

Structural Characterization of the *Pseudomonas aeruginosa* 1244 Pilin Glycan*

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An antigenic similarity between lipopolysaccharide (LPS) and glycosylated pilin of *Pseudomonas aeruginosa* 1244 was noted. We purified a glycan-containing molecule from proteolytically digested pili and showed it to be composed of three sugars and serine. This glycan competed with pure pili and LPS for reaction with an LPS-specific monoclonal antibody, which also inhibited twitching motility by *P. aeruginosa* bearing glycosylated pili. One-dimensional NMR analysis of the glycan indicated the sugars to be 5N β OHC₄7NFmPse, Xyl, and FucNAc. The complete proton assignments of these sugars as well as the serine residue were determined by COSY and TOCSY. Electrospray ionization mass spectrometry (MS) determined the mass of this molecule to be 771.5. The ROESY NMR spectrum, tandem MS/MS analysis, and methylation analysis provided information on linkage and the sequence of oligosaccharide components. These data indicated that the molecule had the following structure: α -5N β OHC₄7NFmPse-(2 \rightarrow 4)- β -Xyl-(1 \rightarrow 3)- β -FucNAc-(1 \rightarrow 3)- β -Ser.

Pseudomonas aeruginosa is an opportunistic pathogen capable of causing severe infections in individuals with compromised defense mechanisms (1). The somatic pili, protein filaments that extend as bundles from one or both of the cell poles, are considered to be a major virulence factor, promoting adherence and invasiveness (2, 3). These fibers are composed of a monomeric subunit, pilin, which has a strain-dependent molecular weight of \sim 16,000. The mature form of this protein is produced by the removal of a six-residue leader sequence, a process that is accompanied by methylation of the nascent amino-terminal phenylalanine (4). Although this had initially been considered the only post-translational modification of this protein, evidence has been generated indicating that *P. aeruginosa* strain 1244 pilin is also glycosylated (5). This process is dependent on *pilO*, a gene present as part of an operon that also contains the pilin structural gene, *pilA*.

Although archeal and eubacterial S-layers commonly contain covalently bound glycan (6–8), other examples of glycosylated prokaryotic surface proteins are rare. Cell wall-associated glycoproteins have been demonstrated in *Streptococcus sanguis*

(9) and *Mycobacterium tuberculosis* (10). Among Gram-negative bacteria, *Campylobacter* species have been shown to contain a general protein glycosylation system that modifies a number of surface proteins including flagellin (11–13). Evidence has been presented that a glycan is associated with *P. aeruginosa* flagellin (14). As with *P. aeruginosa* (5), the pili of *Neisseria meningitidis* and *Neisseria gonorrhoeae* have been shown to be glycosylated. X-ray diffraction studies indicate that the *N. gonorrhoeae* pilin glycan is a disaccharide (15). In addition to glycerol phosphate (16), *N. meningitidis* pilin contains covalently bound trisaccharide (17).

Although the detection of glycosylated bacterial surface proteins is uncommon, the comprehensive determination of their carbohydrate structure has been even more rare. Results presented in this paper provide a complete structural analysis of the *P. aeruginosa* 1244 pilin glycan, showing that it is a serine-linked trisaccharide with the following structure: α -5N β OHC₄7NFmPse-(2 \rightarrow 4)- β -Xyl-(1 \rightarrow 3)- β -FucNAc-(1 \rightarrow 3)-Ser. 5N β OHC₄7NFmPse is an infrequently occurring sugar found in certain *P. aeruginosa* O-antigens (18). All three pilin glycan sugars are repeating unit components of O7 LPS,¹ the serotype to which *P. aeruginosa* 1244 belongs (18).

EXPERIMENTAL PROCEDURES

Bacterial Strains—*P. aeruginosa* strain 1244, a smooth human blood isolate of LPS serotype O7, was originally provided by A. T. McManus, U. S. Army Institute of Surgical Research, San Antonio, and strain 1244N3 was provided by S. Lory, University of Washington, Seattle. *P. aeruginosa* strain 653A was from C. C. Brinton, University of Pittsburgh. All strains were grown aerobically on LB plates or broth cultures at 37 °C. Broth cultures were grown on a rotatory shaker at 275 rpm. Additions to the growth media were as follows: carbenicillin, 200 μ g/ml; tetracycline, 50 μ g/ml; isopropyl- β -D-thiogalactopyranoside, 5.0 mM.

Immunological Procedures—Western blotting was performed as described previously (19). The mAbs used were a gift from J. C. Sadoff, Walter Reed Army Institute of Research, Washington, D. C. mAb 1.2.48 was a hybridoma supernatant fluid, and mAbs 11.14 and 5.44 were ascites fluids. When used in Western blots or twitching inhibition tests, mAbs 11.14 and 5.44 were purified as follows. Ammonium sulfate (0.67 g) was added to 2.0 ml of ascites fluid followed by stirring for 30 min at 4 °C. This material was centrifuged at 12,000 \times g for 15 min at room temperature, and the supernatant fluid was discarded. The precipitate was dissolved in 1.0 ml of 10.0 mM Tris/HCl, pH 8.0, and dialyzed three

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¹ The abbreviations used are: LPS lipopolysaccharide; COSY, ¹H-¹H correlated NMR spectroscopy; ESI-MS, electrospray ionization mass spectrometry; FucNAc, N-acetylfucosamine; GLC-MS, gas-liquid chromatography-electron impact mass spectrometry; LB, Luria-Bertani; mAb, monoclonal antibody; MALDI, matrix-assisted laser desorption/ionization; MS/MS, tandem mass spectrometry; PMAA, partially methylated alditol acetate; 5N β OHC₄7NFmPse, 5-N- β -hydroxybutyryl-7-N-formyl-pseudamino acid; ROESY, rotating frame Overhauser effect NMR spectroscopy; TOCSY, total correlated NMR spectroscopy; Xyl, xylose.

times against one liter of the same buffer at 4 °C. These preparations were applied to a 0.5 × 5.0-cm Mono-Q column equilibrated with this same buffer and eluted with a 0.0–0.4 M NaCl gradient, again in the same buffer, over a volume of 10.0 ml. Eluted protein was monitored by absorption at 280 nm. Fractions (0.25 ml) were taken at a flow rate of 1.0 ml/min and assayed by SDS-polyacrylamide gel electrophoresis using Coomassie Brilliant Blue G-250 stain. The *P. aeruginosa* 1244 LPS standard was a gift from Dr. A. Bhattacharjee, Walter Reed Army Institute of Research. Crude *P. aeruginosa* 653A LPS was prepared using the procedure of Hitchcock and Brown (20).

Blocking enzyme-linked immunosorbent assay employed polystyrene plates coated with either 50 μl of purified native 1244 pili (20 μg/ml) or 50 μl of purified strain 1244 LPS (10 μg/ml). Plates to be coated with LPS as antigen were precoated with 50 μl of polylysine (1 mg/ml). All plates were blocked (2 h at room temperature) with 200 μl of 0.5% casein and 0.5% bovine serum albumin in phosphate-buffered saline. Antibody preparations were diluted 3 × 10⁻⁴ with blocker and incubated for 2 h at room temperature with purified pilin aminoglycan. This material (50 μl) was added to the coated plates and incubated overnight at room temperature with slow shaking. Secondary antibody (50 μl of alkaline phosphatase-labeled goat anti-mouse immunoglobulin G (heavy and light chains), 1:1000 dilution, Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) was applied for 2 h at room temperature with slow shaking. Each step was followed by four washes with phosphate-buffered saline. Plates were assayed by the addition of substrate (150 μl of 10% diethanolamine, 0.05% *p*-nitrophenylphosphate (Sigma), 0.5 mM MgCl₂, pH 9.8) followed by incubation at room temperature with shaking from 10 to 30 min. The reaction was stopped with the addition of 50 μl of 0.1 M ethylenediaminetetraacetic acid, and the A₄₀₅ was measured using a Bio-Rad model 3550 plate reader.

Twitching Motility—The motility assay described by McMichael (21) was carried out as follows. Filter-sterilized antibiotics, isopropyl-β-D-thiogalactopyranoside, or monoclonal antibody were added to 3.3 ml of melted LB agar (containing 1% agar), which was poured into a 15 × 60-mm plastic Petri dish. After solidification, the plate was dried, with lid side up, at 37 °C for 6 h. The medium was then stab-inoculated with the test organism so that the inoculating needle came in contact with the bottom of the plate. The diameter of the zone of growth that radiated from the inoculation point between the agar layer and the interior bottom of the plate was measured after an 18-h incubation at 37 °C.

Preparation of Glycosylated and Nonglycosylated Pili—Strain 1244N3, a mutant that is unable to make pilin (22), could produce nonglycosylated strain 1244 pilin when carrying pPAC24, a plasmid containing the strain 1244 *pilA* gene (which codes for pilin) but lacking the *pilO* gene required for glycosylation. This strain was able to produce glycosylated pilin when carrying pPAC46, a plasmid containing both the strain 1244 *pilA* and *pilO* genes (5). Glycosylated and nonglycosylated pili, produced by hyperexpression of the *pilA* gene of pPAC46 and pPAC24, respectively, were isolated and purified as described previously (5). For use in Western blots, glycosylated and nonglycosylated pilin were purified further to remove traces of LPS. Here, pilin samples were incubated at room temperature for 15 min in 10.0 mM Tris/HCl, 1% (w/v) β-octyl glucoside, pH 8.0, for 20 min. These proteins were next subjected to gel filtration using a 1.0 × 30.0-cm Superose-12 column equilibrated with 10.0 mM Tris/HCl and 1.0% β-octyl glucoside at pH 8.0. Fractions (0.5 ml) were collected at a flow rate of 0.5 ml/min. Glycosylated pilin from this step was applied to an 0.5 × 10.0-cm Mono-P column equilibrated with 0.025 M 2-[Bis(2-hydroxyethyl)imino]-2-(hydroxymethyl)-1,3-propanediol, adjusted to pH 6.25 with HCl, and eluted with 10.0 ml of a solution containing 10% (w/v) Polybuffer 74 (Amersham Pharmacia Biotech) and 1% (w/v) β-octyl glucoside, pH 4.0. Nonglycosylated pilin was applied to a Mono-P column equilibrated with 0.025 M triethanolamine equilibrated to pH 8.3 with acetic acid and eluted with 10.0 ml of a solution containing 0.2% (w/v) Pharmalyte 8–10.5 (Amersham Pharmacia Biotech), 9% (w/v) Polybuffer 96 (Amersham Pharmacia Biotech), 1% (w/v) β-octyl glucoside, pH 6.0. Fractions (0.5 ml) were taken at a flow rate of 1.0 ml/min. Protein elution was monitored by absorption at 280 nm. Glycosylated pilin eluted at approximately pH 4.8, whereas nonglycosylated pilin eluted at pH 6.8. These proteins were dialyzed against 10 mM Tris/HCl, pH 8.0, and stored frozen. No LPS was detected as determined by Western blot using an anti-LPS-specific serum.

Isolation of Pilin Aminoglycan—In a typical preparation, ~12.5 mg of pure glycosylated pili were suspended in 12 ml of a solution containing 5 mM Tris/HCl, 0.5 mM CaCl₂, pH 7.6. To this was added 7.2 mg each of proteinase K and Pronase followed by 40 μl of toluene to suppress microbial growth. This material was incubated at 45 °C for ~18 h, at

which time 1.8 mg each of proteinase K and Pronase were added. This was incubated for an additional 18 h at 55 °C and dried in a stream of filtered air at 45 °C. The digested material was resuspended with 1.0 ml of deionized water, and after removal of precipitate by centrifugation, it was passed through a 1.3 × 20-cm Sephadex G-25 column (calibrated with glucose and lactose) using 25 mM ammonium acetate, pH 8.5, as elution buffer. 0.5-ml fractions at a flow rate of 0.35 ml/min were taken, and protein absorbance was monitored at 280 nm. Aliquots of fractions were incubated with 3.2% orcinol in 80% sulfuric acid at 80 °C for 15 min, and the absorbance at 420 nm was measured. The single peak that appeared in the 400–800 molecular weight range was pooled and dried. Pili in the absence of protease, or protease in the absence of pilin, produced no peak after similar treatment. This dried material was dissolved in a minimal volume of deionized water, loaded onto a 20 × 20-cm Whatman PE SIL G polyester-backed silica gel plate (Fisher), and subjected to TLC using an isopropanol-ammonium hydroxide (2:1) solvent (23).

The position of the glycan-containing band was determined by excising a section of the plate and spraying it with an 0.11% orcinol in 0.3% sulfuric acid solution followed by incubation at 90 °C for 60 min. The area of the plate matching the orcinol-reactive band was scraped and the glycan eluted with deionized water. This material was dried and subjected to a second round of TLC using chloroform-methanol-water (10:10:3; (24)) as solvent. Glycan from this plate was again subjected to TLC using the isopropanol-ammonium hydroxide solvent. The purity of the material resulting from this scheme was tested by two-dimensional high performance TLC using Whatman silica gel glass-backed plates (Fisher) and employing butanol-acetic acid-water (3:1:1) and chloroform-methanol-water (10:10:3) as solvents. Development of these plates with either orcinol or ninhydrin (0.3% ninhydrin in 95% ethanol followed by 90 °C treatment for 30 min) sprays produced a single spot at identical R_f coordinates, indicating that this material was of high purity and contained both a sugar moiety and a free amino group. Approximately 450 μg of this material, as determined by spot test using serine as standard, was obtained using this procedure.

Amino Acid and Sugar Analysis—The amino acid composition of the isolated pilin aminoglycan was determined after hydrolysis in 1 M trifluoroacetic acid for 4 h at 95 °C. Hydrolysis products were subjected to high performance TLC using the isopropanol-ammonium hydroxide solvent described above with L-serine, L-threonine, and L-aspartic acid as standards and ninhydrin treatment for detection. For confirmation, an aminoglycan sample was hydrolyzed *in vacuo* in pre-pyrolyzed 10-mm tubes with 6 N HCl, 0.5% phenol, at 110 °C for 24 h. Amino acid composition of vacuum-dried hydrolysates was carried out at the University of Pittsburgh Protein Sequencing Facility using a Beckman 6300 amino acid analyzer employing post-column ninhydrin detection. The sugar composition of the isolated pilin aminoglycan was analyzed after hydrolysis in 1 M HCl for 2–6 h at 95 °C. Hydrolysis products were subjected to high performance TLC using glucose, lactose, and xylose as standards with acetone-water (9:1 (25)) as solvent and orcinol spray for detection.

Mass Spectrometry—Mass determination of glycosylated pilin was performed at the Mellon Institute Center for Molecular Analysis, Carnegie Mellon University, using a PerSeptive Biosystems Voyager STR with delayed extraction and a high *m/z* detector. Sequence and mass analysis of the pilin aminoglycan was carried out by ESI-MS using a SCIEX API-III mass analyzer operated in the positive ion mode with an orifice potential of 50 V. Spectra are the accumulation of 10–15 scans collected over the mass range of 400 to 2,000. The sample was dissolved in distilled water at a final concentration of 2 μg/μl, and the sample solution was mixed with an equal volume of ESI-MS solution (aqueous 30% methanol containing 1% hydrochloric acid) and pumped into the mass spectrometer at a rate of 3 μl/min. MS/MS was also performed on the SCIEX instrument by selecting the parent ion for collision-induced dissociation using argon as the collision gas.

Nuclear Magnetic Resonance—For NMR analysis, the dried sample was dissolved in D₂O and lyophilized. This procedure was repeated, and then the sample was dissolved in 0.5 ml of D₂O and subjected to both one- and two-dimensional NMR analyses. The spectra were acquired using a Varian 300 MHz instrument. The gradient COSY, TOCSY, and ROESY experiments were performed using the pulse sequences provided by Varian, and processed using Varian software. Chemical shifts (δ) are expressed in parts per million downfield from internal trimethylsilylpropionate with an accuracy of 0.002 ppm.

Xylose Linkage Analysis—The xylosyl linkage was determined by the preparation and GLC-MS analysis of its partially methylated alditol acetate (PMAA). The PMAAs were prepared using the Hakomori method as described by York *et al.* (26). Analysis of the PMAAs was

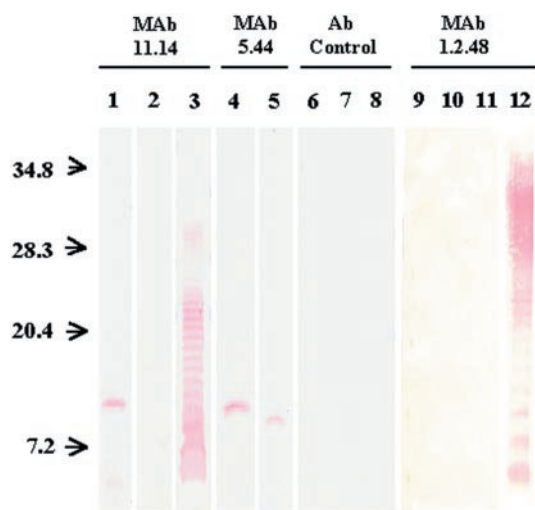


FIG. 1. Western blot of glycosylated and nonglycosylated *P. aeruginosa* 1244 pilin. Lanes 1, 4, 6, and 9 each contained ~0.5 µg of glycosylated pilin/lane. Lanes 2, 5, 7, and 10 each contained ~0.6 µg of nonglycosylated pilin/lane. Lanes 3, 8, and 11 each contained ~1.5 µg of purified *P. aeruginosa* 1244 LPS (serotype O7). Lane 12 contained 10 µl of a crude *P. aeruginosa* 653A LPS (serotype O6) preparation. Lanes 1-3 were probed with O7-specific mAb 11.14. Lanes 4 and 5 were probed with pilin protein-specific monoclonal 5.44. Lanes 6-8 were not treated with primary antibody. Lanes 9-12 were probed with O6-specific mAb 1.2.48. The arrows indicate the position of molecular size standards, with values shown in kDa.

performed by GLC-MS on a Hewlett-Packard 5970 MSD using a 30-m SP2330 capillary column from Supelco (Bellefonte, PA).

RESULTS

Immunological Relationship between Pilin and LPS of P. aeruginosa 1244—Previous work (27) has suggested that the pilin and LPS of *P. aeruginosa* 1244 were immunologically similar. To verify this, glycosylated 1244 pilin was analyzed by Western blot using a mAb 11.14, which is specific for 1244 LPS O-antigen (28). Fig. 1 shows that this antibody reacted with pilin to produce a single band at the anticipated position and also recognized LPS, as indicated by the typical ladder structure produced. To determine whether this mAb was directed against the pilin glycan, the nonglycosylated form of strain 1244 pilin (5) was also tested. Fig. 1 shows that whereas this antibody reacted with glycosylated pilin there was no recognition of the nonglycosylated form, indicating that the pilin glycan was the reactive epitope. Anti-pilus mAb 5.44, previously shown to react with a peptide epitope (29), reacted with both glycosylated and nonglycosylated pilin. Neither form of pilin nor strain 1244 LPS reacted in the absence of primary antibody or in the presence of a mAb directed against O6 serotype *P. aeruginosa* LPS. Western blot analysis of the cytoplasmic, membrane, and periplasmic cell fractions of strain 1244 showed that pilin and LPS were the only antigens present that reacted with mAb 11.14.² A polyclonal typing serum directed against the LPS O antigen and specific for International Antigenic Typing System serotype O7 (30) was found also to recognize glycosylated but not nonglycosylated pilin, as determined by Western blot (results not shown).

An antibody inhibition test was carried out to determine whether the antigen detected by Western blot was present on native functional pili. The pili of *P. aeruginosa* mediate a form of cell motility called twitching (31) that can be inhibited by anti-pilus sera. If the glycan epitope exists on the pilus surface and is accessible to antibody reaction, inhibition of twitching motility by mAb 11.14 would be expected. Table I shows that

TABLE I
Inhibition of *P. aeruginosa* 1244 twitching motility by a glycan-specific mAb

| Antibody | Protein concentration | Motility ^a |
|----------|-----------------------|-----------------------|
| | µg/ml | |
| None | 0.0 | 10.2 |
| 11.14 | 48.2 | 0.0 |
| 11.14 | 16.1 | 0.4 |
| 11.14 | 4.8 | 6.1 |
| 11.14 | 1.6 | 8.6 |

^a Motility zone diameter. The values presented are an average of eight assays.

purified mAb 11.14 inhibited twitching at concentrations as low as 1.6 µg/ml. Because this antibody also reacted with LPS, the possibility existed that twitching motility was inhibited through interaction with and not by directly binding to the pili. To determine whether this was the case, twitching inhibition was tested under conditions where only nonglycosylated pili were produced. *P. aeruginosa* 1244N3, a pilin-negative mutant (22) can produce glycosylated pili when carrying pPAC46, a plasmid bearing the whole *pilAO* operon (5). This organism carrying pPAC24, a plasmid containing *PilA* but not *pilO*, produced only nonglycosylated pilin. Table II shows that twitching motility by the strain producing glycosylated pili was sensitive to inhibition by mAb 11.14, whereas the strain producing nonglycosylated pili was not affected. These results indicate that the 11.14-reactive epitope is present on the surface of the pilus under physiological conditions.

Isolation and Preliminary Characterization of Pilin Aminoglycan—Initial experiments were carried out to confirm the finding (5) that *P. aeruginosa* 1244 pilin was post-translationally modified. Although the sequence of the pilin structural gene, *pilA*, predicted a molecular weight of 15,648 for mature pilin, polyacrylamide gel electrophoresis suggested that the value was ~16,900 (5). In the present study, MALDI analysis of pure strain 1244 pilin produced a value of 16,307 (±25) (results not shown), indicating the presence of covalently bound material with a total mass of ~660 and with no signal seen at the mass predicted by the pilin gene. The use of a saccharide-specific probe indicated that this pilin contained bound glycan (5). In the work presented here, proteolytically digested pure pili were found to contain a glycan material as determined by TLC using orcinol reagent detection. No glycan was seen under the same digestion conditions in the absence of either pili or protease.

The orcinol-reactive material released by pilus degradation was purified by gel filtration and TLC and was found to be homogeneous as determined by two-dimensional high performance TLC using either orcinol or ninhydrin detection. The *R_f* coordinates of the spots produced by these methods were identical. For preliminary identification of the amino-containing component, purified pilin aminoglycan was hydrolyzed (1 M trifluoroacetic acid for 4 h at 95°C) and the hydrolysate subjected to high performance TLC, with undigested aminoglycan, serine, threonine, and aspartic acid as references. The only ninhydrin-reactive spot present in the digested glycan matched the *R_f* of serine, which was absent in undigested aminoglycan (results not shown). These findings were confirmed by amino acid analysis where serine was the sole amino acid present above background. Here it was found that 18.6 nmol of serine (2.0 µg) was produced from 18.8 µg (as determined by sugar analysis) of aminoglycan.

The aminoglycan was treated with 1 M HCl for 6 h at 95°C, and the hydrolysate was subjected to high performance TLC with orcinol detection. This procedure revealed the presence of three sugars, one of which corresponded in mobility to xylose

² J. Rao and P. Castric, unpublished observations.

TABLE II
Pilin glycan-specific inhibition of twitching motility in
P. aeruginosa 1244N3

| Plasmid | Treatment | Motility ^a |
|---------------------|--------------------------------|-----------------------|
| | | <i>mm</i> |
| pPAC24 ^b | None | 0.0 |
| pPAC46 ^c | None | 0.0 |
| pPAC24 | +IPTG ^d | 9.4 |
| pPAC46 | +IPTG | 14.5 |
| pPAC24 | +IPTG, +mAb 11.14 ^e | 8.4 |
| pPAC46 | +IPTG, +mAb 11.14 | 0.0 |

^a Motility zone diameter.

^b pPAC24 contains the *pilA* gene of *P. aeruginosa* 1244 under control of a *tac* promoter. See "Experimental Procedures" for details.

^c pPAC46 contains the *pilAO* operon of *P. aeruginosa* 1244 under control of a *tac* promoter. See "Experimental Procedures" for details.

^d Test medium contains isopropyl- β -D-thiogalactopyranoside (IPTG) at a concentration of 5 mM.

^e Ascites fluid used. Test medium contains approximately 20 μ g/ml mAb 11.14.

(results not shown). Gel filtration of the aminoglycan using a calibrated Sephadex G-25 column indicated a molecular weight in the range of 400 to 800 (results not shown), which would be consistent with the additional mass revealed by the MALDI analysis described above. Altogether, these results suggest that the *P. aeruginosa* 1244 pilin monomer has a single covalently bound trisaccharide *O*-linked to one of the protein's 13 serine residues.

To determine whether the purified aminoglycan carried the mAb 11.14-specific epitope, blocking enzyme-linked immunosorbent assays were carried out in which this molecule was allowed to compete with pure native pili for antibody recognition. Fig. 2 shows that the aminoglycan efficiently inhibited pili binding by mAb 11.14 but failed to interfere with mAb 5.44, which recognizes a pilin protein epitope (29). Competition was also seen between the isolated glycan and pure strain 1244 LPS, supporting the contention that the pilin glycan and strain 1244 LPS *O*-antigen share a common epitope.

Structural Analysis of Pilin Aminoglycan—Results presented above suggested that *P. aeruginosa* 1244 pilin glycan was antigenically related to the LPS *O*-antigen of this organism and was structurally similar to an *O*-antigen repeating unit. Agglutination testing using International Antigenic Typing System sera (results not shown) showed that *P. aeruginosa* 1244 belonged to *O*-antigen group O7, a serogroup that is equivalent to Lanyi-Bergan 7a,7b, 7c (30, 32). The *O*-antigen structure of a *P. aeruginosa* strain from this serogroup has been determined (18, 33). These results provided useful reference information for the structural analysis of the *P. aeruginosa* 1244 pilin aminoglycan.

The proton spectrum of the pilin aminoglycan is shown in Fig. 3. The spectrum is consistent with this oligosaccharide having three of the glycosyl residues reported for the *O*-antigen polysaccharide from the LPS of *P. aeruginosa* (33), namely, Xyl, FucNAc, and 5N β OHC₄7NFmPse. Consistent with the previous report (33), the anomeric resonance at δ 4.54 ($J_{1,2}$ 8.0 Hz) was assigned to FucNAc and that at δ 4.43 ($J_{1,2}$ 7.6 Hz) to Xyl. Both of these glycosyl residues are β -linked. In addition, the 7.6 Hz $J_{1,2}$ coupling of Xyl shows that it is in the pyranose, and not the furanose, form because a β -furanosyl conformation would have a $J_{1,2}$ coupling of less than 1 Hz (34). The resonances at δ 1.52 ($J_{3a,3e}$ = 13.3 Hz) and δ 2.14 ($J_{3a,3e}$ = 13.3 Hz; $J_{3a,4}$ = 4.6 Hz) are consistent with the previously reported axial and equatorial protons, respectively, of 5N β OHC₄7NFmPse. In agreement with knirel *et al.* (33), the chemical shift difference between H3a and H3e of 0.62 shows that the 5N β OHC₄7NFmPse is α -linked. The resonance at δ 2.06 is due to the methyl protons of the *N*-acetyl group of FucNAc. A

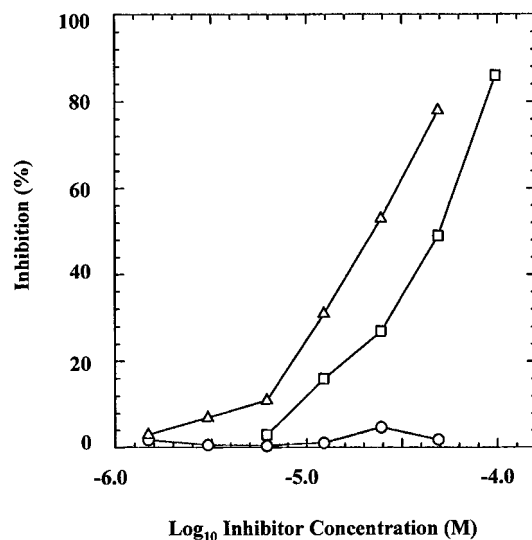


FIG. 2. Blocking enzyme-linked immunosorbent assay using purified *P. aeruginosa* 1244 pilin glycan. Wells were coated with *P. aeruginosa* pili (triangles and circles) or LPS (squares). The test antibody was mAb 11.14 (triangles and squares), and the control was mAb 5.44 (circles). The molar concentration of the inhibitor was approximated using the mass difference between glycosylated and nonglycosylated pilin. This procedure is described under "Experimental Procedures."

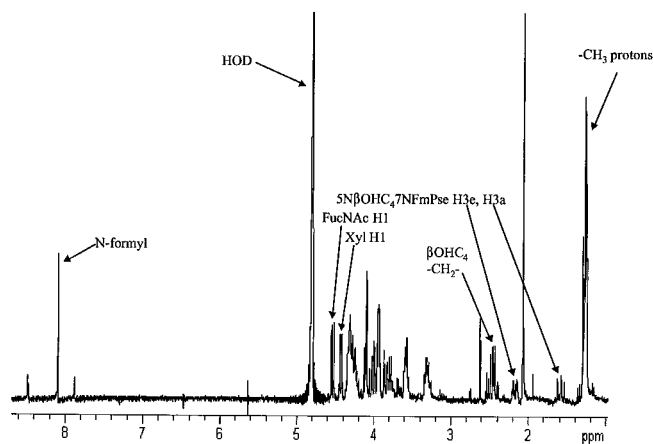


FIG. 3. A proton spectrum of the pilin oligosaccharide.

resonance corresponding to the methyl group of an *O*-acetyl group is not observed indicating that this oligosaccharide is not *O*-acetylated. The resonances from δ 1.23 to δ 1.32 are due to the H6 methyl protons of FucNAc, the H9 methyl protons of Pse5N β OHC₄7NFm, and the H4 methyl protons of a β -hydroxybutyryl group. The resonance at δ 8.09 is due to the proton of the *N*-formyl group at position 7 of Pse5N β OHC₄7NFm. The resonances centered around δ 2.45 are consistent with the H2 protons of the *N*- β -hydroxybutyryl substituent of 5N β OHC₄7NFmPse.

Proton assignments of this oligosaccharide were made by COSY (Fig. 4) and TOCSY (Fig. 5) NMR experiments. These assignments are shown in Table III. The β -Xyl resonances for the pilin oligosaccharide also vary from those reported for the *O*-antigen polysaccharide (33). It is likely that this variation is due to the fact that, in the latter case, all of the *O*-antigen derived oligosaccharides contained Xyl as a terminally linked residue, whereas it is 4-linked in the pilin oligosaccharide (discussed further below). The resonances of the FucNAc are consistent with the lack of an *O*-acetyl substituent. *O*-Acetylation of the FucNAc residue at *O*-4 would have resulted in an H4 chemical shift position of approximately δ 5.2 as reported by

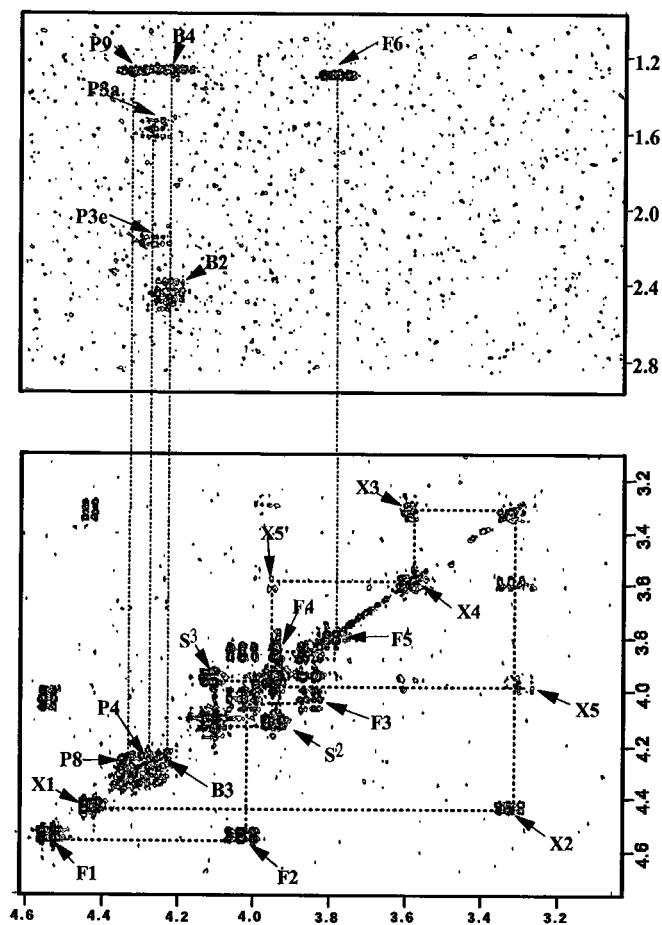


FIG. 4. A COSY spectrum of the oligosaccharide showing the proton assignments of the various residues and substituent groups. F, FucNAc; p, 5N β OHC₄7NFmPse; S, Ser; X, Xyl; B, β -hydroxybutyryl.

Knirel *et al.* (33). However, the FucNAc H4 in the pilin aminoglycan has a chemical shift at δ 3.92. In addition to these differences in the glycosyl residue protons, two other protons (δ 4.10 and δ 3.93) were observed that were coupled to one another but not to any of the other protons in this molecule. These protons are consistent with those of serine, the only amino acid component of this oligosaccharide (discussed above). Thus, the NMR results indicate that this molecule consists of FucNAc, 5N β OHC₄7NFmPse, Xyl, and Ser. The calculated molecular weight for such a molecule is 770, a value that is supported by the mass spectrometric data, which give an [M+H]⁺ of *m/z* 771.5.

The sequence of the glycosyl residues in the pilin oligosaccharide was determined by a ROESY NMR spectrum, tandem MS/MS analysis, and methylation analysis. The ROESY spectrum (Fig. 5) shows that H1 of Xyl (δ 4.43) has a strong nuclear Overhauser effect interaction with the H3 of FucNAc (δ 3.82). H1 of FucNAc (δ 4.54) has a strong nuclear Overhauser effect interaction with the H3 proton of Ser (δ 4.10). Thus, the 5N β OHC₄7NFmPse residue must occupy a terminal position in this molecule. These results indicate the following structure: α -5N β OHC₄7NFmPse-(2 \rightarrow ?) β -Xyl-(1 \rightarrow 3) β -FucNAc-(1 \rightarrow 3) β -Ser. This sequence was confirmed by tandem MS/MS spectrometry, Fig. 6, because fragment ions due to the successive losses of Ser (*m/z* 665.5), FucNAc-Ser (*m/z* 478.5), and Xyl-FucNAc-Ser (*m/z* 347.0) are observed. Methylation analysis was required to determine the linkage position of the xylosyl residue. Partially methylated alditol acetate derivatives were prepared and analyzed by combined GC-MS. This analysis (results not

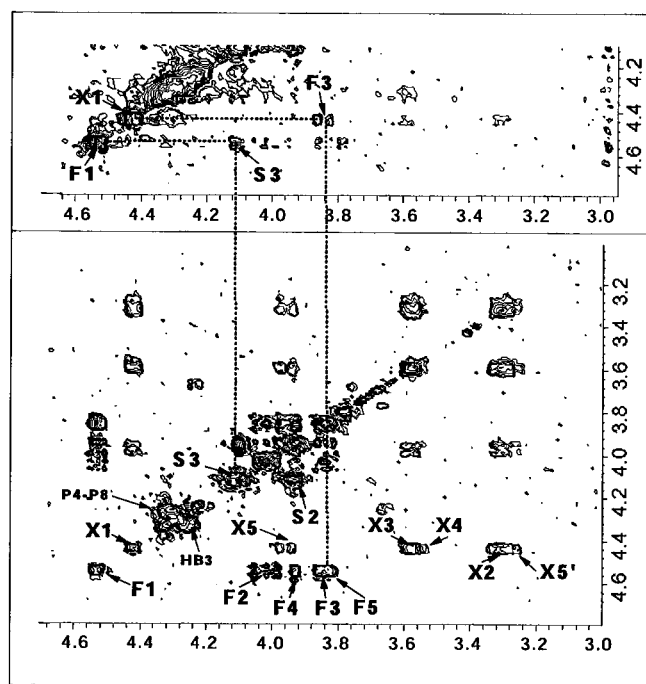


FIG. 5. The top panel is a ROESY spectrum of the anomeric region. The bottom panel is the TOCSY spectrum. The connectivities between the various protons are as indicated in the ROESY and TOCSY spectra. F, FucNAc; X, Xyl; p, 5N β OHC₄7NFmPse; S, serine; and HB, β -hydroxybutyryl. ROESY connectivities: F1/S3, 4.54(FucNAc H1) \rightarrow 4.10 (Ser H3); X1/F3, 4.43(Xyl H1) \rightarrow 3.84 (FucNAc H3).

shown) clearly revealed the presence of a PMAA derived from a 4-linked xylosyl pyranosyl residue or to a 5-linked xylosyl furanosyl residue (*m/z* 118, 189). Because the NMR data clearly showed that the Xyl was present as a β -xylopyranosyl residue, it is concluded that this residue is 4-linked in the oligosaccharide. Based on the above information, the structure of this molecule, presented in Fig. 7, is as follows: α -5N β OHC₄7NFmPse-(2 \rightarrow 4) β -Xyl-(1 \rightarrow 3) β -FucNAc-(1 \rightarrow 3) β -Ser.

DISCUSSION

Results presented in this report have shown that the *P. aeruginosa* 1244 pilin glycan is an O-linked trisaccharide covalently attached through the β -carbon of a serine residue. The calculated mass of the glycan, 666.5, corresponds to the value by which pilin exceeds that predicted by the *pilA* gene. This finding, as well as the absence of the nonmodified form of this protein in cell extracts or glycosylation isoforms (5), shows that each monomer is modified with a single glycan.

Although the glycans of previously described bacterial glycoproteins did not show similarities with common cell saccharides, the *P. aeruginosa* 1244 pilin glycan revealed a structure that has the same sugar composition and sequence as the O-antigen repeating unit of *P. aeruginosa* 170046, a strain belonging to the LPS O7 serotype (33). The pilin glycan differs from the O-antigen only in that the FucNAc residue is not O-acetylated. The structural similarity between the *P. aeruginosa* 1244 pilin glycan and the O7 repeating unit suggests a common biosynthetic origin in which pilin glycosylation might occur as a branch of the pathway of O-antigen production. If this is the case, candidate pilin glycosylation substrates could be nucleotide-sugar intermediates of O-antigen biosynthesis or the complete repeating unit borne on bactoprenyl pyrophosphate (35), a molecule normally anchored on either the inner or outer surfaces of the cytoplasmic membrane. This would be a process similar to the dolichol path of protein glycosylation

TABLE III
NMR assignments

| Sugar | H1 | H2 | H3 | H4 | H5 | H6 | H7 | H8 | H9 |
|----------------------------|------|------|------------------------|------|-------------------|-------------------|-------------------|------|------|
| FucNAc | 4.54 | 4.02 | 3.82 | 3.92 | 3.79 | 1.22 | — ^a | — | — |
| Xyl | 4.43 | 3.27 | 3.55 | 3.53 | 3.94, 3.25 | — | — | — | — |
| Pse | — | — | 1.52, H3a 2.14, H3e | 4.32 | 4.29 ^b | 4.22 ^b | 4.24 ^b | 4.34 | 1.20 |
| β -OHC4 ^c | — | 2.45 | 4.25 | 1.20 | — | — | — | — | — |
| Ser | — | 3.93 | 4.10 | — | — | — | — | — | — |
| Ac ^d | — | 2.06 | — | — | — | — | — | — | — |
| Fm ^e | 8.09 | — | — | — | — | — | — | — | — |

^a —, Not applicable, as these protons do not exist in the respective residues.

^b These resonances cannot be distinguished from one another with certainty.

^c β -OHC4, β -hydroxybutyryl acid.

^d Ac, acetyl.

^e Fm, formyl.

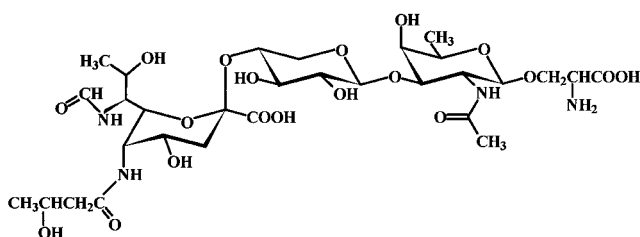


FIG. 6. The tandem MS/MS spectrum of the $[M+H]^+$ 771.5 ion. This spectrum was obtained by ESI-MS analysis. The fragmentation pattern is as indicated in the figure.

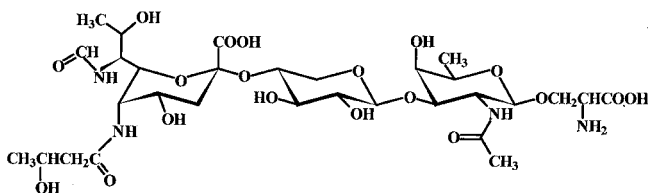


FIG. 7. The *P. aeruginosa* 1244 pilin glycan structure.

present in yeasts and archaeobacteria (6, 36). PilO, which is required for pilin glycosylation (5), is predicted to be a membrane protein that, if located in the cytoplasmic membrane, would be situated correctly for modification of the membrane bound pilin (either following pilin biosynthesis or after transport to the periplasm). If the pilin glycan is produced in this manner, the O-antigen structure should be identical with that of the glycan. Structural analysis of the strain 1244 O-antigen is currently under way to determine this point.

The wall-associated glycan of *S. sanguis*, (9) as well as certain S-layer and flagellin glycans of *Halobacterium halobium* (6–8), are N-linked to the amido nitrogen of an asparagine residue. The S-layer protein glycan of *Thermoanaerobacter thermohydrosulfuricus* is O-linked to a tyrosine residue (37), whereas the *M. tuberculosis* wall glycan is O-linked through a threonine residue (10). By contrast the pilin glycans of *N. meningitidis* and *N. gonorrhoeae*, like that of *P. aeruginosa* 1244, are all O-linked to serine residues (15–17). Although the trisaccharide structure of *N. meningitidis* and the disaccharide of *N. gonorrhoeae* are both attached at pilin serine 63 (15, 17) and the *N. meningitidis* glycerophosphate is covalently bound at serine 93 (16), the site of the *P. aeruginosa* 1244 pilin glycan has not yet been elucidated. Strain 1244 pilin does not share homology with the neisserial proteins in these regions, nor does it have serine residues in the same relative positions.

The strain 1244 pilin belongs to a homogeneous subtype that is commonly found among *P. aeruginosa* clinical isolates (29). Although these findings suggest that this modification is widespread, it is clear that it is not universal because *P. aeruginosa*

strains PAO and PAK produce only nonglycosylated pili (38, 39). The role of pilin glycosylation, particularly as to whether or not it offers a selective advantage with regard to virulence, remains to be determined. It has been proposed that glycosylation of *N. meningitidis* pilin influences adhesion (40). Marceau *et al.* (41) produced evidence that this was not the case, but they showed that pilin glycosylation was associated with the production of S-pilin, a truncated form of this protein. Antibody attachment to the pilus glycan of *N. meningitidis* has been shown to prevent complement binding (42), a response that could protect against phagocytosis. 5N β OHC₄7NfmPse has the same basic structure common to the sialic acid family of sugars, which have been postulated to function as biological masks protecting sensitive protein structures (43). The presence of the pilin glycan may protect the pili from complement binding and phagocytosis or protect potential epitopes from the host B-cell response. The latter has been suggested as a role for glycosylation of *Campylobacter* proteins (13) and the pili of *N. meningitidis* (15). It is also possible that pilin glycosylation functions to protect the pilus against attack from proteolytic enzymes present as part of the host defense or as produced by *P. aeruginosa* itself.

Because the pilin glycan exists on the fiber surface, as demonstrated by its accessibility to antibody binding under physiological conditions, it has the potential for having a significant effect on the relation of the pilus with its chemical and physical environment. However, for this to be the case, the glycan must be uniformly accessible to the pilus fiber surface. For example, if the glycan exists complexed with the pilin subunits in such a way that is partially buried, a situation analogous to the *P. aeruginosa* pilin disulfide loop adhesion site that is a part of each subunit but is available for antibody binding only at the pilus tip (44), it would have limited influence. However, if the glycan extends evenly from the pilus surface, it would be expected to increase the solubility of the pilus fibers by modulating the inherent hydrophobicity (45) of this structure. In addition, the presence of 5N β OHC₄7NfmPse would introduce a negative charge that would lower the pilus isoelectric point, influence solubility, and likely increase ionic interaction among pili and between the pili and extracellular structures. Because the total surface area of the pili is a sizable fraction of the cell surface, it is clear that the pilin glycan has the potential to be a major influence on the interaction of the cell with its environment. This would include especially the ability of *P. aeruginosa* to carry out pilus-dependent functions such as twitching motility and biofilm formation, processes that are important in pathogenesis (46–49).

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