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Glycosylation of *Pseudomonas aeruginosa* Strain Pa5196 Type IV Pilins with Mycobacterium-Like α-1,5-Linked D-Ara*f* Oligosaccharides▽

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Pseudomonas aeruginosa **is a gram-negative bacterium that uses polar type IV pili for adherence to various materials and for rapid colonization of surfaces via twitching motility. Within the *P. aeruginosa* species, five distinct alleles encoding variants of the structural subunit PilA varying in amino acid sequence, length, and presence of posttranslational modifications have been identified. In this work, a combination of mass spectrometry and nuclear magnetic resonance spectroscopy was used to identify a novel glycan modification on the pilins of the group IV strain Pa5196. Group IV pilins continued to be modified in a lipopolysaccharide (*wbpM*) mutant of Pa5196, showing that, unlike group I strains, the pilins of group IV are not modified with the O-antigen unit of the background strain. Instead, the pilin glycan was determined to be an unusual homo-oligomer of α-1,5-linked D-arabinofuranose (D-Ara*f*). This sugar is uncommon in prokaryotes, occurring mainly in the cell wall arabinogalactan and lipoarabinomannan (LAM) polymers of mycobacteria, including *Mycobacterium tuberculosis* and *Mycobacterium leprae*. Antibodies raised against *M. tuberculosis* LAM specifically identified the glycosylated pilins from Pa5196, confirming that the glycan is antigenically, as well as chemically, identical to those of *Mycobacterium*. *P. aeruginosa* Pa5196, a rapidly growing strain of low virulence that expresses large amounts of glycosylated type IV pilins on its surface, represents a genetically tractable model system for elucidation of alternate pathways for biosynthesis of D-Ara*f* and its polymerization into mycobacterium-like α-1,5-linked oligosaccharides.**

Pseudomonas aeruginosa is a gram-negative opportunistic pathogen that can infect immunocompromised, burned, and cystic fibrosis patients (12, 14, 40, 45) but does not typically cause disease in healthy individuals. Among the factors used by *P. aeruginosa* to colonize both living and nonliving surfaces are its polar type IV pili (T4P). T4P are long, thin, flexible, proteinaceous surface appendages required for adherence to a variety of materials and for twitching motility, a unique form of surface translocation (7, 23, 28, 41, 45, 48, 52, 59). The T4P of *P. aeruginosa* are composed of monomeric subunits (pilins) encoded by *pilA* (66) and belong to the NMePhe class of pilins (44, 48, 65). T4P are expressed by many bacteria including *Neisseria* spp., *Escherichia coli*, *Moraxella bovis*, *Dichelobacter nodosus*, *Eikenella corrodens*, *Vibrio cholerae*, *Ralstonia solanacearum*, and *Xanthomonas* spp. (33, 37, 42, 43, 48, 67, 68). The most extensively characterized T4P are those of *Neisseria gonorrhoeae*, *P. aeruginosa*, and *V. cholerae*, for which crystal structures have been determined (17, 30, 53).

In previous work, we showed that the pilins of *P. aeruginosa* could be divided into five distinct phylogenetic groups (designated I to V), based on amino acid sequence and the presence of unique accessory genes immediately downstream of *pilA* (39). The common laboratory strains PAO1 and PAK (both group II) have no accessory genes downstream of *pilA* and produce unmodified mature pilins. However, *P. aeruginosa* strain 1244 (group I) pilins have been shown to be glycosylated with its cognate serotype O7 O-antigen unit (10, 11). The PilO/TfpO glycosyltransferase involved in transfer of the O-antigen unit to the C-terminal Ser residue of PilA is encoded by the gene immediately downstream of *pilA* in group I strains (13). One of the novel pilin types identified in our previous study, from strain Pa5196 (group IV), migrated more slowly during sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) than its predicted molecular mass, suggesting that this pilin may also be posttranslationally modified (39).

Posttranslational modifications of T4P have been most widely studied in *Neisseria* (3). Pilins of *Neisseria meningitidis* and *N. gonorrhoeae* can be O glycosylated at Ser63, modified with an α-glycerophosphate at Ser93 in *N. meningitidis* and phosphorylated at Ser68 in *N. gonorrhoeae*, although this is strain dependent (3, 24, 63, 64). Recent data from the Koomey laboratory (1) showed that *N. gonorrhoeae* pilins can be both glycosylated at Ser63 and modified by phosphoethanolamine and/or phosphocholine at Ser68 and Ser156. These modifications were interdependent, as mutations affecting the occupancy of Ser68 caused corresponding decreases in glycosylation. Marceau et al. (46, 47) demonstrated that glycosylation of Ser63 facilitates the solubilization of pilin monomers but did not appear to play a role in pilus-mediated adhesion, while addition of various phosphoforms is likely to modulate host immune responses to the modified pilins (1, 31).

Group I *P. aeruginosa* pilins are glycosylated on a C-terminal Ser residue (10, 11), whereas in *Neisseria*, the glycan modifications are found on a flexible-loop region within the globular domain of the protein (3, 24, 63, 64). The pilin subunit of

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Pa5196 lacks the terminal Ser residue that is modified in 1244, and previous phylogenetic analyses demonstrated that the pilin of Pa5196 is more closely related to those of *Neisseria* than to those of other *Pseudomonas* strains (39). Therefore, we hypothesized that the Pa5196 pilin would be modified at an internal site, similar to *Neisseria* pilins. We describe here the structural characterization by mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy of the novel carbohydrate modifications on the pilin protein of *P. aeruginosa* strain Pa5196 and discuss their unexpected relevance to the mycobacterial field.

MATERIALS AND METHODS

Unless otherwise stated, all chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Bacterial strains and serotyping. *P. aeruginosa* strains were maintained as glycerol stocks at −80°C and grown and maintained on Luria-Bertani (LB) agar (Difco). Strain 1244 (10) was a gift from P. Castric, serotype O11 strain PA103 (21) was a gift from G. Pier, the International Antigenic Typing Scheme O11 strain was a gift from J. Lam, strain PAK is a laboratory strain originally obtained from J. Boyd, and strain Pa5196 was isolated from a healthy nursing home resident (39). Pa5196 was determined to be serotype O11 by slide agglutination using monoclonal antibodies (MAbs) as described previously (51) and by PCR analysis with the primer sets described by Raymond and colleagues (57). Monoclonal antibody CS-35 (36) and rabbit polyclonal antisera to *Mycobacterium tuberculosis* lipoarabinomannan (LAM) were obtained through the NIH, NIAID contract no. HHSN266200400091C, entitled "Tuberculosis Vaccine Testing and Research Materials," awarded to Colorado State University.

Generation of a *wbpM* mutant of Pa5196. Biosynthesis of the O11 O antigen requires the highly conserved dehydratase WbpM (8, 18, 21). To generate an O-antigen mutant, primers wbpM-up (5′**GAATTC**CCTGGTGTTCAACTACT GGT) and wbpM-down (5′GGG**AAGCTT**CACCGGCGGCCCCATGT) (EcoRI and HindIII restriction sites in boldface) were used to amplify an approximately 1.3-kb fragment of the *wbpM* gene from Pa5196. After digestion with EcoRI and HindIII, the amplicon was cloned into pEX18Ap, linearized with PstI, and ligated with a gentamicin resistance cassette released from pPS856 (32) with PstI. The resulting knockout gentamicin-resistant transformants were selected on LB agar containing 25 mg/liter gentamicin. The mutation was verified by PCR using the above primers. Loss of the Pa5196 O antigen was confirmed with silver-stained SDS-PAGE gel and by Western blotting using rabbit polyclonal antisera to the O11 O antigen (gift from J. Goldberg) as described previously (8, 9).

Pilin isolation. Pilin proteins were isolated using the methods of Castric (10) with modifications. Bacteria were streaked onto LB agar plates in a grid pattern and grown overnight. For SDS-PAGE one plate was used per sample, and for the glycan analysis 50 to 60 plates were used per sample. The bacteria were gently scraped from each plate using a sterile coverslip and resuspended in 2 ml sterile phosphate-buffered saline (pH 7.4) per plate, and then pili were sheared by vigorous vortexing for 1.5 min. The suspension was transferred to two 1.5-ml microcentrifuge tubes and centrifuged for 5 min at maximum speed. The supernatant was transferred to a new tube and centrifuged for an additional 25 min at maximum speed at room temperature. To precipitate the sheared proteins, 1 M $MgCl_2$ was added to the supernatant to a final concentration of 0.1 M and the samples were incubated at 4°C overnight. Samples were centrifuged at maximum speed in a microcentrifuge for 25 min at 4°C. For SDS-PAGE and glycosylation staining, pellets were resuspended in 2× SDS-PAGE loading dye (125 mM Tris, pH 6.8, 2% [wt/vol] 2-mercaptoethanol, 20% [vol/vol] glycerol, 0.001% [wt/vol] bromophenol blue, 4% [wt/vol] SDS), boiled for 5 min, and resolved on a 15% one-dimensional (1D) SDS-PAGE minigel with a prestained benchmark protein ladder (Invitrogen). The protein bands were visualized using colloidal Coomassie blue. For mass spectrometry analysis of the intact protein, the pellets from 50 to 60 plates were pooled into 2 ml of 50 mM NH_4HCO_3, pH 8.5. The suspension was dialyzed overnight using a dialysis cassette (10,000-Da cutoff; Pierce) at 4°C in a total volume of 4 liters of 50 mM NH_4HCO_3, pH 8.5.

Fluorescent glycoprotein detection. Pilins from *P. aeruginosa* strains PAK (nonglycosylated), 1244 (glycosylated), Pa5196, and the Pa5196 *wbpM* mutant were separated on 15% SDS-PAGE gels as described above with the PTM marker (Sigma) containing glycosylated and nonglycosylated protein standards. Glycosylated proteins were detected using the GlycoProfile III fluorescent-glycoprotein detection kit (Sigma) as prescribed by the manufacturer and visualized on a UV transilluminator.

Mass spectrometry analysis of the intact pilin. All mass spectra were acquired on a Q-TOF two-hybrid quadrupole time of flight mass spectrometer (Waters). The dialyzed pilin solution was diluted 1:1 with deionized water and infused at 1 μl/min into the electrospray ionization source. Protein mass spectra were recorded in the range of *m/z* 800 to 3,000. The protein molecular weight profile was generated from the spectra using MaxEnt (Waters).

Enzymatic digestion and nano-LC-MS/MS analysis of the pilin. Pilin proteins were separated from contaminating flagellins by 12% SDS-PAGE. Pilins were excised from the gels and destained using 100 mM ammonium bicarbonate in 30% acetonitrile. The destained gel pieces were washed extensively with deionized water and then dehydrated with acetonitrile. To reduce and block Cys residues, gel pieces were rehydrated by the addition of 10 mM dithiothreitol in 50 mM NH_4HCO_3, pH 8.5, incubated 1 h at 56°C, washed once with 50 mM NH_4HCO_3, and incubated 1 h in the dark at room temperature with 55 mM iodoacetamide in 50 mM NH_4HCO_3. Alkylated proteins were in-gel digested overnight at 37°C with 200 ng of trypsin (Promega) or chymotrypsin (Sigma) in 100 μl of 50 mM NH_4HCO_3. In some instances the bands were doubly digested by adding trypsin after chymotryptic digestion (same ratio) and repeating the overnight incubation. In addition, tryptic digestion was carried out on 20 μg of the original pilin extract, without the reduction/alkylation step using the same incubation conditions.

Nano-liquid chromatography-tandem mass spectrometry (nano-LC-MS/MS) analysis was performed using a CapLC nano-high-pressure liquid chromatography (HPLC) system (Waters) coupled to the Q-TOF2 mass spectrometer. Approximately 0.2 μg of each proteolytic digest was resolved on a 75-μm-inner-diameter by 150-mm Inertsil ODS 3 5-μm nano-HPLC column (Dionex/LC Packings, Sunnyvale, CA) using the following gradient conditions: 5 to 60% acetonitrile, 0.2% formic acid in 30 min; 60 to 90% in 5 min. The mass spectrometer was set for automatic data-dependent MS/MS spectrum acquisition on doubly, triply, and quadruply charged ions. All MS/MS spectra were examined manually for the presence of unusual modifications.

Fractionation of the enzymatic digests and analysis by nESI-MS. Approximately 10 μg of each protein digest was loaded on 20 μl of OligoR3 resin (Applied Biosystems) packed into a gel loader tip in the manner described by Stensballe and coworkers (62). The peptides and glycopeptides were step eluted from the resin by the sequential addition of 0 to 50% aqueous acetonitrile in increments of 5%. Each fraction was diluted 1:1 with 50% methanol and 0.2% formic acid prior to loading into a Picotip Econo12 nano-emitter tip (New Objective, Woburn, MA). The fractions were screened for glycopeptides by nanoelectrospray ionization-tandem mass spectrometry (nESI-MS/MS). The glycopeptides were further interrogated by nESI-MS/MS with front-end-collision-induced dissociation (nESI-feCID-MS/MS) as described previously (69).

Glycopeptide purification for NMR analysis. Intact pilin samples were digested overnight at 37°C with proteinase K (50:1 ratio of pilin to enzyme) in the presence of 2 mM $CaCl_2$. An additional aliquot of proteinase K was added (same ratio), and the sample was incubated at 37°C for a further 24 h. The final digest was applied in turn to a Biogel P4 and a P2 size exclusion column (Bio-Rad) as described previously (72). The eluate from the P2 column was collected in 2.5-ml fractions that were concentrated to 20 μl and analyzed by nESI-MS/MS as described above. Glycan-containing fractions were pooled and analyzed by NMR.

NMR analysis of the pilin glycan. Approximately 160 μg of column-purified pilin glycan was dissolved in 200 μl of D_2O. NMR experiments were carried out at 25°C on an INOVA spectrometer operating at 600 MHz, using a cryogenically cooled probe (Varian Associates Inc., Palo Alto, CA). Standard two-dimensional homonuclear (COSY, TOCSY, and NOESY) and ^{1}H-^{13}C heteronuclear (HSQC and HMBC) spectra were acquired as described previously (27). ^{1}H and ^{13}C chemical shifts were referenced with respect to the methyl group of an internal acetone standard, appearing at 2.225 and 31.1 ppm, respectively. NMR data were processed using the software package TOPSPIN (Bruker Biospin, Billerica, MA).

Determination of the enantiomeric form of the glycan by GC-MS. Both the pilin carbohydrate sample used for NMR analysis and 0.5 mg of D-Ara were derivatized according to the following protocol (64). The carbohydrates were lyophilized and dissolved in 200 μl of *R*-2-butanol plus 30 μl of acetyl chloride. The samples were incubated for 3 h at 100°C and the solvents evaporated under a nitrogen stream. The dried samples were treated with 200 μl of pyridine plus 200 μl of acetic anhydride for 2 h at 100°C and the solvents evaporated under a nitrogen stream. The samples were cleaned twice by addition and evaporation of toluene (0.5 ml). A second batch of D-Ara was derivatized in a similar manner using a racemic mixture of *R,S*-2-butanol instead of *R*-2-butanol. The acetylated samples were dissolved by 50 to 200 μl of methylene chloride for gas chromatography-mass spectrometry (GC-MS) analysis on a Varian 3800 gas chromatography system coupled with a Saturn 2000 ion trap mass spec-

trometer operating in electron impact ionization mode. The analysis conditions were as follows: 2- to 3-μl injection; 1/25 split; inlet temperature, 265°C; column DB 17MS, 30 m by 250 μm, 25-μm film thickness (Agilent, Palo Alto, CA); helium flow, 1 ml/min; starting oven temperature, 180°C, rising at 3.5°C/min to 260°C and then rising at 70°C/min to 280°C. The MS acquisition range was *m/z* 40 to 650.

***Mycobacterium smegmatis* whole-cell lysate preparation and Western blot analysis.** A 9-ml culture of *M. smegmatis* $mc^{2}155$ (gift from J. Liu, University of Toronto) was grown at 37°C in Middlebrook 7H9 broth (Difco) supplemented with 10% oleic acid-albumin-dextrose-catalase (Difco), 0.2% glycerol, and 0.05% Tween 80 for 4 days. The whole-cell lysate used for SDS-PAGE and Western blots was prepared using a modification of the protocol by Pheiffer et al. (54). Cells were harvested by centrifugation for 20 min at 7,000 rpm. Cell pellets were washed two times with phosphate-buffered saline–1% Tween 20 and then resuspended in 750 μl lysis buffer (0.3% [wt/vol] SDS, 5% [wt/vol] 2-mercaptoethanol) and 50 mM Tris-HCl, pH 7.0). The sample was then added to a 2-ml screw-cap tube containing lysing matrix B (0.1-mm silica beads) (BIO101, MP Biomedicals) and vortexed for 30 s. The cells were heat killed at 95°C for 20 min and then disrupted in a Ribolyser (speed, 6.0; 45 seconds; three times at 4°C) (BIO101, MP Biomedicals). Samples were centrifuged in an Eppendorf 5417C centrifuge at 14,000 rpm for 10 min, and 10× SDS-PAGE loading dye was added to a final concentration of 2×. Protein concentration was determined by Bradford assay, and 2.5 μl was used for SDS-PAGE and Western blots.

The whole-cell lysates were separated on 15% SDS-PAGE gels as described above and transferred to nitrocellulose for Western blot analysis. The CS-35 mouse monoclonal antibody (0.001 mg/μl) (raised against *M. tuberculosis* strain H37Rv) was used at 1:1,000 dilution, and the secondary goat anti-mouse AP (Bio-Rad) was used at 1:1,500. The polyclonal anti-LAM antibody (also raised against *M. tuberculosis* strain H37Rv) was used at 1:750 dilution, and the secondary goat anti-rabbit AP (Bio-Rad) was used at 1:1,500. Detection of the alkaline phosphatase antibody conjugates was performed as suggested by the manufacturer (Bio-Rad).

RESULTS

Pa5196 pilins are modified with a novel glycan that is not the O-antigen unit. Pilins from the group IV strain Pa5196 were predicted previously to be posttranslationally modified based on their migration on SDS-polyacrylamide gels (39). DiGiandomenico and colleagues showed that pilins from group I strains of *P. aeruginosa* are modified by addition of the O-antigen subunit of the background strain, regardless of serotype, to the C-terminal Ser residue of the pilin and that abrogation of O-antigen biosynthesis causes loss of pilin glycosylation (22). Strain Pa5196 was determined to be serotype O11, as described in Materials and Methods, using a combination of slide agglutination with specific antisera and PCR with serotype-specific primers. To determine whether the T4P of Pa5196 were modified with the O11 O-antigen unit, the *wbpM* gene, encoding a conserved dehydratase required for O-antigen biosynthesis (8), was disrupted. Although this mutation resulted in loss of the Pa5196 O antigen as determined by silver staining and Western blotting with anti-O11 serum (Fig. 1A), pilins purified from a *wbpM* knockout strain migrated with those of the Pa5196 wild-type strain on SDS-PAGE gel (Fig. 1B). Analysis of sheared pilins using a modified periodic acid-Schiff method revealed fluorescent bands corresponding to glycosylated pilins from group I strain 1244, group IV strain Pa5196, and the Pa5196 *wbpM* mutant, but not the unmodified pilins of group II strain PAK (Fig. 1B). The glycosylated pilins from the Pa5196 wild type are not recognized by the anti-O11 antibody (not shown), confirming that the glycan is not the O11 O unit.

Intact protein mass determination by ESI-MS. Since the pilin appeared to be modified with a novel glycan, we first determined the mass of the modified protein by ESI-MS. Initially, the protein precipitated out of solutions typically used for ESI-MS of more amenable proteins (i.e., 25 to 50% acetonitrile, 0.2% formic acid), but dilution of the pilin solution with deionized water (1:1) rendered it soluble, allowing the mass spectrum shown in Fig. 2 to be obtained. The reconstructed molecular weight profile obtained from this mass spectrum is presented in the inset of Fig. 2. Multiple peaks were observed, ranging in mass from 16,584 to 17,244 Da, larger than the expected 15,109-Da mass of the mature pilin. Furthermore, the peaks were each separated by 132 Da, the mass of a pentose sugar residue. Subtraction of the amino acid-only mass from those observed suggested that the glycan modification(s) could be composed entirely of pentoses. Top-down MS/MS analysis on some of the multiply charged ions of the pilin protein was performed as described by Schirm et al. (60). However, no carbohydrate-related fragment ions were observed, likely because pentose sugars are neutral and do not easily hold a charge during the fragmentation process.

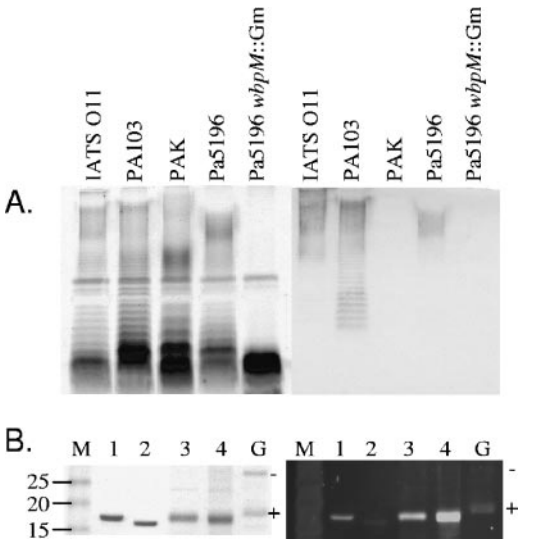

FIG. 1. Pa5196 pilins continue to be glycosylated in a *wbpM* mutant. A: Lipopolysaccharide samples were stained with silver (left) or detected via Western blotting using antibodies against serotype O11 (right). International Antigenic Typing Scheme strain O11 (IATS O11) and strain PA103 (both serotype O11) are positive controls; PAK (serotype O6) is a negative control. The *wbpM* mutant of Pa5196 lacks high-molecular-weight O antigen, as shown by silver staining and Western blotting, while the core region (at the bottom of the gel) is not affected. The band visible in all five lanes on the silver stain is residual proteinase K. B: Pilins from the indicated strains were detected with Coomassie (left) or with a fluorescent glycoprotein stain (right). M, molecular mass markers in kDa; G, glycoprotein controls; −, nonglycosylated (β-casein); +, glycosylated (RNase B). Lane 1, strain 1244 (group I, glycosylated); lane 2, PAK (group II, nonglycosylated); lane 3, Pa5196 (group IV); lane 4, Pa5196 *wbpM* mutant.

Analysis of enzymatic digests by nanoLC-MS/MS. A number of glycopeptides were detected by nanoLC-MS/MS analysis of the pilin tryptic digest, two of which are illustrated in Fig. 3. In both cases it was possible to identify the peptide sequence from the b and y peptide fragment ions in the MS/MS spectra (^{55}NAWPTLVAPTATPGAGQLNATLVGK^{79} and ^{80}YSSVD STIASGYPNGQITVTMTQGK^{104}, respectively). Interestingly, a series of ions corresponding to the sequential neutral loss of 132 Da were observed in the high-*m/z* region of both glyco-

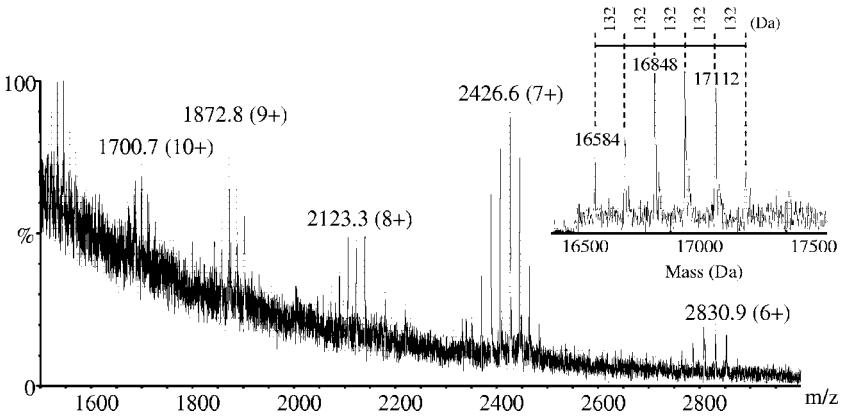

FIG. 2. Analysis of the intact Pa5196 pilin by ESI-MS. The *m/z* value and charge state have been indicated for the most abundant ion in each of the multiply charged protein peak clusters. The reconstructed protein profile is shown in the inset. The interval between any two populations of proteins is 132 Da, equivalent to the mass of a single pentose residue.

peptide MS/MS spectra (Fig. 3), mirroring the glycoform pattern observed for the intact pilin in Fig. 2. Furthermore, oxonium ions corresponding to one and two pentoses were observed at *m/z* 133 and 265, respectively (Fig. 3), suggesting that the peptides were modified with glycan polymers consisting of two or more pentoses. The difference between the observed mass of the $T^{55–79}$ glycopeptide and that of the amino acid sequence alone corresponded exactly to six pentoses (Fig. 3A). Similarly, the mass difference for the $T^{80–104}$ glycopeptide corresponded to six pentoses (Fig. 3B). In fact, MS/MS spectra were obtained for other glycoforms of these peptides corresponding to the addition of three to seven pentoses and occasionally as many as eight.

An exhaustive analysis of all the proteolytic digests (tryptic, chymotryptic, and doubly digested) by nanoLC-MS/MS detected peptides/glycopeptides covering approximately 90% of the amino acid sequence of the mature pilin protein. Peptides from the C-terminal region of the pilin protein were detected only after the disulfide-bonded Cys residues present (Cys123 and Cys147) were reduced and alkylated prior to proteolytic digestion. This finding is consistent with the structure of mature pilins from other *P. aeruginosa* strains, which have a disulfide bond near their C termini (41). In all of the digests analyzed, the only glycopeptides detected originated from the same region of the pilin covered by the two tryptic peptides shown in Fig. 3. None of the three Asn residues in the glycopeptides (N55, N73 and N93) appear within the extended prokaryotic consensus motif for N-linked glycosylation, which was recently shown to require an acidic residue in the −2 position (D/E-Y-N-X-S/T, where Y, X ≠ P) (38). One of the observed chymotryptic peptides, ^{73}NATLVGKY^{80}, overlaps the sequence of the$T^{55–79}$ tryptic glycopeptide but is itself not modified (Fig. 3C) although N73 occurs within a typical eukaryotic N-glycosylation motif (N-X-S/T). Therefore, the glycans were most likely O-linked to serine or threonine residues.

Characterization of the $T^{55–79}$ tryptic glycopeptide by nESI-feCID-MS/MS. To further identify the site of pilin modification, tryptic peptides were screened by nESI-MS and the $T^{55–79}$ glycopeptide was selected for further study by nESI-MS/MS with nESI-feCID-MS/MS. By increasing the orifice voltage from 30 V to 80 V, the glycopeptides were fragmented by feCID, and the doubly protonated fragment ion at *m/z* 1,292.8, composed of the $T^{55–79}$ peptide plus one pentose modification, was then analyzed by MS/MS (Fig. 4). A series of y fragment ions bearing the pentose modification were observed in the upper *m/z* region of this MS/MS spectrum, including Thr64 and Thr66 but not Thr59; therefore, O linkage in this peptide is at Thr64 and/or Thr66 (Fig. 4). At this time, we are unable to determine which of these two sites (or a mixture of both) is occupied. While the adjacent T^{80-104} peptide is clearly glycosylated based on the data in Fig. 3B, it proved difficult to obtain sufficient material for accurate nESI-feCID-MS/MS analysis.

Elucidation of the O-linked pilin glycopeptide structure. Approximately 160 μg of carbohydrate was isolated from 2.75 mg of pilin protein after proteinase K digestion and size exclusion chromatography. NMR characterization of the pilin glycan from *P. aeruginosa* demonstrated that it is composed of a repeating α-(1→5) unit of arabinose. A 1D ^{1}H spectrum of the glycan shows the presence of a major peak in the anomeric region at ~5.08 ppm (Fig. 5A). Anomeric protons from at least two distinct pentose units give rise to this peak (residues *a* and *b* in Table 1). The first corresponds to a terminal αAra*f* moiety (residue *a*), whereas the second corresponds to a subterminal αAra*f* unit that is glycosidically linked at the C5 position (residue *b*). A ^{1}H-^{13}C HSQC spectrum (Fig. 5B) clearly shows that the H5 protons from residue *b* are shifted downfield in the carbon dimension in comparison with those from residue *a* as a result of its participation in a glycosidic bond at C5. Through the acquisition of a ^{1}H-^{13}C HMBC spectrum, we were able to elucidate the interglycosidic linkage pattern, and we also confirmed that the glycan is O linked to the pilin peptide fragments via C1 of αAra*f*. In support of our structural characterization of the glycan, the ^{13}C chemical shift assignments for the αAra*f* units closely match values that have been previously reported for this monosaccharide (5).

There are additional resonances in the spectra of the pilin glycan, likely belonging to αAra*f* units; however, their intensity was significantly weaker in comparison with those of residues *a* and *b*. For instance, in the 1D ^{1}H spectrum (Fig. 5A), there is a minor peak at 5.13 ppm, and we were able to determine that it corresponds to the anomeric proton of a C5-linked αAra*f* moiety. The chemical shift of resonances for any given αAra*f*

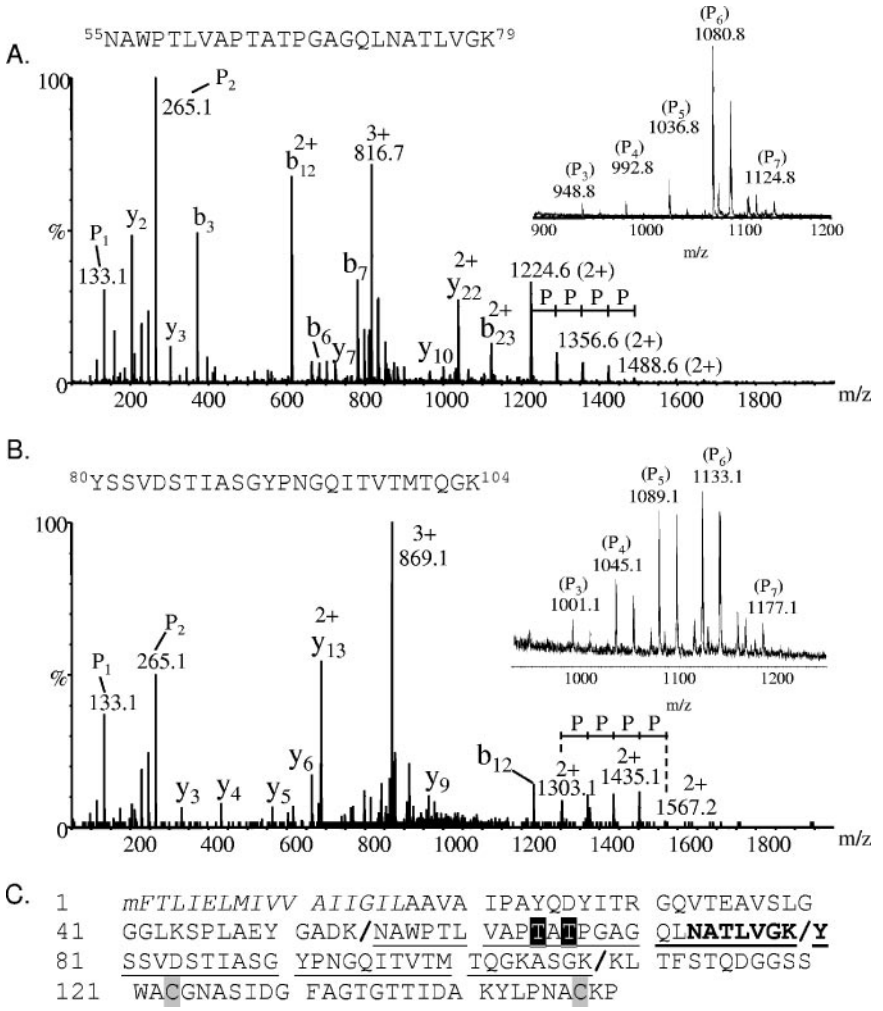

FIG. 3. Identification of pilin glycopeptides by MS/MS. A: LC-MS/MS spectrum of the triply protonated $T^{55–79}$ glycopeptide ion at *m/z* 1,080.8. The b and y fragment ions originate from fragmentation of the peptide backbone. Sequential neutral loss of pentose sugars from the doubly charged peptide ion is observed in the upper part of the MS/MS spectrum (indicated with a "P"). Oxonium ions corresponding to one and two pentoses were observed at *m/z* 133.1 and 265.1, respectively. This ion has six pentose residues and is the most abundant form of this glycopeptide. However, glycoforms ranging from three to seven pentoses were also observed, as is demonstrated in the expanded LC-MS spectrum of triply charged ions provided in the inset. B: LC-MS/MS spectrum of the triply protonated $T^{80–104}$ glycopeptide ion at *m/z* 1,133.1. This ion also contains six pentoses and is the most abundant form of this glycopeptide. MS/MS analysis was performed on many of the other glycoforms observed by LC-MS (inset shows triply charged ions). C: Map of Pa5196 pilin following trypsin and/or chymotrypsin digestion and nanoLC-MS/MS. The protein fragments identified by nanoLC-MS/MS (~90% of the mature protein) are shown in regular font, the hydrophobic N-terminal fragment that could not be detected is in italics, and the two glycosylated tryptic peptides are underlined. The unmodified chymotryptic peptide ^{73}NATLVGKY^{80} (boldface) overlaps both modified peptides. Two linked Cys residues are highlighted in gray, while the two Thr residues, which are the most likely sites of modification, are shown in reverse text. mF, methyl-Phe.

unit within an oligo-αAra*f* structure may change slightly depending on its position within the molecule, and the peak intensity will correspond to its relative population within the sample. The observation of αAra*f* units that give rise to weak peaks in the NMR spectra is reflective of the heterogeneity in the ensemble of pilin-linked oligosaccharides, which, as determined by mass-spectroscopic analysis, have variable numbers of repeating units.

Determination of the chiral orientation of the arabinose glycan. Further studies were performed to determine whether the pilin glycan was composed of the L or D enantiomer of arabinose. Comparison of the GC-MS spectra obtained with D-Ara modified with *R*-2-butanol and with racemic *R*,*S*-2-butanol allowed determination of the chromatographic differences between the two enantiomer forms. L-Ara has the opposite chromatographic behavior compared with D-Ara. Analysis of the alkylated arabinose purified from the Pa5196 pilin gave a GC-MS spectrum similar to the alkylated *R*-2-butanol-D-arabinose (data not shown), demonstrating that the pilins are modified with D-arabinose.

Antigenic identity of the Pa5196 pilin glycan with *Mycobacterium tuberculosis* lipoarabinomannan. A survey of the literature revealed that D-Ara*f* is a rare sugar in gram-negative bacteria, found in some O antigens (i.e., *Stenotrophomonas maltophilia* serotype O21) (25) and on some nodulation factors of rhizobia (49), but not in the linear α-1,5 linked

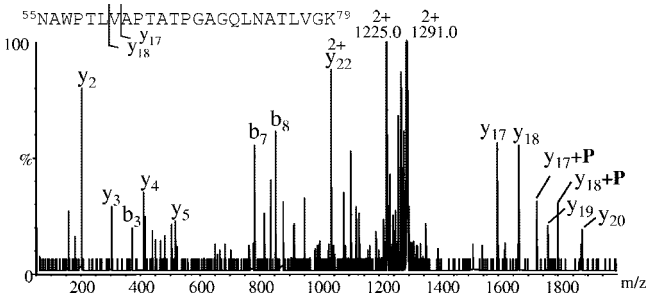

FIG. 4. feCID-MS/MS analysis of the $T^{55–79}$ glycopeptide. The glycopeptides were fragmented by front-end-collision-induced dissociation (orifice voltage = 80 V) as they entered the mass spectrometer. MS/MS analysis was performed on the doubly protonated ion at *m/z* 1,291.1 corresponding to the $T^{55–79}$ peptide plus one pentose moiety. The observation of the pentose-modified y_{17} and y_{18} fragment ions at *m/z* 1,729.5 and 1,799.5 (+P) confirmed that the site of modification is Thr64 or Thr66.

configuration. That particular arrangement is confined mainly to the unusual cell envelopes of the *Corynebacterineae*, including the important human pathogens *Mycobacterium tuberculosis*, *Mycobacterium leprae*, and *Mycobacterium avium* (19, 20). In these bacteria, linear α-1,5-linked oligomers of D-Ara*f*, as well as α-1,2- and α-1,3-linked D-Ara*f* branched structures, occur in both the arabinogalactan (AG) and LAM polymers that form a substantial portion of the cell envelope. Western blot analyses using either MAb CS-35 or a rabbit polyclonal antibody, both raised against purified LAM from *M. tuberculosis*, were used to determine whether the Pa5196 pilin glycan was antigenically, as well as chemically, identical to that found in LAM. Figure 6 shows that rabbit anti-LAM sera specifically recognized glycosylated pilins from Pa5196 but that MAb CS-35, whose epitope was shown previously to encompass a terminal β-D-Ara*f*-(1→2)-α-D-Ara*f*-(1→5)-α-D-Ara*f*-(1→5) moiety (36), did not. These data show that the pilin glycan of Pa5196 is antigenically identical to portions of *M. tuberculosis* LAM and that MAb CS-35 is not capable of recognizing the 1,5-linked linear portion of its epitope in the absence of the terminal branched residue.

TABLE 1. ^{1}H and ^{13}C chemical shift assignments for the two αAra*f* monomers that give rise to the dominant peaks in the NMR spectra of the pilin-associated glycan from *P. aeruginosa*

Residue	Atom	Type	δH (ppm)	δC (ppm)
a	1	CH	5.08	108.3
	2	CH	4.13	81.7
	3	CH	3.95	77.3
	4	CH	4.09	84.8
	5	CH_2	3.71, 3.83	62.0
b	1	CH	5.09	108.3
	2	CH	4.13	81.7
	3	CH	4.01	77.5
	4	CH	4.21	83.1
	5	CH_2	3.80, 3.88	67.6

DISCUSSION

We show here that pilins of *P. aeruginosa* group IV strain Pa5196 are not modified with O-antigen units as previously reported for group I strains, but rather with a homo-oligomer of α-1,5-linked D-Ara*f* units O linked to Thr residues. The oligomer length appears to vary from 3 to 8 units, with an average length of 6 units. To our knowledge, this is the first report of glycosylation of a bacterial protein with a homo-oligosaccharide. This modification is also novel in that it appears to be present on at least two, and possibly more, positions, as both the T4 and T5 tryptic peptides appeared to be modified. As we had insufficient quantities of the T5 peptide to analyze via nESI-feCID-MS/MS, confirmation of its modification awaits site-directed mutagenesis studies.

By examining the closest structural homologue of the Pa5196 pilin, the *N. gonorrhoeae* MS11 pilin (24), we determined that the modification(s) is predicted to be on two different flexible-loop regions in the central part of the protein. Modification of peptide T4 is predicted to be in the αβ-loop region (between the conserved N-terminal α-helix portion of the protein and the globular head domain [17]), and that of peptide T5 is on a predicted loop between the second and third β-strands. Because the total mass distribution of the protein (Fig. 2) is similar to the mass distributions of the T4 and T5 peptides individually (Fig. 3), it is unlikely that both loops are simultaneously modified in any single molecule. Instead, the

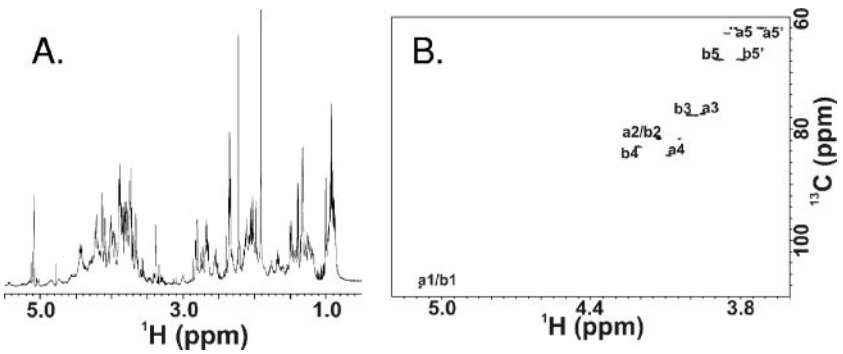

FIG. 5. 1D ^{1}H spectrum (A) and a ^{1}H-^{13}C HSQC spectrum (B) of the O-linked pilin glycan from *P. aeruginosa*. Labeled in the HSQC spectrum are the resonances from the two αAra*f* moieties that give rise to the most prominent glycan-associated peaks.

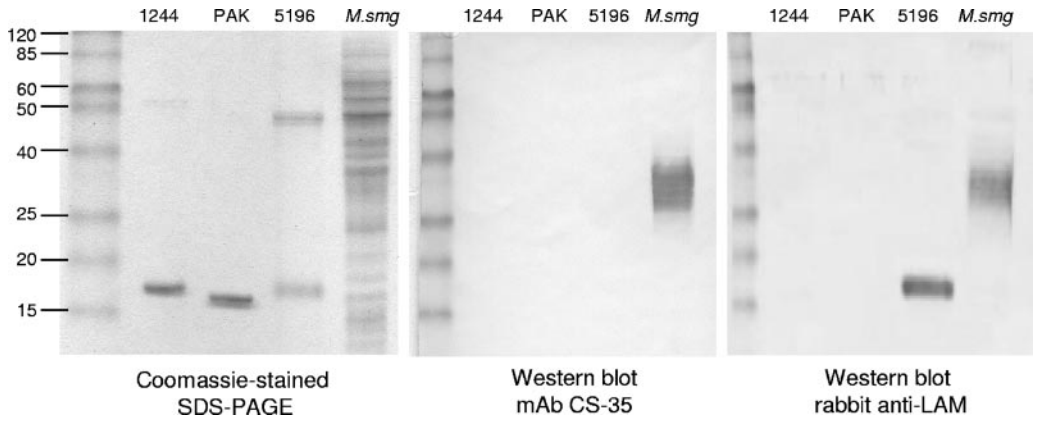

FIG. 6. Recognition of *P. aeruginosa* 5196 pilins by antibodies to lipoarabinomannan. Sheared surface preparations from *P. aeruginosa* strains 1244 (group I), PAK (group II), and 5196 (group IV) were separated on 15% SDS-PAGE gels and stained with Coomassie or transferred to nitrocellulose and probed with either MAb CS-35 or polyclonal rabbit antisera, both raised against LAM purified from *M. tuberculosis*. A whole-cell lysate of *M. smegmatis* (M.smg) was included as a positive control. Masses of the molecular weight markers (in kDa) are indicated at the left.

pilin pool is likely a mixture of proteins modified on either the T4 or T5 peptide, with 3 to 8 D-Ara*f* residues each.

The αβ-loop region (within the T4 peptide) of Pa5196 T4P contains three residues that are potential sites for O-linked glycosylation: Thr59, Thr64, and Thr66. A fourth Thr residue within this peptide, Thr75, was eliminated as a potential site of glycosylation as an overlapping chymotryptic peptide (residues 73 to 80) was shown to be unmodified. The nESI-feCID-MS/MS experiment pointed to Thr64 or Thr66 (or both) as the site of glycosylation, ruling out Thr59 as a modification site. The αβ-loop region of T4P is involved in pilin-pilin subunit interaction in the pilus fiber and is partially exposed on the pilus surface (15–17, 24, 53). The same region is modified in *Neisseria* pilins, where Ser63 can be glycosylated and Ser68 phosphorylated (24, 46, 53). Marceau et al. (46, 47) showed that glycosylation of Ser63 facilitates the solubilization of pilin monomers but does not play a role in pilus-mediated adhesion.

It has been suggested that, in *P. aeruginosa* strain 1244, glycosylation of the C-terminal Ser residue stabilizes the adhesive C-terminal disulfide-bonded loop (DSL), to protect it against proteolytic cleavage or against antibody recognition. Loss of this modification is associated with reduced virulence in a mouse model of infection (34). Recent reconstructions of a type IV pilus fiber, based on cryo-electron microscopy data (16), show that the αβ-loop region interacts with the D region (the C-terminal DSL), and in the case of *N. gonorrhoeae*, the glycan at Ser63 may interact with the DSL (15). Similarly, modification of the T4 peptide would position the Pa5196 pilin glycan to play a protective role for the DSL.

Both of the Thr residues that are potential sites of glycosylation in the T4 peptide are adjacent to Pro residues (Pro63 and Pro67). Although there is presently no known protein consensus sequence for O-linked glycosylation (except for the presence of Thr or Ser), in glycosylated cellulase complexes from *Clostridium thermocellum* and *Bacteroides cellulosolvens*, carbohydrate chains were located in regions rich in Thr and Pro residues (26). The second potential site of glycosylation of the Pa5196 T4P is in the loop connecting β2 and β3 strands of the globular head (peptide T5). This region is also predicted to be on the surface of the pilus fiber (15) and has several Thr and Ser residues (Thr86, Ser89, Thr97, Thr99, Thr101, and Ser106) that could potentially be decorated, although there is only one Pro (Pro92). Unfortunately, we were not able to purify enough T5 material for nESI-feCID-MS/MS to identify which of these residues is modified. In *N. meningitidis* pilins, this region is modified with an α-glycerophosphate, covalently bound to Ser93 (63). Understanding the potential functional and clinical significance of the glycan modifications in Pa5196 will require identification of both the specific sites of pilin modification and the enzymes involved in synthesis of the glycan and its ligation to the pilin protein.

Although the ability of gram-negative bacteria to produce arabinose-containing intermediates, in particular D-arabinose-5-phosphate (epimerized from D-ribose-5-phosphate), for lipid A biosynthesis (58) and 4-amino-4-deoxy-L-arabinose via the *pmr* pathway for lipopolysaccharide biosynthesis and modification (29) is well established, bacterial biosynthetic pathways for D-Ara*f* are not. D-Ara*f* is a major and essential component of mycobacterial cell walls, found both in AG and in LAM. The enzymes of the pathways for AG and LAM biosynthesis are important therapeutic targets, as demonstrated by the clinical utility of the antituberculosis (TB) drug ethambutol, which inhibits specific arabinosyltransferases (2). Despite the significance of D-Ara*f*-containing polymers as anti-TB drug targets, identification of the pathways for their synthesis in mycobacteria has proven to be a challenging task (50), in part due to the difficulties of working with these slow-growing and pathogenic organisms and the requirement for laborious extraction and analysis of complex cell wall components. In the current model for the synthesis of mycobacterial D-Ara*f*-containing polysaccharides, the D-Ara*f* precursor is derived from decaprenylphosphoryl-5-phosphoribose, the first committed intermediate of the pathway (35, 50). The resulting decaprenylphosphoryl–D-Ara*f* molecule is thought to be incorporated directly into the growing AG or LAM by membrane-bound arabinosyltransferases, including those encoded by *embA*, *embB*, and *embC* (2, 4, 6, 61).

The mechanisms for D-Ara*f* biosynthesis and polymerization in *P. aeruginosa* are likely to differ from those in mycobacteria. Based on established pathways for lipopolysaccharide and cap-

sule biosynthesis (56, 70, 71), the glycan is likely to be synthesized from a nucleotide sugar precursor which is polymerized on undecaprenol phosphate through the action of cytoplasmic arabinosyltransferase(s) and subsequently translocated or flipped to the periplasm to be transferred to pilin subunits located in the inner membrane. Recent work by Power and colleagues (55) suggested that *Neisseria* pilin modification occurs in this manner, as homologues of O-antigen flippases (PglF) and ligases (PglL) essential for pilin glycosylation were identified in *N. meningitidis*.

Growth of Pa5196 in high concentrations of ethambutol (200 μg/ml) did not abrogate pilin glycosylation, nor did low-stringency PCR amplification using *embA*, *embB*, or *embC* primers yield any products (data not shown), supporting the notion that the arabinosyltransferases in this strain will be unlike those found in mycobacteria. Despite these anticipated differences, identification of the Pa5196 α-1,5-arabinosyltransferase(s) may be useful to design screens for inhibitors of that specific reaction, as the glycan synthesized by these enzymes is chemically and antigenically identical to that in *Mycobacterium*. Screening would be facilitated by the fact that *P. aeruginosa* Pa5196 pilins are surface exposed and can be easily harvested in large quantities for analysis. Unlike mycobacteria, *P. aeruginosa* is of relatively low virulence, grows rapidly under a variety of conditions, and is highly amenable to genetic manipulation. Also, as immunization of animals with glycosylated *P. aeruginosa* pilins has been shown to raise antibodies to both the protein and glycan components (13), the pilins of Pa5196 could have application as antigens to generate antisera specifically recognizing α-1,5-linked D-Ara*f* oligosaccharides.

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