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Hypomorphic Glycosyltransferase Alleles and Recoding at Contingency Loci Influence Glycan Microheterogeneity in the Protein Glycosylation System of *Neisseria* Species

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As more bacterial protein glycosylation systems are identified and characterized, a central question that arises is, what governs the prevalence of particular glycans associated with them? In addition, accumulating evidence shows that bacterial protein glycans can be subject to the phenomenon of microheterogeneity, in which variant glycan structures are found at specific attachment sites of a given glycoprotein. Although factors underlying microheterogeneity in reconstituted expression systems have been identified and modeled, those impacting natural systems largely remain enigmatic. On the basis of a sensitive and specific glycan serotyping system, microheterogeneity has been reported for the broad-spectrum, *O*-linked protein glycosylation system in species within the genus *Neisseria*. To elucidate the mechanisms involved, a genetic approach was used to identify a hypomorphic allele of *pglA* (encoding the PglA galactosyltransferase) as a significant contributor to simultaneous expression of multiple glycoforms. Moreover, this phenotype was mapped to a single amino acid polymorphism in PglA. Further analyses revealed that many *pglA* phase-off variants (containing out-of-frame configurations in simple nucleotide repeats within the open reading frame) were associated with disproportionally high levels of the *N*,*N*′-diacetylbacillosamine–Gal disaccharide glycoform generated by PglA. This phenotype is emblematic of nonstandard decoding involving programmed ribosomal frameshifting and/or programmed transcriptional realignment. Together, these findings provide new information regarding the mechanisms of neisserial protein glycan microheterogeneity and the anticipatory nature of contingency loci.

Protein glycosylation systems based on both *N*- and *O*-linked modifications are well documented in eubacteria. In addition to the distinctions based on the nature of site attachment, bacterial protein glycosylation systems can be differentiated on the basis of whether they are dedicated to a single class of proteins or target a broad spectrum of protein substrates. These systems can also be classified as to the degree of glycan diversity observed within and between related species. For example, in the type IV pilus (Tfp) *O*-linked glycosylation in *Pseudomonas aeruginosa*, some strains utilize the endogenous *O*-antigen unit found in lipopolysaccharide (LPS; which in itself varies between strains), while others employ D-arabinofuranose moieties synthesized by an apparently dedicated biosynthetic machinery (11, 28). In the *N*-linked, broad-spectrum protein glycosylation system of *Campylobacter jejuni*, a conserved heptasaccharide has been presumed to be highly disseminated. Major disparities in protein glycan diversification have been observed for flagellum-associated, *O*-linked systems, with those in *Campylobacter*, *Helicobacter*, and *Clostridium* exhibiting high levels of intra- and interstrain variation, while the system in *P. aeruginosa* appears to be limited to two major glycoforms (4, 10, 13, 26). Why protein-associated glycans display high intra- and interstrain variation in some systems and not in others remains unknown. Many of these and other systems have only begun to be rigorously characterized, and thus, detailed studies of glycan diversity are still in their infancy.

The broad-spectrum *O*-linked protein glycosylation (*pgl*) system expressed by species within the genus *Neisseria* provides a unique model system in which to study the long-term evolutionary trends and dynamics of protein glycan diversification. The highly related species *Neisseria gonorrhoeae* (the agent of gonorrhea), *Neisseria meningitidis* (an agent of epidemic meningitis), and *Neisseria lactamica* (a commensal colonizing the oropharynx of young children) are of particular importance to human health and disease. The basic genetics and biochemistry of glycan biosynthesis, elaboration, and transfer to protein have begun to be well characterized in these species (7, 8, 27). The nascent glycan chain is first synthesized on the lipid carrier undecaprenyl-pyrophosphate on the inner leaflet of the cytoplasmic membrane and translocated into the periplasm. PglO, a protein-targeting oligosaccharyltransferase structurally related to the WaaL family of O-antigen ligases, then transfers the oligosaccharides en bloc to protein substrates (15). These compartmentalized processes are analogous to those seen for eukaryotic systems in which the transfer of a preassembled oligosaccharide from a dolichyl-pyrophosphate donor to the asparagine side chain of nascent proteins occurs within the endoplasmic reticulum.

The neisserial *pgl* system is unique among broad-spectrum bacterial glycosylation systems in the extreme degree of glycan diversity observed within and between strains. Here, the glycan repertoire is governed by the *pgl* gene content and gene interactions as well as by phase-variable expression of individual components (Fig. 1). Several strains carry a conserved *pgl* core locus

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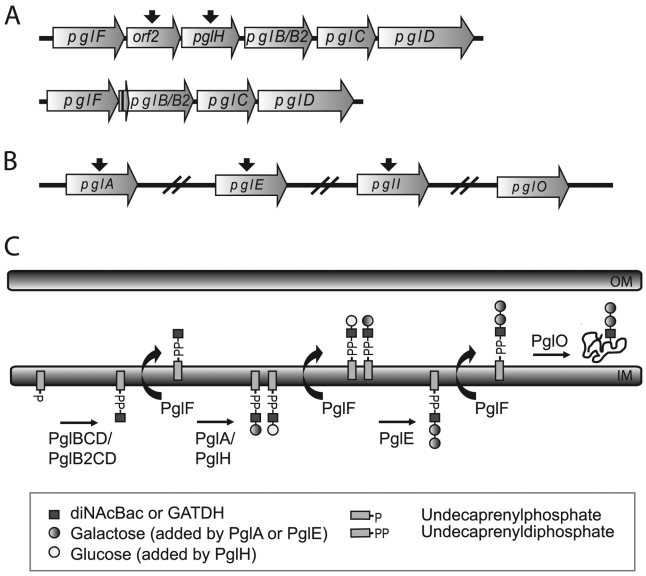


FIG 1 *pgl* genes and glycosylation pathway in *Neisseria*. (A) Polymorphisms at the *pgl* locus of *Neisseria* strains. The ancestral *pgl* locus consists of *pglF*, *orf2*, *pglH*, *pglB-pglB2*, *pglC*, and *pglD*, while other strains lost two genes, *orf2* and *pglH* (see Table S1 in the supplemental material). The first 40 bp of *orf2* and the last 100 bp of *pglH* remain in the deleted *pglFBCD* locus (short ORF between *pglF* and *pglB/B2*). The function of *orf2* is not known at the present. Both ancestral and deleted *pgl* loci are found in combination with both *pglB* and *pglB2* alleles. (B) Additional *pgl* genes, such as *pglA*, *pglE*, *pglI*, and *pglO*, are located elsewhere in the genome. Phase-variable *pgl* genes are indicated with a black arrow above the genes. (C) Current model of the broad-spectrum *O*-linked glycosylation pathway in *Neisseria*. OM, outer membrane; IM, inner membrane.

consisting of *pglB*, *pglC*, and *pglD*, whose products (PglB, PglC, and PglD) function in the synthesis of undecaprenyl diphosphate (UndPP)–*N*,*N*′-diacetylbacillosamine (diNAcBac) (15). Approximately one-half of *N. meningitidis* strains carry an alternative *pglB* allele, namely, *pglB2*, at this locus. *pglB2* is associated with the synthesis of UndPP–glyceramido-acetamido trideoxyhexose (GATDH) rather than UndPP-diNAcBac (12). GATDH differs from diNAcBac by the presence of a glyceroyl moiety instead of an acetyl modification at C-4. Both of these can be further elaborated by the sequential action of two galactosyltransferases, termed PglA and PglE, to form di- and trisaccharide forms (2). The *pglA* and *pglE* genes are found in all strains of *N. gonorrhoeae*, *N. meningitidis*, and *N. lactamica* examined to date. The *pglA* and *pglE* alleles primarily exist in phase-variable forms due to the presence of simple nucleotide repeats within their open reading frames (ORFs) (22). As such repeats are hypermutable due to processes associated with DNA replication and metabolism, this leads to a high rate of indel mutations that in turn alter protein expression. Such genes are referred to as contingency loci, which underlie a bet-hedging strategy in which even small populations carry variants in altered indel configurations. Variations at contingency loci are generally perceived to be dictating on-off phenotypic switching.

We recently identified a third glycosyltransferase gene, *pglH*, that encodes a glucosyltransferase that generates disaccharide products using UndPP-linked monosaccharide substrates (8). However, the resulting glycoform is incapable of being further elaborated by PglE. The *pglH* gene is also a contingency locus, while in some strains, the gene is inactivated due to a large deletion encompassing its 5′ end. Finally, the galactose and glucose residues in the di- and trisaccharide forms are capable of being *O*-acetylated by the activity of PglI, whose gene also exists in phase-variable and non-phase-variable forms (2). Solely on the basis of differences in gene content and polymorphisms, single strains can vary their glycan repertoire from as few as 4 to as many as 7 glycoforms. In the case of *N. meningitidis*, which can carry either the *pglB* or the *pglB2* allele, at least 13 different glycoforms can be expressed within the species (8). These findings indicate that the PglB/PglB2-associated glycosyl-1-phosphate transferase, PglF flippase, and PglO oligosaccharide transferase (which all act downstream of the synthesis of the UndPP-linked saccharides) must each possess relaxed donor specificity.

The phenomenon in which a range of glycan structure variation occurs at a given glycosylation site on a given protein within a defined population of cells is termed microheterogeneity. Although this condition is observed across broad taxonomic boundaries, its biological significance and mechanistic basis remain poorly understood. In part, this situation reflects the technological difficulties in monitoring and quantitating glycan diversity at single sites. In the neisserial *pgl* system, microheterogeneity has been observed in a number of instances (7, 8). For example, microheterogeneity at the level of glycan *O*-acetylation has been documented at a number of occupancy sites. Moreover, some strains have been shown to simultaneously express mono- and disaccharides, di- and trisaccharides, and even all three glycoforms. A unique form of microheterogeneity occurs in strains that carry active alleles of both *pglA* and *pglH*, resulting in the simultaneous expression of a mixture of galactose- and glucose-containing oligosaccharides (8). Here, we investigated the molecular basis for glycan microheterogeneity and identified an important role for hypomorphic glycosyltransferase alleles. We also found evidence for the influence of recoding at these contingency loci, as some phase-off (frameshift-containing) *pglA* alleles were associated with considerable glycosyltransferase activity.

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 (14). Protein glycosylation mutations (*pglA*, $pglE_{on}$) were introduced into various strain backgrounds using transformation, as previously described (1, 2). The following antibiotics were used for selection of transformants at the indicated concentrations: streptomycin, 750 μg/ml; erythromycin, 8 μg/ml; kanamycin, 50 μg/ml.

Allelic exchange of *pglA*. The introduction of the different *pglA* alleles into N400 was performed through a two-step mutagenesis strategy that allowed gene replacement without introducing any selectable marker into the final strain. The method uses a two-gene cassette containing both a selectable marker (*ermC*′) and a counterselectable marker ($rpsL^+$) (18). First, N400 was transformed with pFLOB-*pglA*::*ermC*′-$rpsL^+$ (3), and erythromycin-resistant gonococci were selected. N400 is naturally streptomycin resistant, but the introduction of the $rpsL^+$ allele made the intermediate strain, N400 *pglA*::*ermC*′-$rpsL^+$, streptomycin sensitive. The next step was transformation with genomic DNA from different *N. gonorrhoeae*, *N. meningitidis*, and *N. lactamica* strains, where homologous recombination replaced the *ermC*′-$rpsL^+$ cassette with *pglA* from the genomic DNA and the final strain could be selected on streptomycin

TABLE 1 Strains used in this study

Strain	Parental strain	Relevant genotype	Reference or source
N. gonorrhoeae N400 strains			
KS100 (N400)	VD300	*recA6*[a]	25
KS461	KS100	*recA6 pglE*$_{on}$ *pglA*::*ermC-rpsL*	This study
KS462	KS100	*recA6 pglA*::*ermC-rpsL*	This study
KS488	KS462	*recA6 pglA*$_{5'\ FA1090}$	This study
KS489	KS462	*recA6 pglA*$_{5'\ N400}$	This study
KS477	KS462	*recA6 pglA*$_{ST3787}$	This study
KS478	KS462	*recA6 pglA*$_{ST640}$	This study
KS483	KS462	*recA6 pglA*$_{FA1090}$	This study
KS484	KS462	*recA6 pglA*$_{N400}$	This study
KS742	KS462	*recA6 pglA*$_{Z2491}$	This study
KS743	KS462	*recA6 pglA*$_{MC58}$	This study
KS744	KS462	*recA6 pglA*$_{8013}$	This study
KS745	KS462	*recA6 pglA*$_{FAM18}$	This study
KS473	KS462	*recA6 pglA*$_{GGGG}$	This study
KS474	KS462	*recA6 pglA*$_{GGGG,\ E275A}$	This study
KS492	KS462	*recA6 pglA*$_{G350A}$	This study
KS493	KS461	*recA6 pglE*$_{on}$ *pglA*$_{G350A}$	This study
KS495	KS462	*recA6 pglA*$_{E275A}$	This study
KS101	VD300	*pilE*$_{ind}$	29
KS122	KS101	*pilE*$_{ind}$ *pglA*::*kan*	27
KS773	KS122	*pilE*$_{ind}$ *pglA*::*kan lct*::*pglA*$_{N400}$	This study
KS771	KS122	*pilE*$_{ind}$ *pglA*::*kan lct*::*pglA*$_{FA1090}$	This study
KS772	KS122	*pilE*$_{ind}$ *pglA*::*kan lct*::*pglA*$_{ST3787}$	This study
N. gonorrhoeae FA1090 strains			
KS300	FA1090	*recA6*[a]	25
KS463	KS300	*recA6 pglA*::*ermC-rpsL*	This study
KS464	KS300	*recA6 pglE*$_{off}$ *pglA*::*ermC-rpsL*	This study
KS465	KS463	*recA6 pglA*$_{PRGSG}$	This study
KS466	KS463	*recA6 pglA*$_{PRGSGDLEQPA}$	This study
KS467	KS463	*recA6 pglA*$_{QPA,\ A275E}$	This study
KS468	KS463	*recA6 pglA*$_{PRGSG,\ A275E}$	This study
KS469	KS463	*recA6 pglA*$_{QPA}$	This study
KS470	KS463	*recA6 pglA*$_{GSG,\ A275E}$	This study
KS471	KS463	*recA6 pglA*$_{PRGSGDLEQPA,\ A275E}$	This study
KS472	KS463	*recA6 pglA*$_{GSG}$	This study
KS475	KS463	*recA6 pglA*$_{A146T}$	This study
KS476	KS463	*recA6 pglA*$_{PRGSGDLEQPA,\ A275E,\ A146T}$	This study
KS479	KS463	*recA6 pglA*$_{ST3787}$	This study
KS480	KS463	*recA6 pglA*$_{ST640}$	This study
KS481	KS463	*recA6 pglA*$_{N400}$	This study
KS482	KS463	*recA6 pglA*$_{FA1090}$	This study
KS763	KS463	*recA6 pglA*$_{Z2491}$	This study
KS764	KS463	*recA6 pglA*$_{MC58}$	This study
KS765	KS463	*recA6 pglA*$_{8013}$	This study
KS766	KS463	*recA6 pglA*$_{FAM18}$	This study
KS486	KS463	*recA6 pglA*$_{5'\ N400}$	This study
KS487	KS463	*recA6 pglA*$_{5'\ FA1090}$	This study
KS490	KS463	*recA6 pglA*$_{A350G}$	This study
KS491	KS463	*recA6 pglA*$_{PRGSGDLEQPA,\ A275E,\ A146T,\ A350G}$	This study
KS494	KS464	*recA6 pglE*$_{off}$, *pglA*$_{A350G}$	This study
KS496	KS463	*recA6 pglA*$_{A275E}$	This study
KS769	KS300	*recA6 pglA*::*kan*	This study
KS422	KS300	*recA6 pglH*::*ermC-rpsL*	8
KS459	KS422	*recA6 pglH*$_{H371R}$	8
KS423	KS422	*recA6 pglA pglH*	8
KS460	KS423	*recA6 pglA*::*kan pglH*$_{H371R}$	8
KS770	KS460	*recA6 pglA*$_{N400}$ *pglH*$_{H371R}$	This study

[a] *recA6* is an IPTG-inducible allele of *recA*.

plates. For the derived strains, DNA sequencing was used to confirm the absence of other mutations.

Construction of *pglA* mutant strains. First, the pCRII-*pglA*$_{N400}$ and pCRII-*pglA*$_{FA1090}$ plasmids were constructed by PCR amplifying the whole *pglA* gene and surrounding sequences (3,734 bp) from *N. gonorrhoeae* strains N400 and FA1090 (*pglA*$_{N400}$ and *pglA*$_{FA1090}$, respectively; primers pglA-F-EcoRI [5′-GGAATTCACTAACTGGAATTAACAAATACGGC-3′, where the underlining indicates the restriction site] and pglA-

R-HindIII [5′-CCCAAGCTTAGCAATGCTGAAACAAGCCC-3′]) and inserting the PCR products into the pCRII-TOPO vector (Invitrogen). To exchange the upstream sequences between N400 and FA1090 *pglA*, the pCRII-$pglA_{N400}$, and pCRII-$pglA_{FA1090}$ plasmids were cut with BamHI and MluI and the 5′ sequence fragments were exchanged and ligated with the other pCRII-*pglA* plasmid to generate pCRII-5′$_{N400}$-$pglA_{FA1090}$ and pCRII-5′$_{FA1090}$-$pglA_{N400}$. Since there are very few nucleotides different between N400 and FA1090 in this region, we could use the available restriction enzymes BamHI and MluI, whose fragments include all nucleotides different between the N400 and FA1090 *pglA* 5′ sequences.

To introduce the different mutations into the endogenous site of $pglA_{N400}$ and $pglA_{FA1090}$, the pCRII-*pglA* plasmids were mutated by using specific primers (see Table S2 in the supplemental material) containing the mutation of interest and a QuikChange XL site-directed mutagenesis kit (Agilent Technologies, Inc., 2009), as described in the manual. The mutation was then introduced into the N400 *pglA*::*ermC-rpsL* (KS462) and FA1090 *pglA*::*ermC-rpsL* (KS463) strains by homologous recombination that replaced the *ermC′-rpsL*$^+$ cassette with *pglA* containing the different mutations of interest.

Complementation analysis. The coding regions of the $pglA_{FA1090}$, $pglA_{N400}$, and $pglA_{ST3787}$ genes were amplified from genomic DNA from the respective strains with primers specific for all strains (primers BP114 [5′-CCTTAATTAAATGAAAATCGTTTTTATCAC-3′] and BP115 [5′-AGCTTTGTTTAAACTTACGCCTTCAAAATATCGCG-3′]) containing PacI and PmeI restriction sites and subcloned into plasmid pGCC4 that had been digested with PacI and PmeI. The *pglA* genes from FA1090, N400, and ST3787 were then inserted at an intergenic chromosomal site located between the *lctP* and *aspC* genes and linked to an erythromycin resistance cassette (20, 21) in the pGCC4::*pglA* plasmid. To do complementation analysis, this plasmid was then transformed into strain N400 derivative 4/3/1 *pglA* (KS122) gonococci and selected on plates containing erythromycin.

SDS-PAGE and immunoblotting. Procedures for SDS-PAGE and immunoblotting have been described previously (14). Whole-cell lysates were prepared from equivalent numbers of cells by heating cell suspensions to 65°C for 10 min in SDS-sample loading buffer. Immunoreactive proteins were detected by immunoblotting using the glycan-specific rabbit antibodies pDAb2 (which recognizes diNAcBac-Glc) (8), npg1 (which recognizes diNAcBac), npg2 (which recognizes diNAcBac-Gal), and npg3 (which recognizes diNAcBac-Gal-Gal) (7) and an alkaline phosphatase-coupled goat anti-rabbit secondary antibody (Sigma).

RESULTS

Microheterogeneity is associated with $pglA_{FA1090}$ and $pglA_{N400}$ alleles. Our previous studies of glycosylation revealed a high degree of glycoform variation at both the intra- and interstrain level in *Neisseria* strains (7, 8). Although microheterogeneity was seen in many backgrounds, we were particularly struck by the unique pattern of microheterogeneity in gonococcal strain FA1090, which simultaneously expressed high levels of the diNAcBac monosaccharide glycoform, no disaccharide glycoform, and moderate levels of trisaccharide. This observation was made through the use of the highly specific monoclonal antibodies (MAbs) npg1, npg2, and npg3, which recognize diNAcBac- and diNAcBac-based di- and trisaccharide-associated epitopes, respectively. This result was unanticipated, as FA1090 carries phase-on alleles of *pglA* and *pglE* and is therefore expected to express primarily the diNAcBac trisaccharide glycoform. For example, strain N400 $pglE_{on}$ carries the same functional genotypic background as FA1090 but nevertheless largely synthesizes diNAcBac-Gal-Gal trisaccharide (7, 8). To examine this phenomenon in more detail, glycoform expression in *N. gonorrhoeae* strains FA1090 and N400 and genetically defined derivatives was monitored using the glycan epitope-specific MAbs. As shown earlier, FA1090 expressed high levels of the diNAcBac glycoform and relatively reduced levels of the trisaccharide glycoforms (Fig. 2, lane wt). Replacing the wild-type *pglA* allele with a *pglA*-null allele in this background abolished trisaccharide glycoform expression but had no discernible effect on diNAcBac expression. Reintroduction of the $pglA_{FA1090}$ gene into the null mutant restored the parental phenotype, while reintroduction of the $pglA_{N400}$ gene increased expression of both the di- and trisaccharide forms, which came at the expense of diNAcBac expression, which was nearly abolished. To confirm the inference that the microheterogeneity in FA1090 is related to its *pglA* allele, analogous gene disruption and transfers were carried out in N400. As this background has $pglA_{on}$ and $pglE_{off}$ alleles, it predominantly expresses the disaccharide glycoform, and when a *pglA* disruption was introduced, a major shift to expression of the diNAcBac glycoform was observed. While this alteration was corrected by introduction of the $pglA_{N400}$ gene, the $pglA_{FA1090}$ gene led to a mixture of the diNAcBac and disaccha-

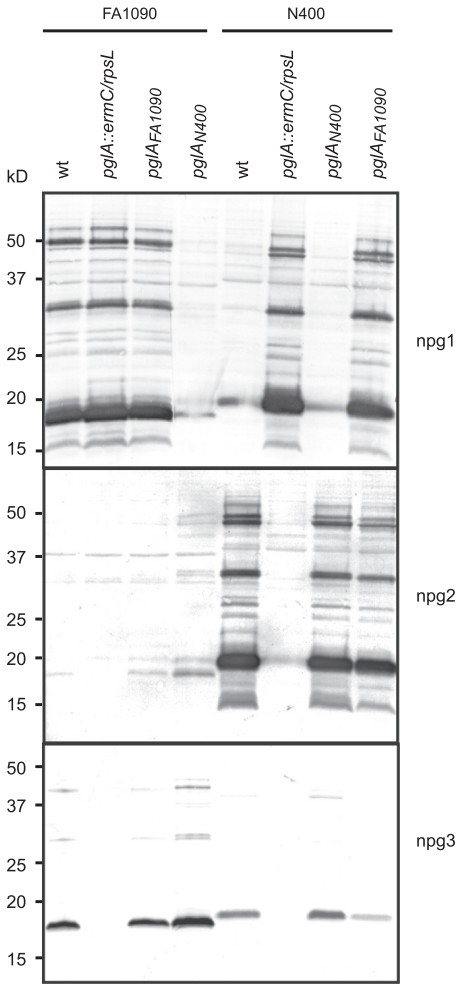


FIG 2 Microheterogeneity is associated with the *pglA* alleles from FA1090 and N400. Immunoblotting of whole-cell lysates from *N. gonorrhoeae* strains FA1090 and N400 and mutant derivatives with glycan-specific monoclonal antibodies (npg1, npg2, and npg3). Note that N400 has $pglE_{off}$ and FA1090 has $pglE_{on}$ and that the glycosylation patterns change according to *pglE* genotype and the presence of the *pglA* allele. Strains used were KS300, KS463, KS482, KS481, KS100, KS462, KS484, and KS483. wt, wild type.

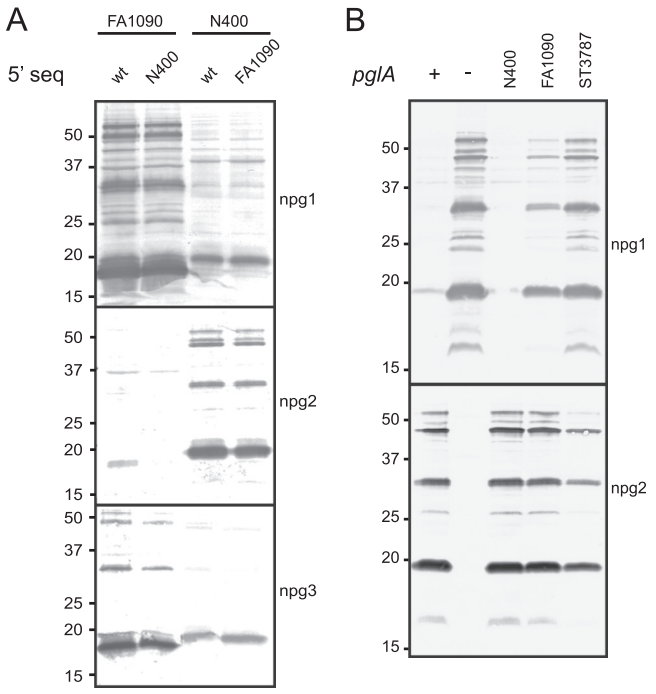


FIG 3 Glycosylation microheterogeneity phenotype is linked to the $pglA_{FA1090}$ ORF. (A) The glycosylation phenotype is not linked to different 5′ sequences of *pglA*. Immunoblotting of whole-cell lysates from *N. gonorrhoeae* strains FA1090 and N400 and mutant strains where only the 5′ noncoding sequences are exchanged using glycan-specific monoclonal antibodies (npg1, npg2 and npg3). Strains used were KS487, KS486, KS489, and KS488. (B) Complementation studies show that the glycosylation phenotype is coupled to the *pglA* ORF. Variant alleles of *pglA* were IPTG (isopropyl-β-D-thiogalactopyranoside) induced and expressed ectopically in the strain N400 derivative 4/3/1 *pglA*, and expression of mono- and disaccharides was monitored by immunoblotting whole-cell lysates with the glycan-specific monoclonal antibodies npg1 and npg2. The immunoblot demonstrates that microheterogeneity is associated with the respective *pglA* ORFs and is not dependent on endogenous expression. Lane −, *pglA*::*kan*; lane +, N400 wild type (*pglA* in the endogenous site). The last three lanes have the respective *pglA* ORFs expressed ectopically. Strains used were KS101, KS122, KS773, KS771, and KS772.

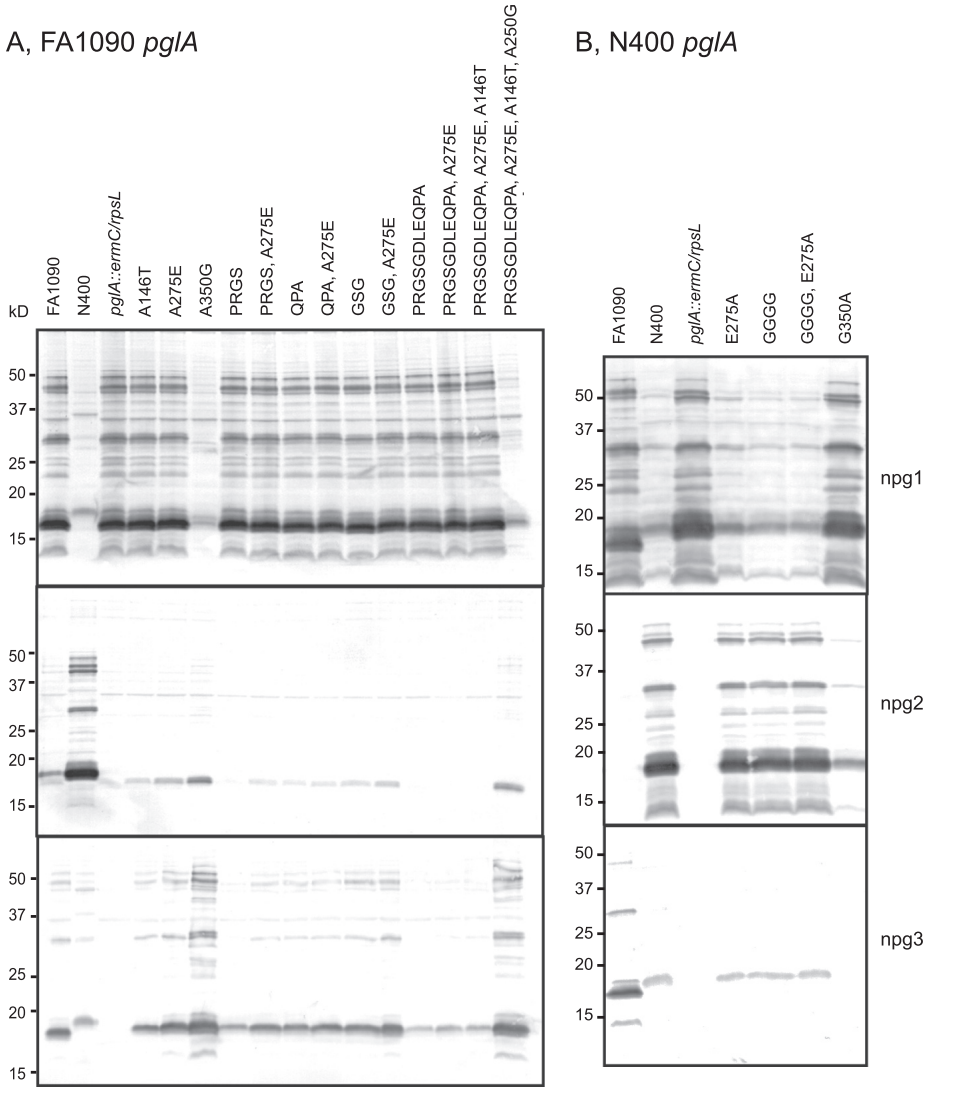
ride glycoforms (Fig. 2). Together, these findings showed that the differences in microheterogeneity could be primarily attributed to *pglA* allele status and, accordingly, that these phenotypes were the result of altered *pglA*-associated activity.

The glycosylation microheterogeneity phenotype is linked to the $pglA_{FA1090}$ ORF. Having established that the distinctive glycosylation phenotypes were linked to *pglA*, we wanted to further elucidate whether this was due to differences in the *pglA* ORF or features mapping 5′ to the ORF. Therefore, we exchanged the upstream sequences between the N400 and FA1090 *pglA* alleles and examined the patterns of glycoform expression. Neither fusion of the $pglA_{N400}$ 5′ sequence to the $pglA_{FA1090}$-coding region nor the reciprocal exchange influenced glycosylation expression (Fig. 3A), proving that the glycosylation phenotype is not linked to sequences 5′ of the *pglA* ORF. To confirm that the outcomes seen in strains carrying different *pglA* loci were ascribed to the coding region of *pglA* and were not indirect effects associated with strain construction, recombinants were created in which the coding region of *pglA* alone was expressed from an ectopic site in a *pglA*-null background. In this experiment, we included the *pglA* allele from *N. lactamica* ST3878, which we previously observed has a glycosylation pattern similar to that of FA1090 (7). Interestingly, this allele is annotated as being in an out-of-frame configuration, and we confirmed this by resequencing. The coding regions of *pglA* from strains FA1090, N400, and ST3787 were placed downstream of a derepressible promoter and operator and expressed in the N400 derivative 4/3/1 *pglA* from an ectopic site. Using immunoblotting and glycan-specific monoclonal antibodies, we confirmed that the coding regions of *pglA* were both necessary and sufficient to obtain the same glycosylation phenotypes observed when exchanging the whole *pglA* allele into the endogenous site. Since *pglE* is phase off in this background, only mono- and disaccharide reactivities are shown in Fig. 3. The complementation studies hence showed that the glycosylation phenotype is coupled to the *pglA* ORF from N400, FA1090, and ST3787 (Fig. 3B).

Identification of a single amino acid responsible for the hypomorphic character of the $pglA_{FA1090}$ allele. Having ascertained the importance of the *pglA*-coding region, we next wanted to examine which features of PglA were responsible for these differences. All amino acids distinct to $PglA_{N400}$ were introduced into $PglA_{FA1090}$ by site-directed mutagenesis in the FA1090 background (Fig. 4A). As anticipated, the simultaneous replacement of all polymorphisms unique to $pglA_{N400}$ in the $pglA_{FA1090}$ allele restored full activity. Of all of these, however, the change from an alanine to a glycine at residue 350 was both necessary and sufficient for the phenotype. Some of the distinguishing amino acids were also examined by mutagenesis of $PglA_{N400}$ in the N400 background (Fig. 4B). Note that this was done in different *pglE* backgrounds, so that the glycosylation patterns cannot be directly compared. When a reciprocal G350A mutation was introduced into $PglA_{N400}$, this resulted in a glycosylation pattern identical to that seen when inserting the whole $pglA_{FA1090}$ allele into N400.

Next, we wanted to alter amino acid 350 in the context of the respective *pglE* backgrounds so that the glycosylation patterns could be directly compared. By introducing $pglA_{A350G}$ in a FA1090 $pglE_{off}$ background, the glycosylation pattern changed accordingly and was identical to that of N400 wild type (Fig. 5; compare the third and the fourth lanes from the left). Furthermore, by introducing $pglA_{G350A}$ in an N400 $pglE_{on}$ background, the glycosylation pattern was identical to that of FA1090 wild type (Fig. 5, first and sixth lanes). In conclusion, the differences between N400 and FA1090 could be mapped to amino acid 350 of PglA. It is worth noting that only PglA from FA1090 has alanine at amino acid 350, while all other strains have glycine in this position (see Fig. S1 in the supplemental material).

Distinct *pglA* alleles are associated with variable levels of microheterogeneity. Having demonstrated the importance of PglA in the distinct FA1090 and N400 glycosylation patterns, we wanted to examine the influence of other *pglA* alleles on microheterogeneity. Therefore, several *pglA* alleles from *N. gonorrhoeae*, *N. meningitidis*, and *N. lactamica* strains were exchanged into the N400 and FA1090 backgrounds using the allelic exchange system. Immunoblotting with the glycan-specific monoclonal antibodies revealed that the different *pglA* alleles accounted for distinct glycosylation patterns (Fig. 6). The *pglA* alleles from strains FA1090, N400, Z2491, and MC58 are all in a phase-on configuration, while the *pglA* alleles from strains 8013, ST3787, ST640, and FAM18 are in a phase-off configuration but the strains nevertheless express



C

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        1                                                                  80
FA1090  MKIVFITTVA SSIYGFRAPV IKKLIGKNHQ VYAFVSEFSD NELDIIREMG VTPVTYRSNR SGVNPFSDIK STFLIFKALK
  N400  MKIVFITTVA SSIYGFRAPV IKKLIGKNHQ VYAFVSEFSD NELDIIREMG VTPVTYRSNR SGVNPFSDIK STFLIFKALK

        81                                                                160
FA1090  KISPDLVFPY FAKPVIFGTF AAKLAGVPRI VGMLEGLGFA FTPQPEGIPL KTKIIKGILI ALYRIALPML ESLIVLNPDD
  N400  KISPDLVFPY FAKPVIFGTF AAKLAGVPRI VGMLEGLGFA FTPQPEGIPL KTKIIKGILI ALYRITLPML ESLIVLNPDD

        161                                                               240
FA1090  KDELLHQYGI KIKNIHILGG IGLDLRQYPY SEADIPDEKE PVKFLFIGRF LKEKGIDDFI RAAEQVKGKY PDTVFTALGA
  N400  KDELLHQYGI KIKNIHILGG IGLDLRQYPY SEADIPDEKE PVKFLFIGRF LKEKGIDDFI RAAEQVKGKY PDTVFTALGA

        241                                                               320
FA1090  IDKSRGGGGD LERFIARDII RFPGFVNNVS EVIKAHHIFV LPSYYREGVP RSTQEAMAVG RAVITTDVPG CRETVADKVN
  N400  IDKPRGSG-D LEQPAARDII RFPGFVNNVS EVIKEHHIFV LPSYYREGVP RSTQEAMAVG RAVITTDVPG CRETVADKVN

        321                                                        376
FA1090  GFLIEPWNPR ILAEKMIYFI ENRAAVRLMA NASYAIAKDK FDAEKVDLKF LDILKA
  N400  GFLIEPWNPR ILAEKMIYFI ENRAAVRLMG NASYAIAKDK FDAEKVDLKF LDILKA
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FIG 4 Mutagenesis identifies one PglA amino acid responsible for diminished galactosyltransferase activity. Expression of different glycans was monitored by immunoblotting with the glycan-specific antibodies npg1, npg2, and npg3. (A) Various amino acid substitutions were introduced into $PglA_{FA1090}$, and the following glycosylation patterns were examined. Mutating only one amino acid, A350G, was sufficient to obtain the same glycosylation pattern obtained when $pglA_{N400}$ was introduced into FA1090 (sixth lane). All amino acids mutated are shown for each lane. Strains used were KS300, KS100, KS462, KS475, KS496, KS490, KS465, KS468, KS469, KS467, KS472, KS470, KS466, KS471, KS476, and KS491. (B) An assortment of amino acid mutations was introduced into $PglA_{N400}$, and the glycosylation patterns that followed were examined. The analogous G350A mutation in $PglA_{N400}$ changed the glycosylation pattern, which was similar to that of $pglA_{FA1090}$ expressed in N400 (last lane). All amino acids mutated are shown for each respective lane. Strains used were KS300, KS100, KS463, KS495, KS473, KS474, and KS492. (C) An alignment of PglA protein sequences from FA1090 and N400, where all dissimilar amino acids are shown in bold.

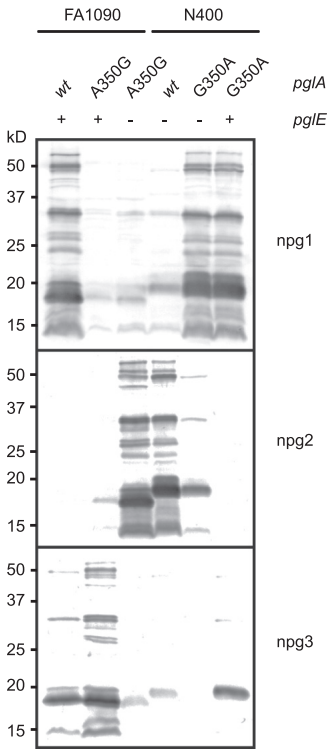

FIG 5 Validation of the roles of amino acid 350 substitutions in common *pglE* backgrounds. Glycosylation patterns were monitored by immunoblotting with the glycan-specific antibodies npg1, npg2, and npg3. By introducing the A350G PglA mutation in the FA1090 $pglE_{off}$ background, the obtained glycosylation was identical to that of N400 wild type. Similarly, by introducing the G350A PglA mutation in the N400 $pglE_{on}$ background, the achieved glycosylation was identical to that of FA1090 wild type. −, *pglE* from strain N400, which is $pglE_{off}$; +, *pglE* from strain FA1090, which is $pglE_{on}$. Strains used were KS300, KS490, KS494, KS100, KS492, and KS493.

detectable levels of the disaccharide glycoform (in the N400 background, as *pglE* is off) or trisaccharide glycoform (in the FA1090 background, as *pglE* is on). Interestingly, the phase-off *pglA* alleles were associated with considerable levels of PglA activity. Furthermore, the levels of activity varied to quite a degree between strains carrying the different $pglA_{off}$ alleles. To investigate the levels of glycoform expression in these strains in more detail, we examined immunoblot signal intensity versus that for standards consisting of admixtures of samples from $pglA_{N400}$ and *pglA*-null backgrounds. In this way, the strain carrying the $pglA_{FA1090}$ allele had about 30% of the npg2-reactive glycoform expression compared to N400, while the strains carrying $pglA_{ST3787}$ and $pglA_{FAM18}$ had about 4% of the npg2-reactive glycoform (Fig. 7). The strain carrying $pglA_{8013}$ had about 1 to 2% of the npg2-reactive glycoform and the strain carrying $pglA_{ST640}$ had about 0.5% of the npg2-reactive glycoform expression compared with the standard. Even $pglA_{ST640}$ (which had the lowest npg2 reactivity level) had reactivity above the background reactivity seen for the *pglA*-null mutant strain.

Interactions of *pglH* and *pglA* alleles and their contributions to microheterogeneity. PglH acts as a glucosyltransferase that further elaborates both UndPP-linked GATDH and diNAcBac to glucose-containing disaccharides (8, 15). During the earlier study, we observed a high degree of *pglH* polymorphisms and found that the $pglH_{FA1090}$ allele is grossly hypomorphic and mapped the reduced glucosyltransferase activity trait to a unique histidine at residue 371. Interestingly, the hypomorphic *pglA* and *pglH* alleles thus exist in the same FA1090 background. Therefore, we wanted to investigate how these hypomorphic glycosyltransferases might influence microheterogeneity when they are expressed together. Subsequently, FA1090-manipulated strains that express the active and/or the less active *pglA* and *pglH* alleles were monitored by immunoblotting with glycan-specific antibodies (Fig. 8). By introducing the most active allele of *pglH* ($pglH_{FA1090\ H371R}$), increased PglH-based disaccharide was detected in backgrounds without PglA or with less active PglA ($pglA_{FA1090}$). By introducing the most active allele of *pglA* ($pglA_{N400}$), PglH-based disaccharide levels were decreased, while a compensatory increase in trisaccharide glycoform (based on PglA and PglE) was detected. Together, these results show how allelic differences and interactions can dramatically modulate glycoform expression.

DISCUSSION

Despite its ubiquitous nature, the mechanisms underlying glycosylation microheterogeneity remain poorly understood. Likewise, the biological significance of these phenomena remains largely unexplored. In systems in which oligosaccharides are transferred *en bloc* to proteins, microheterogeneity may be explained by the network of rapid, sequential reactions taking place in a cellular compartment distinct from that in which substrate transfer occurs. In the absence of any quality control surveillance, simple enzyme kinetics may dictate the degree of oligosaccharide maturation prior to membrane translocation and transfer to substrate. This appears to be the situation operating in the neisserial *O*-linked *pgl* system since it has evolved to tolerate alternate UndPP-linked glycoforms as part of its phase and antigenic variation program. It follows, then, that the relative activities of glycosyltransferases required for synthesis of UndPP-linked oligosaccharides should be a major factor in microheterogeneity. Our data here are entirely congruent with this model.

Based on the extreme specificities and sensitivities of the glycoform-specific MAbs used here, we observed a number of insightful examples of microheterogeneity. In the cases of low-level glycoform expression, an important distinction to be made is whether the signals detected represent those from a small number of cells in which phase expression has been altered or a small amount of expression within each individual cell. For example, gonococcal strain N400 (which carries *pglE* in an off configuration) expresses low levels of diNAcBac and trisaccharide, although it is predicted, on the basis of its genotype, to express solely the disaccharide glycoform. Here, the low levels of trisaccharide are likely attributable to a small number of *pglE* phase-on variants in the cell population, while the low level of diNAcBac expression must be due to incomplete modification in an otherwise wild-type, PglA-expressing cell since the $pglA_{N400}$ allele is not subject to phase variation (as it lacks the extended nucleotide repeat motif) (Fig. 7A; see Table S1 in the supplemental material). Similar concerns also apply to those instances where di- and trisaccharides are detected in strains carrying phase-off *pglA* alleles (such as those from strains 8013, ST3787, ST640, and FAM18). On the basis of the number of guanine residues spanning the simple repeat region (varying from 8 to 11) in these alleles, the frequencies

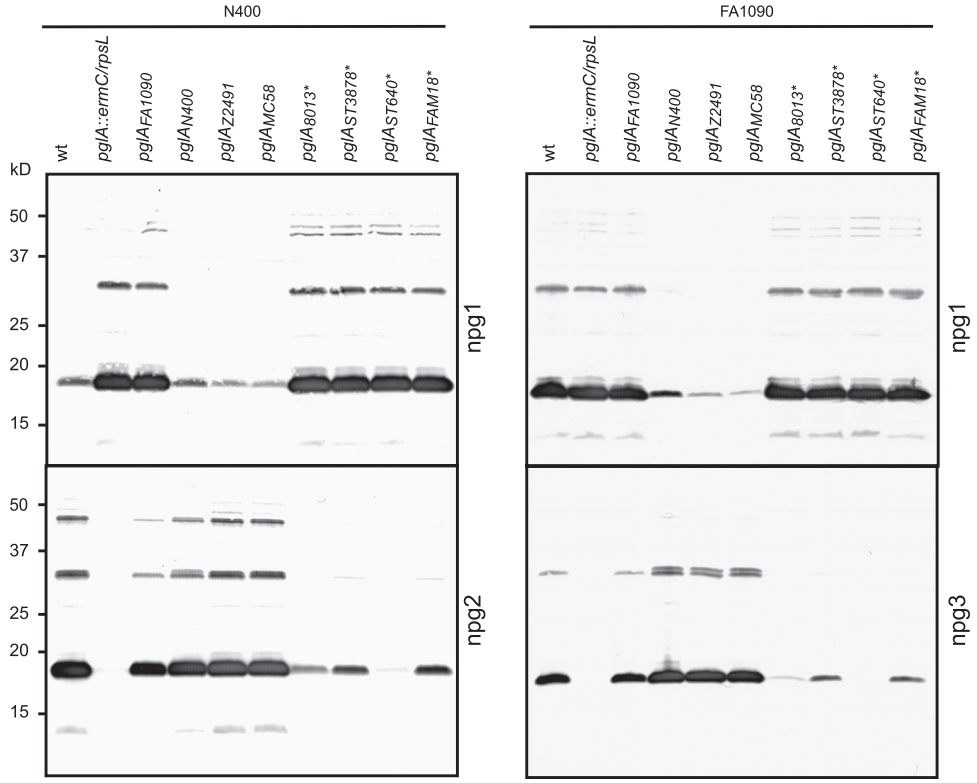

FIG 6 Distinct *pglA* alleles are associated with variable levels of microheterogeneity. Immunoblotting of whole-cell lysates from mutant strains of FA1090 and N400, carrying *pglA* alleles from different *N. gonorrhoeae*, *N. meningitidis*, and *N. lactamica* strains, with glycan-specific monoclonal antibodies (npg1, npg2, and npg3). Note that the *pglA* genes from N400, FA1090, Z2491, and MC58 are in the phase-on configuration, while those from 8013, ST3787, ST640, and FAM18 are in the phase-off configuration and are marked with asterisks (the alignment is shown in Fig. S1 in the supplemental material). N400-derived strains were KS100, KS462, KS483, KS484, KS742, KS743, KS744, KS477, KS478, and KS745. FA1090-derived strains were KS300, KS463, KS482, KS481, KS763, KS764, KS765, KS479, KS480, and KS766.

with which phase-on variants are predicted to arise are orders of magnitude below those required to account for the glycoform levels observed (5). This is especially evident in the case of the $pglA_{ST3787}$ and $pglA_{FAM18}$ alleles, which were associated with levels of disaccharide glycoform expression that were only 20- to 25-fold reduced from the level seen in the $pglA_{N400}$ background. We propose that these alleles are examples of what have been termed "expressed pseudogenes" (16) or "pseudopseudogenes" (6), in which significant levels of gene product are expressed from out-of-frame alleles. Specifically, the poly(G) stretches contained within these *pglA* alleles are sequence patterns for nonstandard decoding at the levels of both programmed ribosomal frameshifting and programmed transcriptional realignment (23). Delineation of these two mechanisms cannot be achieved even at the protein level, as their protein products are identical. Additionally, we documented clear differences in the levels of microheterogeneity among the out-of-frame alleles. This could be due to context-specific, *cis*-acting effects or to other polymorphisms in the *pglA* ORFs. Whatever the situation, our findings indicate that in this system, the contingency locus is not simply a mechanism for on-off expression but, rather, operates to modulate glycoform expression across a broad spectrum from low to high levels. These findings are clearly evocative of findings made in the gonococcal lipooligosaccharide expression system, in which out-of-frame glycosyltransferase alleles are associated with considerable activity (9, 24).

Our observations on protein glycan microheterogeneity in gonococcal strain FA1090 are particularly noteworthy. This strain carries hypomorphic alleles of both *pglA* and *pglH*, and both genes are phase variable, endowing it with particularly unique forms of microheterogeneity. It remains unclear, however, how and why these genotypes have arisen. It is interesting that this strain has been and is continuing to be used in experimental infection of human male volunteers (17). As such, it should be possible to assess how glycoform variation and microheterogeneity might occur *in vivo* in this particular strain.

One important aspect of this work relating to both $PglA_{FA1090}$ and $PglH_{FA1090}$ centers on how single-residue substitutions can have such profound effects on glycosyltransferase activity. It is remarkable that the single substitutions in both proteins are localized to the C terminus and outside the conserved carbohydrate-active enzyme (CAZy) family 1 glycosyltransferase domain (see Fig. S2 in the supplemental material). A trivial explanation would be that these alterations simply reduce steady-state levels of the enzymes. We currently lack the reagents necessary to monitor PglA levels but have previously shown that the single alteration at residue 371 of PglH does not significantly alter protein expression levels (8). An alternative explanation stems from observation made for the WaaJ glycosyltransferase involved in the synthesis of

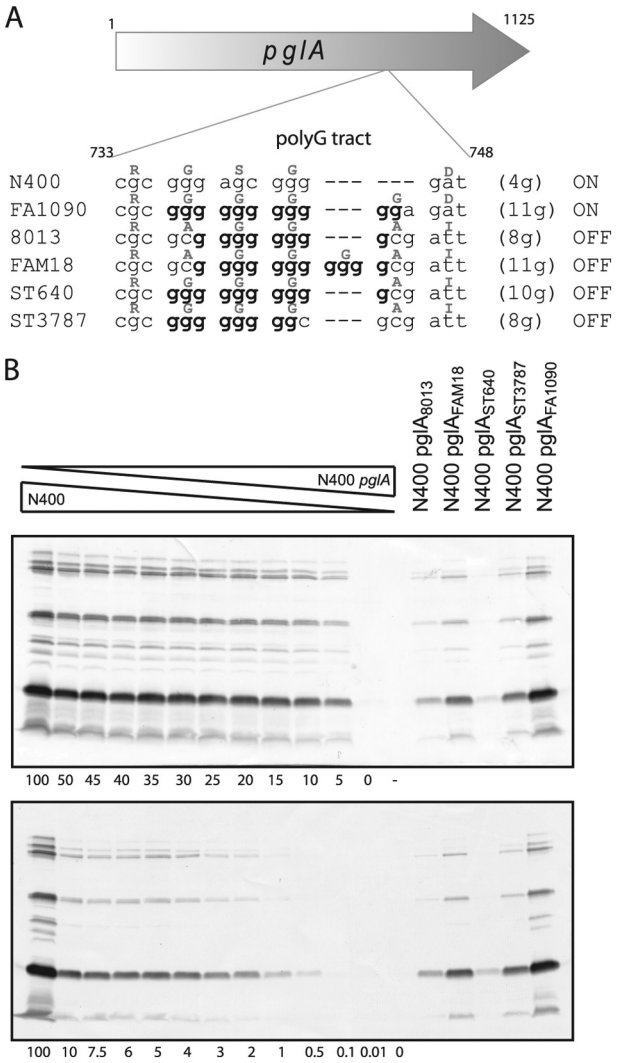

FIG 7 Quantification of the variable levels of microheterogeneity. (A) The poly(G) tracts within the *pglA* ORFs from *N. gonorrhoeae* strains (N400, FA1090), *N. meningitidis* strains (8013, FAM18), and *N. lactamica* strains (ST640, ST3787) are shown with the corresponding amino acids above (gray). The nucleotide numbering is according to $pglA_{N400}$. (B) Immunoblotting of whole-cell lysates from mutant strains of N400 carrying *pglA* alleles from different *N. gonorrhoeae*, *N. meningitidis*, and *N. lactamica* strains with the npg2 glycan-specific monoclonal antibody. The amount of disaccharide glycan in N400 carrying different *pglA* alleles was compared to the level of disaccharide glycan in admixtures of N400 wild type (KS100) and N400 *pglA*::*ermC-rpsL* (KS462). Other strains used were KS744, KS745, KS478, KS477, and KS483. Note that the *pglA* genes from 8013, ST3787, ST640, and FAM18 are in the phase-off configuration.

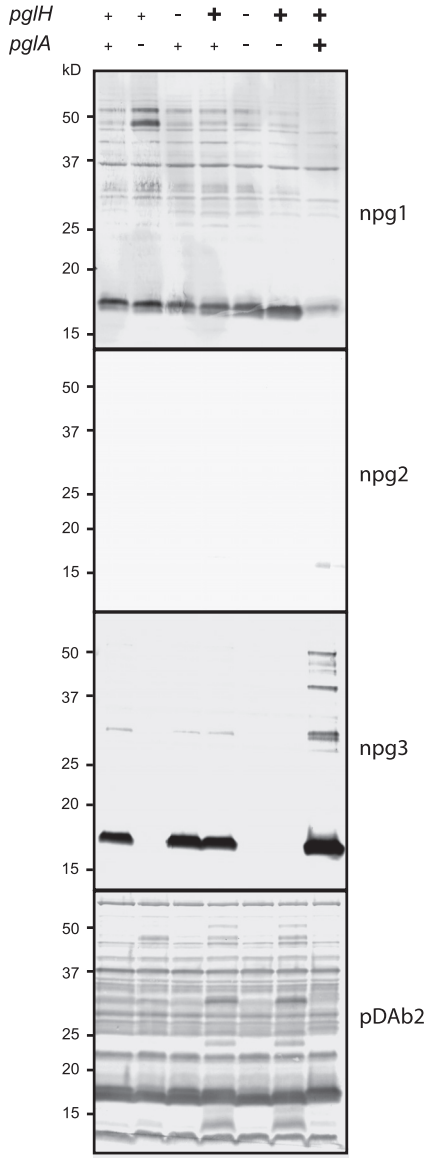

FIG 8 Polymorphic alleles of *pglA* and *pglH* influence microheterogeneity. Immunoblotting of whole-cell lysates from mutant strains of FA1090 carrying different *pglA* and *pglH* alleles with the glycan-specific monoclonal (npg1, npg2, and npg3) and polyclonal (pDAb2, which recognizes diNAcBac-Glc) antibodies. +, wild-type *pglA* and *pglH* alleles; −, null alleles *pglA*::*kan* and *pglH*::*ermC-rpsL*; **+**, hypermorphic alleles $pglH_{FA1090\ H371R}$ and $pglA_{N400}$. Strains with the most active allele of *pglH* had increased diNAcBac-Glc in backgrounds without PglA or with less active PglA (compare the fourth and sixth lanes with the second lane). Strains with most active allele of *pglA* had less diNAcbac-Glc, and instead, more trisaccharide was detected (seventh lane). Strains used were KS300, KS769, KS422, KS459, KS423, KS460, and KS770.

the outer core region of *Escherichia coli* LPS (19). This work reported that the catalytic activity of WaaJ was severely compromised by limited deletions at the very C terminus. This region also maps outside the major catalytic domain but is rich in hydrophobic and positively charged amino acids that were implicated in membrane association. These features are also observed within the C-terminal tail of PglA and PglH. As the acceptors in all these cases are nascent, membrane-associated oligosaccharides, this property might be beneficial for activity, as previously suggested (19). Thus, the single-residue substitutions in the defective PglA and PglH forms might result in diminished membrane targeting. Albeit plausible, more work is needed to firmly address these possibilities.

ACKNOWLEDGMENTS

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