DNA was used for electroporations 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, 50 to 100 ng of supercoiled, covalently closed circular plasmid DNA was used for electroporations 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, 50 to 100 ng of supercoiled, covalently closed circular plasmid DNA was used for electroporations by using a modification of the protocol of Smith and Iglewski (24). 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4.5 h. At this point, the cells were harvested and assayed for 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 10^8 and 2 × 10^7 CFU/ml. Strains containing plasmids 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 antiserum, 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-^32P]ATP by using polynucleotide kinase as instructed by the manufacturer (Be-thesda 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 a product (data not shown). Finally, the products of the sequencing and RT-PCR, using the RT primer and an upstream primer. Only the latter reaction 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 NdeI/BamHI 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_{600} 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
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 NiSO₄ 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 1.3 M 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 MgCl₂·6H₂O with stepwise decreases in urea concentration (4 M, 2 M, 1 M, 0.5 M, no urea).

**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-
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 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.

![Diagram](image1.png)

**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 $P. aeruginosa$ (FleR), and FlbD of $C. crescentus$ (Cflbd). 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.
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 \( \sigma^{54} \) 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 (TGT-N_{10p}-ACA) (5) and was located about 440 bp upstream of the first NifA binding site, within the transcriptional activator FleQ. In addition, the promoter retained wild-type levels of \( \beta \)-galactosidase activities in the fleR mutant strain (PAK-RG), thus suggesting that fleSR is not autoregulated.

The promoter region of the fleQ gene was visually examined for the presence of specific motifs for NtrC, NifA, \( \sigma^{70} \), \( \sigma^{28} \), or \( \sigma^{54} \) binding. We were unable to identify either the NifA binding site (TGT-N_{10p}-ACA) (7) or NtrC binding site (TGCACY-N_{5p}-GGTCCAA) (7) in this region. Neither \( \sigma^{70} \) nor \( \sigma^{28} \) recognition sites could be identified in this upstream region. However, two potential \( \sigma^{54} \) binding sites (GG-N_{5p}-GC) (14) were present between nucleotides 103 and 117 and between nucleotides 189 and 205. To determine the role of \( \sigma^{54} \) 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 \( \beta \)-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-

**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-NPO (fleQ mutant); lane 4, complemented strain PAK-NPO (375Q); fleQ’; lane 5, vector control, PAK-NPO (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.

**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.

**TABLE 2.** Control of the fleSR promoter

<table>
<thead>
<tr>
<th>Host strain</th>
<th>Genetic background</th>
<th>( \beta )-galactosidase activity (Miller units) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAK</td>
<td>Wild type</td>
<td>75 ± 70</td>
</tr>
<tr>
<td>PAK-N1G</td>
<td>rpoN mutant</td>
<td>135 ± 12</td>
</tr>
<tr>
<td>PAK-Q</td>
<td>fleQ mutant</td>
<td>115 ± 2</td>
</tr>
<tr>
<td>PAK-RG</td>
<td>fleR mutant</td>
<td>99 ± 171</td>
</tr>
</tbody>
</table>

Note: The data for PAK-RG is not provided in the table, indicating a potential error in the transcription or data collection process.
NIG 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 NdeI/BamHI 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 chromosomes. 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 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* is discussed.

The translated product of the *P. aeruginosa* *fleQ* gene has many similarities with the transcriptional regulator FliB of *C. crescentus*, which also appears to lack a sensor kinase (17). It is also notable that FliB 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 FliB 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 FliB is that FliB 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

**TABLE 3. Control of the *fleQ* promoter**

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</tr>
</thead>
<tbody>
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<td>PAK</td>
<td>Wild type</td>
<td>75 ± 70, 3,062 ± 298</td>
</tr>
<tr>
<td>PAK-RG</td>
<td>fleR mutant</td>
<td>99 ± 171, 2,493 ± 358</td>
</tr>
<tr>
<td>PAK-N1G</td>
<td>rpoN mutant</td>
<td>62 ± 54, 4,320 ± 194</td>
</tr>
<tr>
<td>PAK-Q</td>
<td>fleQ mutant</td>
<td>123 ± 116, 1,904 ± 325</td>
</tr>
</tbody>
</table>

*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 fle5SR operon. Our results from β-galactosidase assays suggest that this is the case.

The presence of two σ54-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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