

## Modification of *Pseudomonas aeruginosa* Pa5196 Type IV Pilins at Multiple Sites with D-Araf by a Novel GT-C Family Arabinosyltransferase, TfpW<sup>∇</sup>

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Received 1 August 2008/Accepted 5 September 2008

*Pseudomonas aeruginosa* Pa5196 produces type IV pilins modified with unusual  $\alpha$ 1,5-linked D-arabinofuranose ( $\alpha$ 1,5-D-Araf) glycans, identical to those in the lipoarabinomannan and arabinogalactan cell wall polymers from *Mycobacterium* spp. In this work, we identify a second strain of *P. aeruginosa*, PA7, capable of expressing arabinosylated pilins and use a combination of site-directed mutagenesis, electrospray ionization mass spectrometry (MS), and electron transfer dissociation MS to identify the exact sites and extent of pilin modification in strain Pa5196. Unlike previously characterized type IV pilins that are glycosylated at a single position, those from strain Pa5196 were modified at multiple sites, with modifications of  $\alpha\beta$ -loop residues Thr64 and Thr66 being important for normal pilus assembly. Trisaccharides of  $\alpha$ 1,5-D-Araf were the principal modifications at Thr64 and Thr66, with additional mono- and disaccharides identified on Ser residues within the antiparallel beta sheet region of the pilin. TfpW was hypothesized to encode the pilin glycosyltransferase based on its genetic linkage to the pilin, weak similarity to membrane-bound GT-C family glycosyltransferases (which include the *Mycobacterium* arabinosyltransferases EmbA/B/C), and the presence of characteristic motifs. Loss of TfpW or mutation of key residues within the signature GT-C glycosyltransferase motif completely abrogated pilin glycosylation, confirming its involvement in this process. A Pa5196 *pilA* mutant complemented with other *Pseudomonas* pilins containing potential sites of modification expressed nonglycosylated pilins, showing that TfpW's pilin substrate specificity is restricted. TfpW is the prototype of a new type IV pilin posttranslational modification system and the first reported gram-negative member of the GT-C glycosyltransferase family.

*Pseudomonas aeruginosa* is a gram-negative bacterium that inhabits diverse environments and is an important opportunistic pathogen of immunocompromised individuals and cystic fibrosis patients (14, 34, 39, 43, 47, 48). The ubiquity of these bacteria relies in part on their ability to adhere to a wide variety of surfaces via the key virulence factor type IV pili (T4P) (12, 21, 25, 26, 44). T4P are strong and flexible proteinaceous surface filaments expressed by a wide range of bacteria and are involved in twitching motility, adherence, competence, and bacteriophage adsorption (8, 26, 42, 60). The T4P of *P. aeruginosa* are composed primarily of monomeric subunits of pilin encoded by the *pilA* gene (19, 38, 55). We previously identified within the *P. aeruginosa* species five PilA variants (designated groups I through V) which differ in amino acid sequence and length, in their association with specific accessory genes, and in the presence or absence of posttranslational modifications (PTMs) (34).

Glycosylation is now recognized as a common PTM of surface-associated proteins of mucosal pathogens (57). Recent studies have demonstrated roles for this modification in adhe-

sion, protection from proteolytic cleavage, solubility, antigenic variation, and protective immunity (21, 33, 36, 40, 41, 58). Among the best-studied examples of glycosylated bacterial proteins are the T4P of *Neisseria gonorrhoeae*, *Neisseria meningitidis*, and *P. aeruginosa* (reviewed in references 5, 45, and 57). Pilins of *N. meningitidis* are O glycosylated at Ser63 with Gal ( $\beta$ 1-4) Gal ( $\alpha$ 1-3) 2,4-diacetamido-2,4,6-trideoxyhexose (DATDH) (53), and genetic variation in the pilin glycosylation locus can result in modification with 2-acetamido-4-glyceramido-2,4,6-trideoxyhexose (10). Recent data indicate that *N. gonorrhoeae* pilins can be modified at Ser63 with a related glycan, HexNAcDATDH (2, 27). The pilin glycan does not appear to influence pilus-mediated adhesion, but in the case of *N. meningitidis*, plays a role in production of truncated soluble S-pilins (40, 41).

To date, two different pilin glycosylation systems have been identified in *P. aeruginosa*. The first is the well-described system of strain 1244 (group I T4P), in which the oligosaccharide transferase TfpO (also called PilO) attaches an O-antigen subunit of the host lipopolysaccharide to the C-terminal Ser148 of PilA from strain 1244 (PilA<sub>1244</sub>). This site of modification is immediately adjacent to the C-terminal disulfide-bonded loop implicated as the adhesin (15, 62). *tfpO* mutants produce functional, nonglycosylated pili with increased hydrophobicity compared to those of the wild type (49). The mutants exhibit reduced twitching motility and are less virulent than the wild-

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<sup>∇</sup> Published ahead of print on 19 September 2008.

type strain in competitive infection assays, suggesting that the glycan may provide a colonization advantage. The glycan may contribute to T4P adhesiveness or may serve a protective role, shielding the disulfide-bonded loop from proteolytic damage, thus enhancing colonization and virulence over the nonglycosylated mutant strains (15, 49). The pilin glycan may also provide additional antigenic diversity, allowing glycosylated strains to evade immune responses that target nonmodified T4P or to alter their interaction with the immune system.

We recently identified a second pilin glycosylation system in *P. aeruginosa* strain Pa5196 (group IV) (59). Initial studies of strain Pa5196 showed that its pilins were modified at multiple positions with homopolymers of  $\alpha$ -1,5-linked D-arabinofuranose ( $\alpha$ -1,5-D-Araf) (59); this rare sugar configuration is typically found in the lipoarabinomannan (LAM) and arabinogalactan components of the cell walls of mycobacteria. Initial analysis of the glycosylation pattern of PilA<sub>5196</sub> indicated that the sites of modification were likely to be on predicted loops in the antiparallel beta sheet region of the subunits, more similar to pilins of *Neisseria* than to those of *P. aeruginosa* 1244.

In *P. aeruginosa* strain 1244 and other group I strains, the pilin glycosyltransferase TfpO is encoded immediately downstream of *pilA* (9, 20). Strain Pa5196 contains two novel open reading frames, *tfpW* and *tfpX*, in the corresponding locus (34). Based on the location of *tfpW* in the pilin operon and the predicted membrane association and topology of TfpW, which resembles that of TfpO despite the lack of sequence identity between the two proteins, we hypothesized that it may be the arabinosyltransferase responsible for attaching the D-Araf to the pilin subunits.

The present study used a multifaceted approach to specifically map the glycosylation sites within PilA<sub>5196</sub>, using alanine replacement mutagenesis of candidate residues, as well as electron transfer dissociation mass spectrometry (ETD-MS) of tryptic glycopeptides. ETD-MS involves a novel method of peptide fragmentation that cleaves the bond between the amide nitrogen and the alpha carbon of the peptide, while delicate posttranslational modifications, such as O-linked phosphorylation and glycosylation, remain unaltered (16, 29, 56, 63). To our knowledge, this is the first report of this technique being used in the characterization of bacterial glycoproteins and demonstrates that it is an excellent tool for bottom-up studies of complex modified proteins. Finally, we present evidence that TfpW is a glycosyltransferase C family member involved in posttranslational glycosylation of the Pa5196 pilin and propose a model for this novel bacterial protein modification system.

#### MATERIALS AND METHODS

**Bacterial strains and growth conditions.** Bacterial strains used in this work are summarized in Table 1. *Escherichia coli* and *P. aeruginosa* strains were maintained as glycerol stocks at  $-80^{\circ}\text{C}$  and grown on Luria-Bertani (LB) agar (Difco) under standard conditions. For *E. coli* strains in LB, 100  $\mu\text{g/ml}$  ampicillin and 15  $\mu\text{g/ml}$  gentamicin (Gm) were used; for *P. aeruginosa* strains in LB, 50  $\mu\text{g/ml}$  gentamicin, 200  $\mu\text{g/ml}$  carbenicillin (Carb), and 5% sucrose were used. *P. aeruginosa* strains complemented with genes cloned into the pBADGr vector were grown on LB agar supplemented with gentamicin and 0.01% to 0.2% L-arabinose (BioShop). *Mycobacterium smegmatis* strain mc<sup>2</sup>155 was grown, and lysates were prepared as described previously (59).

**Sequence and structural analyses of PilA and TfpW.** DNA sequence information was obtained through direct sequencing, from the J. Craig Venter Institute (www.tigr.org) and NCBI (www.ncbi.nlm.nih.gov). Homology searches were per-

formed with BLASTP and PSI-BLAST (4). Alignments of amino acid sequences were performed by ClustalW (11). Secondary structure predictions for PilA were performed by PHYRE version 2.0 (<http://www.sbg.bio.ic.ac.uk/phyre/>). TfpW transmembrane topology and structural predictions were performed using Transmembrane Hidden Markov Model (TMHMM v.2.0; <http://www.cbs.dtu.dk/services/TMHMM-2.0/>), and TMRPres2D (51). Domain searches of TfpW were done using Pfam 22.0 (<http://pfam.sanger.ac.uk/>) (41). TfpW was classified within glycosyltransferase families using the CAZy database (carbohydrate-active enzymes) ([http://www.cazy.org/fam/acc\\_GT.html](http://www.cazy.org/fam/acc_GT.html)) (17).

**Generation of *pilA* and *tfpW* mutants.** For creation of the *pilA* mutant, a 2,052-bp PCR product, encoding *pilA* and flanking DNA, was amplified from *P. aeruginosa* Pa5196 genomic DNA using the primers 5196B-int-rev3-XbaI and 5196W-int-rev3-HindIII (Table 2) and gel purified (Qiagen). The PCR product was cloned into the XbaI and HindIII sites of the *P. aeruginosa* suicide vector pEX18AP (28), creating pEX18AP+5196BAW, and transformed into *E. coli* DH5 $\alpha$ . A SphI restriction site was introduced into *pilA* of pEX18AP+5196BAW via site-directed mutagenesis (below) using primers 5196A-SDM-SphI\_F and 5196A-SDM-SphI\_R, creating pEX18AP+5196BAW-SphI. A gentamicin resistance cassette flanked by FLP recombination target (FRT) sites was released from pPS856 (28) by digestion with SphI and cloned into the SphI site of pEX18AP+5196BAW-SphI, creating pEX18AP+5196BAW-GmFRT. This construct was electroporated into strain Pa5196 (13), and recombinant strains were selected on LB containing 50  $\mu\text{g/ml}$  Gm. The resulting gentamicin-resistant *pilA* knockout was transformed with pFLP2 (28) to excise the gentamicin marker and plated on LB containing 200  $\mu\text{g/ml}$  Carb. The resulting carbenicillin-resistant, gentamicin-sensitive transformants were cured of pFLP2 by plating on LB plus 5% sucrose, resulting in the unmarked *pilA* mutant 5196NP.

For creation of a *tfpW* mutant, a 2,541-bp PCR product encompassing *pilA* and *tfpW* was amplified from chromosomal DNA from *P. aeruginosa* Pa5196 using the primers 5196pilAup-EcoRI and tfpWdown-HindIII and subsequently cloned into the EcoRI and HindIII sites of pEX18AP, creating pEX18AP-5196AW. A gentamicin-marked transposon was generated in the EZ::Tn5 transposon construction vector pMOD-2 (Epicentre). The gentamicin resistance gene flanked by FRT sites was released from pPS856 (28) using SmaI and cloned into the SacI site within the multiple cloning site of pMOD-2, resulting in pMODEZ::Tn5\_GmFRT. The transposon was released from the vector via digestion with PvuII and gel purified. In vitro mutagenesis of pEX18AP-5196AW with the transposon was performed per the manufacturer's directions. Gentamicin-resistant clones were screened via PCR with the primers 5196tfpWupEZTn and 5196tfpWdown-HindIII, as well as Gm<sub>up</sub> and Gm<sub>down</sub>, to determine the location and orientation of the transposon within *tfpW*. Clone 48, in which the transposon had inserted in the forward orientation and within the first third of the gene, was selected for use as the knockout construct. This construct was electroporated into strain Pa5196, and recombinant strains were selected on LB containing 50  $\mu\text{g/ml}$  Gm. Insertion of the transposon into *tfpW* was verified by PCR of the gentamicin-resistant clones. The gentamicin resistance cassette was removed through the same procedure as described above, resulting in the unmarked *tfpW* insertion mutant *tfpW*::FRT.

**Complementation of mutants.** Mutants were complemented in *trans* by expression of genes cloned into a modified pMLBAD vector (35), pBADGr in which a gentamicin resistance cassette (*aacC1*) had been inserted into the trimethoprim resistance gene *dhfrII*. Pilin variants representing each group, with and without their unique downstream genes, were amplified using the primers listed in Table 2 and cloned into the EcoRI and HindIII sites of the multiple cloning site of pBADGr. The strains used were MPAO1 (group II), 1244 (group I), PA14 (group III), Pa5196 (group IV), and Pa281457 (group V). To create the *pilA*-*tfpX* insert, gene splicing by overlap extension PCR was used to fuse these genes (30). Briefly, in two separate reaction mixtures using *P. aeruginosa* Pa5196 genomic DNA as a template, *pilA* was amplified with a *tfpX* extension (using primers 5196pilAup-EcoRI and tfpXbeg+pilAend<sub>down</sub>), and *tfpX* was amplified with an upstream *pilA* extension (using primers pilAend+tfpXbeg<sub>up</sub> and tRNA<sup>Thr</sup>-down-HindIII). These PCR products were combined in a third PCR with no primers for three cycles in order for the overlapping regions to ligate, and then the flanking primers (5196pilAup-EcoRI and tRNA<sup>Thr</sup>-down-HindIII) were added for an additional 27 cycles to amplify the fused product. The resulting PCR product was cloned as described above and verified by DNA sequence analysis.

**Site-directed mutagenesis.** QuikChange mutagenesis (Stratagene, Cedar Creek, TX) was used for introducing site-directed mutations via PCR, with modifications described by Wang and Malcolm (61). The site-directed mutants (SDMs) generated to map the sites of glycosylation of PilA were created in the pBADGr+5196pilA plasmid. The primers used to generate SDMs are listed in Table 2. To determine whether the Asp43-Asp44 residues of TfpW were required for arabinosylation of pilins, PCR using mutagenic primers containing

TABLE 1. Bacterial strains and constructs used in this work

Strain or plasmid	Relevant characteristic(s)	Source or reference
<i>E. coli</i>		
XL10-Gold	Tet <sup>r</sup> Δ( <i>mcrA</i> )183 Δ( <i>mcrCB-hsdSMR-mrr</i> )173 <i>endA1 supE44 thi-1 recA1 gyrA96 relA1 lac</i> Hte [F' <i>proAB lacI<sup>q</sup>ZΔM15 Tn10</i> (Tet <sup>r</sup> ) Amy Cam <sup>r</sup> ]	Stratagene
DH5α	F <sup>-</sup> φ80 <i>dlacZM15</i> ( <i>lacZYA-argF</i> )U169 <i>deoR recA1 endA1 hsdR17</i> (r <sub>K</sub> <sup>-</sup> m <sub>K</sub> <sup>+</sup> ) <i>phoA supE44 thi-1 gyrA96 relA1 λ<sup>-</sup></i>	Lab stocks
TOP10	F <sup>-</sup> <i>mcrA</i> Δ( <i>mrr-hsdRMS-mcrBC</i> ) φ80 <i>lacZΔM15 ΔlacX74 recA1 araΔ139 Δ(ara-leu)</i> 7697 <i>galU galK rpsL</i> (Str <sup>r</sup> ) <i>endA1 nupG</i>	Invitrogen
<i>M. smegmatis</i> mc <sup>2</sup> 155		
	Avirulent lab strain	J. Liu
<i>P. aeruginosa</i>		
Pa5196	Group IV T4P; rectal isolate	34
PA7	Group IV T4P; multidrug-resistant clinical isolate	P. Roy
5196 <i>pilA</i> +GentFRT	<i>pilA::aacC1FRT</i>	This study
Pa5196 <i>tfpW</i>	EZ::Tn FRT insertion in <i>tfpW</i>	This study
PAO1	Group II T4P; lab strain	J. Boyd
PAK	Group II T4P	P. Castric
1244	Group I T4P; clinical isolate	
PA14	Group III T4P; clinical isolate	
Pa281457	Group V T4P; clinical isolate	34
Plasmids		
pEX18AP	Ap <sup>r</sup> ; <i>oriT</i> <sup>+</sup> <i>sacB</i> <sup>+</sup>	28
pEX18AP+5196BAW	3' end of <i>pilB</i> , <i>pilA</i> , 5' end of <i>tfpW</i>	This study
pEX18AP+5196BAW-SphI	SDM of base pairs C359-T and T360-G to create SphI site in <i>pilA</i>	This study
pEX18AP+5196BAW-GentFRT	Gent-FRT insertion in SphI site of <i>pilA</i>	This study
pMOD EZ::TN 2	Transposon construction plasmid	Epicentre
pMOD EZ::TN 2_Gent-FRT	Insertion of Gent-FRT into SacI site of EZ::TN transposon	This study
pEX18AP+5196 <i>pilA</i> tfpW::EZ::TN Gent-FRT-48	EZ::TN Gent-FRT insertion in <i>tfpW</i>	This study
pBADGr	<i>ori araC-P<sub>BAD</sub></i> Gm <sup>r</sup> <i>mob</i> <sup>+</sup>	H. Harvey et al., unpublished data
pBADGr+5196 <i>pilA</i>	<i>pilA</i> <sub>5196</sub>	This study
pBADGr+5196 <i>pilA</i> - <i>tfpW</i>	<i>pilA</i> <sub>5196</sub> and <i>tfpW</i>	This study
pBADGr+5196 <i>pilA</i> - <i>tfpW</i> - <i>tfpX</i>	<i>pilA</i> <sub>5196</sub> , <i>tfpW</i> , and <i>tfpX</i>	This study
pBADGr+5196 <i>pilA</i> - <i>tfpX</i>	<i>pilA</i> <sub>5196</sub> and <i>tfpX</i>	This study
pBADGr+MPAO1 <i>pilA</i>	<i>pilA</i> <sub>MPAO1</sub>	This study
pBADGr+PAK <i>pilA</i>	<i>pilA</i> <sub>PAK</sub>	This study
pBADGr+1244 <i>pilA</i>	<i>pilA</i> <sub>1244</sub>	This study
pBADGr+1244 <i>pilA</i> - <i>pilO</i>	<i>pilA</i> <sub>1244</sub> and <i>pilO</i> / <i>tfpO</i>	This study
pBADGr+PA14 <i>pilA</i>	<i>pilA</i> <sub>PA14</sub>	This study
pBADGr+1457 <i>pilA</i>	<i>pilA</i> <sub>1457</sub>	This study
pBADGr+5196 <i>pilA</i> T64A	PilA Thr64Ala	This study
pBADGr+5196 <i>pilA</i> T66A	PilA Thr66Ala	This study
pBADGr+5196 <i>pilA</i> T64A and T66A	PilA Thr64Ala, Thr66Ala	This study
pBADGr+5196 <i>pilA</i> T64S and T66S	PilA Thr64Ser, Thr66Ser	This study
pBADGr+5196 <i>pilA</i> T75A	PilA Thr75Ala	This study
pBADGr+5196 <i>pilA</i> T86A	PilA Thr86Ala	This study
pBADGr+5196 <i>pilA</i> T97A	PilA Thr97Ala	This study
pBADGr+5196 <i>pilA</i> T99A	PilA Thr99Ala	This study
pBADGr+5196 <i>pilA</i> T101A	PilA Thr101Ala	This study
pBADGr+5196 <i>pilA</i> T97A, T99A, and T101A	PilA Thr97Ala, Thr99Ala, and Thr101Ala	This study
pBADGr+5196 <i>pilA</i> T86A, T97A, T99A, and T101A	PilA Thr86Ala, Thr97Ala, Thr99Ala, and Thr101Ala	This study
pBADGr+5196 <i>pilA</i> T64A, T66A, T97A, T99A, and T101A	PilA Thr64Ala, Thr66Ala, Thr86Ala, Thr97Ala, Thr99Ala, and Thr101Ala	This study

the desired changes (Table 2) was used to amplify two separate fragments (*pilA-tfpW* and *tfpW-tfpX*), overlapping at the site of mutation. These fragments were then used as overlapping templates in a third PCR to generate a *pilA-tfpW-tfpX* construct with the desired changes, verified by DNA sequencing. The product was cloned into pCR2.1-TOPO and subcloned into pBADGr using XbaI and ScaI, and the resulting construct (*tfpWD43AD44A*) was introduced into the *tfpW* mutant by electroporation.

**Twitching motility assays.** Twitching assays were performed as previously described (23). Briefly, single colonies were stab inoculated with a sterile 200-μl

pipette tip to the bottom of a 1% LB agar plate containing appropriate amounts of antibiotic and L-arabinose where indicated and incubated for 24 h at 37°C and then for a further 24 h at room temperature. The agar was carefully removed, and the adherent twitching zones were visualized by staining with 1% crystal violet for 10 min and then rinsing with tap water to remove unbound dye.

**Surface pilin isolation, SDS-PAGE, and Western blots.** Pili were harvested and analyzed on gels by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie blue staining as described previously (59), and where indicated, transferred to nitrocellulose for Western blot analysis

TABLE 2. Primers used in this work

Primer	Oligonucleotide sequence <sup>a</sup>
5196B-int-rev3-XbaI	5'-AATCTAGATGGTGGTTGGCGGGTCCGGAG
5196W-int-rev3-HindIII	5'-AAAAGCTTGAGAACCACCATCTGCCAGC
5196A_SDM-SphI_F	5'-AGAAGCTGACCTTCAGCATGCAAGACGGGGTTCGTCTTGGGCGTG
5196A_SDM-SphI_R	5'-ACGAACCCCGTCTTGCATGCTGAAGGTTCAGCTTCTTACCAGAAGC
5196pilAup(noSD)-EcoRI	5'-AAGAATTCCAATGAAAGCGCAAAAAGGC
5196tfpWdown (HindIII)	5'-CAGAAGCTTATACTGGAAAAAAGAAGATG
5196tfpWup (EZTN) (for <i>tfpW</i> )	5'-GTTCTGCTTGGGCGTGTGGT
Gm_up	5'-AAGCCTGTTGCGTTCGTAA
Gm_down	5'-TTCTTCCCGTATGCCAA
5196pilAup-EcoRI	5'-AAGAATTCATGAAAGCGCAAAAAGGC
5196pilAdown-HindIII	5'-AGAAGCTTAAAAAGAGACAAGCCCCGCA
tfpWdown-HindIII	5'-AGAAGCTTATACTGGAAAAAAGAAGATG
tRNAThrdown-HindIII	5'-AAAAGCTTTCGAATGAGCTGCTCTACCGACAGAGCT
tfpXbeg+pilAend_down	5'-TCACGTAACCTACCACACCTATTTTGAGCGCTATTACGGC
pilAend+tfpWbeg_up	5'-TAATAGCGCTCAAAATAGGTGTGGTGAGTTACGTGAGC
pilA <sub>II, III, V</sub> up EcoRI	5'-AGAATTCATGAAAGCTCAAAAAGGC
pilA <sub>III</sub> down HindIII	5'-AGAAGCTTTCGCACTCCTCTGCTATTC
pilA <sub>V</sub> down HindIII	5'-AGAAGCTTCTCACAACCTTCCGTCTTTT
pilA <sub>I</sub> up EcoRI	5'-AAGAATTCATGAAAGCTCAGAAGGGT
pilA <sub>I</sub> down HindIII	5'-AGAAGCTTCAAAAACAACCTCAAAAAC
5196pilA SDM T64A fwd	5'-GACTCTGGTTGCCCCAgCTGCTACTCCTGGGGCTGGTCAATTGAATGC
5196pilA SDM T64A rev	5'-GCCCCAGGAGTAGCAGcTGGGGCAACCAGAGTCGGCCAAGCG
5196pilA SDM T66A fwd	5'-GTTGCCCAACTGCTgCTCCTGGGGCTGGTCAATTGAATGC
5196pilA SDM T66A rev	5'-GACCAGCCCCAGGAGcAGCAGTGGGGCAACCAGAGTCGGCC
5196pilA SDM T64A, T66A fwd	5'-GACTCTGGTTGCCCCAgCTGCTgCTCCTGGGGCTGGTCAATTGAATGC
5196pilA SDM T64A, T66A rev	5'-GACCAGCCCCAGGAGcAGCAGcTGGGGCAACCAGAGTCGGCCAAGCG
5196pilA SDM T64/66S fwd	5'-GACTCTGGTTGCCCCAtCTGCTtCTCCTGGGGCTGGTCAATTGAATGC
5196pilA SDM T64/66S rev	5'-GACCAGCCCCAGGAGcAGCAGaTGGGGCAACCAGAGTCGGCCAAGCG
5196PilA T75A_fwd	5'-GGTCAATTGAATGCAGcCTCTGGTTGGCAAGTATTGAGC
5196PilA T75A_rev	5'-GCCAACCAGAGcTGCATTCAATTGACCAGCCCCAGG
5196pilA SDM T86A fwd	5'-CGACGCTAGATAGCgCCATTGCCAGCGGCTATCCTAACC
5196pilA SDM T86A rev	5'-GATAGCCGCTGGCAATGGcGCTATCTACGCTCGAATACTTGCC
5196pilA SDM T97A fwd	5'-CCTAACGGTACAGATGcCTGTCCACCTACTCAAGGTAAGGC
5196pilA SDM T97A rev	5'-GAGTCATGGTGACAGcGATCTGACCGTTAGGATAGCCGCTGGC
5196pilA SDM T99A fwd	5'-GGTCAGATCACTGTgCCATGACTCAAGGTAAGGCTTCTGG
5196pilA SDM T99A rev	5'-CTTACTTGGATGATGGcGACAGTGATCTGACCGTTAGGATAGC
5196pilA SDM T101A fwd	5'-GATCACTGACCATGgCTCAAGGTAAGGCTTCTGGTAAGAAGC
5196pilA SDM T101A rev	5'-GAAGCCTTACCTTGAAGcCATGGTGACAGTGATCTGACCG
5196pilA SDM T97A, T99A, T101A fwd	5'-CCTAACGGTACAGATGcCTGTGcCCATGgCTCAAGGTAAGGCTTCTGGT AAGAAGC
5196pilA SDM T97A, T99A, T101A rev	5'-GAAGCCTTACCTTGAAGcCATGGcGACAGcGATCTGACCGTTAGGATAG CCGC

<sup>a</sup> Nucleic acids in a restriction site (underlined), nucleic acids in a start site (italicized), nucleic acid residues altered in the SDM (lowercase), and codons (boldface) are indicated.

by using standard methods. For Western blots, polyclonal rabbit antibodies against the *P. aeruginosa* Pa5196 pilin were generated by Cedarlane Laboratories. Polyclonal antibodies against *Mycobacterium tuberculosis* LAM were obtained through the NIH, NIAID contract no. HHSN266200400091C, entitled "Tuberculosis Vaccine Testing and Research Materials," awarded to Colorado State University.

**MS analysis of intact pilin.** Pilins for mass spectrometry (MS) analysis were isolated as described previously (59) by shearing surface-expressed pili from bacteria and dissociating these structures into their component subunits. The purified pilin protein tended to form a precipitate that was impossible to resolubilize in the water-based solutions normally used for electrospray ionization MS (ESI-MS). Therefore, a nonaqueous method was developed to overcome this problem. Pilin solutions were evaporated to complete dryness on a Savant centrifugal evaporator and resuspended in concentrated formic acid (10 μl). The proteins were solubilized by the addition of hexafluoroisopropanol (90 μl). All mass spectra were acquired on a Q-TOF2 hybrid quadrupole time-of-flight mass spectrometer (Waters). The nonaqueous pilin solutions were infused into the nanoelectrospray interface at 1 μl/minute, and spectra were recorded in the *m/z* range from 800 to 2,400 (one acquisition per second). Protein molecular weight profiles were derived from the spectra using MaxEnt (Waters).

**Enzymatic digestion and liquid chromatography MS (LC-MS) analysis of the PilA protein from strain 5196NP plus PilA.** The PilA protein from a pilin-less background strain of *P. aeruginosa* Pa5196 (5196NP) (NP for no pili) was re-

duced, alkylated, and tryptically digested according to standard protocols. In summary, approximately 200 μl of 1 μg/μl of PilA was dissolved in 1 M Tris HCl and 6 M guanidine HCl (pH 7.5) containing 2 mM dithiothreitol and incubated for 1 h at 50°C. The reduced cysteines were converted to carboxyamidomethyl derivatives using 10-fold excess of iodoacetamide over dithiothreitol. The protein solution was desalted by concentrating twice on a 3,000 MWL Centricon centrifugal filter. The protein was then diluted to approximately 1 μg/μl with 50 mM ammonium bicarbonate after each concentration step. Trypsin (10 μg) was added to the solution, which was then incubated overnight at 37°C.

The PilA digestion products were analyzed and fractionated by LC-MS using a series 1100 high-performance liquid chromatography (HPLC) system (Agilent) interfaced with the Q-TOF2 mass spectrometer. Approximately 50 μg of the digestion products was injected onto a Jupiter C<sub>18</sub> column (4.6 by 250 mm; 5 μm; 300 Å) (Phenomenex, Torrance, CA). The peptides and glycopeptides were separated using the following gradient: 5% to 60% acetonitrile, 0.2% formic acid in 45 min, increasing to 95% after 50 min. The HPLC eluate (1 ml/min) was split so that 60 μl/min was directed to the electrospray interface of the Q-TOF2 mass spectrometer, while the remainder was collected in 1-min fractions. The Q-TOF2 mass spectrometer was set to acquire one spectrum per second (*m/z*, 400 to 2,000). Glycopeptides were identified by a characteristic pattern of ions separated by 132 Da, the residue mass of a single D-arabinose sugar.

**ETD-MS.** Glycopeptide-containing HPLC fractions were infused at 0.5 μl/minute into the ESI source of an LTQ XL linear ion trap (Thermo Fischer



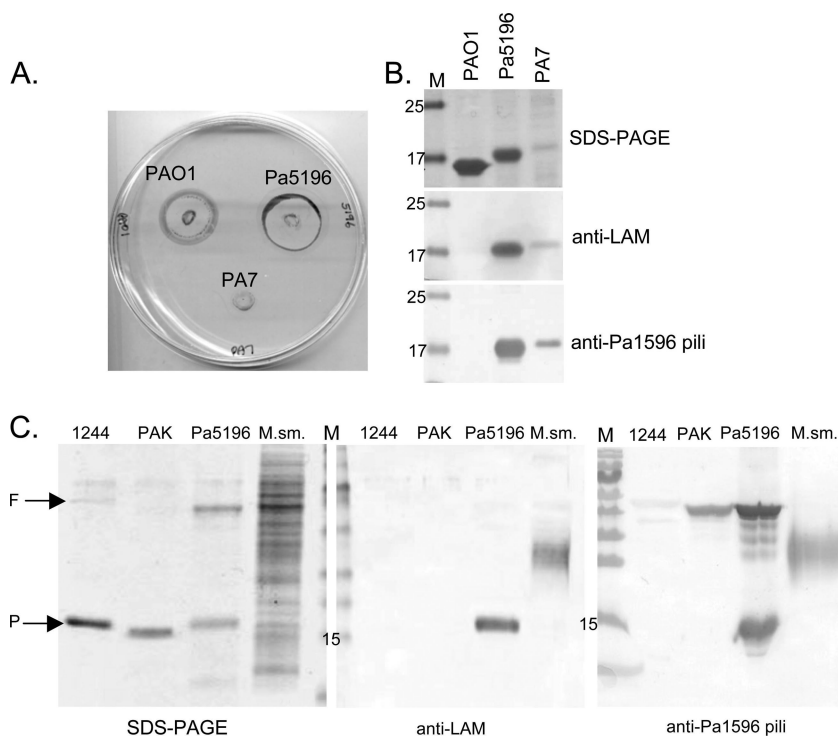


FIG. 2. Pilins from strains Pa5196 and PA7 are modified with D-Araf and elicit antibodies that cross-react with mycobacterial lipoarabinomannan. (A) Strain PA7 produces functional pili when tested for twitching motility by agar subsurface assay, but it is less motile than strain PAO1 or Pa5196; for scale, the dish is 10 cm in diameter. See strain 5196NP in Fig. 3 for an example of a nonmotile strain. (B) SDS-PAGE and Western blot analyses of sheared surface pili demonstrate that Pa5196 and PA7 produce pilins with a larger mass than (nonglycosylated) PAO1, though PA7 produces fewer pili than Pa5196, corresponding with the reduced motility seen in panel A. Both Pa5196 and PA7 pilins react with anti-LAM and anti-PilA<sub>5196</sub> antibodies, while the PAO1 pilins do not, confirming that PA7 pilins are also arabinosylated. The positions (in kilodaltons) of molecular mass markers (lane M) are shown to the left of the gel. (C) Immunization of rabbits with a sheared surface protein preparation from Pa5196 containing pilin and flagellin elicits antibodies that recognize cell wall material from *Mycobacterium smegmatis* (M.sm) of the same size range as anti-LAM sera. The flagellins of Pa5196 are not arabinosylated and do not react with anti-LAM sera. The positions of 15-kDa molecular mass markers (lane M) and of flagellin (F) and pilin (P) are shown to the left of the gel.

Residues Thr97, Thr99, and Thr101 are predicted to occur in a loop region that in *N. meningitidis* pilins is modified at Ser93 with a  $\alpha$ -glycerophosphate (52). We therefore focused our attention first on five candidate residues, Thr64 and Thr66 in the T4 peptide and Thr97, Thr99, and Thr101 in the T5 peptide. Each of these residues, individually and in combination, was changed to Ala via site-directed mutagenesis, and the mutant pilins were expressed from the pBADGr vector in a pilin-less background strain of *P. aeruginosa* Pa5196 (5196NP). To determine whether the pili were being expressed and were functional, twitching motility assays were performed for each SDM (Fig. 3). Twitching motility was moderately reduced in the Thr66Ala and Thr64/66Ala mutants, and the twitching zones appeared to contain more adherent bacteria, as indicated by more intense staining with crystal violet. A Thr64/66Ser mutant had a wild-type twitching phenotype, suggesting that Thr and Ser are interchangeable at these positions. Twitching motility was complemented to wild-type levels for all other SDMs tested, with the exception of the compound mutant Thr64/66/97/99/101Ala, where no twitching zone was observed (Fig. 3).

Sheared surface pili of the SDMs were analyzed by SDS-PAGE and Western blotting to assess the amount of pilin being produced, the mass of the protein, and the presence of

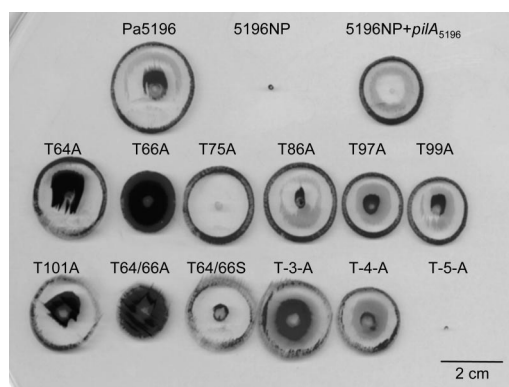


FIG. 3. Twitching motility of PilA<sub>5196</sub> site-directed mutants. All mutant pilins were able to complement twitching motility with the exception of the compound mutant T-5-A. The Thr66Ala and Thr64/Thr66Ala mutants produced slightly smaller, more intensely stained twitching zones, indicating increased adherence of cells to the plastic dish. The wild-type phenotype was restored when select Thr residues were replaced with Ser residues (Thr64/Thr66Ser). Each of the mutant pilins was expressed in the 5196NP (no pili) background from the pBADGr plasmid. T-3-A, Thr97/Thr99/Thr101Ala; T-4-A, Thr86/Thr97/Thr99/Thr101Ala; T-5-A, Thr64/Thr66/Thr97/Thr99/Thr101Ala.

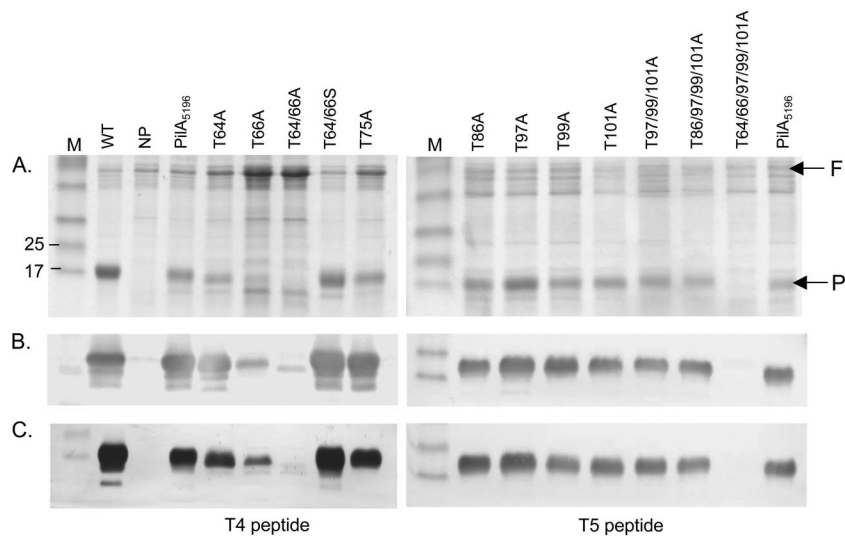


FIG. 4. SDS-PAGE and Western blots of surface T4P from site-directed mutants. (A) Coomassie blue-stained SDS-PAGE of sheared surface pilins expressed in the 5196NP (*no pili*) background. The levels of pili recovered from the Thr66Ala and Thr64/Thr66Ala mutants were reduced compared to those of other mutants. No pili could be recovered from the Thr64/Thr66/Thr97/Thr99/Thr101Ala mutant. WT, wild-type Pa5196 strain; NP, 5196NP (*pilA* mutant). The positions of molecular mass markers (lanes M) (in kilodaltons) and of flagellin (F) and pilin (P) are indicated to the left and right, respectively, of the gels. (B) Western blot with anti-PilA<sub>5196</sub> antibody. Pilins from Thr64Ala, Thr66Ala, and Thr64/Thr66Ala mutants had reduced masses compared with the strain complemented with the wild-type *pilA*<sub>5196</sub> gene. Thr66Ala and Thr64/Thr66Ala mutants also produced less surface pili than other mutants; to better visualize the pilin band, the samples are more concentrated compared with the others (as shown by the increased intensity of the flagellin band). (C) Western blot with anti-LAM antibody. All pilins reacted with the anti-LAM sera, although the Thr64/Thr66Ala mutant reacted weakly. Antibody reactivity is restored to the wild-type reactivity in the Thr64/Thr66Ser mutant.

D-Araf. Equal amounts of sample were loaded in each lane, as determined by the similar intensities of the flagellin band, which is not affected by pilin mutations. SDS-PAGE analysis demonstrated that pili were being produced by all SDMs with the exception of the Thr64/66/97/99/101Ala mutant, suggesting that the quintuple mutant protein was not stable (Fig. 4). More significant was the reduction in both the mass and amount of the pilin isolated from the Thr64Ala, Thr66Ala, and Thr64/66Ala SDMs. There was a decrease in mass of the single mutants compared to the wild-type pilin, and a further decrease in mass was evident in the Thr64/66Ala double mutant, suggesting that both Thr64 and Thr66 were glycosylated in the same molecule. These data were further supported by the weak reaction of the Thr64/66Ala double mutant pilin with the anti-LAM antibody compared with the single mutants and the wild-type protein. In addition to the reduction in anti-LAM reactivity, there was a reduction in the amount of surface pilin isolated from these strains as determined by the reduced intensity of the pilin band on the Coomassie blue-stained gel as well as the Western blot with the anti-PilA<sub>5196</sub> antibody, particularly in variants with the Thr66Ala mutation. The reduced levels of surface pilins were not due to protein instability, as there was no difference in the amount of pilin as determined by anti-PilA Western blotting of whole-cell lysates of the mutants (not shown). The SDMs of the Thr97, Thr99, and Thr101 residues within the T5 tryptic peptide showed no alterations in mass, glycosylation (as determined by Western blotting with anti-LAM antisera), or abundance compared to the wild-type protein. Therefore, the sites that are glycosylated on the second modified peptide were likely to be Ser, not Thr.

MS was used to obtain the molecular weight profile for the

Pa5196 pilin expressed in *trans* in the 5196NP background (positive control for the SDMs), as well as for the Thr64Ala, Thr66Ala, and Thr64/66Ala variants (Fig. 5). The molecular weight profile of the recombinant PilA (Fig. 5A) indicates that it was not as extensively modified with arabinose as we reported previously for the pilin isolated from the wild-type strain (59) and that there is a slightly broader distribution of glycoforms. This modest difference in PTM was most likely due to the change in stoichiometry between the pilin protein, expressed in this case from a multicopy plasmid, and the pilin modification enzyme, expressed from a single copy on the chromosome (see below). The mass profiles of the Thr64Ala and Thr66Ala mutants (Fig. 5B and C, respectively) confirmed the presence of Ala residues in each case and showed that glycosylation was reduced by approximately three or four arabinose residues compared to the wild-type PilA<sub>5196</sub> (Fig. 5A). The molecular weight profile obtained for the double mutant (Fig. 5D) clearly demonstrated loss of six to eight arabinose residues compared with the intact pilin protein, additional evidence that both of these Thr residues are glycosylated in the same protein subunit. The continued presence of arabinose residues on the Thr64/66Ala double mutant protein also confirmed that additional residues in the protein, presumably within the region corresponding to the T5 tryptic peptide, can act as sites of modification.

**ETD-MS analysis of Pa5196 pilins.** To precisely determine the distribution of arabinose sugars on Thr64 and Thr66 and to test our hypothesis that one or more Ser residues are the acceptor residues on the T5 tryptic glycopeptide, we performed ETD-MS on glycopeptides obtained by tryptic digestion of the wild-type pilin expressed in the 5196NP background. This tech-

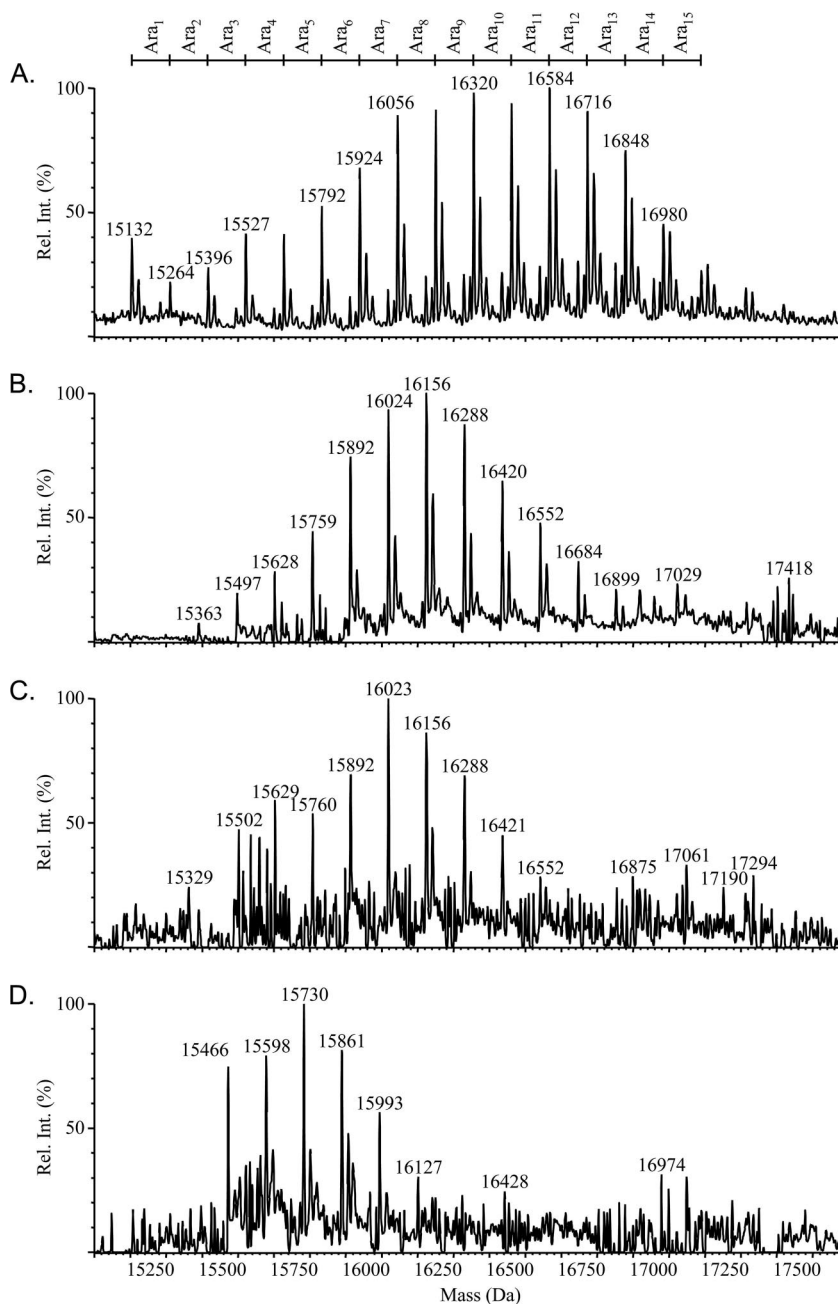


FIG. 5. Mass spectrometry of sheared intact pilins. Pili were isolated and subjected to ESI-MS as described in Materials and Methods to determine the extent of pilin glycosylation. (A) A characteristic pattern of evenly spaced peaks, each separated by 132 Da (the mass of a single D-Araf unit), is observed in the reconstructed molecular mass profile for the wild-type pilin expressed in the nonpilated 5196NP background. The y axis shows the percent relative intensity [Rel. Int. (%)]. (B to D) Mutation of potential acceptor residue Thr64 (B) or Thr66 (C) to Ala causes the loss of approximately three or four D-Araf units each, while mutation of both sites (D) causes a cumulative loss of roughly six to eight D-Araf units. In addition, the glycoform distribution is narrower in the mutants (B to D) relative to the wild type (A), indicating a degree of glycan heterogeneity at these sites in the wild type.

nique fragments the peptides without loss of delicate O-linked glycans, allowing for detailed mapping of the sites of modification. The pilin tryptic digest was fractionated by reverse-phase HPLC-MS, and glycopeptides were observed in two fractions (Fig. 6). The T3-4 glycopeptide dominated the 26- to 27-min fraction (Fig. 6A), while the T5 glycopeptide was eluted in the 21- to 22-min fraction (Fig. 6B). ETD analysis of the

principal glycoform of the T3-4 tryptic peptide, <sup>45</sup>SPLAEYGAD(K/N)AWPTLVAPTATPGAGQLNATLVGK<sup>79</sup>, revealed that both Thr64 and Thr66 are modified, with three D-Araf units each (Fig. 7A).

The ETD tandem MS (MS-MS) spectrum for the T5 peptide <sup>80</sup>YSSVDSTIASGYPNGQITVTMTQKASGK<sup>108</sup>, modified with five arabinose residues is presented in Fig. 7B. Though



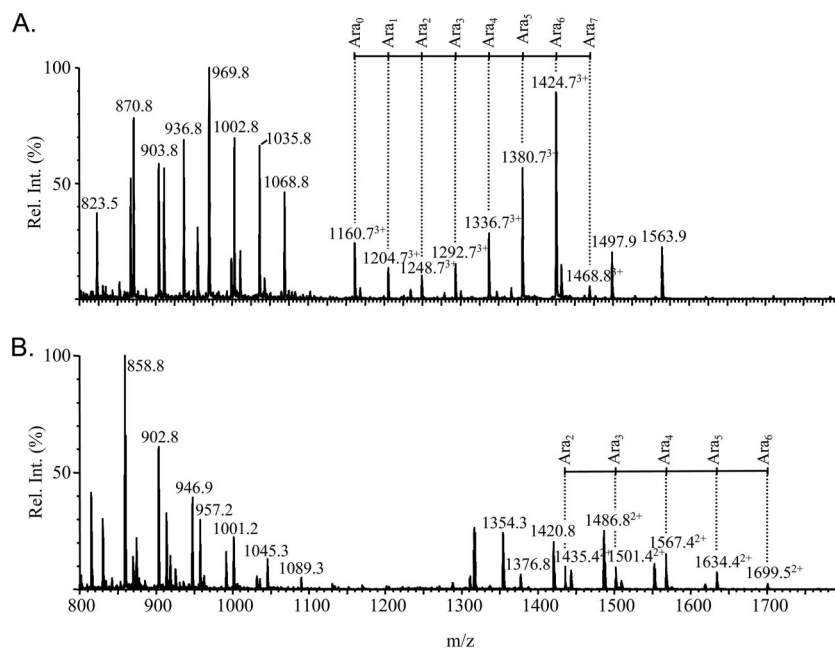


FIG. 6. LC-MS analysis of the tryptic digestion products of pilin protein isolated from *P. aeruginosa* 5196NP plus PilA<sub>5196</sub>. (A) Mass spectrum of the 26- to 27-min HPLC fraction containing the T3-4 glycopeptide. The triply protonated ions of this glycopeptide are indicated and demonstrate the heterogeneity of the glycosylation associated with this peptide. The y axis shows the percent relative intensity [Rel. Int. (%)]. (B) Mass spectrum of the 21- to 22-min fraction containing the T5 glycopeptide. The doubly protonated glycopeptide ions are indicated in this instance.

more complex to interpret, the fragmentation pattern clearly demonstrated that the sites of the D-Araf modification are the four Ser residues, not Thr, as was predicted from the SDM results. The degree of modification at each of the Ser residues is more modest than the Thr glycosylation sites on the T4 glycopeptide, with only one arabinose unit, and sometimes two arabinose units, per Ser residue.

#### Glycosylation of other pilins in the 5196NP background.

There are five known type IVa pilin alleles within *P. aeruginosa* (34) (Fig. 1), but only groups I and IV pilins have been experimentally confirmed to be glycosylated. The group I oligosaccharide transferase TfpO is flexible in terms of the glycan that it can transfer to the pilin and in its protein substrate specificity (15, 22, 31). To investigate the protein substrate specificity of the glycosylation system of strain Pa5196, each of the *P. aeruginosa* pilin variants was expressed in the 5196NP strain. Each of the pilin variants could complement twitching motility in the 5196NP strain; however, the amounts of purifiable surface pili were very low compared to those obtained upon complementation with the cognate pilin. A combination of Western blot analysis with the anti-LAM antibody and intact protein ESI-MS showed that none of the other pilins tested were modified with D-arabinose when expressed in the 5196NP background (not shown), even though all have potential acceptor residues in regions that correspond to those modified in the Pa5196 pilin (Fig. 1). Therefore, additional features of the Pa5196 pilin must be involved in its specific recognition by the group IV glycosylation system.

**Characterization of the putative arabinosyltransferase, TfpW.** Directly downstream of *pilA* in both *P. aeruginosa* Pa5196 and PA7 is a novel open reading frame we named *tfpW*, predicted to encode a 646-residue protein with a cytoplasmic N

terminus, followed by 11 transmembrane segments and a large periplasmic C-terminal region (Fig. 8). BLASTP and PSI-BLAST searches revealed only a few weak matches to the predicted product of *tfpW* (Table 3). Most are hypothetical proteins; however, some similarities of interest include hypothetical tetratricopeptide repeat-containing proteins and putative O-GlcNAc transferases.

To examine the potential role of TfpW in pilin glycosylation, a *tfpW* knockout mutant of Pa5196 was generated as outlined in Materials and Methods. The *tfpW* mutant strain retained twitching motility, although it was reduced compared to the wild type (Fig. 9). Twitching motility was restored to wild-type levels by complementation with a plasmid expressing *pilA-tfpW* or *pilA-tfpW-tfpX*, but not *pilA* alone or *pilA-tfpX*, demonstrating that the reduction in twitching motility was specifically due to the loss of *tfpW* (Fig. 9). Twitching motility was not restored to wild-type levels with the addition of a plasmid containing *tfpO*, demonstrating that *tfpO* cannot complement the *tfpW* mutation (Fig. 9).

SDS-PAGE analysis of sheared pilins from the wild-type Pa5196 strain, the *tfpW* mutant strain, and complemented strains showed that despite only a modest decrease in motility, the *tfpW* mutant strain produced substantially fewer recoverable pili with a reduced mass compared with those of the wild-type strain. The identity of the lower-molecular-mass protein isolated from the *tfpW* mutant strain was confirmed by MS-MS analysis to be the unmodified Pa5196 pilin protein (not shown). Addition of *tfpW* in *trans* restored the wild-type mass and levels of recoverable surface pili in the complemented strain (Fig. 10). A Western blot of sheared T4P with anti-LAM sera showed that pilins from the *tfpW* mutant strain were not recognized by the antibody; reactivity was restored in *tfpW*

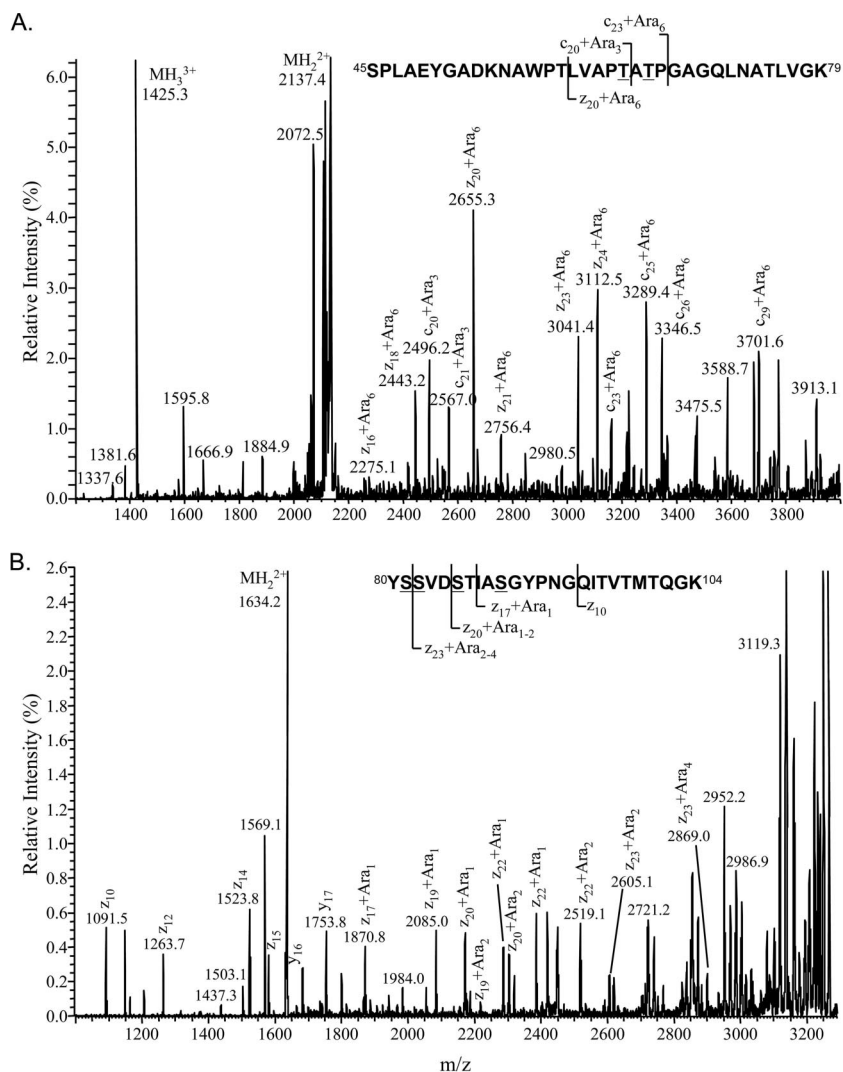


FIG. 7. ETD-MS analysis of glycosylated peptides from strain 5196NP plus *pilA*<sub>5196</sub>. (A) ETD-MS spectrum of the triply protonated ion at  $m/z$  1424.7 corresponding to the T3-4 peptide modified with six D-Araf residues. The major  $c$  and  $z$  fragment ions are indicated in the spectrum and identify Thr64 and Thr66 as the sites of O glycosylation. Furthermore, each site is modified with three D-Araf residues. (B) ETD-MS spectrum of the doubly protonated ion at  $m/z$  1634.2 corresponding to the T5 peptide modified with five D-Araf residues. This fragment ion spectrum indicates that all the serine residues in this peptide are partially modified with one or two D-Araf residues. Thr97, Thr99, and Thr101 are not glycosylated, and it is also very likely that Thr86 is not modified.

mutant strains complemented with *tfpW* in *trans* (Fig. 10A). Together, these data demonstrate that *tfpW* encodes a protein that is necessary for the addition of D-Araf to the pilins of Pa5196.

TfpW could promote pilin glycosylation by synthesizing/polymerizing the D-Araf residues, by transporting them across the inner membrane to the periplasm (flippase), by acting as a pilin glycosyltransferase, or through a combination of these activities. Because all detectable glycosylation was abolished in the *tfpW* mutant, including the addition of single sugars, TfpW is likely to be a glycosyltransferase involved in transfer of one or more residues of D-Araf to PilA. Pfam analysis showed that residues 16 to 249 of TfpW have limited similarity to the dolichyl-phosphate-mannose-protein mannosyltransferase family of O-linked glycosyltransferases, a member of the Carbohydrate-Active Enzymes (CAZy) family GT39 (17). Notably, this family belongs to the

same clan (CAZy family GT22) as the *Mycobacterium* arabinosyltransferases EmbA, -B, and -C (CAZy family GT53). All are members of the small glycosyltransferase GT-C superfamily, defined as integral membrane proteins with 8 to 13 membrane-spanning helices, a proposed active site within the loop between helices 1 and 2, and inverting enzyme activity (37). Although a number of GT-C glycosyltransferases are found in eukaryotes, they are not found in members of the domain *Archaea* and are extremely rare in prokaryotes (6, 32, 37). The proteins contain a flexible binding or catalytic DXD motif (variants include EXD, DXE, DDX, and DEX) in the N-terminal portion of the first or second extracytoplasmic loop, followed by an essential short series of hydrophobic residues (37). In the few GT-C proteins that have been characterized, mutation of either of the conserved acidic residues causes loss of glycosyltransferase activity (37). The first predicted periplasmic loop of TfpW<sub>5196</sub> (between transmem-

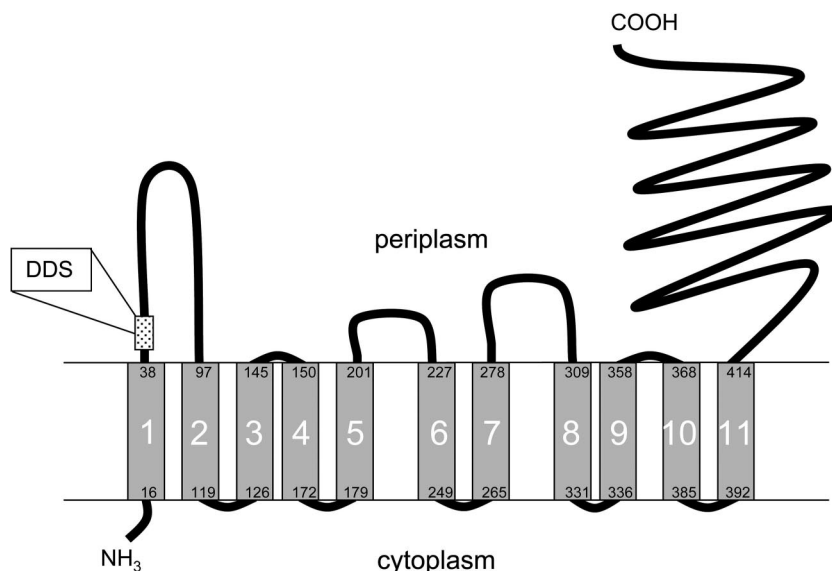


FIG. 8. Topology model of TfpW. TMHMM (<http://www.cbs.dtu.dk/services/TMHMM-2.0>) and TMRPres2D (<http://biophysics.biol.uoa.gr/TMRPres2D>) were used to predict the putative transmembrane domains and topology of TfpW (646 residues). It has a short N-terminal cytoplasmic sequence, followed by 11 predicted membrane-spanning domains (numbered gray boxes) and a large (232-residue) C-terminal periplasmic domain containing predicted tetratricopeptide domains. The small black numbers at the top and bottom of the gray boxes correspond to residue numbers. The putative GT-C DDS motif is located in the first large periplasmic loop between transmembrane domains 1 and 2 and is followed by a characteristic hydrophobic region <sup>45</sup>ILFL<sup>48</sup>.

brane helices 1 and 2; Fig. 8) contains a putative GT-C motif (<sup>42</sup>DDS<sup>44</sup>) followed by hydrophobic residues (<sup>45</sup>ILFL<sup>48</sup>); these features are conserved in the PA7 TfpW orthologue. Figure 10B shows that an Asp42/43Ala double mutant of TfpW<sub>5196</sub> was unable to complement pilin glycosylation in the *tfpW* mutant background, supporting our hypothesis that it is a glycosyltransferase of the GT-C family.

Although TfpW is necessary for pilin glycosylation, it is not sufficient, as transfer of the construct expressing *pilA*<sub>5196-*tfpW*-*tfpX*</sub> (*pilA*<sub>5196</sub> is the *pilA* gene from strain Pa5196) to a *pilA* mutant of the PAO1 laboratory strain of *P. aeruginosa* resulted in the expression of only small amounts of nonglycosylated pilins (not shown). Therefore, additional gene products specific to *P. aeruginosa* Pa5196 and related strains, such as PA7, are likely involved in biosynthesis of the glycan.

## DISCUSSION

Although the basic architecture of type IV pilins is highly conserved, considerable variation exists with respect to amino acid sequence and posttranslational modifications, even within species (9, 18, 34, 50, 54, 57). We previously demonstrated that

*P. aeruginosa* strain Pa5196 produces pili that are modified with O-linked homopolymers of  $\alpha$ -1,5-D-arabinofuranose and postulated that the novel open reading frame downstream of *pilA*<sub>5196</sub>, *tfpW*, encoded the protein involved in this modification (59).

The *P. aeruginosa* arabinans are chemically and immunologically similar to the oligosaccharides within the LAM and arabinogalactan polymers of the cell walls of mycobacteria and other members of the suborder *Corynebacterineae* as determined by nuclear magnetic resonance analysis and reaction of LAM-specific antibodies with glycosylated PilA from strain Pa5196 (PilA<sub>5196</sub>) (59). Antibodies raised against the glycosylated PilA<sub>5196</sub> reacted with cell wall material from *M. smegmatis* and with the pili from strain PA7 (Fig. 2), which has an *P. aeruginosa* Pa5196-like group IV pilin gene as well as *tfpW* and *tfpX* orthologues. We will use the publicly available genome sequence of strain PA7 to identify additional genes that may be involved in the synthesis of D-Araf precursors and any other glycosyltransferase(s) potentially involved in the progressive assembly of the glycan, since transfer of the *pilA*-*tfpW*-*tfpX* gene cassette from Pa5196 to PAO1 did not result in pilin

TABLE 3. Selected BLASTP results for TfpW<sub>5196</sub>

Organism	GenBank accession no.	Homology <sup>a</sup>	Similarity
<i>Geobacter bemidjiensis</i> Bem	ZP_01775296	Putative TPR 2 protein	31% identical, 48% positive over 190 residues
<i>Kueneria stuttgartiensis</i>	CAJ71278	Putative TPR protein	24% identical over 385 residues
<i>Desulfatibacillum alkenivorans</i>	ZP_02130537	Putative TPR protein	30% identical over 206 residues
<i>Desulfococcus oleovorans</i>	ZP_01674191	Putative O-GlcNAc transferase	25% identical over 466 residues
<i>Rhodopirellula baltica</i> SH 1	NP_870507	Putative O-GlcNAc transferase	26% identical over 183 residues

<sup>a</sup> TPR, tetratricopeptide.

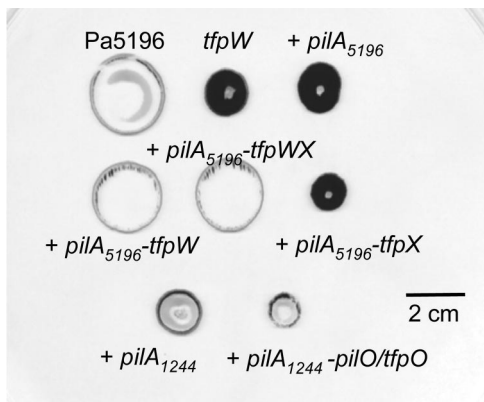


FIG. 9. TfpW influences twitching motility. A *tfpW* mutant displays an altered twitching phenotype compared to that of wild-type strain Pa5196; it produces a smaller, more densely stained twitching zone, suggesting that the mutant is more adherent to plastic than the wild type is. The wild-type twitching phenotype was restored when *tfpW* was expressed in *trans* from either pBADGr+5196pilA-*tfpW* or pBADGr+5196pilA-*tfpW*-*tfpX*, but not with constructs lacking *tfpW*. The *P. aeruginosa* 1244 oligosaccharide transferase PilO/TfpO was not able to complement the *tfpW* mutant. All recombinant strains are on the Pa5196 *tfpW* mutant background, with the exception of the wild-type control, Pa5196.

glycosylation. Preliminary inspection of the PA7 genome did not reveal open reading frames with similarity to *Corynebacterineae* arabinosyltransferase genes, suggesting a novel pathway.

Based on our previous work, PilA<sub>5196</sub> was predicted to have up to 16 arabinose units per pilin subunit (59). Two tryptic peptides encompassing the predicted αβ-loop region of the protein (T4 peptide) and the β-strands 1 and 2 of the anti-parallel beta-sheet domain (T5 peptide) were modified, though the exact sites could not be identified. In this work, SDS-PAGE and Western blot analyses of SDMs of candidate sites Thr64 and Thr66 in the T4 peptide indicated that both residues were

modified in each PilA molecule, while ETD-MS of the principal glycoform of the wild-type pilin showed that each residue is modified with a trisaccharide of D-Araf. Notably, the Thr64Ala Thr66Ala double mutant reacted only weakly with the anti-LAM antibody, despite the MS data showing that the T5 peptide is modified with up to eight sugar residues. In *N. gonorrhoeae*, pilin PTMs were shown to be interdependent; when the modification of Ser68 with phosphoethanolamine and/or phosphocholine was prevented, there was a corresponding decrease in glycosylation at Ser63 (2). In this case, however, it is more likely that the affinity of the anti-LAM antibody for mono- or disaccharides of D-Araf is weaker than for the trisaccharides, leading to reduced reactivity.

On the T5 tryptic peptide, Thr97, Thr99, and Thr101 were considered the most likely sites for modification based on their position in an accessible loop region and approximate correspondence to the Ser93 site of phosphorylation in *Neisseria* pilins (52). To our surprise, mutagenesis and ETD-MS analyses demonstrated that instead, Ser81, Ser82, Ser85, and Ser89 were modified with mono- and disaccharides of D-Araf. Ser81 and Ser82 map to the predicted loop region and the adjacent β-strand region, respectively (Fig. 1), upstream of the first β-strand, while Ser85 is located within the first predicted β-strand, followed by Ser89, which is in the loop between the first and second β-strands. These residues were not uniformly modified, and different glycoforms, likely to represent a natural heterogeneous population of glycosylated pilins, were detected. We previously reported that sheared pilins from strain Pa5196 typically run as a smear on SDS-polyacrylamide gels (34, 59), indicating a range of masses that is likely due to various levels of modification. Further studies will be necessary to understand the mechanisms that control both selection of pilin glycosylation sites and the numbers of sugars added at each position.

The Thr64Ala and Thr66Ala single and double mutants, as well as the nonglycosylated *tfpW* mutant, had fewer surface pili

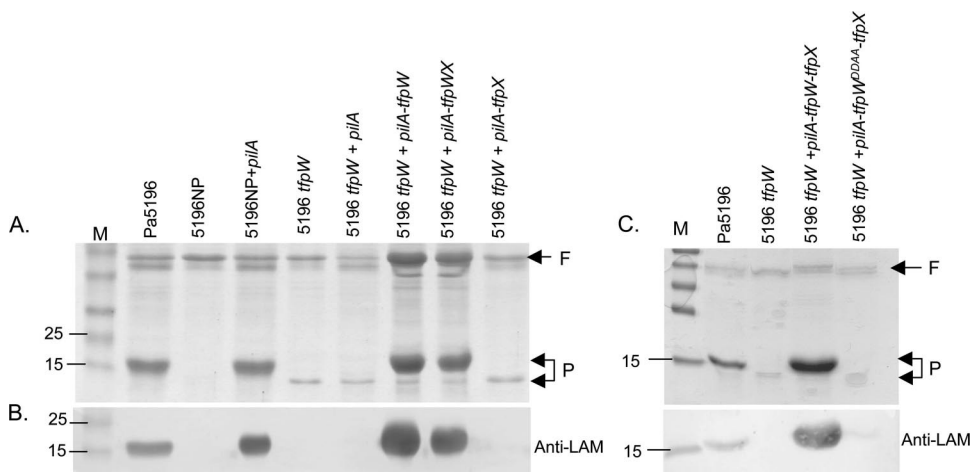


FIG. 10. TfpW is a putative GT-C family glycosyltransferase. (A) Coomassie blue-stained SDS-polyacrylamide gel of sheared surface pili from *P. aeruginosa* Pa5196 and mutant strains. The *tfpW* mutant strain produces reduced amounts of surface pili as well as pilins of reduced mass. These lower-molecular-mass bands were confirmed to be PilA<sub>5196</sub> by MS-MS (not shown). The pilins were restored to wild-type mass with the addition of *tfpW* in *trans*, but not by other genes in the pilin operon. The positions of molecular mass markers (lane M) (in kilodaltons) and of flagellin (F) and pilin (P) are shown to the left and right of the gel, respectively. (B) Western blot using anti-LAM antibody shows that the pilins from the *tfpW* mutant strain fail to react, while those strains complemented with *tfpW* in *trans* regain anti-LAM reactivity. (C) Mutation of the putative GT-C motif within TfpW results in loss of pilin glycosylation.

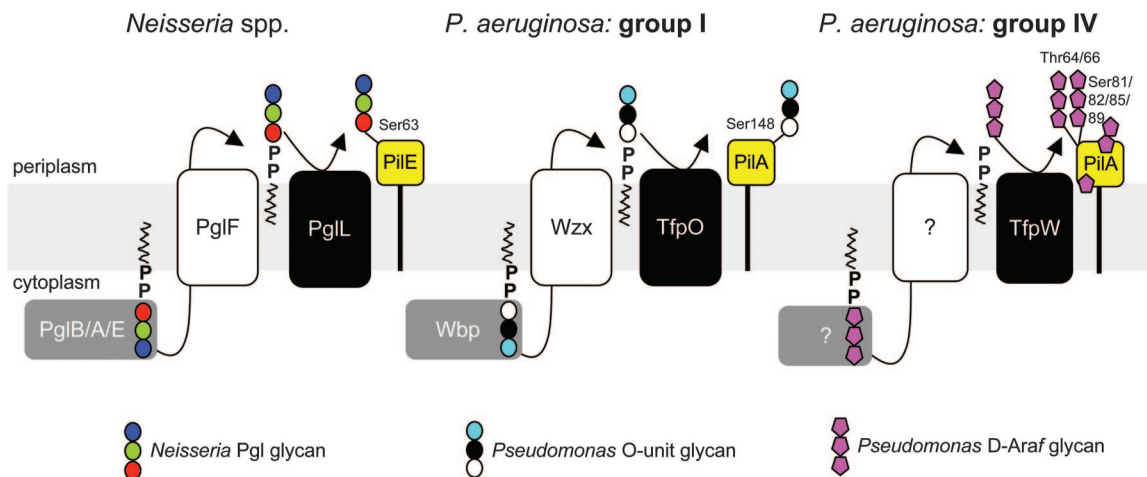


FIG. 11. Models of type IV pilin glycosylation systems. Three systems involved in the glycosylation of type IV pilins have been identified: the Pgl system of *Neisseria* spp., the TfpO system of group I strains of *P. aeruginosa*, and the TfpW system of group IV strains of *P. aeruginosa*. The first two systems attach short hetero-oligosaccharides to a single site on the pilin, while the TfpW system attaches multiple single sugars or short homo-oligosaccharides (two to four units) to seven different acceptor sites on the protein. The Pgl system transfers a heterosaccharide synthesized by dedicated glycosyltransferases, while the TfpO system transfers a lipopolysaccharide O unit corresponding to the serotype of the host strain. The TfpW system transfers D-Araf which is synthesized by an as-yet unknown pathway. P, phosphate.

and reduced motility compared with the wild type, indicating that glycosylation affects pilus assembly or extension/retraction dynamics. This was an unexpected finding, since loss of pilin glycosylation in *P. aeruginosa* 1244 or *Neisseria* does not reduce the amount of recoverable surface pili; in *N. meningitidis*, mutation of Ser63 to Ala actually increased piliation (9, 40). Since the Pa5196 pilins are not degraded in the absence of glycosylation, the D-Araf modifications may influence the pilin's structure or properties, resulting in optimal surface expression, function, or dynamics of T4P. The low levels of recoverable surface pili from the 5196NP strain expressing other *P. aeruginosa* pilin variants compared with levels recovered from their native backgrounds may be similarly due to the observed lack of glycosylation of the heterologous proteins in strain Pa5196.

Our hypothesis that TfpW is the enzyme involved in the modification of PilA with D-Araf (59) was supported by data showing that a *tfpW* mutant produced pilins with reduced mass that no longer reacted with anti-LAM antibodies. In addition to the GT-C motif described above, TfpW is predicted to have a large C-terminal periplasmic region containing tetratricopeptide repeats, a motif typically involved in protein-protein interactions (7). This region of TfpW may be involved in interactions with the pilin subunits and/or components of the pilus assembly complex. The possibility that TfpW could be involved in the biosynthesis or translocation of D-Araf to the periplasm, in addition to acting as a glycosyltransferase, cannot be ruled out at this point. However, the inability of recombinant strain PAO1 carrying the *pilA*<sub>5196</sub>-*tfpW*-*tfpX* genes to express glycosylated pilins argues against the sole involvement of TfpW in arabinose biosynthesis. TfpX does not mediate pilin glycosylation, as complementation of the *tfpW* mutant with *pilA*<sub>5196</sub>-*tfpX* did not restore modification. TfpX has approximately 50% sequence similarity with the pilin accessory proteins TfpY and TfpZ from group III and V strains of *P. aeruginosa* (34), which modulate pilus retraction dynamics (4a).

The current model for type IV pilin glycosylation (Fig. 11)

involves progressive assembly of the glycan on an undecaprenol carrier on the cytoplasmic face of the inner membrane through the action of a series of glycosyltransferases (1, 46). Once the mature glycan is assembled, a flippase translocates the undecaprenol-linked glycan to the periplasmic face of the membrane where the oligosaccharide is transferred to the mature pilin subunit (1, 46). Glycosylated pilin subunits are then shuttled to the pilus assembly complex where they are polymerized to form the fiber. In *Neisseria*, these steps are performed by a dedicated series of Pgl enzymes, while in *P. aeruginosa* 1244, a subset of the O-antigen units translocated by Wzx of the lipopolysaccharide machinery are transferred to PilA<sub>1244</sub> by TfpO (5, 15). In both cases, the glycans are defined hetero-oligosaccharides and are attached to a single acceptor site on the pilin substrate.

In contrast, *P. aeruginosa* Pa5196 expresses the prototype of a new pilin glycosylation system (Fig. 11) where the pilin glycan comprises single D-Araf residues or short homopolymers thereof, attached at multiple sites on each subunit. Gram-negative bacteria typically use cytoplasmic nucleotide sugar substrates to synthesize both hetero- and homopolysaccharides that are attached to either protein or nonprotein acceptors, while studies of the *Corynebacterineae* showed that decaprenol-linked D-ribose precursors are epimerized to D-Araf and incorporated directly into cell wall polymers without a nucleotide sugar intermediate in a process reminiscent of peptidoglycan biosynthesis.

Since no mechanism for the translocation of D-Araf in strain Pa5196 has been identified, it is possible that TfpW could act as a flippase as well as a glycosyltransferase or oligosaccharyltransferase (Fig. 11). Its predicted structure containing multiple transmembrane domains (Fig. 8) is consistent with that of known glycan flippases including Wzx homologues from various species, which have been shown to be capable of translocating one or more isoprenoid-linked sugars. Wzx proteins lack characteristic signature motifs and are in general poorly con-

served at the primary sequence level (3). Investigation of whether D-Araf residues are exposed at the periplasmic face of the inner membrane in a *tfpW* mutant will help to resolve its potential role in glycan translocation.

In summary, strains Pa5196 and PA7 constitute a unique subset of *P. aeruginosa* strains that are of biological and evolutionary interest. TfpW is the gram-negative prototype of an unusual member of the GT-C family that includes the *M. tuberculosis* Emb arabinosyltransferases. Future studies will reveal the specific mechanism by which TfpW glycosylates Pil and the pathway for biosynthesis of its unusual glycan substrate.

#### ACKNOWLEDGMENTS

We thank Ylan Nguyen and Tony Zhang for excellent technical assistance.

This work was supported by grants to L.L.B. from the Canadian Institutes of Health Research (MOP49577) and from the Advanced Food and Materials Network of Centres of Excellence (AFMnet). L.L.B. holds a CIHR New Investigator Award. J.V.K. was a University of Toronto Faculty of Dentistry Harron Scholar and held a studentship with the CIHR Strategic Training Program in Cell Signaling in Mucosal Inflammation and Pain (STP-53877).

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