INFECTION AND IMMUNITY, Mar. 1998, p. 1000–1007 Vol. 66, No. 3
0019-9567/98/$04.00+0
Copyright © 1998, American Society for Microbiology

The *Pseudomonas aeruginosa* Flagellar Cap Protein, FliD, Is Responsible for Mucin Adhesion

SHIWANI K. ARORA,[1] BRUCE W. RITCHINGS,[1] ERNESTO C. ALMIRA,[2] STEPHEN LORY,[3] AND REUBEN RAMPHAL[1]*

Department of Medicine/Infectious Diseases[1] and Interdisciplinary Center for Biotechnology Research,[2] University of Florida, Gainesville, Florida 32610, and Department of Microbiology, University of Washington, Seattle, Washington 98195[3]

Received 9 October 1997/Returned for modification 24 November 1997/Accepted 19 December 1997

Mucin-specific adhesion of *Pseudomonas aeruginosa* plays an important role in the initial colonization of this organism in the airways of cystic fibrosis patients. We report here that the flagellar cap protein, FliD, participates in this adhesion process. A polar chromosomal insertional mutation in the *P. aeruginosa fliD* gene made this organism nonadhesive to mucin in an in vitro mucin adhesion assay. The adhesive phenotype was restored by providing the *fliD* gene alone on a multicopy plasmid, suggesting involvement of this gene in mucin adhesion of *P. aeruginosa*. Further supporting this observation, the in vitro competition experiments demonstrated that purified FliD protein inhibited the mucin adhesion of nonpiliated *P. aeruginosa* PAK-NP, while the same concentrations of PilA and FlaG proteins of *P. aeruginosa* were ineffective in this function. The regulation of the *fliD* gene was studied and was found to be unique in that the transcription of the *fliD* gene was independent of the flagellar sigma factor σ^{28}. Consistent with this finding, no σ^{28} binding sequence could be identified in the *fliD* promoter region. The results of the β-galactosidase assays suggest that the *fliD* gene in *P. aeruginosa* is regulated by the newly described transcriptional regulator FleQ and the alternate sigma factor σ^{54} (RpoN).

Pseudomonas aeruginosa is an opportunistic human pathogen that causes lethal infections in compromised individuals and chronic colonization of the lungs of patients with cystic fibrosis, leading to their death. *P. aeruginosa* has a remarkable ability to persist in the lungs successfully by colonizing respiratory mucus. The molecular mechanisms by which this organism attaches to and colonizes the human airways are poorly understood.

P. aeruginosa has been demonstrated to adhere to intact respiratory epithelial cells in culture (22) as well as to injured respiratory tissue organ culture (32) and injured whole animal trachea (19). Pili present on the surface of *P. aeruginosa* have been shown to contribute significantly to attachment to the epithelial cells (34). However, *P. aeruginosa* mutants lacking pili attach to mucin as efficiently as the wild-type strains (20).

Earlier studies from our laboratory have demonstrated an association between the expression of mucin adhesin(s) and the expression of some flagellar genes in *P. aeruginosa* (24). Mutants defective in the *fliF* gene (2), which codes for the flagellar membrane and supramembrane ring, and the *fliO* gene (25), which codes for one of the proteins of the flagellar export apparatus, were nonmotile and nonadhesive, whereas a *fliC* mutant which is nonmotile and does not make flagellin retains adhesion to mucin (24). These findings suggest that either the mucin adhesin is a structural component of the flagellar apparatus or it utilizes the flagellar export and secretion machinery. Additionally, an alternate sigma factor, RpoN, not only is involved in the transcription of genes specifying bacterial adhesion to mucin and epithelial cells, but also is involved in the expression of flagellar genes (7, 20, 31). Moreover, we have recently identified two regulators, FleR and FleQ, that can potentially work with RpoN to regulate flagellar expression and mucin adhesion (3, 21). However, the specific targets of FleR and FleQ action are still not known. RpoN has also been shown to be important in the regulation of flagellar genes in *Pseudomonas putida* (8), *Caulobacter crescentus* (5), *Vibrio parahaemolyticus* (15), and *Campylobacter coli* (11).

This report presents evidence that the flagellar cap protein (FliD) is directly involved in mucin adhesion. The nucleotide sequence of the *fliD* gene of *P. aeruginosa* was determined, and a chromosomal *fliD* mutant (PAK-NPD) was constructed by mutation in *P. aeruginosa* PAK-NP. This mutant was found to be nonmotile and nonadhesive. The motility and adhesion defects of PAK-NPD were complemented by the *fliD* gene alone, thus suggesting the involvement of FliD in mucin adhesion. The *fliD* gene product was purified from the *Escherichia coli* host and was used as a competitor in an in vitro adherence assay. The pure FliD protein specifically inhibited the binding of *P. aeruginosa* PAK-NP to human respiratory mucins, which indicates direct involvement of this protein in mucin binding. Analysis of the promoter region of the *fliD* gene suggested that this gene is regulated by the regulator FleQ, which works in concert with RpoN, and is independent of σ^{28}, which controls the *fliD* genes of many other bacterial species (13).

* Corresponding author. Mailing address: Department of Medicine/Infectious Diseases, P.O. Box 100277, JHMHC, University of Florida, Gainesville, FL 32610. Phone: (352) 392-2932. Fax: (352) 392-6481. E-mail: Ramphr@medmac.ufl.edu.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. All bacterial strains, plasmid vectors, and their derivatives are shown in Table 1. The bacterial cultures were grown in liquid Luria broth (17), tryptic soy broth (23), or on L agar plates (1.7% agar) with or without antibiotics. The antibiotic concentrations used were as follows: for *E. coli*, ampicillin at 200 μg/ml and gentamicin at 10 μg/ml; for *P. aeruginosa*, carbenicillin at 150 μg/ml, gentamicin at 50 μg/ml, tetracycline at 100 μg/ml, and streptomycin at 300 μg/ml.

Enzymes and chemicals. T4 DNA ligase and all restriction enzymes were purchased from GIBCO-BRL, Inc., Gaithersburg, Md. *Pfu* DNA polymerase was purchased from Stratagene, La Jolla, Calif. The chemicals were purchased either from Sigma Chemical Co., St. Louis, Mo., or from Amresco, Inc., Solon, Ohio. The Bio-Rad protein assay kit was purchased from Bio-Rad Laboratories, Hercules, Calif.

Electroporations. Electroporations were performed by a modification of the protocol of Smith and Iglewski (26). The DNA used for the electroporations was prepared by the alkaline lysis procedure (4). For gene replacement experiments

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype	Source or reference
Strains		
E. coli		
DH5α	*hsdR recA lacZYA* φ80 *lacZ* ΔM15	GIBCO BRL
BL21(DE3)	F^- *ompT hsdS$_B$* (r_B^- m_B^-) *gal dcm* (DE3)	Novagen, Inc., Madison, Wis.
P. aeruginosa		
PAK	Wild-type clinical isolate	D. Bradley
PAK-NP	PAK*pilA*::Tc^r	22
PAK-N1G	PAK*rpoN*::Gm^r	9
PAK-RG	PAK*fleR*::Gm^r	21
PAK-Q	PAK*fleQ*::Gm^r	3
MS540	PAK*fliA*::Gm^r	28
PAK-D	PAK*fliD*::Gm^r	This study
PAK-NPD	PAK*pilA*::Tc^r *fliD*::Gm^r	This study
Plasmids		
pUC18	*E. coli* cloning vector, Amp^r	GIBCO BRL
pLysS	Plasmid containing the T7 lysozyme gene	Novagen
pGEM3Z	Sequencing vector, Amp^r, *lacZ*α peptide	Promega, Madison, Wis.
pBluescript KS(+)	*E. coli* cloning vector, Amp^r	Stratagene, Inc.
pDN19lacΩ	Promoterless *lacZ oriV oriT* Tet^r Str^r Ω fragment	30
pPZ375	*oriV* in pGEM3Z	29
pG10E	pGEM3Z containing a 10-kb *Eco*RI fragment isolated from the cosmid pRR194	This study
pBS7EA	pBluescript KS(+) containing a 7-kb *Eco*RI-*Apa*I insert including the *fliDSorf126orf96* operon	This study
pBS7EAG	pBS7EA with a gentamicin resistance gene inserted into the *Eco*RV site in the *fliD* gene	This study
placΩD	pDN19lacΩ with a 495-bp *Eco*RI-*Bam*HI fragment including the promoter region of the *fliD* gene	This study
pPZ375D	pPZ375 with a complete *fliD* gene	This study
pET15B	Expression vector, T7 promoter, His-tag coding sequence, Amp^r, pBR322 origin	Novagen
pET15BD	*fliD* gene inserted as a PCR product into the *Nde*I-*Bam*HI sites of pET15B	This study

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 or covalently closed-circular plasmid DNA was electroporated into the target strains.

β-Galactosidase assay. Expression of the *lacZ* gene under the control of the putative *fliD* promoter region of the *fliDSorf126orf96* promoter was measured by β-galactosidase assays as described by Miller (17) 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.

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 triphosphates mix, 10% dimethyl sulfoxide, and 0.2 μM primers. Forty cycles were run, each consisting of incubations for 2 min at 95°C, 1 min at 46°C, and 6 min at 72°C. The annealing temperature was kept low because of the low ionic strength of the *Pfu* reaction buffer, and the extension time 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 to the ends of primers (shown 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. RER36 and RER40 were used for the PCR amplification of the *fliD* promoter. RER36 [5′(CCCAAA**GAATTC**ATGGACGTCAGCAATGTC)3′] was located at nucleotide 862 (accession no. L81176); an *Eco*RI site was added to this primer. RER40 [5′(CCCAAA**GGATCC**TGTAGCCGTTGATCGTCG)3′] was located between nucleotides 1339 and 1356 (accession no. L81176); a *Bam*HI site was added to this primer. FliD5p (CCCAAA**GAATTC**CAGGAGAAGCAAGATGG CGAAC) was used as a 5′ primer to amplify the complete *fliD* gene, which was cloned into the vector pPZ375 (29); an *Eco*RI site was added to this primer, which is shown here in boldface. FliD3p (CCCAAA**GGATCC**TCAGCTTTTCT TCACAAGGCC) was used as the 3′ primer to amplify the complete *fliD* gene, which was cloned into the vector pPZ375; a *Bam*HI site was added to this primer, which is shown in boldface. RER39 [5′(CCCAAAAAAAAA**CATATG**GCGAA CAGTACGACG)3′] was used as the 5′ primer to amplify the complete *fliD* gene, which was cloned into the vector pET15B (Novagen, Inc., Madison, Wis.); an *Nde*I site was added to this primer, which is shown here in boldface.

Plasmid constructions. pG10E was constructed by cloning a 10-kb *Eco*RI fragment which was excised from the cosmid pRR194 (21) into the *Eco*RI site of pGEM3Z (Promega, Inc., Madison, Wis.). A 7-kb *Eco*RI-*Apa*I fragment was isolated from pG10E and inserted into the *Eco*RI and *Apa*I sites of pBluescript KS(+), to give pBS7EA. This construct contained the complete *fliDSorf126orf96* operon. The plasmid pBS7EA was partially digested with *Eco*RV (two *Eco*RV sites), and a gentamicin resistance gene cassette was inserted selectively in the *Eco*RV site present in the *fliD* gene, leading to the construction of pBS7EAG. This construct was utilized to generate a chromosomal mutation in the *P. aeruginosa fliD* gene by marker exchange. The plasmid used for complementation of the *fliD* mutation, pPZ375D, was obtained by cloning a 1.4-kb PCR fragment carrying the complete *fliD* gene into pPZ375. The *fliD* expression construct, pET15BD, was constructed by cloning a 1.4-kb PCR fragment containing the complete *fliD* gene into the *Nde*I and *Bam*HI sites of the expression vector pET15B. The promoter fusion construct placΩD was the result of cloning of a 495-bp *Eco*RI-*Bam*HI fragment containing the putative *fliD* promoter region into the *Eco*RI and *Bam*HI sites of the promoter probe vector pDN19lacΩ (30).

DNA sequencing. DNA sequencing was performed according to the *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 with an Applied Biosystems model 373A DNA sequencer. Double-stranded sequences were aligned and assembled by programs in the Sequencher software package (Gene Codes Corp., Ann Arbor, Mich.).

Motility assay. Bacterial strains were grown overnight at 37°C on fresh L 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 (35). 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 1×10^7 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 (33). Bacteria were added to the wells, and the plates were incubated at 37°C for 30 min. 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

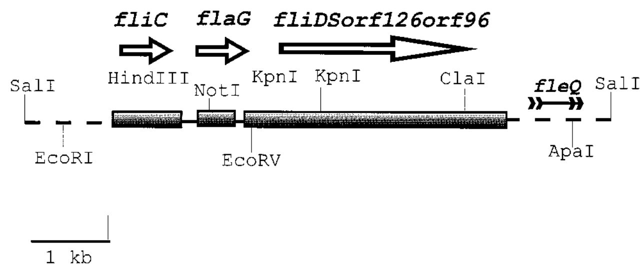

FIG. 1. Map of the *fliDSorf126orf96* region showing the gene arrangement in *P. aeruginosa*. The map is drawn approximately to scale. The shaded rectangles show the coding regions. The arrows beside the shaded rectangles indicate the direction of transcription. The *Eco*RV site used for insertional inactivation of the *fliD* gene is shown.

a minimum of three times. The results are mean values derived from these experiments.

Expression and purification of FliD. The complete *fliD* coding sequence was inserted as a 1.4-kb PCR product into the *Nde*I-*Bam*HI sites of the plasmid pET15B. The resulting plasmid, pET15BD, was introduced into *E. coli* BL21 (pLysS) (Novagen), which contains the T7 polymerase gene on the chromosome under the control of the *lacUV5* promoter. Bacterial cultures were grown to A_{550}s of 0.4 to 0.5, and the T7 promoter was induced by the addition of a 2.0 mM final concentration of isopropyl-β-D-thiogalactopyranoside (IPTG). The cultures were grown for an additional 4 h and then harvested. These pellets were resuspended in binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl [pH 7.9]). The cell lysate was prepared by disrupting the cells in a French pressure cell at 16,000 lb/in^2. The cell debris was removed by centrifugation at 15,000 × *g* for 30 min. 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 $NiSO_4$ according to the pET instruction manual provided by Novagen, Inc. Further steps in the purification of His-FliD were performed according to the pET instruction manual. The His-FliD protein was finally eluted with elution buffer (1 M imidazole, 0.4 M NaCl, 20 mM Tris-HCl [pH 7.9]). The protein was dialyzed against 100 volumes of phosphate-buffered saline (PBS) with three changes. The dialyzed purified His-FliD protein was stored at 4°C.

Thrombin cleavage of His-FliD protein. The 6× His tag was removed from the His-FliD fusion protein by using the thrombin cleavage capture kit (Novagen). Fifty micrograms of the fusion protein was incubated at room temperature with 0.5 U of biotinylated thrombin and thrombin cleavage buffer in a total reaction volume of 150 μl. The incubation was done for 4 h with continuous mixing of the reaction components. The uncleaved His-FliD fusion protein was removed from the reaction mixture by allowing it to bind to the His tag affinity resin at room temperature for 90 min. The supernatant contained essentially the pure FliD protein.

Bio-Rad protein assay. Proteins were quantitated by using the Bio-Rad protein assay kit. The Bio-Rad protein assay is based on the color change of Coomassie brilliant blue G-250 dye in response to various concentrations of protein. The manufacturer's instructions were followed to perform this assay.

Nucleotide sequence accession number. The nucleotide sequence consisting of 3,925 nucleotides containing the complete sequences of *flaG*, *fliD*, *fliS*, *orf126*, and *orf96* has been submitted to GenBank (accession no. L81176).

RESULTS

Nucleotide sequence of the chromosomal region containing *fliD* and *fliS*. The nucleotide sequence of a 3.9-kb fragment of *P. aeruginosa* DNA was determined on both DNA strands. This region contained four open reading frames (ORFs) which appeared to comprise a single operon. The location of these ORFs relative to other flagellar genes is shown in Fig. 1. The first ORF consisted of 1,437 nucleotides, which is predicted to contain a gene that codes for a polypeptide consisting of 478 amino acids (molecular mass, 52.6 kDa). The deduced amino acid sequence of this ORF was compared to known protein sequences in the GenBank, PI, and SWISS-PROT databases by the BLAST program (1). This ORF had strong homology to the *fliD* genes of other bacteria (Fig. 2) and was therefore called the *fliD* gene of *P. aeruginosa*. As shown in Fig. 2, the *P. aeruginosa* FliD (flagellar cap protein) was homologous to the FliDs of other bacteria throughout the ORF, except for a stretch of 12 amino acids (amino acids 196 to 207) which were found in the *P. aeruginosa* FliD but were absent from all the other cap proteins to which it was compared. The functional significance of these amino acids remains to be explored.

The second ORF consisted of 399 nucleotides, which is predicted to contain a gene that codes for a polypeptide consisting of 132 amino acids (molecular mass, 14.5 kDa). This gene has a strong homology to *fliS* genes of other bacteria, and it very likely represents the *P. aeruginosa fliS* homolog. The third ORF consisted of 381 nucleotides, which is predicted to contain a gene that codes for a polypeptide consisting of 126 amino acids (molecular mass, 13.9 kDa). This ORF is also homologous with the *fliS* genes of other bacteria, including that of *P. aeruginosa*. In other bacterial species, these genes are arranged as an operon, consisting of *fliDST* genes (6, 10). In *P. aeruginosa*, the *fliS* gene has apparently undergone a duplication, and the two homologs have evolved separately. The *P. aeruginosa fliS* and *orf126* share 38.5% identity and 59% similarity at the amino acid level. Whether the *fliT* gene is found at another chromosomal location and whether the product of the *orf126* gene performs the same function in *P. aeruginosa* as FliT does in other bacteria are not known at present. The fourth ORF (*orf96*) consisting of 288 nucleotides had no apparent homology to any known proteins in the database.

Upstream of the *fliDSorf126orf96* operon (Fig. 1), an ORF was identified which consisted of 384 nucleotides and was predicted to contain a gene that codes for a polypeptide consisting of 128 amino acids (molecular mass, 14 kDa). This ORF was homologous to the *flaG* genes of *Vibrio anguillarum* (16), *V. parahaemolyticus* (15), and *Bacillus subtilis* (6) and was therefore called the *flaG* gene.

Construction of a *fliD* mutant and its complementation. In order to examine the possible function of FliD, a chromosomal *fliD* mutant was constructed in *P. aeruginosa* PAK-NP by gene replacement. The *P. aeruginosa fliD* gene located on a 7.0-kb *Eco*RI-*Apa*I fragment was inactivated by inserting a gentamicin resistance gene cassette into an *Eco*RV site in the *fliD* gene (Fig. 1). The insertionally inactivated *fliD* gene on the plasmid was electroporated into PAK-NP, where it replaced the corresponding chromosomal copy of the *fliD* gene by double reciprocal recombination, giving rise to a *fliD* mutant strain, PAK-NPD. The replacement of the wild-type *fliD* in PAK-NPD was confirmed by Southern blot analysis (data not shown). Another *fliD* mutant, PAK-D, was constructed in *P. aeruginosa* PAK by the same strategy (data not shown). 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 *fliD* gene was located close to *fleQ*, which has been shown to be involved in motility and mucin adhesion in *P. aeruginosa* (3), we tested this mutant in motility and mucin adhesion assays. These results showed that the *fliD* mutant PAK-NPD was nonmotile (Fig. 3) and nonadhesive (Fig. 4). In order to confirm that the nonmotile and nonadhesive phenotype of PAK-NPD was indeed a result of inactivation of the *fliD* gene and was not due to polar effects on downstream genes, this gene was cloned as a 1.4-kb *Eco*RI-*Bam*HI fragment on a multicopy plasmid, and this construct (pPZ375D) was introduced into PAK-NPD by electroporation. Motility (Fig. 3) and mucin adhesion (Fig. 4) functions were restored in PAK-NPD by a *fliD* gene provided on a plasmid without the need for the downstream genes of the putative operon, while the vector did not complement the *fliD* mutation.

Overexpression of the *fliD* gene and purification of the FliD fusion protein. The *fliD* gene was overexpressed under the control of an inducible T7 promoter on a plasmid in *E. coli*. The complete *fliD* coding sequence was inserted as a PCR

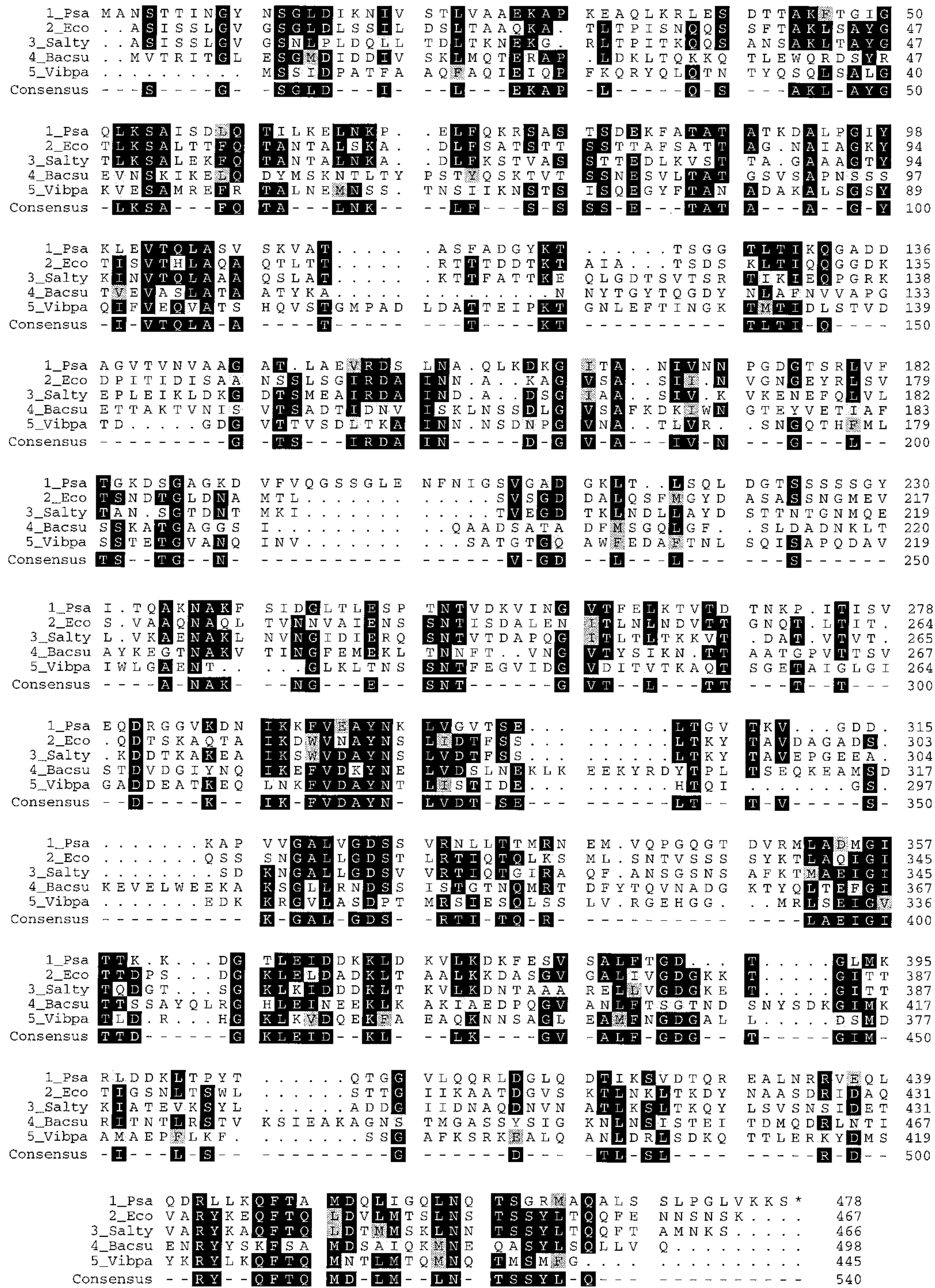

FIG. 2. Computer-generated alignment (Prettybox program [Richard Westerman, Purdue University]) of FliD of *P. aeruginosa* with homologous FliD proteins of other organisms. Dark shading shows identity of amino acids, while the two shades of gray show degrees of similarity (based on the *GCG Comparison Table* [Genetics Computer Group, University of Wisconsin, Madison]). Psa, *P. aeruginosa*; Eco, *E. coli*; Salty, *S. typhimurium*; Bacsu, *B. subtilis*; Vibpa, *V. parahaemolyticus.*

product into the *Nde*I-*Bam*HI sites of the plasmid pET15B. This resulted in an in-frame fusion of six histidine codons to the initiation codon of *fliD*. The resulting plasmid, pET15BD, and the vector control plasmid, pET15BD, were introduced into *E. coli* BL21(DE3), which has the T7 polymerase gene inserted into the chromosome. Bacterial cultures were grown and induced as explained in Materials and Methods. The induced and uninduced whole-cell extracts of *E. coli* BL21(DE3) containing pET15B (vector alone) or pET15BD (vector plus *fliD*) were analyzed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (10% polyacrylamide) (14) (Fig. 5). A new band representing the FliD fusion protein (His-FliD) (in-

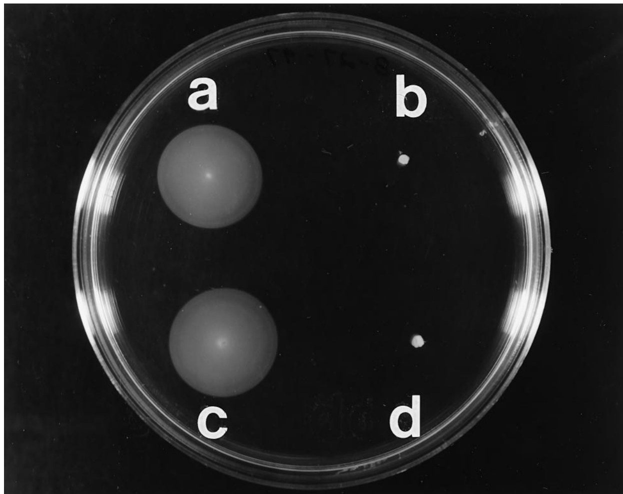

FIG. 3. Soft L agar (0.3%) plate showing the motility phenotype of different *P. aeruginosa* strains. (a) *pilA* mutant PAK-NP. (b) *pilA fliD* mutant PAK-NPD. (c) PAK-NPD containing pPZ375D. (d) PAK-NPD containing the multicopy vector pPZ375.

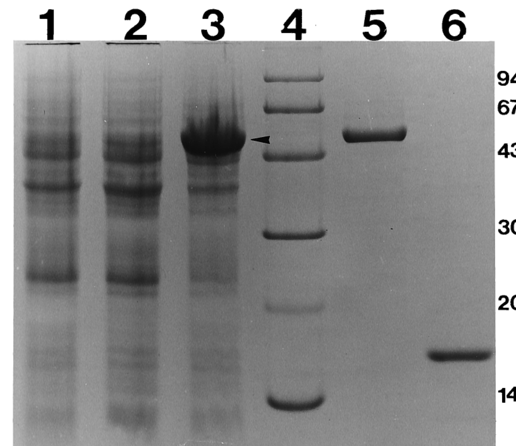

FIG. 5. Overexpression and purification of FliD. FliD was overexpressed in *E. coli* host BL21 with the expression vector pET15B (Novagen, Inc.). Lanes: 1, BL21(pET15B) vector control induced with 2 mM IPTG for 4 h at 37°C; 2, BL21 (pET15BD) vector with FliD insert uninduced; 3, BL21(pET15BD) vector with FliD insert induced with 2 mM IPTG for 4 h at 37°C; 4, Pharmacia low-molecular-mass (kilodaltons) markers; 5, approximately 400 ng of purified His-FliD protein; 6, approximately 400 ng of purified His-FlaG protein.

dicated by an arrow) was observed at the expected location (Fig. 5, lane 3).

The His-FliD protein was purified from the cell lysates of *E. coli* BL21(DE3) carrying pET15BD as described in Materials and Methods. A small aliquot of the purified His-FliD was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% polyacrylamide) (Fig. 5, lane 5). A single band was observed which migrated at the same location as the induced *fliD* gene product in the whole-cell extracts of *E. coli* BL21(DE3) carrying pET15BD (indicated by an arrow in Fig. 5). The 6× His tag attached to the purified FliD fusion protein was removed by thrombin cleavage as explained in Materials and Methods. The purified FliD protein without the 6× His tag was analyzed again by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% polyacrylamide) to ascertain the purity of the FliD protein. The cleavage presented a single band with mobility slightly faster than that of FliD carrying the N-terminal His tag (data not shown). The protein concentration of this purified preparation of the FliD protein was determined by using the Bio-Rad protein assay kit as described in Materials and Methods.

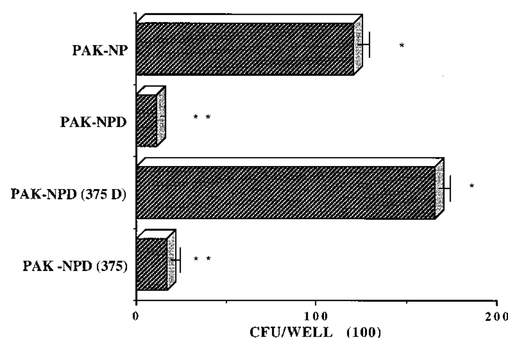

FIG. 4. Adhesion of *pilA* and *fliD* mutants of *P. aeruginosa* PAK to mucin. PAK-NP, *pilA* mutant of PAK; PAK-NPD, *pilA fliD* mutant of PAK; PAK-NPD (375D), PAK-NPD complemented with the complete *fliD* gene on a multicopy plasmid vector, pPZ375; PAK-NPD (375), PAK-NPD with the vector pPZ375. Differences shown by asterisks are significant (* versus **, $P < 0.001$) by Student's *t* test.

The *flaG* gene was overexpressed by the same expression system as that used for the *fliD* gene (data not shown). Subsequently, the FlaG protein was purified in the same manner as the FliD protein (Fig. 5, lane 6).

Effect of the FliD protein on mucin adhesion of strain PAK-NP. The purified FliD protein without the 6× His tag, was utilized in the in vitro competition assays to test whether it could compete with *P. aeruginosa* PAK-NP cells for binding to mucin. Two *P. aeruginosa* control proteins were used in this assay: purified pili (PilA protein) and purified FlaG protein. Based on our previous observations, it is clear that neither *pilA* (20) nor *flaG* (data not shown) is involved in flagellar formation or adhesion to mucin. *P. aeruginosa* pili were purified by the method of Frost and Paranchych (7a), while *P. aeruginosa* FlaG protein was expressed and purified exactly the same way as the FliD protein. Equimolar (1.83 μM) concentrations of purified proteins and their dilutions were allowed to bind to mucin for 30 min at 37°C, and the excess, unbound protein was washed away. The bacterial adhesion assay was then performed as explained in Materials and Methods.

As shown in Fig. 6, only FliD protein inhibited *P. aeruginosa* PAK-NP binding to mucin. At the same molar concentration, the two control proteins PilA and FlaG did not inhibit the binding of *P. aeruginosa* PAK-NP to mucin.

Regulation of the *fliD* gene. The *fliD* upstream sequence was visually analyzed for the presence of consensus σ^{28} (TAAA-N_{15}-GCCGATAA), σ^{54} (YTGGYAYR-N_3-YYTGCW), and σ^{70} (TTGACA-N_{17}-TATAAT) recognition sites. Figure 7 shows the 495-bp sequence, including the *fliD* promoter. No σ^{28} binding site could be detected in this region. However, four putative σ^{54} binding sites were located at nucleotides 74, 98, 148, and 334. Finally, a sequence homologous to the σ^{70} promoter was identified at nucleotides 418 (−10 box) and 394 (−35 box), (Fig. 7).

To understand the regulation of the *fliD* gene, the *fliD* promoter (495-bp sequence shown in Fig. 7) was fused with the promoterless *lacZ* gene, and the activity of the *fliD* promoter was measured in a number of *P. aeruginosa* strains. Table 2 shows the results of these β-galactosidase assays. The β-galactosidase activity in the wild-type PAK was 10 times that of the

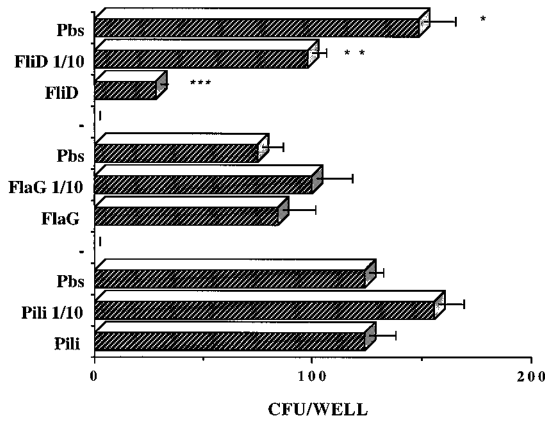

FIG. 6. Effect of FliD protein on *P. aeruginosa* binding to mucin. The mucin adhesion of *P. aeruginosa* PAK-NP in PBS (Pbs) is compared to mucin adhesion in the presence of different amounts of *P. aeruginosa* proteins. Two control *P. aeruginosa* proteins, FlaG and PilA, were used at the same molar concentrations as FliD to test for any nonspecific effects of a protein. Differences shown by asterisks are significant (* versus **, $P < 0.01$; * versus ***, $P < 0.001$) by Student's *t* test.

pDN19lacΩ vector control, demonstrating the existence of a promoter in the *fliD* upstream sequence. This promoter was insensitive to the transcriptional regulator FleR and the flagellar sigma factor σ^{28}, since the *fleR* and the σ^{28} (*fliA*) mutants had β-galactosidase activities as high as that of the wild-type PAK (Table 2). Both *rpoN* (PAK-N1G) and *fleQ* (PAK-Q) mutants had significantly reduced activity of the *fliD* promoter, indicating that the transcription from the *fliD* promoter requires σ^{54} (product of the *rpoN* gene) and the transcriptional activator FleQ. However, the *fliD* promoter still had a basal activity in the absence of either σ^{54} or FleQ, suggesting a dual regulation, perhaps involving σ^{70}, of the *fliD* promoter.

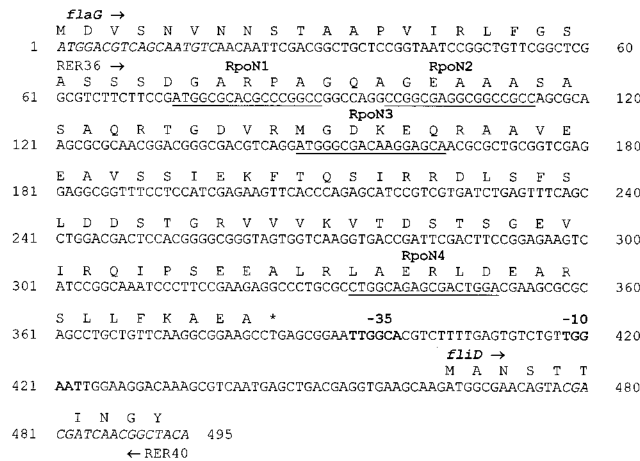

FIG. 7. Promoter region of *fliDSorf126orf96* operon. The promoter region of *fliDSorf126orf96* used for β-galactosidase assays included the complete coding sequence of the *flaG* gene and extended into the coding region of *fliD*. The primers used for the β-galactosidase experiments, RER36 and RER40, are shown. In the upstream sequence, the four putative RpoN binding sites (YTGGYAYR-N_3-YYTGCW) are underlined and the −10 and −35 elements of the σ^{70} binding site (TTGACA-N_{17}-TATAAT) are in boldface.

TABLE 2. Control of the *fliD* promoter in *P. aeruginosa*

Host strain	Genetic background	Mean β-galactosidase activity (Miller units) ± SD	
		Vector alone	*fliD* promoter
PAK	Wild type	112 ± 15	1,154 ± 9
PAK-N1G	*rpoN* mutant	102 ± 13	166 ± 8
PAK-Q	*fleQ* mutant	65 ± 16	331 ± 4
PAK-RG	*fleR* mutant	87 ± 1	1,107 ± 48
MS540	*fliA* mutant	112 ± 34	1,798 ± 73

DISCUSSION

The adhesion of *P. aeruginosa* to mucin is one of the earlier steps in the process of *P. aeruginosa* colonization of the human airways. In pursuit of a specific adhesin responsible for this interaction, we have discovered that a structural component of the flagellum, FliD, exhibits a direct association with mucin. A polar insertional mutation in the *P. aeruginosa fliD* gene abolished the motility and mucin adhesion functions of the organism. Both the motility and the mucin adhesion functions were restored when the *P. aeruginosa fliD* gene alone was provided on a multicopy plasmid. In vitro competition assays demonstrated that the purified FliD protein was capable of specifically inhibiting the association of *P. aeruginosa* cells with mucin.

The *fliD* genes of *E. coli* and *Salmonella typhimurium* encode the filament cap protein, also called the hook-associated protein 2 (HAP2), and form an operon, *fliDST* (10, 12). The role of FliD in these organisms is to facilitate the polymerization of endogenous flagellin at the tips of the growing flagellar filaments. However, there are conflicting reports regarding the role played by the *fliS* and *fliT* genes in flagellar formation (6, 10, 36). The *fliS* gene has been implicated as a chaperone involved in the export of flagellin (36), while *fliT* apparently has no effect on flagellar formation. All three genes of the *fliDST* operon have been shown to negatively regulate the export of FlgM, the flagellum-specific anti-sigma factor (37). *fliD* mutants of *E. coli* and *S. typhimurium* secrete excess amounts of FlgM into the culture medium. Our studies show that the gene arrangement of the *fliDST* operon in *P. aeruginosa* is quite different. The *fliT* gene seems to be absent from this operon, and instead there is a duplication of the *fliS* gene. The fact that the *fliD* gene alone could complement the motility and mucin adhesion defects of the *P. aeruginosa fliD* mutant suggests that the *fliS* and *fliT* genes are not important for these two functions. Whether there is excess excretion of FlgM in the *P. aeruginosa fliD* mutant as in the *fliD* mutants of *E. coli* and *S. typhimurium* remains to be tested. However, by analogy, we expect a similar phenotype of this mutant. The adhesion-negative phenotype of the *P. aeruginosa fliD* mutant is probably not due to excess export of FlgM (lower intracellular concentration of FlgM and hence upregulation of class 2 and class 3 genes [e.g., *fliC*]), since our earlier reports have shown that the flagellin (*fliC*)-negative mutant is adhesive to mucin (24).

The alignment of the deduced amino acid sequence of the *P. aeruginosa fliD* gene with those of the other FliD proteins shows that the structure of this protein is conserved through the entire ORF. However, it is interesting that a stretch of 12 amino acids (amino acids 196 to 207) was absent from the other FliD proteins. The significance of this stretch of amino acids is not clear at present.

Our studies indicate that the regulation of the *fliD* gene in *P. aeruginosa* is quite different from that in other organisms. It

has been suggested that the *fliD* gene of *S. typhimurium* belongs to class 3A, since it is dually regulated by σ^{28} (*fliA*) and the master regulator FlhD (13). However, our analysis of the sequence upstream of the *fliD* gene revealed that there is no σ^{28} binding sequence present in this region. Consistent with these data, the promoter studies showed that the transcription of the *fliD* gene was independent of σ^{28}. Furthermore, we have previously shown that a σ^{28}-deficient *P. aeruginosa fliA* mutant still adheres to mucin (24); if the *fliD* gene were σ^{28} regulated, then one would have expected a loss of adhesion in the *fliA* mutant. The promoter studies also demonstrated the requirement of the transcriptional regulator, FleQ, and the sigma factor RpoN. The transcriptional regulator FleQ belongs to the group of transcriptional activators which work in concert with RpoN and was previously shown to be involved in the regulation of motility and mucin adhesion in *P. aeruginosa* (3). Upstream of the *fliD* gene, we detected several putative σ^{54} binding sites and a strong σ^{70} promoter as well. The precise promoter assignment still awaits determinations based on the mapping of the transcriptional start site for this gene. Given the observed basal-level expression of *fliD-lacZ* fusions in *fleQ* and *rpoN* mutant backgrounds, it is conceivable that *fliD* is transcribed by two species of RNA polymerase, one containing σ^{54} and the other containing σ^{70}. FleQ could be directly regulating *fliD* gene expression in conjunction with RpoN. Alternatively, FleQ could regulate the expression of another regulator which may be involved in transcription of *fliD* with either σ^{54} or σ^{70}. This dual regulation would imply that the same gene is expressed under different conditions, responding to different needs of the bacterial cell, such as motility and adherence.

How does a flagellar cap protein function in adherence? One possible scenario involves an initial interaction of the flagellar tip with mucin. In fact, scanning electron photomicrographs of the surface of CF epithelia suggest that this might be the case in reality (27). This fragile interaction can be then strengthened by further attachment with additional FliD proteins located in the outer membrane, synthesized as a consequence of flagellar breakage following the initial binding step. Alternatively, other signals provided by the host may direct the synthesis of additional FliD exclusively for function in mucin adherence. This could explain the existence of two regulatory mechanisms for *fliD* expression, as implied by our analysis of the *fliD* gene promoter.

Interestingly, FliD has been implicated in virulence of *Proteus mirabilis*. A *fliD* mutant of *P. mirabilis* was shown to be attenuated in the colonization of the urinary tract and in virulence in a mouse model of ascending urinary tract infection (18). Further studies are geared towards finding the role for *P. aeruginosa fliD* in vivo. In summary, we postulate that the flagellar cap protein FliD is directly involved in the binding of *P. aeruginosa* to mucin. These findings will allow us to answer questions pertaining to the structure and role of this protein in the colonization process. Identification of the precise mucin binding site and finding the receptor that is recognized by this adhesin are among the challenges of the future. This information would prove useful in the development of new approaches to the prevention of colonization of the respiratory tract by *P. aeruginosa.*

ACKNOWLEDGMENTS

We acknowledge the Interdisciplinary Center for Biotechnology Research (ICBR) computer facilities of the University of Florida for use of the VAX computers for DNA sequence analyses. This work was supported by NIH grants HL33622 (R.R.) and AI32624 (S.L.).

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. **Arora, S. K., B. W. Ritchings, E. C. Almira, S. Lory, and R. Ramphal.** 1996. Cloning and characterization of *Pseudomonas aeruginosa fliF*, necessary for flagellar assembly and bacterial adherence to mucin. Infect. Immun. **64:** 2130–2136.
3. **Arora, S. K., B. W. Ritchings, E. C. Almira, S. Lory, and R. Ramphal.** 1997. A transcriptional activator, FleQ, regulates mucin adhesion and flagellar gene expression in *Pseudomonas aeruginosa* in a cascade manner. J. Bacteriol. **179:**5574–5581.
4. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
5. **Brun, Y. V., and L. Shapiro.** 1992. A temporally controlled sigma-factor is required for polar morphogenesis and normal cell division in *Caulobacter*. Genes Dev. **6:**2395–2408.
6. **Chen, L., and J. D. Helmann.** 1994. The *Bacillus subtilis* σ^{D}-dependent operon encoding the flagellar proteins FliD, FliS, and FliT. J. Bacteriol. **176:**3093–3101.
7. **Chi, E., T. Mehl, D. Nunn, and S. Lory.** 1991. Interaction of *Pseudomonas aeruginosa* with A549 pneumocyte cells. Infect. Immun. **59:**822–828.

7a. **Frost, L. S., and W. Paranchych.** 1977. Composition and molecular weight of pili purified from *Pseudomonas aeruginosa* K. J. Bacteriol. **131:**259–269.

8. **Inouye, S., M. Kimoto, A. Nakazawa, and T. Nakazawa.** 1990. Presence of flagella in *Pseudomonas putida* is dependent on the *ntrA* (*rpoN*) gene. Mol. Gen. Genet. **221:**295–298.
9. **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.
10. **Kawagishi, I., V. Muller, A. W. Williams, V. M. Irikura, and R. M. Macnab.** 1992. Subdivision of flagellar region III of the *Escherichia coli* and *Salmonella typhimurium* chromosomes and identification of two additional flagellar genes. J. Gen. Microbiol. **138:**1051–1065.
11. **Kinsella, N., P. Guerry, J. Cooney, and T. J. Trust.** 1997. The *flgE* gene of *Campylobacter coli* is under the control of the alternative sigma factor σ^{54}. J. Bacteriol. **179:**4647–4653.
12. **Kutsukake, K., Y. Ohya, S. Yamaguchi, and T. Iino.** 1988. Operon structure of flagellar genes in *Salmonella typhimurium*. Mol. Gen. Genet. **214:**11–15.
13. **Kutsukake, K., and N. Ide.** 1995. Transcriptional analysis of the *flgK* and *fliD* operons of *Salmonella typhimurium* which encode flagellar hook-associated proteins. Mol. Gen. Genet. **247:**275–281.
14. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
15. **McCarter, L. L.** 1995. Genetic and molecular characterization of the polar flagellum of *Vibrio parahaemolyticus*. J. Bacteriol. **177:**1595–1609.
16. **McGee, K., P. Hörstedt, and D. L. Milton.** 1996. Identification and characterization of additional flagellin genes from *Vibrio anguillarum*. J. Bacteriol. **178:**5188–5198.
17. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Mobley, H. L. T., R. Belas, V. Lockatell, G. Chippendale, A. L. Trifillis, D. E. Johnson, and J. W. Warren.** 1996. Construction of a flagellum-negative mutant of *Proteus mirabilis*: effect of internalization by human renal epithelial cells and virulence in a mouse model of ascending urinary tract infection. Infect. Immun. **64:**5332–5340.

18a. **Paranchych et al.**

19. **Ramphal, R., and M. Pyle.** 1983. Adherence of mucoid and nonmucoid *Pseudomonas aeruginosa* to acid-injured tracheal epithelium. Infect. Immun. **41:**345–351.
20. **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.
21. **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.
22. **Saiman, L., K. Ishimoto, S. Lory, and A. Prince.** 1990. The effect of piliation and exoproduct expression on the adherence of *Pseudomonas aeruginosa* to respiratory epithelial monolayers. J. Infect. Dis. **161:**541–548.
23. **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.
24. **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.
25. **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.
26. **Smith, A. W., and B. H. Iglewski.** 1989. Transformation of *Pseudomonas aeruginosa* by electroporation. Nucleic Acids Res. **17:**10509.
27. **Smith, J. J., S. M. Travis, E. P. Greenberg, and M. J. Welsh.** 1996. Cystic

fibrosis airway epithelia fail to kill bacteria because of abnormal airway surface fluid. Cell **85:**229–236.
28. **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.
29. **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.
30. **Totten, P. A., and S. Lory.** 1990. Characterization of the type a flagellin gene from *Pseudomonas aeruginosa* PAK. J. Bacteriol. **172:**7188–7199.
31. **Totten, P. A., J. C. Lara, and S. Lory.** 1990. The *rpoN* gene product of *Pseudomonas aeruginosa* is required for expression of diverse genes, including the flagellin gene. J. Bacteriol. **172:**389–396.
32. **Tsang, K. W., A. Rutman, E. Tanaka, V. Lund, A. Dewar, P. J. Cole, and R. Wilson.** 1994. Interactions of *Pseudomonas aeruginosa* with human respiratory mucosa *in vitro*. Eur. Respir. J. **7:**1746–1753.
33. **Vishwanath, S., and R. Ramphal.** 1984. Adherence of *Pseudomonas aeruginosa* to human tracheobronchial mucin. Infect. Immun. **45:**197–202.
34. **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.
35. **Woodward, H., B. Horsey, V. P. Bhavanandan, and E. A. Davidson.** 1982. Isolation, purification, and properties of respiratory glycoproteins. Biochemistry **21:**694–701.
36. **Yokoseki, T., K. Kutsukake, K. Ohnishi, and T. Iino.** 1995. Functional analysis of the flagellar genes in the *fliD* operon of *Salmonella typhimurium*. Microbiology **141:**1715–1722.
37. **Yokoseki, T., T. Iino, and K. Kutsukake.** 1996. Negative regulation by FliD, FliS, and FliT of the export of the flagellum-specific anti-sigma factor, FlgM, in *Salmonella typhimurium*. J. Bacteriol. **178:**899–901.

Editor: P. E. Orndorff