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Identification of Two Genes with Prepilin-Like Leader Sequences Involved in Type 4 Fimbrial Biogenesis in *Pseudomonas aeruginosa*

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Type 4 fimbriae are surface filaments produced by a range of bacterial pathogens for colonization of host epithelial surfaces. In *Pseudomonas aeruginosa*, they are involved in adhesion as well as in a form of surface translocation called twitching motility, and sensitivity to infection by fimbria-specific bacteriophage. Analysis of the 2.5-kb intergenic region between the previously defined *pilR* and *pilV* genes on *P. aeruginosa* genomic *Spe*I fragment E has identified three new genes, *fimT*, *fimU*, and *dadA. The predicted 18.5-kDa products of the *fimT* and *fimU* genes contain prepilin-like leader sequences, whereas the third gene, *dadA**, encodes a protein similar to the D-amino acid dehydrogenase of *Escherichia coli*. Isogenic mutants constructed by allelic exchange demonstrated that the *fimU* gene was required for fimbrial biogenesis and twitching motility, whereas the *fimT* and *dada** mutants retained wild-type phenotypes. However, overexpression of the *fimT* gene was found to be able to functionally replace the lack of a *fimU* gene product, suggesting a subtle role in fimbrial biogenesis. The identification of these proteins increases the similarity between type 4 fimbrial biogenesis and the supersystems involved in macromolecular traffic, such as extracellular protein secretion and DNA uptake, all of which now possess multiple protein species that possess prepilin-like leader sequences.**

Type 4 fimbriae are polar organelles that occur on the surface of a number of bacterial pathogens including *Pseudomonas aeruginosa*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Dichelobacter nodosus*, and *Moraxella bovis* (7, 39, 51). These fimbriae are involved in attachment and colonization of host epithelial surfaces, as well as a form of flagellum-independent surface translocation across solid surfaces termed twitching motility (21), which is proposed to occur via extension and retraction of the fimbrial strand (5). In *P. aeruginosa*, the expression of the fimbrial subunit gene (*pilA*) is also required to enable infection by fimbria-dependent bacteriophages (55). However, the recent identification of nonfimbriated mutants that remain sensitive to bacteriophage infection suggests that the PilA protein does not need to be assembled into an extracellular filament and may only be required to be presented at the cell surface (8, 9).

The biogenesis and function of type 4 fimbriae in *P. aeruginosa* involve the expression and regulation of a large network of genes. These genes have been physically mapped to six separate regions on the *P. aeruginosa* genome. The largest group is located on *Spe*I fragment E at 75 min on the genetic map and contains the major fimbrial subunit gene (*pilA*), two ancillary genes (*pilBC*), and the leader peptidase gene (*pilD*) (23, 37, 38). Located approximately 20 kb downstream are the two-component sensor-regulator system that controls *pilA* transcription (*pilSR*) (22) and two recently identified genes whose products possess prepilin-like leader sequences (*pilV* and *pilE*) (2, 44). The alternative sigma factor σ^{54}, encoded by the *rpoN* gene, is required for *pilA* transcription and is located downstream of *pilA* (18, 27). The second region is located on *Spe*I fragment H at 20 min on the genetic map and contains two tandemly orientated nucleotide-binding proteins, *pilT* and *pilU* (58, 59), and a cluster of genes, *pilGHIJK*, which encode proteins which display homology to the enteric chemotaxis system (8–10) and which are thought to control fimbrial function in response to environmental stimuli transduced via this Che-like network. A third gene cluster encoding five genes required for fimbrial assembly, *pilMNOPQ*, has been identified on *Spe*I fragment B located at 5 min on the genetic map (32, 33). Another two-component regulatory system, encoded by the *fimS* and *algR* genes, located at 10 min on the physical map on *SpeI* fragment G has recently been shown to be required for type 4 fimbrial biogenesis, suggesting complex regulatory controls integrating fimbrial biogenesis with the expression of other virulence factors (57). The last two loci at 40 min (*Spe*I fragment I) and 30 min (*Spe*I fragment S) on the genomic map contain the genes *pilZ* and *pilF*, respectively, whose specific functions are unknown but which are required for the correct export and assembly of the fimbrial subunit (1, 54).

Many similarities exist between the biogenesis of type 4 fimbriae and the assembly of systems required for the export of extracellular proteins or the import of DNA in a large range of gram-negative and gram-positive bacterial species (2, 24, 34). A characteristic of these networks is the presence of multiple genes which possess prepilin-like signal sequences (3, 14, 26, 31, 41). It has been proposed that type 4 fimbriae may be assembled via a pathway analogous to these complexes involved in macromolecular trafficking (24, 41, 58). This hypothesis has been strengthened by the recent identification of two additional genes (*pilV* and *pilE*) which have prepilin-like leader sequences and which are required for fimbrial biogenesis (2, 34, 44).

In this report, we have characterized the area between the *pilR* and *pilV* genes on *Spe*I fragment E and identified three additional open reading frames (ORFs). Two of these have been shown to be involved in the biogenesis of type 4 fimbriae by the construction of isogenic mutants. Further, they also

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TABLE 1. Plasmids used in this study

Plasmid	Description	Source or reference
pEB15	*P. aeruginosa* T7 expression vector	6
pUCP18/19	*P. aeruginosa-E. coli* shuttle vectors	47
pSM-TET	Source of Tc^r cassette	36
pOK12	Km^r cloning vector	53
pMOB3	Source of *Not*I cassette carrying *sacBR* and oriT	48
pAW103	PAK *pilA* gene carried in pUCP19	55
pRIC333	*Bam*HI-*Bgl*II fragment carrying *pilV*	2
pRIC338	*Sal*I-*Bgl*II fragment carrying *fimU pilV*	This study
pRIC351	*Xho*I fragment from 3′ *pilR* to 3′ *pilV* in pBSK	This study
pRIC375	*Xho*I fragment from 3′ *pilR* to 3′ *pilV* in pUCP18	This study
pRIC380	pBSK carrying the *Not*I cassette from pMOB3	This study
pRIC384	*Xho*I-*Bgl*II fragment from 3′ *pilR* to 3′ *pilV* in pOK12	This study
pRIC388	pRIC380 carrying *fimT*:Tc^r (in *Sal*I) on *Spe*I fragment	This study
pRIC389	pRIC380 carrying *dadA**:Tc^r (in *Pst*I) on *Spe*I fragment	This study
pRIC390	pRIC380 carrying *fimU*:Tc^r (in *Pst*I) on *Spe*I fragment	This study
pRIC393	*fimU* in pEB15 in direction of T7 promoter	This study
pRIC395	*dadA** in pEB15 in direction of T7 promoter	This study
pRIC399	*dadA** (*Nco*I-*Xho*I) in pUCP19 (against *lac*)	This study
pRIC402	*fimT* in pEB15 in direction of T7 promoter	This study
pRIC408	pRIC380 carrying *dadA** *fimTU*:Tc^r (in *Pst*I) on *Spe*I fragment	This study
pRIC420	*Pst*I fragment carrying *fimT* in pUCP18 (with *lac*)	This study
pRIC421	*Pst*I fragment carrying mutated *fimT*(D-1) in pUCP18 (with *lac*)	This study
pRIC423	*Sph*I fragment carrying *fimU* in pUCP19 (with *lac*)	This study

contain N-terminal sequences that are characteristic of the fimbrial subunit and display homology to other members involved in the extracellular protein export pathways of gram-negative bacteria. This increases the parallels between protein export systems and the biogenesis of type 4 fimbriae and strengthens the conclusion that the structures involved have a common ancestor and are assembled by a similar mechanism.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *P. aeruginosa* PAK (David Bradley, Memorial University of Newfoundland, St. John's, Canada) and PAO1 (Bruce Holloway, Monash University, Clayton, Australia) were used to construct the mutants described in this study. *Escherichia coli* JM109 was used as a host for all manipulations, including generation of single-stranded DNA templates prior to DNA sequencing, and *E. coli* S17-1 was used as the donor strain in the conjugal matings. The DNA inserts of recombinant plasmids used throughout this study are listed in Table 1. Complementation experiments were performed in plasmids pUCP18 and -19 (kindly provided by H. Schweizer), which replicate stably in both *Pseudomonas* and *E. coli* hosts (47). Bacteriophage PO4 is a PAK fimbria-specific bacteriophage described elsewhere (4). The D3112 and B3 bacteriophages have been described elsewhere (43) and were kindly provided by A. Darzins. All bacterial strains were maintained on Luria-Bertani medium (45) containing 100 μg of kanamycin or ampicillin (*E. coli*) per ml or 500 μg of carbenicillin (*P. aeruginosa*) per ml if maintenance of plasmids was required.

Recombinant DNA techniques and transformation. Preparation of plasmid DNA, digestion with restriction endonucleases (New England Biolabs, Beverly, Mass.), ligations, and transformation in *E. coli* hosts were carried out under standard conditions as described by Sambrook et al. (45). Preparation of *P. aeruginosa* competent cells and subsequent transformation were performed as described previously (2).

Construction of isogenic mutants. Transconjugants were generated by plate conjugation of rifampin-resistant *P. aeruginosa* PAO1 and PAK with *E. coli* S17-1 (carrying the desired constructs in pRIC380) on nonselective media overnight at 37°C. The mating mixture was resuspended in L broth and plated on rifampin (20 μg/ml) plates containing tetracycline (200 μg/ml). Individual colonies were streaked onto L agar containing 5% sucrose and tetracycline in order to isolate colonies which were carbenicillin sensitive and tetracycline resistant.

Bacteriophage sensitivity. Bacteriophage PO4 was diluted from stock preparation in SM buffer (45), and 10 ml of the appropriate dilutions was added to 0.1 ml of logarithmic-phase culture of the *Pseudomonas* strain to be tested and allowed to absorb for 10 min at room temperature. Overlay agar, cooled to 40°C, was then added, and the mixture was poured onto a fresh Luria-Bertani plate. Both the base and overlay agar contained 500 μg of carbenicillin per ml where appropriate. Growth curves were performed by inoculating L broth with a 1/100 dilution of overnight culture. The cells were grown at 37°C to logarithmic phase (A_{600} of 0.5), and the culture was split in half. PO4 bacteriophage stock was added to a multiplicity of infection of 10 to one of each pair of cultures, and A_{600} readings of the cultures with and without phage were compared at regular intervals.

Subsurface twitching assay. The quantitative assay for twitching motility was an adaptation of the subsurface stab assay in which twitching motility is elaborated as a zone of cellular motility in the interstitial area between the bottom of the agar and the petri dish (2, 35). The twitching zone was fixed and stained with Coomassie blue R250 (in 20% methanol–10% acetic acid) after the 1% agar had been compressed and was photographed after destaining.

Protein expression. Plasmid-encoded proteins were visualized by using the T7 expression vector pEB15 and *P. aeruginosa* ADD1976, which contains a T7 RNA polymerase gene under *lac* control introduced into the chromosome (6). The conditions for induction and labelling were as described previously (2).

Sequencing and protein analysis. A series of subclones and deletions were constructed for DNA sequence analysis, and single-stranded DNA sequencing was achieved by the dideoxy chain termination method (46) using the Pharmacia T7 deazaG/A sequencing kit. Sequence analysis was performed with the MacVector 4.0.1 software program. Analysis of the deduced protein sequences was performed with the Genetics Computer Group (Madison, Wis.) software packages (12). TFASTA and BLAST searches of the GenBank databases were performed through the Australian National Genome Information Service.

Pilin preparation and immunoblotting. Surface preparations of fimbriae were made as described previously (2). All samples were heated to 100°C for 5 min prior to analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 15% gel (Bio-Rad) by the method of Laemmli (29). The proteins were then transferred electrophoretically to Hybond-C nitrocellulose paper (Amersham) in the Tris-glycine buffer system of Towbin et al. (52) and detected with anti-fimbrial subunit antiserum (1:5,000) and goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase (1:5,000).

Cell fractionation. After removal of surface fimbrial filaments as described above, the bacterial cells were fractionated as follows. Periplasmic contents were released according to the $MgCl_2$ treatment method of Hoshino and Kageyama (25), and the cells were further separated into membrane and cytoplasmic fractions as described by Hancock and Nikaido (19).

ELISA. The enzyme-linked immunosorbent assay (ELISA) was done essentially by the method of Engvall and Perlmann (15). Bacterial cells were bound overnight, and the developing antibody was alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin. The A_{405} was read with a Bio-Rad ELISA reader. Bovine serum albumin was used as an antigen control. Viable bacterial counts were performed to confirm that the overnight incubation in coating buffer did not result in appreciable cell lysis.

Nucleotide sequence accession number. The complete nucleotide sequence of the *pilR-pilV* region appears in the EMBL and GenBank databases under accession number L48934.

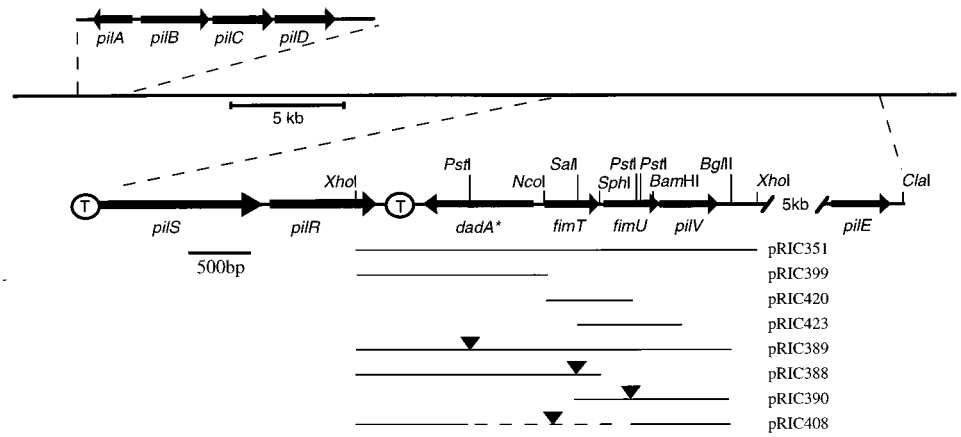

FIG. 1. The relative location of the genes associated with type 4 fimbrial biogenesis on *Spe*I fragment E located at 70 to 74 min on the genomic map. The *pilA* to *-D* cluster (37) is located approximately 20 kb upstream of the *pilSR* regulatory genes (22). The *pilE* gene (44) is located approximately 5 kb downstream of the *pilV* gene (2). The arrows represent the relative transcriptional orientations of the coding regions. A schematic representation of the restriction map of the intergenic region between *pilR* and *pilV* diagrammatically represents some of the relevant plasmid inserts listed in Table 1. The "T" represents putative bidirectional transcriptional terminators, and the black triangle represents the Tc^r cassette insertion point.

RESULTS

Cloning and sequence analysis of the *pilR-pilV* intergenic region. The cosmid pMO013441 was obtained from a wild-type *P. aeruginosa* PAO1 genomic library and contains the *pilSR* genes and DNA sequences distal to this locus (22). The recently identified *pilV* gene is also located on this cosmid (2), and Southern hybridization analyses estimated the intergenic region between *pilSR* and *pilV* to be approximately 2.5 kb (data not shown). A 3.5-kb *Xho*I fragment that hybridized specifically to a *pilV* probe (pRIC333, Table 1) was subcloned from pMO013441 into pBluescriptSK, generating pRIC351 (Fig. 1). Preliminary sequence analyses of this *Xho*I fragment identified sequences identical to the 3′ end of the *pilR* coding region, confirming that pRIC351 represented the *pilR-pilV* intergenic region.

The complete nucleotide sequence of the *pilR-pilV* region was determined for both strands (see above for accession number). The percent G+C for this region was 65, which is in excellent agreement with the value of 67% estimated for the *Pseudomonas* genome (56). An ORF search of this region found three putative coding regions with the characteristic high bias for G+C residues in the third position of the coding triplet. The largest ORF was found in the orientation opposite to both *pilR* and *pilV* and is followed by a putative bidirectional transcriptional terminator structure downstream of *pilR* (Fig. 1). This ORF exhibited a third-position G+C bias of 83% and encoded a protein of 364 amino acids with a predicted M_r of 39,387. This ORF has a good ribosome binding site (GGAGG) located 5 bp upstream of a GTG start codon. When the predicted amino acid sequence was used to perform BLAST and TFASTA searches of the GenBank database, this ORF, designated *dadA**, displayed high homology (29% identical and 49% conservative) with the D-amino acid dehydrogenase (*dadA*) gene of *E. coli*, especially at the NH_2 and COOH termini. (Note that we use the asterisk to indicate similarity, but not necessarily homology, of biological function.)

The other two putative ORFs were located in the orientation opposite to *dadA** in the same direction as *pilV* (Fig. 1). The first ORF, located 134 bp away from the *dadA** initiation codon, encodes a putative protein of 169 amino acids with a predicted M_r of 18,946. Located a further 105 bp downstream was another ORF which encodes a product of 168 amino acids (*M*r, 18,128) and whose 3′ coding region overlapped the *pilV* initiation codon by 10 bp. Both these ORFs were preceded by potential ribosome binding sites and also possessed high G+C bias in the third coding position, with values of 76 and 83%, respectively. The translated protein sequences of these two similarly sized ORFs, named *fimT* and *fimU*, were used to perform database searches. FimT and FimU were found to possess a high degree of homology to both type 4 fimbrial subunits and members of extracellular protein secretion networks found in a number of bacteria. Both possessed the extremely hydrophobic N-terminal region that is characteristic of this class of protein (Fig. 2). FimU contained the highly conserved G−1F+1/E+5 motif in the predicted mature protein that is required for both cleavage of the leader sequence and subsequent methylation of the mature protein by the specific endoprotease PilD (or its equivalent) (50) (Fig. 2). However, the residues around the potential cleavage site of FimT were not as conserved, being A−1L+1/E+5. Although all known

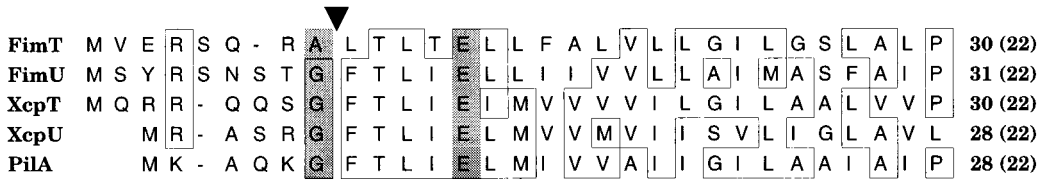

FIG. 2. Alignment of the hydrophobic N-terminal regions of FimT, FimU, XcpT, XcpU, and PilA. The identical residues among at least three members are boxed, although many conservative substitutions are also evident. The amino acid number is shown on the right, with the position with respect to the mature protein shown in parentheses. The cleavage point (after the Gly-1 or Ala-1 residue) of the prepilin-like leader sequence is indicated by an arrowhead. The critical residues for cleavage (−1 position) and methylation (+5 position) are shaded.

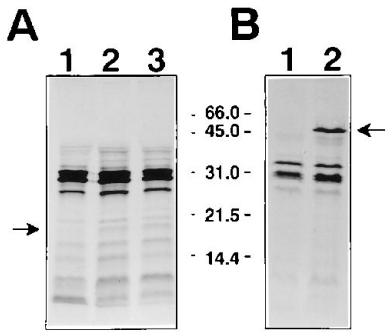

FIG. 3. Expression of the *fimT* and *fimU* (A) and *dadA** (B) genes in *P. aeruginosa* ADD1976. Plasmid-encoded proteins produced from the T7 promoter in plasmid pEB15 were labelled with [^{35}S]methionine and separated on a 15% polyacrylamide gel. The plasmids used were as follows: (A) pEB15 (vector alone; lane 1), pRIC393 (expressing *fimU*; lane 2), and pRIC402 (expressing *fimT*; lane 3); (B) pEB15 (vector alone; lane 1) and pRIC395 (expressing *dada**, lane 2). The molecular mass (in kilodaltons) markers are indicated between the panels, and the unique gene products are indicated by arrows.

members of this protein class identified to date have a G−1 residue, mutational analyses of the PilA protein have shown that Ala at position −1 does allow cleavage of the signal sequence (49). Site-directed mutagenesis has shown that F+1 to L+1 is also a conservative substitution (49), and some other natural members of this class of proteins possess a Met (PulI, OutI, and TcpA) or a Leu (BfpA) in this position (13, 16, 20, 42). Therefore, it remained a possibility that both FimT and FimU would be processed by the PilD endoprotease in a similar fashion as PilA and PilV (2, 50). Another common feature within the fimbrial subunits is a disulfide-bonded domain, usually found towards the C terminus. Both the predicted FimT and FimU proteins have a pair of Cys residues in this region separated by 31 and 30 residues, respectively.

Extensive similarities exist over the entire length of the prepilin-like leader sequence containing components of the protein secretion networks (2, 24). It has been noted that the members can further be distinguished by several group-specific residues distal to the E+5 residue in the hydrophobic core (2). The FimT and FimU proteins are highly related to each other (27% identity and 43% similarity), and although neither contains the absolute group-specific identity distal to the E+5 residue, FimT and FimU do display considerable overall homology to the protein secretion components XcpT and XcpU, being 34 and 43% similar, respectively.

Expression of *dadA, *fimT*, and *fimU* genes.** To confirm the predicted ORFs, the *dadA**, *fimT*, and *fimU* genes were separately cloned into the broad-host-range T7 expression vector pEB15 in an attempt to visualize the protein products and confirm the predicted ORFs. After induction with IPTG (isopropyl-β-D-thiogalactopyranoside) and labelling with [^{35}S]methionine, *P. aeruginosa* ADD1976 carrying plasmid pRIC395, which contained the *dadA** gene in the correct orientation with respect to the T7 promoter, produced a unique protein with an approximate M_r of 40,000 (Fig. 3). Introduction of the *fimT* gene (pRIC402) or the *fimU* gene (pRIC393) into pEB15 resulted in the identification of unique proteins after prolonged exposure to compensate for the fact that these proteins contained only two and three methionine residues, respectively (Fig. 3).

Generation and phenotypic characterization of isogenic *dadA, *fimT*, and *fimU* mutants.** To evaluate the role that these three genes play in type 4 fimbrial biogenesis, construction of isogenic chromosomal mutants was required. Although our laboratory possesses an extensive library of transposon-generated fimbrial mutants that have lost their twitching motility phenotype, Southern analysis revealed that none of our mutants localized to this area (data not shown). Therefore, the unique *Pst*I and *Sal*I restriction sites in *dadA** and *fimT*, respectively, and the two closely spaced *Pst*I sites in *fimU* were selected as suitable positions to inactivate the relevant coding sequences (Fig. 1).

The plasmids required for construction of the mutants are shown in Fig. 1 and listed in Table 1. Briefly, the Tc^r gene cassette from pSM-TET (36) was inserted in the unique sites stated above. The plasmids were all constructed in the pOK12 vector (Table 1), whose multiple cloning site is flanked by *Spe*I sites. Once the Tc^r cartridge had been inserted into the gene to be inactivated, the entire insert was removed as a *Spe*I fragment and inserted into the unique *Spe*I site in pRIC380. Plasmid pRIC380 is a suicide delivery vector that is essentially pBluescriptSK$^+$ (Ap^r or Cb^r) containing the *Not*I cassette from pMOB3, which carries *oriT* and *sacBR* genes (Table 1). The subsequent recombinant plasmids carrying inactivated *dadA** (pRIC389), *fimT* (pRIC388), and *fimU* (pRIC390) genes were transformed into the donor *E. coli* strain S17-1 and conjugally introduced into wild-type *P. aeruginosa* PAK and PAO1. The inability of these plasmids to replicate in *P. aeruginosa* implies that any Tc^r Cb^s transconjugants should represent a mutated chromosomal gene. The homologous segments of DNA represented an ample amount of homology for recombination into both strains, and Tc^r Cb^r transconjugants due to plasmid integration appeared at a frequency of 10^{-6} and 10^{-8} for *P. aeruginosa* PAK and PAO1, respectively. These isolates were then grown on tetracycline plates containing 5% sucrose to select for excision of the plasmid while leaving a mutated gene copy on the chromosome. Two mutants of each class (except PAO1*dadA**) were selected, and their genotypes were confirmed by Southern hybridization analysis (data not shown).

Infectivity by fimbria-dependent bacteriophages is a characteristic that has been associated with the expression of a functional fimbrial strand. The PAK mutants were assayed against the PAK-specific bacteriophage PO4 (4) and also against the bacteriophages D3112 and B3 that had been generated on PAK, as they have been previously shown to be subject to host restriction (43, 55). Likewise, the PAO1 mutants were assayed against B3 and D3112 that had been generated on PAO1, and the titers are shown in Table 2. It is clear that the *fimT* and *dadA** mutants still allow a productive bacteriophage infection against all bacteriophage examined to levels comparable to those of the respective wild-type parents (Table 2). However, the PAO1*fimU* mutant was totally resistant to infection by D3112$_{(O)}$ and B3$_{(O)}$, whereas the PAK*fimU* mutant remained

TABLE 2. Bacteriophage titers

Bacterial strain	Titer of bacteriophage:				
	PO4	B3*cts*$_{(O)}$	B3*cts*$_{(K)}$	D3112*cts*$_{(O)}$	D3112*cts*$_{(K)}$
PAK	1×10^{11}	hr[a]	1×10^6	hr	7.1×10^7
PAK*dadA**	3×10^{11}	hr	2×10^6	hr	7×10^7
PAK*fimT*	2×10^{11}	hr	1×10^6	hr	6.8×10^7
PAK*fimU*	9×10^{10}[b]	hr	9×10^4	hr	1.4×10^6
PAK*ATU*	0	hr	0	hr	0
PAO1	ND[c]	4×10^9	hr	6.6×10^9	hr
PAO1*fimT*	ND	2×10^9	hr	5.4×10^9	hr
PAO1*fimU*	ND	0	hr	0	hr

[a] These bacteriophages show host restriction (hr).
[b] Plaques were turbid.
[c] ND, not done.

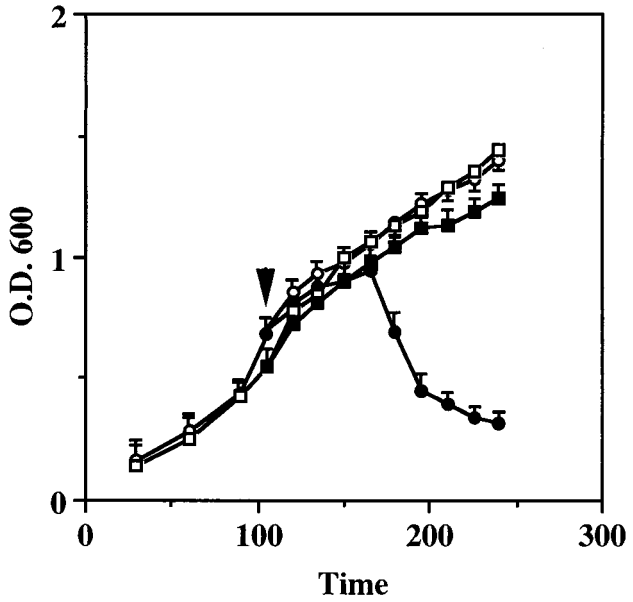

FIG. 4. A growth curve examining the effect of the addition of the bacteriophage PO4 on the growth of *P. aeruginosa* PAK and the PAK*fimU* mutant. The points represent the average of three such experiments, and the standard errors are indicated by bars. The arrow represents the time point of addition of the bacteriophage to the bacterial culture. PAK with PO4 (●) showed rapid cell lysis in comparison with PAK alone (○). In contrast, PAK*fimU* with PO4 (■) appeared to demonstrate retarded cell growth in comparison with PAK*fimU* (□).

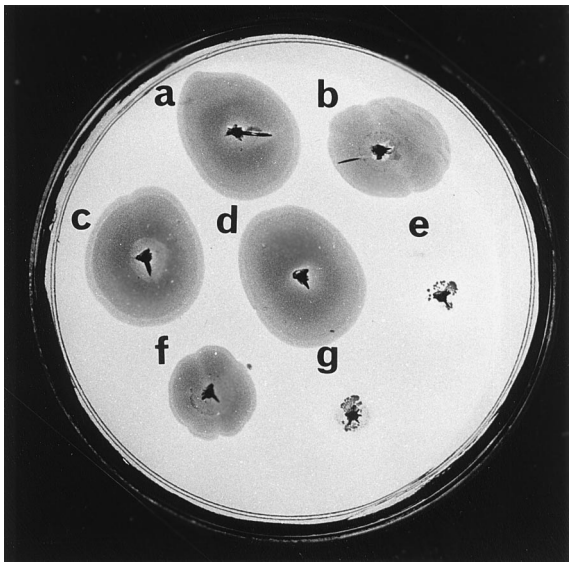

FIG. 5. The subagar surface translocation assay used to determine twitching motility. Zones of motile cells are as follows: a, *P. aeruginosa* PAK; b, *P. aeruginosa* PAO1; c, *P. aeruginosa* PAK*dadA**; d, *P. aeruginosa* PAK*fimT*; e, *P. aeruginosa* PAK*fimU*; f, *P. aeruginosa* PAO1*fimT*; g, *P. aeruginosa* PAO1*fimU*.

sensitive to $D3112_{(K)}$ and $B3_{(K)}$ infection, albeit at a level slightly reduced from wild-type PAK. Recent data have provided evidence that infection by both the D3112 and B3 bacteriophages is reliant on some other cellular factor in conjunction with expression of a type 4 fimbrial subunit, although the serotypic class of the subunit is irrelevant (55). The data presented here with respect to the *fimU* mutants imply that either the cellular factor involved or the mode of D3112 and B3 infection in PAK and PAO1 differs subtly, or that the *fimU* gene product performs a slightly different function in the two wild-type strains. When the titer of bacteriophage PO4 against the PAK*fimU* mutant was determined, it possessed a wild-type titer (Table 2), although the plaques generated were smaller and extremely turbid compared with infection of wild-type PAK. To investigate the effect that bacteriophage PO4 has on the PAK*fimU* mutant, growth curves were determined (Fig. 4). After addition of bacteriophage PO4 to logarithmic-phase PAK cells, cell lysis was observed as monitored by readings of optical density at 600 nm (Fig. 4). In contrast, the addition of PO4 to PAK*fimU* cells consistently resulted in some retardation of cell growth similar to that seen previously for strain PAO1 (55). Thus, we believe that the presence of these small, turbid zones may represent a slight productive infection by PO4 but that the bacteriophage is unable to product an effective lytic infection in PAK*fimU*.

A further phenotypic assay used to classify these mutants was the subsurface stab test to assay twitching motility. The PAK*dadA**, PAK*fimT*, and PAO1*fimT* mutants were all unaltered in their ability to translocate via twitching motility and produced motility zones equivalent to those of their respective wild-type strains (Fig. 5). In contrast, however, the *fimU* mutant from both serotypes produced no detectable motility zones, which is characteristic of nonfunctional fimbrial mutants (Fig. 5). Subcellular fractionation on the mutants was performed, and the fractions were assayed via immunoblotting for the presence of the PilA fimbrial subunit. All of the mutants lacked PilA in the cytoplasmic or periplasmic fractions (Fig. 6) but had a membrane pool of processed PilA (Fig. 6). As expected from the twitching assay, the PAK*dadA** and PAK*fimT* mutants possessed fimbriae on the cell surface. In contrast, the PAK*fimU* mutant produced no detectable surface fimbriae (Fig. 6). The results for the *fimT* and *fimU* mutants of PAO1 displayed the same fractionation profile (data not shown).

Finally, these mutants were analyzed for their ability to export extracellular proteins to determine whether these mutations had any affect on the closely related Xcp pathway. All the mutants were assayed for the export of elastase, and it was found that they were identical to their respective wild-type parental strains (data not shown), suggesting that these effects observed were specific for the type 4 fimbrial biogenesis system.

Complementation of a *fimU* mutant. As the PAK*fimU* mutant remained sensitive to infection by PO4 bacteriophage, it was likely that disruption of this gene does not have any polar effect on the *pilV* gene that lies immediately downstream and whose initiation codon is contained within the 3′ *fimU* coding sequence, as *pilV* mutants are resistant to bacteriophage PO4 infection (2). Complementation data obtained during the characterization of the *pilV* gene suggested that, although the native *pilV* promoter lay upstream of the *Pst*I site internal to *fimU* (2) (Fig. 1), there was a weak promoter that transcribed the *pilV* gene which was located downstream of the *Bam*HI site in the 3′ coding region of *fimU* (2) (Fig. 1). Transcription driven

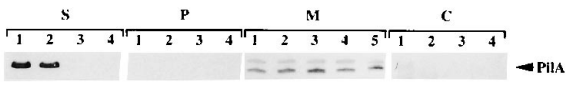

FIG. 6. Immunoblot of PilA subunit found in cellular fractions of *P. aeruginosa* PAK*dadA** (lanes 1), *fimT* (lanes 2), *fimU* (lanes 3), and *ATU* (lanes 4) mutants. Cell fractions are as follows: extracellular shear fraction (S), periplasm (P), membrane (M), and cytoplasm (C). The PilA subunit is indicated by an arrow. A PAK*pilD* mutant is shown in lane 5 of the membrane fraction as an unprocessed PilA control and migrates slightly more slowly.

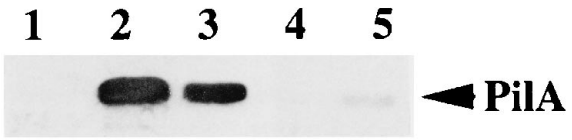

FIG. 7. Immunoblot of surface-located pilin located in samples of *P. aeruginosa* PAK*fimU*[pUCP19] (lane 1), *P. aeruginosa* PAK*fimU*[pRIC338] (lane 2), *P. aeruginosa* PAK*fimU*[pRIC423] (lane 3), *P. aeruginosa* PAK*fimU*[pRIC421] (lane 4), and *P. aeruginosa* PAK*fimU*[pRIC420] (lane 5) reacted with a 1:5,000 dilution of antipilin antiserum. The PilA protein is indicated on the side of the immunoblot.

from this promoter (without the aid of an external vector promoter) restored the phenotype of bacteriophage sensitivity but not that of surface-extractable fimbriae or twitching motility to *pilV* mutants. The phenotype of the PAK*fimU* mutant lends support to this hypothesis.

Complementation of the PAK*fimU* mutant with plasmid pRIC338 fully restored the wild-type phenotypes of twitching motility, complete sensitivity to PO4 infection (data not shown), and the presence of surface-extractable fimbriae (Fig. 7). In order to confirm that the *fimU* mutation was having no effect on downstream genes, plasmid pRIC423, which contains only the *fimU* gene, was introduced into the *fimU* mutant. This plasmid was also able to restore the full spectrum of wild-type phenotypes including export of PilA to the cell surface (Fig. 7). Similarly, the pRIC338 and pRIC423 plasmids were all able to restore wild-type twitching, bacteriophage sensitivity, and surface fimbriae to the PAO1*fimU* mutant (data not shown).

Construction and characterization of a *dadA* fimT fimU* triplet mutant. The *fimT* and *fimU* genes encode peptides that display similarity to the XcpTU proteins. This observation, in conjunction with the fact that a *fimT* mutant demonstrated no detectable impairment of type 4 fimbrial biogenesis and that the *fimU* mutant appeared only to lose the twitching motility phenotype yet remained sensitive (albeit impaired) to bacteriophage infection, led us to consider the possibility that these two proteins may be performing similar roles. To investigate this possibility further, a triple mutant defective in all of the *dadA**, *fimT*, and *fimU* genes was constructed. This was done by cloning the *Xho*I-*Bgl*II fragment covering this area into the *Sal*I-*Bgl*II sites of pOK12, generating pRIC384. The ~1.1-kb internal *Pst*I fragment that spans the three genes was then replaced with the Tc^r gene cassette. This entire construct was then transferred as a *Spe*I fragment into pRIC380, generating pRIC408 (Table 1), which was then mobilized into the wild-type PAK strain as detailed above. A number of Tc^r mutants were analyzed by Southern hybridization and displayed the expected genotype of a *dadA* fimT fimU* mutant (data not shown).

As expected, the *ATU* (*dadA* fimT fimU*) mutant was unable to translocate via twitching motility (Fig. 8) and produced no surface-extractable PilA protein (Fig. 7). Indeed, the processed PilA subunits were trapped in the membrane fraction (Fig. 6), as seen previously with the *fimU* mutant. However, when the *ATU* mutant was analyzed for sensitivity to bacteriophage PO4 infection, it was found to be totally resistant (Table 2). Therefore, while a *fimU* mutant remained sensitive to infection by bacteriophages PO4, B3, and D3112, concurrent removal of the *fimT* gene resulted in the loss of this bacteriophage sensitivity. This suggests firstly that the *fimT* gene product is subtly involved in the type 4 fimbrial biogenesis system and does not simply represent a redundant or nonexpressed (silent) gene and secondly that the FimT and FimU proteins may be acting in a similar fashion.

The *ATU* mutant was complemented with various plasmid constructs, and the resultant transformants were tested for bacteriophage sensitivity, surface fimbriae, and twitching motility. Complementation with all three genes (pRIC375) was able to fully restore the wild-type phenotypes, whereas the introduction of *dadA** (pRIC399) had no effect on the phenotypes tested, confirming that the *dadA** gene is not directly involved in the biogenesis and function of type 4 fimbriae. However, complementation with the *fimT* gene alone (pRIC420) not only restored full bacteriophage PO4 sensitivity but was also able to restore twitching motility (Fig. 8). When the *fimU* gene alone was introduced (pRIC423), the transformants also displayed wild-type twitching motility phenotypes (Fig. 8). These data imply that overexpression (due to plasmid copy number) of the *fimT* gene can overcome the absence of a functional *fimU* gene product. To confirm this, pRIC420 was introduced into the PAK*fimU* and PAO1*fimU* mutants, where it was found to restore twitching motility (data not shown). Interestingly, however, although both the PAK*fimU* and *ATU* mutants carrying pRIC420 and overexpressing the *fimT* gene regained their twitching motility, immunoblotting of surface fimbriae preparations demonstrated a substantial reduction in the amount of surface-extractable fimbriae (data not shown; Fig. 7).

In an attempt to quantitate more precisely the relative amounts of PilA on the cell surface, whole-cell ELISA was performed with an antifimbrial antiserum. This showed that *ATU* mutant cells overexpressing *fimT* (plasmid pRIC420) produced significantly less than wild-type amounts of cell surface fimbriae (Fig. 9A). Indeed, compared with a *pilA* mutant which produces no PilA protein at all (as a control for the nonspecific immunoreactivity of the antiserum), the level is only marginally, albeit reproducibly, higher. This not only lends support to the faint protein band detected by immunoblotting surface preparations of these cells but also suggests that the zone of twitching motility observed with *P. aeruginosa* strains is not directly correlated with the numbers of fimbriae exposed beyond the cell surface. The overexpression of a part of the

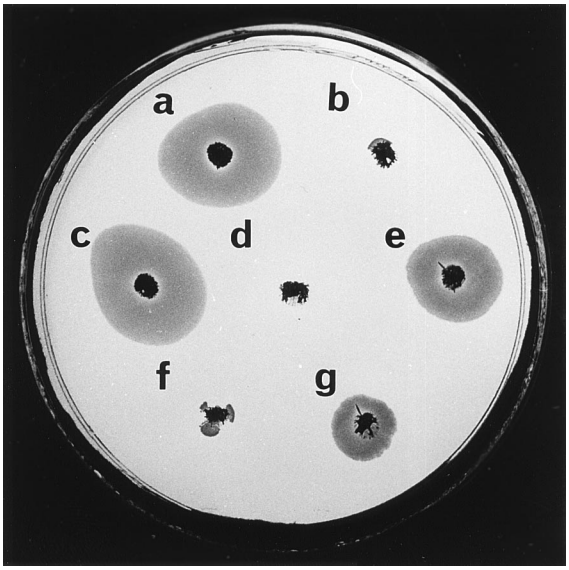

FIG. 8. The subagar surface translocation assay used to determine twitching motility. Zones of motile cells are as follows: a, *P. aeruginosa* PAK[pUCP19]; b, *P. aeruginosa* PAK*ATU*[UCP19]; c, *P. aeruginosa* PAK*ATU*[pRIC375]; d, *P. aeruginosa* PAK*ATU*[pRIC399]; e, *P. aeruginosa* PAK*ATU*[pRIC423]; f, *P. aeruginosa* PAK*ATU*[pRIC421]; g, *P. aeruginosa* PAK*ATU*[pRIC420].

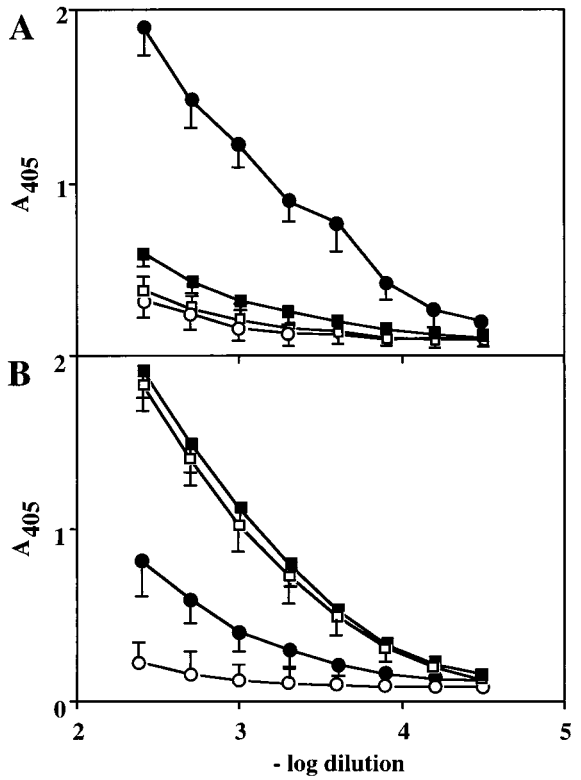

FIG. 9. ELISA against whole cells of the following *Pseudomonas* strains: (A) PAK (●), PAK*pilA* (○), PAK*ATU*[pRIC375] (■), and PAK*ATU*[pRIC420] (□); (B) PAK (●), PAK*pilA* (○), PAK*pilA*[pAW103] (■), and PAK*pilT* (□) (58). The reaction in panel B was stopped early to ensure that PAK*pilA*[pAW103] and PAK*pilT* remained on-scale. The points are the averages of four independent sets of duplicates, and the standard errors are indicated.

export machinery of the fimbrial subunit may disturb the stoichiometric levels of all the components required, which may result in reduced levels of surface fimbriae. Indeed the *ATU*-[pRIC375] cells also displayed severely reduced numbers of surface fimbriae as detected by ELISA (Fig. 9A). The PAK*pilA* mutant transformed with the *pilA* gene restored all the wild-type phenotypes associated with functional surface fimbriae, although there is a reduction in the size of the twitching zone (55). An ELISA on this strain displayed an increased level of surface PilA compared with the wild-type PAK cells and a level similar to that of a hyperfimbriated PilT mutant (Fig. 9B). This suggests that only selective overexpression of biogenesis and/or export components will disturb and/or reduce the total number of surface fimbriae and that twitching motility (possibly via fimbrial extension and retraction) is not directly related to the number of exposed assembled fimbriae.

As noted earlier, the predicted amino acid sequence of the *fimT* gene contains a A−1L+1/E+5 motif at its N terminus which differs from the classical G−1F+1/E+5 motif seen with most prepilin-like containing proteins. Site-directed mutagenesis was employed to construct a *fimT* mutant which changed the A−1 residue to E−1. Such a mutation has previously been shown to prevent the cleavage of both PilA and PilV (2, 49). The mutant was constructed by PCR, completely sequenced to ensure that no other mutations had been introduced, and cloned into pUCP18, generating pRIC421. Introduction of pRIC421 into the *ATU* or *fimU* mutants did not result in restoration of twitching motility (Fig. 8), bacteriophage sensitivity, or surface-extractable fimbriae (Fig. 7), which confirms that the A−1 residue is critical to the function of the FimT protein.

The data presented here demonstrate that overexpression of FimT can overcome the absence of FimU. To investigate whether this functional substitution could be extended to the downstream PilV gene, the plasmids pRIC420 (*fimT*) and pRIC423 (*fimU*) were introduced into the R306 (*pilV*) mutant (2). Overexpression of either of these genes in mutant R306 was unable to functionally compensate for the absence of the PilV protein, as evidenced by the lack of twitching zones (data not shown).

DISCUSSION

The identification of two further genes that are required for correct biogenesis and function of type 4 fimbriae in *P. aeruginosa* and that possess prepilin-like leader sequences increases the level of similarity between this system and those for extracellular protein secretion and DNA uptake. These systems all contain three to four proteins which possess the short, cleavable, hydrophobic leader sequences homologous to that first defined in the major subunit of type 4 fimbriae, PilA (or pilin). The *fimT-fimU-pilV* cluster in conjunction with the downstream *pilE* gene brings to four the number of such proteins (apart from PilA) involved in type 4 fimbrial biogenesis. Furthermore, it increases the overall complexity of the type 4 network, as it brings the total number of genes known to be required to 27.

The FimT and FimU proteins fall in the XcpT and XcpU-like class on the basis of the similarity of the hydrophobic core regions situated directly after the predicted PilD cleavage site, although they display marked similarity to each other over their entire sequence length. Further, their overall hydropathic nature bears striking resemblance to both XcpT/U and PilV, suggesting that like these proteins they reside in the membrane fraction. The results presented here suggest that the FimT and FimU proteins play slightly distinct roles, as isogenic mutants do not carry the same phenotype, although their function is proposed to be similar on the basis of the observation that overexpression of FimT can compensate for a lack of FimU. This is not a general phenomenon, as neither of them could compensate for the absence of PilV or vice versa. The lack of any polar effects on *fimU* in the *fimT* mutants suggests that these genes are located on separate transcriptional units. If high levels of FimT can compensate for the loss of FimU, the wild-type twitching motility phenotype of the *fimT* mutants may also be a result of a naturally high level of expression of the *fimU* gene. However, in a previous study a *pilV* (and therefore *fimU*) mRNA transcript could not be detected (2). The physical nature of the complexes formed by proteins that have type 4 leader sequences and are processed by PilD has so far been refractory to biochemical analysis, but we expect that they will be somewhat different for protein secretion, DNA uptake, and fimbrial assembly, given that each of these systems is composed of proteins with little homology beyond their conserved amino-terminal sequence. A common link between the structural subunits is the presence of a disulfide bonded loop, usually proximal to the C terminus. In *P. aeruginosa*, the PilA protein has a 12-amino-acid C-terminal loop that has been shown to be responsible for binding to epithelial cell receptors (17, 30). The other prepilin-like proteins, PilV, PilE, FimT, and FimU, all possess these characteristic residues, although they are spaced by 12, 25, 31, and 30 residues, respectively. In contrast, however, all the prepilin-like proteins from the protein secretion pathways (*Klebsiella oxytoca* PulG to -J, *Erwinia* spp. OutG to -J, *Vibrio cholerae* EpsG to -J, *Aeromonas hy-*

drophila ExeG to -J, *Xanthomonas campestris* XpsG to -J, and *P. aeruginosa* XcpT to-W) (3, 14, 26, 31, 40, 41) do not contain any Cys residues. Moreover, the *V. cholerae* mannose-sensitive hemagglutinin, itself a type 4 prepilin like protein, is found within a cluster of three other similar proteins, all of which possess C-terminal Cys residues (28). The absence or presence of disulfide loops in these ancillary proteins may represent the foundation for slightly different export-assembly scaffolds for specific functions of either protein secretion or fimbrial assembly. Preliminary evidence in our laboratory suggests that the function of the FimU protein can be decreased or abolished by decreasing the spacing of the C-terminal disulfide loop, based on the ability to restore twitching motility to a *fimU* mutant. The importance of these Cys residues in the prepilin-like proteins is currently under investigation.

The results presented here suggest that the overexpression of the export machinery in an incorrect stoichiometric ratio results in a significant decrease in the level of PilA protein presented at the cell surface. However, this decrease in surface PilA levels is not necessarily mirrored by a corresponding degree of loss of twitching motility. This raises the possibility that the *P. aeruginosa* cell needs very few fimbrial filaments in order to be motile or that the level of surface fimbriae is not a reflection of their functional activity, which is loosely consistent with the extension-retraction model. Overexpression of the *pilA* gene results in a twofold increase of surface fimbrial subunits (which is similar to a hyperfimbriated *pilT* mutant) and only results in a slight decrease of twitching motility (55). This also suggests that, whereas the export machinery can tolerate the production of high levels of the subunit protein, disruption of the relative levels of the export components severely diminishes the number of fimbriae. This fact should be taken into account when analyzing complementation data, as restoration of phenotypes such as twitching motility and surface-extractable and/or exposed fimbriae should be measured in parallel.

The expression of the major fimbrial subunit PilA is tightly linked to sensitivity to fimbriae-dependent bacteriophages. However, data that suggest that PilA need not necessarily be visible extracellularly or extractable from the cell surface to enable infection by these bacteriophages are accumulating. The nonfimbriated yet bacteriophage-sensitive *fimU* mutant is yet another that possesses these characteristics, along with mutants in the *fimS* gene and the *pilGHIJK* cluster (8, 9, 57). Interestingly, the effect of the two bacteriophages D3112*cts* and B3*cts* differs between the PAK and PAO1 *fimU* mutants. This paradox along with the complex relationship between bacteriophage sensitivity and fimbrial expression remains under investigation and appears to be different between each class of bacteriophage.

The discovery of two additional minor components with type 4 leader sequences that are required for fimbrial assembly or function increases the similarities between this system and those for protein secretion and DNA uptake. There are now five proteins with type 4 leader sequences which are known to be required for fimbrial biogenesis in *P. aeruginosa*: PilA, PilE, PilV, FimT, and FimU. Four similar genes have also been identified in the *V. cholerae* MsH cluster (28) and are likely to be found in other type 4 fimbriated species. We believe that these observations provide a firm basis for predicting how fimbriae are formed. We suggest that most if not all of these ancillary proteins will be involved informing some sort of basal complex similar to that proposed for protein secretion and which probably connects the inner and outer membranes. These proteins are expressed only at low levels, and recent data suggest that PilV and PilE are localized to the cell membrane (2, 11). In contrast, PilA is expressed at high levels and is extruded beyond the cell surface. We suggest therefore that rather than being a substrate for secretion in the conventional sense, PilA is an infrastructural component of the system that has been extended beyond the cell to form an organelle. This organelle has in turn evolved the capacity for adhesion and movement in the colonization of epithelial cells in eukaryotic hosts by type 4 fimbriated bacteria. The supersystems involved in type 4 fimbrial biogenesis, protein secretion, and DNA uptake have many similarities, and while these may simply represent common architecture, they may also represent a similarity in the mode by which they function. (34).

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