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Genetic, Structural, and Antigenic Analyses of Glycan Diversity in the O-Linked Protein Glycosylation Systems of Human *Neisseria* Species▽†

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Bacterial capsular polysaccharides and lipopolysaccharides are well-established ligands of innate and adaptive immune effectors and often exhibit structural and antigenic variability. Although many surface-localized glycoproteins have been identified in bacterial pathogens and symbionts, it not clear if and how selection impacts associated glycoform structure. Here, a systematic approach was devised to correlate gene repertoire with protein-associated glycoform structure in *Neisseria* species important to human health and disease. By manipulating the protein glycosylation (*pgl*) gene content and assessing the glycan structure by mass spectrometry and reactivity with monoclonal antibodies, it was established that protein-associated glycans are antigenically variable and that at least nine distinct glycoforms can be expressed *in vitro*. These studies also revealed that in addition to *Neisseria gonorrhoeae* strain N400, one other gonococcal strain and isolates of *Neisseria meningitidis* and *Neisseria lactamica* exhibit broad-spectrum O-linked protein glycosylation. Although a strong correlation between *pgl* gene content, glycoform expression, and serological profile was observed, there were significant exceptions, particularly with regard to levels of microheterogeneity. This work provides a technological platform for molecular serotyping of neisserial protein glycans and for elucidating *pgl* gene evolution.

It is now well established that protein glycosylation based on both N- and O-linked modifications occurs in bacterial species. In N-linked systems exemplified by the system in *Campylobacter jejuni*, large numbers of proteins that are translocated to the periplasm are glycosylated based on the presence of sequon elements and asparagine-targeting oligosaccharyltransferases related to those that operate in eukaryotes (21, 36, 69, 73). Two O-linked systems associated with covalent modification of type IV pilin subunits in pathogenic *Neisseria* species and in selected strains of *Pseudomonas aeruginosa* have been particularly well characterized (2, 16, 46–48, 54). The latter systems are remarkably similar to the N-linked system characterized in *C. jejuni* in that oligosaccharides are synthesized cytoplasmically as lipid-linked precursors that are then flipped into the periplasm. Protein-targeting oligosaccharyltransferases structurally related to the WaaL family of O-antigen ligases then transfer the oligosaccharides to protein substrates (2, 18, 49). The similarities between these N- and O-linked systems are perhaps best illustrated by genetic and functional interactions between components of the *C. jejuni* oligosaccharide biosynthetic machinery and elements of the neisserial pilin glycosylation pathway (2, 18). In contrast, the mechanisms operating in other bacterial O-linked systems are not completely understood yet, and there appears to be considerable diversity in the mechanisms of oligosaccharide synthesis, transfer of the glycan to the protein, and the cellular compartment in which glycan addition takes place. Prime examples of this diversity include the glycosylation of major subunits of S-layers (53), flagella (40), and type IV pili, as well as nonpilus adhesins, such as autotransporters (7, 51) and a family of serine-rich proteins identified in Gram-positive species (72). Recently, the pilin glycosylation system in the Gram-negative species *Neisseria gonorrhoeae* (the etiological agent of gonorrhea) was shown to be a general O-linked system in which a large set of structurally distinct periplasmic proteins undergo glycosylation (64). Likewise, a general O-linked glycosylation system targeting periplasmic and surface-exposed proteins has been documented in *Bacteroides fragilis* (19). In addition, an increasing number of lipoproteins in *Mycobacterium tuberculosis* have been found to be O glycosylated, and current evidence suggests that a single glycosylation pathway operates with these proteins (50).

The large number of bacterial protein glycosylation systems strongly suggests that these systems are advantageous and affect fitness. In fact, mutants with mutations in the general glycosylation systems of *C. jejuni* and *B. fragilis* are defective in mucosal colonization, although the fundamental basis for the observations is unclear (19, 23). In some cases, defects in protein stability and trafficking have been documented. Examples of the latter have been reported for the Aida and Ag43 autotransporter adhesins of *Escherichia coli* and the serine-rich Fap1 streptococcal adhesin (11, 35, 72). In these cases, the glycosylation status appears to influence protein integrity along with intracellular or membrane trafficking events.

Glycosylation may also influence protein structure and func-

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tion or activity at the extracellular level. In the context of host-symbiont and host-pathogen interactions, bacterial cell surface polysaccharides and glycolipid glycans are well-established targets of both innate and adaptive immune responses (13, 61). However, the potential influence of protein-linked carbohydrate on immune recognition and signaling is only beginning to be investigated. Given the well-established effect of conjugating protein to carbohydrate on glycan-related immunogenicity, glycoproteins could be predicted to promote a robust T-cell-dependent antibody response directed toward glycan epitopes. In line with this, immunization of mice with O-glycosylated type IV pilin from *P. aeruginosa* strain 1244 (which bears a single repeat unit of the O antigen, the dominant component of its lipopolysaccharide) resulted in protection against challenge with immunological specificity for the O-polysaccharide (27). In addition, structural heterogeneity of carbohydrate modifications has been shown to affect the serospecificity of *Campylobacter* flagellins (41). With regard to innate immunity, the N-linked protein glycans of *C. jejuni* have been shown to influence interleukin-6 production by human dendritic cells via interaction with the macrophage galactose-type lectin (MGL) (62). Also, flagellin glycosylation of the phytopathogenic bacteria *Pseudomonas syringae* pv. glycinea and *P. syringae* pv. tomato appears to play an important role in hypersensitive cell death in nonhost plants and in host cell recognition (56, 57). Similarly, the flagellin glycosylation status in *P. aeruginosa* influences proinflammatory responses in human cell cultures (63).

Studies of O-linked flagellar glycosylation in *P. aeruginosa*, *C. jejuni*, and a number of Gram-positive species have revealed considerable variability in genomic glycosylation islands (40). In addition to differences in gene content, some genes localized in these loci are subject to phase (on-off) variation involving slipped-strand mispairing events. Similar findings have been obtained for the O-linked glycosylation system in *N. gonorrhoeae* and a related system in *Neisseria meningitidis* (2, 4, 29, 48). These observations strongly suggest that protein-associated glycans are positively selected. However, attempts to elucidate the evolutionary processes impacting these systems are complicated by difficulties in connecting genotype with phenotype. For example, predicting enzymatic activities of components involved in glycan biosynthesis based on the sequence alone is notoriously difficult. Therefore, glycosylation-related functions are characterized best by using purified components in *in vitro* assays. Moreover, despite recent advances in mass spectrometric (MS) and nuclear magnetic resonance (NMR) technologies, glycoprotein structural analysis is still arduous, particularly when proteins are expressed at low levels. Thus, current methodologies are not optimized for studies of large numbers of strains and mutants.

The broad-spectrum O-linked protein glycosylation system of *N. gonorrhoeae* is particularly well characterized with regard to the genetics of oligosaccharide biosynthesis, modification, and transfer to protein via the PglO/PglL oligosaccharyltransferase. As shown using strain N400, combined genetic and MS analyses, including interspecies complementation, have revealed that this system (designated the *pgl* [protein glycosylation] system) is remarkably similar to the N-linked system of *C. jejuni* with respect to the use of a peptide-proximal 2,4-diacetamido-2,4,6-trideoxyhexose (DATDH) sugar and related biosynthetic pathways for generating lipid-linked glycan substrates (2, 18, 39). The lipid-linked DATDH sugar can be further converted successively into hexose (Hex)-DATDH disaccharide and Hex-Hex-DATDH trisaccharide forms by the PglA and PglE glycosyltransferases, respectively (2). The hexoses in both the di- and trisaccharide forms can also undergo O acetylation by the PglI enzyme (2, 70). As *pglA*, *pglE*, and *pglI* are each predicted to be subject to phase variation in some backgrounds, strains have the potential to express five distinct glycoforms (2, 4, 29, 48, 70). A similar system operates in *N. meningitidis* strain c311, although to date only pilin and the AniA nitrite reductase proteins have been shown to be glycosylated (37). Pioneering analyses of pilin from this strain identified a trisaccharide with a terminal alpha-1-4-linked digalactose moiety attached to DATDH (54). Interestingly, nearly one-half of *N. meningitidis* isolates are reported to have a unique allele of *pglB* designated *pglB2* associated with synthesis of a proximal glyceramido-acetamido trideoxyhexose (GATDH) rather than DATDH (10). In addition, some strains of both *N. gonorrhoeae* and *N. meningitidis* have been reported to contain additional genes predicted to encode glycosyltransferases linked to the core locus that includes the *pglF*, *pglB*, *pglC*, and *pglD* genes (32, 48). Thus, it appears that the number of protein-associated glycans may be far greater than currently perceived. The genus *Neisseria* also includes a number of related species that colonize humans, including *Neisseria lactamica*, which is closely related to *N. gonorrhoeae* and *N. meningitidis* but is rarely associated with disease (24), as well as other, more divergent commensal species. An examination of recently available genome sequences of these nonpathogenic species revealed that they contain open reading frames (ORFs) whose products share high levels of amino acid identity with many of the protein glycosylation components found in *N. gonorrhoeae* and *N. meningitidis* and with many of the *N. gonorrhoeae* proteins targeted for glycosylation. However, protein glycosylation has not been documented in any of these species yet.

Here, we developed a systematic approach for elucidating intra- and interstrain glycan diversity and its genetic basis in neisserial O-linked glycans by employing serotyping, mass spectrometric analyses, and genetically defined recombinant backgrounds. We then used these tools to demonstrate that protein-associated glycans are antigenically variable and that isolates of *N. meningitidis* and *N. lactamica* also exhibit broad-spectrum O-linked protein glycosylation.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are described in Table 1 and were grown on conventional GC medium as described previously (20). Protein glycosylation mutations (*pglA*, *pglC*, $pglE_{on}$, *pglI*) were introduced into various strain backgrounds using transformation as previously described (1, 2). Antibiotics were used for selection of transformants at the following concentrations: streptomycin, 750 μg/ml; erythromycin, 8 μg/ml; and kanamycin, 50 μg/ml. In the case of meningococcal transformants, 100 μg/ml kanamycin was used. In the high-pH treatment experiments, cells were incubated in GC media containing 50 mM NaOH at 37°C for 45 min.

Allelic exchange of *pglB*/*pglB2*. The MC58 and 8013 *pglB* alleles were introduced into N400 by using a two-step mutagenesis method that allowed gene replacement without introduction of a selectable marker into the final strain. This method uses a two-gene cassette containing both a selectable marker (*ermC'*) and a counterselectable marker ($rpsL^{+}$) (31). First, the pCRII-pgl plas-

TABLE 1. *Neisseria* strains used in this study

Strain	Parent strain	Relevant genotype	Main glycan modification (identified by MS)	Reference
N. gonorrhoeae strains				
FA1090 (ST-1899)				ATCC 700825
KS300 (FA1090)	FA1090	*recA6*	Ac-Hex-Hex-DATDH	59
KS301	FA1090	*pglC*::*kan*	No glycan	This study
KS302	FA1090	*pglA*::*kan*	DATDH	This study
KS303	FA1090	*pglI*::*kan*	Hex-Hex-DATDH	This study
KS100 (N400)	VD300	*recA6*	Ac-Hex-DATDH	59
KS104 (GGC)	N400	*pglC*::*kan*	No glycan	22
KS141	N400	*pglA*::*kan*	DATDH	2
KS142	N400	$pglE_{on}$	Ac-Hex-Hex-DATDH	2
KS144	N400	*pglI*::*kan*	Hex-DATDH	2
KS304	N400	$pglE_{on}$ *pglI*::*kan*	Hex-Hex-DATDH	This study
KS305	N400	$pglB_{MC58}$	Ac-Hex-DATDH	This study
KS306	KS305	$pglB_{MC58}$ *pglA*::*kan*	DATDH	This study
KS307	KS142	$pglB_{MC58}$ $pglE_{on}$	Ac-Hex-Hex-DATDH	This study
KS308	N400	$pglB2_{8013}$	Hex-GATDH	This study
KS309	KS308	$pglB2_{8013}$ *pglA*::*kan*	GATDH	This study
KS310	KS142	$pglB2_{8013}$ $pglE_{on}$	Hex-Hex-GATDH	This study
KS101 (4/3/1)	VD300	*pilE ind*		71
KS105	4/3/1	*pglC*::*kan*		64
KS122	4/3/1	*pglA*::*kan*		64
KS127	4/3/1	$pglE_{on}$		65
KS311	4/3/1	$pglB2_{8013}$		This study
KS312	4/3/1	$pglB2_{8013}$ *pglA*::*kan*		This study
KS313	4/3/1	$pglB2_{8013}$ $pglE_{on}$		This study
KS314	4/3/1	*pglI*::*kan*		This study
KS315	4/3/1	$pglE_{on}$ *pglI*::*kan*		This study
N. meningitidis strains				
KS316 (MC58, ST-74)				58
KS317	MC58	*pglC*::*kan*		This study
KS318	MC58	*pglA*::*kan*		This study
KS319 (H44/76, ST-32)				26
KS320 (Z2491, ST-4)				44
KS321 (8013, ST-177)				33
KS322 (FAM18, ST-11)				6
KS323 (BZ 10, ST-8)				33
KS324 (BZ 198, ST-41)				33
N. lactamica strains				
KS325 (ST-3787)				5, ATCC 23970
KS326 (ST-640)				5

mid was constructed by PCR amplifying the whole N400 *pgl* locus with primers pgl-F (5′-AGCATATTGACGGGCTTGTCGC-3′) and pgl-R-(5′-AAGCGAAA TCCTCGGACACG-3′) and inserting the PCR product into the pCRII-TOPO vector (Invitrogen). Then the $ermC'/rpsL^+$ cassette from pFLOB4300 was amplified with primers pUC-F-*MluI* (5′-CCGACGCGTCCCAGTCACGACGTTG TAAAACG-3′) and pUC-R-*MluI* (5′-CCGACGCGTAGCGGATAACAATTT CACACAGG-3′) (MluI sites are underlined) and subsequently cloned into the MluI sites in *pglF* and *pglB* of the pCRII-pgl plasmid, which replaced some of the N400 *pgl* locus with the $ermC'/rpsL^+$ cassette and resulted in the pCRII-*pgl*::$ermC'/rpsL^+$ plasmid.

N400 was then transformed with pCRII-*pgl*::$ermC'/rpsL^+$, and erythromycin-resistant gonococci were selected. N400 is naturally streptomycin resistant, but introduction of the $rpsL^+$ allele made the intermediate strain, N400 *pgl*::$ermC'/rpsL^+$, streptomycin sensitive. The next step was transformation with MC58 or 8013 genomic DNA, in which homologous recombination replaced the $ermC'/rpsL^+$ cassette with the locus from the meningococcal genomic DNA, and the final strain was selected on streptomycin plates. For derived strains N400 $pglB2_{8013}$ and N400 $pglB_{MC58}$ (in which the locus was exchanged without leaving any remaining antibiotic resistance cassette), DNA sequencing was used to confirm that no other mutations were present. Derivatives of strain 4/3/1 carrying $pglB2_{8013}$ were constructed by using the same two-step mutagenesis strategy.

SDS-PAGE and immunoblotting. The procedures used for SDS-PAGE and immunoblotting have been described previously (20). Whole-cell lysates were prepared from equivalent numbers of cells by heating cell suspensions at 65°C for 10 min in SDS sample loading buffer. A rabbit polyclonal antibody against the nitrite reductase AniA was used at a 1:10,000 dilution. The mouse SM1 monoclonal antibody (MAb) (67), which specifically recognizes class I pilin types, was used at a 1:1,000 dilution. A rabbit polyclonal antiserum raised against the PilE-derived peptide [44]KSAVTEYYLNHGKWPENNTSA[64] (Research Genetics), which reacts with all pilin types, was used at a 1:10,000 dilution (3). Immunoreactive proteins were detected by immunoblotting using the glycan-specific rabbit antibodies described below and an alkaline phosphatase-coupled goat anti-rabbit secondary antibody (Sigma).

Development of glycan-specific rabbit polyclonal and monoclonal antibodies. Rabbits were immunized with purified pili from strains N400 *pglA*, N400, and N400 $pglE_{on}$, which express the DATDH-based monosaccharide, disaccharide, and trisaccharide glycoforms, respectively. Following a regimen with at least three immunizations (100 μg antigen/immunization), immune sera were harvested. These sera were designated pAb1, pAb2, and pAb3, respectively. To generate rabbit hybridomas, splenocytes from immunized rabbits were isolated and fused with a rabbit hybridoma fusion partner (34, 52). The antibodies were produced by Epitomics, Inc. (Burlingame, CA). Clones secreting glycan epitope-recognizing antibodies were selected by enzyme-linked immunosorbent assay (ELISA) screening of hybridoma supernatants using purified pili. Further screening of the ELISA-positive clones using immunoblotting identified hybridoma clones suitable for differentiating between the specific glycans. These clones

were designated MAbs npg1 (neisseria protein glycan 1), npg2, and npg3, respectively. The rabbit monoclonal antibodies (10 mg/ml) were used at a 1:20,000 dilution for immunoblotting.

Development of GATDH-specific rabbit polyclonal antibodies. Polyclonal antibodies (designated pGAb) were generated by rabbit immunization as described above using pili purified from strain N400 *pglB2*$_{8013}$ *pglA*, which expressed the protein-linked monosaccharide GATDH (Agrisera, Sweden). Solid-phase affinity purification was performed by immunoblotting a whole-cell lysate from the strain expressing the GATDH glycan (N400 *pglB2*$_{8013}$ *pglA*) and incubating it with pGAb (1:2,000 dilution). Specifically reactive glycoprotein bands were localized by developing strips cut from the edge of a polyvinylidene difluoride (PVDF) membrane, realigning them, and then excising the relevant area of the membrane. Antibodies were eluted from the membrane by two washes with 1 ml glycine-HCl buffer (5 mM, pH 2.3), which were collected and neutralized to physiologic pH by addition of 50 μl Tris-HCl (1 M, pH 7.8). The solution was then finally diluted in Tris-buffered saline (TBS) (1:3) before it was used for immunoblotting.

Sample preparation and ESI-MS analysis of intact PilE. Type IV pili were isolated and treated using a methanol-chloroform washing and precipitation procedure, as described previously (2). Samples were subjected immediately to MS analysis or frozen at −80°C. Data were acquired with a quadrupole time of flight mass spectrometer (Q-Tof micro; Waters Corporation, Milford, MA) equipped with the standard Z-spray electrospray ionization (ESI) source as previously described (2).

Membrane protein fraction preparation and in-gel digestion. *N. meningitidis* strain MC58 cells were harvested and washed with phosphate-buffered saline (PBS), and cell lysis was performed using a French press. Cellular debris was removed by centrifugation at 5,000 × *g* for 20 min, and the crude membranes were recovered from the supernatant by centrifugation at 100,000 × *g* for 60 min. The recovered membranes were washed two times in PBS and stored at −80°C.

Coomassie blue-stained protein bands were serially rehydrated and washed with 150 μl of high-performance liquid chromatography (HPLC)-grade water, 150 μl of acetonitrile-water (1:1, vol/vol), and 100% acetonitrile at room temperature. Protein reduction was carried out by addition of 50 μl of 10 mM dithiothreitol-0.05 M NH_4HCO_3 (60 min, 56°C) to the dehydrated gel pieces. Then the excess reduction buffer was removed, and thiol groups were alkylated by addition of 50 mM iodoacetamide and 0.05 M NH_4HCO_3 and incubation for 45 min at room temperature in the dark. The gel pieces were washed twice with 150 μl of acetonitrile-water (1:1, vol/vol) and 100% acetonitrile at room temperature. Digestion buffer (5 to 10 μl) containing 16 ng/μl trypsin (pig; modified; sequencing grade; Sigma-Aldrich) in 0.05 M NH_4HCO_3 was added, and samples were kept on ice for 30 min to allow rehydration of the gel pieces. To limit autoproteolysis of trypsin, the remaining buffer was removed and replaced with 35 to 50 μl of 0.05 M NH_4HCO_3, and digestion was carried out overnight at 37°C. Peptides were extracted successively with 5% formic acid, 5% formic acid-acetonitrile (1:1, vol/vol), and acetonitrile. The combined supernatants were dried with a SpeedVac and then redissolved in 0.1% formic acid. Samples were subjected immediately to mass spectrometric analysis or frozen at −80°C.

Nanoflow online liquid chromatographic MS analysis of proteolytic peptides. Reverse-phase (C_{18}) nano online liquid chromatographic MS/MS analyses of proteolytic peptides were performed using an HPLC system consisting of two Agilent 1200 HPLC binary pumps (one nano pump and one capillary pump) with a corresponding autosampler, column heater, and integrated switching valve. This liquid chromatography system was coupled via a nanoelectrospray ion source to an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). For analyses, 4 μl of a peptide solution was injected onto the extraction column (5 by 0.3 mm) filled with Zorbax 300 SB-C18 (particle size, 5 μm; Agilent). Samples were washed with a mobile phase consisting of 0.1% formic acid, and 3% acetonitrile. The flow rate, provided by the capillary pump, was 4 μl/min. After 7 min, the integrated switching valve was activated, and the peptides were eluted in the back-flush mode from the extraction column onto a C_{18} 3-μm resin column (150 by 0.075 mm; GlycproSIL C_{18}-80Å; Glycpromass, Stove, Germany). The mobile phase consisted of acetonitrile and MS-grade water, both containing 0.1% formic acid. Chromatographic separation was obtained using a binary 5 to 55% acetonitrile gradient for 60 or 210 min. The flow rate, provided by the nanoflow pump, was 0.2 μl min^{-1}.

Mass spectra were acquired in the positive-ion mode by applying a data-dependent automatic switch between the survey scan and tandem mass spectrum (MS/MS) acquisition. Peptide samples were analyzed by a high-energy collision dissociation (HCD) fragmentation method by acquiring one Orbitrap survey scan in the mass range from *m/z* 300 to 2000, followed by MS/MS of the three most intense ions in the Orbitrap. The target value in the LTQ-Orbitrap was 1,000,000 for a survey scan at a resolution of 30,000 at *m/z* 400 using lock masses for recalibration to improve the mass accuracy of precursor ions. Fragmentation in the C-trap was performed by collision-induced dissociation with a target value of 5,000 ions. The ion selection threshold was 500 counts. Selected sequenced ions were dynamically excluded for 180 s.

Data analysis. Mass spectrometric data were analyzed with an in-house neisserial protein sequence database using SEQUEST. The mass tolerances of a fragment ion and a parent ion were defined as 0.05 Da and 5 ppm, respectively. Methionine oxidation and cysteine carbamidomethylation were selected as a variable or fixed modification. A false discovery rate of 0.01 was required for proteins and peptides with a minimum length of 6 amino acids. Neisserial glycopeptide MS/MS spectra were manually searched by using Qual Browser, version 2.0.7.

Immunogold and transmission electron microscopy. Sample grids were prepared by touching carbon-coated Formvar copper grids to individual bacterial colonies grown on GC agar (18 h, 37°C, 5% CO_2) and were fixed with 1% glutaraldehyde in phosphate-buffered saline (PBS) (pH 7.4) for 4 min and four washes on drops of PBS containing 0.15% glycine. For immunogold labeling, the grids with fixed bacteria were first blocked with 0.8% bovine serum albumin (BSA) and then incubated with the rabbit MAbs (dilution, 1:100) for 30 min at room temperature. After four washes on drops of PBS containing 0.15% glycine, the grids were incubated with gold-conjugated protein A (10 nm) for 30 min. After six rinses on drops of water, the grids were stained for 3 min with uranyl acetate (2% aqueous solution) before they were viewed with a Philips CM100 transmission electron microscope.

RESULTS

DATDH-based glycoforms are immunogenic and antigenic. We observed previously that immunization of rabbits with glycosylated PilE protein (in purified type IV pili) resulted in antibodies that reacted in a glycan-specific manner with several other glycoproteins in *N. gonorrhoeae* (64). To examine glycan-related immunogenicity and antigenicity in more detail, rabbits were immunized with glycosylated PilE proteins derived from bacteria with different *pgl* backgrounds synthesizing either DATDH monosaccharide (*pglA*), acetyl (Ac)-Hex-DATDH disaccharide (wild type, *pglE*$_{off}$), or Ac-Hex-Hex-DATDH trisaccharide (*pglE*$_{on}$). Immunoblotting was then used to examine the patterns of reactivity using whole-cell lysates from strains expressing the different glycoforms (Fig. 1A). To eliminate the potential confounding influence of antibodies directed to PilE, polypeptide backgrounds in which *pilE* was conditionally repressed were used. As a negative control, a *pglC* null mutant was used as glycosylation is disrupted in this background due to the obligatory role of PglC in synthesis of the basal DATDH sugar (2). Antibodies raised against PilE from the *pglA* strain displayed a specific pattern, reacting only with glycoproteins from the strain expressing DATDH monosaccharide, whereas antibodies raised against PilE from wild-type N400 (*pglE*$_{off}$) reacted specifically with glycoproteins from the strain synthesizing the Ac-Hex-DATDH disaccharide. Likewise, antibodies raised against trisaccharide PilE detected glycoproteins in the *pglE*$_{on}$ background but also showed weak reactivity with some glycoproteins in the disaccharide-expressing strain. The glycoprotein patterns seen by the antibodies were strikingly similar except for minor perturbations in relative mobility that coincided with glycan size. Similar effects were seen for the strains that differed in O-acetylation status, and they were most dramatic for glycoproteins in the lower-molecular-weight range. Taken together, the results indicate that there was a specific polyclonal antibody response in rabbits to epitopes associated with each glycoform. In addition, changes in glycoform expression were not associated with discernible changes at the macroheterogeneity level (protein substrate targeting).

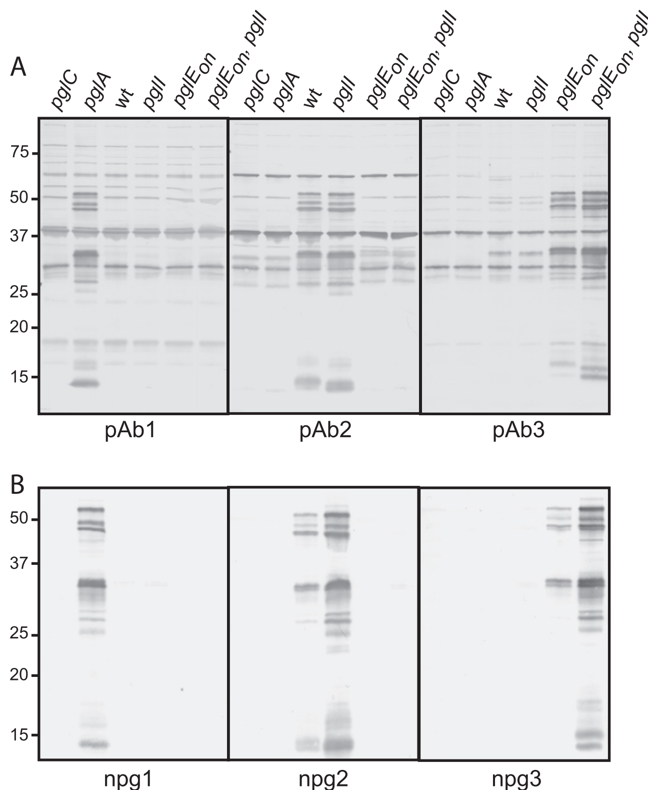

FIG. 1. DATDH-based glycans are immunogenic and antigenic: immunoblotting of whole-cell lysates of *N. gonorrhoeae pgl* mutant strains and variants with (A) the rabbit polyclonal antibodies pAb1, pAb2, and pAb3 raised against purified PilE bearing the DATDH, Ac-Hex-DATDH and Ac-Hex-Hex-DATDH glycans, respectively, and (B) the rabbit monoclonal antibodies npg1, npg2 and npg3 raised against the corresponding glycans. The strains used all had a 4/3/1 background in which *pilE* was conditionally repressed and were KS105 (*pglC*), KS122 (*pglA*), KS101 (wt), KS314 (*pglI*), KS127 (*pglE*$_{on}$), and KS315 (*pglE*$_{on}$ *pglI*). wt, wild type.

Given these findings and our goal of generating more specific glycan-recognizing reagents, monoclonal antibody (MAb)-producing cell lines were derived from immunized rabbits (52). Following screening for the cell lines with glycan specificity, three cell lines and corresponding MAbs were chosen for further study based on recognition of mono-, di-, and trisaccharide forms. When used for immunoblotting, these MAbs produced reactivity patterns that largely mimicked the reactivity patterns seen with the corresponding polyclonal antisera (Fig. 1B). However, the signals seen with the MAbs specific for the di- and trisaccharides were stronger for the *pglI* backgrounds lacking O acetylation. Thus, the epitopes recognized by these MAbs may be masked or otherwise compromised by acetylation of the hexose sugars.

Neisserial glycoprotein profiles and diversity in selected strains and species. Although broad-spectrum O-linked protein glycosylation has been well characterized in *N. gonorrhoeae* N400, it is not clear if general protein glycosylation occurs in other neisserial strains and species. To begin to address this possibility, selected strains were subjected to immunoblotting using the three glycan-specific MAbs. The strains were chosen primarily on the basis of the availability of their genome sequences or previous *pgl* characterization and included one *N. gonorrhoeae* strain, seven *N. meningitidis* strains, and two strains of *N. lactamica*. For strains reactive with one of the MAbs, the major reactive species was PilE (as shown by immunoblotting using the SM1 pilin MAb and a pilin peptide-directed antiserum that reacts with all PilE forms). As shown in Fig. 2, diverse reactivity patterns were detected, which nonetheless could be placed into groups based on similarities and levels of microheterogeneity. For example, *N. gonorrhoeae* N400 showed the strongest reactivity with the npg2 MAb and weaker reactivity with the mono- and trisaccharide-directed MAbs. This pattern was consistent with the results of MS analyses of N400 glycoproteins, which revealed that the disaccharide glycoform was the predominant glycoform (2), and with the presumed presence of less abundant cells carrying phase-on variants of *pglE*. This pattern was also seen for *N. meningitidis* strains MC58, H44/76, and Z2491, although the latter strain was negative for reactivity with the trisaccharide MAb. Also, the disaccharide-directed MAb detected multiple proteins in all three of these strains, and the overall patterns were similar to one another but somewhat distinct from those for the *N. gonorrhoeae* strains. *N. meningitidis* strains 8013, FAM18, BZ 10, and BZ 198 were essentially nonreactive with all three MAbs, except for weak signals for the monosaccharide epitope associated with PilE in 8013 and BZ 10. The overall lack of reactivity observed with these strains appears to correlate with the presence of the *pglB2* gene, which was implicated in the synthesis of the lipid-linked GATDH sugar intermediate. *N. lactamica* strain ST-640 reacted only with the monosaccharide MAb, as expected since the *pglA* allele is phase off. Finally, *N. gonorrhoeae* FA1090 and *N. lactamica* ST-3787 displayed similar patterns marked by predominant reactivity with the monosaccharide MAb, no reactivity with the disaccharide MAb, and weak reactivity with the trisaccharide MAb.

The unique pattern of microheterogeneity observed for strain FA1090 was not anticipated as this strain carries the *pglB* gene and phase-on alleles of *pglA* and *pglE*. Thus, one would predict that this strain should express the DATDH trisaccharide form and thus behave phenotypically like the N400 *pglE*$_{on}$ strain, but this was not the case. To examine this finding in more detail, we constructed mutants of FA1090 carrying null alleles of either *pglA* or *pglC* and compared them to equivalent mutants of *N. gonorrhoeae* N400 and *N. meningitidis* MC58. As shown Fig. 3A, introduction of the *pglC* mutation into FA1090 eliminated reactivity with the monosaccharide-specific MAb, as it did in N400. However, while introduction of the null allele of *pglA* into FA1090 had no effect on reactivity with the monosaccharide MAb, the same mutation in N400 resulted in a dramatic increase in reactivity. Also, comparison of the *pglA* null-allele mutants revealed remarkably similar glycoprotein profiles for the N400 and FA1090 mutants (Fig. 3A). Evaluation of the equivalent mutants of *N. meningitidis* MC58 revealed a pattern identical to that of N400 with respect to glycan antigenicity, although the relative mobilities of the predominant glycoproteins appeared to be distinct from those observed for the two *N. gonorrhoeae* strains (Fig. 3B).

Direct effect of O acetylation on protein-associated glycan antigenicity. To distinguish whether the effect of O acetylation on the reactivity of the di- and trisaccharide-recognizing MAbs was due to its influence on macroheterogeneity or due to

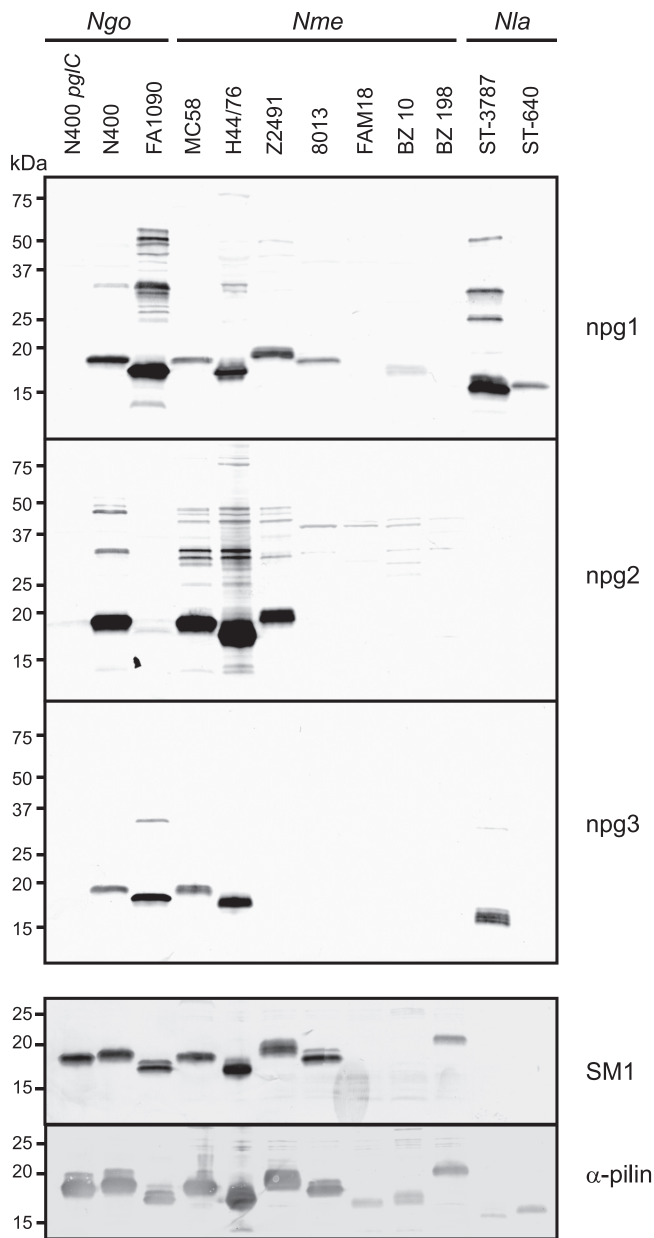

FIG. 2. Neisserial glycoprotein profiles and glycan diversity. (Top panels) Immunoblotting of whole-cell lysates from strains of *N. gonorrhoeae* (*Ngo*) (N400 *pglC*, N400, and FA1090), *N. meningitidis* (*Nme*) (MC58, H44/76, Z2491, 8013, FAM18, BZ 10, and BZ 198), and *N. lactamica* (*Nla*) (ST-3787 and ST-640) with glycan-specific monoclonal antibodies. (Bottom panels) Immunoblotting using the SM1 MAb specifically recognizing class I pilin types and a polyclonal antiserum raised against the PilE-derived peptide [44]KSAVTEYYLNHGKWPE NNTSA[64], which reacts with all pilin types. Thus, strains FAM18, BZ 10, ST-3787, and ST-640 all express class II pilins. Also, strains 8013, FAM18, BZ 10, and BZ 198 carry the *pglB2* allele that is associated with synthesis of GATDH glycans, while all of the other strains have the *pglB* allele that is associated with synthesis of DATDH glycans.

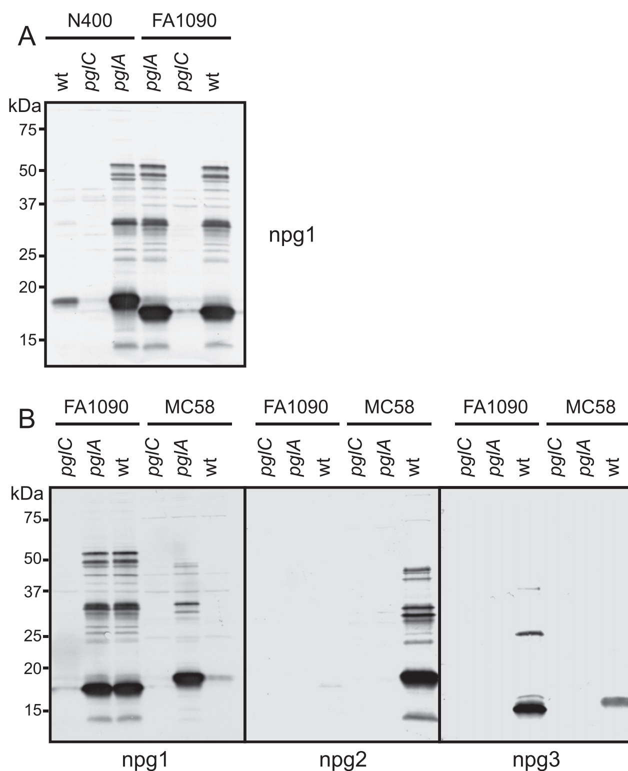

FIG. 3. Glycoprotein profiles and glycan antigenicity in strains N400, FA1090, and MC58. (A) Whole-cell lysates of *N. gonorrhoeae* N400 and FA1090 wild-type (wt) strains and *pglA* (monosaccharide-expressing) and *pglC* (glycosylation-null) mutants probed with MAb npg1. The strains used were KS100 (N400 wt), KS104 (N400 *pglC*), KS141 (N400 *pglA*), KS302 (FA1090 *pglA*), KS301 (FA1090 *pglC*), and KS300 (FA1090 wt). (B) Immunoblotting of whole-cell lysates of *N. gonorrhoeae* FA1090 and *N. meningitidis* MC58 wild-type strains and *pglA* (monosaccharide-expressing) and *pglC* (glycosylation-null) mutants probed with MAbs npg1, npg2, and npg3. The strains used were KS301 (FA1090 *pglC*), KS302 (FA1090 *pglA*), KS300 (FA1090 wt), KS317 (MC58 *pglC*), KS318 (MC58 *pglA*), and KS316 (MC58 wt).

masking of the epitopes, we exploited the established lability of O-linked acetate bonds under high-pH conditions. As shown in Fig. 4, suspending cells of N400 in medium with 50 mM NaOH prior to sample preparation increased the immunoreactivity of the disaccharide-recognizing MAb to the level observed for the untreated *pglI* mutant. In addition, a novel band was detected, which corresponded to an aberrantly migrating form of PilE (as it was not present in treated samples of a strain in which *pilE* was conditionally repressed and it reacted with the pilin-specific antibody [data not shown]). Both increased immunoreactivity and the aberrantly migrating form of PilE were observed for the treated samples of FA1090 probed with the trisaccharide-recognizing MAb (Fig. 4) and of MC58 probed with the di- and trisaccharide-recognizing MAbs (data not shown). Similar to what has been observed previously for capsular and O antigens (9, 43), in this study O acetylation had a documented effect on glycan antigenicity, presumably by blocking epitope accessibility.

Although our data clearly document that there are multiple glycoproteins in *N. meningitidis* strains MC58, H44/76, and Z2491 and *N. lactamica* strain ST-3787, the repertoire of glycoproteins in each strain has not been characterized. There has been only one report of an *N. meningitidis* glycoprotein other than PilE, and this glycoprotein is the AniA nitrite reductase of strain C311, which was identified by its immunoreactivity with

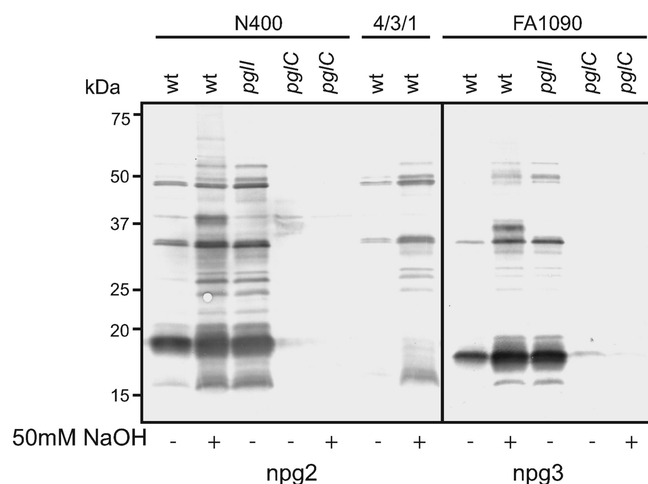

FIG. 4. Increased npg2 and npg3 immunoreactivity at high pH and in *pglI* mutants. Immunoblotting of whole-cell lysates of wild-type strains, *pglI* mutants, and high-pH-treated wild-type strains revealed increased reactivity with the npg2 and npg3 monoclonal antibodies due to the lack of an *O*-acetyl group in *pglI* mutants or with the high-pH treatment. The extraneous band that appeared during high-pH treatment is PilE (as determined by immunoblotting with SM1 [data not shown]). This band was not present with the 4/3/1 background, in which *pilE* was conditionally repressed. The strains used were KS100 (N400 wt), KS144 (N400 *pglI*), KS104 (N400 *pglC*), KS101 (4/3/1 wt), KS300 (FA1090 wt), KS303 (FA1090 *pglI*), and KS301 (FA1090 *pglC*). wt, wild type.

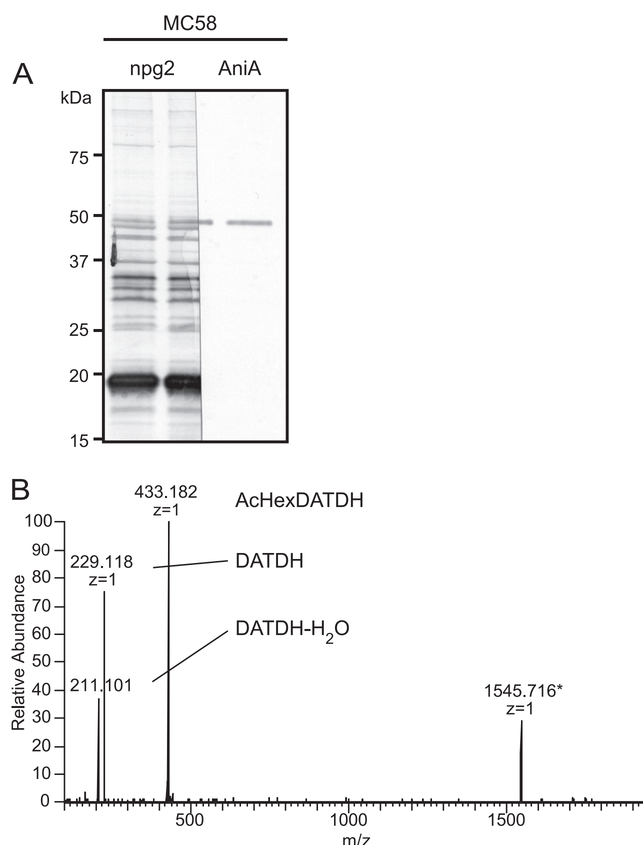

FIG. 5. AniA and GNA1946 are glycosylated in MC58. (A) Equivalent whole-cell lysate samples of *N. meningitidis* MC58 were electrophoresed in adjacent lanes, and following transfer to a PVDF membrane, the membrane was divided in half lengthwise. The two pieces were then probed with MAb npg2 and antibodies to AniA. The results demonstrate that the same band corresponding to AniA was identified with both antibodies. (B) Identification of the glycopeptide ^{6}DSAPA ASASAAADNGAAK^{23} from lipoprotein GNA1946 derived by tryptic cleavage of a membrane protein-enriched fraction of MC58. High-energy collision dissociation (HCD) fragmentation of $[M+2H]^{2+}$ at *m/z* 989.4505 revealed characteristic oxonium ions at *m/z* 433.182 and *m/z* 229.118 corresponding to *O*-Ac-Hex-DATDH and DATDH glycans, respectively. The signal at *m/z* 1545.716 is a signal from the peptide backbone after loss of the glycan moiety; the monoisotopic theoretical molecular mass is 1,545.714 Da.

antibodies raised against glycosylated PilE and was characterized by MS (37). Concurrently, AniA was also shown to be glycosylated in *N. gonorrhoeae* N400 (64). To examine the AniA glycosylation status in MC58, immunoblotting analyses using the disaccharide-recognizing MAb and antibodies to AniA were carried out in parallel. As shown in Fig. 5A, the AniA band was readily identified as the slowest-migrating MAb-specific band, at approximately 48 kDa. Moreover, this protein reacts with the disaccharide-specific MAb, and equivalent signals were observed for the H44/76 and Z2491 strains using the disaccharide-specific MAb (Fig. 2). Using an alternative "shotgun" approach in which tryptic peptides in gel slices from SDS-PAGE of an MC58 membrane protein fraction were directly examined for glycan-related signals by using MS2, a glycopeptide from protein GNA1946 was identified (Fig. 5B). This glycopeptide was equivalent to that reported for the orthologous Ng1237 glycoprotein in *N. gonorrhoeae* N400 (64). A more detailed analysis of the meningococcal glycoproteomes will be presented elsewhere.

Influence of the *pglB2* allele on glycan structure and antigenicity. Immunoblotting of *N. meningitidis* strains established that there was a negative correlation between MAb reactivity and the presence of the *pglB2* gene. In order to assess the nature of this association in more detail, we used an allelic replacement strategy related to that used originally to establish the connection between the GATDH proximal sugar and *pglB2*. We replaced the *N. gonorrhoeae* N400 *pgl* locus with the corresponding loci from *N. meningitidis* MC58 and 8013, which harbor *pglB* and *pglB2*, respectively, using a counterselectable marker (*rpsL*$^{+}$) that allows gene replacement without the presence of selectable markers in the resulting recombinant strain. By carrying out these replacements with the *pglA* and *pglE*$_{on}$ backgrounds of N400, we also examined if the corresponding glycosyltransferases were capable of transferring the first and second hexoses onto the lipid carrier-linked basal sugars to generate the di- and trisaccharide forms of GATDH. We examined purified, intact PilE derived from the recombinants using a "top-down" electrospray infusion (ESI) MS approach developed previously (1). The reconstructed molecular mass profile of the corresponding spectrum for the *pglB*$_{MC58}$ background revealed a distribution of species very similar to that reported for the parental background (expressing endogenous PglB) (Fig. 6). These species corresponded to PilE modified with either a 432-Da glycan moiety (O-acetylated Hex-DATDH [Ac-Hex-DATDH]) or a 391-Da glycan moiety (Hex-DATDH) and carrying either one or two phosphoethanolamine (PE) moieties (see Table S1 in the supplemental material). Likewise, in the *pglA* and *pglE*$_{on}$

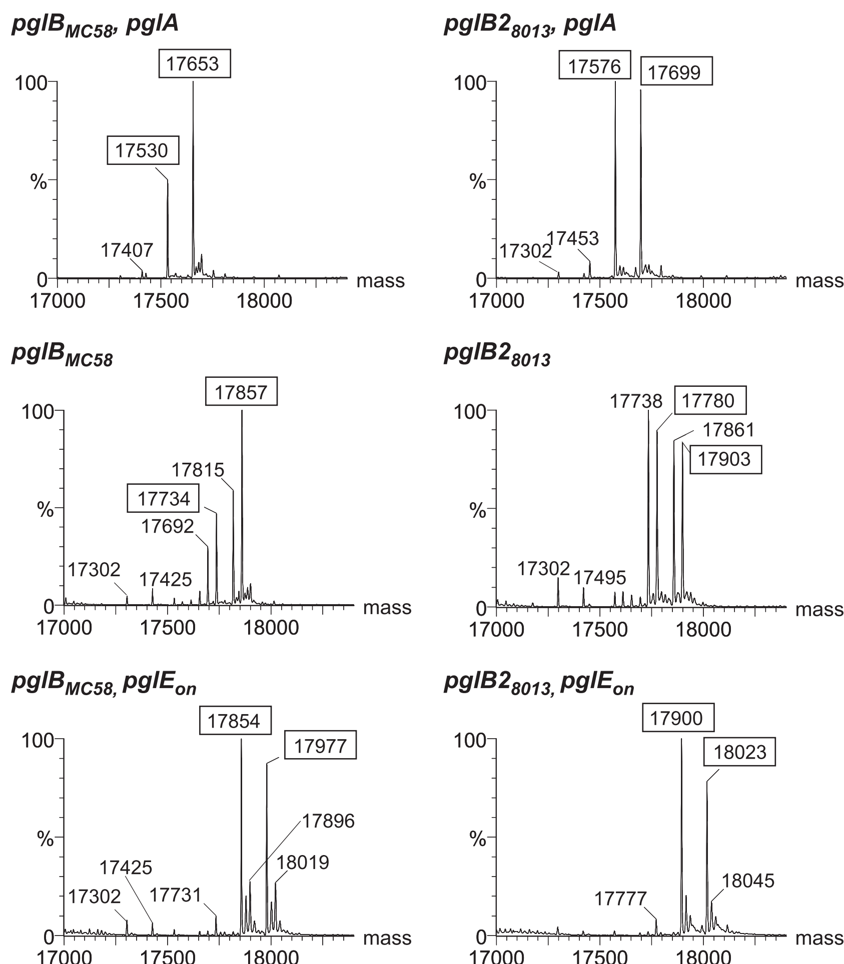

FIG. 6. MS analysis of intact PilE carrying DATDH- or GATDH-based glycan forms. ESI-MS analyses of intact PilE utilizing pili from strains carrying either $pglB_{MC58}$ or $pglB2_{8013}$ in different *pgl* backgrounds were carried out to characterize the glycan structure. N400 $pglB_{MC58}$ *pglA* produced two major signals that are enclosed in boxes and represent PilE carrying the DATDH monosaccharide with one (*m*/*z* 17530) or two (*m*/*z* 17653) PE modifications. The N400 $pglB2_{8013}$ *pglA* signals represent PilE carrying the GATDH monosaccharide with one (*m*/*z* 17576) or two (*m*/*z* 17699) PEs. For N400 $pglB_{MC58}$ and N400 $pglB2_{8013}$, the major signals represent PilE with one or two PE modifications in conjunction with the Ac-Hex-DATDH and Ac-Hex-GATDH disaccharides, respectively (indicated by *m*/*z* values in boxes). For N400 $pglB_{MC58}$ $pglE_{on}$, major signals correspond to PilE carrying Hex-Hex-DATDH with one or two PE modifications (boxes). N400 $pglB2_{8013}$ $pglE_{on}$ major signals are enclosed in boxes and represent Hex-Hex-GATDH with one or two PEs. Table S1 in the supplemental material shows all of the ion species along with the *m*/*z* values and corresponding molecular weights. The strains used were KS306 (N400 $pglB_{MC58}$ *pglA*), KS309 (N400 $pglB2_{8013}$ *pglA*), KS305 (N400 $pglB_{MC58}$), KS308 (N400 $pglB2_{8013}$), KS307 (N400 $pglB_{MC58}$ $pglE_{on}$), and KS310 (N400 $pglB2_{8013}$ $pglE_{on}$).

backgrounds, $PglB_{MC58}$ expression produced the MS PilE profiles observed for the parental N400 strain. In contrast, the equivalent molecular mass profiles of the corresponding spectra for the $pglB2_{8013}$ background were consistent with the presence of the basal GATDH sugar. In the samples from the wild-type recombinant, the predominant species corresponded to PilE modified with either a 478-Da glycan moiety (O-acetylated Hex-GATDH [Ac-Hex-GATDH]) or a 437-Da glycan moiety (Hex-GATDH) and carrying either one or two PE moieties. Identification of the GATDH sugar was further substantiated by the presence in the ESI mass spectrum of the glycan oxonium ion at *m*/*z* 479.2 and its pattern of fragmentation during subsequent analysis by collision-induced dissociation (CID) MS/MS (see Fig. S1a in the supplemental material). The O-acetylated Hex-GATDH disaccharide characterized here is different from an *N*-acetylhexosamine GATDH glycoform described previously despite the fact that the basic methodologies and strains employed in the two studies were the same (10). The difference can best be explained by the inability of the workers in the previous study to resolve the mass difference between O-acetylated hexose (204 Da) and *N*-acetylhexosamine (203 Da). In the *pglA* background, the profiles were indicative of modification with GATDH together with one or two PE moieties. Again, identification of the GATDH sugar was confirmed by the presence in the ESI mass spectrum of the glycan oxonium ion at *m*/*z* 275.2 and its patterns of fragmentation during subsequent analysis by collision-induced dissociation (CID) MS/MS (see Fig. S1b in the supplemental material). In the $pglE_{on}$ background, the profiles were indicative of modification with a Hex-Hex-GATDH trisaccharide

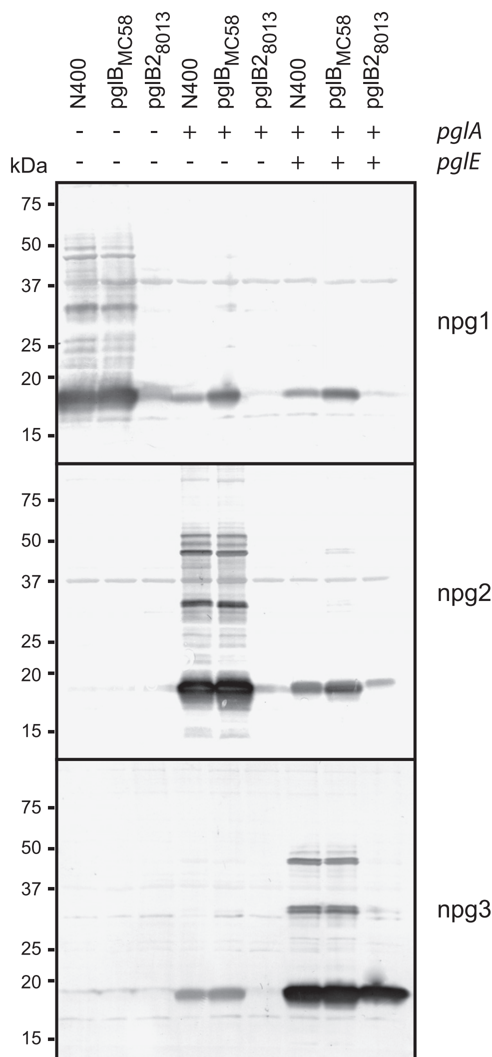

FIG. 7. GATDH-based glycans are antigenically distinct from DATDH-based forms. Western blots of N400 (*pglB*), N400 *pglB*$_{MC58}$, and N400 *pglB2*$_{8013}$ in different *pgl* backgrounds were incubated with the npg1, npg2, and npg3 monoclonal antibodies. The strains used were KS141 (N400 *pglA*), KS306 (N400 *pglB*$_{MC58}$ *pglA*), KS309 (N400 *pglB2*$_{8013}$ *pglA*), KS100 (N400), KS305 (N400 *pglB*$_{MC58}$), KS308 (N400 *pglB2*$_{8013}$), KS142 (N400 *pglE*$_{on}$), KS307 (N400 *pglB*$_{MC58}$ *pglE*$_{on}$), and KS310 (N400 *pglB2*$_{8013}$ *pglE*$_{on}$).

with one or two PE moieties. These findings confirm the previously described association of PglB2 with GATDH synthesis and demonstrate for the first time that PglA and PglE are necessary for synthesizing di- and trisaccharides having GATDH at the reducing end. Furthermore, the Hex-GATDH glycoform is susceptible to O acetylation, although there were significantly lower levels of this form than there were in the DATDH-expressing background (2). Somewhat surprisingly, signals indicative of O acetylation were not detectable in the Hex-Hex-GATDH-expressing background.

Using strains expressing defined glycoforms in a single background, MAb reactivity was examined (Fig. 7). First, the patterns of reactivity exhibited by the strains expressing endogenous and MC58 PglB were identical for the three MAbs. This included detection of low levels of modified PilE with mono- and trisaccharide-recognizing MAbs in the disaccharide-expressing strains and of low levels of modified PilE with mono- and disaccharide-recognizing MAbs in the trisaccharide-expressing strains. For the trisaccharide reactivity in the disaccharide-expressing strains and the disaccharide reactivity in the trisaccharide-expressing strains, this could have been due to the presence of *pglE* phase variants that arose in the populations. For the monosaccharide reactivity in the disaccharide- and trisaccharide-expressing strains, it was unlikely due to a minor population of *pglA* phase-off variants as the allele in this background is not predicted to be subject to phase variability. Therefore, microheterogeneity in these cases may have reflected instances in which lipid-linked DATDH was flipped across the membrane prior to its modification by PglA.

The results for the GATDH-expressing backgrounds were strikingly distinct, and the levels of MAb reactivity were dramatically reduced. Nonetheless, weak PilE reactivity was seen for the monosaccharide-expressing strain with the monosaccharide-specific MAb and for the disaccharide-expressing strain with the disaccharide-specific MAb. It is noteworthy that moderate PilE reactivity was detected for the GATDH-based trisaccharide-expressing strain with the trisaccharide-specific MAb.

Immunochemical analysis of GATDH-based saccharides. To examine GATDH immunogenicity and antigenicity, PilE bearing the GATDH monosaccharide (in the form of purified pili) was used to immunize rabbits, and the response was monitored by immunoblotting with the full suite of *N. gonorrhoeae* strains and recombinants (Fig. 8). The response observed was specific, and reactivity was detected only with the GATDH-expressing strain. In this case, four major immunoreactive proteins were detected, and three of them were identified as the AniA nitrite reductase and the Ng1494 and Ng1043 lipoproteins (data not shown). To compare this pattern with those observed for the other glycan-expressing strains, the same immunoblot filter was probed sequentially with the MAbs. Taking into account the minor shifts in mobilities associated with the different chain lengths of the glycoforms, the overall patterns of glycosylated proteins were remarkably similar. Moreover, npg3 detected an antigen pattern in the Hex-Hex-GATDH background analogous to that seen in the other strains, although the intensity was lower. Along with data for reactivity with the equivalently modified PilE, this confirms that npg3 has a diminished but specific ability to recognize an epitope associated with Hex-Hex-GATDH glycan. Taken together, the results show that protein-linked GATDH glycan is immunogenic and antigenic and that at least the GATDH and Hex-Hex-GATDH glycoforms can act as donor substrates in general, broad-spectrum protein glycosylation.

Glycan epitopes are exposed on type IV pili. Modeling based on structural data suggested that the glycan attached at Ser63 of PilE is exposed on the pilus polymer surface (15). We directly tested this hypothesis using the MAbs and strains expressing DATDH-based glycans in transmission immunoelectron microscopy. Transmission electron micrographs demonstrated that decoration of pili with the MAbs occurred in a glycan-specific manner, with the caveat that in the case of the di- and trisaccharide-reactive MAbs the reactivity was dramatically increased in the absence of O acetylation (in the *pglI* background) (Fig. 9).

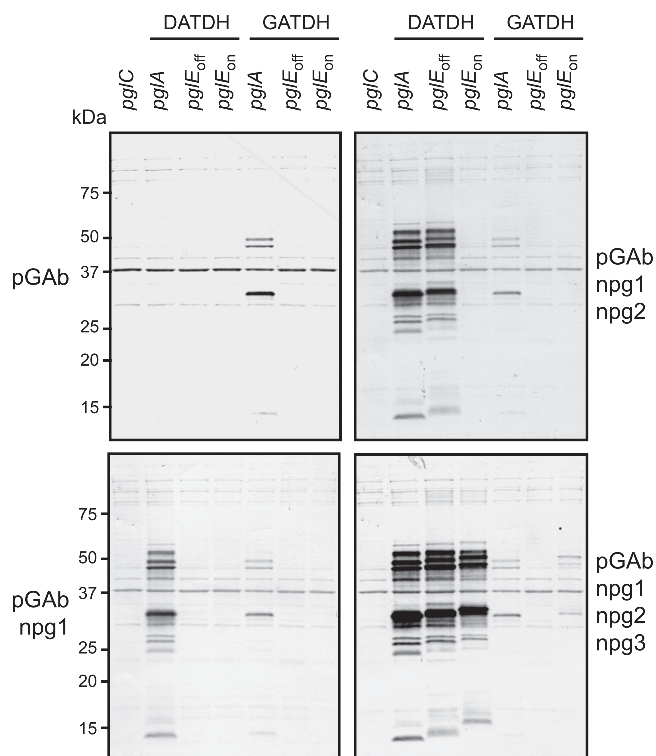

FIG. 8. Protein-associated GATDH monosaccharide is both immunogenic and antigenic. An immunoblot of whole-cell lysates from strains with defined *pgl* backgrounds was first incubated with pGAb, a polyclonal antiserum raised against pili bearing the GATDH monosaccharide. The same filter was subsequently reprobed with the npg1, npg2, and npg3 MAbs with washing and developing steps between exposures. The strains used all had a 4/3/1 background, in which *pilE* was conditionally repressed, and were KS105 (4/3/1 *pglC*), KS122 (4/3/1 *pglA*), KS101 (4/3/1), KS127 (4/3/1 $pglE_{on}$), KS312 (4/3/1 $pglB2_{8013}$ *pglA*), KS311 (4/3/1 $pglB2_{8013}$), and KS313 (4/3/1 $pglB2_{8013}$ $pglE_{on}$).

DISCUSSION

Pathogenic *Neisseria* species express a large number of cell surface components that are subject to intrastrain antigenic and phase variation. The evolution of the genetic systems underlying diversification of these surface molecules is likely driven by selection imposed by host adaptive and innate immunity. Previous studies have suggested that PilE-associated glycans might be immunogenic, as well as antigenically variable within a strain and between strains. Until now, however, the evidence for this has been somewhat limited as it has been obtained primarily with modified PilE as the immunogen and antigen and thus the specific contributions of polypeptide and glycan to the epitopes involved have been difficult to differentiate. Using neisserial glycoproteins modified with defined carbohydrates and immunization of rabbits, we demonstrated here that protein-linked glycans are both immunogenic and antigenic. In addition, the immune response was quite specific despite the use of structurally related glycans. The fact that the npg2 and npg3 MAb reactivities were dramatically enhanced by both the *pglI* null allele and chemical treatment strongly suggests that the epitopes recognized are sterically masked by O acetylation. As the *pglI* allele in *N. gonorrhoeae* N400 is not predicted to give rise to phase-off variants (70), the weak sig-

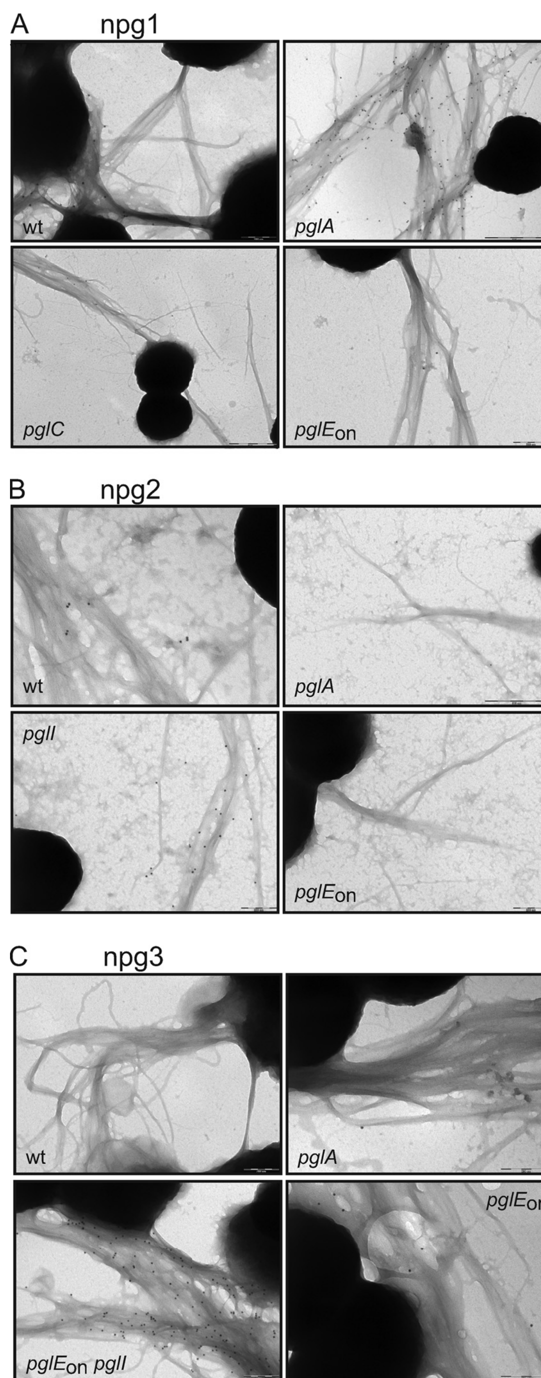

FIG. 9. Glycan MAbs react specifically with intact pili as determined by immunoelectron microscopy. Immunogold labeling and transmission electron microscopy using the monoclonal antibodies (npg1, npg2, and npg3) against different N400 *pgl* strains demonstrated their specificity by binding to pili of the corresponding strains. The strains used were KS100 (N400) (wild type [wt]), KS141 (N400 *pglA*), KS104 (N400 *pglC*), KS142 (N400 $pglE_{on}$), KS144 (N400 *pglI*), and KS304 (N400 $pglE_{on}$ *pglI*). Note that for the npg2 and npg3 MAbs, the levels of immunoreactivity were dramatically enhanced in the *pglI* background, in which glycan O acetylation was not present.

TABLE 2. Protein glycosylation genotypes and phenotypes

Strain	Genotype				Predicted glycan based on genotype	Main glycan identified[a]
	pglA	*pglE*	*pglI*	*pglB*		
N400	On, 4G[b]	Off, 14x[c]	On, 6G[b]	*pglB*	Ac-Hex-DATDH	Ac-Hex-DATDH
FA1090	On, 11G	On[b]	On, 6G[b]	*pglB*	Ac-Hex-Hex-DATDH	DATDH
MC58	On, 11G	Off, 34x	On, 13G	*pglB*	Ac-Hex-DATDH	Ac-Hex-DATDH
H44/76	On, 11G	Off, 23x	On, 10G	*pglB*	Ac-Hex-DATDH	Ac-Hex-DATDH
Z2491	On, 14G	On, 25x	On, 10G	*pglB*	Ac-Hex-Hex-DATDH	Ac-Hex-DATDH
8013	Off, 8G	Off, 39x	On, 10G	*pglB2*	GATDH	
FAM18	Off, 11G	Off, 29x	Off, 14G	*pglB2*	GATDH	
BZ 10	Off, 12G	Off, 24x	Off, 9G	*pglB2*	Hex-GATDH	
BZ 198	On, 8G	Off, 22x	Off, 11G	*pglB2*	Hex-GATDH	
ST-3787	On, 8G	On, 16x	On, 12G	*pglB*	Ac-Hex-Hex-DATDH	DATDH
ST-640	Off, 10G	On, 25x	On, 15G	*pglB*	DATDH	DATDH

[a] See Fig. 2.
[b] Not phase variable.
[c] x indicates the presence of a CAAACAA repeat.

nals seen in di- and trisaccharide-expressing backgrounds with these MAbs thus likely reflect microheterogeneity due to incomplete O acetylation. Together, the results of this work prove that a single *N. gonorrhoeae* strain can express at least five distinct antigenic glycoforms: DATDH, Hex-DATDH, Hex-Hex-DATDH, and O-acetylated variants of the latter two glycoforms.

The MAbs recognizing defined glycan epitopes made it possible to probe other fundamental aspects of neisserial protein glycosylation. Most importantly, broad-spectrum, general O-linked protein glycosylation appears to be a common feature in the three species of *Neisseria* most important for humans and is not limited to *N. gonorrhoeae* strain N400. Given its presence in the nonpathogenic species *N. lactamica*, general O-linked protein glycosylation does not appear to be a virulence factor *per se*. Second, changes in glycan structure are manifested globally, as shown by the conservation of glycoprotein patterns in *pgl* variants and mutants of *N. gonorrhoeae* N400 and *N. gonorrhoeae* FA1090, as well as *N. meningitidis* MC58. Third, the overall repertoire of predominant glycoproteins appears to be more similar within a species than between species. Analyses of more isolates of all three species are warranted in order to corroborate and extend these findings.

Although the association between PglB2 and synthesis of the basal GATDH sugar is well established, it is not clear what effect the GATDH moiety has on glycan diversification and glycan-associated phenotypes. The *pglB2* allele has been documented only for *N. meningitidis* strains and likely arose via import into a *pglB* background (48). Together with the presence of this allele in approximately 50% of isolates, it seems plausible that the prevalence of GATDH reflects the influence of positive selection. Perhaps most important in this regard is the finding that the GATDH moiety can be further modified by PglA and PglE glycosyltransferases, yielding Hex-GATDH and Hex-Hex-GATDH oligosaccharides. Since all three of these forms are antigenically distinct from the forms bearing the basal DATDH sugar, as shown by their lack of MAb reactivity, the introduction of *pglB2* into *N. meningitidis* strains and its spread may have been due to immune selection. In addition, our data suggest that GATDH may have a negative impact on acetylation of hexoses linked to it or may promote lability of the acetate bonds. Given the manner in which the strains were constructed, we cannot formally rule out the possibility that the unique O-acetylation phenotypes are not related directly to the *pglB2* allele but result from the altered gene organization and composition 3′ of *pglB2*. Together with the variable O acetylation of the GATDH disaccharide form, single *N. meningitidis* strains can express either four distinct GATDH glycoforms or five DATDH glycoforms.

Predicting the glycosylation phenotype based solely on the genotype is notoriously difficult (14). Despite this, there was a surprising degree of concordance between gene content and glycan phenotype as determined by both MAb reactivity and MS. Not only did reactivity with the MAbs correlate with the presence of *pglB*, *pglA*, and *pglE*, but minor levels of reactivity with the trisaccharide-recognizing MAb also correlated with the presence of phase-off alleles of *pglE*. Nonetheless, significant exceptions were found (Table 2). For example, on the basis of its *pglB*, $pglA_{on}$, and $pglE_{on}$ genotypes, FA1090 would have been predicted to have expressed predominantly the DATDH-based trisaccharide and thus have an immunoblot pattern similar to that observed for the N400 $pglE_{on}$ background. Instead, it has a reactivity pattern consistent with a mixture of DATDH-based monosaccharide and trisaccharide forms. In addition, a similar pattern was seen for *N. lactamica* strain ST-3787, which, based on its genotype, would be expected to express the DATDH-based trisaccharide. Together with the reactivity with the trisaccharide MAb in FA1090 and ST-3787, this microheterogeneity likely reflects reduced PglA activity, which could be due to either an altered PglA structure or the presence of an alternative activity that competes with PglA for the lipid-linked DATDH substrate. Also, these two scenarios are not mutually exclusive. Furthermore, the *pglB* $pglA_{on}$ $pglE_{off}$ genotype of *N. meningitidis* strain Z2491 resulted in the prediction that it should have an MAb reactivity pattern identical to that of MC58 and H44/76. However, unlike the results for the other two strains, no reactivity with the trisaccharide recognizing MAb was seen for Z2491 even though *pglE* is phase on in this strain. Further studies are needed to resolve the basis for these discrepancies.

The finding that immunization with glycosylated Tfp from *N. gonorrhoeae* produces a humoral immune response specific to the glycan moiety is interesting in a number of ways. First, a large number of studies in the 1960s to 1990s examined the

immunogenicity and antigenicity of *N. gonorrhoeae* pili in order to assess their potential as a gonococcal vaccine component (8, 30, 42, 60). Together with analogous studies of *N. meningitidis* pili, these studies provided serological evidence for pilus antigenic diversity, which was subsequently interpreted as a reflection of wholesale changes in pilin primary structure resulting from gene conversion-like events involving multiple pilin gene copies (55, 74). Although not appreciated at the time, it seems very likely that glycosylated pili were used in these studies and that antiglycan antibodies may have influenced at least some of the observations made. In this context, it is also worth noting that although a large number of MAbs and sera (including those raised against pilin-based peptides) have been used to define conserved and variable domains on pili, the surface-exposed epitopes involved have been precisely defined in only a very few instances (17, 55, 65, 66, 68). We suggest that glycan modification at a site in pilin which is both surface exposed and constrained at the level of primary structure may directly mask conserved epitopes at two levels. First, the glycan may physically block accessibility of polypeptide-directed antibodies. Second, the glycan may perturb antigen processing and peptide presentation such that humoral responses to conserved domains are diminished and instead directed toward the glycan, which is antigenically variable itself.

Glycan-directed antibodies might also exert an effect through glycoproteins other than PilE. In this context, it is interesting that other studies have reported evidence indicating that some of the glycoproteins identified in *N. gonorrhoeae* and now in *N. meningitidis* are exposed at the bacterial surface. These glycoproteins include a putative peptidyl-prolylisomerase (NGO1225) (38), GNA1946 (45), Ag473 (NGO1043) (28), and the nitrite reductase AniA (NGO1276) (37). It is also worth mentioning that the original evidence that AniA is expressed *in vivo* was derived from detection of AniA-reactive antibodies in diseased patients but not in normal human sera (12). Based on our findings, it is possible that the reactions observed for AniA reflect a more general antiglycan response rather than a specific AniA response.

In summary, these studies lay the foundation for detailed elucidation of the genotype-phenotype relationships underlying protein glycan diversity in important neisserial species. Given the high degree of *pgl* gene polymorphism and the presence of uncharacterized genes linked to known *pgl*-related loci, we hypothesize that the total repertoire of protein glycans may be significantly larger than that identified so far. In fact, the protein-associated glycan diversity may exceed that documented for neisserial lipooligosaccharides and meningococcal capsular polysaccharides. The findings of this study and the reagents used should also facilitate efforts to evaluate the potential presence of glycoproteins in outer membrane vesicle (OMV)-based vaccines currently in use or being tested (25) and to evaluate whether humans generate either humoral or innate immune responses to the protein glycan moieties during carriage or disease.

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