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Characterization of *Pseudomonas aeruginosa fliO*, a Gene Involved in Flagellar Biosynthesis and Adherence

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Pseudomonas aeruginosa **binds to eukaryotic cells via both pilus and nonpilus adhesins, while binding of *P. aeruginosa* to mucin is pilus independent. To characterize genes involved in non-pilus-mediated adherence, transposon mutants of the nonpiliated strain *P. aeruginosa* PAK-NP that are unable to bind to cells or mucins were isolated. Two such mutants, *P. aeruginosa* B164 and *P. aeruginosa* RR18, were identified previously as deficient in binding to eukaryotic cells or mucins as well as nonmotile. The transposon insertion in each of these strains was mapped to the same gene. Sequence analysis of both DNA flanking the transposons and plasmids that could complement the mutations indicated that this open reading frame encodes a putative protein homolog of both *Escherichia coli* FliO and *Erwinia carotovora* subsp. *atroseptica* MopB. The transposons in both of these mutants are nonpolar, since the addition of the *P. aeruginosa fliO* gene in *trans* restored adherence to both cells and mucins to these mutants. The cloned *fliO* gene also complemented the motility defect of both B164 and RR18. A 1.6-kb *Kpn*I fragment from the PAK chromosome that contained the *fliO* gene was sequenced. The *fliO* gene appears to be part of an operon with a complete open reading frame upstream of the FliO homolog encoding a putative protein homolog of FliN of both *E. coli* and *Salmonella typhimurium*. The partial open reading frame downstream of *fliO* encodes a putative homolog of both *E. coli* and *S. typhimurium* FliP. The *fliN* gene is flanked on its 5 -end by the 3 -end of a homolog of a *fliM* gene. The *P. aeruginosa* FliN protein was identified with a T7 expression system, while all attempts to identify the *P. aeruginosa* FliO protein were unsuccessful. Homologs of *P. aeruginosa* FliO are involved in the biosynthesis of flagella, but the function of FliO in this biosynthetic process remains unknown. Further study should reveal the precise role of *P. aeruginosa* FliO in non-pilus-mediated adherence, which could include regulation of expression or localization of a nonpilus adhesin.**

The infection of the respiratory tract of patients with cystic fibrosis by *Pseudomonas aeruginosa* follows a pattern of colonization which characterizes mucosal infections by many extracellular pathogens. Bacteria first attach to specific tissue receptors, elaborate extracellular toxins which cause tissue damage or alterations in the host metabolism and, furthermore, frequently elicit an inflammatory response. The first stage of colonization, i.e., adhesion, involves a complex interaction of a number of bacterial adhesins with host receptors. A number of putative *P. aeruginosa* adhesins have been characterized to date, including pili (10, 11, 36), alginate (27), and exoenzyme S (3). Various receptors for *P. aeruginosa* adhesins have also been described, such as asialo GM1 (16, 19), cholesterol (28), and lactosyl ceramide (2), all of which are present in the cell membrane. In addition, oligosaccharide moieties present in respiratory mucins and possibly lactoferrin have been implicated as receptors for this organism (8, 25).

We had previously used a combination of screening methods to isolate mutants of a nonpiliated *P. aeruginosa* strain altered in adherence to both epithelial cells and mucin which were defective in a nonpilus adhesin (33). These mutants defined two classes of adhesins. One class apparently recognized receptors on both epithelial cells and mucins, while another class of *P. aeruginosa* adhesin was mucin specific. The former class utilized random insertions of transposon Tn*5*G into the bacterial chromosome, which resulted in loss of the adhesive phenotype and also led to loss of motility. The latter class of mutants was isolated by introduction of a promoter into a random site in the chromosome of a nonadhesive *rpoN* mutant of *P. aeruginosa*, resulting in restoration of adherence.

Here we report characterization of the gene affected by insertion of Tn*5*G into closely linked sites in mutants *P. aeruginosa* B164 and *P. aeruginosa* RR18. The gene was cloned and sequenced, and its putative protein product was identified. The predicted FliO protein is homologous to components involved in flagellar biosynthesis in a number of bacterial species. The *fliO* gene is flanked downstream of a putative *fliN* gene and is present upstream of a putative *fliP* gene. Introduction of the cloned *fliO* gene into B164 and RR18 restored both motility and adhesion to epithelial cells and mucin.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. All bacterial strains, plasmid vectors, and their derivatives are shown in Table 1. All cultures were routinely grown on Luria broth agar or minimal A salts (9) supplemented with 50 mM monosodium glutamate and 1% glycerol. Antibiotic concentrations for *P. aeruginosa* were as follows: tetracycline, 100 μg/ml; gentamicin, 20 μg/ml; neomycin, 300 to 600 μg/ml. Antibiotic concentrations for *E. coli* were as follows: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml. Motility was measured on Luria broth–0.3% agar containing the appropriate antibiotics.

Cell culture and adherence assay. A549 cells were obtained from the American Type Culture Collection. The cells were maintained as described previously (33). All bacteria were grown in Luria broth (containing the appropriate antibiotics) overnight with mild shaking at 37°C. Prior to use in adhesion assays, the A_{600} of the cultures was recorded. An optical density of 1.0 at 600 nm corresponds to 6.0×10^8 bacteria per ml as determined by plating dilutions of bacterial suspension onto L agar plates and counting the number of CFU following overnight incubation of the plates at 37°C. One-milliliter aliquots of each culture were centrifuged at 13,000 rpm in a microcentrifuge for 2 min to sediment bacteria. The bacterial pellet was then resuspended in 1 ml of Hanks'

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype[a]	Source or reference
E. coli strains		
DH5α	*hsdR recA lacZYA* ϕ80 *lacZΔM15*	
XL1-Blue	*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac*[F′ *proA*$^+$*B*$^+$ *lacI*^{q} *lacZΔM15* Tn*10*(*tet*)]	BRL[b]
BL21(DE3)	F$^-$ *ompT* r_B^- m_B^-(DE3)	Novagen
P. aeruginosa strains		
PAK	Wild type	D. Bradley
PAK-NP	PAK *pilA*::*tet*	29
B164	Nonadherent mutant, *fliO*::Tn*5*G	33
RR18	Nonadherent mutant, *fliO*::Tn*5*G	33
Plasmids		
pSP329Km	Wide-host-range vector, Km^r	This study
pMMB67HE	Wide-host-range vector, Amp^r	14
pBluescriptII SK/KS$^{+/-}$	Phagemid	Stratagene
pDS6	9.0-kb *Eco*RI fragment from B164 chromosome containing Tn*5*G, cloned into pBluescriptII SK$^+$	This study
pDS6.4	3.3-kb *Bgl*II-*Kpn*I fragment from pDS1 cloned into *Kpn*I-*Bam*HI-digested pBluescriptII SK$^-$; contains part of transposon and flanking DNA	This study
pDS118	9.0-kb *Eco*RI fragment from RR18 chromosome containing Tn*5*G, cloned into pBluescriptII SK$^+$	This study
pDS118.4	3.1-kb *Bgl*II-*Kpn*I fragment from pDS118 cloned into *Kpn*I-*Bam*HI-digested pBluescriptII SK$^-$; contains part of transposon and flanking DNA	This study
pDS31B	Adherence-complementing cosmid	33
pDS5	4-kb *Bgl*II fragment from pDS31B, cloned in *Bam*HI site of pMMB67HE	This study
pDS18	pDS5 derivative, 1.6-kb *Kpn*I fragment containing both *fliN* and *fliO* genes in pBluescriptII KS$^+$	This study
pDS18.3	1.6-kb *Kpn*I fragment from pDS18 cloned into pSP329Km	This study
pDS21.3	2.1-kb *Kpn*I fragment of pDS5 cloned into pSP329Km harboring part of *fliP* gene	This study
pDS52	1.1-kb *Kpn*I-*Eco*RI subclone of pDS18.3, cloned into *Kpn*I-*Eco*RI-restricted pSP329Km, contains *fliN* gene and all but 27 nucleotides of *fliO* gene	This study
pDS59.4	0.7-kb *Kpn*I-*Bss*HII fragment of pDS18 harboring *fliN* gene, cloned into pSP329Km	This study
pDS9.1	0.9-kb *Pst*I fragment from pDS18, cloned into pSP329Km, contains *fliO* gene	This study
pDS9.2	Same insert as that of pDS9.1, *fliO* gene in opposite orientation to pDS9.1	This study
pDS188	0.9-kb *Pst*I fragment from pDS18 harboring *fliO* gene, cloned into *Pst*I-restricted pBluescriptII SK$^+$; opposite orientation from that of T7 and *lac* promoters	This study
pDS190	0.9-kb *Pst*I fragment from pDS18 harboring *fliO* gene, cloned into *Pst*I-restricted pBluescriptII SK$^+$; same orientation as that of T7 and *lac* promoters	This study

[a] Abbreviations: pilA, PAK pilin structural gene; *fliC*, PAK flagellin structural gene; *tet*, tetracycline resistance gene; Km, kanamycin; Amp, ampicillin.
[b] BRL, Bethesda Research Laboratories.

balanced salt solution supplemented with 10 mM sodium phosphate buffer (pH 7.4; HBS). Bacteria were again sedimented by centrifugation and resuspended in HBS. They were then diluted in HBS to give approximately 7.5×10^7 bacteria per ml. The adherence assay was performed as described previously (33), with the bacteria centrifuged onto the monolayer because of the motility defect present in many of the strains that were examined. Since this procedure does not distinguish internalized from surface-bound bacteria, all data are presented as the total number of bacteria associated with the monolayer. Adherence of bacteria to mucins was determined as described previously (33).

DNA manipulations and analyses. Plasmid DNA was prepared by the method of Birnboim and Doly (5). Agarose gel electrophoresis, DNA restriction digests, DNA ligations, and Southern hybridizations (under high-stringency conditions) were done essentially as described by Sambrook et al. (30). Subclones of pDS31B were constructed as follows (Fig. 1). A 5-kb *Sal*I fragment was subcloned into *Sal*I-restricted pMMB67HE to form pDS8. A 4.2-kb *Bgl*II fragment from pDS31B was subcloned into *Bam*HI-digested pMMB67HE to form pDS5. A 1.6-kb *Kpn*I fragment from pDS5 was cloned into either *Kpn*I-restricted pBluescriptII KS$^+$ or pSP329Km to form pDS18 or pDS18.3, respectively. A 2.1-kb *Kpn*I fragment from pDS5 was cloned into either *Kpn*I-restricted pBluescriptII KS$^+$ or pSP329Km to form pDS21 or pDS21.3, respectively. A 1.1-kb *Kpn*I-*Eco*RI fragment from pDS18 was cloned into pSP329Km to form pDS52. A 0.8-kb *Bss*HII-*Kpn*I blunt-ended fragment from pDS18 was cloned into pSP329Km to form pDS59.4. A 1.0-kb *Pst*I fragment from pDS18 was cloned into both pBluescriptII KS$^+$ and pSP329Km in both orientations to form pDS188, pDS190, pDS9.1, and pDS9.2.

Identification of site of transposon insertions in the chromosomes of *P. aeruginosa* B164 and *P. aeruginosa* RR18. For each mutant, a 9.0-kb *Eco*RI fragment containing the entire transposon Tn*5*G was cloned into pBluescriptII SK$^+$ to form pDS6 (from B164) and pDS118 (from RR18). A unique *Bgl*II site in the gentamicin cassette of transposon Tn*5*G allowed us to clone one end of the transposon plus flanking DNA into pBluescript vectors for both mutants. A 3.3-kb *Bgl*II-*Kpn*I fragment from pDS1 was cloned into *Kpn*I-*Bam*HI-restricted pBluescriptII SK$^-$ to form pDS6.4. A 3.1-kb *Bgl*II-*Kpn*I fragment from pDS118 was cloned into *Kpn*I-*Bam*HI-restricted pBluescriptII SK$^-$ to form pDS118.4. Sequencing of a single-stranded template from these plasmids was performed as described below with an oligonucleotide primer specific for the IS*50* element of transposon Tn*5* (5′-GTCACATGGAAGTCAGATC-3′).

DNA sequence determination. Subclones of pDS18 in the vectors pBluescriptII (SK/KS)$^{+/-}$ were constructed from appropriate restriction sites and transformed into *E. coli* XL1-Blue. DNA sequencing of either single-stranded or double-stranded templates was performed by the dideoxy chain termination method (31) with the Sequenase DNA sequencing kit (U.S. Biochemical Corp., Cleveland, Ohio). Both strands of the fragment were sequenced. Sequencing was performed with either the T7 or T3 primer or synthetic oligonucleotides as primers. Some reactions were treated with terminal deoxynucleotidyltransferase (Gibco-BRL, Gaithersburg, Md.) to help resolve compressions (12). Reaction mixtures were separated in either 6.0% acrylamide gels containing 8 M urea and 30% formamide or 6.0% gels containing 50% formamide (17).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Whole cells were denatured by boiling in 2% SDS, 1% β-mercaptoethanol, and 50 mM Tris (pH 7.5). Preparations were separated on 15% polyacrylamide Laemmli gels (20), and the proteins were transferred electrophoretically to nitrocellulose by the method of Towbin et al. (34). Monoclonal antiflagellin was kindly provided by A. Siadak (Oncogen, Seattle, Wash.). Antiflagellin antibody bound to the blot was visualized with ^{125}I-protein A followed by autoradiography.

T7 RNA polymerase expression. Plasmids were transformed into *E. coli* BL21(DE3) for use in the T7 expression system. This strain is lysogenic for λDE3, which carries the T7 RNA polymerase gene under *lacUV5* control. Expression of genes controlled by the T7 promoter was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 1 to 2 h. Host RNA polymerase was inactivated by the addition of 200 μg of rifampin per ml for 45

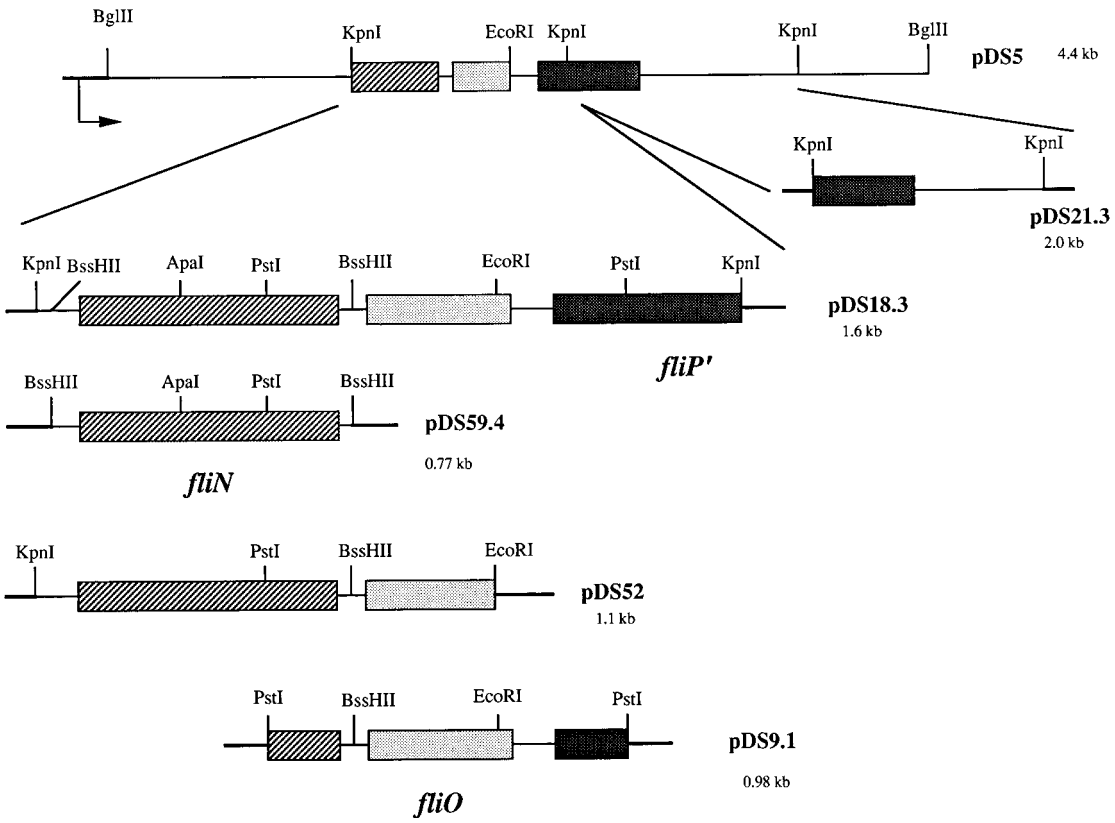

FIG. 1. Subcloning and localization of genes complementing the adherence defect of *P. aeruginosa* B164 and *P. aeruginosa* RR18. Subcloning of large fragments from pDS5 (a fragment cloned from the cosmid pDS31B) was performed as described in the text. All inserts were cloned into the broad-host-range vector pSP329Km prior to conjugation into the *P. aeruginosa* mutants. Hatched boxes indicate positions of putative *fliN*, *fliO*, and *fliP* genes. (The figure is not drawn to scale.)

min. Proteins were labelled with [^{35}S]-Express (DuPont-New England Nuclear, Boston, Mass.) for 10 min. Whole cells were denatured and boiled for 4 min in SDS-PAGE sample buffer prior to separation in SDS-polyacrylamide gels. After electrophoresis, the gels were fixed in 10% methanol–10% acetic acid for 30 min, washed in distilled water (twice for 15 min each time), and then placed in 0.5 M salicylic acid–1.5% glycerol for fluorographic enhancement prior to drying.

Nucleotide sequence accession number. The sequence of pDS18, including the *fliN*, *fliO*, and partial *fliP* genes, has been submitted to GenBank and assigned the accession number L39832.

RESULTS

Cloning of the region of the *P. aeruginosa* chromosome containing determinants of mucin and epithelial cell adherence. We have previously described transposon-induced adherence-defective mutants of *P. aeruginosa* PAK (33). One such mutant, B164, was identified by screening a transposon bank in an epithelial cell binding assay, while mutant RR18 was isolated after a similar screen on mucin-coated microtiter plates. B164 was also unable to adhere to mucin, and conversely, RR18 was defective in binding to epithelial cells. In addition, B164 and RR18 were nonmotile as a result of the absence of flagellar synthesis. Southern analysis indicated that two of these mutants, B164 and RR18, carried their respective transposon Tn*5*G insertions in closely linked regions of the chromosome. We had identified three cosmid clones that could restore the phenotypic defect of both B164 and RR18 (33). With DNA flanking the transposon insertion of B164 used as a probe, subclones from one of the cosmids (pDS31B) were constructed (Fig. 1); they were then tested for restoration of the adherence phenotype of both B164 and RR18 (Fig. 2).

A 4.0-kb *Bgl*II fragment from the original complementing cosmid pDS31B was subcloned into the broad-host-range vector pMMB67HE to form pDS5. Transconjugates of both B164 and RR18 containing plasmid pDS5 were adherent to A549 cells (data not shown). Additional subclones were then generated by use of restriction sites within pDS5, and these resulted in a series of plasmids, some of which restored adherence to epithelial cells when introduced into B164 (Fig. 2A). Plasmid pDS18.3 complemented the adhesion defect of B164 in the epithelial cell binding assay. Three additional subclones (pDS9.1, pDS52, and pDS59.4) were constructed from pDS18.3 (Fig. 2). Of these three subclones, both pDS52 and pDS9.1 were capable of complementing the epithelial cell adhesion defect in B164. When pDS9.1 was introduced into RR18, this plasmid restored the adherence of RR18 to both mucin (Fig. 2B) and cells (data not shown). Furthermore, pDS9.1 complemented the adhesion defect of B164 to mucin (Fig. 2B). These results further confirmed that the original transposon insertions in B164 and RR18 are indeed closely linked and that the gene or genes inactivated by the transposon are contained within the 0.9-kb *Pst*I fragment in pDS9.1. Sequence analysis (see below) indicated that a single complete gene, a putative homolog of *E. coli fliO*, is located in this fragment. Furthermore, a plasmid containing the same insert as pDS9.1, but with a kanamycin cassette inserted into the *fliO* gene at a unique *Rsr*II site, did not restore the phenotype to either mutant (data not shown).

Sequence analysis of the DNA complementing the adhesion defect. The entire DNA sequence of the 1.6-kb *Kpn*I insert in pDS18.3 is shown in Fig. 3. This region contains two complete open reading frames, both of which possess significant homology to bacterial flagellar biosynthetic genes. In addition, two

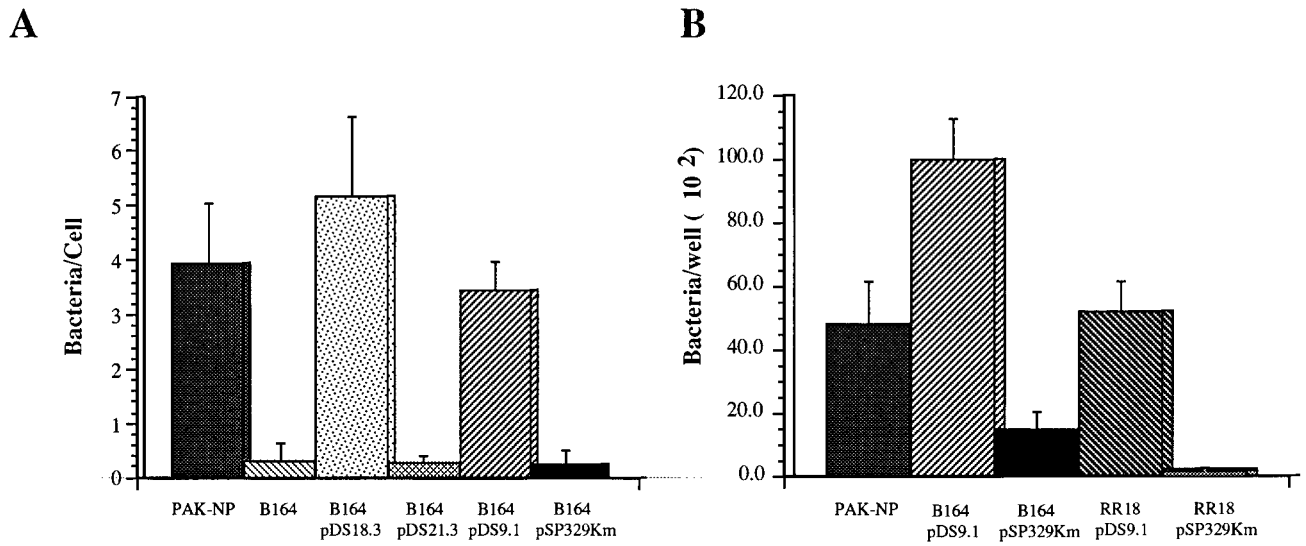

FIG. 2. Adherence of strains to either A549 epithelial cells (A) or mucins (B). Adherence assays were performed as described in Materials and Methods. Numbers represent means of duplicates of at least three experiments. Error bars represent 1 standard deviation.

partial open reading frames which are interrupted by the *Kpn*I sites used in cloning of pDS18.3 were identified. Because the smallest complementing region (in plasmid pDS9.1) contained a single complete open reading frame, it was inferred that the adhesion defect was therefore due to disruption of this gene. The sites of the transposon insertions were further verified by sequencing of the cloned region flanking Tn*5*G in B164 and RR18, and these sites are also indicated in Fig. 3. The insertions are separated by 236 bp and are entirely within the gene homologous to *fliO* of *E. coli*. The insertion in RR18 was between nucleotides 765 and 766 (of pDS18) in the *fliO* gene (between the codons for Cys-21 and Ala-22), and the B164 insertion was between nucleotides 1001 and 1002 (interrupting the Glu-100 codon) (Fig. 3). The second complete open reading frame upstream of the putative *fliO* homolog encodes a protein homolog of FliN of both *E. coli* and *Salmonella typhimurium*. The partial open reading frame downstream of *fliO* encodes a homolog of both *E. coli* and *S. typhimurium* FliP. Finally, *fliN* is flanked on its 5′-end by the 3′-end of a homolog of a *fliM* gene from *E. coli* and *S. typhimurium.*

The sequence of *fliO* indicates that this gene is 425 nucleotides in length (Fig. 3). The ATG initiation codon for the *fliO* gene is 4 bp downstream of the termination codon of the *fliN* gene at position 703 and is preceded by a potential Shine-Dalgarno sequence 11 bp upstream. There are no other in-frame ATG initiation codons in this gene. The *fliO* gene codes for a predicted protein of 142 amino acids with a molecular mass of 14,842 Da. Analysis of the deduced FliO protein indicates that it is very basic, with a pI of 11.48. *P. aeruginosa* FliO is also quite hydrophobic, containing 54% hydrophobic amino acid residues. Two regions of high hydrophobicity near the N terminus of the polypeptide may represent membrane-spanning segments.

Computer-assisted searches of GenBank, PIR, and SWISS-PROT databases by use of the BLAST program (1) with the *P. aeruginosa fliO* sequence revealed significant homology to only two entries in GenBank. *P. aeruginosa* FliO is 35% identical and 58% similar to *Erwinia carotovara* subsp. *atroseptica* MopB protein (23) over a 107-amino-acid stretch and 44% identical and 67% similar to *E. coli* FliO (21) over 61 amino acids in the central portion of the polypeptide (Fig. 4).

The nucleotide sequence of pDS18 indicates that the *fliN* gene of *P. aeruginosa* is 473 nucleotides in size. The ATG initiation codon at position 228 is 30 bp downstream from the end of a putative *fliM* gene. This initiation codon is preceded by the sequence AAGA, which is a putative Shine-Dalgarno element. Two other potential in-frame ATG initiation codons are at nucleotides 336 and 390; however, neither is preceded by a reasonable Shine-Dalgarno sequence. The 473-nucleotide open reading frame codes for a predicted protein of 158 amino acids with a molecular mass of 16,608 Da. Analysis of this amino acid sequence indicates that the FliN protein has a pI of 4.06. The amino acid sequence of FliN indicates that there is no signal sequence at the N-terminal region of the protein.

Analysis of the data bank entries with the putative *P. aeruginosa* FliN sequence revealed similarities to sequences of a number of bacterial proteins involved in motility. Figure 5 shows the alignment of the FliN homologs from *P. aeruginosa*, *E. coli* (22), *S. typhimurium* (18), *Caulobacter crescentus*, and *Agrobacterium tumefaciens*, the FliY protein of *Bacillus subtilis* (6), and the MopA protein of *E. carotovora* subsp. *atroseptica* (23). In all cases, the regions of significant region of similarity are in the C-terminal domains of the respective proteins, while the N termini show virtually no similarity. This C-terminal region of *P. aeruginosa* FliN is also homologous to components involved in the secretion of virulence factors, including SpaO of *S. typhimurium*, Spa33 of *Shigella flexneri*, and YscQ of *Yersinia pestis* and *Y. enterocolitica* (Fig. 5).

The ATG initiation codon for a putative *fliP* gene is present 22 nucleotides downstream of the *fliO* stop codon at nucleotide 1150 (Fig. 3). This open reading frame continues to the end of the *Kpn*I fragment in pDS18. The open reading frame codes for the first 141 amino acids of a putative FliP homolog. Sequence analysis indicates that plasmid pDS21.3 contains the remainder of the *fliP* gene (as well as a portion of a putative *fliQ* gene) (data not shown). The N terminus of the putative FliP protein appears to contain a sequence with all of the features of a secretion signal peptide. Inhibition of signal peptide processing of *E. coli* FliP by metabolic inhibitors has been demonstrated previously (21). This putative signal sequence has a long stretch of hydrophobic amino acids which is preceded by a 4-amino-acid positively charged domain. The po-

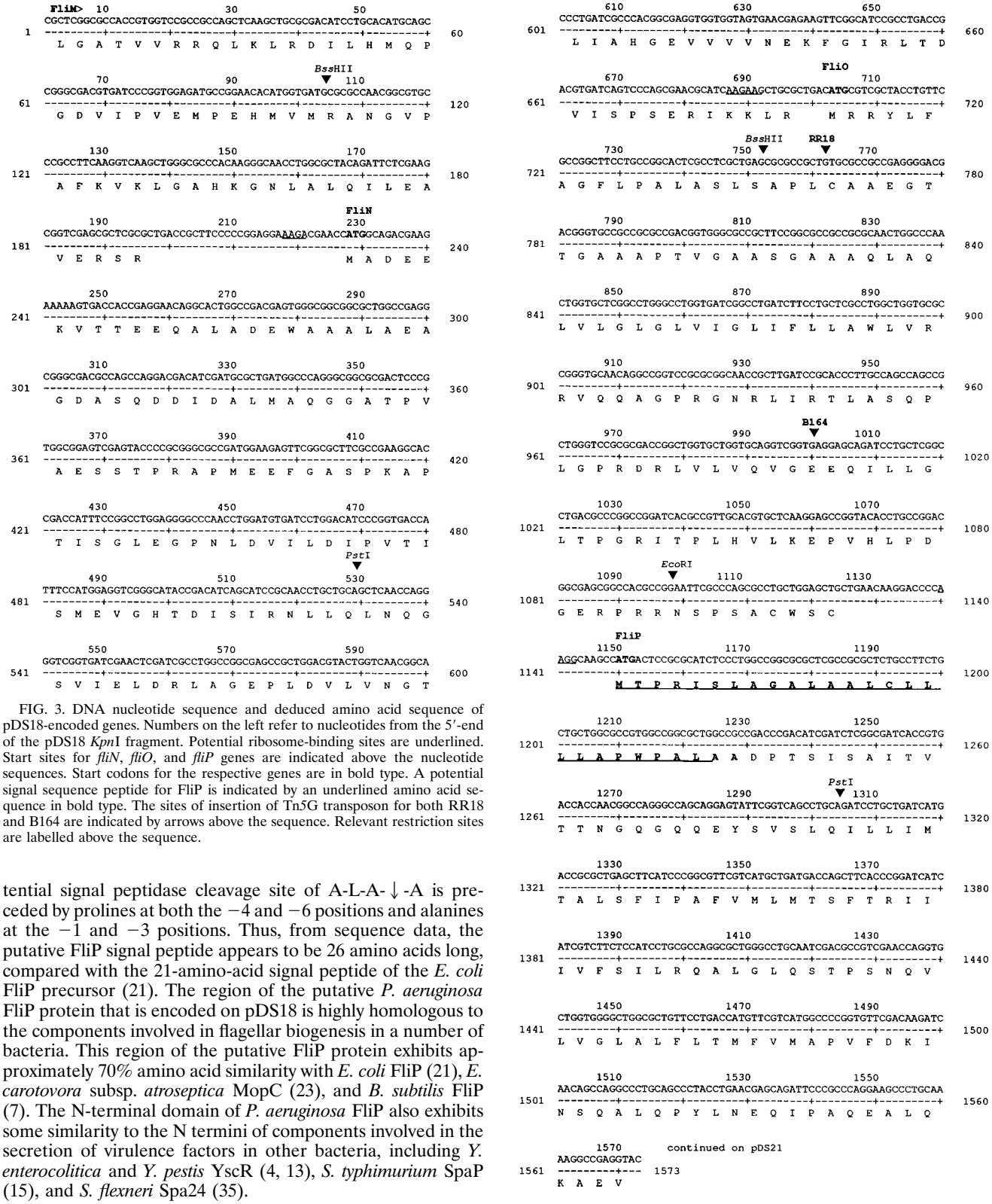

FIG. 3. DNA nucleotide sequence and deduced amino acid sequence of pDS18-encoded genes. Numbers on the left refer to nucleotides from the 5′-end of the pDS18 *Kpn*I fragment. Potential ribosome-binding sites are underlined. Start sites for *fliN*, *fliO*, and *fliP* genes are indicated above the nucleotide sequences. Start codons for the respective genes are in bold type. A potential signal sequence peptide for FliP is indicated by an underlined amino acid sequence in bold type. The sites of insertion of Tn*5*G transposon for both RR18 and B164 are indicated by arrows above the sequence. Relevant restriction sites are labelled above the sequence.

tential signal peptidase cleavage site of A-L-A-↓-A is preceded by prolines at both the −4 and −6 positions and alanines at the −1 and −3 positions. Thus, from sequence data, the putative FliP signal peptide appears to be 26 amino acids long, compared with the 21-amino-acid signal peptide of the *E. coli* FliP precursor (21). The region of the putative *P. aeruginosa* FliP protein that is encoded on pDS18 is highly homologous to the components involved in flagellar biogenesis in a number of bacteria. This region of the putative FliP protein exhibits approximately 70% amino acid similarity with *E. coli* FliP (21), *E. carotovora* subsp. *atroseptica* MopC (23), and *B. subtilis* FliP (7). The N-terminal domain of *P. aeruginosa* FliP also exhibits some similarity to the N termini of components involved in the secretion of virulence factors in other bacteria, including *Y. enterocolitica* and *Y. pestis* YscR (4, 13), *S. typhimurium* SpaP (15), and *S. flexneri* Spa24 (35).

Identification of plasmid-encoded FliN and FliO polypeptides. Plasmids containing the complete *fliN* and *fliO* genes were used in the T7 expression system. All genes were cloned downstream of the T7 promoter in both orientations in the pBluescriptII vectors. What appeared to be one insert-specific protein with a molecular mass of approximately 18.5 kDa was produced by *E. coli* BL21(DE3) carrying pDS18 (Fig. 6). When pDS60, containing *fliN* alone, was expressed in this system, a unique band of approximately 18.5 kDa was observed. This suggests that *fliN* utilizes the ATG start codon at nucleotide position 228 (Fig. 3). This open reading frame codes for a FliN

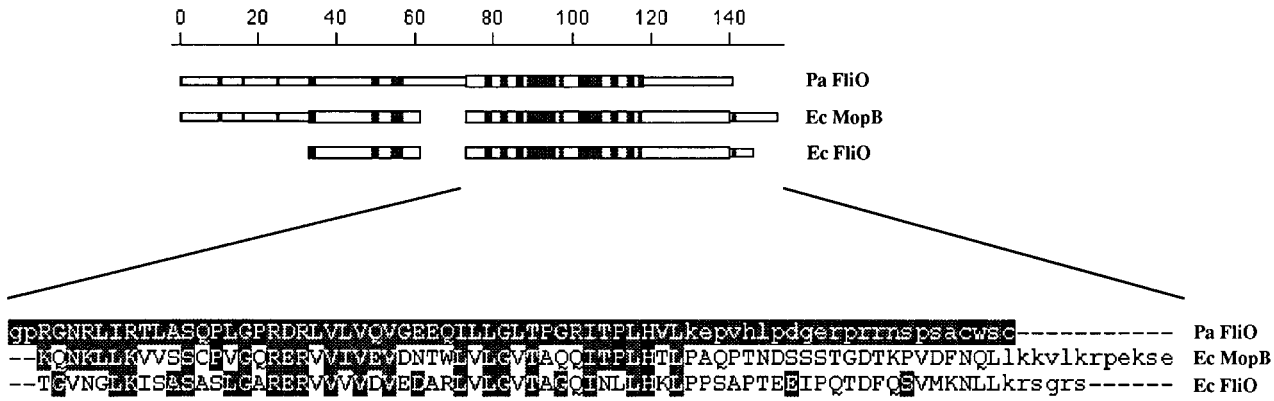

FIG. 4. MACAW alignment of *P. aeruginosa* (Pa) FliO homologs, namely, *E. carotovora* subsp. *atroseptica* MopB and *E. coli* FliO. The upper portion of each panel represents a schematic of the indicated alignment. Values above the figure indicate the numbers of amino acids. Gaps were introduced in the proteins to more closely align the regions of similarity. The larger rectangles represent blocks of similarity. Dark shading in the schematic represents areas of similarity between all FliO homologs. The bottom portion of each panel illustrates the single-amino-acid code alignment of the region selected. For each residue, a shade is selected on the basis of the score matrix entry of that residue compared with the one in the same column as the *P. aeruginosa* FliO. Regions with a single-letter amino acid code in capital letters indicates blocks of highest similarity. The shading of each amino acid indicates the degree of similarity. White letters on a black background indicate that the amino acid residue is identical to the *P. aeruginosa* FliO at an unmutable residue. White letters on a gray background indicate identical or similar residues.

protein with a predicted molecular mass of 16,608 Da. When plasmid pDS91, which contains the *fliO* gene, was expressed in *E. coli* BL21(DE3), no insert-specific bands were observed (Fig. 6). The putative protein sequence of FliO contains only two cysteines at amino acid positions 138 and 141. Phenotypic analysis indicated that plasmid pDS52, which codes for all but the C-terminal 8 amino acids of FliO, was sufficient to complement the phenotypic defects in both adherence and flagellin production in both B164 and RR18. The two cysteines in positions 138 and 141 are in this 8-amino-acid C-terminal region. It is possible that a portion of the C terminus of FliO is being processed in *E. coli* and would not be identified in this T7

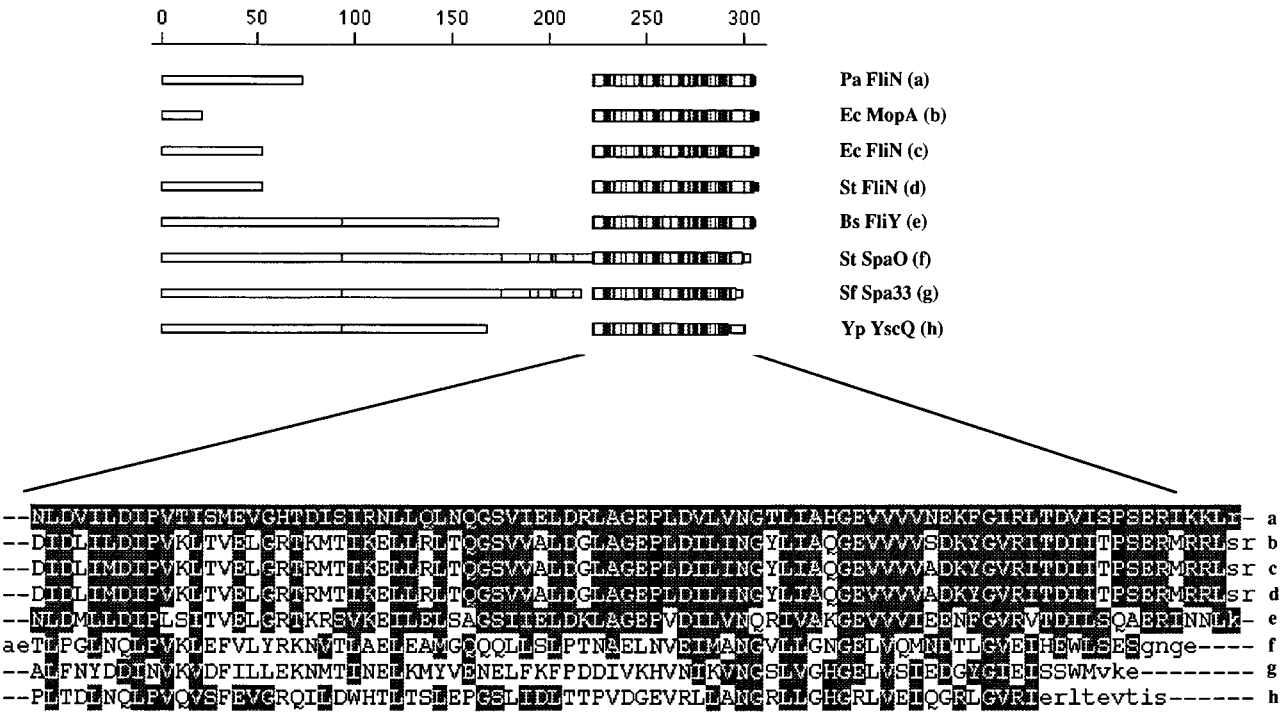

FIG. 5. MACAW alignment of FliN homologs. The upper portion of each panel represents a schematic of the indicated alignment. Values above the figure indicate the numbers of amino acids. Gaps were introduced in the proteins to more closely align the regions of similarity. The larger rectangles represent blocks of similarity. Dark shading in the schematic represents areas of similarity between all FliN homologs. The bottom portion of each panel illustrates the single-amino-acid code alignment of the region selected. For each residue, a shade is selected on the basis of the score matrix entry of that residue compared with the one in the same column as the *P. aeruginosa* FliN. Regions with the single-letter amino acid code in capital letters indicate blocks of highest similarity. The shading of each amino acid indicates the degree of similarity. White letters on a black background indicate that the amino acid residue is identical to the *P. aeruginosa* FliN at an unmutable residue. White letters on a gray background indicate identical or similar residues. Abbreviations: Pa, *P. aeruginosa*; Ec MopA, *E. carotovora* subsp. *atroseptica* MopA; Ec FliN, *E. coli* FliN; St, *S. typhimurium*; Bs, *B. subtilis*; Sf, *S. flexneri*; Yp, *Y. pestis.*

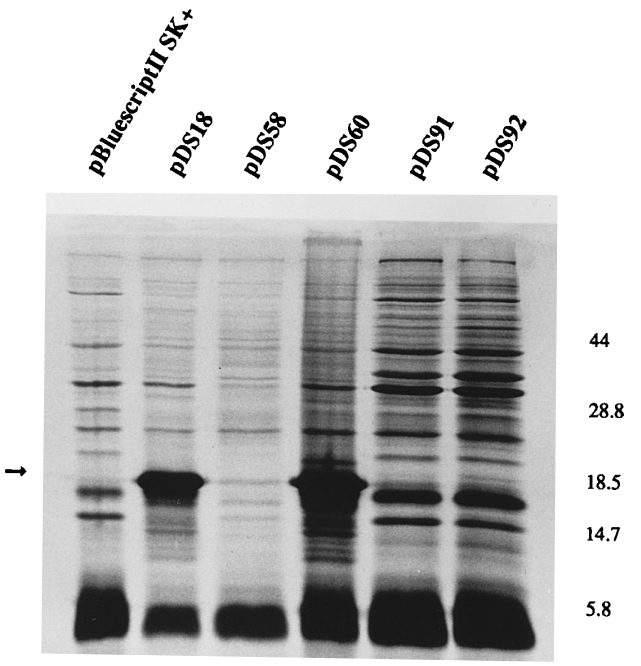

FIG. 6. Autoradiograph of plasmid-encoded polypeptides labelled with ^{35}S-methionine and -cysteine in the T7 expression system. *E. coli* BL21(DE3) containing the plasmids indicated above each lane were utilized for T7 expression, and whole-cell preparations were subjected to SDS-PAGE with 15% gels as described in Materials and Methods. The arrow on the left indicates the labelled FliN polypeptide, and the numbers on the right represent molecular masses in kilodaltons.

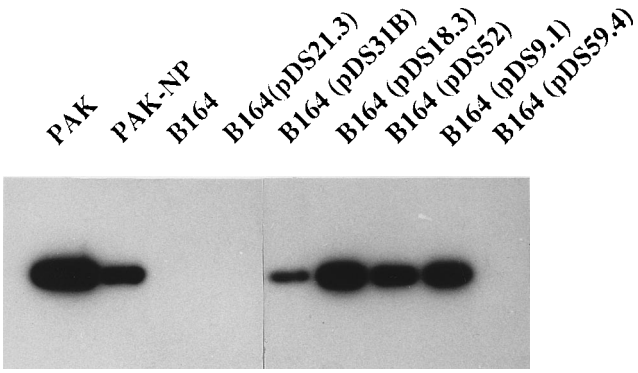

FIG. 7. Western blot (immunoblot) analysis of transconjugates. Cells were removed from plates grown overnight at 37°C and resuspended in phosphate-buffered saline to approximately the same density. The cells were then added to SDS-PAGE sample buffer and boiled prior to electrophoresis through SDS–10% polyacrylamide gels (20). The proteins were transferred to nitrocellulose, and flagellin was detected with a monoclonal anti-PAK flagellin antibody, followed by ^{125}I-protein A. Similar results were observed when these plasmids were introduced into *P. aeruginosa* RR18. Clones containing the *fliO* gene alone restored flagellin antigen to *P. aeruginosa* B164.

system. Plasmids were constructed to allow us to use the T7 expression system in *P. aeruginosa* PAK. The FliN that was visualized in this system possessed a similar molecular mass as that observed in *E. coli*, while no FliO protein was observed (data not shown). We could not label FliO in either *E. coli* or *P. aeruginosa* with [^{3}H]leucine in place of ^{35}S-methionine. It is possible that either the FliO protein is extremely unstable and is degraded quickly in this system or FliO is a secreted polypeptide and would not be detected by this labelling procedure.

Complementation of flagellin production and motility of *P. aeruginosa* B164 and RR18. Mutants B164 and RR18 are nonmotile and unable to synthesize flagellin. Plasmid pDS9.1 restored synthesis of flagellin antigen in both of the mutant backgrounds (Fig. 7), and transconjugates of both B164 and RR18 containing pDS9.1 became motile (data not shown). This observation confirmed that the motility and adherence defects in B164 and in RR18 are due to inactivation of the same single gene, *fliO*.

DISCUSSION

We have previously isolated two linked transposon-insertion mutants of *P. aeruginosa* PAK-NP, termed B164 and RR18, which were defective in adhesion to both epithelial cells and mucins and which were also defective in flagellin synthesis. Here we describe molecular characterization of a gene inactivated by the two transposon insertions, which led to loss of motility and adherence. The predicted amino acid sequence of this gene product possesses strong homology to the proteins encoded by both *fliO*, a previously described gene involved in flagellar synthesis in *E. coli*, and *mopB* of *E. carotovora*. Although we have performed no functional assays, we assume that the gene described here represents the *P. aeruginosa* homolog of these genes and refer to it as *fliO*. Additional sequence analysis of the flanking regions of *P. aeruginosa fliO* shows regions that encode proteins that may possess similar flagellar biosynthetic functions. The sequence 5′ of *P. aeruginosa fliO* encodes the putative homologs of both FliM and FliN, while downstream of *fliO* is the coding sequence for FliP. The genetic organization of the corresponding gene cluster (*fliMNOP*) is therefore conserved between *E. coli*, *S. typhimurium*, and *P. aeruginosa.*

We have mapped the sites of insertions of B164 and RR18 within the coding region of *fliO*. A plasmid (pDS9.1) which contains only intact *fliO* was capable of complementing the adhesion and motility defects of both B164 and RR18. Since *fliO* in other bacteria is contained within a multigenic operon, the lack of polar effect of insertions in *fliO* on downstream genes suggests that the role of FliO in adherence is independent of the downstream genes. Interestingly, restoration of flagellin synthesis and motility was also attained with the same adherence-complementing clone, indicating that downstream genes were transcribed from internal promoters or a promoter provided by the transposon.

The function of the *E. coli fliO* gene product in flagellar biogenesis is unknown (21). Since it is located in the operon containing genes of the flagellar switch but does not appear to be a structural protein, one possible function could relate to this protein assisting the proper localization of the various flagellar components. Therefore, it could also serve a similar role in the localization and assembly of the adhesin on the bacterial surface. The amino-terminal half of the predicted FliO polypeptide contains several potential membrane-spanning regions, and therefore FliO could be a membrane protein and it would be this compartment from which FliO could act during localization of both the flagellar structure and the adhesin. Previously, we had shown that both *fliA-pilA* and *fliC-pilA* mutants retained the adhesive phenotype (33). This suggests that the mutations in *fliO* that result in an adhesion defect are independent from the motility defect observed in these mutants.

A family of proteins in gram-negative pathogens, which all possess a high degree of similarity in their carboxy-terminal domains, are related to the putative *P. aeruginosa* flagellar switch protein FliN. These proteins include YscQ of *Y. pestis*

and *Y. enterocolitica*, SpaO of *S. typhimurium*, and Spa33 of *S. flexneri*. The function of these proteins in virulence is not known; however, it is believed that they play a role in secretion of virulence factors by *Yersinia*, *Shigella*, and *Salmonella* species. A comparison of the regions of homology of FliN homologs was represented graphically by comparing the amino acid sequences in the Multiple Alignment Construction and Analysis Workbench (MACAW) program from the National Center for Biotechnology Information for the Macintosh. This program identifies and aligns regions of functional similarity in proteins by a strategy of pairwise comparison of multiple sequences (32). As can be seen in Fig. 7, the MACAW alignment illustrates that there is a block of similarity at the C terminus of each FliN homolog. This block may indicate some functional similarity among these components. Similarly, the N-terminal region of *P. aeruginosa* FliP possesses homology with both other FliP proteins and components of putative secretion machinery of several bacteria (*Y. enterocolitica* YscR, *S. typhimurium* SpaP, and *S. flexneri* Spa24). This suggests that there are a number of proteins which are structurally related to flagellar biosynthetic components that play other roles in the expression of bacterial virulence.

The close relationship between adhesion and motility in *P. aeruginosa* suggests that these two functions are coordinately regulated. The alternative sigma factor RpoN controls transcription of both pilin and nonpilus adhesins and is also required for synthesis of flagellin. This linkage between motility and adherence has been supported by observations that *P. aeruginosa* shows chemotaxis towards mucin and binds to mucin by a nonpilus adhesive mechanism (24, 26).

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