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The *pilG* Gene Product, Required for *Pseudomonas aeruginosa* Pilus Production and Twitching Motility, Is Homologous to the Enteric, Single-Domain Response Regulator CheY

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The *Pseudomonas aeruginosa pilG* gene, encoding a protein which is involved in pilus production, was cloned by phenotypic complementation of a unique, pilus-defective mutant of strain PAO1. This mutant, designated FA2, although resistant to the pilus-specific phage D3112 was sensitive to the pilus-specific phages B3 and F116L. In spite of the unusual phage sensitivity pattern, FA2 lacked the ability to produce functional polar pili (*pil*) and was incapable of twitching motility (*twt*). Genetic analysis revealed that the FA2 *pil* mutation, designated *pilG1*, mapped near the *met-28* marker located at 20 min and was distinct from the previously described *pilT* mutation. This map location was confirmed by localization of a 6.2-kb *Eco*RI fragment that complemented FA2 on the *Spe*I and *Dpn*I physical map of the *P. aeruginosa* PAO1 chromosome. A 700-bp region encompassing the *pilG* gene was sequenced, and a 405-bp open reading frame, with characteristic *P. aeruginosa* codon bias, was identified. The molecular weight of the protein predicted from the amino acid sequence of PilG, which was determined to be 14,717, corresponded very closely to that of a polypeptide with the apparent molecular weight of 15,000 detected after expression of *pilG* from the T7 promoter in *Escherichia coli*. Moreover, the predicted amino acid sequence of PilG showed significant homology to that of the enteric CheY protein, a single-domain response regulator. A chromosomal *pilG* insertion mutant, constructed by allele replacement of the wild-type gene, was not capable of pilus production or twitching motility but displayed normal flagellum-mediated motility. These results, therefore, suggest that PilG may be an important part of the signal transduction system involved in the elaboration of *P. aeruginosa* pili.

Pseudomonas aeruginosa pathogenesis is multifactorial and involves a number of cell-associated and extracellular virulence factors (31). Both in vivo and in vitro studies have implicated the type IV (NMePhe) pili of *P. aeruginosa* as important cell-associated virulence determinants that play a crucial role in bacterial adherence and colonization of mucosal epithelium (18, 34, 54, 56, 57, 65, 83). The pili of *P. aeruginosa* also serve as receptors for various bacteriophages, such as the transposable Mu-like phages D3112 and B3 (13, 14, 37, 58, 60), the temperate transducing phage F116 (55), and the virulent phage PO4 (7). Furthermore, the polar pili of *P. aeruginosa* are also responsible for a novel mode of flagellum-independent surface translocation known as twitching motility (10, 26). The exact role of pili in this unique form of surface translocation, however, has not been elucidated.

Genetic analysis of *P. aeruginosa* pilus production and twitching motility has revealed the requirement for several *pil* loci which have been localized to different regions of the chromosome. The pili of *P. aeruginosa* are composed of protein subunits encoded by the *pilA* structural gene (64), the expression of which requires both the alternative sigma factor RpoN (32) and the transcriptional activator PilR (33). Three additional nearby genes are also required for pilus production. These genes, designated *pilB*, *pilC*, and *pilD*, are transcribed in the direction opposite that of *pilA* (49). While the functions of the proteins encoded by the *pilB* and *pilC* genes have not yet been fully established, the *pilD* gene encodes a protein with pilin-specific leader peptidase activity which is required for the successful translocation of pilin subunits across the inner membrane (50). The PilD protein has also been shown to process components of the machinery necessary for the secretion of several *P. aeruginosa* extracellular products (51, 75). Recent mapping studies have shown that the *pilA*, *-B*, *-C*, *-D*, *-R*, and *rpoN* genes reside relatively close to one another, between 71.3 and 75 min, on the PAO1 chromosome (21).

The *P. aeruginosa pilT* gene, which is essential for twitching motility, has been localized near the *proC* gene at 20 min on the PAO chromosome (82). *pilT* mutants are characterized by the presence of multiple, nonretractile pili (*pil*^{NR}) and, as a result, are incapable of translocating across solid surfaces. The *pilT* gene product, which is presumably required for pilus retraction, encodes a protein that exhibits a significant degree of homology to PilB and to certain proteins in other bacterial systems involved in DNA transport and protein secretion (82).

Furthermore, since pilus production and twitching motility are likely to be complex processes, it is not surprising that a preliminary analysis of several independent pilus-specific phage-resistant mutants from this laboratory has revealed the presence of additional *pil* loci. In addition to the genes located within the 71- to 75-min and 20-min regions described previously (21, 82), at least three additional *pil* loci have been localized to approximately 7, 27, and 41 min (16, 20).

This report describes the identification, mapping, and characterization of a new gene, designated *pilG*, which is involved in *P. aeruginosa* pilus production. I present evidence that PilG has significant homology to a subfamily of bacterial response regulator proteins, which includes such members as CheY and Spo0F, that retain only the conserved response regulator N-terminal domain (73). Since these proteins are believed to directly control the adaptive re-

TABLE 1. Strains, phages, and plasmids used in this study

Strain, phage, or plasmid	Relevant characteristics[a]	Source or reference
E. coli		
DH5α	*recA endA1 gyrA96 thi-1 hsdR17 sup44 relA1 lacZΔM15*	N. Pagratis
S17-1	RP4 2-Tc::Mu-Km::Tn*7 pro* r^- m^+ (Tp^r Sm^r)	70
BL21(DE3)	F^- *ompT* r_B^- m_B^-, lambda D69 lysogen carrying phage T7 gene *1* under control of P_{lacUV5}	76
CBK940	Nal^r derivative of DH10B	C. Berg
D1210	HB101 $lacI^q$ $lacY^+$	62
D1210HP	D1210, lambda *xis kil* cI857	25; C. Berg
DH10B	F^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80d*lacZΔM15* Δ*lacX74 endA1 recA1 deoR* Δ(*ara leu*)*7697 araD139 galU galK nupG rpsL*	Bio-Rad
P. aeruginosa		
CD10	PAO4141::D3112 *cts*	13
FA1	D3112^r F116^r B3^r	This study
FA2	*pilG1* D3112^r F116^s B3^s	This study
FA3	PAO4141 *pilG*::mγδ-1 Km^r D3112^r F116^r B3^r	This study
HOD1	PAO1 *pilT* mutant, D3112^r F116^r B3^r	16, 79
PA103	Fla^-	D. Galloway
PAO1	Prototroph, FP^-	27
PAO222	*ilv-226 his-4 lys-12 proA82 met-28 trp-6* FP^-	24
PAO381	*leu-10 strA* $FP2^+$	R. V. Miller
PAO4141	*pro-9024 met-9020 blaP9202 blaJ9111 aph-9001* FP^-	H. Matsumoto
Phages		
D3112 *cts*	*cts* (temperature-sensitive repressor)	58
B3 *cts*	*cts* (temperature-sensitive repressor)	A. Darzins
F116L *cts53*	*cts* (temperature-sensitive repressor)	36; R. V. Miller
G101	Generalized transducing phage	28
Plasmids		
pADD948	Mini-D948 in vivo cloning replicon, Ap^r Cm^r	14
pADD1619	pRO1614 with 6.0-kb *Bam*HI fragment (*pilT*$^+$)	A. Darzins
pCP13	IncP Tc^r Km^r (23 kb) *cos*	15
pIF200	pOX38::miniγδ-1 Km^r	2
pIF402	pACYC184-γδ*tnpA* Cm^r	C. Berg
pMOB	Miniplasmid vector, 1.8 kb, Ap^r	74; Gold Biotech
pMOB3	pHSS21 *sacB sacR oriT* Km^r Cm^r	69
pNOT19	pUC19 with 10-bp *Nde*I-*Not*I adaptor in *Nde*I site, Ap^r	69
pRK2013	rep_{pMB1} Tra^+ (RK2) Km^r	22
pRO1614	Broad-host-range vector, 6.2 kb, Ap^r (Cb^r) Tc^r	53
pT7-2	T7 φ10 promoter, 2.777 kb, Ap^r	United States Biochemical Corp.
FP2	Hg^r Tra^+ Cma^+	29

[a] Ap^r, ampicillin resistance; Cb^r, carbenicillin resistance; Cm^r, chloramphenicol resistance; Cma^+, chromosome mobilization ability; Hg^r, mercury resistance; Km^r, kanamycin resistance; Nal^r, naladixic acid resistance; Tc^r, tetracycline resistance; Tp^r, trimethoprim resistance; Tra^+, self-transmissible; *oriT*, RK2 origin of transfer; rep_{pMB1}, replicon from pMB1; Sm^r, streptomycin resistance. *P. aeruginosa* gene designations are as described by Royle et al. (61).

sponse, by analogy PilG may play an equally important role in the signal transduction system that regulates the production of *P. aeruginosa* pili.

MATERIALS AND METHODS

Bacterial strains, phages, and plasmids. The bacterial strains, phages, and plasmids used in this study are described in Table 1.

Media. LB medium was routinely used to propagate *P. aeruginosa* and *Escherichia coli*. LB broth was 1% tryptone (Difco)–0.5% yeast extract (Difco)–0.5% NaCl. Minimal medium for the selection of *P. aeruginosa* recombinants was as described by Brammer and Clarke (11) except that the trace element solution was omitted. Amino acids were added to a final concentration of 1 mM, and glucose was added to a final concentration of 50 mM. For most solid media, agar (Difco) was added at a concentration of 1.5%. LB top agar for determining titers of phage lysates contained agar at a concentration of 0.7% and 1 mM $MgSO_4$. The antibiotic concentrations for *E. coli* were as follows: ampicillin, 100 μg/ml; chloramphenicol, 30 μg/ml; kanamycin, 30 μg/ml; naladixic acid, 20 μg/ml; and tetracycline, 25 μg/ml. For selection of *P. aeruginosa* drug-resistant transconjugants or transductants, Pseudomonas Isolation Agar (Difco) was supplemented with carbenicillin (1 mg/ml), tetracycline (300 μg/ml), or kanamycin (200 μg/ml).

DNA methods. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs, Inc. (Beverly, Mass.) and used as recommended by the supplier. Chromosomal and plasmid DNAs were isolated from *P. aeruginosa* by the methods of Marmur (42) and Birboim and Doly (3), respectively. Rapid small-scale and large-scale isolations of plasmid DNA from *E. coli* were done as previously described (30, 38). DNA fragments were purified from agarose gels after electrophoretic separation by the method of Vogelstein and Gillespie (80). Procedures for Southern hybridization analysis and the generation of cosmid banks of *P. aeruginosa* PAO1 by using plasmid pCP13 were performed as previously described (15).

Genetic procedures. Uninterrupted liquid matings with FP2 were carried out as previously described (17). Recombinants were tested for phage sensitivity by repatching individual colonies onto the same minimal selective medium and placing 10 μl of a D3112 phage suspension (5×10^9 PFU/ml) on each patch. Any evidence of cell killing as detected by zones of lysis were scored as sensitive. *E. coli* cells were transformed by the method of Mandel and Higa (40) as modified by Morrison (47). Recombinant plasmids in *E. coli* were introduced into *P. aeruginosa* by using triparental matings with pRK2013 (22) as the mobilizing plasmid or with strain S17-1 as the mobilizer (70). Transductions with G101 (43) and D3112 (14) were performed as described previously. Gene replacement procedures were carried out as recently described by Schweizer (69).

Isolation of phage-resistant, pilus-defective (*pil*) mutants. Lysates of D3112 *c*ts were prepared as previously described (13, 46). An overnight culture of PAO1 cells was diluted, and 3×10^7 cells were mixed with 3×10^{10} D3112 *c*ts phage. Following addition of soft agar, the mixture was poured onto LB agar plates and incubated for 24 h at 42°C. Surviving colonies were streaked onto individual LB agar plates. Phage sensitivity was assayed by two methods. For screening purposes, strains were streaked onto LB agar media and spotted with 10 μl of a phage lysate (10^9 to 10^{10} PFU/ml). Zones of clearing were indicative of phage sensitivity. Phage sensitivity was also measured by the plaque assay. Ten-microliter spots of diluted phage lysates were spotted onto LB top agar seeded with 0.2 ml of a culture of indicator bacteria grown to stationary phase in LB broth.

Motility assays. Twitching motility was assayed by two methods. LB agar (1%) plates poured to an average depth of 3 mm were dried briefly, and the strains to be tested were stab inoculated with a needle to the bottom of the polystyrene dish. Plates were incubated at 37°C for 24 h, after which time the zone between the agar and polystyrene was measured (45). The second procedure used to visualize twitching motility was the slide culture method. Strains were point inoculated onto the surface of a LB agar (1%) slice placed on a microscope slide. The inoculum was covered with a coverslip, and the slide cultures were incubated at 37°C for 1 to 12 h in a humid environment to prevent drying of the medium. Twitching motility was visualized under a Zeiss phase microscope, using the 40× objective. Strain PAO1 (Pil^+ Twt^+) or PAO4141 (Pil^+ Twt^+) was included as a positive control in both assays.

Flagellum-mediated motility was assayed by inoculating the strains to be tested into the center of a LB agar (0.3%) plate. After 8 to 12 h of incubation at 37°C, the plates were inspected for radial zones of bacterial growth indicating a motile response. Strain PAO1 (Fla^+) or PAO4141 (Fla^+) was included as a positive control. Strain PA103 (Fla^-) was included as a negative control.

DNA sequence analysis. The 705-bp *Eco*RI-*Bam*HI fragment from pADD2670 was subcloned into the miniplasmid pMOB. mγδ-1 (Km^r) insertions were introduced into the cloned fragment and mapped by the procedure described previously (2). Individual insertions that were spaced roughly 100 to 200 bp apart were chosen for sequence analysis (see Fig. 4B). Double-stranded DNA sequencing was accomplished with the dideoxy chain termination method (63) with the Sequenase 2.0 sequencing kit (United States Biochemical). The oligonucleotide primers used to determine the sequences adjacent to the mγδ-1 insertions (*res* and *kan* primers) have been described previously (2). 7-Deaza-dGTP was used in the place of dGTP to reduce the number of sequencing artifacts. ^{35}S-dCTP-labeled samples were run in 8 M urea–6% polyacrylamide gels. Sequence analysis was performed with the IBI MacVector software program. Nucleotide and derived amino acid sequences were also analyzed with the Wisconsin Genetics Computer Group software (version 7.1). The TFASTA algorithm for protein homology was used to compare the sequence of the deduced protein product of *pilG* with sequences in the GenBank data base (release 73.0). PUBLISH and GAP were used to generate the *pilG* sequence (see Fig. 5) and for comparison between PilG and CheY (see Fig. 6), respectively.

Expression studies with the T7 RNA polymerase-promoter system. The T7 expression system was used as described by Tabor and Richardson (77). A 705-bp *Hin*dIII-*Bam*HI fragment containing the *pilG* gene was cloned into the identical sites of the T7 promoter-containing vector pT7-2. The resulting plasmid was transformed into BL21(DE3), and expression of the cloned fragment was analyzed by labeling the induced gene products with ^{3}H-labeled amino acids (Amersham; 1 mCi/ml) as described by Groisman et al. (23). The labeled polypeptides were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12.5% acrylamide [wt/vol]) (19) and visualized by fluorography (12).

Electron microscopy. Bacterial strains were examined by electron microscopy for the presence or absence of pili by the following method. LB agar plates were seeded from overnight cultures of bacteria grown in LB broth. Following 6 to 8 h of growth, bacterial cells were removed by gentle washing in 3% gluteraldehyde in phosphate-buffered saline (PBS) and deposited on carbon-coated 400-mesh grids. After 5 min, the grid was blotted, air dried, shadow casted with palladium, and observed under a Zeiss 10C electron microscope.

Immunoblotting. Solubilized proteins were electrophoretically separated by SDS-PAGE (12.5% acrylamide [wt/vol]) as described above. Prestained molecular weight standards (Bio-Rad) were used to assess the efficiency of protein transfer to nitrocellulose membranes. Western immunoblotting was performed as described by Towbin et al. (78), using antipilin rabbit polyclonal serum.

Nucleotide sequence accession number. The nucleotide sequence of the *pilG* gene has been deposited in the GenBank data library under accession number L10831.

RESULTS

Isolation and characterization of a unique PAO pilus-defective (*pil*) mutant. Selection for resistance to pilus-specific phage is a simple strategy that has been used to isolate pilus-defective mutants of *P. aeruginosa* (6, 8, 9, 35). The *P. aeruginosa* Mu-like phages D3112 and B3 have been previously shown to require pili for the infection process and have been used successfully as selective agents in isolating pilus-defective mutants of PAO1 (60). By employing this selection method, a total of 12 spontaneous PAO1 D3112-resistant mutants from a single experiment were isolated. A preliminary analysis of these 12 mutants revealed that they all failed to release viable phage, grew equally well as the parent strain PAO1 at 30, 37, and 42°C, were motile (Fla^+), and remained prototrophic. Macroscopic analysis also revealed that the D3112-resistant mutants had lost the ability to spread on agar plates and grew as compact, domed colonies with smooth and well-defined edges. By comparison, the parent PAO1 grew as flat, spreading colonies with

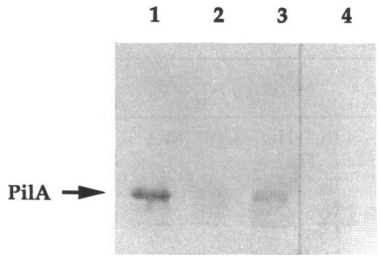

FIG. 1. Immunoblot analysis of pili isolated from *P. aeruginosa*. The blot was incubated with antiserum to *P. aeruginosa* PAO pilin. Lanes: 1, PAO1; 2, FA2; 3, FA2/pADD693; 4, FA3.

ragged edges (10). Phage spot screening assays revealed that 11 of these mutants were broadly resistant to all of the pilus-specific phages used in this study (D3112 *c*ts, B3 *c*ts3, and F116L). However, one D3112-resistant mutant remained sensitive to B3 *c*ts3 and F116L. This unique mutant, designated FA2, was chosen for further analysis.

Plaque assays revealed that phages B3 and F116L were able to form plaques on FA2 just as well as on the parental strain PAO1, with no significant difference in plating efficiency (data not shown). In contrast, phage D3112 was not able to form recognizable plaques on lawns of FA2. However, a slight zone of lysis was noted at a high multiplicity of infection. This suggested that FA2 might be able to support a D3112 infection, albeit at a reduced level.

The ability of phage D3112 to infect FA2 was analyzed more quantitatively by using the D3112 transduction system described previously (14). PAO1, FA2, and FA1, a mutant broadly resistant to the pilus-specific phages isolated in this study, were infected with a phage lysate prepared from CD10 harboring the D3112 in vivo cloning replicon pADD948 (14) at a multiplicity of infection of 1. The transduction frequency (i.e., number of carbenicillin-resistant transductants per PFU) for each strain tested was determined. The transduction frequency for the D3112-sensitive parental strain PAO1 was 1.4×10^{-3}. The transduction frequency for mutant FA1, however, was 5.7×10^{-8}, which represented a 2.4×10^{4}-fold decrease compared with PAO1. This low transduction frequency is typical of mutants which are broadly resistant to the pilus-specific phages and incapable of producing pili (60). By comparison, the transduction frequency for mutant FA2 was 4.8×10^{-5}, which represented only a 29-fold decrease compared with PAO1. This result demonstrated that FA2 was not nearly as resistant to D3112 as FA1, since a significant number of FA2 cells could still be transduced. More importantly, the results of this transduction experiment suggested that FA2 retained a functional phage receptor which was apparently being differentially recognized by the pilus-specific phages used in this study.

Immunoblot and electron microscopic analyses were used to determine the ability of FA2 to produce pili. Cultures of PAO1 and FA2 grown on agar plates were collected in a minimal volume of PBS, vortexed vigorously to shear the pili, and centrifuged to remove cells. The supernatant fluid was concentrated and subjected to SDS-PAGE and immunoblot analysis. Figure 1 (lane 1) shows that the parent PAO1 was capable of producing pili under these conditions, as demonstrated by the recognition of the pilin protein subunit (15 kDa) by the PAO antipilin antibody. However, under identical growth conditions little or no detectable pilin protein could be found in the FA2 sample (Fig. 1, lane 2).

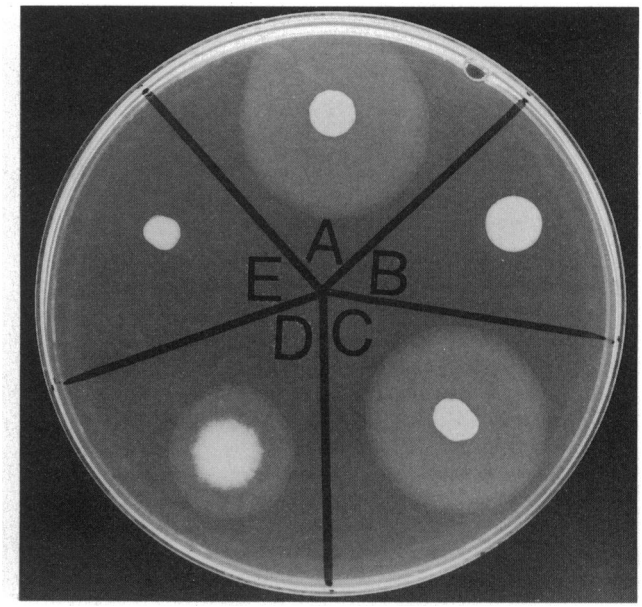

FIG. 2. Stab assay for twitching motility. LB agar (1.0%) plates were stab inoculated with the appropriate strains and incubated for 24 h at 37°C. The diffuse bacterial zone between the agar layer and the bottom of the polystyrene petri dish is a measure of twitching motility (45). The smaller white zones in the center represent cells growing on the top surface of the agar. Sectors: A, PAO1 (Pil^+ Twt^+); B, FA2 (Pil^- Twt^-); C, FA2/pADD693 (Pil^+ Twt^+); D, PAO4141 (Pil^+ Twt^+); E, FA3 (Pil^- Twt^-).

This finding was consistent with electron microscopic analysis of FA2, which revealed the absence of filamentous polar pili (data not shown).

Twitching motility, a novel mode of flagellum-independent surface translocation, has been correlated with the presence of functional polar pili (10, 26). Mutants of *P. aeruginosa* that lack pili or contain nonretractile pili (pil^{NR}) are incapable of twitching motility (10, 82). In order to determine whether FA2 could translocate across surfaces, a simple macroscopic screening assay was used to assess twitching motility. This assay is based on the previous observation that twitching-proficient (Twt^+) strains of *Moraxella bovis* (45) and *P. aeruginosa* (12a) are capable of producing a measurable zone of spreading growth when inoculated at the bottom of an agar plate. In this assay, cells with functional pili move in a flagellum-independent manner along the interface between the agar and the bottom of the polystyrene petri dish. However, pilus-defective mutants of *P. aeruginosa*, which are incapable of twitching motility, do not migrate from the point of inoculation. Figure 2, sector A, shows that the piliated and twitching-proficient parental strain PAO1 (Pil^+ Twt^+) was capable of producing a spreading zone of cells in such an assay. FA2, on the other hand, was unable to spread from the point of inoculation, even after prolonged incubation (Fig. 2, sector B).

Microscopic analysis of *P. aeruginosa* slide cultures has been useful in differentiating twitching motility from other forms of surface translocation (10, 26). An examination of a PAO1 slide culture revealed the presence of characteristic motile rafts of cells at the leading edge of the moving zone (Fig. 3A) and the unique twitching movement of individual cells (10, 26). However, since FA2 was unable to spread significantly from the point of inoculation (Fig. 2, sector B),

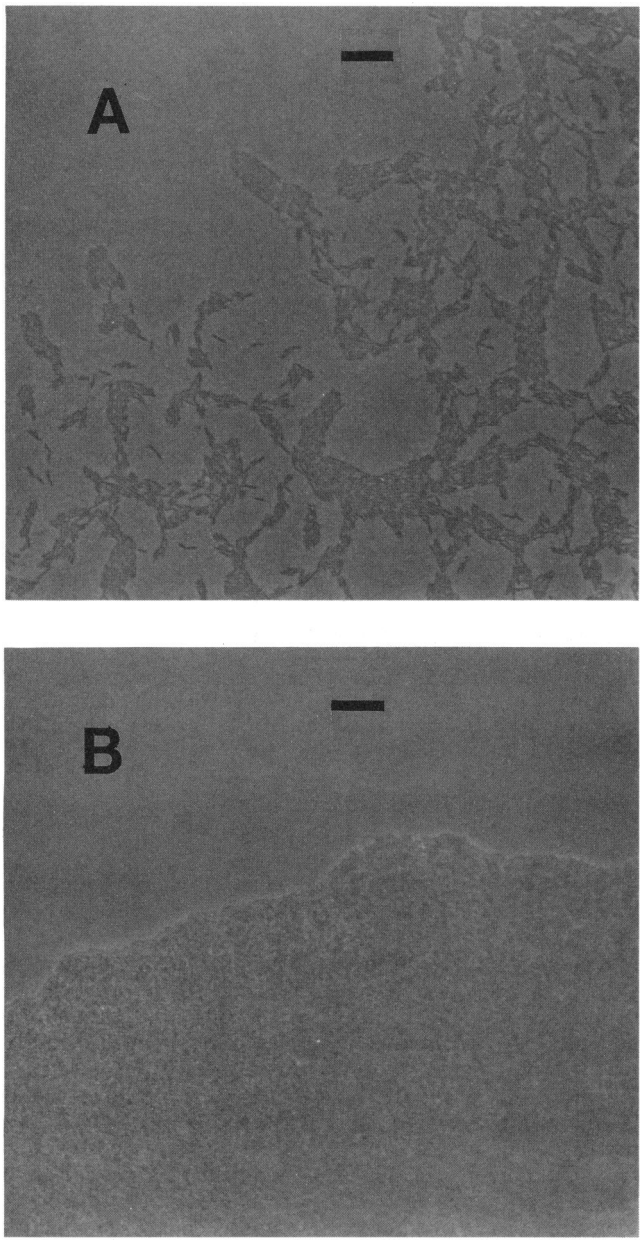

FIG. 3. Slide culture assay for twitching motility. *P. aeruginosa* PAO1 (A) and FA2 (B) were inoculated onto slide cultures and incubated for 6 to 12 h at 37°C. The areas photographed represent the outermost end of cell growth (magnification, ca. ×400). Bar, 5 μm.

the outer boundary of the slide culture microcolony was characterized by multiple layers of nonmotile cells forming a well-defined edge (Fig. 3B). Therefore, from these results, it was concluded that the inability of FA2 to translocate across surfaces by twitching motility was due to the lack of functional pili (Pil^- Twt^-). Nevertheless, FA2 retained the ability to produce a receptor that could still function in the pilus-specific phage infection process.

Mapping of the FA2 *pil* mutation. Several loci involved in pilus biogenesis and twitching motility have been mapped to different regions of the PAO chromosome (16, 21, 82). In FP2-mediated matings, the FA2 mutation responsible for partial resistance to phage D3112 was coinherited at a high frequency with auxotrophic markers located within the first 23 min of the PAO chromosome (Table 2). Markers located later on the PAO chromosome showed no linkage to the FA2 *pil* mutation (data not shown). The D3112 resistance mutation of FA2 was coinherited at a low frequency when both the *ilv* and *his* markers (0 to 7 min) were selected. However, selection for both the *lys* and *met* markers (10 to 20 min) demonstrated a very high coinheritance with the FA2 D3112 resistance mutation (Table 2). Subsequent selection for the individual markers *lys* and *met* revealed that the D3112 phage resistance mutation was apparently tightly linked to the *met-28* allele located at 20 min. The approximate map location of the FA2 *pil* mutation, as determined by FP2-mediated conjugation, was confirmed with G101-mediated transduction, which showed that the FA2 D3112 resistance mutation was cotransduced with the *met-28* marker (Table 2).

TABLE 2. Conjugational and transductional mapping of the FA2 mutation

Donor[a]	Marker(s) selected	Marker location[b]	Coinheritance frequency (%)[c]
FA2/FP2	Ilv^+ His^+ Lys^+ Met^+ Trp^+	0–23	97 (60/62)
	Ilv^+ His^+	0–7	9 (4/44)
	Lys^+ Met^+	10–20	95 (38/40)
	Lys^+	10	8 (7/87)
	Met^+	20	92 (61/66)
FA2(G101)	Met^+	20	16 (15/97)
HOD1(G101)	Met^+	20	73 (77/106)

[a] In every case the recipient was PAO222 Nal-1.
[b] Numbers indicate marker locations in minutes on the revised chromosomal map (52).
[c] Numbers in parentheses indicate the number of transconjugants containing the unselected marker (phage D3112 resistance) per number of transconjugants scored.

The *P. aeruginosa pilT* locus has been previously shown to map near the *proC* gene (82), which is close to the *met-28* marker located at 20 min. G101 transductional analysis with the *pilT* mutant (pil^{NR}), HOD1 (16), revealed that the pilus-specific phage resistance associated with the *pilT* mutation was even more tightly linked with the *met-28* marker than the FA2 *pil* mutation (Table 2). Furthermore, a plasmid, designated pADD1619, which harbors the *pilT* gene on a 6.0-kb *Bam*HI fragment (79, 82), was not capable of complementing FA2. These results, therefore, suggested that the FA2 *pil* locus was close to, but distinct from, the *pilT* locus. As a result, the FA2 *pil* locus was designated *pilG1*.

Complementation of the FA2 *pilG1* mutation. The *pilG* locus was cloned by phenotypic complementation of FA2. Briefly, a *Hin*dIII-generated cosmid (pCP13) library of PAO1 DNA was mobilized into FA2, and tetracycline-resistant (Tc^r) transconjugants were screened visually for the presence of the distinctive wild-type colony morphology associated with pilus production and twitching motility. Several colonies with the wild-type colony morphology were identified after screening of approximately 10^4 Tc^r transconjugants. Three of these colonies were streak purified and, upon testing, were found to have regained sensitivity to phage D3112 and twitching motility, as determined by phage plaque and stab assays, respectively. Preliminary analysis of the plasmid DNA from the three phenotypically complemented transconjugants revealed that each contained, in addition to the pCP13 vector, a large (ca. 30-kb) *Hin*dIII

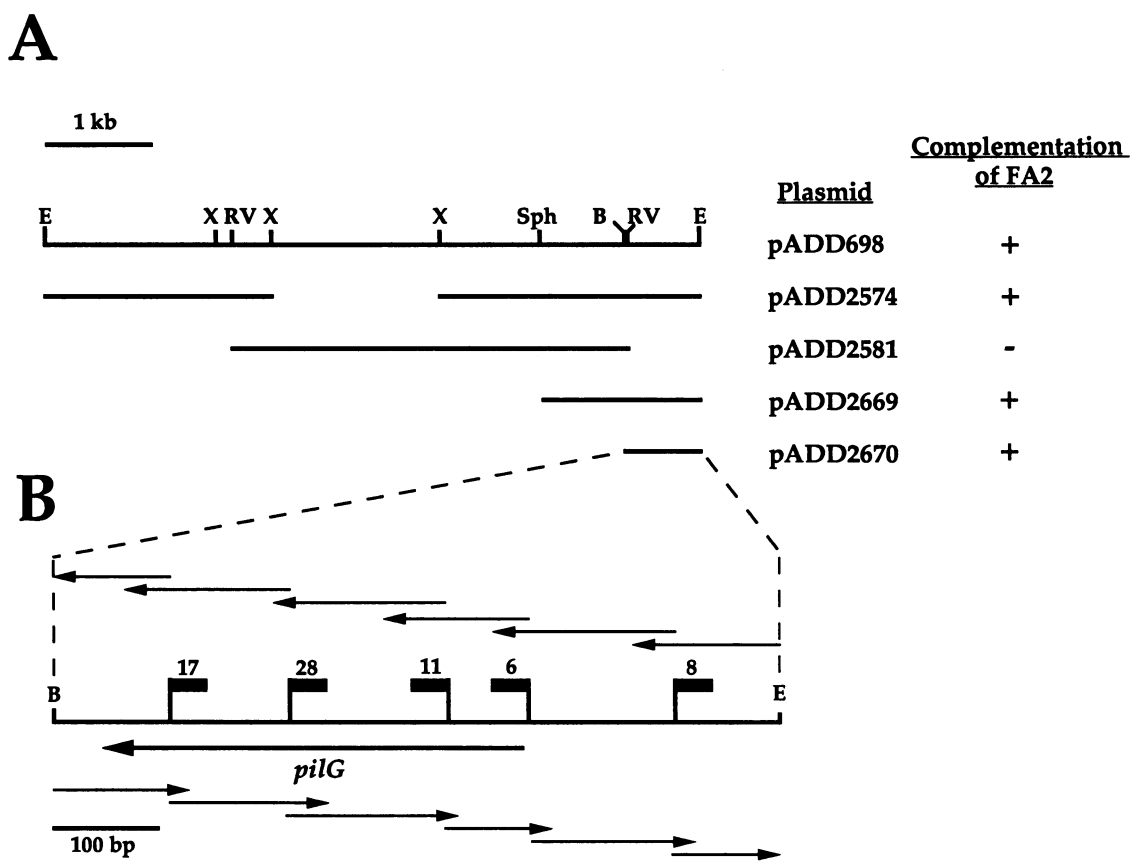

FIG. 4. Analysis of the *pilG* locus. (A) Restriction map of the 6.2-kb *Eco*RI insert in pADD2564 which complements FA2 (*pilG1*). Lines under the restriction map indicate sequences retained in the subcloning analysis. Only pertinent restriction endonuclease sites are shown. Abbreviations: B, *Bam*HI; E, *Eco*RI; RV, *Eco*RV; Sph, *Sph*I; X, *Xho*I. (B) Sequencing strategy for the *pilG* gene. The nucleotide sequence of the entire 705-bp *pilG* region was determined by using the indicated mγδ-1 insertions. The orientation of each insertion is indicated by the rectangle at each insertion point. The rightmost part of each rectangle indicates the *res* terminus (2).

fragment. Additional restriction analysis of these recombinant plasmids, however, suggested that they were most likely identical. Therefore, one of these recombinant plasmids, designated pADD693, was transformed into *E. coli* and reintroduced into FA2 by mating. All of the FA2/pADD693 transconjugants tested were complemented to the wild-type phenotype, as judged by restoration of colony morphology, D3112 sensitivity, twitching motility (Fig. 2, sector C), and PilA production (Fig. 1, lane 3), indicating that complementation was plasmid linked.

Subclones of pADD693 were generated to determine the locations of the sequences responsible for complementing the FA2 *pilG* mutation. From this analysis a recombinant plasmid, designated pADD698, which contained a 6.2-kb *Eco*RI fragment from pADD693 cloned into the unique *Eco*RI site of the broad-host-range vector pRO1614, was found to specifically complement the *pilG1* mutation in *trans* (Fig. 4A). This 6.2-kb *Eco*RI fragment was used as a probe in Southern hybridization analysis of *Eco*RI and *Hin*dIII digests of PAO1 chromosomal DNA. As predicted, this probe identified *Eco*RI and *Hin*dIII fragments of 6.2 and >20 kb, respectively (data not shown). The 6.2-kb *Eco*RI fragment was also used as a probe to localize the *pilG* gene on the *P. aeruginosa* PAO physical map in order to confirm the genetic mapping data. Contour-clamped homogeneous electric field pulsed-field gel electrophoresis and hybridization analysis localized the *pilG* gene to the *Spe*I H and *Dpn*I E fragments located at 16 to 19 min on the PAO1 chromosome (20). This result was consistent with the data presented in Table 2 and confirmed the map location of *pilG*.

Subsequent subcloning experiments were aimed at more precisely localizing the *pilG* locus within the 6.2-kb *Eco*RI fragment. Removal of two interior *Xho*I fragments from pADD698 yielded pADD2574, which retained the ability to complement the *pilG1* mutation (Fig. 4A). Cloning of a 3.8-kb *Eco*RV fragment into the *Ssp*I site of pRO1614 resulted in a plasmid, designated pADD2581, which lost the ability to complement FA2 and suggested that the *pilG* locus was located near one of the ends of the 6.2-kb *Eco*RI fragment. The *pilG* locus was eventually localized first to a 1.6-kb *Eco*RI-*Sph*I fragment (pADD2669) and then to an approximately 700-bp *Eco*RI-*Bam*HI fragment (pADD2670) (Fig. 4A).

DNA sequence analysis of the *pilG* gene. The 705-bp DNA region spanning the *pilG* locus was sequenced on both strands by using five individual mγδ-1 insertions (Fig. 4B). Several possible open reading frames (ORFs) contained entirely within the 705-bp fragment were found. However, of these, only the 405-bp ORF was likely to be the correct PilG reading frame. This determination was based on the findings that the 405-bp ORF (i) contained 53, 37, and 82% G+C at codon positions 1, 2, and 3, respectively, (ii) contained the

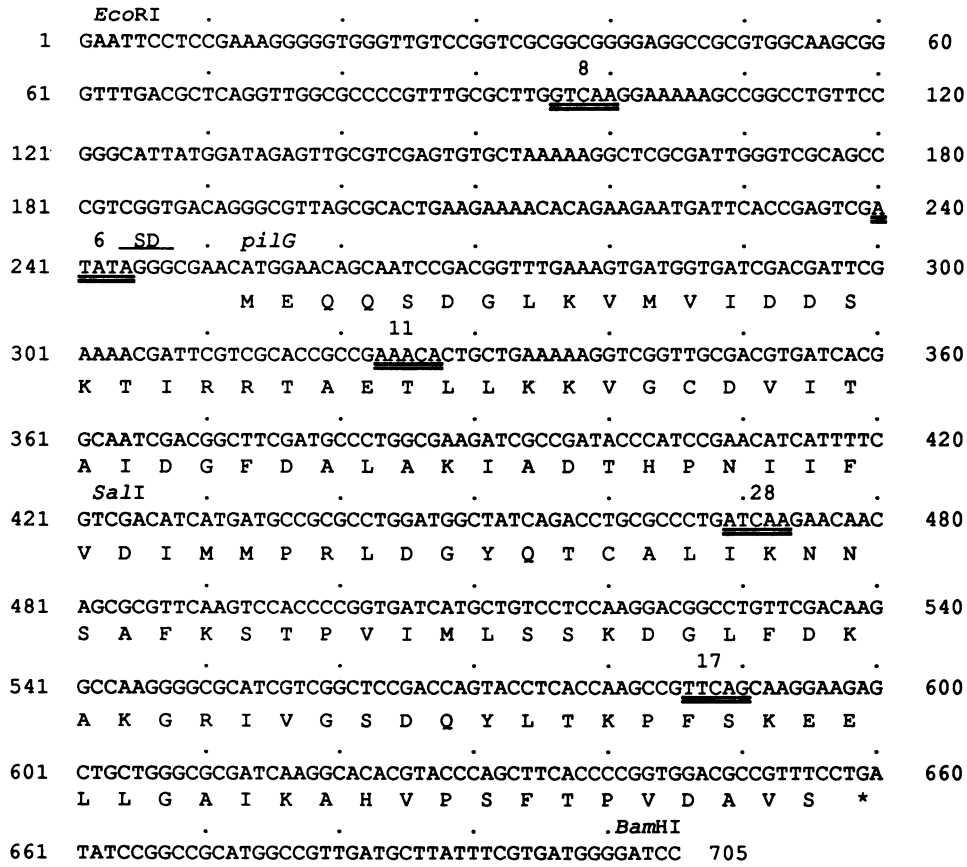

FIG. 5. Nucleotide sequence of the *P. aeruginosa pilG* coding region and flanking sequences. Numbering starts at the *Eco*RI site and ends at the *Bam*HI site. The start codon is located below the gene designation, and the stop codon is designated with an asterisk. A putative ribosome-binding site is indicated by SD. Amino acids deduced from the nucleotide sequence are specified by standard one-letter abbreviations. The double-underlined sequences indicate the target sites and positions of individual mγδ-1 insertions used for sequencing. Relevant restriction sites are located above the sequence.

codon bias pattern characteristic of microorganisms with a high G+C content (81), and (iii) was in the direction of transcription determined in T7 expression studies (*Eco*RI site towards the *Bam*HI site) (Fig. 5; see Fig. 7). This 405-bp ORF was predicted to encode a protein of 135 amino acids with a molecular weight of 14,717. A possible Shine-Dalgarno sequence was found at positions 244 to 247 (AGGG), adjacent to a putative ATG translational start (Fig. 5).

Homology of PilG to the enteric response regulator CheY. The putative PilG amino acid sequence translated from the nucleotide sequence was used to conduct a TFASTA search of the GenBank data base. The PilG sequence showed significant similarity to a large group of bacterial proteins known as response regulators. However, when the size of PilG (135 amino acids) and the fact that the homology between PilG and the response regulators was limited to the N-terminal domains of these proteins are taken into account, then PilG appears to belong to a subclass of response regulators that consist of only the single, conserved N-terminal domain (e.g., CheY and Spo0F [1, 73]).

A 32% amino acid identity to the enteric chemotaxis protein CheY was found (Fig. 6) (44, 48, 72). The predicted protein sequence of PilG revealed the conservation of several critical CheY structural and functional features. On the structural side, PilG contained residues that correspond to the hydrophobic core of CheY. Most notable are the three β-strand regions that contain clusters of four hydrophobic amino acid residues. But more importantly, on the functional side, PilG retained the critical amino acid residues located near to and a part of the phosphorylation active site of CheY (Asp-13, Asp-57, and Lys-109) (Fig. 6) (73).

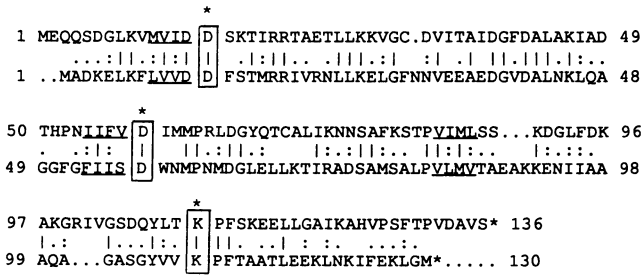

FIG. 6. Sequence alignment of PilG (top line) and *Salmonella typhimurium* CheY (bottom line). The conserved Asp-13, Asp-57, and Lys-109 residues of *S. typhimurium* CheY are boxed. The underlined residues correspond to those clusters of four hydrophobic residues that correspond to the three internal β-strands of CheY (73). The GAP program from the University of Wisconsin Genetics Computer Group was used to generate the alignment. The vertical bars indicate identical amino acids, and colons and periods indicate two degrees of similarity of amino acids.

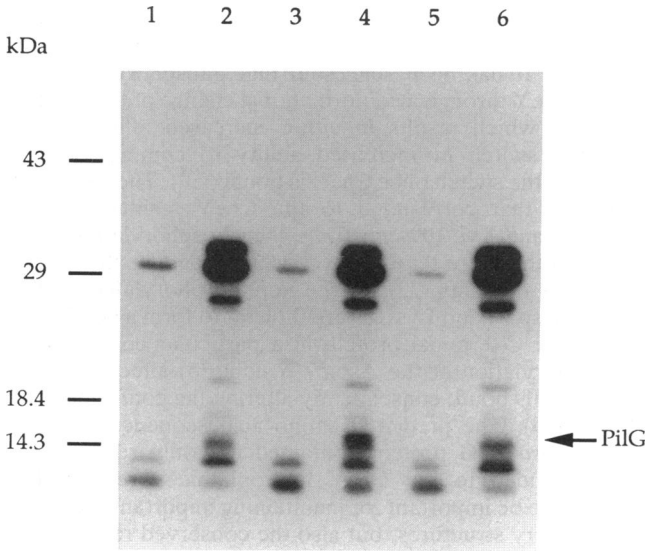

FIG. 7. Expression of the *pilG* gene in *E. coli*. An autoradiograph of ^{3}H-amino-acid-labeled polypeptides expressed with the T7 system in *E. coli* BL21(DE3) is shown. Lanes: 1, pT7-2; 2, pT7-2 plus IPTG; 3, pADD2638; 4, pADD2638 plus IPTG; 5, pADD2664; 6, pADD2664 plus IPTG. Positions of molecular mass markers (in kilodaltons) are indicated on the left. The location of the *pilG* gene product is indicated by the arrow.

Expression of *pilG* in *E. coli*. To verify that the 405-bp ORF (Fig. 5) encodes a protein of the size predicted from the nucleotide sequence, the *pilG* gene was expressed by using the T7 RNA polymerase-promoter system (76, 77). The 705-bp fragment harboring the entire PilG coding region was cloned into vector pT7-2, and the resulting recombinant plasmid, pADD2638, was transformed into BL21(DE3). Following induction with IPTG (isopropyl-β-D-thiogalactopyranoside), proteins were labeled with ^{3}H-amino acids and analyzed by SDS-PAGE and fluorography. Compared with the IPTG-induced pT7-2 control (Fig. 7, lane 2), extracts from cells harboring pADD2638 contained one additional labeled protein (Fig. 7, lane 4). The M_r of this protein band (15,000) closely approximated the predicted molecular weight of PilG (14,717) from the amino acid sequence, suggesting that the PilG protein was produced in *E. coli*. No insert-specific labeled polypeptides were detected when the T7 promoter was placed at the opposite end of the 705-bp fragment adjacent to the *Bam*HI site (data not shown). The deletion of approximately 230 bp from the 3′ end of the predicted ORF (pADD2664) resulted in the apparent loss of the 15,000-Da protein (Fig. 7, lane 6). This result correlated well with the apparent loss of complementing activity when the deleted fragment was introduced into FA2 (data not shown).

Construction and analysis of a marker exchange *pilG* mutant. An allele replacement technique (69) was used to create a genetically defined *pilG* mutant. Briefly, plasmid pADD2692, which contained an mγδ-1 insertion (Km^{r}) in the PilG coding region (insertion 11 in Fig. 3B), was linearized with *Not*I and ligated with the *oriT-sacB*-containing *Not*I cassette of pMOB3 (69). The resulting plasmid, designated pADD2703, was mobilized into PAO4141, and plasmid integration events were detected by plating mating mixtures on selective media containing kanamycin. Curing of the unwanted DNA sequences was facilitated by streaking isolated Km^{r} colonies onto LB-kanamycin plates containing 5% sucrose. This procedure yielded approximately 100 sucrose-resistant (Suc^{r}) colonies. Twenty of these Suc^{r} colonies were tested in phage spot assays, and all were found to be D3112 resistant. Therefore, one colony, designated FA3, was chosen for further analysis.

Southern hybridization analysis was used to verify the replacement of the wild-type allele for the *pilG*::mγδ-1 insertion. *Eco*RV and *Xho*I genomic digests of PAO4141 and FA3 were blotted to a nylon filter and probed with the ^{32}P-labeled 705-bp *Eco*RI-*Bam*HI *pilG*-containing fragment. These enzymes were chosen for this analysis because they do not cut within the 1.8-kb mγδ-1 element. Therefore, it was predicted that the probe should recognize PAO4141 *Eco*RV and *Xho*I fragments with sizes of 4.9 and 3.1 kb, respectively, and FA3 *Eco*RV and *Xho*I fragments with sizes of 6.7 and 4.9 kb, respectively. The results of the Southern analysis revealed that there was an approximately 1.8-kb shift in the hybridization pattern from PAO4141 to FA3 (data not shown). This shift in the FA3 hybridization pattern was consistent with the predicted fragment sizes of the gene replacement mutant.

Further characterization of FA3 revealed that it (i) remained auxotrophic for only methionine and proline, like the parental strain PAO4141, (ii) was broadly resistant to all of the pilus-specific phage used in this study, (iii) was nonmotile (Twt^{-}) in twitching motility assays (Fig. 2, sector E) compared with the parental strain PAO4141 (Fig. 2, sector D), (iv) was unable to produce pili as determined by Western blot (Fig. 1, lane 4) and electron microscopic analysis (data not shown), and (v) was just as motile (Fla^{+}) as the parent PAO4141 in semisolid agar.

To rule out possible polar effects due to the mγδ-1 insertion, pADD2670, which contains the 705-bp *Eco*RI-*Bam*HI *pilG*-containing fragment (Fig. 4A), was introduced into FA3, and transconjugants were tested for the restoration of twitching motility and pilus production. The presence of pADD2670 was found to restore twitching motility, sensitivity to the pilus-specific phages, and pilus production to FA3 (data not shown). This result indicated that if additional *pil* genes were located immediately downstream of *pilG*, the mγδ-1 insertion had no significant polar effects on these downstream sequences.

DISCUSSION

This report describes the characterization of *pilG*, a new gene involved in *P. aeruginosa* pilus biosynthesis and twitching motility. The cloning of the *pilG* gene was facilitated by the isolation of a unique mutant which was identified initially from its resistance to the pilus-specific phage D3112. Although immunological and electron microscopic analyses of FA2 indicated that it was not capable of producing intact functional pili, phage plaque and D3112 transduction studies revealed that FA2 was being differentially recognized by the pilus-specific phages used in this study. Specifically, FA2 was shown to be only partially resistant to phage D3112, but it remained as sensitive as PAO1 to the pilus-specific phages B3 and F116L. This suggested that, in spite of the lack of intact pili, a functional phage receptor(s), in some form or another, was still capable of participating in the infection process.

The unique phenotype of FA2 may be explained by a possible defect in the process of pilus elongation. Even though this mutant was incapable of producing assembled filaments, it might still be capable of forming some sort of

preliminary structure which consists simply of an exposed pilus tip at the cell surface. It is conceivable that in this abbreviated or shortened state the altered pilus structure may have the ability to limit or restrict the access of certain pilus-specific phages to the cell. The degree to which this mutant is able restrict the access of phages would, in a large part, depend upon exactly how much of the pilus tip remains exposed at the cell surface. Phages like D3112, for example, may normally adsorb to regions of the pilin subunit that are no longer readily accessible when placed in the context of an exposed tip. B3 and F116L, on the other hand, may bind to different regions of the pilin subunit that remain accessible near the surface of the cell with an exposed pilus tip. Such a selective recognition of phage by an exposed pilus tip could explain the observed differences in the phage sensitivity patterns.

The hypothesis that FA2 is producing an exposed pilus tip is similar, in many respects, to a model previously proposed to explain the phenotype of a strain of *E. coli* containing an F plasmid harboring a specific *traC* mutation, *traC1044* (66). TraC, a 99-kDa cytoplasmic protein (41, 67), has been implicated along with several other proteins specified by the F *tra* region in the assembly of the F pilus. Schandel et al. (66) showed that cells carrying the *traC1044* mutation (Arg-to-Cys substitution) failed to produce intact F pili. More importantly, they showed that the *traC1044*-containing mutant was temperature-sensitive for infection by the filamentous bacteriophage f1, which uses the tip of the pilus as a receptor, but was resistant to the icosahedral RNA phage f2, which initiates an infection by attaching to the sides of the pilus. Therefore, it seemed likely that the *traC1044* mutant was forming an exposed F-pilus tip at the cell surface instead of an extended filament (66).

More-recent studies have shown that TraC has the ability to interact with the membrane in the presence of other F-factor Tra proteins (68). This interaction with the membrane assembly complex is influenced by the structure of the carboxyl-terminal end of TraC, since the TraC1044 mutant protein, which contains the amino acid substitution in the C-terminal end, exhibits a weaker interaction with the membrane complex than the wild-type protein (68). The interaction of TraC with the assembly complex was apparently abolished in a *traC* amber mutant (66), which is broadly resistant to both phages f1 and f2. Therefore, even though the exact role of TraC in F-pilus production has not been elucidated, the results to date suggest that TraC may function in the assembly and extension of the pilus after the formation of some preliminary pilus structure (66).

The nature of the *pilG1* mutation is not known. However, if it was a simple amino acid substitution (as was the case for TraC1044), it is possible that the PilG1 mutant protein might still be capable of interacting, either directly or indirectly, with the cytoplasmic face of the *P. aeruginosa* pilus assembly complex. Even though this interaction would not be sufficient to produce assembled pili, it might still allow the cell to form an exposed pilus tip. A result consistent with this hypothesis was the discovery that the insertionally inactivated *pilG* mutant FA3 was broadly resistant to the pilus-specific phages, presumably because it was incapable of forming the altered pilus structure (i.e., exposed pilus tip). This result is very similar to the situation seen with the F*traC* amber mutant (66).

The predicted amino acid sequence of PilG revealed significant homology to the enteric, single-domain response regulator CheY (44, 72), whose activity has been shown to be controlled by phosphorylation (4). Phosphorylation causes CheY to interact with the flagellar motor-switch components and is the signal required for clockwise flagellar rotation. It has been suggested that phosphorylation activates CheY through a conformational change of the signaling surface, which results in either increased affinity to the flagellar switch or increased ability to communicate the signal to the switch once CheY is bound (59). The amino acid residues that correspond to the CheY residues Asp-13, Asp-57, and Lys-109 constitute the phosphorylation active site and therefore tend to be conserved among all response regulator domains (73). In the nonphosphorylated form of CheY, Asp-57 and Lys-109 are thought to form a bond which holds the C-terminal protein in a particular conformation. Phosphorylation at the Asp-57 residue (5) affects the Asp-57–Lys-109 bond, consequently altering the conformation of CheY (39). The predicted amino acid sequence of the *P. aeruginosa* PilG protein contained not only residues that corresponded to the CheY hydrophobic core, which is thought to be important for maintaining important secondary and tertiary structures, but also the conserved residues that define the response regulator active site (Fig. 6). Therefore, even in the absence of direct biochemical evidence of phosphorylation, the extensive sequence similarity that defines the response regulator domain suggests that PilG may retain a conserved phosphotransfer enzymology and, like CheY, may also function as a response regulator by directing pilus production within its respective signal transduction network.

In spite of the homology to the enterobacterial chemotaxis protein CheY, two lines of evidence suggest that PilG is involved in pilus biosynthesis and not in directing flagellar rotation. First, the *P. aeruginosa* homolog of the enterobacterial *cheY* gene has been recently cloned and sequenced (71). The *P. aeruginosa cheY* homolog was found immediately downstream of the *fliA* gene, which encodes the sigma factor of RNA polymerase used to transcribe the flagellin gene (71). This *fliA-cheY* gene region has recently been localized to a region of the *P. aeruginosa* PAO physical map that corresponds to 59 to 61 min on the genetic map (21), a considerable distance from the *pilG* locus. Second, enteric *cheY* mutants are counterclockwise biased (i.e., they swim for extended periods without tumbling) and show only a slight degree of spreading in swarm plate assays. However, both of the *pilG* mutants used in this study (FA2 and FA3) were just as motile in semisolid-agar motility (flagellar) assays as the parental strains. Therefore, on the basis of the observations in this study, it is unlikely that PilG is involved in flagellum-mediated chemotaxis.

In conclusion, the results of this study suggest that PilG is likely to play a major role in directing the assembly of the *P. aeruginosa* pilin subunits. The striking sequence similarity to CheY suggests that PilG acts as a single-domain response regulator whose function may be to receive a certain environmental signal(s) and then transduce that signal to the pilus assembly machinery via specific protein-protein interactions. Experiments aimed at determining the ability of PilG to be phosphorylated, the nature of the environmental signal that induces the phosphorylation of PilG, the identity of its cognate sensor-protein kinase, and the specific interaction of PilG with protein complexes are in progress. Furthermore, preliminary nucleotide analysis and expression studies of sequences downstream of *pilG* have revealed the presence of several additional genes that may be cotranscribed along with *pilG* as a polycistronic message. Therefore, these downstream sequences are also currently being

investigated for their involvement in pilus production and twitching motility.

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