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Assembly of Mutant Pilins in *Pseudomonas aeruginosa*: Formation of Pili Composed of Heterologous Subunits

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Recently, we reported the degree of N-terminal processing within the cytoplasmic membranes of three mutant pilins from *Pseudomonas aeruginosa* PAK with respect to leader peptide removal and the methylation of the N-terminal phenylalanine (B. L. Pasloske and W. Paranchych, Mol. Microbiol. 2:489–495, 1988). The results of those experiments showed that the deletion of 4 or 8 amino acids within the highly conserved N terminus greatly inhibited leader peptide removal. On the other hand, the mutation of the glutamate at position 5 to a lysine permitted leader peptide cleavage but inhibited transmethylase activity. In this report, we have examined the effects of these mutant pilins upon pilus assembly in a *P. aeruginosa* PAO host with or without the chromosomally encoded pilin gene present. Pilins with deletions of 4 or 8 amino acids in the N-terminal region were not incorporated into pili. Interestingly, pilin subunits containing the glutamate-to-lysine mutation were incorporated into compound pili together with PAO wild-type subunits. However, the mutant pilins were unable to polymerize as a homopolymer. When wild-type PAK and PAO pilin subunits were expressed in the same bacterial strain, the pilin subunits assembled into homopolymeric pili containing one or the other type of subunit.

Pseudomonas aeruginosa is an opportunistic pathogen which causes local infections at burn sites or in damaged corneas as well as systemic infections in cancer patients or burn victims (1). It is also the major pathogen of nosocomial pneumoniaes and chronic lung infections in patients with cystic fibrosis (14).

P. aeruginosa produces polar, flexible filaments called pili which have a diameter of 5.2 nm and an average length of 2,500 nm (3, 9). These pili produce a unique form of locomotion called twitching motility (13) and act as the receptors for a number of pilus-specific bacteriophages (5). Recent work with a mouse burn model has demonstrated that unpiliated *P. aeruginosa* cells are 10-fold less virulent than piliated cells (23), presumably because of their ability to bind epithelial cells and promote colonization. It has also been shown that the pili of *P. aeruginosa* bind to specific receptors on buccal and tracheal epithelial cells (7).

The only detectable subunit of *P. aeruginosa* pili is the 15,000-dalton protein, pilin. The pilin gene is chromosomally encoded as a single copy (19). Pilin subunits are assembled in a helical array of five subunits per turn, with a pitch of 4.1 nm (9, 24). The pilin of *P. aeruginosa* belongs to a class of pilins characterized by the N-terminal residue, N-methylphenylalanine (NMePhe), and by a highly conserved, hydrophobic stretch of 30 amino acids at the N terminus. Such pili have been classified as NMePhe pili (17) and are produced by a number of gram-negative pathogens, including *Neisseria gonorrhoeae*, *Moraxella bovis*, *Moraxella nonliquefaciens*, and *Bacteroides nodosus*. These pilins are translated as a precursor (prepilin) with an amino-terminal extension of 6 or 7 amino acids. The positively charged leader peptide is removed enzymatically, leaving phenylalanine (Phe) as the new N-terminal amino acid. Subsequent methylation produces NMePhe.

The highly conserved N terminus of the NMePhe pili suggests that this region is involved in an important function(s). It is likely that the N terminus acts as a signal sequence for membrane targeting. Another possibility is that the necessary stabilizing forces for subunit-subunit interaction during pilus assembly are provided by this part of the molecule (6, 24). Recently, mutations were made within the N-terminal region of the pilin gene and their effects on pilin stability and processing within *P. aeruginosa* were determined (20). Deletions of 4 or 8 residues (amino acids 4 to 7 or 4 to 11, respectively) abolished leader peptide cleavage; however, substitution of a lysine (Lys) for the glutamate at position 5 (Glu-5) in mature pilin resulted in complete leader peptide removal without any detectable methylation of the N-terminal Phe.

This paper describes the effects that these pilin mutations have upon pilus assembly. For example, pilins with deletions of 4 or 8 amino acids were not incorporated into pili at all. In contrast, pilin having the Glu-5-to-Lys mutation was incorporated heterogeneously into pili in the presence of wild-type pilin, but it was unable to assemble as a homopolymer. When viewed with the electron microscope, these mixed pili exhibited a corkscrewlike morphology compared with the straight filaments of wild-type pili. These observations indicate that NMePhe or Glu-5 or both play an integral role in the polymerization of the pilin subunits.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. The bacterial strains and plasmids used are listed in Table 1. The media used were Luria-Bertani (LB) medium (15), *Pseudomonas* isolation agar, and MacConkey agar (MA). *Pseudomonas* isolation agar and MA were purchased from Difco Laboratories, Detroit, Mich. Lactose was added to 0.3% (wt/vol) in MA agar. Antibiotic concentrations were as follows: mercuric chloride (Hg), 15 μg ml^{-1}; chloramphenicol and streptomycin, 200 μg ml^{-1}; nalidixic acid, 40 μg ml^{-1}; carbenicillin, 300 μg ml^{-1}; tetracycline, 10 and 150 μg ml^{-1} for *Escherichia coli* and *P. aeruginosa*, respectively.

Pilus preparations. The transconjugants of *P. aeruginosa* DB2 were grown on eight cookie sheets (27 by 390 cm) in LB agar overnight at 30°C. The cells were scraped off into 150 ml

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TABLE 1. Strains and plasmids

Strain or plasmid	Relevant genotype or property	Source or reference
E. coli		
HB101	F⁻ Sm^r *recA13*	2
CSH56	Nal^r	Cold Spring Harbor Laboratory[a]
ED2602	*lac* (F$^+$ *lac*$^+$)	Neil Willetts[b]
MM294(pRK2013)	Mobilizing plasmid	21
BLP1	Tn*501* transposed into the HB101 genome	This study
P. aeruginosa		
2pfs	Multipiliated strain PAK	4
PAKp	Pilin-lacking mutant PAK	20
PAO1-leu	*leu* mutant of PAO1 (ATCC 25247)	W. Paranchych
DB2	Multipiliated strain PAO1	22
BLP4	Tn*501*::*pilA* strain DB2	This study
Plasmids		
pLAFR	Cosmid cloning vector, Tc^r	10
pBP310	PAK *pilA* clone in pLAFR	This study
pBP311	Tn*501*::*pilA* in pBP310	This study
pBP400	Control plasmid, chimera of pT7-4 and pKT210; Cb^r Cm^r	20
pBP500	PAK pilin gene clone	20
pBP540	PAKΔ4 pilin gene clone	20
pBP580	PAKΔ8 pilin gene clone	20
pBP500$_9$	PAK Glu-5-to-Lys pilin gene clone	20

[a] Cold Spring Harbor, N.Y.
[b] 28 Barcoo St., Roseville, New South Wales, Box 20, 2069, Australia.

of 1× SSC buffer (150 mM NaCl plus 15 mM sodium citrate, pH 7.0) and subjected to vigorous mixing with a magnetic stirrer at 4°C for 1 h. The cells were centrifuged several times at 6,000 × *g* for 10 min until a pellet was no longer visible after centrifugation. The pH of the supernatant was adjusted to 4.3 with 1 M citric acid. The pili were allowed to precipitate at 4°C for 4 h, subjected to centrifugation (6,000 × *g*) for 15 min, and suspended in 0.05 M phosphate buffer (pH 7.0).

Phage sensitivity assay. The *P. aeruginosa* transconjugants were streaked onto tetracycline-LB agar, and 2 μl of the pilus-specific bacteriophage PO4 (10^9 PFU/ml) (5) was pipetted onto the center of the streak. The cells were incubated overnight at 30°C. A clear zone within the growing cells indicated lysis and the presence of retractile pili.

Immunoblotting. Immunoblotting was performed as previously described (18).

Plasmid transfer by conjugation. Triparental matings were performed as described by Pasloske and Paranchych (20). Donor and recipient cells were incubated together with *E. coli* MM294, which contains the helper plasmid pRK2013. The helper plasmid has all the gene products necessary for plasmid conjugation with compatible plasmids. In a triparental mating, pRK2013 transfers itself into the donor cell and is then able to transfer the donor's plasmid, which has an origin of transfer but lacks the other transfer genes needed for conjugation (26).

In order to transfer the F *lac* plasmid by conjugation, donor and recipient strains were each grown overnight without shaking at 37°C in 2 ml of LB broth. The mating involved adding 0.2 ml of the donor and 0.2 ml of the recipient strains to 2 ml of LB broth and incubating them at 37°C for 30 min. The mating solution was plated onto the appropriate selective medium and incubated at 37°C.

Electron microscopy and immunolabeling. The transconjugants of *P. aeruginosa* DB2 were grown on chloramphenicol-LB agar overnight at 30°C. A loopful of the cells was suspended in 50 μl of phosphate-buffered saline, pH 7.0, and 5 μl of the suspension was placed onto a hydrophilic carbon film supported by a copper mesh grid. If the cells were not incubated with antibody, they were then washed twice with reticulocyte standard buffer (10 mM Tris hydrochloride, 10 mM NaCl, 1.5 mM $MgCl_2$, pH 7.0) and stained with 0.75% sodium phosphotungstate (pH 7.0).

If the cells were to be coated with antibody, a 1:50 dilution of either anti-PAK or anti-PAO pili serum in phosphate-buffered saline was added to the cells on the grid and incubated for 5 min at room temperature. The grid was then washed twice with reticulocyte standard buffer and stained with 0.75% sodium phosphotungstate.

The grids were examined with a Philips EM240 electron microscope.

Protein sequencing. N-terminal sequencing was performed by the method of Matsudaira (16) with the following modifications. Purified pili were solubilized by adding approximately a 0.5× volume of 0.1 M ammonium hydroxide. A 1-cm square of polyvinylidene difluoride membrane (Millipore Corp., Bedford, Mass.) was soaked with 100% methanol and rinsed with distilled water for 5 min. The membrane was placed on blotting paper, and 2 nmol of pili was applied to the membrane and allowed to completely dry. The membrane was rewetted with 50% methanol for a few seconds and then rinsed in distilled water over a 24-h period with the water being changed every few hours. The membrane was dried, and automated Edman degradation was performed as previously described (18).

Transposition of Tn*501* into the chromosome of *E. coli* HB101. Tn*501*::RSF1010 was transformed into *E. coli* ED2602, and red colonies were selected on Hg-lactose-MA. *E. coli* ED2602 (Tn*501*::RSF1010) was mated with *E. coli* CSH56, and red transconjugants were selected on nalidixic acid-Hg-lactose-MA plates. *E. coli* CSH56 (Tn*501*::F *lac*) was crossed with HB101, and red transconjugants were selected on Hg-streptomycin-lactose-MA medium. Spontaneous loss of the F *lac* plasmid with a coincidental integration of Tn*501* into the chromosome of *E. coli* HB101 was achieved by growing *E. coli* HB101 (Tn*501*::F *lac*) in LB broth without antibiotic selection, plating these cells onto Hg-streptomycin-lactose-MA, and looking for white colonies. One white colony which had the required phenotype was selected and was named *E. coli* BLP1.

Cloning a cosmid containing the *P. aeruginosa* PAK pilin gene. A cosmid bank of *P. aeruginosa* was prepared as outlined by Goldberg and Ohman (11), except that a Packagene Lambda DNA Packaging System (Bio/Can Scientific, Mississauga, Ontario, Canada) was used to package the cosmid DNA. The cosmid bank of *P. aeruginosa* PAK harbored in *E. coli* HB101 was plated on tetracycline-LB agar at a dilution sufficient to yield single, discrete colonies. Nitrocellulose filters were laid over the colonies and then lifted off the plates. The filters with the cosmid bank were probed with the nick-translated 1.2-kilobase *Hin*dIII fragment containing the PAK pilin gene. One colony was detected harboring a cosmid (pBP310) having a 7-kilobase *Eco*RI fragment containing the pilin gene. This *Eco*RI fragment was the same size as that detected in Southern blots of the PAK genome digested with *Eco*RI (19). In total, pBP310

had seven *Eco*RI fragments, adding up to approximately 26 kilobases, cloned into the *Eco*RI site of pLAFR.

Construction of BLP4, a pilin-lacking derivative of *P. aeruginosa* DB2. pBP310 was transformed into *E. coli* BLP1 and then mobilized into *P. aeruginosa* PAKp. Transconjugants which grew on tetracyline-Hg-*Pseudomonas* isolation agar plates were tested for their sensitivity to the pilus-specific bacteriophage PO4. *P. aeruginosa* PAKp is resistant to PO4 but was complemented for phage sensitivity by pBP310. Transconjugants resistant to the phage were candidates for cosmids with a disrupted pilin gene. Cosmid DNA (pBP311) was purified from one isolate which was phage resistant and transformed into *E. coli* HB101. Restriction mapping indicated that Tn*501* had inserted into the 1.2-kilobase *Hin*dIII fragment of pBP310 containing the pilin gene. Further analysis by DNA sequencing (19) revealed that the transposon had integrated between the Phe-1 and Thr-2 codons of the pilin gene.

A gene replacement of the chromosomal PAO pilin gene with the disrupted PAK pilin gene was completed by performing a triparental mating of *E. coli* HB101(pBP311) and *P. aeruginosa* DB2 and selecting transconjugants on Hg-*Pseudomonas* isolation agar medium. The colonies of multipiliated *P. aeruginosa* DB2 were small compared with those of the wild-type strain of *P. aeruginosa*. Approximately 10% of the transconjugant colonies were much larger than the other colonies; these large colonies had lost their tetracycline resistance, while the small colonies had not. The loss of tetracycline resistance indicated that pBP311 had been lost from the host. Direct examination of one of these large colonies (*P. aeruginosa* BLP4) by electron microscopy confirmed that the cells did not have any pili. The strongest evidence for homologous recombination was provided by restriction mapping of the total DNA from these isolates. Four different restriction digests of genomic DNA from the mutant isolates were probed with the PAK pilin gene probe. The mutant restriction patterns differed from the equivalent wild-type patterns and produced the fragment sizes expected of chromosomal DNA having undergone the anticipated exchange of the Tn*501*-mutated PAK pilin gene for the PAO pilin gene (data not shown).

RESULTS

Electron microscopy of *P. aeruginosa* PAO1-leu expressing the mutant pilins. The chimeras containing the different PAK pilin genes were mobilized into *P. aeruginosa* PAO1-leu, and each transconjugant was examined for piliation by electron microscopy. The strains containing the vector control (pBP400) and the PAK pilin clone (pBP500) assembled pili; however, the pBP500 strain produced two- to threefold more pili (about three pili per cell) than did the PAO background strain containing the vector alone. Transconjugants carrying pBP540 or pBP580 were unpiliated. These plasmids carry deletions that eliminate residues 4 to 7 (Δ4; pBP540) or 4 to 11 (Δ8; pBP580) of the mature pilin polypeptide. The pBP580 clone was quite lethal to *P. aeruginosa* PAO1-leu, resulting in about a 10,000-fold reduction in the number of transconjugants. The majority of the $pBP500_9$ transconjugants were unpiliated, although some cells (~5%) produced one or two very short pili (data not shown).

Electron microscopy of the multipiliated and pilin-lacking *P. aeruginosa* strains DB2 and BLP4 expressing the mutant pilins. The same PAK pilin mutant constructs were mobilized into the multipiliated strain *P. aeruginosa* DB2. As observed by electron microscopy, the piliation of these strains differed from that seen with strain PAO1-leu. Transconjugants containing pBP400, pBP500, pBP540, and $pBP500_9$ were all multipiliated, whereas it was not possible to procure a viable *P. aeruginosa* harboring pBP580. It is likely that the pilin Δ8 gene product was lethal to the multipiliated mutant. To establish the pilus composition of each of the transconjugants, the cells were incubated in the presence of rabbit anti-PAK or -PAO pili serum and observed by electron microscopy. The results are summarized in Table 2.

TABLE 2. Relative amounts of the PAK and PAO pilins detected in the pili of the multipiliated and pilin-lacking *P. aeruginosa* transconjugants

Plasmid (pilin phenotype)	Relative amounts of pilin types in *P. aeruginosa* host strain:			
	DB2		BLP4	
	PAK	PAO	PAK	PAO
pBP400 (none)	−	+++	−	−
pBP500 (PAK)	+++	+	+++	−
pBP540 (PAK Δ4)	−	+++	−	−
$pBP500_9$ (PAK Glu-5 to Lys)	+++	+	−	−

[a] −, No pilin; +, minor pilin type; +++, major pilin type.

As expected, the pBP400 transconjugant had pili which adsorbed only anti-PAO serum. However, regardless of whether anti-PAK or -PAO pili serum was used, immunolabeling of the pBP500 transconjugant resulted in two types of pili: those coated with antibody along their entire length and those not coated at all (Fig. 1). The predominant serotype observed was PAK. Antibody-coated pili were easily distinguished from naked pili in the micrographs. These data indicate that each pilus was a homopolymer, composed of either PAK or PAO subunits arising from the cloned PAK or chromosomally encoded PAO pilin genes, respectively.

Pili expressed by transconjugants carrying pBP540 were coated with anti-PAO pili serum but not with anti-PAK pili serum, suggesting that these pili consisted only of the chromosomally encoded PAO pilin subunits (data not shown). N-terminal sequence results for pili purified from this strain were consistent with this observation. A single NMePhe peak was detected in the first cycle of sequencing, and only Glu was observed in the fifth cycle. Thus, the only detectable subunit was PAO pilin.

The pili of the $pBP500_9$ (Glu-5-to-Lys pilin) transconjugants had a wavy, braided appearance (Fig. 2A) not typical of wild-type PAO pili. These pili interacted with both anti-PAK and -PAO pili sera, although the anti-PAK pili serum coated the pili more heavily than did the anti-PAO pili serum (Fig. 2B and C). These results suggest that each individual pilus contained both PAK and PAO subunits. N-terminal amino acid analysis of the pili indicated that unmethylated Phe and NMePhe were present in a 3-to-1 ratio, respectively. Therefore, it is highly probable that Phe originated from the unmethylated mutant PAK pilin incorporated into the same pili as the PAO subunit in a 3-to-1 ratio. Both glutamate and lysine were detected in the fifth cycle of the sequence analysis.

When the same clones were mobilized into the pilin-lacking *P. aeruginosa* BLP4, only bacteria containing pBP500 expressed pili; those containing pBP400, pBP540, and $pBP500_9$ were bald. It was not possible to grow a pBP580 derivative of this *P. aeruginosa* strain.

Immunoblots of the purified pili. Purified pili were prepared from cultures of *P. aeruginosa* DB2 transconjugants

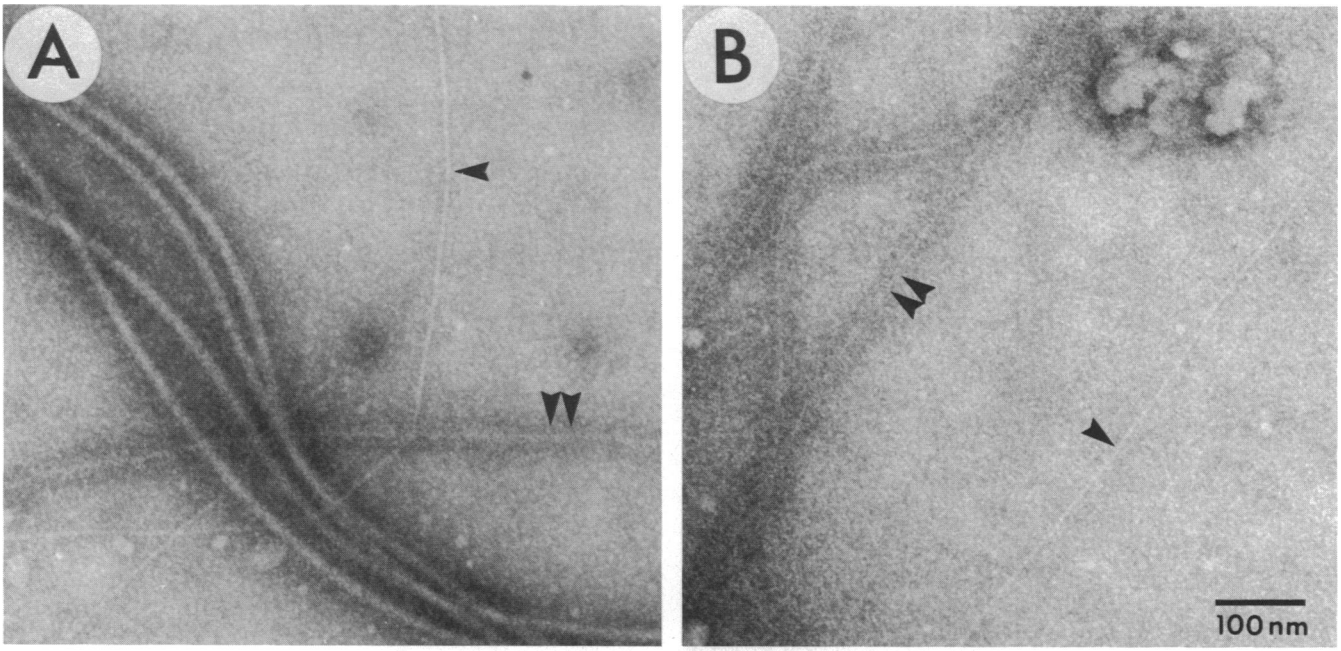

FIG. 1. Immunolabeling of the pili assembled by the transconjugants of *P. aeruginosa* DB2 carrying pBP500. *P. aeruginosa* DB2 harboring pBP500 was incubated with either anti-PAO (A) or anti-PAK (B) pili serum, negatively stained, and observed with the electron microscope. Single arrowheads point to naked pili; double arrowheads point to a pilus coated with antibodies.

containing pBP500, pBP540, and pBP500$_9$ grown without antibiotic present in medium. Immunoblots from the pBP500 and pBP540 strains reacted equally well with anti-PAO serum, whereas the anti-PAK pili serum reacted weakly (Fig. 3A).

The weak reactivity of the pili from the pBP500 cells with the anti-PAK pili serum did not agree with the results of the immunolabeling experiments (Fig. 1). Therefore, pili were purified from pBP500 cells grown in the same medium which had been used for the immunolabeling experiments, that is, in the presence of chloramphenicol. Immunoblots of these pili showed strong reactivity with the anti-PAK pili serum and weak reactivity with the anti-PAO pili serum, thus duplicating the immunolabeling results (Fib. 3B).

The purified pili from the pBP500$_9$ transconjugant reacted more strongly with anti-PAK pili serum than with the anti-PAO pili serum, consistent with the 3-to-1 ratio of PAK to PAO pilin determined by the N-terminal sequencing of the purified pili.

DISCUSSION

Recent experiments revealed that deletions within the highly conserved N terminus of *P. aeruginosa* NMePhe pilin resulted in poor leader peptide cleavage; a Glu-to-Lys mutation at position 5 resulted in a loss in detectable methylation of the N-terminal Phe, although leader peptide removal was not affected (20). To assess the effect of these pilin mutations on pilus assembly, the piliation of various transconjugants was observed by electron microscopy and the compositions of the pili were determined by three different methods (immunoblotting and N-terminal sequencing of purified pili and immunolabeling of pili on whole cells).

The mutant pilin clones were mobilized into three different strains of *P. aeruginosa*, and each strain of transconjugant was examined. The pBP400 (control vector) transconjugants behaved as expected, producing only endogenous pili (or no pili at all in the pilin-lacking mutant). In all three hosts harboring the PAK pilin clone (pPB500), not only was the wild-type PAK subunit properly processed in the membrane, but it was assembled into pili in a host which normally assembles PAO pili. This was not surprising, since it has recently been demonstrated that *B. nodosus* pili are assembled in *P. aeruginosa* DB2 when *B. nodosus* pilin subunits are provided (8). These data demonstrate that the ancillary factors involved in NMePhe pilus assembly have a broad specificity for NMePhe pilins. Since the homology of *P. aeruginosa* pilin with the *B. nodosus* pilin is essentially confined to the first 30 residues, and PAK and PAO subunits have about as much overall homology with each other as they do with the *B. nodosus* pilins, it is likely that the conserved N-terminal sequence plays an important role in NMePhe subunit assembly.

Immunolabeling revealed that each pilus of *P. aeruginosa* DB2(pBP500) was homogeneous, that is, each pilus was composed entirely of either PAK or PAO pilin. This phenomenon was previously observed in the case of two serotypically different *B. nodosus* pilins assembled in *P. aeruginosa* DB2 (8). Elleman and Peterson hypothesized that the pili were homogeneous because translation and assembly were closely linked temporally and spatially. As a result, one mRNA molecule might produce all the pilin needed for one pilus at a single assembly site. However, our observation that the Glu-5-to-Lys mutant PAK pilin was assembled into heterologous PAO pili contradicts this concept. Our data indicate that a pilin pool localized in the membranes of *P. aeruginosa* (20, 25) can be utilized for pilus assembly. They further suggest that the key component to the interaction of NMePhe subunits is the conserved N-terminal sequence, since two subunits with only 65% overall homology to each other were able to assemble into a pilus. It is interesting that the Glu-5-to-Lys mutant pilin assembled in the presence of the wild-type PAO pilin into mosaic pili but was incapable of

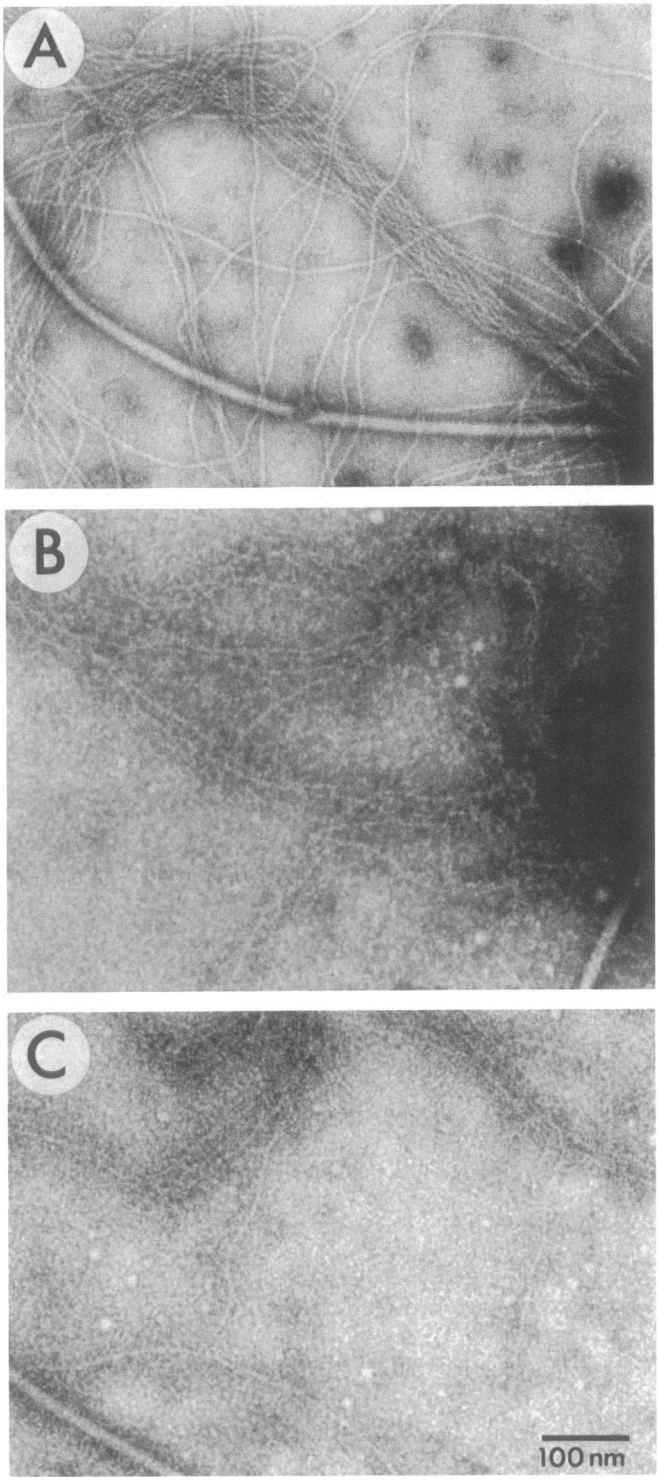

FIG. 2. Immunolabeling of the pili assembled by the pBP500$_9$ transconjugants of *P. aeruginosa* DB2. Micrographs of the pili produced by *P. aeruginosa* DB2 harboring pBP500$_9$ were taken under three different conditions: (A) without the addition of anti-pili serum, (B) with anti-PAK pili serum, and (C) with anti-PAO pili serum.

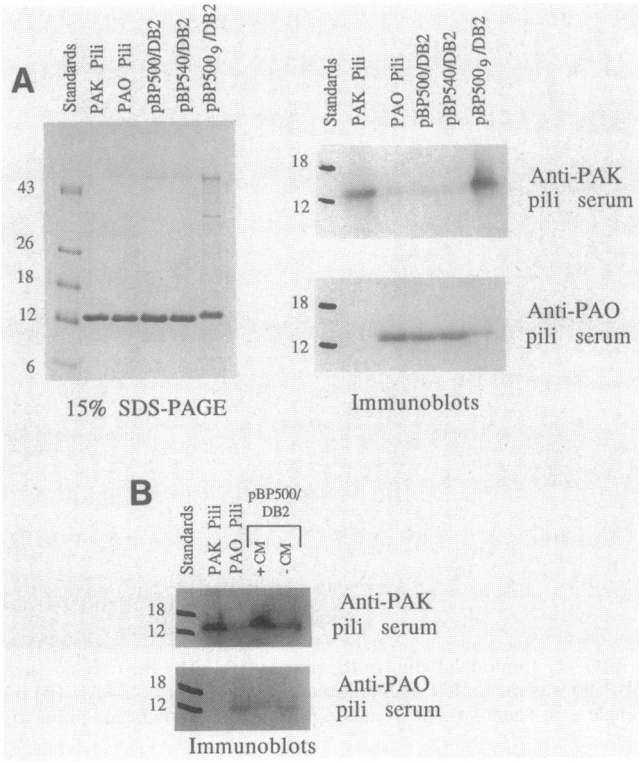

FIG. 3. Electrophoresis and immunoblots of the pili purified from *P. aeruginosa* DB2 transconjugants. The pili from the *P. aeruginosa* DB2 transconjugants harboring either pBP500, pBP540, or pBP500$_9$ were purified, fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblotted with either anti-PAK or anti-PAO pili serum. (A) Electrophoresis and immunoblots of transconjugants grown without antibiotic selection. (B) Immunoblots of pili purified from pBP500 transconjugants grown either in the absence (−CM) or in the presence (+CM) of chloramphenicol. The PAK and PAO pili were purified from *P. aeruginosa* 2pfs and DB2, respectively. Molecular weight standards are marked (in thousands) along the sides of the gel and the immunoblots.

self-polymerization in the pilin-lacking mutant. It is not clear from these results whether it is the Glu-5 or NMePhe or both residues which are critical to pilus formation.

The inability of the pilin Δ4 subunits to assemble in the pilin-lacking host (*P. aeruginosa* BLP4) was consistent with the observation that only PAO pilin was assembled in *P. aeruginosa* DB2(pBP540). Even though a small proportion of the pilin Δ4 subunits have their leader peptides cleaved (20), the deletion in the N terminus still leads to aberrant assembly. Therefore, it is likely that the deleted residues are required either for recognition by the assembly machinery or for stabilization of subunit interactions.

Assembly of each of the mutant PAK pilins was tested in the presence of the wild-type PAO pilin but not in the presence of the wild-type PAK pilin. Lacking the antisera, which could distinguish between wild-type and mutant PAK pilin subunits within the assembled pilus by the immunolabeling strategy, it was not feasible to do the same experiments with the wild-type PAK pilin. However, we suspect that results similar to those of the PAO studies would be obtained by using the PAK wild-type pilin subunit.

In *P. aeruginosa* PAO1-leu, essentially no pili were assembled from the Glu-5-to-Lys mutant, whereas in the multipiliated strain DB2 this mutant pilin was efficiently assembled. This observation might be explained by a concentration-dependent model for pilus assembly. Grasberger et al. (12) postulated a theoretical membrane-protein system in which the probability of interactive species associating is enhanced by increasing protein concentrations in the mem-

brane. This model does not exclude the possibility that pilin translation and assembly are closely linked (8), in which case pilin levels would be high due to the localization of nascently translated pilin from single mRNA transcripts at the assembly sites. If the pilin subunit was inherently incapable of self-polymerization, as in the case of the Glu-5-to-Lys mutant pilin, assembly would not be linked to translation. This would lead to an increased pilin concentration in the membrane. In the multipiliated strain, the mutant pilin concentration would reach the critical level for polymerization postulated by Grasberger et al., and pili would assemble provided PAO pilin was present to catalyze the reaction. However, in *P. aeruginosa* PAO1-leu, the pilin would not reach the concentration necessary for assembly to occur.

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