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Cloning and Characterization of *Pseudomonas aeruginosa fliF*, Necessary for Flagellar Assembly and Bacterial Adherence to Mucin

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***Pseudomonas aeruginosa* adheres to the mucosal surfaces of the lungs. This process appears to be mediated by nonpilus adhesins which bind to mucin. To find this nonpilus adhesin(s), mutagenesis of a nonpiliated mutant of *P. aeruginosa* with transposon Tn*5*G, followed by a screen for mucin adhesion, was used to isolate a series of mutants unable to adhere to mucin. All of these mutants were also found to be defective in motility. One such mutant, PAK-RR20, is characterized here. The site of the transposon insertion in PAK-RR20 was localized to a gene which is homologous to the *fliF* gene of other organisms and was flanked by other motility-related genes, *fliE* and *fliG*. Both adhesion and motility defects in PAK-RR20 were complemented by providing the *fliF* gene in *trans*. Since complementation could have been due to the presence of an internal promoter in the *fliF* gene or in the Tn*5*G transposon, which allowed the transcription of the downstream genes, another chromosomal mutant of the *fliF* gene was constructed by insertional inactivation with an antibiotic resistance cassette. This mutant was also nonmotile and nonadhesive. However, the two defects in this new mutant could not be complemented by the *fliF* gene in *trans*, consistent with the interpretation that there is no internal *fliF* promoter but possibly a functional promoter in the Tn*5*G transposon. The complete nucleotide sequences of the *fliE* and *fliF* genes and a partial nucleotide sequence of the *fliG* gene of *P. aeruginosa* were determined. Control of the promoter upstream of the *fliE* gene was analyzed by construction of a *fliE-lacZ* fusion and the introduction of this construct into strains of *P. aeruginosa* with mutations in several regulatory genes. β-Galactosidase expression measurements indicated that the *fliE* promoter does not utilize RpoF (σ^{28}) or RpoN (σ^{54}) sigma factors. The characterization of this gene as being responsible for the loss of adhesion indicates that basal body structures are probably important for localization of the adhesin.**

Pseudomonas aeruginosa is the predominant opportunistic bacterial pathogen that colonizes the lungs of patients with cystic fibrosis, ultimately leading to patient mortality. This organism inhabits the respiratory secretions of these patients (2) and has been shown to adhere to human mucin in vitro (24). Genetic studies have revealed that the expression of the bacterial components required for adhesion to mucin is under the control of the product of the *rpoN* gene (14), which is known to be required for the expression of pilin and unknown flagellar component(s) (5). A direct role for pili in the adhesion of *P. aeruginosa* to mucin appears to have been ruled out since mutants of the *P. aeruginosa pilA* gene, which codes for the structural subunit of pili, are just as adhesive to mucins as are their isogenic parental strains (14). This observation contrasts with the important role that pili seem to play in adhesion to cells (15, 25).

In regard to the other possible adhesins, it is conceivable that some component of the flagellar system is responsible for nonpilus-mediated adhesion, since a number of transposon-induced mutants identified by their inability to adhere to mucin in vitro also lacked flagella (19). However, it is clear that flagellin itself is not responsible for adhesion since mutants that are defective either in flagellin (*fliC*) or in the sigma factor required for flagellin transcription (*fliA*) are still adhesive (19). Thus, by an unknown mechanism, adhesion is related to the flagellar system. Two of these nonadhesive, nonmotile mutants have been recently characterized (20). Both of these mutants, B164 and RR18, were found to be transposon insertions into a single gene, *fliO*, a gene of the flagellar biogenesis pathway. The *fliO* gene product is believed to be involved in the export of flagellar proteins (11); therefore, it is possible that the nonpilus adhesin is exported via this system, implicating a flagellar component that is synthesized after this protein or alternately an entirely unrelated protein that is exported via this system.

During the screening of a *P. aeruginosa* transposon insertion bank for nonadhesive mutants, another mutant, PAK-RR20, was isolated (19). Southern analysis showed that this transposon was in a gene different from *fliO* of the previously characterized mutant (19, 20). We have, therefore, undertaken a detailed analysis of this mutant in order to better understand the relationship between the flagellum and adhesion and to possibly identify the genetic determinant responsible for nonpilus-mediated adhesion. The studies reported here show that this transposon is inserted into an open reading frame (ORF) that is homologous to the *fliF* gene of other bacteria. In the enteric bacteria *Escherichia coli* and *Salmonella typhimurium*, the *fliF* gene is a part of an operon consisting of *fliFGHIJK* genes and *fliE* is transcribed divergently. In the case of *P. aeruginosa*, we have found that the *fliE*, *fliF*, and *fliG* genes are transcribed in the same direction along the chromosome and perhaps are part of a single operon. The *fliE*, *fliF*, and *fliG*

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TABLE 1. Bacterial strains and plasmids used in this study

Strain, plasmid, or cosmid	Relevant genotype or phenotype	Source or reference
Strains		
E. coli DH5α	*hsdR recA lacZYA* φ80 *lacZ*Δ*M15*	GIBCO-BRL
P. aeruginosa		
PAK	Wild-type clinical isolate	D. Bradley
PAK-NP	PAK*pilA*::Tc^r	17
PAK-N1G	PAK*rpoN*::Gm^r	6
MS540	PAK*fliA*::Gm^r	21
MS540-NT	PAK*fliA*::Gm^r *rpoN*::Tet^r	This study
PAK-RR20	Nonadherent Tn*5*G insertion mutant	19
PAK-NPF	PAK*pilA*::Tet^r *fliF*::Gm^r	This study
Plasmids		
pUC18	*E. coli* cloning vector	
pGEM3Z	Sequencing vector, Amp^r, *lacZ* peptide	Promega, Madison, Wis.
pPZ375	*oriV* in pGEM	22
pPZ375F	pPZ375 with complete *fliF* gene	This study
pDN19lacΩ	Promoterless *lacZ*, *oriV*, *oriT*, Tet^r, Ω fragment	23
placΩE	*fliE* promoter in pDN19lacΩ	This study
pRR24	pUC18 with an *Eco*RI fragment containing Tn*5*G and 1.75 kb of flanking *P. aeruginosa* DNA	This study
pSAE5	pGEM with 5-kb *P. aeruginosa* DNA insert	This study
pKI11	pUC18 containing the *P. aeruginosa rpoN* gene inactivated with a Tet^r gene cassette	5
Cosmids		
pVK102	Cosmid vector, Nm^r Tc^r	7
pRR194	pVK102 with 20-kb *P. aeruginosa* DNA insert containing the *fliE* and *fliF* genes	This study
pSA238	pVK102 with 20-kb *P. aeruginosa* DNA insert containing the *fliG* gene	This study

genes code for three structural proteins, a basal body component, the membrane and supramembrane (MS) ring, and the switch component of the flagellum structure, respectively (10). Thus, the relationship between the flagellum and adhesion in *P. aeruginosa* appears to be a more complex one, involving more than one of the class 2 genes of the flagellar system.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. All the bacterial strains, plasmid vectors, and derivatives used in this study are shown in Table 1. All cultures were grown in liquid L broth (13), Terrific Broth (18), or on agar plates (1.7% agar) with or without antibiotics. The antibiotic concentrations used were as follows (in micrograms per milliliter): for *E. coli*, ampicillin (200), tetracycline (25), and kanamycin (30); for *P. aeruginosa*, carbenicillin (300), tetracycline (200), gentamicin (50), and streptomycin (300).

Enzymes and chemicals. All restriction enzymes and T4 DNA ligase were purchased from GIBCO-BRL, Inc., Gaithersburg, Md. Pfu DNA polymerase was purchased from Stratagene, La Jolla, Calif. Chemicals were purchased either from Sigma Chemical Co., St. Louis, Mo., or from Amresco Inc., Solon, Ohio.

DNA isolation and analysis. Small-scale plasmid DNA was prepared by the method of Birnboim and Doly (3). Larger-scale preparations of plasmid DNA were made by using a Qiagen plasmid kit (Qiagen Inc., Chatsworth, Calif.). Agarose gel electrophoresis, DNA restriction digests, and DNA ligations were performed by the method of Sambrook et al. (18). Southern hybridizations were performed either by using radiolabeled probes, as described by Sambrook et al. (18), or by using nonradioactive Southern hybridization Genius Kit 1 (Boehringer Mannheim, Indianapolis, Ind.).

PCR amplification. PCRs were performed in a DNA Thermal Cycler 480 (Perkin-Elmer Cetus). The reactions were performed in 100-μl volumes with Pfu polymerase. Each reaction mixture contained a final concentration of 50 ng of DNA template, 2.5 U of Pfu polymerase, 2.0 mM $MgCl_2$, 0.1 mM deoxynucleoside triphosphate mix, 10% dimethyl sulfoxide, and 0.2 μM (each) primers. Thirty cycles were performed for each reaction. Each cycle consisted of incubations for 1 min at 94°C, 1 min at 55°C, and 3 min at 72°C. The primers used for PCRs were RER19, RER20, RER21, and RER22. These primers were purchased from GIBCO-BRL. Restriction sites were added at the ends of each primer to facilitate subsequent cloning of the PCR products. Additional nucleotides were added 5′ to the restriction sites to ensure efficient cleavage. The location and nucleotide sequence of each primer used in PCRs was as follows: RER19 (5′CCC**GAATTC**CGACGCGGGGATGGAC3′), nucleotides 15 to 30, the *Eco*RI site added is shown in bold; RER20 (5′CCC**GGATCC**CGCATTTCCAGCATCA3′), nucleotides 330 to 346, the *Bam*HI site added is shown in bold; RER21 (5′CCC**GGATCC**GCGCAACAAACTGGTC3′), nucleotides 580 to 596, the *Bam*HI site added is shown in bold; and RER22 (5′CCCAAA**AAGCTT**ACGCCGAGGCTGGTCT3′), nucleotides 2673 to 2689, the *Hin*dIII site added is shown in bold.

Plasmid constructions. All subclones used for sequencing were derived from cosmid pRR194 (see Fig. 1) by cloning small restriction fragments into plasmid vector pGEM3Z or pUC18. Plasmid pPZ375, a derivative of pGEM3Z with *oriV*, was used for cloning the DNA fragment containing the *fliF* gene. In brief, a 2.0-kb DNA fragment with *Bam*HI and *Hin*dIII ends was ligated into the *Bam*HI and *Hin*dIII sites of pPZ375 to give rise to pPZ375F. This 2.0-kb DNA fragment was produced by PCR using primers RER21 and RER22 and contained the complete *fliF* structural gene from nucleotides 655 to 2454 (see Fig. 2). Plasmid placΩE was generated by ligation of a 331-bp piece of DNA with *Eco*RI and *Bam*HI ends into the *Eco*RI and *Bam*HI sites of pDN19lacΩ. This 331-bp DNA fragment was produced by PCR using primers RER19 and RER20. This DNA contained the entire upstream region of the *fliE* gene from nucleotides 15 to 346 (see Fig. 2). Plasmid pSAE5 was constructed by cloning a 5.0-kb *Eco*RI fragment containing the *fliG* gene into the *Eco*RI site of pGEM3Z.

DNA sequencing and data analysis. 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, respectively. The labeled extension products were analyzed on a model 373A DNA sequencer (Applied Biosystems). Double-stranded sequences were aligned and assembled by using programs in the Sequencher software package (Gene Codes Corp., Ann Arbor, Mich.). The deduced amino acid sequences of the genes identified by DNA sequence analysis were compared with known protein sequences in the GenBank, PIR, and SWISS-PROT databases by using the BLAST program (1).

β-Galactosidase assay. The expression of the *lacZ* gene under the control of the putative *fliE* promoter region was measured by β-galactosidase assay, as described by Miller (13). Plasmid-containing bacteria were grown in L broth and streptomycin.

Bacterial transformations. *E. coli* DH5α was transformed by the $CaCl_2$ method described by Sambrook et al. (18). *P. aeruginosa* strains were transformed either by the protocol of Mercer and Loutit (12) with minor modifications or by electroporation, as previously described (16).

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.

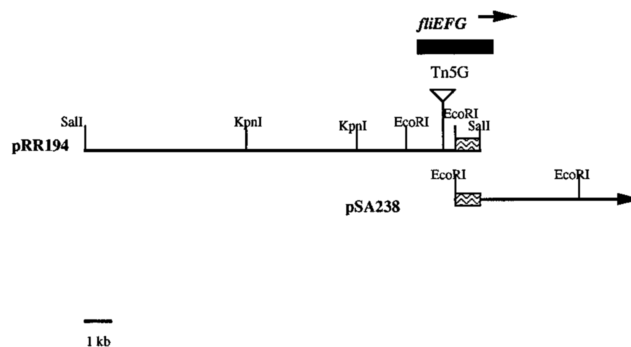

FIG. 1. Maps of the relevant regions of cosmids pRR194 and pSA238 containing the *P. aeruginosa fliEFG* operon. Maps are drawn approximately to scale. The 1-kb PCR-generated probe is shown as a rectangle filled with wavy lines. The *P. aeruginosa fliEFG* operon is shown as a solid rectangle and is divided between the two cosmids. The arrow beside the solid rectangle indicates the direction of transcription. Complete *fliE* and *fliF* genes are located on pRR194, while the *fliG* gene is present on pSA238. The triangle labeled Tn*5*G shows the approximate location of the insertion of transposon Tn*5*G in mutant PAK-RR20.

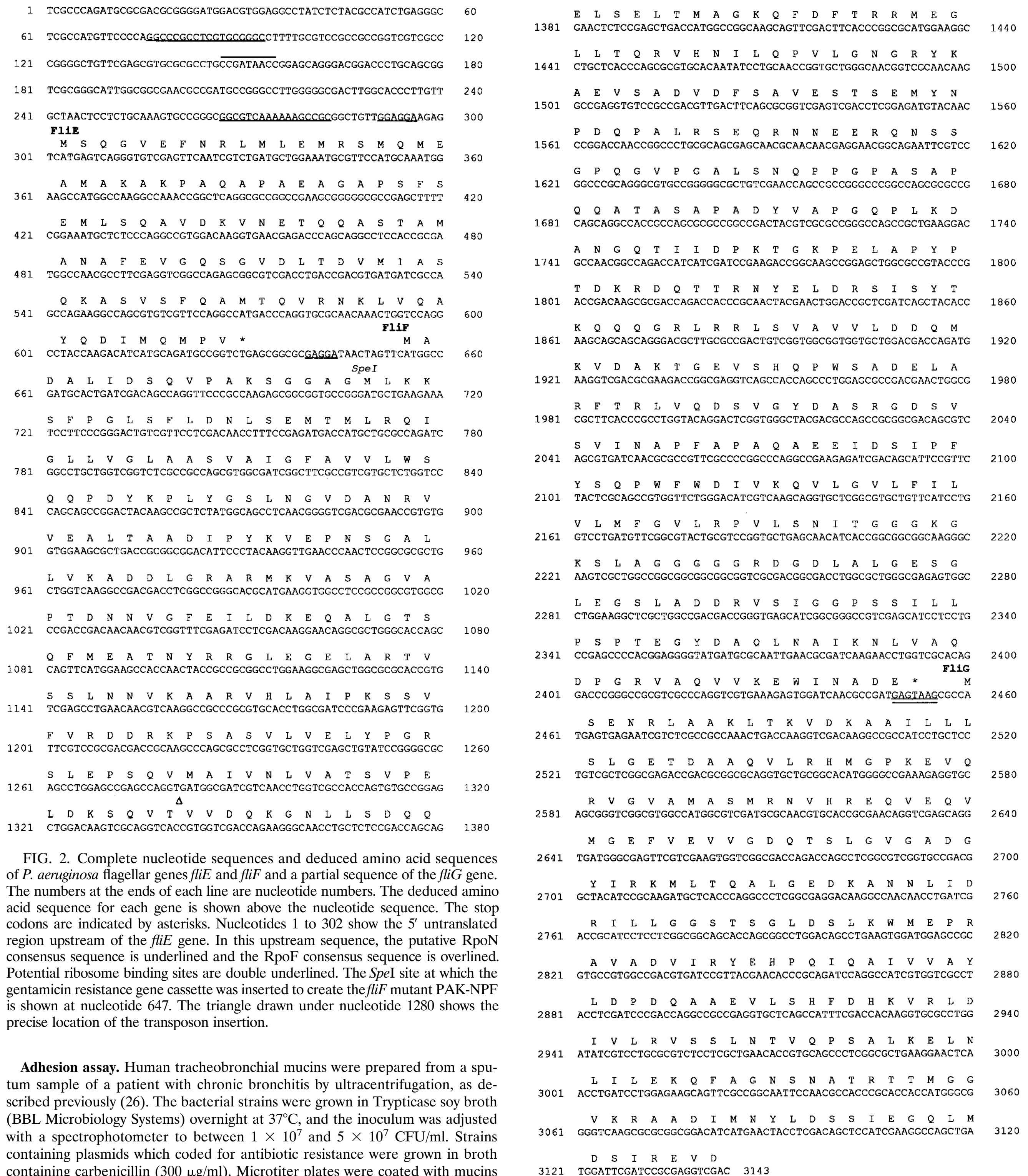

FIG. 2. Complete nucleotide sequences and deduced amino acid sequences of *P. aeruginosa* flagellar genes *fliE* and *fliF* and a partial sequence of the *fliG* gene. The numbers at the ends of each line are nucleotide numbers. The deduced amino acid sequence for each gene is shown above the nucleotide sequence. The stop codons are indicated by asterisks. Nucleotides 1 to 302 show the 5′ untranslated region upstream of the *fliE* gene. In this upstream sequence, the putative RpoN consensus sequence is underlined and the RpoF consensus sequence is overlined. Potential ribosome binding sites are double underlined. The *Spe*I site at which the gentamicin resistance gene cassette was inserted to create the *fliF* mutant PAK-NPF is shown at nucleotide 647. The triangle drawn under nucleotide 1280 shows the precise location of the transposon insertion.

Adhesion assay. Human tracheobronchial mucins were prepared from a sputum sample of a patient with chronic bronchitis by ultracentrifugation, as described previously (26). The bacterial strains were grown in Trypticase soy broth (BBL Microbiology Systems) overnight at 37°C, and the inoculum was adjusted with a spectrophotometer to between 1×10^7 and 5×10^7 CFU/ml. Strains containing plasmids which coded for antibiotic resistance were grown in broth containing carbenicillin (300 μg/ml). Microtiter plates were coated with mucins at a concentration of 50 μg/ml (24). Bacteria were added to wells, and plates were incubated at 37°C. Wells were washed 15 times in a manually operated microtiter plate washer, and the bacteria bound to wells were desorbed with Triton X-100 and plated for enumeration. Each strain was tested a minimum of three times. The results presented are the mean values derived from these experiments.

Nucleotide sequence accession number. The nucleotide sequence of 3,143 nucleotides containing the complete sequences of the *fliE* and *fliF* genes and a partial sequence of the *fliG* gene was submitted to the GenBank database (accession number L43507).

RESULTS

Cloning of a region of the *P. aeruginosa* chromosome containing *fliEFG*. *P. aeruginosa* mutant PAK-RR20 was obtained by transposon Tn*5*G mutagenesis of strain PAK-NP and en-

richment for nonadherent bacteria by several passages of a bank of mutants over mucin-coated polystyrene plates (19). Chromosomal DNA prepared from *P. aeruginosa* mutant PAK-RR20 was probed with a radiolabeled gentamicin resistance gene cassette to locate the insertion site of Tn*5*G in PAK-RR20. Transposon Tn*5*G with 1.75 kb of flanking *P. aeruginosa* DNA was cloned into pUC18 as an *Eco*RI fragment to give plasmid pRR24. By using DNA templates from pRR24 and primers hybridizing to sequences within the IS*50* element of Tn*5*G, the precise site of the insertion of Tn*5*G in the nonadherent mutant PAK-RR20 was determined. The transposon inserted within the coding sequence of the *fliF* gene at nucleotide 1280 (see Fig. 2). The same *Eco*RI fragment was also used as a probe to screen a cosmid bank of *P. aeruginosa* DNA in the broad-host-range vector pVK102 (7) by colony hybridization. One probe-reactive clone, pRR194, which contained about 20 kb of DNA insert was identified (Fig. 1). Restriction fragments were subcloned from pRR194, and the resulting subclones were used for sequencing.

In order to obtain DNA contiguous with pRR194, a 1-kb probe was prepared from the 3′ end of the insert in pRR194 by PCR. The same cosmid bank was screened again with this new 1-kb PCR-generated probe. One probe-reactive cosmid clone, pSA238, was identified (Fig. 1). The cosmid DNA from this clone was purified, and a 5.0-kb *Eco*RI fragment which hybridized with the same probe was isolated from this cosmid. This *Eco*RI fragment containing part of the *fliF* gene and the *fliG* gene was cloned into pGEM to generate plasmid pSAE5, which was utilized for sequencing.

Sequence analyses of the *fliE*, *fliF*, and *fliG* genes. Firstly, the *fliE*, *fliF*, and *fliG* genes are preceded by the *fleSR* operon, which has been recently described (16). The nucleotide sequence of a 3.2-kb piece of *P. aeruginosa* DNA was determined on both DNA strands (Fig. 2). Two complete ORFs and one partial ORF were identified in this sequence. The polypeptides deduced for the two complete ORFs have significant homologies to the *fliE* and *fliF* gene products of other organisms (Table 2; Fig. 3). A partial ORF is located downstream of the *fliF* ORF, and it specifies a protein homologous with the products of other bacterial *fliG* genes (Table 2).

The first ORF, with homology to the *fliE* gene, consists of 330 nucleotides. This ORF begins with an ATG codon at nucleotide 303 and ends with a stop codon (TGA) at nucleotide 630. A potential ribosome binding site is located 6 bp upstream of the ATG start codon. The deduced molecular mass of this protein is 12.1 kDa. In contrast to the *fliE* genes of *E. coli* and *S. typhimurium*, the *P. aeruginosa* homolog appears to be part of an operon rather than being a separate gene and is oriented in the same direction as the *fliF* gene. The second ORF, a homolog of the *fliF* gene, is 1,797 nucleotides in length, beginning with an ATG codon at nucleotide 655 and ending with a stop codon (TAA) at nucleotide 2452. This ORF is predicted to encode a protein with a molecular mass of 65.9 kDa. A potential ribosome binding site is located 10 bp upstream of the ATG start site. The third ORF, which was partially sequenced, has significant homologies to the *fliG* genes of other bacteria. This ORF begins with an ATG at nucleotide 2460 and has a potential ribosome binding site 4 bp upstream of the ATG start site. There are 23 nucleotides between the TGA codon of the *fliE* ORF and the ATG codon of the *fliF* ORF; no transcriptional terminators could be located within this region. There are only 5 nucleotides between the TAA codon of the *fliF* ORF and the ATG codon of the *fliG* ORF. The reading frames switch from frame 3 in *fliE* to frame 1 in *fliF* and then to frame 3 in the *fliG* ORF.

Complementation of PAK-RR20. *P. aeruginosa fliF* mutant PAK-RR20 was transformed with pPZ375F, a plasmid containing the complete *fliF* gene, and vector pPZ375. Bacteria carrying either pPZ375F or pPZ375 were then tested for the restoration of motility and adhesion. The nonmotile and nonadhesive phenotype of PAK-RR20 was complemented with pPZ375F, but not with vector pPZ375 (Table 3).

TABLE 2. Homologies of *P. aeruginosa* flagellar proteins to related proteins of other organisms

Protein	Organism	% Identity	% Similarity
FliE	*S. typhimurium*	43.1	61.8
	E. coli	42.3	59.6
	Bacillus subtilis	37.7	55.7
FliF	*S. typhimurium*	37.2	56.3
	Caulobacter crescentus	30.9	55.9
	Bacillus subtilis	21.0	50.1
FliG	*S. typhimurium*	39.4	63.8
	E. coli	39.3	63.9
	Bacillus subtilis	29.9	57.7
	Caulobacter crescentus	26.3	53.1

Construction and complementation of PAK-NPF. Since the transposon insertion into the *fliF* gene did not produce the expected polar effect, it was important to construct another mutant of the *fliF* gene. This mutation was created by inserting a gentamicin resistance gene cassette into the *Spe*I site between the putative ribosome binding site and the putative translational initiation site of the *fliF* gene (Fig. 2). This insertion mutant, PAK-NPF, was also nonmotile and nonadhesive (Table 3). However, in contrast to transposon mutant PAK-RR20, the nonmotile and nonadhesive phenotype of PAK-NPF was not complemented by providing the *fliF* gene on the same multicopy plasmid which complemented PAK-RR20 (Table 3).

Construction of a double sigma factor mutant, MS540-NT. Strain MS540, which has the *fliA* (*rpoF*) gene inactivated (21), was used as the recipient in a triparental mating with *E. coli* DH5α containing plasmid pKI11 (5) and *E. coli* DH5α containing helper plasmid pRK2013 (4). Plasmid pKI11 contains a copy of the *P. aeruginosa rpoN* gene insertionally inactivated with a tetracycline resistance gene cassette. Transconjugants were initially selected on agar plates containing tetracycline (200 μg/ml) and then tested for carbenicillin sensitivity. One such Tet^r Cb^s transconjugant was picked and further characterized. The colonies formed by this mutant were small, grew slowly in rich growth medium, and were nonmotile. They were also auxotrophic for glutamine as are other *P. aeruginosa rpoN* mutants (5). The insertion of the tetracycline resistance gene cassette in the *rpoN* gene of this mutant, MS540-NT, was then confirmed by Southern blot analysis (data not shown).

Analysis of the promoter region of the *fliE* gene. As shown in Fig. 2, two potential RpoN recognition sequences (underlined) and a sequence with a perfect match to the RpoF recognition sequence (overlined) were identified upstream of the *fliE* ORF. No consensus −10 or −35 sequence could be identified in this region, and no promoter-like sequences could be identified in the intergenic spaces between *fliE* and *fliF* or between *fliF* and *fliG* ORFs, suggesting that *fliE*, *fliF*, and *fliG* may be organized in an operon.

In order to test whether the identified binding sites for the alternative sigma factors RpoN and/or RpoF are functional promoters, a 331-bp DNA fragment which contained the DNA sequence upstream from the end of the *fleR* gene to the *fliE*

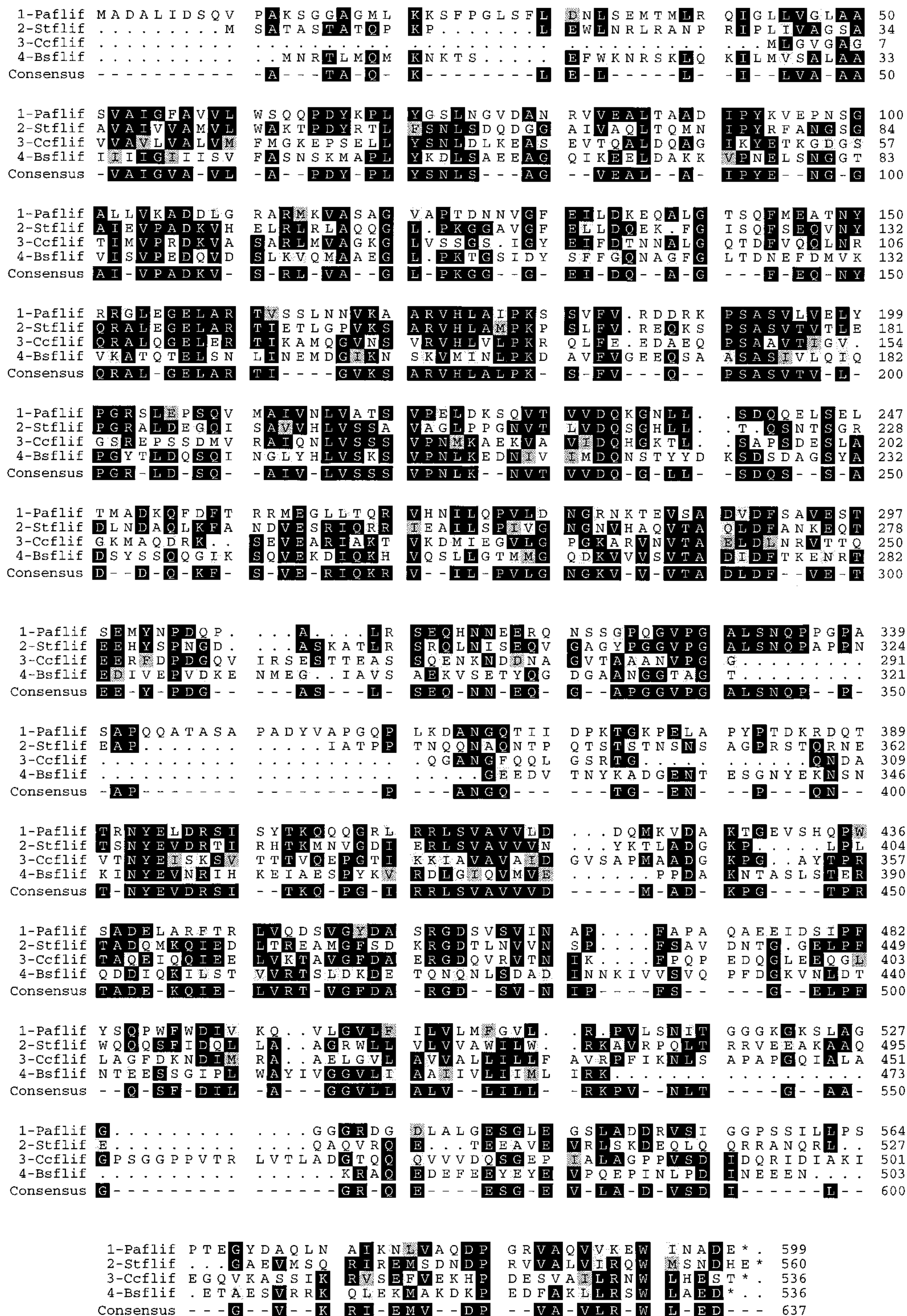

FIG. 3. Computer-generated alignment (Prettybox program; Richard Westerman, Purdue University) of *P. aeruginosa* FliF (Paflif) with homologous FliF proteins of other organisms. Black shading shows identical amino acids, and different shades of gray show the degrees of similarity of other amino acids to the black-shaded ones (on the basis of the Genetics Computer Group comparison table). The bottom row shows the computer-generated consensus sequence. Stflif, *S. typhimurium* FliF; Ccflif, *C. crescentus* FliF; Bsflif, *B. subtilis* FliF.

ORF was first obtained by PCR. This DNA fragment was then inserted upstream of a promoterless *lacZ* gene in plasmid pDN 19lacΩ (23) to give plasmid placΩE. Plasmids pDN19lacΩ (vector) and placΩE were introduced into *P. aeruginosa* PAK (wild type), PAK-N1G (*rpoN* mutant), MS540 (*rpoF* mutant), and MS540-NT (*rpoN* and *rpoF* mutant) by transformation, and the transformants were tested for β-galactosidase activity. As shown in Table 4, the levels of β-galactosidase activity were

TABLE 3. Complementation of the adhesion defects in strains PAK-RR20 and PAK-NPF

Complementation of adhesion defect	Adhesion to mucin (10^2 CFU/well)
In PAK-RR20	
PAK-NP (control)	84 ± 16
PAK-RR20	7 ± 5
PAK-RR20(pPZ375F)	34 ± 12
PAK-RR20(pPZ375)	7 ± 3
In PAK-NPF	
PAK-NP (control)	103 ± 15
PAK-NPF	3 ± 1
PAK-NPF(pPZ375F)	3 ± 1
PAK-NPF(pPZ375)	2 ± 1

not significantly different between the wild-type *P. aeruginosa* strain, PAK, and mutant strains PAK-N1G, MS540, and MS540-NT, each carrying placΩE. The same strains carrying vector pDN19lacΩ had low β-galactosidase activities. This suggests that under the growth conditions utilized, neither RpoN nor RpoF was used in transcription of the *fliEFG* operon.

DISCUSSION

We previously isolated a nonmotile and nonadhesive mutant (PAK-RR20) of *P. aeruginosa* PAK-NP (19). The parental strain used to isolate PAK-RR20 carried a mutation in the pilin structural gene; therefore, the adhesion defect was due to lack of expression of a nonpilus adhesin. In this report, we have identified the site of the transposon insertion in PAK-RR20. Transposon Tn*5*G was inserted into a *P. aeruginosa* homolog of the *fliF* gene and resulted in the simultaneous loss of motility and adhesion. The complete nucleotide sequences of the *fliE* and *fliF* genes and a partial nucleotide sequence of the downstream *fliG* gene were ascertained. The predicted protein products of these genes show significant sequence homologies with the corresponding genes of both gram-negative and gram-positive bacteria. Two putative RpoN binding sites and one putative RpoF binding site were identified upstream of the *fliE* gene. When a promoter fusion to the *lacZ* gene was made by using the *fliE* upstream region and introduced into *P. aeruginosa* strains defective in RpoN, RpoF, or both sigma factors simultaneously, there were no differences in β-galactosidase activity between these mutants and the parent strain. These data suggest that transcription of the *fliEFG* operon does not depend on RpoN or RpoF.

In contrast to *E. coli* and *S. typhimurium*, in which the *fliE* gene is transcribed as a single gene divergently from the *fliF* gene (10), we have found in *P. aeruginosa* that both the *fliE* and *fliF* genes are part of a gene cluster together with *fliG*, perhaps in an operon. Since it was possible to complement the motility and mucin adherence defect in PAK-RR20 by the cloned *fliF* gene alone in *trans*, it seemed that the other genes in the *fliEFG* operon required for the completion of flagellar structure were transcribed either from a promoter within the Tn*5* transposon or from an internal promoter in the 3′ end of the *fliF* gene. In order to test this hypothesis and determine whether the defects were due to the *fliF* gene alone, another mutant, PAK-NPF, was made by using a gene replacement strategy. This mutant was not complemented by the very plasmid that complemented the transposon mutant. This is consistent with the presence of an internal promoter in Tn*5*G, promoting the transcription of genes in the *fliEFG* operon, downstream of the *fliF* gene. A similar nonpolar transposon insertion in the *P. aeruginosa fliO* gene was recently reported (20). Thus, insertional inactivation of the *fliF* gene causes a polar effect on the operon, but there are other structural considerations which could have affected the complementation of adhesion. The MS ring, which is the product of the *fliF* gene, is the first structure to be laid down (8). If this is not present, the subsequent gene products are not found in *E. coli*. Though the effect is polar from the point of view of insertional inactivation, other flagellar gene products of other later operons would also not be transcribed.

The role of putative RpoN and RpoF binding sites upstream of the *fliE* gene is not clear. RpoF binding sequences have been identified in the promoter regions of many class 2 flagellar genes of other bacteria (10), but inexplicably they do not appear to be under the control of RpoF. Since transcription of the *fliE* gene does not require a functional RpoF or RpoN, another unidentified sigma factor which is involved in the regulation of class 2 flagellar genes probably exists in *P. aeruginosa*. In *E. coli* and *S. typhimurium*, *flhC* and *flhD* coordinately regulate the expression of class 2 flagellar genes (9). It is possible that homologs of *flhC* and/or *flhD* regulate the expression of the *fliEFG* operon in *P. aeruginosa*.

The *fliF* gene product (MS ring) is inserted into the cytoplasmic membrane (10). On the basis of its cellular location, it is highly unlikely that it can physically interact with an exogenous receptor in mucin, since an adhesin would have to be surface exposed. It is clear, however, that the *fliF* gene product is a platform in the cell membrane that is required for the addition of various proteins during assembly of the flagellum (8). If the adhesin is a late class 2 flagellar gene product, it would not be expressed in a *fliF* mutant, since the subsequent flagellar components would not be made. Alternately, if the MS ring was made but the export pore was not assembled, then the adhesin would probably not be exported. We believe that this is the situation in the previously described *fliO* mutant (20).

The localization of these mutations has provided a better framework in which to understand the localization and identity of the nonpilus mucin adhesin. There are now several possibilities to consider. Our recently published work indicates that adhesion and motility are regulated by a two-component system called *fleS-fleR*. Thus, *fleS-fleR* could regulate the synthesis of the adhesin, the export apparatus, or both in addition to other flagellar proteins. Another possibility is that the adhesin is not a flagellar protein but that it requires an intact flagellar export apparatus for its localization, which in turn requires the MS ring for its assembly. Sequential mutagenesis of flagellar genes beginning with the flagellar hook gene and going down-

TABLE 4. Expression of β-galactosidase from the *fliE* promoter fusion to *lacZ*

Strain	Genotype or description	Plasmid	β-Galactosidase activity (Miller units)[a]
PAK	Wild-type clinical isolate	pDN19lacΩ	70 ± 41
		placΩE	5,407 ± 138
PAK-N1G	PAK*rpoN*::Gm^r	pDN19lacΩ	312 ± 47
		placΩE	4,788 ± 303
MS540	PAK*fliA*::Gm^r	pDN19lacΩ	145 ± 33
		placΩE	4,814 ± 157
MS540-NT	MS540*rpoN*::Tc^r	pDN19lacΩ	245 ± 107
		placΩE	4,965 ± 60

[a] Determined as described by Miller (13).

ward into the export apparatus should localize the stage at which adhesion to mucins is conferred. Alternately, elucidation of the genes controlled by FleR may assist in limiting the choice of possible genes involved in adhesion.

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REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Baltimore, R. S., C. D. C. Christie, and G. J. Smith.** 1989. Immunohistopathologic localization of *Pseudomonas aeruginosa* in lungs from patients with cystic fibrosis. Am. Rev. Respir. Dis. **140:**1650–1661.
3. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
4. **Figurski, D., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent upon plasmid functions provided *in trans*. Proc. Natl. Acad. Sci. USA **76:**1648–1652.
5. **Ishimoto, K. S., and S. Lory.** 1989. Formation of pilin in *Pseudomonas aeruginosa* requires the alternative sigma factor (RpoN) of RNA polymerase. Proc. Natl. Acad. Sci. USA **86:**1954–1957.
6. **Ishimoto, K. S., and S. Lory.** 1992. Identification of *pilR*, which encodes a transcriptional activator of the *Pseudomonas aeruginosa* pilin gene. J. Bacteriol. **174:**3514–3521.
7. **Knauf, V. C., and E. W. Nester.** 1982. Wide host-range cloning vectors: a cosmid clone bank of an agrobacterium Ti plasmid. Plasmid **8:**45–54.
8. **Kubori, T., N. Shimamoto, S. Yamaguchi, K. Namba, and S.-I. Aizawa.** 1992. Morphological pathway of flagellar assembly in *Salmonella typhimurium*. J. Mol. Biol. **226:**433–446.
9. **Liu, X., and P. Matsumura.** 1994. The FlhD/FlhC complex, a transcriptional activator of the *Escherichia coli* flagellar class II operons. J. Bacteriol. **176:** 7345–7351.
10. **Macnab, R. M.** 1992. Genetics and biogenesis of bacterial flagella. Annu. Rev. Genet. **26:**131–158.
11. **Malakooti, J., B. Ely, and P. Matsumura.** 1994. Molecular characterization, nucleotide sequence, and expression of the *fliO*, *fliP*, *fliQ*, and *fliR* genes of *Escherichia coli*. J. Bacteriol. **176:**189–197.
12. **Mercer, A. A., and J. S. Loutit.** 1979. Transformation and transfection of *Pseudomonas aeruginosa*: effects of metal ions. J. Bacteriol. **140:**37–42.
13. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14. **Ramphal, R., L. Koo, K. S. Ishimoto, P. A. Totten, J. C. Lara, and S. Lory.** 1991. Adhesion of *Pseudomonas aeruginosa* pilin-deficient mutants to mucin. Infect. Immun. **59:**1307–1311.
15. **Ramphal, R., J. C. Sadoff, M. Pyle, and J. D. Silipigni.** 1984. Role of pili in the adherence of *Pseudomonas aeruginosa* to injured tracheal epithelium. Infect. Immun. **44:**38–40.
16. **Ritchings, B. W., E. C. Almira, S. Lory, and R. Ramphal.** 1995. Cloning and phenotypic characterization of *fleS* and *fleR*, new response regulators of *Pseudomonas aeruginosa* which regulate motility and adhesion to mucin. Infect. Immun. **63:**4868–4876.
17. **Saiman, L., K. Ishimoto, S. Lory, and A. Prince.** 1990. The effect of piliation and exoproduct expression on adherence of *Pseudomonas aeruginosa* to respiratory epithelial cells. J. Infect. Dis. **161:**541–548.
18. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
19. **Simpson, D. A., R. Ramphal, and S. Lory.** 1992. Genetic analysis of *Pseudomonas aeruginosa* adherence: distinct genetic loci control attachment to epithelial cells and mucins. Infect. Immun. **60:**3771–3779.
20. **Simpson, D. A., R. Ramphal, and S. Lory.** 1995. Characterization of *Pseudomonas aeruginosa fliO*, a gene involved in flagellar biosynthesis and adherence. Infect. Immun. **63:**2950–2957.
21. **Starnbach, M. N., and S. Lory.** 1992. The *fliA* (*rpoF*) gene of *Pseudomonas aeruginosa* encodes alternative sigma factor required for flagellin synthesis. Mol. Microbiol. **6:**459–469.
22. **Temple, L., A. Sage, G. E. Christie, and P. V. Phibbs, Jr.** 1994. Two genes for carbohydrate catabolism are divergently transcribed from a region of DNA containing the *hexC* locus in *Pseudomonas aeruginosa* PAO1. J. Bacteriol. **176:**4700–4709.
23. **Totten, P. A., and S. Lory.** 1990. Characterization of the type a flagellin gene from *Pseudomonas aeruginosa* PAK. J. Bacteriol. **172:**7188–7199.
24. **Vishwanath, S., and R. Ramphal.** 1984. Adherence of *Pseudomonas aeruginosa* to human tracheobronchial mucin. Infect. Immun. **45:**197–202.
25. **Woods, D. E., D. C. Straus, W. G. Johanson, Jr., V. K. Berry, and J. A. Bass.** 1980. Role of pili in adherence of *Pseudomonas aeruginosa* to mammalian buccal epithelial cells. Infect. Immun. **29:**1146–1151.
26. **Woodward, H., B. Horsey, V. P. Bhavanandan, and E. A. Davidson.** 1982. Isolation, purification, and properties of respiratory glycoproteins. Biochemistry **21:**694–701.

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