

## Glycosylation of Pilin and Nonpilin Protein Constructs by *Pseudomonas aeruginosa* 1244<sup>∇†</sup>

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PilO is an oligosaccharyl transferase (OTase) that catalyzes the O-glycosylation of *Pseudomonas aeruginosa* 1244 pilin by adding a single O-antigen repeating unit to the  $\beta$  carbon of the C-terminal residue (a serine). While PilO has an absolute requirement for Ser/Thr at this position, it is unclear if this enzyme must recognize other pilin features. To test this, pilin constructs containing peptide extensions terminating with serine were tested for the ability to support glycosylation. It was found that a 15-residue peptide, which had been modeled on the C-proximal region of strain 1244 pilin, served as a PilO substrate when it was expressed on either group II or group III pilins. In addition, adding a 3-residue extension culminating in serine to the C terminus of a group III pilin supported PilO activity. A protein fusion composed of strain 1244 pilin linked at its C terminus with *Escherichia coli* alkaline phosphatase (which, in turn, contained the above-mentioned 15 amino acids at its C terminus) was glycosylated by PilO. *E. coli* alkaline phosphatase lacking the pilin membrane anchor and containing the 15-residue peptide was also glycosylated by PilO. Addition of the 3-residue extension did not allow glycosylation of either of these constructs. Site-directed mutagenesis of strain 1244 pilin residues of the C-proximal region common to the group I proteins showed that this structure was not required for glycosylation. These experiments indicate that pilin common sequence is not required for glycosylation and show that nonpilin protein can be engineered to be a PilO substrate.

Colonization and dissemination of the opportunistic pathogen *Pseudomonas aeruginosa* rely to a large extent on the ability of this organism to produce functional type IV pili (26). These protein fibers, which radiate from the cell pole, are adhesion factors (51), mediate a form of surface translocation referred to as twitching motility (10, 37), and are important in biofilm formation (39). The pili of this organism are primarily composed of a monomeric subunit called pilin (PilA). Type IV pili can be differentiated into two classes (a or b) on the basis of the PilA sequence and structure (23). Although they display considerable sequence variation, the majority of the type IVa pilins of *P. aeruginosa* can be placed into one of three groups on the basis of primary structure and antigenicity, as well as by the presence of auxiliary pilin genes found immediately downstream from *pilA* (8, 33). We previously determined that pilin from *P. aeruginosa* 1244, which belongs to group I (8), contained an O-antigen repeating unit covalently attached to the  $\beta$ -hydroxyl group of a serine residing at the C terminus of this protein (7). While the specific physiological role of the pilin glycan in this organism is not clear, the presence of this saccharide influences pilus hydrophobicity and has a pronounced effect on virulence, as determined in a mouse respiratory model (47). The metabolic origin of the pilin saccharide is the O-antigen biosynthetic pathway (14), and its attachment is

catalyzed by an oligosaccharyl transferase (OTase) called PilO (6). Specific regions of this cytoplasmic membrane protein necessary for glycosylation activity have been identified (42). Topological studies of PilO have shown that these regions face the periplasm, suggesting that pilin glycosylation takes place in this chamber (42). Here the glycan substrate is the O-antigen repeating unit covalently linked to the undecaprenol carrier lipid.

PilO has a very relaxed glycan substrate specificity, as indicated by the evidence that it is able to utilize a number of structurally dissimilar O-antigen repeating units as substrate (14), and requires only features of the reducing end sugar to carry out pilin glycosylation (28). WaaL, the enzyme that transfers polymerized O antigen to core lipid A, from *Escherichia coli* also has a similar broad glycan specificity (19). Recent studies (18) provided evidence that PglL, an OTase of *Neisseria meningitidis*, recognized only the carrier lipid and was able to attach a variety of saccharides to the pilin of this organism. Although the glycan specificity of PilO is relaxed, this enzyme will not attach other carrier lipid-bound saccharides, such as the peptidoglycan subunit or polymerized O-antigen repeating unit, to pilin. This is indicated by the absence of pilins with increased mass in O-antigen-negative mutants or the production of multiple pilin sizes in the wild-type strain (6).

*In vivo* analysis of mutagenized *P. aeruginosa* 1244 pilin showed that the C-terminal serine of this protein was a major pilin glycosylation recognition feature of PilO (27). In addition, modification (substitution of the C-terminal amino acid with a 3-residue sequence terminating in serine) of a group II pilin allowed PilO-dependent attachment of the O-antigen repeating unit (27). While these results suggested that the preponderance of pilin structural information was not required for glycosylation, it was not clear whether regions common among

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the *P. aeruginosa* pilins were needed. In the present study three types of experiments were carried out in order to answer this question. First, the glycosylation site was extended away from the pilin surface with the addition of a 15-residue peptide which terminates with serine. Second, an engineered periplasmic protein containing the glycosylation residue at its C terminus was fused with pilin and tested for PilO activity. Finally, this periplasmic protein containing no pilin common region was constructed and tested. Evidence presented in this paper suggests that PilO requires only the glycosylation target residue.

The work presented also indicated that, in addition to pilins, nonpilin protein free in the periplasm or anchored to the cytoplasmic membrane could be engineered so as to serve as a PilO substrate. These results suggest that a wide range of pilins and nonpilin proteins can be engineered to serve as substrate for glycosylation, a finding that would potentially have practical value, particularly in the area of vaccine construction. In addition to elucidating the protein specificity of the PilO system, the present work determined that the peptide extension used can supply functional epitope information to the modified protein, in addition to providing a site for glycosylation. Altogether, the results presented suggest that engineering of pilins and nonpilin proteins for the biological generation of protein-peptide-saccharide constructs is a potentially important strategy in vaccine design.

#### MATERIALS AND METHODS

**Culture conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. Cultures were grown at 37°C on solid or liquid (with agitation at 250 rpm) medium. Bacteria were grown on LB medium for general culturing, on CAYE medium (2% agar, 0.75% Casamino Acids, 0.15% yeast extract) for production of pili, and on Trypticase soy broth for expression of PhoA fusions. The concentrations of antibiotics used in the selective media were as follows: ampicillin at 50 µg/ml for *Escherichia coli*, carbenicillin at 250 µg/ml for *P. aeruginosa*, kanamycin at 35 µg/ml for *E. coli* and 100 µg/ml for *P. aeruginosa*, and tetracycline at 100 µg/ml for *P. aeruginosa*. When added, isopropyl-β-D-thiogalactopyranoside (IPTG) was present in a final concentration of either 0.1 mM (*E. coli*) or 5.0 mM (*P. aeruginosa*).

**Plasmid construction.** Details of plasmid construction, including the oligonucleotide primers used, are presented in the supplemental material.

**Cellular alkaline phosphatase determination.** Cellular alkaline phosphatase determination employed the protocol of Daniels et al. (12). Culture absorbance at 600 nm was determined using a Spectronic 20 spectrometer. Absorbances at 415 and 595 nm were measured using a Bio-Rad model 3550 microplate reader. PhoA enzyme units were calculated using a standard equation (36).

**Protein sample preparation.** Strains expressing pilin constructs were grown on solid media in the presence of necessary antibiotics. Cell extracts were prepared as described previously (8).

For PilAP, PilAPI, and PilAPII, broth starter cultures of *P. aeruginosa* PA103 *wzy<sub>PaO11</sub>::aacC1* (where *wzy<sub>PaO11</sub>* is *wzy* of *P. aeruginosa* serotype O11) containing the plasmids of interest were grown overnight. Seven milliliters of the starter cultures was used to inoculate 300 ml of the same medium containing 5.0 mM IPTG. These cultures were grown overnight and centrifuged at 5,000 rpm for 30 min in a GSA rotor. The pellet was resuspended in 100 ml of 0.05 M Tris HCl, pH 7.3, and centrifuged at 5,000 rpm for 30 min in a GSA rotor. These cells were resuspended in 10 ml of lysis buffer (10 mM phosphate, 30 mM NaCl, 0.25% Tween 20, 10 mM β-mercaptoethanol, 10 mM EDTA, 10 mM EGTA) and subjected to sonication on ice with three 15-s bursts using a Biosonik III sonicator (Bronwill Scientific) and then centrifuged at 10,000 rpm for 30 min in an SS-34 rotor. The supernatant was subjected to centrifugation at 30,000 rpm for 1 h in an SW60Ti rotor, after which the pellets were rinsed with 5 ml of ice-cold phosphate-buffered saline. This pellet was dissolved with 200 µl of lysis buffer. The protein concentration was determined using the bicinchoninic acid assay (Pierce).

In order to analyze ECAP, ECAP1, and ECAP2, broth starter cultures of *P.*

*aeruginosa* PA103 *wzy<sub>PaO11</sub>::aacC1* containing the plasmids of interest and 5 mM IPTG were grown overnight as described above. The periplasmic fraction of these cells was isolated using the protocol of Poole and Hancock (41). This material was centrifuged at 35,000 rpm for 1.5 h in a SW-60 Ti rotor, and the supernatant was dialyzed overnight against 2 liters of 0.05 M Tris HCl, pH 7.3. The sample was lyophilized and dissolved with 1 ml of deionized water. This solution was then fractionated by ammonium sulfate precipitation (17), followed by gel filtration by fast-performance liquid chromatography using a Superose 12 column. Fractions containing PhoA were determined using the alkaline phosphatase assay of Garen and Levinthal (21).

**Mass spectrometric analysis.** Analysis of ECAP and ECAP1 was carried out on GluC-digested samples. Thirty micrograms of purified protein in 50 µl of 0.1 M ammonium bicarbonate was treated according to the manufacturer's directions. In brief, the protein was reduced with the addition of dithiothreitol to 10 mM, followed by alkylation with the addition of iodoacetamide to 20 mM. This material was digested with 1.5 µg GluC (Thermo Scientific) for 22 h at 37°C. The peptide fragments produced were separated by capillary C<sub>18</sub> high-pressure liquid chromatography using a ThermoElectron Surveyor liquid chromatograph with a Micro AS autosampler. Direct analysis of the column effluent was carried out using a ThermoElectron LCQ Deca XP Plus quadrupole ion trap mass spectrometer utilizing a nanospray ionization source. This instrument was set up to perform both mass spectrometry (MS) and MS/MS analysis. The computer algorithm controlling these experiments selected the three largest ions from an MS spectrum for MS/MS analysis in triplicate. This process was repeated throughout the chromatographic separation. MS and MS/MS data analysis employed the SEQUEST program, in which the data obtained were compared with those from an *in silico* digestion of *Escherichia coli* alkaline phosphatase. Pilin mass analysis was carried out by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) MS using a PerSeptive Biosystems Voyager STR with DE and a high-*m/z* detector.

**Electrophoresis and Western blot methods.** The electrophoresis and Western blot methods described previously (42) were followed. Polyacrylamide gels with 16% T and 3% C were used when PilA constructs were analyzed. Polyacrylamide gels with 10% T and 3% C were used when PhoA, PilAPhoA, and derivatives of these proteins were examined. All primary antibodies were mouse IgG. A monoclonal antibody (Sigma-Aldrich Co.) was used to detect PhoA. A *P. aeruginosa* IATS (International Antigenic Typing Scheme) (35) serotype O11-specific monoclonal antibody (ERFA Biotech) was used to detect proteins bearing this saccharide. Monoclonal antibody 11.14 (46) was used to detect proteins bearing the O7 repeating unit. Monoclonal antibody 5.44 (8), which was specific for the α-β loop region, or monoclonal antibody 6.45 (8), which was specific for the disulfide loop region, was used to detect *P. aeruginosa* strain 1244-specific pilin epitopes. Monoclonal antibody 2.97 (46) was used to recognize strain PA103 pilin. Secondary antibodies (anti-mouse IgG) were either phosphatase (Kirkegaard & Perry Laboratories) or Alexa Fluor (Sigma-Aldrich) labeled. The latter label was detected using a Molecular Dynamics 595 fluorimager, as described previously (42). This protocol was also used to quantitate PhoA fusions. Here, protein samples were separated by polyacrylamide gel electrophoresis using 1-mm 10% T and 3% C gels. The separated proteins were electroblotted for 23 min to 0.45-µm-pore-size nitrocellulose paper at 100 V using a 25 mM Tris HCl–192 mM glycine–20% methanol buffer. Under these conditions, staining with Coomassie brilliant blue showed that no protein with the apparent molecular weight of the fusion remained in the gel. After the gels were blocked and the Alexa Fluor secondary antibody was applied, relative fluorescence levels were determined using the ImageQuant program. Utilizing a double layer of membrane showed that no fusion passed through the first nitrocellulose paper.

**Site-directed mutagenesis.** Mutagenesis of DNA coding for the *P. aeruginosa* 1244 pilin disulfide loop residues was carried out as described in the supplemental material. The plasmid encoding the protein to be tested also contained a functional *pilO* gene. The mutagenized *pilA* was expressed in *P. aeruginosa* 1244N3, where the glycosylation state was indicated by a shift in apparent molecular weight, as determined by Western blotting using a pilin-specific monoclonal antibody.

**Generation of polyclonal anti-683 pilin serum.** The protocol used for the generation of polyclonal anti-683 pilin serum was approved by Duquesne University's Institutional Animal Care and Use Committee (IACUC). All animal storage and experimentation took place at the USDA-approved Duquesne University Animal Care Facility. Preparation of strain 683 pili followed a standard methodology (29). A total of 6 female BALB/c mice (Hilltop Lab Animals, Scottsdale, PA), approximately 6 to 8 weeks old and weighing 25 g, were immunized using a protocol described previously (29).

**Twitching motility.** The twitching motility protocol was carried out as described previously (7).

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Reference or source
<b>Strains</b>		
<i>P. aeruginosa</i>		
PA1244	Wild-type strain, pilin group I	44
PA1244N3	Interruption of sigma factor <i>rpoN</i> with tetracycline cassette, Tc <sup>r</sup>	43
PA103	Wild-type strain, pilin group II	34
PA103	Interruption of <i>wzy</i> gene with a gentamicin cassette ( <i>aacC1</i> ), Gm <sup>r</sup> <i>wzy</i> <sub>PA011</sub> :: <i>aacC1</i>	13
PA683	Wild-type strain, pilin group III	This study
<i>E. coli</i>		
XL-Gold	<i>endA1 supE44 thi-1 recA1 gryA96 relA1 lacHte</i>	Stratagene
HB101	<i>supE44 hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1</i>	4
<b>Plasmids</b>		
pHK734	pTrc99a with <i>E. coli phoA</i>	31
pCR2.1-TOPO	3.9-kb cloning vector, Ap <sup>r</sup>	InVitrogen
pECAP	pMMB66EH with pHK734 <i>phoA</i>	This study
pECAPaas	pMMB66EH with mutated <i>E. coli phoA</i> that had an AAS stop extension added to the C-terminal lysine residue	This study
pECAPpep	pECAP with DNA coding for C-terminal TAWKPNYAPANAPKS stop	This study
pMMB66EH	8,807-bp broad-host-range expression vector, IPTG inducible <i>tac</i> promoter, Amp <sup>r</sup> Cb <sup>r</sup>	20
pUCP26PilO	pUCP26 with <i>pilO</i> under the control of <i>tac</i> promoter, Tc <sup>r</sup>	27
pPAC24	pMMB66EH with 1244 <i>pilA</i>	6
pPAC46	pMMB66EH with 1244 <i>pilAO</i>	6
pUC46	<i>pilAO</i> in pUC19	9
pW136A	pPAC46 with <i>pilA</i> codon for W136 converted to A	This study
pK137A	pPAC46 with <i>pilA</i> codon for K137 converted to A	This study
pN139A	pPAC46 with <i>pilA</i> codon for N139 converted to A	This study
pY140A	pPAC46 with <i>pilA</i> codon for Y140 converted to A	This study
pP142A	pPAC46 with <i>pilA</i> codon for P142 converted to A	This study
pN144A	pPAC46 with <i>pilA</i> codon for N144 converted to A	This study
pP146A	pPAC46 with <i>pilA</i> codon for P146 converted to A	This study
pK147A	pPAC46 with <i>pilA</i> codon for K147 converted to A	This study
pS148A	pPAC46 with <i>pilA</i> codon for S148 converted to A	9
pRMCD28	Ap <sup>r</sup> , PhoA fusion vector	12
pPilAPhoA	pRMCD28 with 1244 <i>pilA</i>	This study
pPilAP	pMMB66EH with 1244 <i>pilA-E. coli phoA</i> fusion	This study
pPilAPaas	pPilAP with DNA coding for C-terminal AAS stop	This study
pPilAPpep	pPilAP with DNA coding for C-terminal TAWKPNYAPANAPKS stop	This study
pSAD100	pUC18 with PA103 <i>pilA</i>	27
pSD5	pMMB66EH with PA103 <i>pilA</i>	27
pMAM	pSD5 with DNA coding for C-terminal TAWKPNYAPANAPKS stop	This study
pPAL100	pMal-cRI containing DNA coding for a <i>malE-pilO</i> gene fusion	42
pUCP24	Broad-host-range cloning vector, pUC18 derived, Gm <sup>r</sup>	50
pUCP24PilO	pUCP24 with <i>pilO</i> under control of <i>tac</i> promoter	This study
p683PilA	pMMB66EH with PA683 <i>pilA</i>	This study
p683aas	p683pilA with DNA coding for C-terminal AAS stop	This study
p683pep	p683pilA with DNA coding for C-terminal TAWKPNYAPANAPKS stop	This study

**Homology modeling.** The *E. coli* PhoA molecular structure was obtained from the Protein Data Bank (<http://www.rcsb.org/pdb/explore/explore.do?structureId=1ALK>). PhoA homology models of the PhoA constructs were generated using 3D-PSSM software online (<http://www.sbg.bio.ic.ac.uk/3dpssm/index2.html>). The tertiary homology structural output was analyzed using the DS Viewer Pro program (version 6.0) for surface-exposed C-terminal residues and surface charge.

## RESULTS

**Glycosylation of engineered pilins.** We have previously shown that the *P. aeruginosa* PA103 pilin, a protein that is normally not glycosylated and that is a member of the group II family, could be engineered to act as a glycosylation substrate by substituting three residues (AAS) for the C-

terminal amino acid (27). These and other experiments indicated the importance of the C-terminal serine but did not eliminate the possibility that PilO required other pilin components that are common to both proteins. Representatives of the three major pilin groups of *P. aeruginosa* show strong sequence identity over the N-proximal region of this protein and, to a lesser extent, in the C-proximal region (Fig. 1), a characteristic shared with other type IV pilins (25). Experiments were carried out to determine whether the glycosylation site could function if it was extended a significant distance from the protein surface or if its close proximity with the pilin common regions was required. To do this, the *pilA* gene of strain PA103 was extended so as to code for 15 more residues. The sequence chosen (Fig. 2A) was based on

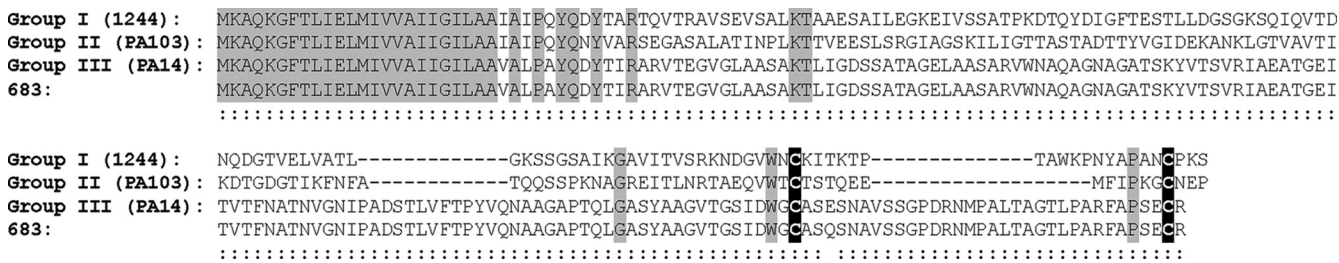


FIG. 1. Comparison of the *P. aeruginosa* 683 PilA primary structure with pilin group I, II, and III representatives. Sequence identity is indicated by gray highlighting. The alignment of the three representative pilin groups is based on the common sequence of the N-proximal region and the disulfide loop region. Cysteines forming the disulfide loop are in white with a black background. Identity between PA14 and 683 pilin residues is indicated by a colon. The GenBank accession numbers for strains 1244, PA103, and PA14 are CAA58768.1, P08015.1, and ABJ13792.1, respectively.

the strain 1244 C-proximal region (TAWKPNYAPANCPKS). The cysteine of this sequence was replaced with an alanine in order to avoid potential incorrect disulfide formation. This sequence was selected in part because it is a component of a stable protein, which suggested that the fusion might be resistant to degradation. It was also picked because it is a known B-cell epitope and is the target of characterized monoclonal antibodies (8, 9), suggesting that fusions containing this structure could be detected by immunoblotting. A group II pilin was chosen as the platform over the 1244 protein to avoid potential DNA excision problems which could occur with a repetitive contiguous sequence.

This protein construct, which was coded for by pMAM and which was termed P103I (Fig. 2A), was assayed by Western

blotting in a PilAO-negative (PilAO<sup>-</sup>) background using a PA103 pilin-specific monoclonal antibody as the probe (Fig. 3A). PilO was delivered from a second plasmid. The pilin produced in the absence of PilO had an increased size, as would be expected from the presence of the added peptide. The stability of this protein construct was indicated by the absence of pilin degradation products. Pilin generated in the presence of a functional *pilO* gene produced an antigen species of even greater apparent molecular weight, suggesting that this protein had been modified by PilO. A blot produced in an identical manner was probed with an antibody specific for the O-antigen repeating unit of the host strain (Fig. 3B). While the lipopolysaccharide ladder was seen in all cases, the presence of PilO gave an additional band which was at an identical position as the putatively modified P103I, suggesting that



FIG. 2. Features of the fusions employed in this study. (A) Parent and modified pilins; (B) pilin-alkaline phosphatase constructs (modified and unmodified); (C) parent and modified alkaline phosphatase. The cartoons show the relative sizes and orientations of construct components (gray boxes, PilA; white boxes, PhoA) as well as the modification site (black boxes). The protein product name is listed in the first column of each panel. The plasmid containing the gene expressing this protein is in the second. The third column shows essential features of the protein construct. Here, the C-terminal additions are in white with a black background. The PilA-PhoA junctions are shown in black with a gray background.

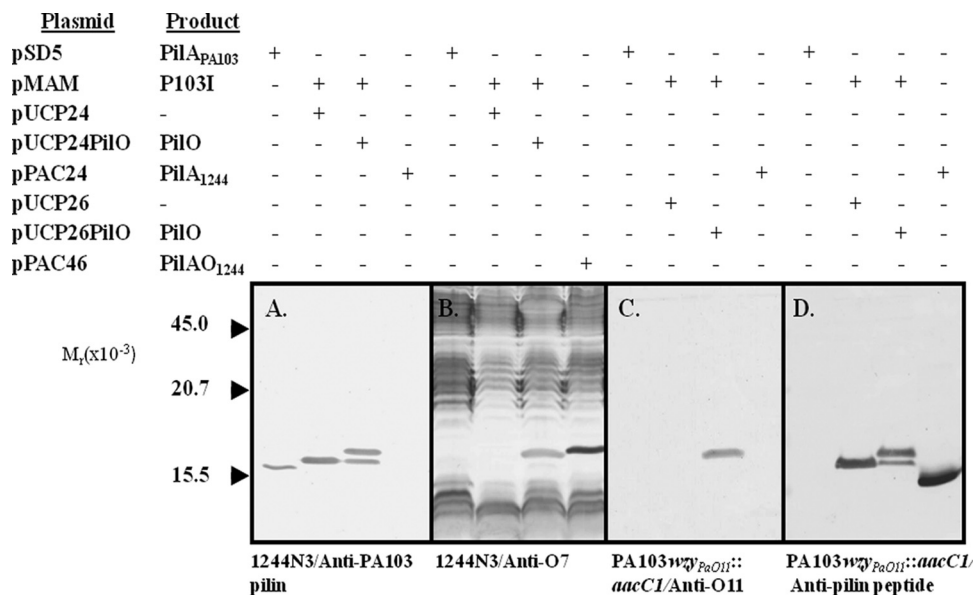


FIG. 3. Western blot of natural and modified PilA of *Pseudomonas aeruginosa* PA103 in the presence and absence of PilO. Antibodies used were monoclonal antibody 2.97, which is specific for strain *P. aeruginosa* PA103 pilin (A); monoclonal antibody 11.14, which is specific for the O antigen of *P. aeruginosa* IATS serotype O7 (B); monoclonal antibody O11, which is specific for the O antigen of *P. aeruginosa* IATS serotype O11 (C); and monoclonal antibody 6.45, which is specific for the disulfide loop region of strain 1244 pilin (D). The plasmids of panels A and B were expressed in *P. aeruginosa* 1244N3. The plasmids of panels C and D were expressed in *P. aeruginosa* PA103wzy<sub>PaO11</sub>::aacCI. Glycosylated and nonglycosylated 1244 pilin was produced from pPAC46 and pPAC24, respectively.

this protein contains bound O-antigen repeating unit. In order to confirm this, pMAM was expressed in *P. aeruginosa* PA103wzy<sub>PaO11</sub>::aacCI, which produced an antigenically dissimilar pilin and carrier-lipid-bound O-antigen repeating unit (but no polymerized O antigen). This system allowed Western blot detection of the pilin glycan with an O-antigen-specific antibody without visual interference from the lipopolysaccharide ladder. Here a reaction was seen only when pMAM was expressed in the presence of a functional *pilO* gene (Fig. 3C). In addition, the apparent molecular weight of this antigen matched that of the putative glycosylated construct seen in Fig. 3A and B. The presence of the peptide extension in both the putative glycosylated and the nonglycosylated constructs was confirmed by Western blotting (Fig. 3D) using monoclonal antibody 6.45, which has previously been determined to be specific for the C-terminal region of strain 1244 pilin (8). The control for this response is the reaction of monoclonal antibody 6.45 with nonglycosylated 1244 pilin produced by pPAC24 (Fig. 3D). A twitching assay showed that while cloned PA103 *pilA* expressed in a PilAO<sup>-</sup> mutant of strain 1244 supported motility, expression of pMAM did not (results not shown). In addition, electrophoretic analysis of the supernatant of sheared cells showed the absence of pilin protein (results not shown), indicating that the construct is not assembled into a pilus. Altogether, these results suggest that moving the glycosylation site away from the pilin surface does not interfere with PilO action and is evidence that the pilin common regions are not necessary for glycosylation.

The *P. aeruginosa* group III pilins are the third most common type produced by this organism (33). Nucleotide sequencing of the *pilA* of *P. aeruginosa* 683 revealed that the pilin coded for by this gene was nearly identical to the group III pilin

of strain PA14 and that these pilins, while they share core sequence features, were considerably larger than members of the other two groups (Fig. 1). In addition, accessory gene *tfpY*, typically found immediately downstream of the group III *pilA* (33), was present in this position in strain 683 (results not shown). Published results have shown that PA14 pilin is not glycosylated (2). We determined, using MALDI-TOF MS, that the strain 683 pilin isolated had a mass of 17,475 kDa, which agreed well with the predicted value (17,453 kDa), further suggesting that this protein was also not modified.

Experiments were carried out to determine (i) if the strain 683 pilin could be engineered to be a PilO substrate and (ii), if so, whether the glycosylation site could be functional if it is extended away from the protein. In order to do this, two modified pilins were constructed. The first, which was analogous to a group II construct that was shown to support glycosylation (27), was p683aas (Fig. 2A). This plasmid produced P683I, which was strain 683 pilin with an AAS extension at its C terminus. The second, p683pep, coded for P683II, which contained the C-terminal 15-residue epitope described above (Fig. 2A). Expression of these constructs in *P. aeruginosa* PA103wzy<sub>PaO11</sub>::aacCI was analyzed by Western blotting using the procedure described above and employing either a 683 pilin-specific polyclonal antibody or the O-antigen-specific monoclonal antibody as probe. Figure 4A shows that both p683aas and p683pep, expressed in the absence of a functional *pilO* gene, produced antigen of the expected increased apparent molecular weight as well as a form matching unmodified pilin. In the presence of functional *pilO*, a larger form yet was seen with both constructs, suggesting that these pilins had undergone posttranslational modification. To test this, a blot run with identical strain 683 pilin samples was probed with the

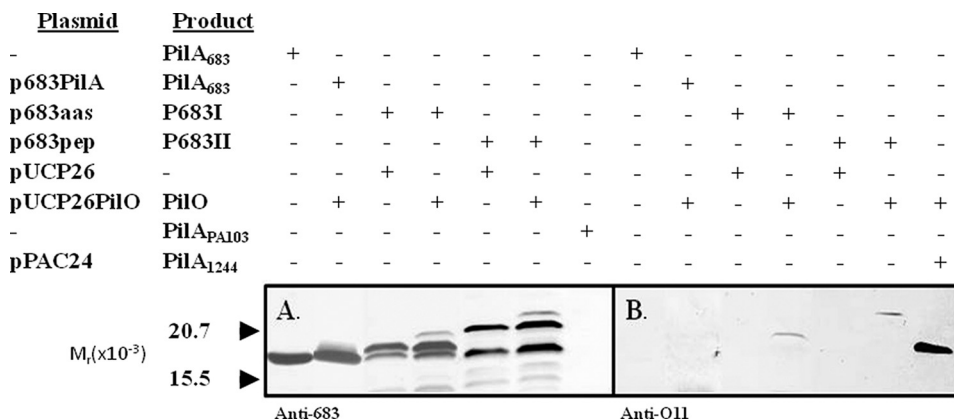


FIG. 4. Western blot of natural and modified *P. aeruginosa* 683 PilA in the presence or absence of PilO. The antibodies used were a polyclonal preparation raised against pure 683 pilin (A) or a monoclonal antibody against IATS serotype O11 (B). All plasmids were expressed in *P. aeruginosa* PA103wz<sub>y</sub><sub>PaO11</sub>::aacC1. Strain 683 pilin and strain PA103 pilin were supplied as purified proteins. pPAC24 contains a functional *pilA* gene but no *pilO*.

O-antigen-specific monoclonal antibody. Here it can be seen (Fig. 4B) that a band of the same apparent molecular weight as the largest pilin structure detected by the anti-683 pilin serum was produced only in the presence of functional *pilO*. pPAC24 expressed in the presence of pPUCP26 produced the glycosylated pilin control of Fig. 4B. A twitching assay of these constructs showed that while p683PilA supported motility when it was expressed in a PilA<sup>-</sup> *P. aeruginosa* strain, neither of the plasmids producing modified pilins did so (results not shown). Further, only trace amounts of pili were present in the supernatants of sheared cells (results not shown). Overall, these results indicate that group III pilin, in spite of size and sequence differences, can be engineered to function as a glycosylation substrate for PilO by adding either a short peptide extension or the 15-residue structure. Further, moving the glycosylation site away from the pilin surface, as with P103I, supports PilO function, which suggests that the pilin common sequences are not needed for modification.

**Glycosylation of engineered nonpilin protein.** To further test the PilO substrate requirements, experiments were carried out to determine whether nonpilin protein could be engineered to function as a glycosylation target. In the first of two strategies, we constructed a protein fusion that targeted the glycosylation site to the periplasmic side of the cytoplasmic membrane, the location of PilO's catalytic region and glycan substrates (42). To accomplish this, the C-terminal residue of *P. aeruginosa* 1244 PilA was fused with the N terminus of *E. coli* PhoA lacking a leader sequence, thus producing PilAP (Fig. 2B). PhoA from *Escherichia coli* was chosen because it shared with strain 1244 pilin surface characteristics that were necessary for glycosylation (27). Specifically, it has a C-proximal region that extends away from the protein surface and a net positive charge in this region (results not shown). PilAP was engineered so as to have a C-terminal AAS (PilAPI, coded by pPilAPaas) or the pilin peptide described above (PilAPII, coded by pPilAPpep) at its C terminus (Fig. 2B). High levels of alkaline phosphatase (grown under conditions which suppress expression of the chromosomal *phoA* gene) were produced by *P. aeruginosa* strains expressing these plasmids, indicating that the fusion was active and that the PilA component of the

construct was able to target the PhoA portion to the periplasm of this organism (Table 2). The membrane fraction of *P. aeruginosa* PA103wz<sub>y</sub><sub>PaO11</sub>::aacC1 expressing these constructs (along with a plasmid producing functional PilO) was analyzed by Western blotting using an anti-PhoA serum as a probe. The results (Fig. 5A) showed antigens of the expected size and indicated that, although degradation was seen, the fusion targeted as anticipated. A Western blot of the periplasmic fraction of these strains using the same probe showed antigens of lower molecular weight, suggesting that the fusions were subject to degradation, resulting in the release of PhoA (results not shown). The presence of the PilA component of this fusion was confirmed by Western blotting (Fig. 5B) using a monoclonal antibody specific for a central region of the strain 1244 pilin primary structure. These samples were also assayed by Western blotting using the O-antigen-specific monoclonal antibody as a probe in order to test for the presence of O-antigen repeating unit (Fig. 5C). Before this step was carried out, the membrane fractions were normalized after determination of relative antigen levels using a quantitative Western blot. Figure 5C shows that neither the control construct nor the fusion containing the AAS extension produced a reaction, indicating that these proteins were not suitable glycosylation substrates for PilO. However, fusion PilAPII produced a strong response, suggesting that, as above, the added peptide allowed this protein to function as a PilO substrate. Altogether, these results

TABLE 2. Alkaline phosphatase levels

Plasmid <sup>a</sup>	Alkaline phosphatase activity <sup>b</sup>
pPAC24	0.0
pPilAP	11,507.1
pPilAPaas	11,836.4
pPilAPpep	10,413.1
pECAP	15,629.1
pECAPaas	20,569.1
pECAPpep	9,675.1

<sup>a</sup> Plasmids were expressed in *P. aeruginosa* PA103 wz<sub>y</sub><sub>PaO11</sub>::aacC1/pUCP26PilO.  
<sup>b</sup> Activity is given in standard units (36).

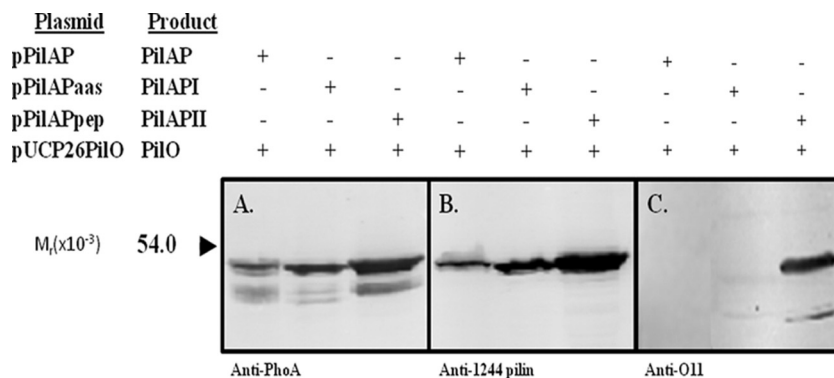


FIG. 5. Western blot of natural and modified PilA-PhoA fusions in the presence of PilO. The antibodies used were a monoclonal antibody specific for *E. coli* PhoA (A); monoclonal antibody 5.44, which was specific for a *P. aeruginosa* 1244 pilin epitope (B); and monoclonal antibody O11, which was specific for *P. aeruginosa* IATS serotype O11 (C). All plasmids were expressed in *P. aeruginosa* PA103wzy<sub>PaO11</sub>::aacC1 containing pUCP26PilO. See the legend to Fig. 2 for construct details.

suggest that a nonpilin protein anchored to the outer surface of the cytoplasmic membrane can be engineered to act as substrate for this enzyme. This is further evidence that the pilin common region is not required for PilO activity. The second strategy employed aimed to determine whether a nonpilin periplasmic protein which was not fused to pilin (and not anchored in the cytoplasmic membrane) could serve as a glycosylation substrate. Here, *E. coli* PhoA (PECAP, coded for by pECAP), a protein that normally targets to the periplasm, was engineered so as to have a C-terminal AAS (PECAPI, coded by pECAPaas) or the pilin peptide described above (PECAPII, coded by pECAPpep) at its C terminus (Fig. 2C). To test for protein glycosylation, plasmids coding for these constructs were expressed in *P. aeruginosa* PA103wzy<sub>PaO11</sub>::aacC1, which, as described above, allowed Western blot assay of glycosylated protein without lipopolysaccharide interference. Here, PilO was supplied from a plasmid carrying a functional *pilO* gene. Table 2 shows that high levels of alkaline phosphatase activity were produced under growth conditions suppressing expression of the chromosomal *phoA*, indicating that the PhoA constructs were active and targeted correctly. These proteins were purified from the periplasmic fraction using salt fractionation and gel filtration, which resulted in preparations containing only minor contamination, as determined by polyacrylamide gel electrophoresis followed by protein staining with Coomassie brilliant blue (results not shown). Aliquots of this material containing equivalent amounts of protein were then subjected to Western blot analysis using an anti-PhoA antibody as a probe. Here, antigens of the anticipated size were seen for each sample (Fig. 6A). When an identical blot was probed with the O-antigen-specific monoclonal antibody, a strong reaction was seen with PECAPII, indicating that, as with PilAPII, this construct was a glycosylation substrate (Fig. 6B). No reaction was seen with the PECAPI construct. In order to clarify whether the engineered portion of PECAPI was intact and a good potential substrate, this construct, as well as the control protein, was digested with GluC and subjected to liquid chromatography followed by mass spectrometry (MS and MS/MS). An intact C-terminal fragment (DLFYTMKAALGLK) of the control protein was detected with a cross-correlation score of 2.65. While this fragment was absent in PECAPI, another

fragment (DLFYTMKAALGLKAAS) which was not present in the control digestion and which had a cross-correlation score of 3.38 was seen. These results indicated that this protein contains the potential glycosylation site and that the absence of modification indicated that PilO was not able to recognize this structure.

The finding that PECAPII is glycosylated while PECAPI is not raises the possibility that PilO recognizes a portion of the group I disulfide loop region, in addition to the C-terminal residue. This is unlikely, since we have previously shown that mutant 1244 pilin lacking the entire disulfide loop is glycosylated (27). In order to reexamine this point, eight of the conserved group I disulfide loop and tail residues (see Fig. S1 in the supplemental material) found in this portion of *P. aeruginosa* 1244 PilA were replaced with alanines and tested for their ability to support glycosylation. Figure 7 shows that this treatment did not influence the ability of PilO to glycosylate pilin. These results confirm the findings of previous studies (27) and suggest that, other than the C-terminal serine/threonine, no pilin components are required for protein glycosylation by PilO. Altogether, the results presented here provide evidence that the pilin constant region is not required for glycosylation and suggest that the potential for the use of PilO in glycoengi-

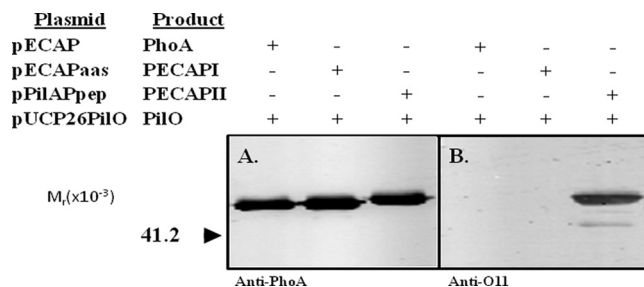


FIG. 6. Western blot of natural and modified *E. coli* PhoA produced in the presence of PilO. The antibodies used were a monoclonal antibody specific for *E. coli* PhoA (A) and monoclonal antibody O11, which was specific for *P. aeruginosa* IATS serotype O11 (B). All plasmids were expressed in *P. aeruginosa* PA103wzy<sub>PaO11</sub>::aacC1 containing pUCP26PilO. See the legend to Fig. 2 for construct details.

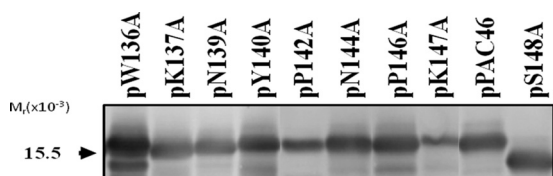


FIG. 7. Glycosylation of mutant *P. aeruginosa* 1244 pilins. Cloned *pilA* from this organism was subjected to site-directed mutagenesis, resulting in clones which produced the pilins seen. The primary antibody employed was monoclonal antibody 5.44. Pilin produced from pPAC46 was the glycosylated control, while pilin from pS148A, which has been shown to be nonglycosylated (9), was the negative control.

neering is considerably broadened with the finding that non-pilin proteins can be employed as substrates.

## DISCUSSION

The type IV pilins make up a protein family that is characterized by common sequence and structural features (11). While previous work (27) has shown that PilO requires an unblocked serine or threonine occupying the pilin C-terminal position, shared pilin components are also potential recognition features for pilin glycosylation. The evidence offered in the present paper suggests that this is not the case. Here it was shown that extension of the PilO site away from the pilin surface by 15 residues does not prevent glycosylation. In addition, insertion of a protein between the pilin C terminus and this peptide extension likewise results in active protein glycosylation. Finally, attaching this peptide modification to the carboxyl end of a native periplasmic protein also results in efficient production of a stably glycosylated product. The peptide extension used in each of these experiments was designed from the C-proximal portion of the disulfide loop and the pilin tail region of strain 1244 pilin, which left open the possibility that PilO recognized portions of the peptide used for extension of the glycosylation site. The finding that these residues, as present in native pilin, were not required for PilO activity suggested that this region was not a determinant in glycosylation. Overall, the results presented here suggest that PilO recognizes only the C-terminal serine. While the C-terminal residue is crucial to PilO activity, other factors are also important. Previous studies showed that a minimum distance from the pilin surface as well as a compatible surface charge is required for activity (27). Interference of enzyme activity by protein surface properties may be responsible for the finding that while attachment of both the AAS and the peptide extensions to the pilins allowed glycosylation, the shorter structure did not support modification of either the PilAPI or the PECAPI fusions. These results may be due to steric hindrance caused by protein surface structures or to a charge effect caused by the presence of the required metal or the noncovalent attachment of substrate (or product) to the PhoA catalytic site (32). The extension of the glycosylation site well away from the protein surface may have allowed modification of PilAPII and PECAPII. A longer C-terminal extension may produce a more efficient interaction between PilO and P683I and P683II. However, the inefficient glycosylation of both P683I and P683II may also reflect an interference caused by the bulky disulfide region of this protein or a construct instability (since glycosyl-

ation was not improved using P683II) introduced by modification at the C terminus. The physical environment of the glycosylation site and the composition of any peptide extension will be important considerations in the future design of both pilin and nonpilin constructs.

PglO, an OTase, of *Neisseria gonorrhoeae* and related species was found to be able to glycosylate a number of cellular proteins, in addition to pilin (5, 49). While the simple protein substrate requirements of PilO and the ready availability of its O-antigen substrate raise the possibility that this enzyme has other protein substrates as well, the absence of reactive antigens seen when *pilO* was expressed in a Wzy-negative strain (Fig. 2C) suggests that this is not the case. One reason might be that while PglO is able to modify internal serines (1), PilO is able to glycosylate only a C-terminal serine or threonine (9, 27). Thus, the PilO protein substrate requirements are, while simple, likely found naturally on very few proteins. Another is that the oligosaccharides utilized by PilO vary in charge, size, and hydrophobicity, depending on the lipopolysaccharide serotype. The glycan substrate for the general glycosylation systems of *Neisseria* and *Campylobacter* are structurally less variable (22, 48). While this variable modification is clearly tolerated on the surface of a structural protein such as pilin, as indicated by the finding of a variety of pilin-associated O antigens among *P. aeruginosa* strains (unpublished observations), the presence of this diversity of O-antigen repeating units could have a differentially negative effect on the activity of an enzyme or chaperone protein. Overall, these results point to dissimilarity in protein substrate specificities between PglO and PilO, which suggests that there is a difference in the physiological role of protein glycosylation in the organisms that produce these enzymes.

The ability of PilO to attach heterologous O-antigen repeating units to pilins (14) suggests the possibility of constructing a vaccine that could provide both pilin and O-antigen-specific protection. Both of these structures are strongly immunogenic and have been considered vaccine candidates (16). Immunization with either pili (38) or O antigen (15, 40, 52) from *P. aeruginosa* has been shown to be protective, as determined in murine models. We have shown that the *P. aeruginosa* strain 1244 pilin-bound O-antigen repeating unit is immunogenic (9) and that this structure provides O-antigen-specific protection (29). A T-cell-dependent response against this saccharide is indicated by the strong IgG reaction seen (29). This vaccine target is not limited to the lipopolysaccharide of *P. aeruginosa*, since the minimal PilO glycan requirement is found in the O-antigen repeating units of many bacterial pathogens (14, 28). In addition, the evidence presented here that PilO requires no pilin component other than the C-terminal residue suggests that structurally different pilins from a variety of pathogens could be engineered to act as glycosylation substrates. Candidates for research in this area would be the type IVb pilins (23), including the structurally unusual Flp fimbriae (3), as well as the chaperone-assisted pili (45).

The present work demonstrates that it is possible to insert a peptide epitope between pilin and the associated O-antigen repeating unit. This addition allows the incorporation of a distinctly different vaccine constituent that could target a pathogen component structurally unrelated to pilin or the O antigen. For example, it may be possible to construct a single

vaccine that could protect against colonization and dissemination (pilin response), have a neutralizing effect (from a peptide based on a toxin epitope), and stimulate opsonization (due to production of O-antigen-specific antibodies). Using a similar line of reasoning, a tripartite construct could be made that was composed of a nonpilin protein platform to which a glycosylated peptide was attached. Again, this structure could potentially stimulate three separate types of response. The glycosylation of PECAPII suggests that other free periplasmic proteins can be engineered for such activity, while modification of the PilAPII fusion indicates that membrane-anchored nonpilin proteins can serve as substrates for PilO. It will be of particular interest to determine whether transiently periplasmic species such as proteins transported by the type II or type IV secretion systems can also be engineered for O-antigen glycosylation.

Delivery of multiple forms of the same immunogen can produce a suppressed response against each component. The pilins of *Dichelobacter nodosus* (30) and the O antigen of *P. aeruginosa* (24) are examples of this phenomenon. A further disadvantage of numerous forms of a single immunogen is that many preparations are required to produce a single vaccine cocktail. These problems could potentially be avoided by incorporating antigenically unrelated epitopes in the same vaccine structure using the tripartite strategy described above. Cocktails of these preparations incorporating only the most common immunogen forms could potentially avoid the immunogenic suppression response. The targeting of three separate epitopes could compensate for incomplete immunogen coverage. Studies are required to determine the relative immunogenicity and protection afforded by such preparations.

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