INFECTION AND IMMUNITY, Dec. 1995, p. 4868–4876 Vol. 63, No. 12
0019-9567/95/$04.00+0


# Cloning and Phenotypic Characterization of *fleS* and *fleR*, New Response Regulators of *Pseudomonas aeruginosa* Which Regulate Motility and Adhesion to Mucin

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Received 28 July 1995/Returned for modification 7 September 1995/Accepted 26 September 1995

**This work has identified two genes (designated *fleS* and *fleR*) in *Pseudomonas aeruginosa* which are highly homologous to members of the subclass of two-component systems involved in transcriptional regulation of a diverse array of genes from $\sigma^{54}$ promoters. The genes are located upstream from *fliE*, a flagellar gene of *P. aeruginosa*, and they are arranged in a putative *fleSR* operon. FleS has a predicted molecular mass of 43.87 kDa and shows strong homology to histidine kinases which in other two-component systems have been shown to be sensor proteins. FleR has a predicted molecular mass of 51.26 kDa and is homologous to other regulatory proteins that bind to specific upstream activating elements to enhance transcription of genes with $\sigma^{54}$ promoters. The *fleSR* system is believed to control both flagellar synthesis and adhesion to mucin. Several lines of evidence are presented. (i) A nonpiliated mutant of *P. aeruginosa* PAK containing a gentamicin cassette in *fleR* is nonmotile and nonadhesive. (ii) The *fleR* mutant regained motility and adhesion when complemented with a wild-type copy of *fleR*. (iii) A Western blot (immunoblot) of the *fleR* mutant showed no synthesis of flagellin, and electron microscopy of the *fleR* mutant confirmed the lack of flagella. Previous work has shown that flagellar mutants with mutations in *fliA* ($\sigma^{28}$) or *fliC* (the structural gene for flagellin) retain adhesion; therefore, these new observations suggest that FleSR regulates both the expression of flagella and the nonpilus adhesin(s) for mucin or that one of the flagellar proteins (other than flagellin) may be responsible for adhesion to mucins.**

The opportunistic pathogen *Pseudomonas aeruginosa* is the most frequent colonizer of the respiratory tracts of patients with cystic fibrosis. The infection and the subsequent inflammatory response result in the severe pulmonary compromise which is characteristic of most cases of this disease. The pathogenesis of the various steps which lead to this outcome have not been fully elucidated, but the infection is clearly initiated by adhesion to and colonization of the airways by *P. aeruginosa*. The evidence available from studies of clinical material suggests that the mucus secretions of these patients are the principal sites of colonization (3, 10, 27) and therefore the site of adhesion of this organism. These clinical observations have also been supported by demonstration of specific binding of *P. aeruginosa* to human airway mucin in vitro (35). While binding of *P. aeruginosa* to injured airway epithelial cells (7, 23) and to intact cells in culture has been demonstrated (25), direct evidence for bacterial adhesion to airway cells of infected individuals has not been found to date.

Several bacterial surface proteins on nonmucoid strains have been implicated in the initial interaction of *P. aeruginosa* with the host. Pili (fimbriae) were the first *Pseudomonas* components identified as adhesins (24, 37), but they appear to be principally mediating the adhesion to cells (22). Nonpilus adhesins, however, appear to be the mediators of adhesion to mucin (21, 22). Little is known about the nature of these nonpilus adhesins, other than the observations that their expression is controlled by the alternative sigma factor RpoN (22) and that the adhesins recognize certain disaccharides found in mucins (21). Recent findings also confirm that the expression of these adhesins is linked to flagellar expression since transposon insertions into at least two flagellar genes resulted in the simultaneous loss of motility and adhesion (28). It is clear that flagellin itself is not the nonpilus adhesin, since mutations in *fliC*, the flagellin structural gene, or in *fliA*, the regulator of flagellin expression, do not cause a loss of adherence (28). These observations therefore suggest several possibilities: (i) one of the genes in the flagellar biosynthetic pathway encodes a nonpilus adhesin, (ii) the nonpilus adhesin(s) and some components of the flagellar system are coregulated, (iii) export of the adhesin(s) to the bacterial cell surface uses the flagellar export pathway. We cannot yet distinguish among these alternatives, but adhesion and motility are apparently coregulated at the transcriptional level, because a mutation in *rpoN*, which leads to the loss of mucin adherence, also results in lack of flagellar expression. Most RpoN-controlled systems work in concert with a group of two-component regulators, the NtrC subfamily (13). In *P. aeruginosa*, for example, the expression of the pilin adhesin is controlled by the PilS/PilR regulatory pair (4). We have therefore undertaken a molecular study of the regulatory network which controls expression of the nonpilus adhesin(s) of *P. aeruginosa*. In this manuscript we report the identification of a new two-component regulatory system which controls both motility and adhesion to mucin. The regulatory component is highly homologous to the NtrC subfamily of transcriptional activators. In particular, there is striking homology of the regulator to FlbD of *Caulobacter crescentus* which is known to regulate the synthesis of three specific classes of flagellar genes of this organism (20). To date,

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TABLE 1. Bacterial strains and plasmids used in this study

| Strain, vector, or plasmid | Genotype or description | Source or reference |
|---|---|---|
| *P. aeruginosa* strains | | |
| PAK | Wild-type clinical isolate | D. Bradley |
| PAK-NP | Nonpiliated PAK (*pilA* mutant) | 25 |
| PAK-FG | PAK-NP with chromosomal inactivation of *fleR* | This study |
| PAK-RG | PAK with chromosomal inactivation of *fleR* | This study |
| PAK-FG (KS) | PAK-FG complemented with *fleR* on a low-copy-number plasmid, pMMB67KS, which restores wild-type phenotype | This study |
| PAK-FG (67) | PAK-FG containing the vector alone, pMMB67EH, which does not restore wild-type phenotype | This study |
| PAK-RG (KS) | PAK-RG complemented with fleR on a low-copy-number plasmid, pMMB67KS, which restores wild-type phenotype | This study |
| PAK-RG (67) | PAK-RG containing the vector alone, pMMB67EH, which does not restore wild-type phenotype | This study |
| PAK-RR20 | PAK-NP with Tn*5G* insertional inactivation of *fliF* gene 3′ to *fleR* gene | 27 |
| Cloning vector | | |
| pMMB67EH | Broad-host-range cloning vector, $Chl^r$ | S. Lory |
| pUC19 | *E. coli* cloning vector, $Amp^r$ | |
| pBluescript KS(+) | *E. coli* cloning vector, $Amp^r$ | Stratagene, Inc. |
| pVK102 | Broad-host-range cloning cosmid, $Kan^r$ | S. Lory |
| Recombinant plasmids | | |
| pRR194 | pVK102 with ca. 20-kb partial *Sal*I insert from PAK genomic DNA | This study |
| pMMB67KS | pMMB67EH with 2.1-kb *Kpn*I-*Sal*I insert from pRR194 containing the *fleR* gene | This study |
| pUBK4.5 | pUC19 containing a 4.5-kb *Kpn*I insert from pRR194 | This study |
| pUR194Sal | pUC19 containing a 3.1-kb *Sal*I insert from pRR194 | This study |

no homologs of these *P. aeruginosa* genes have been found in the extensively characterized *Escherichia coli* and *Salmonella typhimurium* flagellar systems. The nucleic acid and protein sequences, homologies, and some phenotypic characteristics of this system are described.

## MATERIALS AND METHODS

**Enzymes and reagents.** Enzymes were purchased from Life Technologies (Gaithersburg, Md.) and New England Biolabs (Beverly, Mass.). Reagents were purchased from Sigma Chemical Co. (St. Louis, Mo.) and Amresco Inc. (Solon, Ohio). The Genius nonradioactive detection kit is a product of Boehringer-Mannheim (Indianapolis, Ind.).

**Bacterial strains and plasmids.** A list of all strains and plasmids used in this work is presented in Table 1. Bacterial strains were grown in Luria broth or Terrific broth (26) for plasmid or cosmid DNA preparation. The appropriate antibiotics were used to maintain the plasmids or chromosomal insertions in the following concentrations: ampicillin, 200 μg/ml for pUC19 and pBluescript-based plasmids; kanamycin, 30 μg/ml for cosmids; chloramphenicol, 30 μg/ml for plasmids in *E. coli* and 300 μg/ml for plasmids in *P. aeruginosa*; tetracycline, 50 μg/ml for strain PAK-NP; gentamicin, 15 μg/ml for plasmids in *E. coli* and 50 μg/ml for plasmids and chromosomal insertions in strains of *P. aeruginosa*. Motility studies were done on 0.3% Luria broth agar containing the appropriate antibiotic when necessary.

**Electroporation.** DNA was introduced into *P. aeruginosa* by electroporation following the protocol of Smith and Iglewski (30). Plasmids were purified by the alkaline lysis or boiling method (16). For gene replacements, a pUC19 plasmid carrying an insertionally inactivated *fleR* gene was linearized at a restriction site within the vector and was concatamerized by ligation at a high concentration of DNA (>1.0 μg/20-μl ligation reaction). After the ligation, the DNA was ethanol precipitated, resuspended in distilled water, and electroporated into the appropriate strain of *P. aeruginosa.*

**Motility assay.** Strains were grown overnight at 37°C on fresh plates containing the appropriate antibiotics and then transferred to 0.3% soft agar plates containing the same antibiotics. These plates were then incubated at 37°C, and the resulting bacterial growth was examined for motility after 18 h.

**Electron microscopy.** Cells were negatively stained by mixing an equal volume of the bacterial suspension in 50 mM sodium phosphate–10 mM $MgCl_2$ with a 2% aqueous solution of phosphotungstic acid (pH 7.4). A drop was spotted on a Parlodion (Mallinckrodt, Inc., St. Louis, Mo.) carbon-coated grid (300 mesh), and after 15 s, the liquid was withdrawn by being drained against filter paper. Samples were examined with a JEOL 100B transmission electron microscope operating at 60 kV.

**Adhesion assay.** Bacterial adherence to mucin was tested by using static cultures grown overnight in tryptic soy broth containing the appropriate antibiotic. The inoculum was adjusted spectrophotometrically to an optical density at 580 nm of 0.05, and the CFU were determined after plating on MacConkey agar. The range of bacteria used was between $2 \times 10^7$ and $5 \times 10^7$ CFU/ml. The microtiter plate wells were coated with respiratory mucins that were prepared as previously described (38), and the remainder of the adhesion assay was performed as previously described (35). Wells without mucin coating were used as controls for nonspecific binding.

**Sequencing.** DNA sequencing was accomplished by using the Taq DyeDeoxy Terminator and DyePrimer cycle sequencing protocols developed by Applied Biosystems (Perkin-Elmer Corp., Foster City, Calif.) using fluorescence-labelled dideoxynucleotides and primers, respectively. The labelled extension products were analyzed on an Applied Biosystems Model 373A DNA Sequencer. Double-stranded sequences were aligned and assembled by using programs in the Sequencher software package (Gene Codes Corp., Ann Arbor, Mich.).

The nucleotide sequence of the *fleSR* locus reported in this study was submitted to GenBank and assigned the accession number L41213.

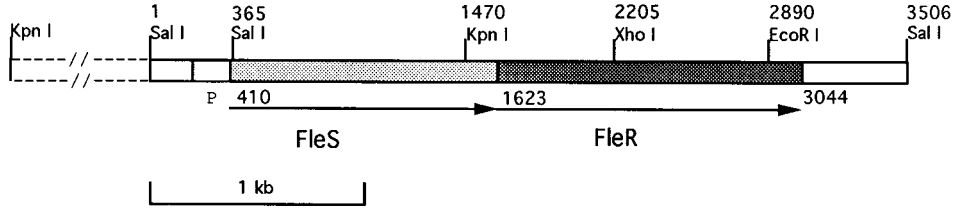


FIG. 1. Map of the *fleSR* region, showing the location of the promoter (P) of the putative *fleSR* operon and the two open reading frames, *fleS* and *fleR*. The unique *Xho*I site in *fleR* was used to insertionally inactivate *fleR* with a gentamicin gene cassette.

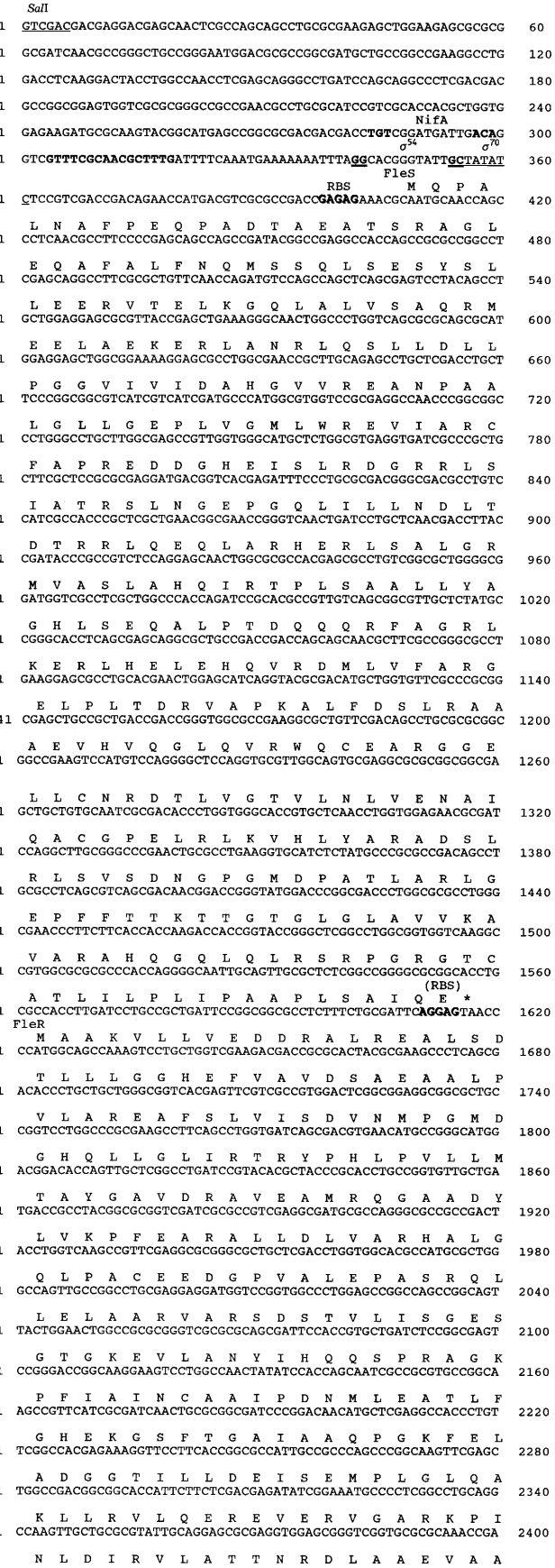


FIG. 2. Nucleotide sequence of 3.5 kb of DNA from *P. aeruginosa* containing the putative *fleSR* operon and promoter region. Also shown are the deduced amino acid sequences of the two open reading frames, FleS and FleR. Putative NifA, IHF, and $\sigma^{54}$ and $\sigma^{70}$ binding sites are shown in the promoter region. Potential ribosome binding sites precede the putative methionine start sites of both FleS and FleR. A transcriptional stop site is shown downstream of the *fleR* TGA stop codon.

**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 (15), and the proteins were electrophoretically transferred to nitrocellulose (34). The filters were treated with 2% nonfat dry milk in Tris-buffered saline, incubated with antisera, washed, and probed with horseradish peroxidase-labelled anti-mouse immunoglobulin G (IgG) and IgM (Kirkegaard and Perry, Gaithersburg, Md.). Monoclonal antiflagellin was kindly provided by A. Siadak, Oncogen, Seattle, Wash.

## RESULTS

**Genetic techniques.** The adhesion- and motility-defective strain PAK-RR20 has been previously described (28). This strain was isolated by Tn*5G* transposon mutagenesis of PAK-NP and selecting for the loss of adhesion to mucin (28). The Tn*5G* transposon with about 1.75 kb of flanking DNA was cloned on an *Eco*RI fragment to obtain a probe. A cosmid bank of PAK DNA in the vector pVK102 (11) was probed with the radiolabelled Tn*5G* probe, and a single probe-reactive clone was isolated. This clone, pRR194, contained about 18 to 20 kb of PAK DNA. The cosmid DNA was digested with several restriction enzymes and probed by Southern blot analysis using the Genius nonradioactive detection kit, following the manufacturer's instructions (Boehringer-Mannheim). Several *Sal*I fragments hybridized with the probe DNA, the largest being 3.1 kb. This fragment was used to obtain DNA sequence information. Since this *Sal*I fragment did not appear to contain the promoter of the first open reading frame, the 4.5-kb *Kpn*I fragment, which overlaps with the 3.1-kb *Sal*I fragment at the 5′ end of the first open reading frame, was used to obtain additional upstream sequence (Fig. 1).

**DNA sequence and amino acid analysis.** The nucleotide sequence of 3.5 kb of DNA from pRR194 and the deduced amino acid sequence revealed a putative promoter region, two complete open reading frames, and an additional downstream sequence (Fig. 2). The first open reading frame begins with an

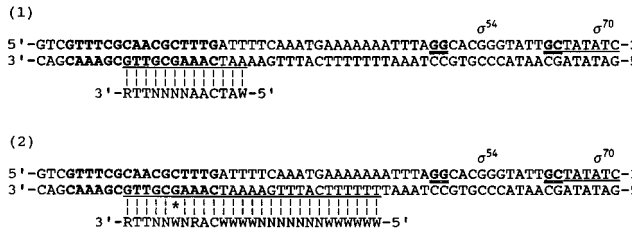


FIG. 3. Portion of *fleS* promoter region from bp 301 to 360, showing overlap between a palindrome and the two consensus sequences of IHF. Both sequences 1 and 2 show the palindrome (in bold). Sequence 1 shows perfect match with the IHF consensus binding site described by Friedman (8), and sequence 2 shows a 26-of-27-nucleotide with the IHF binding site described by Kur et al. (12). W, dATP or dTTP; N, any deoxynucleoside triphosphate; R, dATP or dGTP. The putative $\sigma^{54}$ and $\sigma^{70}$ binding sites are also shown.

ATG at nucleotide 410 and is predicted to encode a protein of 402 amino acids, or 43.87 kDa. A second reading frame begins with an ATG codon at nucleotide 1623 and is predicted to encode a protein of 473 amino acids or 51.26 kDa. Since these two reading frames are located adjacent to each other, with the ribosome binding site of ORF2 located within the coding sequence of ORF1 and no obvious promoter elements upstream from ORF2, it is very likely that these two genes are transcribed as an operon. The upstream region of ORF1 contains a putative ribosome binding site (GAGAG) and several potential transcriptional regulatory sequences. These include a potential $\sigma^{70}$ promoter motif (TATATC), a GG-$N_{10}$-GC sequence, which is the promoter sequence for genes transcribed by $\sigma^{54}$-containing RNA polymerase, a long A/T region, and a palindrome which overlaps with a putative integration host factor (IHF) DNA-binding site (Fig. 3) (8, 12). Finally this region also contains a sequence, TGT-$N_{10}$-ACA, which is the recognition site for the nitrogen fixation transcriptional regulator NifA (5). The existence of these latter three motifs suggests that the genes encoded by this putative operon may be under control of $\sigma^{54}$ and its cognate transcriptional activators. However, the presence of the putative $-10$ promoter element, in the absence of recognizable $-35$ sequence, leaves the possibility that these genes are positively regulated and transcribed by $\sigma^{70}$ (19).

A search of the GenBank database using the BLAST program (1) revealed that these two open reading frames possessed considerable homology to the family of two component regulatory systems. ORF1 was homologous to the sensor kinases, and ORF2 was homologous to the response regulators. Neither of the genes has been described for *P. aeruginosa*. On the basis of phenotypic characterization (described below), which indicates that these genes are involved in the expression of flagella, these two open reading frames are being called *fleS* and *fleR* for flagellar expression sensor and regulator, respectively. There are no previously described *E. coli* or *S. typhimurium* homologs of *fleS* or *fleR* which control synthesis of flagella; therefore, the current flagellar gene nomenclature system cannot be used (9). Moreover, the highest level of similarity of the FleR protein (Table 2) is with FlbD of *C. crescentus*, a regulator of flagellum synthesis, which is part of the *FliF* operon in *C. crescentus* (20). FlbD is a positive regulator of the hook operon as well as a negative regulator of the *fliF* gene (20). However, FlbD is not known to have a cognate sensor protein.

**FleS and FleR are members of two-component regulatory systems.** Computer analysis revealed that FleS and FleR are homologous to the family of protein pairs involved in signal transduction. These two-component regulatory systems include sensory components, homologs of FleS (Fig. 4 and Table 2), and transcriptional factors homologous with FleR (Fig. 5 and Table 2). The mechanism of signal transduction involves autophosphorylation of the sensor followed by transfer of the phosphate to the regulator (32). FleS, the sensory component, contains the conserved histidine (His-191) and asparagine (Asn-302) residues and the conserved pattern of glycines (Gly-332, -334, -356, -358) (4). FleR is homologous to several regulator proteins which have been shown to activate transcription from $\sigma^{54}$ promoters. FleR contains all of the domains found in this family of proteins, including (i) the amino-terminal acid pocket (Glu-10, Asp-11, Asp-53) which contains the site of phosphorylation at Asp-53 (32), (ii) the c1 to c7 central domains involved in ATP binding and activation of $\sigma^{54}$ (18), and (iii) the carboxy-terminal helix-turn-helix DNA-binding motif (ca. residues 425 to 470) (6). Of note, there are six amino acids (Ser-48, Ser-137, Ala-197, Thr-198, Ser-292, Arg-299) in the FleR sequence which do not conform to the sequence in regions which are highly conserved. These regions have been sequenced multiple times in both directions, with no obvious sequence anomalies being found, and the codons conform to the codon usage table for *P. aeruginosa* (36). Unlike most sensors of the two-component regulatory systems, FleS lacks stretches of hydrophobic amino acids which would form transmembrane domains. The hydrophobicity analysis according to Kyte and Doolittle (14) indicates a relatively hydrophilic protein (data not shown), and therefore it is likely that FleS is a soluble cytoplasmic protein. Similar analysis of FleR (data not shown) suggests that it is also a cytoplasmic protein, as is the case of all regulators of this family.

**FleR control of motility and adherence to mucin.** In order to examine the possible function of FleR, a chromosomal knockout of the *fleR* gene was created as follows. A 2.036-kb *Kpn*I-*Sal*I fragment containing the *fleR* gene was cloned into pUC19, and a gentamicin resistance cassette was introduced into the *Xho*I site within the *fleR* gene (Fig. 1). The resulting plasmid was then linearized and electroporated into strain PAK-NP, and allelic replacement of the chromosomal copy of *fleR* with the gentamicin-containing mutant copy was demonstrated by Southern hybridization (data not shown). The mutant strain, PAK-FG, grew normally in Luria broth and M9 medium and did not show any unusual colony morphology on L-agar plates. Since additional downstream sequencing had shown that *fleR* was located directly upstream of a flagellar operon (2), the PAK-FG mutant was tested for motility. Figure 6 shows that the *fleR* mutant PAK-FG was nonmotile. The motility defect was due to the absence of flagella as shown by electron microscopy (Fig. 7). When extracts of PAK-FG were analyzed by Western immunoblots with anti-flagellin antibody (33), no

TABLE 2. Homology of FleS and FleR to related proteins

| Protein and related protein | Organism | % Similarity | % Identity |
|---|---|---|---|
| FleS | | | |
| HydH | *E. coli* | 54.8 | 34.8 |
| NtrB | *Bradyrhizobium* sp. | 52.1 | 27.7 |
| PilS | *P. aeruginosa* | 51.3 | 26.5 |
| NtrB | *K. pneumoniae* | 50.0 | 24.9 |
| FleR | | | |
| FlbD | *C. crescentus* | 63.3 | 43.7 |
| PilR | *P. aeruginosa* | 63.2 | 42.0 |
| HydG | *E. coli* | 61.1 | 41.7 |
| NtrC | *K. pneumoniae* | 59.8 | 39.7 |

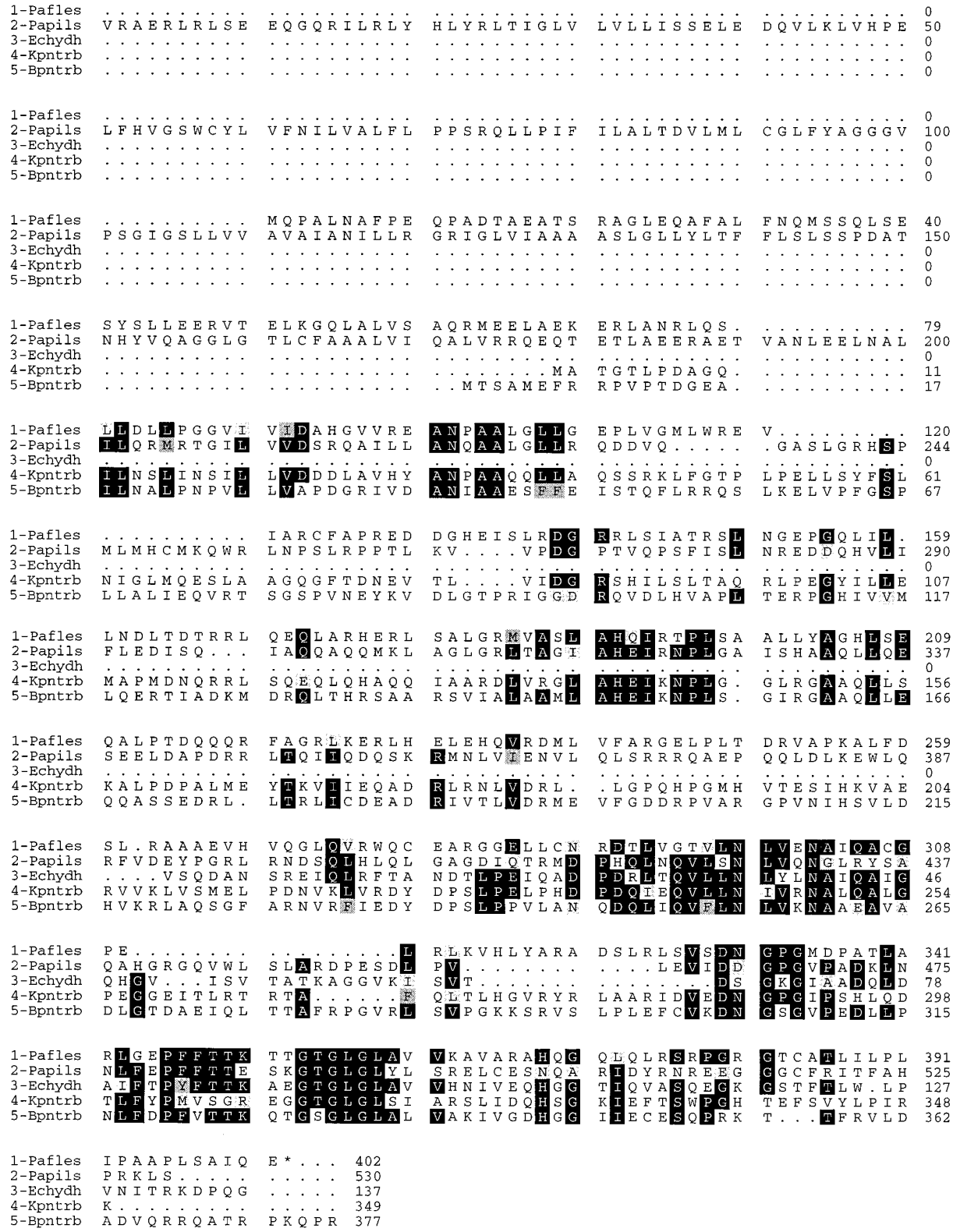


FIG. 4. Computer-generated alignment (Prettybox program, Richard Westerman, Purdue University) of FleS with homologous sensor proteins from other two-component systems. Dark shading shows identity, while light shading indicates similarity among amino acid residues.

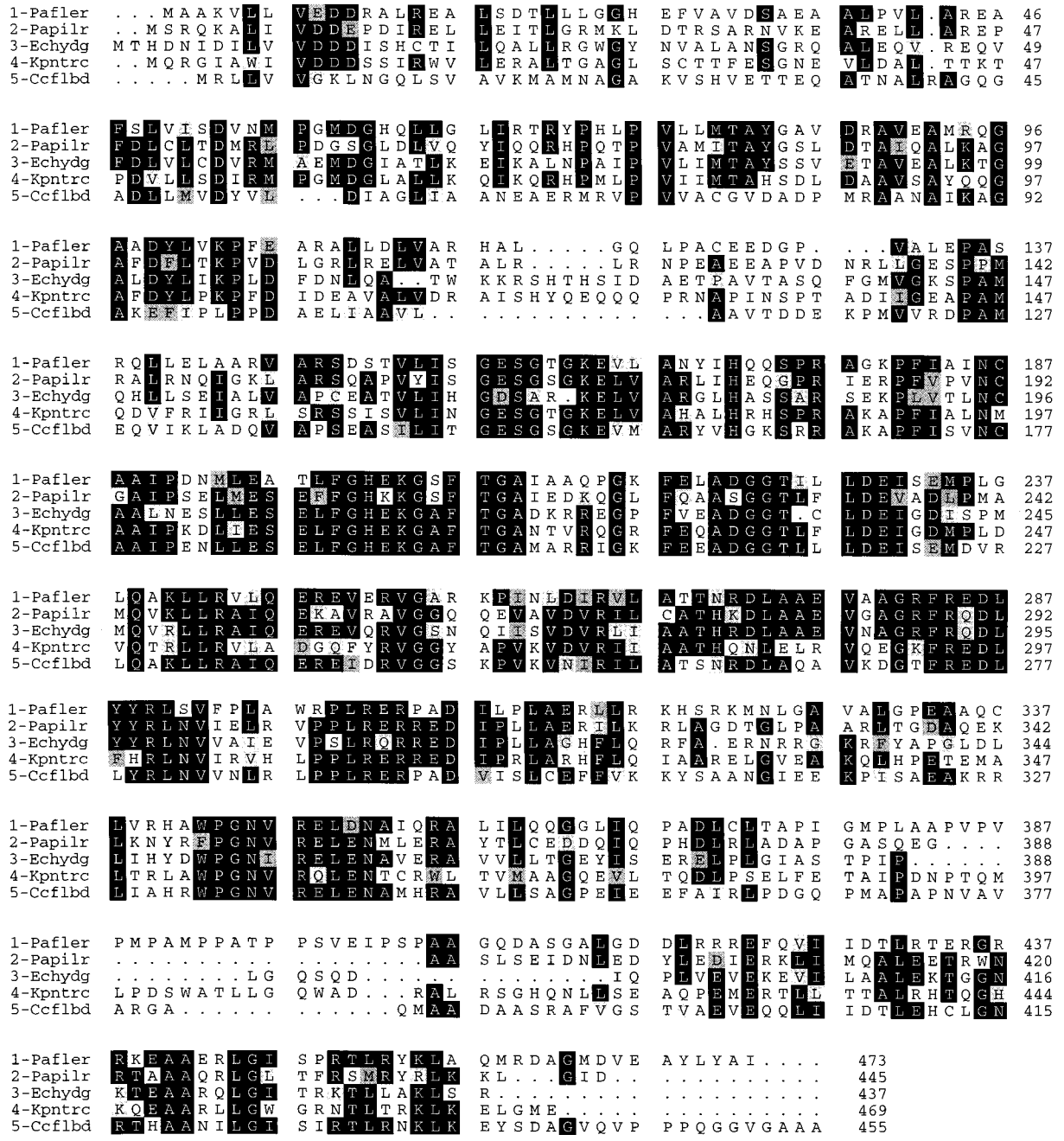


FIG. 5. Computer-generated alignment (Prettybox program, Richard Westerman, Purdue University) of FleR with homologous regulator proteins from other two-component systems. Dark shading shows identity, while light shading indicates similarity among amino acid residues.

flagellin antigen was detected (Fig. 8). In order to confirm that the mutation of this gene alone was responsible for the observed effects, a wild-type copy of *fleR* was cloned into plasmid pMMB67EH, resulting in the plasmid construct pMMB67KS.

Complementation of the mutant strain PAK-FG with this construct resulted in the restoration of motility (Fig. 6), appearance of the polar flagellum (Fig. 7), and restoration of flagellin synthesis (Fig. 8). The effect of the *fleR* mutation on adherence to mucin was also examined. Results in Fig. 9 show that the *fleR* mutant PAK-FG adheres poorly to mucin, and the adherence phenotype is restored by the complementing plasmid pMMB67KS. Furthermore, the *fleR* mutation was engineered in wild-type piliated *P. aeruginosa* PAK. This mutant strain PAK-RG was piliated when examined by electron microscopy and remained sensitive to the pilus-specific phage PO4 (data not shown). However, it showed greatly reduced adhesion to mucin, thus demonstrating that mucin adherence is almost exclusively mediated by the *fleR*-regulated nonpilus adhesin.

## DISCUSSION

This manuscript describes the cloning, sequencing, and phenotypic characterization of a putative two-component regula-

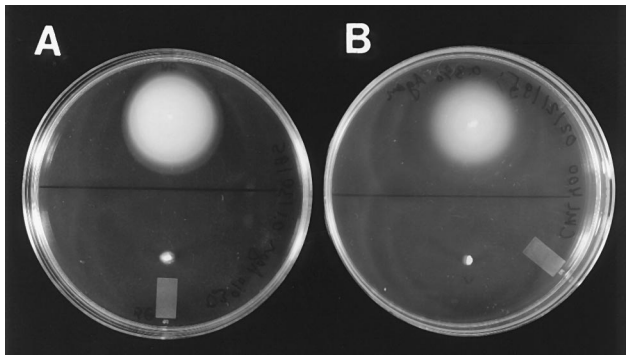


FIG. 6. Soft agar (0.3%) motility plates with appropriate antibiotics showing motility of FleR mutants. (A) Top, motile parental strain, PAK-NP; bottom, nonmotile *fleR* mutant strain PAK-FG. (B) Top, motile *fleR*$^+$ strain PAK-FG (KS), in which the *fleR* chromosomal mutation has been complemented with a functional copy of *fleR* on a low-copy-number plasmid, pMMB67KS; bottom, nonmotile *fleR* mutant strain PAK-FG (67), demonstrating that *fleR* chromosomal mutation is not complemented with the vector alone (pMMB67).

tory system in *P. aeruginosa* designated as FleS/FleR. We had previously described a transposon insertion mutant of *P. aeruginosa* (PAK-RR20) which was defective in adherence to mucins and epithelial cells (33). The genes specifying this two-component system were discovered after sequence analysis of DNA from a cosmid clone which contains a DNA fragment corresponding to the site of transposon insertion in PAK-RR20. However, these genes proved to be upstream of the site of the transposon insertion, in a different operon (2). Insertional inactivation of *fleR* resulted in a strain which was defective in motility because of its inability to produce flagella and was unable to bind to mucin. Complementation with a clone encoding *fleR* confirmed that the observed phenotypes were due to the disruption of *fleR* and not due to polar effects on downstream genes.

This sensor/regulator pair belongs to a subclass of two-component systems which includes PilS/PilR, another regulatory pair which in *P. aeruginosa* controls the expression of the pilin gene, encoding an epithelial cell adhesin (4). The regulatory component FleR (as well as PilR) belong to a related family of transcriptional activators (the NtrC subfamily), which regulate gene expression utilizing RNA polymerase containing $\sigma^{54}$ (13).

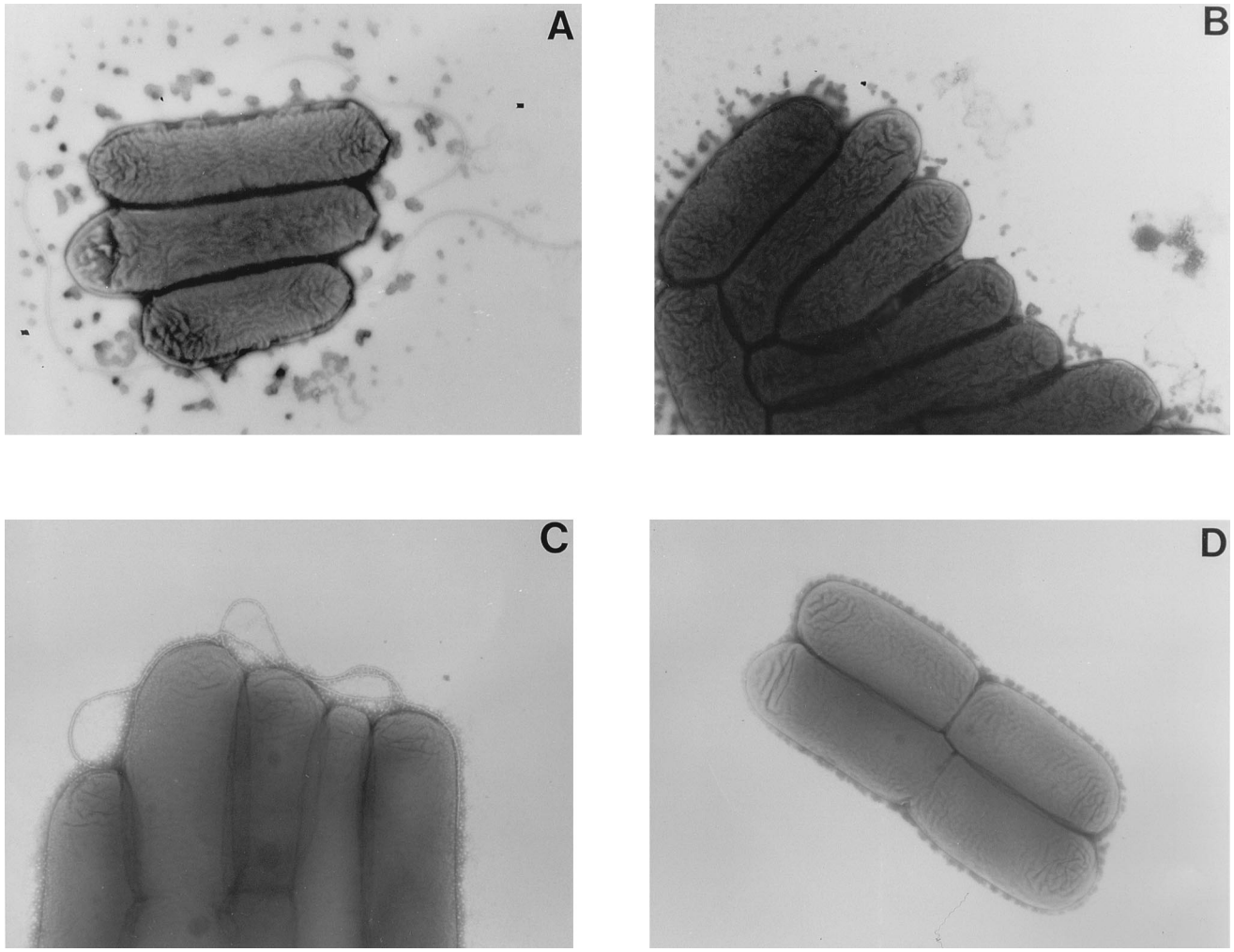


FIG. 7. Electron micrographs of wild-type PAK and *fleR* mutants. (A) Parental strain PAK-NP, showing intact flagella. (B) *fleR* mutant strain, PAK-FG, lacking flagella. (C) *fleR*$^+$ strain PAK-FG (KS), in which the *fleR* chromosomal mutation has been complemented with a functional copy of *fleR* on a low-copy-number plasmid, pMMB67KS, showing restoration of flagella. (D) *fleR* mutant strain PAK-FG (67), demonstrating that *fleR* chromosomal mutation is not complemented with the vector alone (pMMB67).

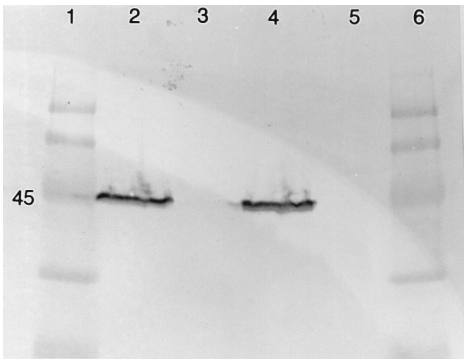


FIG. 8. Western immunoblots of PAK and *fleR* mutants, probed with monoclonal antibody raised against purified flagellin. Lanes 1 and 6, protein molecular mass markers. Bands in lanes 2 and 4 are adjacent to the 45-kDa marker, which is the expected size for the flagellin protein in *P. aeruginosa*. Lane 2, parental strain, PAK-NP. Lane 3, PAK-FG (*fleR* mutant). Lane 4, complemented strain PAK-FG (KS) (*fleR*$^+$). Lane 5, strain PAK-FG (67) (*fleR* mutant), with the vector pMMB67.

FleR also shares striking homology to FlbD of *C. crescentus*, another transcriptional regulator of $\sigma^{54}$-dependent genes that is involved in the synthesis of some of the flagellar components of this organism (20). The primary function of FlbD appears to be control of the temporal expression of genes for flagellar components, essential for ordered assembly of flagella. The role of FleR may be analogous in *P. aeruginosa*, since a mutation in *fleR* leads to absence of flagella. However, the inability of the *fleR* mutant to adhere to mucin suggests that there may be additional genes, differing from those specifying flagellar components, under the control of FleR. No two-component system which regulates these particular diverse phenotypic characteristics has been previously described. This apparent coordinate regulation of motility and adherence may be advantagous during early stages of colonization of the respiratory tract.

Expression of pili was unaffected when a mutation was made in the *fleR* gene of a piliated strain, but adhesion to mucins was markedly diminished to about 10% of that seen with the isogenic parental strain. This observation is consistent with the hypothesis that adhesion to mucins is mediated primarily by nonpilus adhesin(s), and it suggests that *fleR* also controls the expression of the nonpilus adhesin(s). While the existence of nonpilus adhesin(s) in *P. aeruginosa* has been hypothesized for some time (22), this is the first class of mutant that lacks nonpilus adhesin(s) and a flagellum but is still able to make pili. This mutant should prove useful in examining the functions of pili independent of the other surface organelles that may be involved in adhesion.

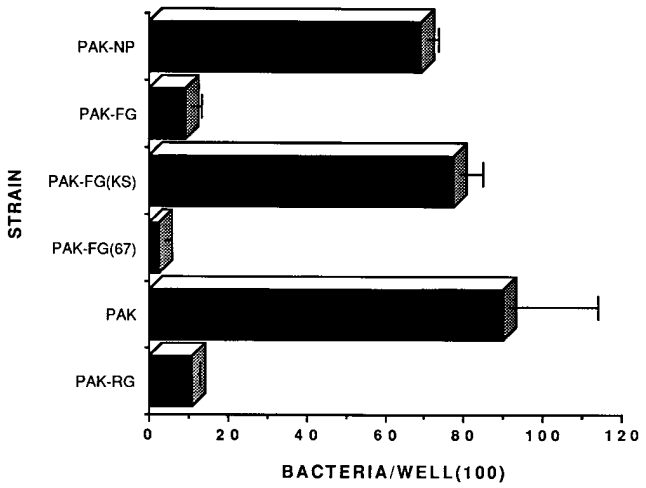


FIG. 9. Adhesion of *pilA* and *fleR* mutants of strain PAK to mucin. PAK-NP, *pilA* mutant of PAK. PAK-FG, *pilA fleR* mutant of PAK. PAK-FG (KS), *pilA fleR*$^+$ mutant complemented with functional copy of *fleR* on a low-copy-number plasmid, pMMB67KS. PAK-FG (67), *pilA fleR* mutant with the vector pMMB67. PAK-RG, *pilA*$^+$ *fleR* mutant of PAK.

The exact site of action of FleR has yet to be determined since the hierarchical expression and organization of *P. aeruginosa* flagellar genes are unknown. In fact, very few *P. aeruginosa* flagellar genes have been identified. Among those described are *fliC*, which encodes the flagellin subunit (33); *fliA*, the gene for the sigma factor, which controls flagellin synthesis (31); and two chemotaxis components encoded by *cheY* and *cheZ* (17, 31). More recently, FliN, FliO, and FliP, which are apparently involved in export of flagellar proteins (29), and FliE, FliF, and FliG, which form internal flagellar structures, have also been described (2). However, none of the *P. aeruginosa* genes described to date has been shown to have functional $\sigma^{54}$ promoters, and they are therefore unlikely to be regulated by FleR. The existing paradigms for flagellar gene expression are the well-described *E. coli* and *S. typhimurium* models, but since none of the flagellar genes of these organisms is known to be $\sigma^{54}$ regulated, it is unlikely that *P. aeruginosa* flagellar gene expression will fit these models. However, in *C. crescentus*, which does have a FleR homolog, some genes for flagellar components, such as the basal body, the hook complex, and flagellin, are under the direct control of $\sigma^{54}$ (20). It is, however, unlikely that FleR acts in an identical manner to FlbD since in *P. aeruginosa* the flagellin gene is regulated by $\sigma^{28}$ (31) and the *P. aeruginosa fliEF* operon which encodes two components of the basal body does not have a requirement for $\sigma^{54}$ (2). Thus, if there is a similarity of flagellar genes in *C. crescentus* and *P. aeruginosa*, by exclusion, it would probably be at the level of transcription of the genes encoding the hook, rod, or axial proteins.

Examination of the putative regulatory region adjacent to the *fleS* gene suggested that *fleS* and *fleR* are part of a regulated operon. This is inferred from the presence of a putative $\sigma^{54}$ promoter at the $-24/-12$ region, which, in analogy with other genes controlled by RpoN, is activated by transcriptional factors of the NtrC type (13). Furthermore, existence of a conserved recognition site for IHF and the binding site for the nitrogen fixation regulator NifA suggests that similar regulatory proteins may be involved in the control of *fleS*/*fleR* expression. Additionally, the presence of a putative $-10$ promoter region and the absence of a conserved $-35$ sequence, an arrangement which is commonly found in positively regulated genes (19), suggests that the putative *fleSR* operon could be transcribed from this $\sigma^{70}$ promoter, or these genes may be under a dual control involving $\sigma^{70}$ and $\sigma^{54}$. Precise mapping of the transcriptional start sites under a variety of conditions should answer the question of promoter usage. These data add to the mounting body of evidence intimately linking the adhesion and motility systems of *P. aeruginosa*. These data also provide incontrovertible evidence of the minimal role that pili play in adhesion to mucins. Whether the connection between motility and mucin adherence is at the level of regulation of gene expression, protein localization, or dual use of the same structural component remains to be elucidated.

## ACKNOWLEDGMENTS

This work was supported by PHS grants HL33622 to R.R. and AI32624 to S.L. R.R. was also supported by the Cystic Fibrosis Foundation and the American Lung Association of Florida.

We thank J. C. Lara for performing the electron microscopy.

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*Editor:* B. I. Eisenstein