Amino Acid Substitutions in PilD, a Bifunctional Enzyme of Pseudomonas aeruginosa

EFFECT ON LEADER PEPTIDASE AND N-METHYLTRANSFERASE ACTIVITIES IN VITRO AND IN VIVO*

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Subunits of type IV pili and a subset of proteins of the type II extracellular protein secretion apparatus undergo two consecutive post-translational modifications: leader peptide cleavage, followed by methylation of the newly created N-terminal amino acid. These two reactions are carried out by a single bifunctional enzyme encoded in Pseudomonas aeruginosa by the pilD gene. Properties of PilD mutants at positions Gly⁹⁵ and/or Lys⁹⁶ which were differentially affected in leader peptidase and N-methyltransferase function were characterized. None of the single amino acid substitutions showed a significant alteration in their ability to cleave the prepilin leader peptide; however, two double mutants did exhibit a modest reduction in the efficiency of cleavage. In contrast, a significant decrease of N-methyltransferase activity was detected in PilD having substitutions at Gly⁹⁵. Mutants with substitutions at position Lys⁹⁶ showed a variable effect on N-methyltransferase activity with an apparent requirement for any charged amino acid at this position. Absence of N-methyltransferase activity did not appear to interfere with the ability of P. aeruginosa to assemble functional pili. Moreover, pilin monomers isolated from P. aeruginosa expressing PilD with Gly⁹⁵ substitutions were not methylated. Although complete methylation does not appear to be absolutely required for pilus assembly in P. aeruginosa, this modification may be important for pilus function in its natural habitat.

The respiratory distress and morbidity in individuals suffering from cystic fibrosis is most often caused by the opportunistic pathogen Pseudomonas aeruginosa (1). This organism has at its disposal a variety of adhesins (2), including type IV pili, to establish an infection in a susceptible host (3). Upon attachment and multiplication, P. aeruginosa can secrete a variety of hydrolytic enzymes and exotoxins (4). Central to these two processes (attachment and secretion) is PilD (also called XcpA), a bifunctional enzyme that catalyzes post-translational modifications of several proteins required for assembly of type IV pilin subunits into filaments and for assembly of a functional extracellular protein secretory apparatus (5, 6).

Type IV pili are made up of polymerized pilin monomers that are synthesized as precursors with a basic, short leader peptide. Other proteins required for pilus biogenesis (PilE, PilV, PilW, and PilX), as well as some components of the type II general secretory apparatus (XcpT, XcpU, XcpV, XcpW), are synthesized with a type IV pilin-like leader sequence and share extensive sequence similarity with the N-terminal hydrophobic portion of mature type IV pilin subunit (7, 8). PilD proteolytically removes the leader sequence from each of these substrate proteins and subsequently monomethylates the α-amino group of the newly exposed N-terminal phenylalanine (9, 10). Although removal of the leader peptide by PilD is essential for each of its substrates to be functional in formation of pili or for extracellular protein secretion, it has not been established whether N-methylation is essential for function of these related proteins as well.

Structure and function analysis of PilD, an integral membrane protein, has shown that 4 cysteines in the cytoplasmic domain are required for both proteolysis and methylation (11). However, cleavage and methylation can occur independently in vitro, and methylation can be inhibited without affecting cleavage, which indicates that these two functions are separable (10, 11). The initial structure and function study was extended here to identify further amino acid residues of PilD that constitute each of the active sites. We report that the type IV leader peptidase and N-methyltransferase active sites are indeed functionally separable by introducing single amino acid substitutions at predetermined positions. The cleavage activity of several different site-specific PilD mutants was unaffected as determined by kinetic analysis. In contrast, the N-methyltransferase activity of these same PilD mutants varied from undetectable to wild-type levels. Specifically, the glycine at position 95 of PilD, and to a lesser extent lysine at position 96, appeared to be essential for wild-type N-methyltransferase activity. With the exception of one mutant, the leader peptidase and N-methyltransferase activities of the PilD mutants were essentially identical when examined in vitro and in vivo; however, the methylation defect observed in vivo was dependent on PilD levels. Interestingly, P. aeruginosa expressing any one of the different PilD mutants showed no defect in pilus biogenesis or extracellular protein secretion. This indicates that PilD substrates may not need to be fully N-methylated for these proteins to be functional.

MATERIALS AND METHODS

Bacterial Strains, Phage, Plasmids, and Growth Conditions—All bacterial strains, phage, and plasmids used in this study are listed in Table I. Escherichia coli S17-1 [pirim (12)] was used to conjugally transfer all plasmids to P. aeruginosa. E. coli were grown in 2% YT medium when single-stranded DNA template was to be prepared for mutagenesis or sequencing. All other bacterial cultures were grown with aeration at 37 °C in Luria broth (13). The following antibiotic concentrations (in µg/ml) were used when appropriate: for P. aeruginosa, carbenicillin (150), neomycin (200); for E. coli, ampicillin (150), chloramphenicol (20).
IPTG 1 was used at a final concentration of 1 mM unless otherwise indicated. Oligonucleotide-directed Site-specific Mutagenesis and DNA Sequencing—Mutations in the Gly95 and Lys96 codons of pilD (14) were generated by oligo-directed site-specific mutagenesis of the phagemid pB18CC, which had been converted to a plasmid by KpnI digestion and subsequent ligation as described previously (16). Two mutagenic oligodeoxynucleotides, one for the first two positions of each codon such that random changes occurred in the Gly95 codon alone, in the Lys96 codon alone, or in both the Gly95 and Lys96 codons. The mutagenic oligonucleotide used was DGR-1 (5′-GGCGCTGGGNNCNNGTGCTCGTCCTGC-3′); the underlined codons were originally GGC (Gly95) and AAG (Lys96). Each mutagenic oligodeoxynucleotide was separately annealed immediately before transfection of the tac promoter on plasmid pMMB66EH (Table 1), which is a broad-host range plasmid that can replicate in *P. aeruginosa*.

**Preparation of Enzymes and Substrates—** *P. aeruginosa* PAK-2B18CC (*pilD::Tn5*) harboring either the cloned wild-type or mutant pilD genes was grown for 14–16 h in the presence of IPTG to overexpress PilD. Total membranes containing PilD were prepared as described previously (16), resuspended to approximately 30 mg/ml in 25 mM triethanolamine HCl, pH 7.5, 10% glycerol and stored at 50–70°C at −20 °C. The amount of PilD present was determined by comparison with known amounts of purified PilD on Western immunoblots or by comparison to known amounts of carbonic anhydrase resolved by SDS-PAGE and stained with Coomassie Blue. The concentration of membrane-associated PilD recovered was on average 0.19 mg/ml, which constituted approximately 0.55% of the total membrane protein.

Prepilin substrate used for leader peptidase and *N*-methyltransferase assays was obtained by overexpressing the pilIA gene from the tac promoter on the plasmid pMStac27P1D in *P. aeruginosa* PAK-2B18CC. Total membranes containing prepilin, prepared as described above, were either used as the source of substrate for the enzyme assays or the prepilin was purified as described previously (17). The amount of membrane-associated pilin was determined by comparison with known amounts of purified pilin on Western immunoblots or by comparison to known amounts of lysozyme resolved by SDS-PAGE and stained with Coomassie Blue. Purified prepilin was resuspended in 25 mM triethanolamine HCl, pH 7.5, 10% glycerol to 10 mg/ml and stored at −20 °C.

Unmethylated mature pilin was generated by cleaving purified or membrane-associated prepilin with wild-type PilD in a 2-h leader peptidase assay (see below). PilD was inactivated by heating to 80 °C for 20 min, and then the pilin product was concentrated to approximately 10 mg/ml by acetone precipitation. Complete *in vitro* processing of prepilin to mature pilin was verified by SDS-PAGE, which was then used as a substrate for *N*-methyltransferase reactions (see below).

In *vivo* generated pilin substrate for *N*-methyltransferase assays was isolated as follows. *P. aeruginosa* strains grown on Luria broth agar plates at 37 °C in the presence or absence of IPTG were resuspended in 10 mM MgCl2 and pili were sheared from the cell surface by vortexing for 30 s, then isolated away from whole cells by centrifugation and concentrated to approximately 10 mg/ml in a speed-vac.

**Kinetics of Leader Peptidase Activity—** Leader peptidase reactions were carried out over several intervals with saturating levels of prepilin and varying concentrations of PilD. Cleavage reactions containing 7.56 nmol PilD and 30–180 μM (i.e. 0.1–1 Kcat) prepilin, and proceeding for 15 min, were used for kinetic studies since these reactions were linear as a function of time and enzyme concentration. The velocity of each reaction was calculated by determining what fraction of prepilin was converted to mature pilin as measured by laser densitometry. Previously measured PilD catalysis was found to follow normal Michaelis-Menten kinetics with an apparent substrate affinity (Km) found that PilD has a slightly higher affinity for prepilin (3-fold) and a turnover rate (kcat) of 180 min−1 (17); however, in this study it was found that PilD has a slightly higher affinity for prepilin (3-fold) and that the kcat for PilD was 1300 min−1, which means this enzyme is actually 7-fold more effective at cleaving its substrate than originally postulated.

Enzyme activities were measured on preparations of prepilin with a minimum of six different substrate concentrations and at least three separate reactions per concentration. Values for Km (μM) and Vmax (mmol/min/mmol of enzyme) were derived from substrate concentrations and velocities by using the program Enzyme Kinetics, version 1.0c (Hypercard Stack for Macintosh)(18) which calculates these values in a two-step procedure of first direct linear estimates (19) followed by a maximum likelihood estimation method (20). By using these units for Vmax directly gives the substrate turnover rate per min (kcat).

**SDS-PAGE and Western Immunoblot Analysis—** The leader peptidase reaction mixtures were separated by 18% SDS-PAGE (21). After staining the gels with Coomassie Brilliant Blue R250, the percent methylation of pilin, the gels were prepared for fluorography after staining with 50% methanol in water for 30 min, treating with 0.5 M salicylate, 1.5% glycerol for 30 min, then drying them under vacuum at 52 °C, and exposing the gels to X-ray film (Kodak X-Omat).

For measuring PilD levels, samples were separated by 12% SDS-PAGE and then electrophoretically transferred to nitrocellulose. Nitrocellulose membranes were incubated with rabbit polyclonal anti-PilD antibody at a dilution of 1:500. Binding of the primary antibody was visualized by incubating with goat anti-rabbit IgG conjugated to alkaline phosphatase and then developing with 5-bromo-4-chloro-3-indoly phosphate and nitro blue tetrazolium as described (22). PilD detected by immunoblot analysis was quantitated by densitometry with the Foto/Analyst image analysis system (Fotodyne).

**Bacteriophage PO4 Sensitivity, Exoprotein Secretion Assays, and Adherence Assay—** For the determination of bacteriophage PO4 sensitivity, single colony forming units of *P. aeruginosa* PAK-2B18CC harboring either the cloned wild-type or mutant pilD genes were streaked in a single line on Luria broth-carbenicillin agar plates with (5, 10, 25, and 50 μg/ml) and without IPTG. The plates were incubated at 37 °C for 24 h after spotting PO4 (approximately 1011 plaque-forming units/ml) in the center of the streak. *P. aeruginosa* that are assembling functional pili will be lysed by bacteriophage PO4; therefore, phage sensitivity was scored as a zone of clearing in the area of phage inoculation.

To quantitatively examine the extracellular secretion of *P. aeruginosa* PAK-2B18CC harboring either the cloned wild-type or mutant pilD genes, the bacterial cultures were grown on Luria broth agar plates containing 0.8% sheep red blood cells or 1% skim milk, respectively. A zone of clearing around individual colony-forming units indicated extracellular secretion.

**Adherence Assay—** To quantitatively examine the extracellular secretion of wild-type *P. aeruginosa* PAK-2B18CC expressing wild-type PilD, we used a subconfluent monolayers at a multiplicity of infection of approximately 100. Cell-associated bacteria were released with 1% Triton X-100, and viable counts were determined by plating on Luria broth agar plates. Typically, 3% of the original inoculum of wild-type *P. aeruginosa* PAK, or 20% of *P. aeruginosa* PAK-2B18CC expressing wild-type PilD would adhere to the monolayer, whereas 100-fold less (0.05%) *P. aeruginosa* PAK-2B18CC with or without pMMB66EH would adhere.

Electron Microscopy—*P. aeruginosa* PAK-2B18 (pilD::Tn5) harboring either the cloned wild-type or mutant pilD genes were grown on LB-carbenicillin agar plates with (10 μg or 1 μg) or without IPTG for 14–16 h at 37 °C, gently resuspended in 10 mM MgCl2, and adsorbed to 200-mesh Formvar, carbon-coated grids for 2 min. Samples were

1 The abbreviations used are: IPTG, isopropyl-1-β-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; [3H]AdoMet, S-adenosyl-l-[methyl-3H]methionine; PLC, phospholipase C; L-Pase, leader peptidase.
washed with saline and water, negatively stained with either 1% uranyl acetate (4 min) or 2% phosphotungstic acid, pH 7.2 (30 s), and air-dried after excess stain was drained against filter paper. Samples were examined with a JEOL 1200 transmission electron microscope operating at 60 kV.

RESULTS

Site-directed Mutagenesis of Conserved Amino Acids within a Potential N-Methyltransferase Box of PilD—The post-translational modifications carried out by the bifunctional enzyme PilD of *P. aeruginosa* are necessary for type IV pilus biogenesis and for the assembly of a functional apparatus of the general secretory pathway. PilD is a leader peptidase and a N-methyltransferase, and both activities appear to involve enzymatic mechanisms that are unrelated to other known enzymes. It has been clearly established that the leader peptidase activity of PilD is essential for pilus assembly and exoprotein secretion; however, it has not been determined whether N-methylation by PilD is biologically essential (9–11).

Initial structure and function analysis of the 290-amino acid PilD polypeptide showed that four cysteine residues (Cys75, Cys78, Cys79, and Cys100) are required for full peptidase and methyltransferase activities (11). Limited homology to other thiol methyltransferases was found in a six-amino acid sequence of PilD, which is located within the most highly conserved domain of the family of PilD homologs (11). Interestingly, included in the potential methyltransferase box is Cys97 of PilD (Leu-Gly-Gly-Lys-Cys97-Ser) (11), and similarly, a cysteine is found in the same position of the homologous regions from the *E. coli* EcoRI (INGKCP) (24) and EcoRII (INGKCS) (25) methyltransferases. Alignment of 14 PilD homologs shows that two of the six amino acids within this putative methyltransferase box are highly conserved (Fig. 1). The glycine at position 95 (Gly95) is invariant, and the lysine at position 96 (Lys96) was found in half of the homologs, whereas the other half had a conserved change to another basic amino acid, arginine. One notable exception is the *E. coli* homolog BfpP, which has glutamic acid in the position equivalent to Lys96 of PilD. Regardless, Gly95 and Lys96 are the most highly conserved amino acids in the putative methyltransferase box of PilD; therefore, oligonucleotide-directed site-specific mutagenesis of Gly95 and Lys96 was carried out to determine whether these two residues are part of the PilD N-methyltransferase active site.

A degenerate oligonucleotide was used to introduce single or double mutations into the first two nucleotides of the Gly95 and/or Lys96 codons of pilD by the method of Kunkel et al. (15). A total of 18 unique pilD mutants was identified by DNA sequencing, including 12 single mutants (3 at Gly95 and 9 at Lys96) and 6 double mutants. In order to analyze the effect of the different mutations on PilD function, 11 of the mutant genes were subcloned from M13mpRI into pMMB66EH for further analysis (Table 1). The pMMB66EH plasmid was used since the various mutant pilD genes could be cloned downstream of the IPTG-inducible tac promoter, which allowed for controlled expression, and because the plasmid could replicate in *P. aeruginosa*.

Subsequently, the 10 plasmids capable of expressing comparable levels of mutant PilD protein in *E. coli* were mobilized into the *P. aeruginosa* pilD mutant PAK-2B18CC (pilD::Tn5) by conjugation. It was then necessary to determine whether any of the Gly95/Lys96 mutations in PilD affected the stability or proper localization of the enzyme to the inner membrane of *P. aeruginosa*. The envelope fraction isolated from *P. aeruginosa* PAK-2B18CC harboring the recombinant plasmids and grown in the presence of IPTG was analyzed by Western blot using anti-PilD antibody. It was found that similar amounts of mutant and wild-type PilD were detectable in the *P. aeruginosa* PAK-2B18CC membrane preparations (Fig. 2). This showed that the mutations had no detectable effect on the stability or membrane localization of PilD.

In Vitro Analysis of the Leader Peptidase and N-Methyltransferase Activities of the Mutant PilD—Membrane fractions from *P. aeruginosa* PAK-2B18CC expressing the Gly95/Lys96 pilD mutants were analyzed for leader peptidase and N-methyltransferase activities, employing assays previously developed in this laboratory (9, 10). Purified PilD was previously shown to cleave one of its substrates (prepilin) in the presence of a non-ionic detergent and acidic phospholipids (9); however, the most efficient reactions occurred when PilD and prepilin were part of total crude membrane preparations (17). In this study, membrane-associated Gly95/Lys96 PilD mutants with either purified or membrane-associated prepilin as substrate were used in the leader peptidase assay as described under “Materials and Methods.” Cleavage was assessed by the increased mobility of the mature pilin by SDS-PAGE, when compared with prepilin. All of the Gly95/Lys96 PilD mutants were able to process prepilin into mature pilin as efficiently as wild-type PilD (Fig. 3); therefore, these two amino acids are not essential for leader peptide cleavage activity.

The *in vitro* N-methylation assay is essentially identical to the leader peptidase assay except that the radioactive methyl donor [14C]AdoMet was added to the reaction, with incorporation of radioactivity into mature pilin being determined by SDS-PAGE and fluorography. There was no detectable methylation of pilin by any of the three Gly95 PilD mutants (PilD-
G95D, -G95S, and -G95Y) (Fig. 4). In contrast, there was a variable degree of pilin methylation by the four Lys\(^{96}\) PilD mutants as compared with wild-type PilD, and some could be detected only after longer exposure of the fluorographs (Fig. 4, lower panel). PilD-K96R was capable of methylating substrate as well as wild-type PilD, and the activity of PilD-K96E was estimated to be ~5% of the wild-type levels. Pilin methylation by PilD-K96L was very poor, and it was estimated to correspond to approximately 1% of wild-type activity, whereas N-methyltransferase activity was detected in strains expressing PilD-K96G. It is interesting to note that the two mutants with the most N-methyltransferase activity were PilD-K96R and PilD-K96E, which reflect changes that occur naturally in other PilD homologs (Fig. 1). All of the PilD double mutants (PilD-G95D, K96E; G95S, K96G, and -G95SK96R) were not detectably methylating pilin, presumably due to the Gly\(^{95}\) mutations being dominant. Thus, a single amino acid substitution was sufficient to abolish the in vitro N-methyltransferase activity of PilD without detectably affecting its ability to cleave substrate.

The Gly\(^{95}\)/Lys\(^{96}\) PilD mutants had no detectable decrease in leader peptidase function, but their ability to methylate substrate was quite variable, which might have been due to a decrease in functional stability over the 20-min to 1-h time course of the in vitro cleavage and N-methyltransferase assays, respectively. To determine whether the heat stability of the Gly\(^{95}\)/Lys\(^{96}\) PilD mutants had been altered, the enzymatic activity of each mutant was assessed after preincubation for 20 min at various temperatures, ranging from 37 to 80 °C. The pretreated PilD mutants were subsequently used in a prepilin cleavage reaction that included an additional 20-min incubation at 37 °C, and then the percent conversion of prepilin to mature pilin was quantitated by densitometry. It was found that wild-type PilD remained fully active even when preheated to 50 °C, it retained on average 45% activity when

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**Fig. 2.** Western immunoblot of total membrane extracts containing mutant PilD. *P. aeruginosa* 2B18CC (pilD::Tn5), harboring wild-type or mutant pilD genes cloned into pMMB66EH under the control of the tac promoter, were grown in the presence of 1 mM IPTG. Western blots of total membrane fractions, containing approximately 0.1 mg of PilD per lane, were probed with anti-PilD. Purified PilD (0.015 mg) was used as a positive control in lane 1 and *P. aeruginosa* 2B18CC harboring the vector pMMB66EH alone was used as a negative control in lane 2.

**Fig. 3.** In vitro leader peptidase assay of the Gly\(^{95}\)/Lys\(^{96}\) PilD mutants. The leader peptidase reactions were carried out for 20 min at 37 °C with 200 ng of prepilin and 8.0 ng of membrane-associated PilD (molar ratio of 50:1, substrate to enzyme). The reaction products were separated by 18% SDS-PAGE and stained with Coomassie Blue. Puriﬁed pilD protein (0.015 mg) was used as a positive control in lane 1 and in vitro generated pilin (200 ng, lane 2) were included as reference size standards for substrate and product, respectively. The negative control for peptidase activity was total membrane fractions from *P. aeruginosa* 2B18CC harboring the vector pMMB66EH alone, which lacks the PilD protein (lane 3).
N-Methyltransferase-deficient Mutants of PilD

heated to 65 °C, and it was finally rendered inactive when heated to 80 °C (Fig. 5). Each of the Gly95/Lys96 PilD mutants showed a level of heat stability similar to wild-type PilD (data not shown), although at 65 °C the cleavage activity of the mutants was reduced 75–85% as opposed to 55% for wild type, as shown for PilDG95D (Fig. 5). In addition, the N-methyltransferase activity of wild-type PilD and of the Gly95/Lys96 PilD mutants was not affected by pre-heating the enzymes at 50 °C (data not shown). The N-methyltransferase activity of enzymes heated at 65 °C or higher was not analyzed since cleavage is required for methylation to occur. Therefore, the only functionally significant difference between the Gly95/Lys96 PilD mutants and wild-type PilD appears to be in their ability to methylate substrate in vitro.

Kinetic Analysis of the Leader Peptidase Activity of the Mutant PilD—PilD must remove the leader peptide from its substrate(s) in order to monomethylate the α-amino group of the newly exposed N-terminal phenylalanine; consequently, leader peptide cleavage by PilD is a rate-limiting step for the N-methyltransferase activity of PilD. A kinetic analysis of the leader peptidase activity for the Gly95/Lys96 PilD mutants was performed to determine whether the reduced activity was due to a decrease in catalytic efficiency of cleavage.

To initiate these studies, the reaction rate of substrate cleavage for each of the Gly95/Lys96 PilD mutants was determined first. The rate of prepeptidase processing for each Gly95/Lys96 PilD mutant was similar to wild-type PilD in that they were linear as a function of enzyme concentration and time when the substrate concentration was at saturating levels. The results from a typical experiment are shown in Fig. 6, and in this particular experiment the molar ratio of substrate (prepilin) to enzyme (PilD) in each reaction was 4000:1. However, the maximum reaction rate for 7 of the 10 Gly95/Lys96 PilD mutants was reduced 25–65% relative to wild-type PilD. Three exceptions showed a similar rate of reaction to wild-type PilD: 1) PilDG95S (Fig. 6A), which is interesting because this mutant was unable to methylate pilin in vitro (Fig. 4); 2) PilDK96R (Fig. 6B), which represents a substitution that is found naturally in many of the PilD homologs (Fig. 1); and 3) PilDG95S, K96R (Fig. 6C), which includes the previous two mutations together. The above measured initial reaction rates for several substrate concentrations were used in a steady state kinetic analysis to quantitatively determine whether substrate affinity and/or maximum rate of catalysis of the Gly95/Lys96 PilD mutants were significantly affected.

It was previously shown that PilD-catalyzed cleavage of prepilin follows normal Michaelis-Menten kinetics with the measured reaction velocity being dependent on substrate concentration (17). The kcat and Km values for each of the Gly95/Lys96 PilD mutants are listed in Table II. Only 2 of the 10 Gly95/Lys96 PilD mutants, PilD-G95D,K96E and PilD-G95S,K96G, showed a significant decrease in catalytic efficiency in comparison to wild-type PilD, as evidenced by the kcat/Km ratio. These two double mutants showed a slightly higher affinity for substrate relative to wild-type PilD, but their turnover rates were reduced by more than 25-fold (Table II). The inability of double mutants PilD-G95D,K96E and PilD-G95S,K96G to methylate substrate in vitro may have been due to an 18-fold reduction in the catalytic efficiency (kcat/Km ratio) of cleavage in comparison to wild-type PilD; however, in this case, the inability to methylate pilin in vitro was probably not caused by a reduction in cleavage efficiency because three of the four substitutions introduced singly (G95D, G95S, and K96G) were essentially as efficient as wild-type PilD in catalyzing cleavage of their substrates (Table II), yet these mutants could not methylate pilin (Fig. 4).

Previous work from this laboratory has shown that PilD methylates prepilin and mature pilin with similar kinetics, whereas the former reaction requires only cleavage and methylation, whereas the latter reaction requires only methylation. Thus, the N-methyltransferase activity of the Gly95/Lys96 PilD mutants was assessed by using mature pilin as substrate to bypass the rate-limiting cleavage reaction. It was found that the altered methylation phenotype of the Gly95/Lys96 PilD mutants was identical whether the substrate was prepilin (Fig. 4) or mature pilin (data not shown), which is consistent with the results from the kinetic analysis above. Overall, these data indicate that the Gly95/Lys96 PilD mutant deficiency in N-methyltransferase activity is not due to an inability or reduced ability to cleave the prepilin substrate.

Effect of Amino Acid Substitutions in PilD on Pili Biogenesis and Extracellular Protein Secretion—The Gly95/Lys96 mutant PilD enzymes were examined for their ability to complement the type IV pili biogenesis and type II protein export defects of P. aeruginosa PAK-2B18CC. Complementation of pilus assembly was probed with the pilus-specific bacteriophage PO4 since pilulated P. aeruginosa are sensitive to killing by this phage, whereas non-piliated P. aeruginosa, such as strain PAK-2B18CC, are resistant to killing by PO4. P. aeruginosa PAK-2B18CC expressing each of the 10 Gly95/Lys96 pilD mutant clones at a high level (i.e. when induced by IPTG) or low level (i.e. in the absence of IPTG) were sensitive to phage PO4.
...and 16.4 nM PilD, which is equivalent to a molar ratio of 4000:1 (substrateenzyme). A, rate of reaction for the Gly95 PilD mutants. B, rate of reaction for the Lys96 PilD mutants. C, rate of reaction for the PilD double mutants at positions Gly95 and Lys96.

(data not shown). Furthermore, electron microscopic examination of the recombinant strains showed that the pilD mutant P. aeruginosa PAK-2B18CC (Fig. 7A) can form pili when expressing pilDG95S (Fig. 7C), and they are indistinguishable from bacteria expressing wild-type pilD (Fig. 7B) in average number per cell and number and in morphology. Complementation of the P. aeruginosa PAK-2B18CC protein export defect can be detected when this normally non-hemolytic strain becomes hemolytic on blood agar plates due to the secretion of phospholipase C (PLC). As with pilus biogenesis, each of the Gly95/Lys96 PilD mutants could complement the PLC secretion defect of P. aeruginosa PAK-2B18CC (data not shown). It appears that the substitutions at Gly95 or Lys96 did not significantly affect PilD function; however, it was still not known whether the Gly95/Lys96 mutant PilD retained their ability to N-methylate substrate in vivo.

Enzymatic Activity of the PilD Mutants in Vivo—The above results showed that most of the Gly95/Lys96 PilD mutants cleaved prepilin as efficiently as wild-type in vitro and that P. aeruginosa PAK-2B18CC expressing either wild-type or mutant PilD could assemble functionally similar pili. Most of the Gly95/Lys96 PilD mutants had a moderate to severe defect in their ability to N-methylate pilin in vitro, but it was still unknown whether the mutated enzymes had a similar defect when functioning in vivo.

The expression of the plasmid-encoded mutant pilD genes is under the control of the IPTG-inducible tac promoter; however, low levels of PilD are produced even in the absence of IPTG (Fig. 8). The level of PilD being produced in the absence or presence of IPTG was quantitated by Western blot analysis combined with densitometry. As compared with the levels of PilD produced by wild-type P. aeruginosa PAK, it was determined that in P. aeruginosa PAK-2B18CC (a mutant with an insertional disruption of the pilD gene), approximately 20-fold less (Fig. 8) and 12-fold more (data not shown) plasmid-encoded PilD was produced in the absence and presence of 1 mM IPTG, respectively. The leader peptidase activity of the wild-type and mutant PilDs was unaltered when present at 20-fold below chromosomal levels (IPTG) since prepilin and preXcpT were fully processed (Fig. 9). Furthermore, P. aeruginosa PAK-2B18CC, expressing mutant PilD in the absence of induction by IPTG were sensitive to killing by bacteriophage PO4, and their export of PLC and elastase was similar to wild-type as was their adherence to A549 lung epithelial cells (data not shown).

For an assay of the methylation in P. aeruginosa (i.e. in vivo) was developed based on an earlier observation of Strom et al. (10) who showed that in vitro PilD could not further methylate a previously methylated substrate. This work also established that the N-methyl modification of PilD substrates was very stable. In our assay, pili sheared from the surface of P. aeruginosa were harvested, and then the methylation state of the in vivo modified pilin subunits was probed with wild-type PilD in an in vitro N-methyltransferase assay.
In this assay, wild-type PilD would not be capable of methylating pilin subunits \textit{in vitro} if they were already methylated, but wild-type PilD would further methylate \textit{in vivo} generated pilin that was unmethylated or only partially methylated.

By using this \textit{in vivo} N\textsubscript{-}methyltransferase assay it was found that the reduced level of wild-type PilD, 20-fold less than normal level, in the absence of IPTG induction results in 80–95% of substrate being methylated (Fig. 10), which shows that the \textit{in vivo} N\textsubscript{-}methyltransferase of pilin, as was observed \textit{in vitro}, was kinetically slower than cleavage of the leader peptide (compare Figs. 9 and 10). In contrast, reduced amounts of some Gly\textsuperscript{95}/Lys\textsuperscript{96} PilD mutants resulted in pilin that was not detectably methylated (Fig. 10). However, the methylation defect of the Gly\textsuperscript{95}/Lys\textsuperscript{96} PilD mutants could be mitigated when these enzymes were overproduced by the addition of IPTG (Fig. 10). Plasmid pMDK96R constitutively expresses PilD-K96R; therefore, \textit{P. aeruginosa} PAK-2B18CC(pMDK96R) overproduces PilD-K96R in the presence or absence of IPTG, which results in fully methylated pilin under either condition (Fig. 10). The only Gly\textsuperscript{95}/Lys\textsuperscript{96} PilD mutant that showed a different N\textsubscript{-}methyltransferase phenotype \textit{in vitro} versus \textit{in vivo} was PilD-K96G, where \textit{in vitro} it showed no detectable N\textsubscript{-}methyltransferase activity and \textit{in vivo} it methylated pilin as efficiently as wild-type PilD (Fig. 10). Although several of the Gly\textsuperscript{95}/Lys\textsuperscript{96} PilD mutants had a defect in their N\textsubscript{-}methyltransferase activity \textit{in vitro}, this defect did not appear to be as prominent \textit{in vivo}; however, the level of pilin methylation \textit{in vivo} did correlate with the level of mutant enzyme being produced.

In \textit{Vivo} N\textsubscript{-}Methyltransferase Activity of the PilD Mutants Expressed at Chromosomal Levels—The induced level of plasmid-encoded PilD was modulated by varying the IPTG concentration in growing cultures to determine whether \textit{“native”} levels of mutant PilD were sufficient to fully N\textsubscript{-}methylate pilin \textit{in vivo}. It was found that 50 \(\mu\text{M}\) IPTG was enough to maximally induce plasmid-encoded PilD expression, which was approximately 7–12-fold above wild-type \textit{P. aeruginosa} PAK chromosomal PilD levels (Fig. 8). In addition, it was found that 10 and 25 \(\mu\text{M}\) IPTG induced expression comparable to and 2-fold above chromosomally produced PilD levels, respectively (Fig. 8).
A range of IPTG concentrations (0, 10, 25, and 50 μM) was used to induce mutant PilD expression in P. aeruginosa PAK-2B18CC, and then assembled pili were sheared from the bacterial cell surface, and their methylation state was probed with wild-type PilD in an in vitro N-methyltransferase assay. The same cultures used to harvest pili were also probed for PilD expression by Western blot analysis using anti-PilD antibody to verify enzyme levels being produced under the growth conditions examined (Fig. 8). When plasmid-encoded wild-type PilD was induced with as little as 5 μM, which yielded enzyme at 3-fold below chromosomal levels, pilin subunits were always 100% methylated (i.e. these pilin subunits would not be further methylated by wild-type PilD in an in vitro N-methyltransferase assay). The PilD-Lys<sup>96</sup> mutants methylated 65–85% of substrate when expressed 20-fold below chromosomal levels as compared with 80–95% by wild-type PilD at similar levels (i.e. in the absence of IPTG induction). This difference represents only a slight defect in the N-methyltransferase activity of the mutant enzyme (Fig. 11). In order for the pilin subunits to be fully methylated by the PilD-K96 mutants, enzyme expression needed to be induced with 10 μM IPTG or greater (Fig. 11). In contrast, pilin subunits that had been processed by PilD-Gly<sup>95</sup> mutants or PilD-Gly<sup>95</sup>精品<sup>96</sup> double mutants in vivo remained unmethylated when the mutant enzyme was present below chromosomal levels (Figs. 10 and 11), but chromosomal levels of mutant enzyme methylated 50–60% of the pilin subunits, and mutant enzyme present at 2-fold above chromosomal levels methylated 75–80% of the pilin subunits (Fig. 11). In general, then, a Gly<sup>95</sup>精品<sup>96</sup> PilD mutant that shows a methylation defect in vitro also shows a similar defect in vivo; therefore, there is a strong correlation between the in vitro and in vivo N-methyltransferase activities of these PilD mutants.

**DISCUSSION**

P. aeruginosa and many other bacteria simultaneously carry two specialized localization processes, secretion of extracellular enzyme and assembly of type IV pili. It has been recognized in recent years that the basic mechanisms of extracellular protein secretion via the general secretory pathway and assembly of pilin subunits are related based on similarities of individual components that comprise the machinery of protein secretion and pilus assembly. Moreover, in P. aeruginosa, the two export pathways require the activity of one common enzyme, the product of the pilD gene, which specifies a bifunctional leader peptidase/N-methyltransferase (10).

Comparison of in vitro cleavage and methylation reactions, catalyzed by PilD, has shown that the rate of cleavage of prepilin substrates is significantly faster than N-methylation. Under optimal conditions, complete cleavage was observed after 10 min, while complete N-methylation required 60 min (10). We have extended the previous work to show that the difference in the two reactions rates can also be observed in vivo by limiting pilD expression, and by examining the extent of methylation of pilin subunits that were assembled into pili. PilD produced at 20-fold below normal chromosomal levels in P. aeruginosa could fully cleave prepilin, but as much as 20% of the pilin subunits isolated from assembled pili were not N-methylated. Under these conditions, PilD cleaved many more prepilin subunits than it was capable of methylating, yet the number, morphology, and function of the type IV pili produced by P. aeruginosa was unaffected. This result indicates that not every subunit has to be methylated for assembly of functional pili. In addition, this result showed that the in vitro leader peptidase and N-methyltransferase assays accurately reflect the relative reaction rates measured in vivo.

We had also previously shown that the two post-translational modifications carried out by PilD can occur independently in vitro and that both active sites for prepilin processing and for N-methylation are adjacent within a relatively large (approximately 68 amino acid) cytoplasmic loop domain (10, 11). This work also suggested that the two active sites are non-overlapping. The use of sulfhydryl-reactive alkylating agents and site-directed mutagenesis also showed that both enzymatic activities of PilD were dependent on four highly conserved cysteine (Cys<sup>72</sup>, Cys<sup>75</sup>, Cys<sup>97</sup>, and Cys<sup>190</sup>) located within the cytoplasmic loop (11). However, it was not clear if the cysteines were directly involved in leader peptidase activity or if they were required for the formation or the maintenance of the active site conformation because there was only a slight stimulation of cleavage by the addition of thiols to the reaction mixture (10).

The involvement of cysteines in the methylation reaction catalyzed by PilD led to a comparison with other thiol methyltransferases and a region of limited homology, restricted to a 6-amino acid region of PilD that included one cysteine (Leu-Gly-Lys-Cys<sup>97</sup>-Ser), was identified (11). Comparison of this region with other members of the PilD family of homologs showed that Gly<sup>95</sup>精品<sup>96</sup> and Lys<sup>96</sup>精品<sup>96</sup> were the most highly conserved amino acids of this potential methyltransferase box, which were targeted for site-directed mutagenesis in this study. In order to assess the role of N-methylation in the assembly
and/or function of the type II secretion apparatus and type IV pilus, a PilD mutant that lacks N-methyltransferase activity and is unaffected for leader peptidase activity is required. This is the exact functional phenotype the Gly95/Lys96 PilD mutants showed, which for the most part was reproducible under both in vitro and in vivo conditions.

By using an in vitro functional analysis of 10 different single and double Gly95/Lys96 PilD mutants, we showed that the leader peptidase and N-methyltransferase functions were separable with a single amino acid change. Any substitution for Gly95, which is invariant in the 14 known PilD homologs, abolished the N-methyltransferase activity of PilD in vitro. In contrast, the Lys96 mutants showed a variable degree of N-methyltransferase activity depending on the amino acid change. Two substitutions for Lys96, arginine and glutamic acid, resulted in mutant PilD that retained significant N-methyltransferase activity in vitro, with the former being a conservative change that is found in approximately half of the PilD homologs, whereas the latter changes the charge and is found in only one PilD homolog, BfpP of enteropathogenic E. coli (26). A substitution to glycine was the only change in Lys96 that completely abolished N-methyltransferase activity in vitro. Amino acid Gly95 appears to be essential for the N-methyltransferase activity of PilD, but, in contrast, Lys96 can be substituted for other amino acids with mutant PilD showing a range of N-methyltransferase activities. Additionally, it appears that PilD retains more of its N-methyltransferase activity when Lys96 is substituted with a charged amino acid. These data clearly establish that the leader peptidase and N-methyltransferase active sites are non-overlapping within the large cytoplasmic loop domain of PilD.

PilD must function as a leader peptidase before it can N-methylate its substrate; therefore, each PilD mutant was examined for any alterations in leader peptidase activity as compared with wild-type PilD. In contrast to the PilD mutants in the cysteine residues (11), the kinetic parameters of the leader peptidase activity for most of the Gly95/Lys96 PilD mutants were unaltered. By using membrane-associated PilD and prepilin, measured catalysis was previously shown to follow normal Michaelis-Menten kinetics with an apparent substrate affinity ($K_m$) of 650 $\mu$M and a turnover rate ($k_{cat}$) of 180 min$^{-1}$ (17). Here we found that the $K_m$ and $k_{cat}$ for wild-type PilD to be 210 $\mu$M and 1300 min$^{-1}$, respectively. Hence, PilD has a slightly higher affinity for substrate and is actually 7-fold more efficient at cleaving substrate than originally postulated. All of the PilD mutants that had a single mutation in either Gly95 or Lys96 were as efficient at cleaving prepilin as wild-type PilD; however, two of the three double Gly95/Lys96 PilD mutants showed a significant reduction in leader peptidase activity. The inability of the double mutants to methylate pilin in vitro was not likely due to a reduction in cleavage efficiency since the four substitutions introduced singly (G95D, G95S, K96E, and K96G) essentially catalyzed cleavage as efficiently as wild-type PilD, yet three of these mutants singly could not methylate pilin. When the cleavage reaction is bypassed by providing mature pilin as substrate, which is N-methylated as efficiently as prepilin by wild-type PilD, each of the 10 PilD mutants showed a similar defective N-methyltransferase phenotype. In vitro, each of the Gly95/Lys96 PilD mutants was found to cleave prepilin and preXcpT as efficiently as wild-type PilD, even when mutant PilD expression was 20-fold below normal chromosomal levels.

In addition to PilD, two other bacterial leader peptidases, catalyzing the cleavage of signal peptides from secreted proteins, have been biochemically characterized. The typical signal sequence of proteins entering the sec-dependent secretion system are cleaved by either leader peptidase I or II (LPaseI or LPaseII). LPaseI acts on a wide variety of substrates with a reasonable affinity ($K_m$ = 16.5 $\mu$m) and has a modest turnover rate ($k_{cat}$ = 520 min$^{-1}$) (27). LPaseII specifically recognizes and cleaves lipoproteins; this enzyme has a high affinity ($K_m$ = 6 $\mu$m) for its substrates in addition to an efficient turnover rate ($k_{cat}$ = 1800 min$^{-1}$) (28). In comparison, PilD has a lower affinity for its substrates than the LPases that may reflect the variety of related but not identical substrates that have to be processed by this enzyme, yet it efficiently cleaves one class of highly abundant proteins (type IV pilin precursors), each pilus filament containing between 500 and 1000 subunits.

We also compared the functional phenotypes of the Gly95/Lys96 PilD mutants in vitro. Because assembled, fully methylated pilins cannot function as substrates for further methylation (10) the extent of in vivo methylation can be accurately assessed by examining the ability of pilin subunits of pilin, isolated from various PilD mutants, to accept methyl groups in vitro. By using this knowledge, we developed an assay where wild-type PilD was used in an in vitro N-methyltransferase assay to probe the methylation state of pilin that had been processed and modified in vivo by wild-type or mutant PilD. If pilin is N-methylated in vivo, then PilD would not further methylate this substrate in the in vitro assay. Conversely, if pilin is only partially methylated, or not methylated at all, then PilD will N-methylate the population of pilin subunits that was not N-methylated in vivo, with the corresponding signal produced in vitro being inversely proportional to the amount of substrate that was N-methylated in vivo.

By using this in vivo N-methyltransferase assay we found a strong correlation between in vitro and in vivo activities for the mutant PilD, but the in vivo activity depends on the amount of mutant enzyme being produced. At the reduced levels of wild-type PilD (approximately 5% of the levels made by wild-type bacteria expressing a chromosomal pilD gene) 80–95% of the pilin subunits isolated from assembled pilus filaments were methylated. At the same expression level, the Gly95 PilD mutants were not detectably N-methylating pilin in P. aeruginosa, whereas the Lys96 PilD mutants were able to methylate approximately 15–35% of the processed pilin subunits. However, in contrast to the in vitro activities, the Gly95 PilD mutants were able to fully N-methylate pilin in vivo when expressed at approximately 5-fold above chromosomal PilD levels, but when expressed at chromosomal levels, only 50–60% of the pilus subunits were N-methylated. Similarly, the Lys96 PilD mutants could overcome their defect in N-methylating substrate when expressed above chromosomal levels, but these mutants showed a consistently moderate defect in N-methyltransferase activity in vivo as opposed to variable levels of activity in vitro. These data indicate that the Gly95 PilD mutants possess some residual N-methyltransferase activity that is detectable in vivo, but very little, if any, methylation is required for the assembly and function of type IV pili or the type II secretion apparatus.

The currently available data suggest a model for a series of interactions between PilD and its substrates, which takes place on the cytoplasmic face of the inner (cytoplasmic) membrane. The positively charged leader peptide and N-terminal domain of prepilin are necessary and sufficient to promote translocation across the cytoplasmic membrane, as shown previously. The basic leader in front of the N-terminal hydrophobic domain may very likely function to orient and anchor the prepilin in the membrane. Because of the predicted orientation, the cleavage of the substrate takes place in the cytoplasmic side of the membrane, where methylation of mature pilin takes place as well. The site of the two modification reactions is consistent
with the topological location of the PilD domain containing the putative active site residues (11) and the presence of the methyl donor AdoMet. Although PilD is a bifunctional enzyme, the comparison of kinetics of processing and methylation (10) suggests that the two reactions are not necessarily coupled, and it is conceivable that methylation is not carried out by the same PilD molecule that was responsible for leader peptide cleavage.

*P. aeruginosa* produces several extracellular and surface-associated proteins, and the ability to export these virulence determinants is primarily dependent on PilD. Clearly the maturation of prepilin and prepilin-like proteins is essential for extracellular secretion, but it is not clear what consequences the two modification steps have on the subsequent fate and function of the processed proteins. Processing of signal peptide anchors by LPase I is responsible for the release of secreted proteins from the cytoplasmic membrane (29); however, it is unlikely that processed pilin monomers similarly dissociate from the membrane. Following cleavage of the short, basic leader peptide, the hydrophobic N-terminal domain of pilin would very likely prevent the release of the mature subunits from the membrane. Following cleavage of the short, basic leader peptide, the hydrophobic N-terminal domain of pilin would very likely prevent the release of the mature subunits from the membrane. Following cleavage of the short, basic leader peptide, the hydrophobic N-terminal domain of pilin would very likely prevent the release of the mature subunits from the membrane.

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