



# Genotypic and Phenotypic Characterization of the O-Linked Protein Glycosylation System Reveals High Glycan Diversity in Paired Meningococcal Carriage Isolates

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**ABSTRACT** Species within the genus *Neisseria* display significant glycan diversity associated with the O-linked protein glycosylation (*pgl*) systems due to phase variation and polymorphic genes and gene content. The aim of this study was to examine in detail the *pgl* genotype and glycosylation phenotype in meningococcal isolates and the changes occurring during short-term asymptomatic carriage. Paired meningococcal isolates derived from 50 asymptomatic meningococcal carriers, taken about 2 months apart, were analyzed with whole-genome sequencing. The O-linked protein glycosylation genes were characterized in detail using the Genome Comparator tool at the <https://pubmlst.org/> database. Immunoblotting with glycan-specific antibodies (Abs) was used to investigate the protein glycosylation phenotype. All major *pgl* locus polymorphisms identified in *Neisseria meningitidis* to date were present in our isolate collection, with the variable presence of *pglG* and *pglH*, both in combination with either *pglB* or *pglB2*. We identified significant changes and diversity in the *pgl* genotype and/or glycan phenotype in 96% of the paired isolates. There was also a high degree of glycan microheterogeneity, in which different variants of glycan structures were found at a given glycoprotein. The main mechanism responsible for the observed differences was phase-variable expression of the involved glycosyltransferases and the O-acetyltransferase. To our knowledge, this is the first characterization of the *pgl* genotype and glycosylation phenotype in a larger strain collection. This report thus provides important insight into glycan diversity in *N. meningitidis* and into the phase variability changes that influence the expressed glycoform repertoire during meningococcal carriage.

**IMPORTANCE** Bacterial meningitis is a serious global health problem, and one of the major causative organisms is *Neisseria meningitidis*, which is also a common commensal in the upper respiratory tract of healthy humans. In bacteria, numerous loci involved in biosynthesis of surface-exposed antigenic structures that are involved in the interaction between bacteria and host are frequently subjected to homologous recombination and phase variation. These mechanisms are well described in *Neisseria*, and phase variation provides the ability to change these structures reversibly in response to the environment. Protein glycosylation systems are becoming widely identified in bacteria, and yet little is known about the mechanisms and evolutionary forces influencing glycan composition during carriage and disease.

**KEYWORDS** *Neisseria meningitidis*, carriage, whole-genome sequencing, O-linked protein glycosylation, glycan diversity, microheterogeneity

*Neisseria meningitidis* is a Gram-negative bacterium colonizing the human oropharynx, normally without causing disease. The carriage prevalence has been shown to range widely (from 3% to 20%) and is influenced by factors such as geography, age,

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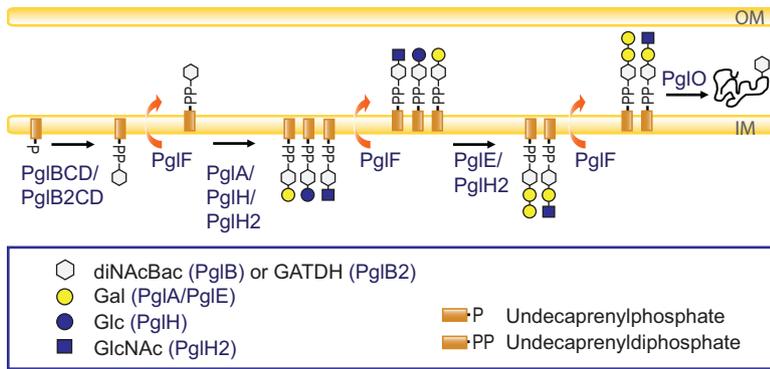
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**FIG 1** Simplified overview of the *O*-linked protein glycosylation pathway in *Neisseria*. The current model of the broad-spectrum *O*-linked glycosylation pathway expressed by species within the genus *Neisseria* is shown. OM, outer membrane; IM, inner membrane.

crowded societies, contact with a case, and the epidemic/endemic situation (1). Meningococci may occasionally invade the bloodstream and progress to meningococcal disease with severe meningitis and septicemia.

Bacteria covalently attach different glycan structures to diverse molecules such as lipids and proteins. *N. meningitidis* exhibits a general *O*-linked glycosylation system in which several surface-exposed and periplasmic proteins are glycosylated. The major glycoprotein, PilE, is the building subunit of pili, which are important virulence factors. In addition, the meningococci produce lipopolysaccharides (LPS), peptidoglycan, and capsular polysaccharide. These molecules are synthesized by distinct but similar pathways where glycosyltransferases perform the sequential addition of monosaccharides onto a lipid carrier.

*N. meningitidis* isolates can be classified into 12 different serogroups based on their polysaccharide capsule. The majority of disease cases is caused by the members of serogroups A, B, C, W, X, and Y (2, 3). Some strains of *N. meningitidis*, especially those isolated from the oropharynx of healthy carriers, can also be rendered noncapsulated through regulation of the capsule production, either due to phase variation of the capsule synthesis genes (4) or due to inactivation of genes in the capsule gene cluster (*cps*) (5). Other strains lack the genes for capsule synthesis, modification, and transport (*cps* operon) (6). In these strains, the *cps* operon is replaced by a noncoding region (*cnl* region) that resembles that of other neisserial species, such as *Neisseria gonorrhoeae* and *Neisseria lactamica*. The capsule is believed to represent an advantage in invasive disease by helping the bacteria to evade complement-mediated and phagocytic killing by the host (2), but cases where noncapsulated meningococci have caused disease have also been reported (7–10). On the other hand, loss of capsule seems to enhance colonization (11).

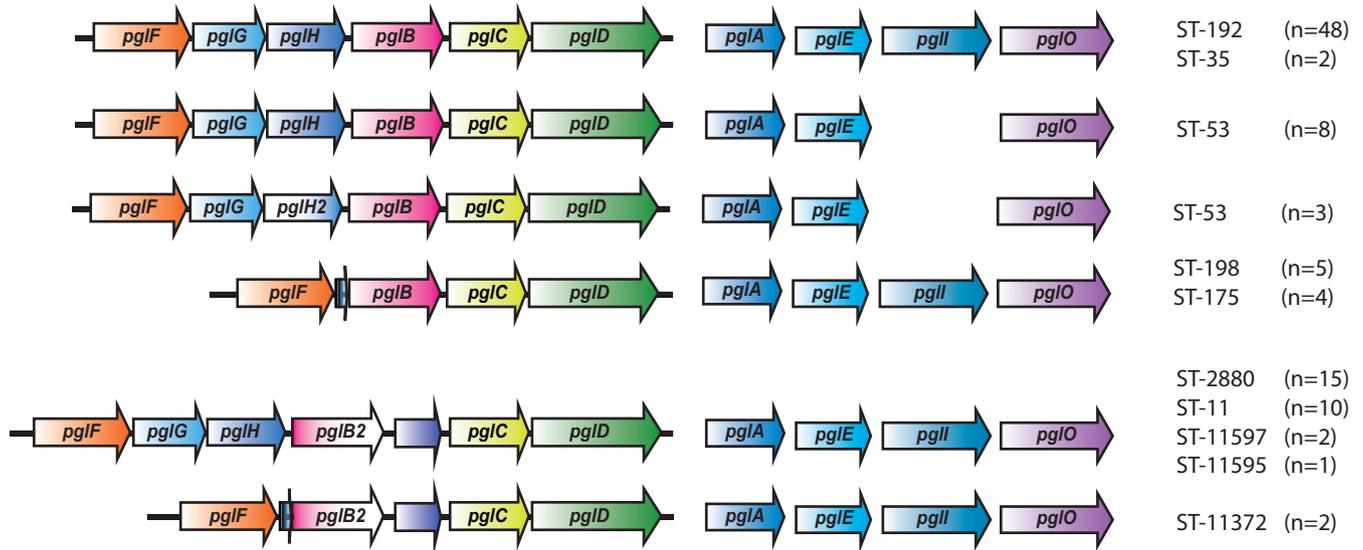
*Neisseria* species express a broad spectrum of *O*-linked protein glycosylation (12–14). The glycoproteins identified in *N. gonorrhoeae* are predicted to be lipoproteins or transmembrane proteins localized in the periplasm or cell surface (13, 15). The closely related *N. meningitidis* species is predicted to have a similar glycoprotein repertoire. *Neisseria* species display high glycoform variability (12, 13, 16–18), and a simplified overview of the glycosylation pathway and the glycosyltransferases that are involved is shown in Fig. 1. The *pgl* core locus products function in the synthesis of undecaprenyldiphosphate (UndPP) monosaccharides (PglB/PglB2, PglC, and PglD) and in the translocation to the periplasm (PglF). PglB is a bifunctional protein with an acetyltransferase domain and a phosphoglycosyltransferase domain responsible for synthesis of *N,N'*-diacetylbaucillosamine (diNacBac). The variant bifunctional PglB2 protein has an ATP grasp domain responsible for synthesis of glyceramido-acetamido trideoxyhexose (GATDH). The N-terminal phosphoglycosyltransferase domains in PglB and PglB2 are, however, identical. PglA is a galactosyltransferase that acts on diNacBac and GATDH,

whereas PglE is a galactosyltransferase that elongates the PglA-generated disaccharide to a trisaccharide. PglH is a glucosyltransferase that acts on both diNAcBac and GATDH to generate glucose (Glc)-containing disaccharides. Recently, a PglH2 variant that acts on both diNAcBac and GATDH to generate GlcNAc-containing disaccharides was identified (19). In addition, PglG was characterized as a glycosyltransferase that elaborates the undecaprenyl diphosphate-linked disaccharide in *Neisseria elongata* subsp. *glycolytica* with di-*N*-acetyl hexuronic acid (20), but no PglG activity has been detected in pathogenic *Neisseria* species. The neisserial glycoforms can be further modified via O-acetylation mediated by the acetyltransferase PglI to further increase neisserial glycan diversity (see Fig. S1 in the supplemental material). To date, evidence has shown that more than 30 different glycoforms can be synthesized by combinations of glycosyltransferases and the O-acetylase within neisserial species (21).

Whole-genome sequencing (WGS) of *Neisseria* isolates has revealed extensive genomic variation both in gene sequence diversity and in gene content, as a consequence of homologous recombination (22). This is also the case for *pgl* genes. First, there are two gross polymorphisms at the *pgl* loci, namely, the variable presence of *pglG* and *pglH* and the mutually exclusive presence of *pglB* and *pglB2*. The variable presence of two open reading frames (ORFs) in the *pgl* locus includes a putative glycosyltransferase gene, *pglG*, in addition to a glucosyltransferase-expressing gene, *pglH*. Strains lacking these two ORFs retain the first 40 bp of *pglG* and the last 100 bp of *pglH*. The *pglB2* allele is found among commensal neisserial species and *N. meningitidis* strains but not among *N. gonorrhoeae* strains. Second, *pglA*, *pglE*, and *pglI* are absent in a significant number of commensal species, except *N. lactamica*. Third, polymorphisms also exist at the gene level as described for *pglH* and *pglH2*, where only one nonsynonymous mutation is accountable for the glycoform switch from Glc to GlcNAc (19). Fourth, several *pgl* genes contain mononucleotide or polynucleotide repeat tracts subjected to phase variation and, thus, to reversible on/off expression. Changes in the number of repeats arise by slipped-strand mispairing during DNA replication. The *pgl* genes have the phase-variable tract within their coding region; consequently, an off configuration results in a nonfunctional, truncated protein due to frameshifting of the correct reading frame. Differential on/off expression can then facilitate the adaptation of bacteria to fluctuations in the environment.

Genetic variation is believed to facilitate escape of the adaptive immune response. A recent study detected homologous recombination within the *pgl* loci through genomic analysis of 100 African serogroup A isolates representing the clonal replacement of hypervirulent meningococcal clone sequence type 7 (ST-7) by the ST-2859 descendant clone. It was suggested that this recombination event emphasizes the role of protein glycosylation diversity in immune evasion (23). It has also been shown that *N. meningitidis* isolates that express the antigenic invariable class II pilin display additional pilin glycosylation sites, which supports a model where these strains evade the immune system by changing their sugar structure rather than their pilin structure (24).

The isolates analyzed in this study were collected as part of a longitudinal carriage study in Ethiopia in 2014 (25). A group of individuals identified as asymptomatic carriers of *N. meningitidis* in a cross-sectional study were followed weekly for asymptomatic carriage in a 2-month period. Paired meningococcal carriage isolates collected 6 to 9 weeks apart from 50 individuals were analyzed by WGS. A high degree of within-host genetic changes was revealed, with genes involved in glycosylation being among those showing the highest degree of genetic variation. All these genes showed phase-variable mononucleotide differences resulting in differential on/off expression within the paired isolates. This observation led us to further characterize each of the *pgl* genes, as well as how their genetic variability alters the protein glycan repertoire expressed within these paired isolates.



**FIG 2** Protein glycosylation gene variants in meningococcal carriage isolates. An overview of the *pgl* genes present in the collection is shown. There are two major polymorphisms documented in the *pgl* locus of strains of *Neisseria*. The configuration associated with *pglG* and *pglH* represents the ancestral form, and the other corresponds to the deleted form. Ancestral and deleted forms are found in combination with both *pglB* and *pglB2* allele variants.

**RESULTS**

**Major polymorphism in *pgl* gene content within a small geographic area.** The whole genomes of paired *N. meningitidis* isolates collected approximately 2 months apart from the same asymptomatic carrier were shown to have a high degree of within-host genetic changes (25). As three genes involved in the O-linked protein glycosylation system were identified among the genes that differed in  $\geq 30\%$  of paired isolates, we have here extended the analysis to include all *pgl* genes in these 100 meningococcal carriage isolates belonging to 10 STs.

The *pgl* gene compositions represented in our strain collection are shown in Fig. 2. There are two known major polymorphisms within the *pgl* locus. First, the monosaccharide can be either diNAcBac or GATDH, depending on the presence of *pglB* or *pglB2* alleles, respectively. Within our collection, 70 isolates, belonging to ST-35, ST-53, ST-175, ST-192, and ST-198, carried the *pglB* allele and thus expressed diNAcBac glycoforms, and 30 isolates, belonging to ST-11, ST-2880, ST-11372, ST-11595, and ST-11597, carried the *pglB2* allele variant responsible for GATDH glycoforms (Table 1). A second form of polymorphism at the *pgl* locus involves the variable presence of two linked ORFs, *pglG* together with the *pglH* or the *pglH2* variant. Both diNAcBac and GATDH glycoforms can be further extended by addition of Glc or GlcNAc, as determined by the presence of *pglH* or *pglH2* variant alleles, respectively. In our collection, *pglH* was present in 86 isolates and *pglH2* in only three ST-53 isolates, while the linked *pglG* and *pglH* ORFs were absent in all 11 isolates belonging to ST-175, ST-198, or ST-11372 (Table 1 and Fig. 2).

The *pgl* locus composition was conserved within the different STs, except for ST-53, where both *pglH* and *pglH2* variants were observed, as shown in Fig. 2. The ST-53

**TABLE 1** Major polymorphism in the *pgl* locus

Isolate category	No. of isolates with indicated status with respect to <sup>a</sup> :	
	<i>pglH</i> and <i>pglH2</i>	<i>pglB</i> and <i>pglB2</i>
Gene absent	11	0
Allele variant	89 (86 + 3)	100 (70 + 30)
Total	100	100

<sup>a</sup>Values in parentheses represent numbers of isolates with the indicated status with respect to the genes listed in the column headings.

isolates were previously found to form two different subclusters by core genome multilocus sequence typing (cgMLST), Bayesian analysis of population structure (BAPS), and single nucleotide polymorphism (SNP) analysis (25), and our analysis revealed that one subcluster had *pglH* while the other had the *pglH2* variant allele. In addition, *pglI* was absent in both ST-53 subclusters, eliminating the possibility of O-acetylation of the different glycoforms synthesized in this sequence type.

**Allelic variation in the *pgl* genes within the paired isolates.** We compared the allelic differences in each of the *pgl* genes within the paired isolates ( $n = 46$ ) (Table 2), excluding four pairs of isolates that previously were shown to belong to different strains. Three individuals (individuals 47, 48, and 50) carried strains with different STs at the two time points, and a fourth individual (individual 49) clearly carried different strains belonging to the same ST, ST-53 (25) (see Table S1 in the supplemental material). The three pairs with a change of ST differed in all *pgl* genes. In individual 49, all *pgl* genes but *pglC* and *pglD* differed between the two isolates. In fact, different gene compositions were observed in the isolates consecutively colonizing these four individuals, individual 47 (with or without *pglG* and *pglH*), individual 48 (with or without *pglI*, *pglB*, and *pglB2*), individual 49 (with *pglH* and *pglH2*) and individual 50 (with *pglB* and *pglB2*) (Table S1).

The sequence was incomplete for one or both of the isolates of the pair for some genes, and those genes were therefore excluded from the analysis. Alignment and comparison of all *pgl* gene sequences were performed, and the mechanisms involved in the genetic differences observed were assigned as point mutations, deletions, or homologous recombinations. Table 2 shows that the genetic differences between pairs ranged from 60% to no change and that phase variation was the most frequent mechanism. Moreover, all phase-variable *pgl* genes showed a high degree of allelic differences due to their heptanucleotide (*pglE*) or mononucleotide (*pglA*, *pglH*, *pglG*, *pglI*) repeat tract variations. The heptanucleotide variation in *pglE* was the most extensive, with variation in 60% of the pairs. The levels of variation between pairs in the other phase-variable genes ranged from 20% to 44%. The numbers of repeats also differed between the *pgl* genes, where *pglE* had 9 to 59 heptanucleotide repeats, whereas all the other *pgl* genes had shorter mononucleotide repeats (8 to 19 cytosines or guanines) (Fig. 3). The largest switches within pairs were observed in the *pglE* gene of individuals 8, 23, and 34.

The *pgl* core locus genes *pglB/B2*, *pglC*, and *pglD*, responsible for synthesis of monosaccharide, were the most highly conserved *pgl* genes, with no genetic changes between the paired isolates (Table 2).

**Polymorphisms within and adjacent to phase-variable repeat tracts.** In addition to the phase variation mechanism accountable for many of the genetic differences between pairs, there were additional polymorphisms (mechanism assigned as point mutation, deletion, or homologous recombination) observed around the phase-variable regions of *pglG*, *pglH*, *pglI*, and *pglA* (Table 2).

There were some differences between and within STs adjacent to the polyguanosine [poly(G)] tract in *pglA*, while the flanking sequences were highly similar (see Fig. S2 in the supplemental material). One of the nine isolates had a deletion of 13 nucleotides just prior to the poly(G) tract in ST-11, and this deletion was not observed in isolates from any of the other STs. Both ST-35 isolates had a guanosine (G)-to-adenine (A) point mutation in the middle of the poly(G) tract, thus interrupting the mononucleotide repeat of the *pglA* gene and presumably resulting in constitutive PglA expression. Two subjects (individuals 15 and 44) carrying ST-2880 isolates had a two-nucleotide deletion upstream of the poly(G) tract at the second time point.

In ST-2880 isolates, we observed an AGG deletion 3' to the poly(G) tract of *pglI* for both time points for individuals 15 and 17 and at the second time point for individuals 2, 5, and 10 (Fig. S3).

The *pglH* locus showed polymorphisms between STs along the whole gene (Fig. S4). A 5' point mutation (guanosine to cytosine) adjacent to the polycytosine [poly(C)] tract was found in individuals 20, 22, and 33 carrying ST-192 isolates.

**TABLE 2** Overview of genetic changes in *pgl* genes in paired meningococcal carriage isolates<sup>a</sup>

NEIS no. <sup>b</sup>	Gene	Gene product	PV tract	% of pairs <sup>c</sup> with genetic differences (n = 46)	Mechanism(s) and proportion(s) of pairs <sup>d</sup> with genetic differences					
					All STs (n = 46)	ST-11 (n = 5)	ST-53 (n = 4)	ST-192 (n = 23)	ST-2880 (n = 7)	
NEIS0568	<i>pglE</i>	Glycosyltransferase	CAACAAA <sup>e</sup>	60	PV, 60%	PV, 100%	PV, 50%	PV, 50%	PV, 71%	
NEIS0400	<i>pglH/H2</i>	Glycosyltransferase	Poly(C)	44	PV, 44 %; PM, 4%	PV, 60%	No change <sup>f</sup>	PV, 70%; PM, <sup>g,h</sup> 9%	PV, 14%	
NEIS0380	<i>pglI</i>	O-Acetyltransferase	Poly(G)	41	PV, 41 %; PM, 9%	PV, 60%; PM, <sup>h</sup> 40%	Gene absent	PV, 52%	PV, 43%; D, <sup>g,h</sup> 43%	
NEIS0401	<i>pglG</i>	Putative glycosyltransferase	Poly(C)	33	PV, 30 %; R, 4%; PM, 2%	PV, 40%; R, 20%	PV, 50%	PV, 70%; R, <sup>h</sup> 9%; PM, <sup>h</sup> 4%	No change	
NEIS0213	<i>pglA</i>	Glycosyltransferase	Poly(G)	22	PV, 20%; PM, 4%; R, 2%; D, 2%	PV, 20%; D, <sup>h</sup> 20%	No change	PV, 13%	PV, 43%; PM, <sup>g,h</sup> 29%	
NEIS0402	<i>pglF</i>	Flippase		11	PM, 11%	PM, 20%	PM, <sup>g</sup> 75%	No change	No change	
NEIS0539	<i>pglO</i>	Oligosaccharyltransferase		9	PM, 7%; R, 2%	No change	PM, 25%	PM, <sup>g</sup> 9%; R, 4.5%	No change	
NEIS0399/ NEIS2838	<i>pglB/B2</i>	N-Acetyltransferase/phospho-glycosyltransferase		No change	No change	No change	No change	No change	No change	
NEIS0397	<i>pglC</i>	Aminotransferase		No change	No change	No change	No change	No change	No change	
NEIS0396	<i>pglD</i>	Dehydratase		No change	No change	No change	No change	No change	No change	

<sup>a</sup>D, deletion; PM, point mutation; PV, phase variation; R, recombination.

<sup>b</sup>The annotated genes are accessible in Genome Comparator (<https://pubmlst.org/>).

<sup>c</sup>Data represent pairs with any genetic differences. Pairs from individuals carrying different strains at the two time points were excluded from the analysis.

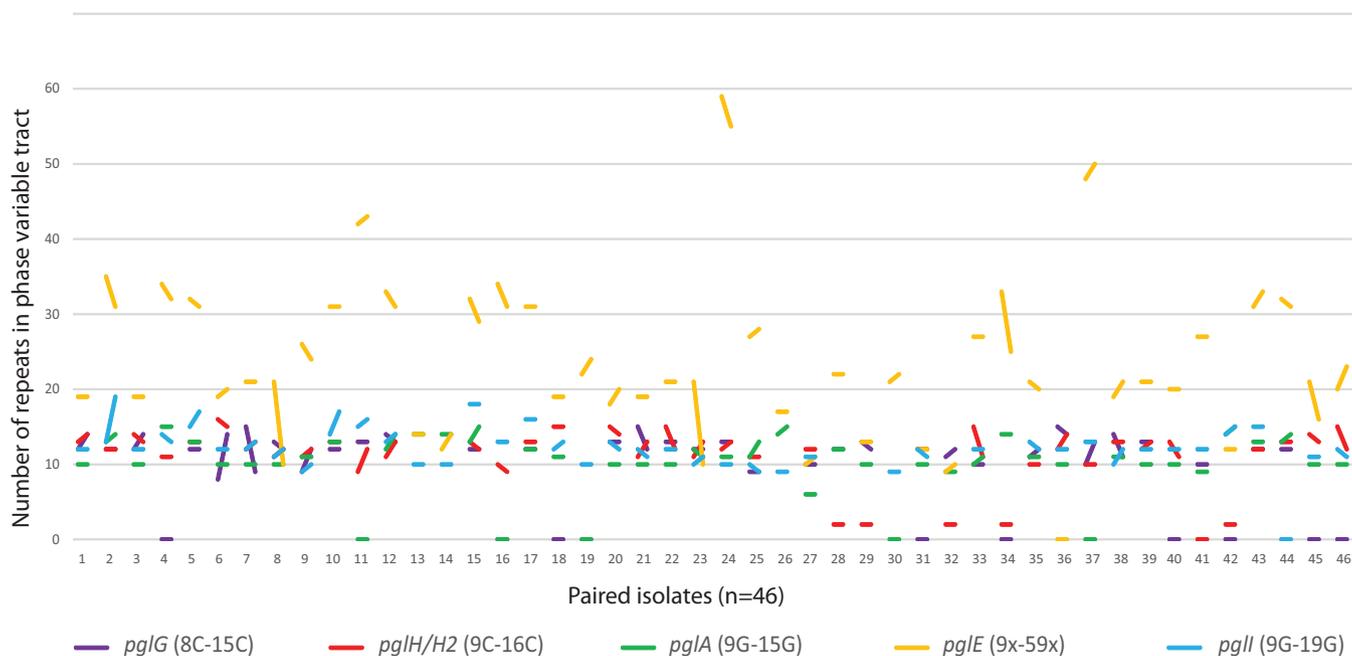
<sup>d</sup>In some pairs, two or more mechanisms of genetic change were observed.

<sup>e</sup>ST-192 and ST-198 have different repeats as detailed in Table S5.

<sup>f</sup>No PV tract.

<sup>g</sup>The same in all pairs, probably due to recombination.

<sup>h</sup>Close to PV tract.

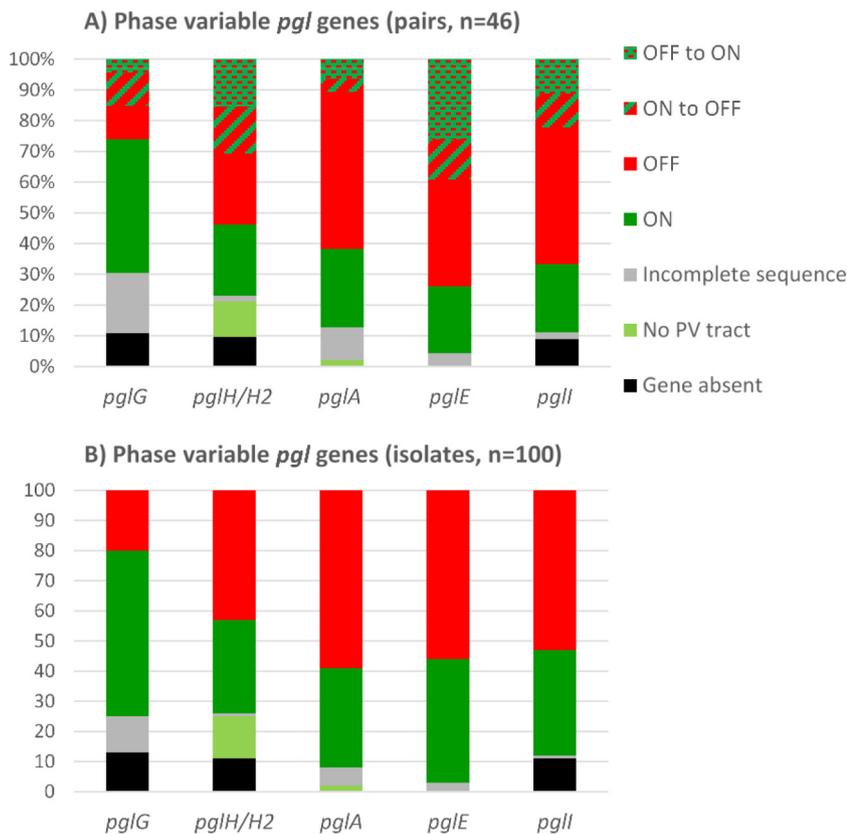


**FIG 3** Phase variation of *pgl* genes within paired isolates. The variations in the lengths of the phase-variable tracts between all paired isolates ( $n = 46$ ) are represented by a line for each of the *pgl* genes. The poly(C) tract in *pglG* ranges between 8 Cs and 15 Cs for all individuals, with the largest change seen in individuals 6 and 7. The poly(C) tract in *pglH/H2* ranges between 9 Cs and 16 Cs for all individuals, with the largest change seen in individual 33. Individuals 28, 29, 32, 34, and 42 lacked the poly(C) tract in *pglH* (which has only three cytosines in this position). The poly(G) tract in *pglA* ranges between 9 Gs and 15 Gs, with the largest change seen in individual 2. The poly(G) tract in *pgl* ranges between 9 Gs and 19 Gs for all individuals. The heptanucleotide phase-variable tract in *pglE* has between 9 and 59 repeats, with the largest change seen in individuals 8, 23, and 34. The line between the two isolates is horizontal for identical repeat numbers. Pairs where the sequence(s) of one or both isolates was incomplete are indicated at the bottom, along the x axis. Note that a missing line for *pglG*, *pglH*, or *pglI* within an individual indicates the absence of the gene.

The *pglE* gene contained the heptanucleotide CAACAAA repeat for most isolates, except the ST-198 isolates and some of the isolates within ST-192 (Fig. S5). Most of the ST-192 isolates (46/48) had a longer sequence of CAACAAA repeats together with one or two repeat variants (CAATAAA) included in the same tract, possibly representing a mechanism that stabilizes the phase-variable region. One ST-192 isolate had a CAC CCGA repeat sequence variant. In ST-198, there was also an alternative heptanucleotide repeat unit, CACCCAA.

**Homologous recombination in *pgl* genes.** Several studies have shown that the *pgl* locus is subject to high genetic variation due to homologous recombination. Although the isolates used in this study were sampled only 6 to 9 weeks apart, homologous recombination was observed in a variety of genes. In individual 40, the *pglO* gene had two nucleotide substitutions within a 26-bp region (Fig. S6), and in individuals 7 and 46, blocks of nucleotides 3' to the poly(C) region were changed in the *pglG* gene (Fig. S7).

Some of the changes observed in close proximity to the phase-variable tracts were probably also due to homologous recombination events since they were identical in different isolate pairs. An identical two-nucleotide difference was observed 5' in *pglA* of ST-2880 isolates in individuals 15 and 44. In contrast, a two-nucleotide insertion in the *pglA* gene was seen 3' in ST-11372 isolates of individual 26 (Fig. S2). We also observed a two-nucleotide insertion in *pglH* 5' to the poly(C) in individuals 20, 22, and 33 carrying ST-192 isolates (Fig. S4) and in *pglI* for individuals 2, 5, and 10 (ST-2880 isolates) and 11 (ST-11 isolates) (Fig. S3). Altogether, these data indicate that the phase-variable *pgl* gene regions are subjected to both phase variability and genetic variation likely introduced by homologous recombination. Furthermore, we observed the same point mutation within the *pglF* gene in three of four paired ST-53 isolates and within *pglO* in four (9%) of paired ST-192 isolates. All these paired isolates within STs had the exact same variation between the two sampling points, thus probably excluding the possibility of a random insertion/deletion.



**FIG 4** Phase-variable *pgl* genes. Overviews of the status of phase-variable *pgl* genes (on/off configurations) for paired isolates ( $n = 46$ ) (A) and individual isolates ( $n = 100$ ) (B). The distributions of absent genes, incomplete sequences, on configurations, and off configurations are shown for *pglA*, *pglE*, *pglI*, *pglG*, and *pglH*. In addition, *pglA* and *pglH* have some alleles without the phase-variable tract and these are constitutively on (No PV tract) (i.e., no phase variation tract).

**Phase variation of *pgl* genes and their impact on protein expression.** The *pgl* genes contain mononucleotide or polynucleotide repeat tracts within their coding regions. A change in the number of repeats is reversible, and these genes can therefore change between functional protein expression (on configuration) and nonfunctional protein expression because of premature stop codons and truncation in the noncoding reading frame (off configuration). For all phase-variable genes, sequence alignments and assessments of the homopolymeric tracts were performed (Fig. 3), and the status of each phase-variable gene (on/off configuration) is summarized based both on paired isolates (Fig. 4A; pairs,  $n = 46$ ) and on all isolates (Fig. 4B; isolates,  $n = 100$ ). This analysis revealed that all isolates, except two ST-35 isolates, had poly(G) tracts in *pglA* and that the lengths ranged between 9 and 15 Gs. *pglA* genes without poly(G) tracts are constitutively in an on configuration. For *pglA*, 33 isolates had the on configuration while 59 isolates were off; furthermore, both isolates were on in 12 pairs, both isolates were off in 24 pairs, and a change of poly(G) tract length resulted in a switch in on/off status in five pairs. The *pglA* gene was turned off in two of these individuals, while the *pglA* gene was turned on in three individuals.

All of the *pglE* alleles contained heptanucleotide repeat tracts with various lengths. We observed between 9 and 59 repeat units. However, the Illumina technology is not capable of covering these long repetitive sequences; therefore, we could not determine the on/off status of this gene by WGS. The *pglE* gene was therefore PCR amplified and analyzed by Sanger sequencing in all isolates. Comparing the Sanger and Illumina MiSeq sequencing data, the results were consistent only for the shorter *pglE* repeat tracts (17% of the isolates). The Sanger analysis revealed that for *pglE*, 41 isolates (10 pairs) had the on configuration while 56 (16 pairs) were off. Among the paired isolates,

27 had a change of heptanucleotide tract length and 18 of these resulted in a switch in on/off status. Interestingly, the *pglE* genes were switched from on to off in 6 individuals, whereas this gene was switched from off to on in 12 individuals.

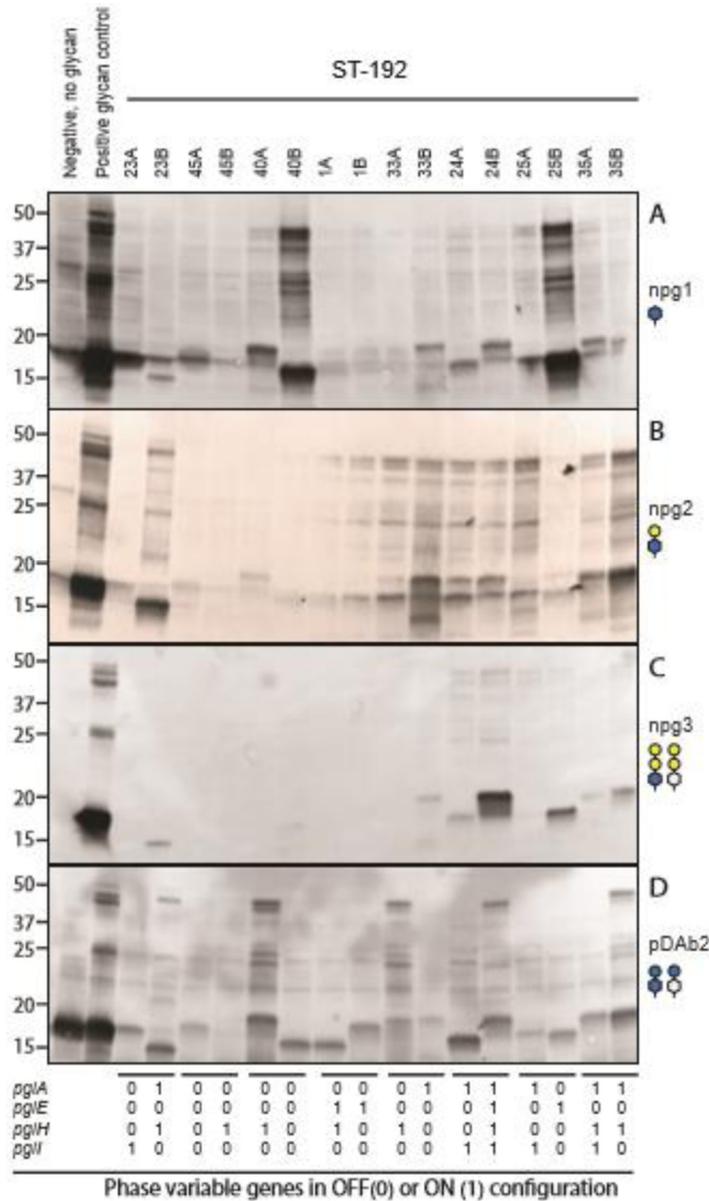
The *pglI* gene was absent in 11 isolates, all belonging to ST-53. The *pglI* alleles were all phase variable, containing poly(G) tracts with 9 to 19 Gs. In this collection, 35 isolates (10 pairs) had the *pglI* gene switched on and 53 (20 pairs) had this gene switched off, and there was a change in the on/off configuration in 10 of the paired isolates. Here, we observed equal numbers of changes of configuration in the two directions.

The *pglG* and *pglH* insertion was variably present in the isolates as detailed above. Although *pglG* is not shown to be functional in meningococci, the phase-variable poly(C) tract consists of 8 to 15 cytosines (Cs) and was presumably in the on and off configurations in 55 and 20 isolates (20 and 5 pairs), respectively. Fourteen of the paired isolates had a change of poly(G) tract length, and 7 of these resulted in a switch in predicted on/off status. The *pglG* gene was turned off in five of these individuals, and the *pglG* gene was turned on in two individuals. The *pglH* gene lacked the phase-variable tract in 14 isolates. These were isolates within ST-53, ST-11595, and ST-11597. Of the 75 strains that had the homopolymeric poly(C) tract (9 to 16 Cs), there were changes in 20 of the pairs; 31 isolates (12 pairs) had *pglH* on and 43 (12 pairs) had *pglH* off. We observed an on-to-off switch in 16 of the pairs, and there were equal numbers of changes of configuration in the two directions.

#### **The glycosylation phenotype revealed a high degree of microheterogeneity.**

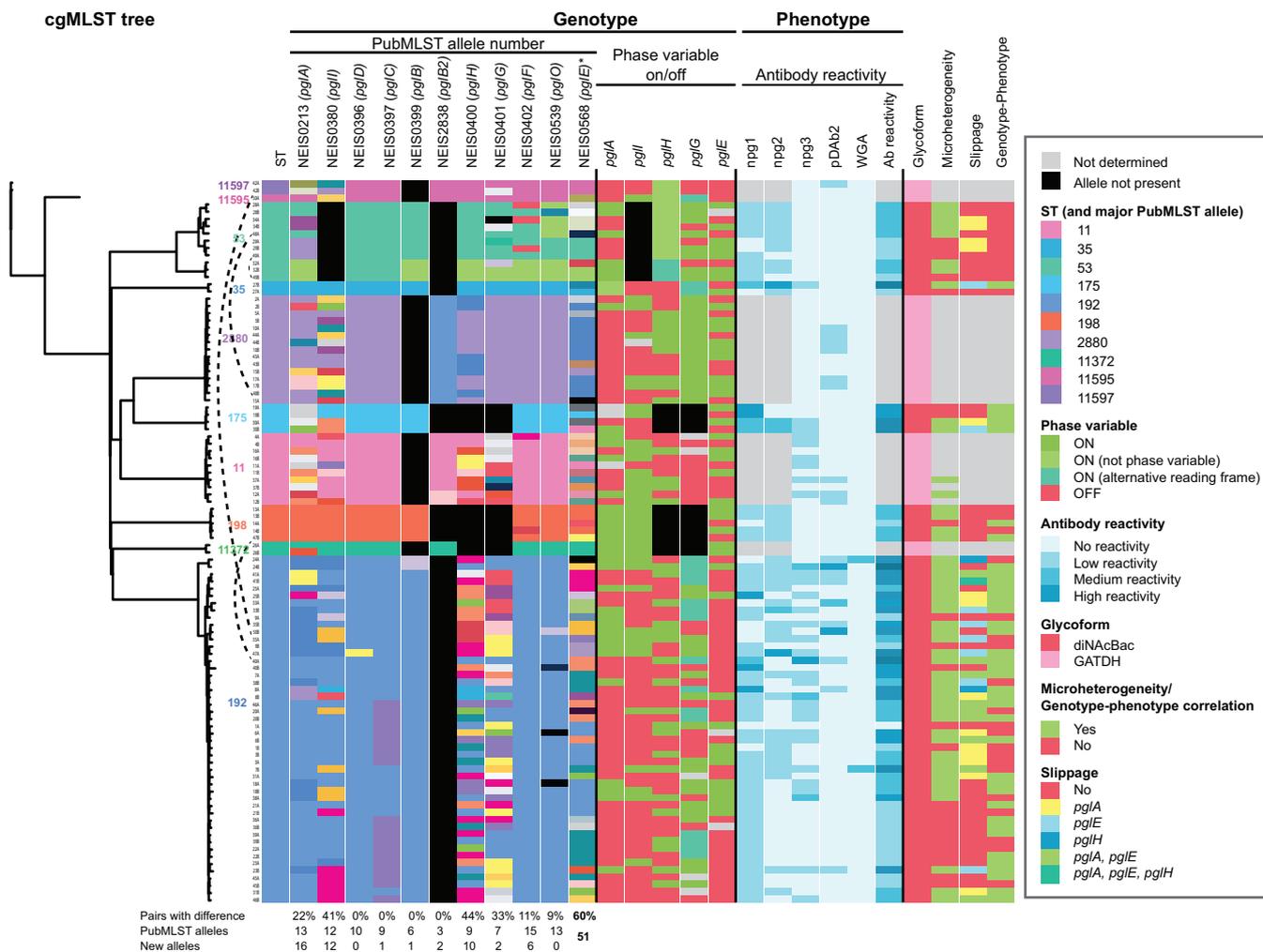
All isolates were screened for the protein glycosylation phenotype using immunoblotting with glycan-specific antibodies. For strains carrying *pglB*, we used glycan-specific monoclonal antibodies (MAbs) (npg1, npg2, and npg3), polyclonal antibody pDAb2, and the lectin wheat germ agglutinin (WGA) which together recognize all known diNAcBac-based glycoforms. The strains with *pglB2* could not be analyzed in such detail, as only pDAb2/WGA and npg3 can recognize both diNAcBac- and GATDH-based *pglH/H2* disaccharides and trisaccharides, respectively. From the immunoblots, we assigned and scored reactivity for each strain as no reactivity (score of 0), low reactivity (score of 1), moderate reactivity (score of 2), or high reactivity (score of 3). We scored no reactivity (0) when the level of reactivity was comparable to that seen with the glycosylation null mutant and high reactivity (3) when it was comparable to that seen with the positive glycosylation mutants that have previously been well characterized (12, 19). We also summarized the scoring for all immunoblots to compare the overall levels of observed glycosylation in these strains. An overview of all phenotypic glycosylation results is shown in Table S1 (see also Fig. 6) together with the genotypic data for these isolates.

Analysis and interpretation of the glycosylation phenotype can be complicated, and as an example, we present all details from the immunoblots with selected pairs of representative ST-192 isolates in Fig. 5. ST-192 isolates have the *pglB* allele, and immunoblotting showed variable reactivity with all antibodies investigated. Overall, the ST-192 isolates displayed a high degree of glycan microheterogeneity, as most isolates (67%) reacted with two or more antibodies (Table S1). As shown in Fig. 5, changes in glycosylation patterns of the paired isolates were observed between the two different time points as a consequence of phase variation in individuals 23, 40, 33, 24, 25, and 35. For individual 23, an off-to-on configuration switch in both *pglA* and *pglH* changed the synthesis from mainly diNAcBac (npg1) to both *pglA* disaccharide diNAcBac-Gal (npg2) and *pglH* disaccharide diNAcBac-Glc (pDAb2). In isolates from individual 40, *pglH* was turned off and the synthesis changed from diNAcBac-Glc (pDAb2) to diNAcBac (npg1). For individual 33, *pglA* was turned on and *pglH* off and synthesis subsequently changed from mainly diNAcBac-Glc (pDAb2) to diNAcBac-Gal (npg2). In individual 24, both *pglE* and *pglH* were turned on and both diNAcBac-Gal-Gal (npg3) and diNAcBac-Glc (pDAb2) appeared at a later time point. In individual 25, *pglA* was turned off and a change from diNAcBac-Gal (npg2) to diNAcBac (npg1) was subsequently seen. In addition, *pglE* was turned on. However, since *pglA* was off in this isolate, there were only small amounts of the trisaccharide diNAcBac-Gal-Gal (npg3). For individual 35, an on-to-off configu-



**FIG 5** Protein glycosylation in ST-192 isolates. Data represent the reactivity of endogenous glycoproteins following immunoblotting with the glycan-specific monoclonal antibodies npg1 (specific for diNAcBac) (A), npg2 (specific for diNAcBac-Gal) (B), and npg3 (specific for both diNAcBac and GATDH trisaccharides) (C) and pDAb2 polyclonal antibody (specific for diNAcBac-Glc) (D). The strains used were as follows: lane 1, KS104 (*pgI*C; negative control with no glycan synthesized); lane 2, positive glycan controls, including KS141 (N400 *pgIA*) (A), KS100 (N400) (B), KS142 (N400 *pgIE<sub>on</sub>*) (C), and KS966 (*pgIA pgII lct::pgIH2<sub>SK-03-1035</sub>*) (D); lanes 3 to 18, *N. meningitidis* ST-192 isolates from time points A and B as indicated. The *pgIA*, *pgIE*, *pgIH*, and *pgII* genotype configurations for each isolate are shown below the immunoblots.

ration switch in *pgII* increased the detection of *pgIA* disaccharide diNAcBac-Gal (npg2), *pgIH* disaccharide diNAcBac-Glc (pDAb2), and trisaccharide diNAcBac-Gal-Gal (npg3). Forty percent of the ST-192 isolates had npg2 reactivity although *pgIA* was in an off configuration. This result can be explained by the presence of some degree of expression through transcriptional slippage such that a small amount of transcript was made in an on configuration and thus translated into a small amount of functional PglA proteins. This phenomenon has previously been observed in *pgIA* alleles from other strains (18). Although they were less abundant, we also observed npg3 expression in 33% and 6% of isolates that had *pgIE* off and *pgIH* off, respectively (Table S1).



**FIG 6** Core genome MLST, *pgl* genotype, and protein glycosylation phenotype. An overview of phylogenetic relationships together with genotypic and phenotypic results for this strain collection is presented. The genotypic data include the allelic numbers assigned by the PubMLST genome comparator protein glycosylation typing scheme and the on/off configuration of the phase-variable genes. The color codes are shown in the figure legend. Not determined, incomplete sequence, analysis not performed, or not possible to evaluate. All details presented in this figure are listed in Table S1.

Although half of the isolates had *pglH* on, only five of these had pDAb2 reactivity (Fig. 5). In particular, isolates 45B, 1A, and 1B had no competing glycosyltransferases for PglH and were therefore expected to synthesize PglH disaccharide. The overall glycosylation level appeared to be low, suggesting either that there was impaired glycosylation in these isolates or that the glycoforms expressed were not recognized in our analysis. Further genetic, phenotypic, and structural characterization is needed to be able to understand the discrepancies.

**Correlation between *pgl* genotype and glycosylation phenotype.** There was not a complete correlation between glycosylation genotype and phenotype in the ST-192 isolates described above (Fig. 5) or in the other isolates (summarized in Fig. 6 and Table S1). Figure 6 contains phylogeny data together with genotypic and phenotypic results for this strain collection. The STs are indicated by different colors and are in agreement with the clustering of the cgMLST tree. The genotypic data include the allelic numbers assigned by the PubMLST genome comparator typing scheme and the on/off configuration of the phase-variable genes. The alleles are colored with the same code as the STs of the dominant allele within the subgroup, while all other alleles are randomly colored. By using this approach, the color patterns demonstrated that the alleles were highly associated with the STs and cgMLST. Another striking observation is the number of allele variants in the phase-variable genes (*pglA*, *pglI*, *pglE*, *pglG*, *pglH*). This was

predominantly due to variations in repeat numbers rather than to other nucleotide differences.

The O-linked glycosylation system is at present characterized to such an extent that we can make justified hypotheses concerning the nature of the molecular mechanisms behind most divergences. The phenotypic data include the scoring of antibody reactivity as described above.

In addition, we have interpreted the genotypic and phenotypic data and added table columns corresponding to glycoform (diNAcBac or GATDH based), microheterogeneity (two or more glycoforms present), phase-variable slippage (expression from the off configuration), and correlation between genotypic and phenotypic data. Microheterogeneity was observed in 67% of the isolates and transcriptional slippage in 49% of the isolates with diNAcBac glycoforms. In examining the level of correlation between genotypic and phenotypic data, we included transcriptional slippage as an accepted mechanism involved in this system and found correlations in 54% of the diNAcBac-synthesizing isolates. The remaining isolates had very low (level 0 to 2) reactivity with the glycan-specific antibodies and/or glycosyltransferases that did not show any detectable activity. The lack of typing reagents for GADTH makes it difficult to determine the levels of microheterogeneity, transcriptional slippage, and correlation in the remaining isolates.

## DISCUSSION

WGS analysis revealed a high degree of within-host genetic changes in meningococcal isolates taken 6 to 9 weeks apart, and phase-variable *pgl* genes were among the genes showing the highest degree of genetic variation (25). This study was intended to unravel the degree of direct conformity between the *pgl* genotype and the glycosylation phenotype, as well as the level of glycan complexity expressed by this system. That has, to our knowledge, been done for only a very few *Neisseria* strains. Our isolate collection included all known *pgl* locus variants identified to date. As the events leading to the major *pgl* locus variants are believed to have occurred in one location and then spread (26), finding all known variants in a small geographic area in a rural/semirural part of Ethiopia therefore underlines the global spread of the meningococcus and its genes. In addition, the correlation between the *pgl* genotype and protein glycosylation is complicated by allele polymorphisms and phase variation. Together, these observations pointed to a more thorough investigation of the *pgl* genotype and protein glycosylation within these isolates.

Analyzing paired carriage isolates gives insight into genetic evolution within the host in short time periods. Genetic and phenotypic changes in the meningococcus have been shown to happen over a very short time *in vivo*, directly impacting the adaptation of the bacterium to evade the host immune system and to increase its virulence (27). The accidental human passage of a meningococcal strain acquired in the laboratory showed that both *pglA* and *pglI* were turned off in the patient whereas they were on in the parental strain. Minor variation in sequence repeat tract leads to entirely altered glycoform expression, resulting in that case in a change from diNAcBac-Gal-AcGal to diNAcBac-Glc expression after human passage of the isolate. Those authors proposed that this change, among others, could confer an advantage to the bacteria to escape the immune system (27).

Phase variation was the predominant mechanism accounting for the genetic differences observed between pairs for all phase-variable *pgl* genes. The phase variation in *pglE* was the most extensive, with variation in 60% of the pairs compared to 20% to 44% of the pairs for the other *pgl* loci. The expansions and extensions were also more extensive in *pglE* than in the other *pgl* loci. The heptanucleotide repeat and the longer phase-variable tracts presumably make *pglE* more unstable, as phase variation rates has been believed to increase proportionally with the tract length. The frequencies of phase variation in the *pgl* genes, *in vitro* and *in vivo*, have not been studied. However, for other phase-variable genes in the meningococcus, such as hemoglobin-binding protein genes *hpuAB* and *hmbR*, the *in vitro* switching rates seen under conditions of growth on

agar plates ranged from  $\sim 10^{-6}$  to  $10^{-2}$  CFU $^{-1}$  (28) and also differed between different serogroups (29). In our study, we did not investigate phase variation during culturing and therefore cannot exclude the possibility that some of the changes observed happened *in vitro* during processing of the samples. Other possibilities are that multiple phase variants exist *in vivo* and that the changes do not represent differences over time but rather represent natural selection *in vivo* of the dominant bacteria colonizing the host. Phase variation during isolation and culture has been investigated in *hpuAB* and *hmbR*, using contemporaneously acquired clinical specimens (blood/cerebrospinal fluid), and a protocol for determining *in vivo* phase variation status has been developed (30). Using such a method to investigate the *pgl* genes would be highly interesting.

Recombination events were found within paired meningococcal carriage isolates in *pglG*, *pglA*, and *pglO*. In addition, several paired isolates within one ST had exactly the same variation between the two sampling points in *pglA*, *pglH*, *pglI*, and *pglF*, which was thus likely introduced by homologous recombination. Several studies have detected homologous recombination within the *pgl* loci, emphasizing a role of protein glycosylation diversity in immune evasion (23, 31). Krauland et al. compared genomes of serogroup Y ST-23 clonal complex early and late strain types and hypothesized that emergence of a late strain type was primarily due to antigenic changes that allowed escape from population immunity. They found that genes exhibiting differences included antigenic outer membrane protein genes; genes involved in iron acquisition and uptake; and genes involved in pilus structure, function, and glycosylation (31). However, the phenotypic outcomes of the observed genetic *pgl* gene differences were not investigated in any of those studies.

Information on gene content, polymorphisms, and phase variation is crucial to be able to predict glycosylation phenotype and glycan diversity. To date, we have known that a meningococcal strain has the capacity to express between 7 and 21 different glycans. Protein glycosylation genotype and phenotype correlations have been difficult within this system because glycans are not directly encoded by the genome but are enzymatic products that are somewhat more difficult to predict. This is further complicated by the fact that *Neisseria* exhibits a high degree of polymorphism both at the gene content level and with regard to polymorphic glycosyltransferases. In addition, the neisserial protein glycans can be subject to the phenomenon of microheterogeneity (12, 17), in which variant glycan structures are found at specific attachment sites of a given glycoprotein. Even though the study was somewhat hampered by the lack of a complete set of antibodies to recognize all GADTH glycoforms, we were able to study the glycan diversity. ST-11 isolates in our collection express antigenic invariable class II pilin. Gault et al. suggested that *N. meningitidis* isolates with a class II pilin display additional pilin glycosylation sites and proposed a model where these strains evade the immune system by changing their sugar structure rather than their pilin structure (24). We were not able to investigate the glycosylation phenotype in ST-11 isolates in detail here since they synthesize GADTH glycoforms, but the genotype indicates that these isolates have the capacity to express up to 21 different glycans. Additional pilin glycosylation sites would therefore also increase the magnitude of microheterogeneity in ST-11 isolates.

Extensive acetylation complexity and microheterogeneity of *pgl* glycoform O-acetylation by PglI were recently characterized using mass spectrometry (21). In this study, we screened the isolates using immunoblotting, and the complexed acetylation pattern therefore cannot be depicted. The functional implications of *pgl* oligosaccharide O-acetylation are currently unknown, but the O-acetylation has been shown to alter *pgl* glycan antigenicity. These studies involved both immunoblotting and immunogold labeling with glycoform-specific MAbs, and the altered reactivity was suggested to have been caused by the acetyl group masking the recognition epitope present on sugar moieties (12). This can be difficult to evaluate in single isolates, as the levels of glycosylation also differ due to the level of activity expressed by each of the involved glycosyltransferases. For individual 35, however, the paired isolates shared the same *pgl*

genotype, with the exception of *pgII*, and we observed an overall increase in reactivity at time point B that can be explained only by the *pgII* off configuration. In ST-53 isolates, the number of possible glycoforms is only five since isolates within this sequence type do not have the *pgII* gene responsible for O-acetylation of the glycans. The majority of pathogenic *Neisseria* isolates and some commensal *Neisseria* species (*N. lactamica*, *N. mucosa*, and *N. polysaccharea*) have the *pgII* gene, while it is missing in the related distal commensal *Neisseria* species such as *N. cinerea*, *N. flavescens*, *N. subflava*, *N. sicca*, and *N. elongata* (32). Interestingly, the two species of pathogenic *Neisseria* have multiple, dedicated O-acetylation systems targeting phosphatidylglycerol (PG), lipooligosaccharides (LOS), and, in the case of *N. meningitidis*, capsule polysaccharides (CPS) (33–35). The *pgII* gene is also predicted to be subject to phase-variable expression in meningococci and *N. lactamica* but not in gonococci and other commensal species (32).

The number of genes with allelic differences between isolates from different individuals within the same ST was about twice as high as the number of differences between paired isolates from the same carrier (25). A recent study looking at within-host evolution by genomic comparison of throat and blood strain pairs from four patients with meningococcal disease also found changes in *pilE*, *modA*, and *pgII* (36), which were also among the most frequently changed genes in our previous study. Those authors hypothesized that genomic variants arise during carriage and that invasive variants occasionally emerge and cause disease (36). The role of glycosylation in meningococcal pathogenesis has not been resolved, and thus, neither has the influence of phase variation in *pgI* genes on disease and possibly on vaccine immune responses. Further characterization employing genetic manipulations and mass spectrometry as used in earlier studies (17, 19) will most likely further add to the data showing high glycan variability predicted from the genotype. We suspect that such an outcome is likely since a high number of strains showed low reactivity to the glycan-specific antibodies and since only a restricted number of *N. meningitidis* strains have been investigated for the glycosylation phenotype thus far. Either new functions assigned to already-characterized glycosyltransferases or novel glycosyltransferases might conceivably be identified. Another plausible explanation for the low level of glycosylation is the competition for substrate between the glycosyltransferases (PglA/PglH or PglH/PglE competition) as previously documented (17, 21).

Here we have shown that glycoform diversity is caused by *pgI* gene variation, polymorphism, and phase variation and that the glycoproteins can be subject to the phenomenon of microheterogeneity. Taking the data together, the complexity of the O-linked protein glycosylation system requires further studies to understand how *N. meningitidis* utilizes the high variation in *pgI* gene content to produce high glycoform diversity and to evade the human immune response in both carriage and disease.

## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** The isolates were obtained from a cross-sectional meningococcal carriage study conducted among healthy 1- to 29-year-old individuals in the Arba Minch area in southern Ethiopia in 2014 (37), where a subgroup of individuals identified as asymptomatic carriers were followed using repeated (weekly) collection of oropharyngeal samples over a period of 9 weeks as previously described (25). All control bacterial strains used in this study were *N. gonorrhoeae* (see Table S2 in the supplemental material). Isolates were grown overnight on Colombia agar base plates (Oxoid Ltd., Basingstoke, United Kingdom) containing 5% defibrinated horse blood (TCS Biosciences Ltd., Buckingham, United Kingdom) at 37°C in an atmosphere of 5% CO<sub>2</sub>.

**Next-generation sequencing.** Genomic DNA was extracted using an automated MagNAPure isolation station and a MagNAPure 96 DNA and viral neuraminidase (NA) small-volume kit (Roche, Basel, Switzerland), according to the manufacturer's instructions. The sequencing libraries were made using a Kapa HyperPlus kit (Kapa Biosystems, Wilmington, MA); sequencing (500 cycles) was performed on an Illumina MiSeq platform with MiSeq Reagent kits (v2; Illumina Inc., San Diego, CA) with 250-bp paired-end run modes; and the reads were subsequently trimmed, filtered, and assembled as previously described (25).

**Sanger sequencing.** The *pgIE* gene in all isolates was PCR amplified using primers BP009 (TCGAR AGTTATTGGGAAGCGTGT) and BP010 (TATTMCACGCCGAACCCGGA). The PCR products were sequenced

using a BigDye Terminator v1.1 cycle sequencing kit and an ABI 3730 DNA analyzer (Applied Biosystems) according to the manufacturer's recommendation.

**Genome comparison, *pgl* genotype, and phylogenetic analyses.** Genomes were uploaded to and are available from the PubMLST.org database (<http://pubmlst.org/neisseria/>) (Table S1), which is served by the Bacterial Isolate Genome Sequence Database (BIGSdb) platform (38). For *pgl* allelic comparisons, gene-by-gene analyses were performed using the Genome Comparator tool and the glycosylation scheme within the PubMLST website. For core genome MLST analysis, the 1,605 loci defined as the core genome in the database (*N. meningitidis* cgMLST v1.0) were used (39). Incomplete loci were removed from individual pairs prior to calculations. Distance matrices based on the allelic differences were created using the Neighbor-net method (40). To visualize the phylogenetic network involving all 100 isolates from the 50 individuals, SplitsTree4 v4.14.4 was used (41).

For determinations of the mechanisms of differences between paired isolates, sequences were uploaded, aligned, and compared manually in MEGA7 (42). Mechanisms were classified as representing point mutations where only a single nucleotide difference was present, as recombinations where multiple nucleotide differences were present in the same area, and as phase variations where various lengths of repeat nucleotides were seen, and deletions or insertions were determined in relation to the majority of isolates. In cases where alleles were incomplete or missing in BIGSdb, missed gene sequences were retrieved to some extent in performing BLAST searches (43). For determination of the on/off configuration for the phase-variable genes, the sequenced were translated and compared manually. To confirm that the observed differences in isolate pairs around the *pgl* locus were supported by the raw data rather than being artifacts introduced by spurious assemblies, we mapped raw FASTQ reads back to previously produced contigs using BWA (44), converted the output to the BAM format (45), and manually inspected the pileup of reads around the observed differences using the program artemis v.16.0.11 (46). A read was considered to span a repeat tract if it contiguously spanned the entire tract and was anchored on each side of the repeat region with at least five nonambiguous, nonrepetitive nucleotides. The repeat number of a region was called by simple majority, i.e., was supported by at least 50% of all reads spanning the repeat tract. Additionally, a minimum of two reads had to span the repeat tract.

**SDS-PAGE and immunoblotting.** The procedures used for SDS-PAGE and immunoblotting have been described previously (47). 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. Immunoreactive proteins were detected by immunoblotting by using glycan-specific monoclonal antibodies npg1, npg2, and npg3 (12); the rabbit polyclonal antibody pDAb2 (17); and an alkaline phosphatase-coupled goat anti-rabbit secondary antibody (Sigma). To identify glycoproteins bearing a terminal GlcNAc, alkaline phosphatase-conjugated succinylated wheat germ agglutinin (sWGA) lectin (EY Labs) was used as described previously (19, 48).

**Data availability.** The sequences generated and analyzed during the current study are available at the pubMLST.org website (see identification numbers in Table S1).

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00794-17>.

**SUPPLEMENTAL FILE 1**, PDF file, 0.7 MB.

**SUPPLEMENTAL FILE 2**, CSV file, 0.1 MB.

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