Combining FAIMS based Glycoproteomics and DIA Proteomics reveals widespread proteome alterations in response to glycosylation occupancy changes in *N. gonorrhoeae*.

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Abstract

Protein glycosylation is increasingly recognized as a common protein modification across bacterial species. Within the Neisseria genus O-linked protein glycosylation is conserved yet closely related Neisseria species express O-oligosaccharyltransferases (PglOs) with distinct targeting activities. Within this work, we explore the targeting capacity of different PglOs using Field Asymmetric Waveform Ion Mobility Spectrometry (FAIMS) fractionation and Data-Independent Acquisition (DIA) to allow the characterization of the impact of changes in glycosylation on the proteome of N. gonorrhoeae. We demonstrate FAIMS expands the known glycoproteome of wild type N. gonorrhoeae MS11 and enables differences in glycosylation to be assessed across strains expressing different pglO allelic chimeras with unique substrate targeting activities. Combining glycoproteomic insights with DIA proteomics, we demonstrate that alterations within pglO alleles have widespread impacts on the proteome of N. gonorrhoeae. Examination of peptides known to be targeted by glycosylation using DIA analysis supports alterations in glycosylation occupancy occurs independently of changes in protein levels and that the occupancy of glycosylation is generally low on most glycoproteins. This work thus expands our understanding of the N. gonorrhoeae glycoproteome and the roles that pglO allelic variation may play in governing genus-level protein glycosylation.

Introduction

Protein glycosylation is a conserved process observed across all domains of life which allows the augmentation of protein properties including solubility, enzymatic activity and protein stability [1-4]. While once thought to be absent in bacterial systems it is increasingly recognized that numerous diverse protein glycosylation pathways exist across bacterial species ranging from dedicated systems responsible for modifying single proteins, such as flagellin [5] and autotransporters [6], to those responsible for modifying hundreds of proteins known as general glycosylation systems [1, 2, 7]. Within Gram-negative species, multiple general glycosylation systems have now been identified with the PglO (also known as PglL) family of oligosaccharyltransferases emerging as a widespread class of *O*- linked glycosylation systems [8]. This family of enzymes is increasingly recognized to be functionally diverse with multiple variants identified that possess unique properties including glycan specificities and substrate ranges [9-11]. While multiple PglO enzymes have been experimentally confirmed to drive *O*- glycosylation in genera including *Moraxella* [9]*Acinetobacter* [11, 12], *Burkholderia*[13-15], *Francisella*[16], *Pseudomonas*[17], *Ralstonia*[18] and *Neisseria*[19-21] the glycoproteomes of these bacterial species are still poorly defined with MS based approaches increasingly used to characterize bacterial glycoproteomes.

The characterization of glycopeptides using liquid chromatography–mass spectrometry (LC-MS) has become an indispensable tool for glycoproteomics [22]. While isolation of bacterial glycopeptides using offline enrich-

ment such as hydrophilic [11, 12, 15, 18, 23, 24], charge [20] or affinity [25, 26] chromatography approaches followed by LC-MS have been ubiquitously used for bacterial glycoproteomic studies, these approaches increase the complexity of experimental designs and, as highlighted within comparisons of conventional multidimensional fractionation approaches [27], suffer from significant sample loss. An alternative to offline fractionation techniques is the use of online separation approaches with Ion mobility spectrometry (IMS) based separation/fractionation emerging as a powerful technique to streamline the analysis of biomolecules [28]. IMS enables the separation of analytes based on a combination of charge and collisional cross-section making it uniquely suited for separating mixtures containing diverse analytes [29]. Previously it has been demonstrated that IMS allows the enrichment of populations of cross-linked peptides [30, 31], the fractionation of combinations of post-translational modifications (PTMs) to dissect PTM crosstalk [32] and even the ability to distinguish glycopeptides from non-glycosylated components based on their collisional cross-section alone [33]. Leveraging IMS, multiple groups have shown different IMS platforms are advantageous for glycoproteomic analysis including trapped ion mobility separation (TIMS) [34] as well as high-field asymmetric waveform ion mobility spectrometry (FAIMS) [35-38]. Using FAIMS - based glycopeptide enrichment, we previously demonstrated that unique glycopeptides within bacterial glycoproteomes inaccessible to hydrophilic enrichment chromatography could be identified, allowing the identification of novel glycosylation events without offline glycopeptide enrichment [35]. While we have used FAIMS - based glycopeptide enrichment to characterize both bacterial *N* - and *O* -linked glycopeptides [35, 39], it is still unclear if this approach is uniformly applicable to all bacterial glycosylation systems due to the inherent chemical diversity of bacterial glycans [40].

Within members of the *Neisseria* genus, *O*- linked glycosylation was first confirmed nearly 30 years ago with protein crystallography and MS-based analysis revealing the presence of glycosylation events within the major pilin subunit (PilE) of*Neisseria gonorrhoeae* [41] and*Neisseria meningitidis* [42]. Future work expanded understanding of the genetics [19, 20, 43-45] and the biochemical processes [45-47] associated with*O* -linked glycosylation in *Neisseria* to reveal that diverse arrays of glycans can be utilized [20, 44] to glycosylate multiple extracytoplasmic proteins [48, 49]. Within *N. meningitidis* and*N. gonorrhoeae, O*- linked glycosylation has been shown to contribute to antigenic variation allowing the evasion of the humoral immune response [43, 50, 51] yet its conservation across *Neisseria* species suggests that glycosylation may play roles beyond this function. Within *N. gonorrhoeae,* arguably the best characterized *Neisseria*glycoproteome to date, glycan serotyping [48] and immunoaffinity protein enrichment [26] coupled with LC-MS analysis have identified at least 19 glycoproteins [26] yet the true extent of the*N. gonorrhoeae* glycoproteome remains unclear. A critical barrier to glycoproteomic studies within *N. gonorrhoeae* has been the dependency on bespoke affinity reagents such as monoclonal antibodies to facilitate the identification or enrichment of glycoproteins prior to LC-MS analysis. Recently, we demonstrated that *N. gonorrhoeae*glycopeptides could be readily identified from whole cell lysates using LC-MS allowing the assessment of the *N. gonorrhoeae* glycoproteome without the need for offline glycopeptide enrichment [52]. Notably, we also observed proteome and glycoproteomic changes in *N. gonorrhoeae* in response to the expression of an exogenous *pglOO*- oligosaccharyltransferase from the distantly related species*Neisseria elongata* [52]. Thus, these findings suggest alterations in *pglO* alleles within*N. gonorrhoeae* may drive both proteome as well as glycoproteome alterations. However, a systematic assessment of how different*pglO* alleles influence the proteome/glycoproteome of *N. gonorrhoeae* is required to confirm if this is a general phenomenon.

Within this work, we explore the use of FAIMS fractionation to expand our understanding of the glycoproteome of *N. gonorrhoeae* . Leveraging FAIMS based enrichment, we explore differences in glycosylation patterns across *N. gonorrhoeae* strains expressing different *pglO* allelic chimeras with unique substrate targeting activities identifying 44 unique glycoproteins. To further understand the proteomic changes driven by alterations in PglOs, we utilized Data-Independent Acquisition (DIA) [53, 54] revealing proteomic alterations across *N. gonorrhoeae* strains expressing different *pglO*alleles. These findings support the expression of different *pglO*alleles drive glycoproteome and proteome alterations within*Neisseria* species as well as that the expression of a glycoprotein alone, even if known to be compatible with other PglO enzymes, may be insufficient to predict glycosylation efficiency with a given PglO. Combined, this work expands our under-

standing of the*N. gonorrhoeae* glycoproteome and supports that the distinct targeting activities of different *pglO* alleles appears to drive proteomic changes independent of the glycoproteins targeted for glycosylation.

Methods

Bacterial strains and culturing conditions : *N. gonorrhoeae* MS11 [55] and *N. gonorrhoeae* strains expressing chimeric *pglO* alleles [52] used within this study are listed in Supplementary Materials Table 1. *N. gonorrhoeae* strains were grown for 16-18hr (at 37°C with 5% CO_2) on GC medium (Difco) supplemented with Kellogg's supplement [56]. Cells were pelleted (4°C, 2800 g , 10 minutes), washed with ice-cold PBS (4°C, 10,000g) and then snapped frozen.

SDS-PAGE and immunoblotting: SDS-PAGE and immunoblotting of*N. gonorrhoeae* glycoproteins was undertaken as previously described [48]. Briefly, whole-cell lysates were prepared from equivalent numbers of cells by heating cell suspensions to 65°C in SDS–sample loading buffer and then lysates separated on a 15% SDS-PAGE gel prior to being transferred to a nitrocellulose membrane. Glycoproteins were detected by immunoblotting with the npg3 monoclonal rabbit antibody reactive to the*N. gonorrhoeae* di-N-acetylbacillosamine-galactose-galactose glycan [43] at a 1:10,000 dilution and detected using a alkaline phosphatase-coupled goat anti-rabbit secondary antibody (Sigma).

Proteome sample preparation: Snap frozen cells were resuspended in 4% sodium dodecyl sulfate (SDS), 100 mM Tris pH 8.5, and boiled at 95°C for 10 minutes with shaking (2000 rpm; Eppendorf ThermoMixer®) and ˜1mg of protein precipitated by mixing with 4x volume of ice-cold acetone and incubating overnight at -20°C. Protein samples were then pelleted at 0°C, 4000 g , 10 minutes, the acetone discarded and samples air dried. Precipitated proteome samples were prepared using S-trap mini columns (Protifi, USA) according to the manufacturer's instructions. Briefly samples were resuspended in 4% SDS, boiled and then protein amounts quantified using a BCA assay (Thermo Fisher Scientific). 200 µg of each sample was then reduced with 10mM DTT at 95°C for 10 minutes, allowed to cool to room temperature then alkylated with 40mM of Iodoacetamide for 30 minutes in the dark. Samples were then acidified with phosphoric acid to a final concentration of 1.2%, then mixed with seven volumes of 90% methanol/100mM TEAB pH 7.1 before being applied to S-trap mini columns. Samples were washed four times with 90% methanol/100mM TEAB pH 7.1 to remove SDS then Trypsin/Lys-c (1:100, Promega, USA) in 100mM TEAB pH 8.5 spun through the S-trap columns. Samples were digested overnight at 37°C then collected from the S-traps by washing with 100mM TEAB pH 8.5 followed by 0.2% FA followed by 0.2% FA/50% ACN. Peptide washes were pooled, dried and then resuspended in Buffer A* (0.1% TFA, 2% ACN) before being cleaned up with home-made high-capacity StageTips composed of 1 mg Empore C18 material (3M) and 5 mg of OLIGO R3 reverse phase resin (Thermo Fisher Scientific, USA) as previously described [57, 58]. Columns were wet with Buffer B (0.1% FA, 80% ACN) and conditioned with Buffer A* prior to use. Resuspended samples were loaded onto conditioned columns, washed with 10 bed volumes of Buffer A* and bound peptides were eluted with Buffer B before being dried then stored at -20@C. For glycoproteomic analysis of*N. gonorrhoeae* strains possessing chimeric *pglO* alleles, pooled references were created by mixing individual replicates of each*N. gonorrhoeae* strain expressing a single chimeric *pglO*allele in equal amounts resulting in 15 pooled references in total, in line with the gas phase fractionation library approach of Pino *et al.* used for DIA analysis [53].

LC-MS FAIMS fractionation based glycoproteomics : Purified peptide samples of either biological replicates of *N. gonorrhoeae*MS11 or the pooled references of *N. gonorrhoeae* strains expressing chimeric *pglO* alleles were re-suspended in Buffer A* and separated using a two-column chromatography set up composed of a PepMap100 C18 20 mm x 75 µm trap and a PepMap C18 500 mm x 75 µm analytical column (Thermo Fisher Scientific) coupled to a Orbitrap Fusion Lumos Tribrid Mass Spectrometer equipped with a FAIMS Pro interface (Thermo Fisher Scientific). 125-minute gradients were run for each sample altering the buffer composition from 2% Buffer B to 28% B over 106 minutes, then from 28% B to 40% B over 9 minutes, then from 40% B to 80% B over 3 minutes, the composition was held at 80% B for 2 minutes, and then dropped to 2% B over 2 minutes and held at 2% B for another 3 minutes. The Lumos Mass Spectrometer was operated in a FAIMS data-dependent mode with individual analytical runs collected at FAIMS Compensation Voltages (CVs) -20, -30, -40, -50, -60, -70 and -80 for *N. gonorrhoeae* MS11 as previously described [35] or FAIMS

CVs -25, -35, -45, -55, -65 and -75 for *N. gonorrhoeae* strains possessing chimeric*pglO* alleles. For each FAIMS CV, a single Orbitrap MS scan (350-2000 *m/z* , maximal injection time of 50 ms, an Automated Gain Control (AGC) of maximum of $4*10^5$ ions and a resolution of 120k) was acquired every 3 seconds followed by Orbitrap MS/MS HCD scans of precursors (NCE 30%, maximal injection time of 80 ms, an AGC set to a maximum of $2*10^5$ ions and a resolution of 30k). HCD scans containing the oxonium ions (204.0867; 138.0545, 366.1396 as well as the diNAcBac specific ions 229.1189 and 211.1082 *m/z*) triggered three additional product-dependent MS/MS scans of potential glycopeptides; a Orbitrap EThcD scan (NCE 15%, maximal injection time of 300 ms, AGC set to a maximum of $2*10^5$ ions with a resolution of 30k and using the extended mass range setting to improve the detection of high mass glycopeptide fragment ions [59]); a ion trap CID scan (NCE 35%, maximal injection time of 40 ms, an AGC set to a maximum of $5*10^4$ ions) and a stepped collision energy HCD scan (using NCE 35% with 5% Stepping, maximal injection time of 300 ms, an AGC set to a maximum of $2*10^5$ ions and a resolution of 30k).

LC-MS Data-independent acquisition (DIA) proteome analysis: Proteome samples from individual biological replicates of *N. gonorrhoeae* strains expressing chimeric *pglO* alleles were re-suspended in Buffer A* and separated using a Vanquish Neo (Thermo Fisher Scientific) equipped with a ACQUITY UPLC Peptide BEH C18 Column (300Å, 1.7 µm, 1 mm X 100 mm, Waters Corporation) coupled directly to an Orbitrap Exploris 480 (Thermo Fisher Scientific). 5 µg of proteome digest was loaded directly on to the ACQUITY column for 1 minute at 50 µL/min with Buffer A (0.1% formic acid, 2% DMSO) and the buffer composition altered from 2% Buffer B (0.1% formic acid, 77.9% acetonitrile, 2% DMSO) to 26% B over 70 minutes, then from 26% B to 99% B over 2 minutes and then was held at 99% B for 1.5 minutes. The Orbitrap 480 Mass Spectrometer was operated in a data-independent mode automatically switching between the acquisition of a single Orbitrap MS scan (350-951 m/z, maximal injection time of 50 ms, an AGC set to a maximum of 250% and a resolution of 120k) and the collection of 16 m/z DIA windows between 350 and 951 m/z (200-2000 m/z, NCE 28%, maximal injection time of 54 ms, a AGC set to 2000% and a resolution of 30k).

Glycoproteomic Data Analysis:FAIMS experiments were searched with FragPipe version 19 [60-64] using a modified "glyco-O-HCD" workflow allowing Carbamidomethyl as a fixed modification of cysteine in addition to oxidation of Methionine, N-terminal acetylation and the addition of the trisaccharide diNAcBac-Gal-Gal (mass 552.2167 Da) on Serines as variable modifications. Searches were performed against the *N. gonorrhoeae*MS11 proteome (Uniprot Accession: UP000016457, 2047 proteins) allowing a 1% FDR. The resulting "psm.tsv" files for each FAIMS fraction were combined using R retaining only glycopeptides with a MSfragger Hyperscore >20. To further ensure high data quality assigned glycopeptides were manually assessed and the HCD and EThcD spectra assigned to the best scoring unique glycopeptide annotated with the Interactive Peptide Spectral Annotator [65]. Only glycopeptides which generated high confident HCD and EThcD MS/MS events, defined as spectra containing multiple b/y ions for HCD or c/z/y for EThcD as well as evidence of glycan oxoniums and characteristic loss of glycans within HCD spectra or the expected charge reduced masses for EThcD, were considered for further analysis. Manually validated glycopeptides are provided within Supplementary Data 1 and 2 and only these glycopeptides were used for analysis and data visualization.

DIA Proteomic analyses : DIA data were searched using Spectronaut (Biognosys, Switzerland) versions 17.1.221229 using the DIA-library free analysis workflow allowing oxidation of Methionine and N-terminal acetylation as variable modifications as well as Carbamidomethyl as a fixed modification of cysteine. The protein LFQ method set to MaxLFQ, single hit proteins excluded, and the precursor PEP cut-off was altered to 0.05 from the default 0.2 to improve quantitative accuracy. Searches were performed against the *N. gonorrhoeae* MS11 proteome (Uniprot Accession: UP000016457) supplemented with the chimeric PglO sequences. Protein outputs from Spectronaut were processed using Perseus (version 1.6.0.7) [66] with missing values imputed based on the total observed protein intensities with a range of 0.3 σ and a downshift of 1.8 σ. Statistical analysis was undertaken within Perseus by grouping biological replicates together which were assessed using ANOVA analysis to compare changes across all groups or using student t-tests to compare the proteome changes between the $pglO_{meningitidis}$ group to all other combinations of *pglO* groups with a minimum fold change of +/- 1 considered for further analysis. Multiple hypothesis correction was undertaken

using a permutation-based FDR approach allowing an FDR of 5%. Matching of protein homologs between *N. gonorrhoeae* MS11 and the reference *N. gonorrhoeae* FA 1090 strain (Uniprot accession: UP000000535) was undertaken using the Proteome comparison function of PATRIC, the bacterial bioinformatics database and analysis resource [67] with the *N. gonorrhoeae*FA 1090 gene names, ORF assignments and Gene ontology information used to enable Enrichment analysis using Fisher exact tests in Perseus.

Data visualization and availability: Visualization of proteomic data was undertaken using R (version 4.2.1) using the tidyverse [68] collection of packages. All mass spectrometry data (RAW files, FragPipe outputs, Spectronaut experiment files, Rmarkdown scripts, and input tables) have been deposited into the PRIDE ProteomeXchange repository [69, 70]. All PRIDE accession numbers, descriptions of the associated experiments are provided within Supplementary Materials Table 2.

Results

FAIMS fractionation allows monitoring of the *N. gonorrhoeae* glycoproteome.

The identification of *N. gonorrhoeae* glycoproteins has traditionally relied on the detection and/or enrichment of glycoproteins using bespoke antibodies which, while effective [26, 48], has restricted the characterization of the *N. gonorrhoeae* glycoproteomes to specialized laboratories. Currently at least 19 proteins are known to be glycosylated in *N. gonorrhoeae*[26] yet the full extent of protein glycosylation within this species is unclear. Utilizing FAIMS instrumentation, we previously demonstrated bacterial glycopeptides could be isolated from complex lysates providing a complementary technique to affinity enrichment approaches for assessing bacterial glycoproteomes [35]. Thus, to improve our understanding of the *N. gonorrhoeae* glycoproteome and to further benchmark the abilities of FAIMS to isolate bacterial glycopeptides, we assessed the identification of *N. gonorrhoeae*MS11 glycopeptides across differing FAIMS CVs from -20 to -80 (Figure 1A-D, Supplementary Table 1 & Supplementary Data 1). Consistent with our previous studies [35], we observe that FAIMS fractionation readily allows the identification of glycopeptides across different FAIMS CVs (Figure 1A) with the majority of glycopeptides identified within CVs above -50 and a CV of -30 enabling access to the highest number of unique glycopeptides across replicates (Figure 1A-C). Across three biological replicates, a total of 48 unique glycopeptides were identified with >70% of identified glycopeptides (34 glycopeptides) observed across all replicates (Supplementary figure 1A). At the protein level, a total of 26 unique glycoproteins were identified with 13 corresponding to previously identified glycoproteins [26] (Figure 1D). Interestingly, across the 48 unique glycopeptides only 7 glycosylation sites were definitively localized reflecting the low charge density of these glycopeptides and supporting previous studies demonstrating the preference for serine glycosylation by PglO (Supplementary Figure 1B &C, Supplementary Data 1). Taken together, these results support FAIMS fractionation enables glycoproteomic analysis of *N. gonorrhoeae*to a comparable depth to previous approaches and allows the identification of novel glycoproteins.

FAIMS enabled glycoproteomic analysis expands the known glycoproteome of *N. gonorrhoeae* expressing chimeric PglOs.

With the ability of FAIMS fractionation to allow the identification of*N. gonorrhoeae* glycopeptides established, we sought to further probe the utility of this approach to understand glycoproteome diversity utilizing a recently reported panel of chimeric *pglO* alleles generated from the fusion of $pglO_{cinerea}$ and$pglO_{meningitidis}$ expressed within *N. gonorrhoeae* [52]. Across this panel of 15 *pglO* alleles dramatic changes in glycosylation patterns are observed (Figure 2A), yet the individual glycoproteins altered, if these changes are due to changes in occupancy or glycoprotein levels, as well as how these alterations impact the broader proteome remain unclear. To assess these questions, we utilized a pooled reference approach [53] generating reference pools of each *pglO* allele and surveyed these glycoproteomes using FAIMS fractionation revealing dramatic differences in the number of glycopeptides and glycoproteins identified across strains (Figure 2A, Supplementary table 2 and Supplementary Data 2). Consistent with the changes observed by glycan – specific, western analysis, FAIMS based glycoproteomics supports a reduction in glycoprotein diversity within the majority of chimeric *pglO* alleles compared to *N. gonorrhoeae* expressing $pglO_{meningitidis}$ (Figure 2B). At the glycopeptide level, a total of 66 unique glycopeptides were identified across all *pglO* alleles with the ma-

jority of FAIMS glycoproteome references only containing a subset of glycopeptides (Supplementary Figure 2). Combined, a total of 37 unique glycoproteins were identified across this panel including 26 previously uncharacterized glycoproteins (Supplementary Figure 3). Across this panel, only ˜40% of glycoproteins were identified in more than 3 strains (16 glycoproteins, Figure 2C) with upset analysis revealing only five glycoproteins uniformly identified across all strains and the *N. gonorrhoeae* reference generated from strains expressing $pglO_{meningitidis}$ possessing the highest number of unique glycoproteins (9 unique glycoproteins, Figure 2D). Importantly, FAIMS analysis recapitulates the trends in the levels of glycosylation observed by glycan specific blotting, for example, western blotting supports a marked decrease in glycosylation within *N. gonorrhoeae* expressing the *pglO* hybrid 4 and consistent with this FAIMS analysis demonstrates only five glycoproteins were identified within this strain (Figure 2A and D). Combined, these results expand the number of substrates known to be able to be glycosylated within *N. gonorrhoeae* to 44 glycoproteins (Supplementary Table 4, Supplementary Figure 4) and supports that the expression of different *pglO* alleles results in the alteration of the glycoproteome of *N. gonorrhoeae* .

DIA analysis supports the expression of different *pglO* alleles results in widespread proteomic alterations in *N. gonorrhoeae*.

While FAIMS enabled glycoproteomics and western analysis supports changes in the glycoproteome of strains expressing chimeric *pglO* alleles, how this impacts the *N. gonorrhoeae* proteome as well as individual glycoproteins is unclear. To explore this, we undertook a quantitative assessment of the proteomes of *N. gonorrhoeae* expressing different *pglO* alleles seeking to understand changes both in identified glycoproteins as well as the broader proteome. Using DIA analysis, four biological replicates of each strain were assessed leading to the identification of 1459 proteins with > 96% of proteins observed in each replicate and all biological replicates examined demonstrating a Pearson correlation of >0.9 (Supplementary Figure 5A and B, Supplementary table 3). PCA analysis reveals modest separation of *pglO* alleles yet clustering of most biological groups (Figure 3A) with ANOVA analysis supporting diverse proteome changes across the panel with 481 proteins differentially altered within at least one biological group (Figure 3B and C). Clustering of these 481 differentially altered proteins reveals the separation of *pglO* alleles into three groups, denoted as group one, composed of $pglO_{meningitidis}$ and *pglO* hybrid 16; group two, composed of $pglO_{cinerea}$ as well as hybrid 1, 2, 3, 4; and group three, *pglO* hybrid 5, 6, 7, 8, 9, 10, 11 and 12 (Figure 3C). Enrichment analysis of these differentially abundant proteins demonstrates a small enrichment within proteins assigned as "integral component of membrane [GO:0016021]" (Fisher exact test, enrichment value 1.6041, p-value = $7.01*10^{-11}$, Supplementary table 5) supporting the enrichment in changes within the membrane proteome in response to changes in *pglO* alleles. To further understand the differential changes between *pglO* alleles, we examined protein alterations (defined as proteins with a fold change of ± 1-fold ($\log_2$) and a -log10(p-value) >2.0 respectively, Supplementary table 3) observed between $pglO_{meningitidis}$ and the remaining members of the panel revealing 76 differentially altered proteins (Figure 4A). Akin to protein alterations enriched within the ANOVA analysis, multiple differentially abundant proteins corresponded to membrane proteins including permeases (NGO_0196, NGO_0216, NGO_2011 and NGO_2012, highlighted in blue) and transporters (NGO_0395, NGO_1957 and NGO_1435 highlighted in red) again supporting differential changes in the membrane proteome in response to changes in *pglO* alleles (Figure 4B). Surprisingly, across these 76 altered proteins only a single known glycoprotein was observed to be differentially impacted (NGO_1577, highlighted in green Figure 4B) supporting that despite alterations in glycosylation in response to changes in *pglO* alleles, most glycoproteins appear unaffected. Combined these finding support that across *N. gonorrhoeae* strains expressing different *pglO* alleles, notable changes in the proteome, but not known glycoproteins, are observed with these alterations being enriched for membrane components.

DIA analysis supports alterations in glycosylation occupancy between *pglO* alleles.

The observation that across the *N. gonorrhoeae* proteome only a single glycoprotein is impacted by the expression of different *pglO* alleles supports alterations in glycosylation reflect changes in occupancy rather than changes in glycoprotein levels. Across the 44 glycoproteins identified within *N. gonorrhoeae* using DIA analysis, we observed 40 of these glycoproteins are quantified with > 10 unique precursors highlighting

the ability of DIA analysis to provide high protein sequence coverage of the *N. gonorrhoeae* glycoproteome (Supplementary Figure 6). Due to this high coverage, we sought to directly gauge the relative levels of glycosylation using the non-glycosylated forms of glycopeptides inspired by recent studies highlighting the ability of PTM status and isoform specific information to be retrievable using peptide-centric analysis [71-73]. Within our DIA dataset, 59.5% (50 out of 84) peptide sequences observed glycosylated within FAIMS experiments (Supplementary table 1 and 2) were identifiable in their non-glycosylated forms with 39.3% (33 out of 84) corresponding to fully cleaved peptide sequences suitable for quantification assessments (Figure 5A, Supplementary Table 6). Overlaying protein and peptide quantification information in combination with information on the glycosylation status as determined from FAIMS analysis, we observe that while the protein levels of glycoproteins remain constant across*N. gonorrhoeae* strains expressing different *pglO* alleles, peptides observed to undergo glycosylation display alterations across biological groups consistent with changes in occupancy (Figure 5B-D, Supplementary Figure 7-10). For example, within the glycoprotein NGO_1365, the peptide^{384}EWAPSENQAAAPQAGVQTASEAKPASEAK^{412}shows consistent and similar measurements within biological groups but across groups notable variations within the peptide abundance are observed (Figure 5B). Consistent with changes in glycosylation, we also observed the absence of unglycosylated peptides within some biological groups suggesting potential enhanced occupancy of these peptides with specific *pglO* alleles. For example, within the glycoproteins NGO_1800, the peptide^{173}VAVGVQTASGAQTVR^{187} was observed across all biological groups but is absent in 3 out of the 4 biologicals of $pglO_{meningitidis}$ expressed within *N. gonorrhoeae* (Figure 5C). Similarly, for the glycoprotein NGO_2092 we observe the peptide^{31}EQAVSAAQSESASVTVK^{50} can be consistently identified within biological groups corresponding to*pglO* hybrid 1, 2, 4, 5, 6, 8, 9 and 11 but is absent in all other groups with glycosylation being observed on this peptide in$pglO_{cinerea}$ and *pglO* hybrid 3, hybrid 7, 9, 10 and 12 (Figure 5D). These results support that the alterations observed within the *N. gonorrhoeae* glycoproteome by western and FAIMS analysis correspond to changes in occupancy with the intensity / the detectability of multiple non-glycosylated forms of known glycopeptide altered across strains expressing different *pglO*alleles.

Discussion

Within this work, we demonstrate the utility of FAIMS based fractionation and DIA analysis for probing bacterial glycosylation systems revealing glycosylation changes observed in response to chimeric*pglO* alleles in *N. gonorrhoeae* occur largely independent of changes in glycoprotein levels. Benchmarking the use of FAIMS fractionation for *N. gonorrhoeae* we show the glycoproteome of*N. gonorrhoeae* can effectively be studied without the need for bespoke affinity reagents and that novel glycopeptides can be readily identified using this approach. Importantly this work shows that despite the absence of protein abundance changes in glycoproteins alterations within the broader proteome of *N. gonorrhoeae* are readily identifiable in response to changes in glycosylation patterns.This work builds on our previous exploration of PglO targeting specificities [52] and supports that PglO protein substrate recognition is discrete yet reprogrammable by the manipulation of PglO. While early studies have noted differences in the compatibility of *O*- oligosaccharyltransferases to target substrates [74], our observations here expand on these findings demonstrating this also occurs outside of heterologous expression systems. Our work here supports that differences in PglO targeting ranges may be more common than previously thought which has important ramifications for predicting the targets of bacterial glycosylation across genera as well as unlocking the potential of these enzymes for glycoengineering [75].

While the use of FAIMS fractionation has been noted to be advantageous for glycoproteomics typically improving glycoprotein/glycopeptide coverage by as much as 75% [35, 38], within this study we observed a >200% increase in the number of identified glycoproteins in *N. gonorrhoeae* compared to earlier studies [26, 48]. While it is likely that this increase is due to multiple factors including the use of current generation MS instrumentation, as well as the glycosylation of proteins not natively glycosylated within *N. gonorrhoeae* by chimeric*pglO* alleles, it is noteworthy that many novel glycoproteins identified here correspond to integral membrane proteins containing multiple transmembrane domains (Supplementary table 4). Previous studies exploring the *N. gonorrhoeae* glycoproteome have traditionally used protein centric approaches including protein level antibody-based enrichment [26] or 2D gel electrophoresis coupled with antibody-based detec-

tion [48] which while effective are known to be incompatible with poorly soluble hydrophobic membrane proteins [76, 77]. The use of peptide-centric analysis, as undertaken here, is known to enhance both the detection as well as the characterization of modifications within bacterial membrane glycoproteins [24]. While most previously known *N. gonorrhoeae* glycoproteins were identified using FAIMS fractionation, glycopeptides derived from four previously identified glycoproteins failed to be detected with two of these proteins (NGO_1237 and NGO_0994) detected by DIA analysis and the remaining two glycoproteins either not detected (NGO_0983) or absent due to being disrupted within these backgrounds (PilE, NGO_1177). Importantly, while NGO_1237 and NGO_0994 are both known to be glycosylated the precise location of glycosylation within NGO_0994 is yet to be confirmed being predicted to be located in a region lacking Lysines / Arginines [52] and glycosylation within NGO_1237 requiring alternative enzymes to Trypsin to be accessible [48, 78]. Combined, these results support that for the Trypsin accessible glycoproteome, FAIMS based analysis provides broad coverage of the known glycoproteome and provides access to novel membrane glycoproteins.

The identification that changes in glycosylation patterns impact the*N. gonorrhoeae* proteome contributes to a growing body of work linking proteome alterations with changes in glycosylation occupancy across a range of Gram-negative species [79-81]. Interestingly while previous studies have explored the impact of the abolishment of glycosylation few studies have assessed how changes in glycosylation occupancy or glycosylation patterns impact bacterial proteomes. Recently it was demonstrated that within Burkholderia species the silencing of glycosylation using CRISPRi reduced occupancy but resulted in only modest proteomic impacts [82] yet in contrast our findings here support the *N. gonorrhoeae* proteome is extensively impacted by changes in glycosylation patterns. While*O*- linked glycosylation within *B. cenocepacia* has been shown to be important for the stability of multiple proteins [81, 83] we observed no change in the protein abundance of glycoproteins within this work suggesting that within *N. gonorrhoeae O*- linked glycosylation may exert its effects by modulating protein interactions or protein activities. Consistent with this previous work has shown for *N. gonorrhoeae*PilE glycosylation is not required for Pilin formation yet appears to enhance the stability of this structure [84] and for other forms of bacterial glycosylation, such as N-linked glycosylation within *C. jejuni,*glycosylation has also been shown to stabilise protein complexes with this effect occurring independent of changes in protein abundance [85]. Thus, while we find the abundance of glycoproteins are unaffected future work may seek to confirm if the glycosylation events identified here also play roles in modulating protein functions and/or the stabilization of protein complexes driving the proteomic effects observed here.

Within this study, our implementation of DIA analysis allowed access to proteome as well as glycosylation occupancy information yet, it is important to note that many glycopeptides observed by FAIMS fractionation are not observable within our DIA dataset due to the imperfect overlap in the peptides observed between these two approaches. As previously noted, FAIMS fractionation preferentially enables access to large (>1000 m/z), low charge density glycopeptides [35] with a similar trend also observed for *N. gonorrhoeae* (Supplementary Figure 1B). Due to this bias, most *N. gonorrhoeae* glycopeptides fall outside the m/z range utilised in our DIA analysis (350 to 951 m/z) which has been tailored to maximize the proteome coverage [53]. While DIA methods compatible with glycopeptides are emerging [86, 87], these methods consciously utilize wider DIA m/z ranges which improve access to glycopeptides yet this can lead to more congested MS/MS spectra reducing DIA depth [53]. While enzymatic [88, 89] and genetic [90] approaches can be used to reduce glycan sizes and heterogeneity to enhance the detection of both glycosylated and non-glycosylated forms of peptides the size of the native *N. gonorrhoeae* trisaccharide (552Da) limits the feasibility of using identical analysis approaches. Despite this caveat we find the non-glycosylated forms of 50 peptides subjected to glycosylation could be observed using DIA supporting that while not optimized to allow coverage of both the glycoproteome and proteome information on glycosylation occupancy is still readily accessible. Interestingly within this analysis, we observed the changes in the non-glycosylated forms of peptides is generally modest, less than a <40% reduction in intensity with only a few examples of peptides unable to be identified between different strains (Figure 5 and Supplementary Figure 7 to 10). Thus, this relatively small impact on abundance suggests that the overall glycosylation occupancy may be modest for many *N. gonorrhoeae* glycoproteins observed here.

In summary, this work furthers our understanding of the utility of FAIMS based fractionation for bacterial glycoproteomics and expanse the known glycoproteome of *N. gonorrhoeae* . This work contributes to a growing body of studies that demonstrates that bacterial oligosaccharyltransferases possess discrete targeting ranges and that the manipulation of these enzymes can vary the observed glycosylation pattern within bacterial systems. Our identification that changes in *O* -linked glycosylation patterns leads to changes in the proteome further supports that glycosylation plays multiple roles in *N. gonorrhoeae* biology. Finally, this work demonstrates how glycoproteomic insights and peptide centric DIA information can be integrated to provide support for changes in occupancy across *N. gonorrhoeae*strains expressing different *pglO* chimeras. These changes in glycosylation patterns largely appear independent of changes in glycoprotein levels and peptide analysis supports that glycosylation occupancy is generally low within most observed glycoproteins.

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Figure legends

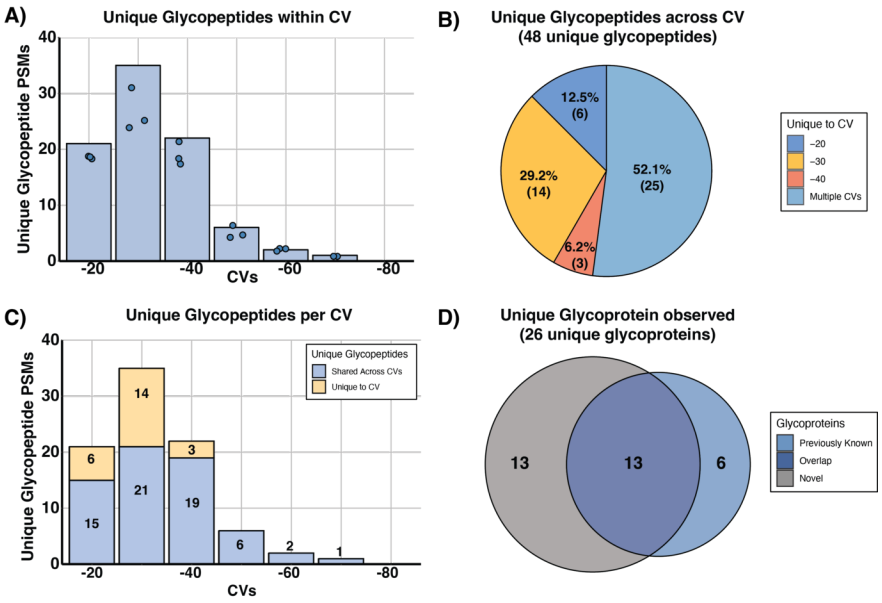

Figure 1. FAIMS fractionation enables the enrichment of glycopeptides within *N. gonorrhoeae* MS11. A) FAIMS fractionation reveals the enrichment of glycopeptides at high FAIMS CVs. Blue dots denote the numbers of unique glycopeptides across individual biological replicates at each FAIMS CV. **B & C)** Pie chart and bar plots demonstrating that high FAIMS CVs contained the highest number of unique glycopeptides identified across biological replicates with a FAIMS CV of -30 resulting in the highest number of unique glycopeptides not observed in other CVs.**D)** Venn diagram of FAIMS identified glycoproteins compared to previously reported *N. gonorrhoeae* glycoproteins [26] supports that FAIMS analysis allows the identification of multiple novel *N. gonorrhoeae*glycoproteins.

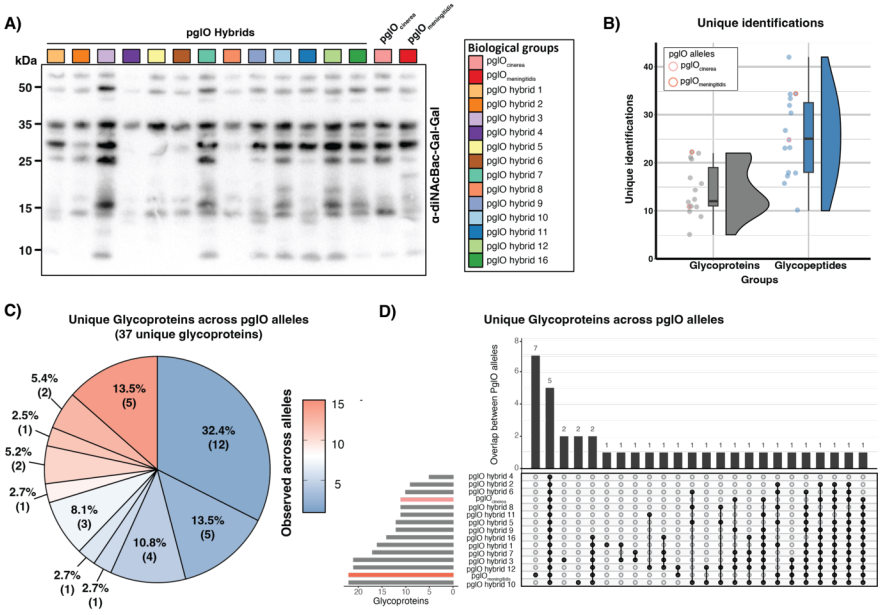

Figure 2. FAIMS enabled Glycoproteomic analysis of chimeric *pglOs* within *N. gonorrhoeae*. A) Glycan specific immunoblotting supports changes in glycosylation with *N. gonorrhoeae* strains expressing chimeric pglOs. **B)** Raincloud plots of unique *N. gonorrhoeae* glycoproteins and glycopeptides observed within strains expressing different pglOs with $pglO_{meningitidis}$ and $pglO_{cinerea}$ alleles denoted in red and pink. **C & D)** Pie chart and Upset plots of glycoproteins observed across *N. gonorrhoeae* expressing chimeric PglOs reveals most glycoproteins are observed in a limited number of strains with the highest number of unique glycoproteins observed within $pglO_{meningitidis}$ and only five glycoproteins identified across all strains.

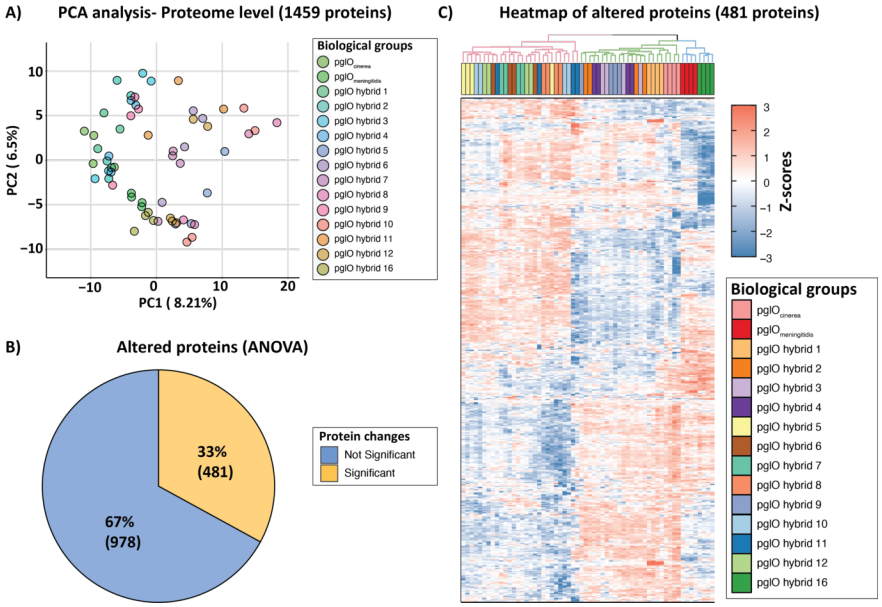

Figure 3. Proteomic alterations observed across *N. gonorrhoeae* strains expressing different *pglO* alleles.A) PCA analysis of strains expressing different *pglO*alleles reveals clustering of biological

groups and minimal separation of groups across the PC1 and PC2 dimensions. **B)** Pie chart of proteins observed altered across the proteome supports that >30% of proteins appear impacted within at least one*pglO* alleles by ANOVA analysis. **C)** Heatmap of unsupervised clustered Z-scored proteomic alterations supports widespread changes across biological groups revealing different*pglO* alleles impact the proteome in different ways with three broad clusters of proteome alterations observed across strains. Unsupervised clustering of biological replicates denoted by coloring of the dendrogram branches.

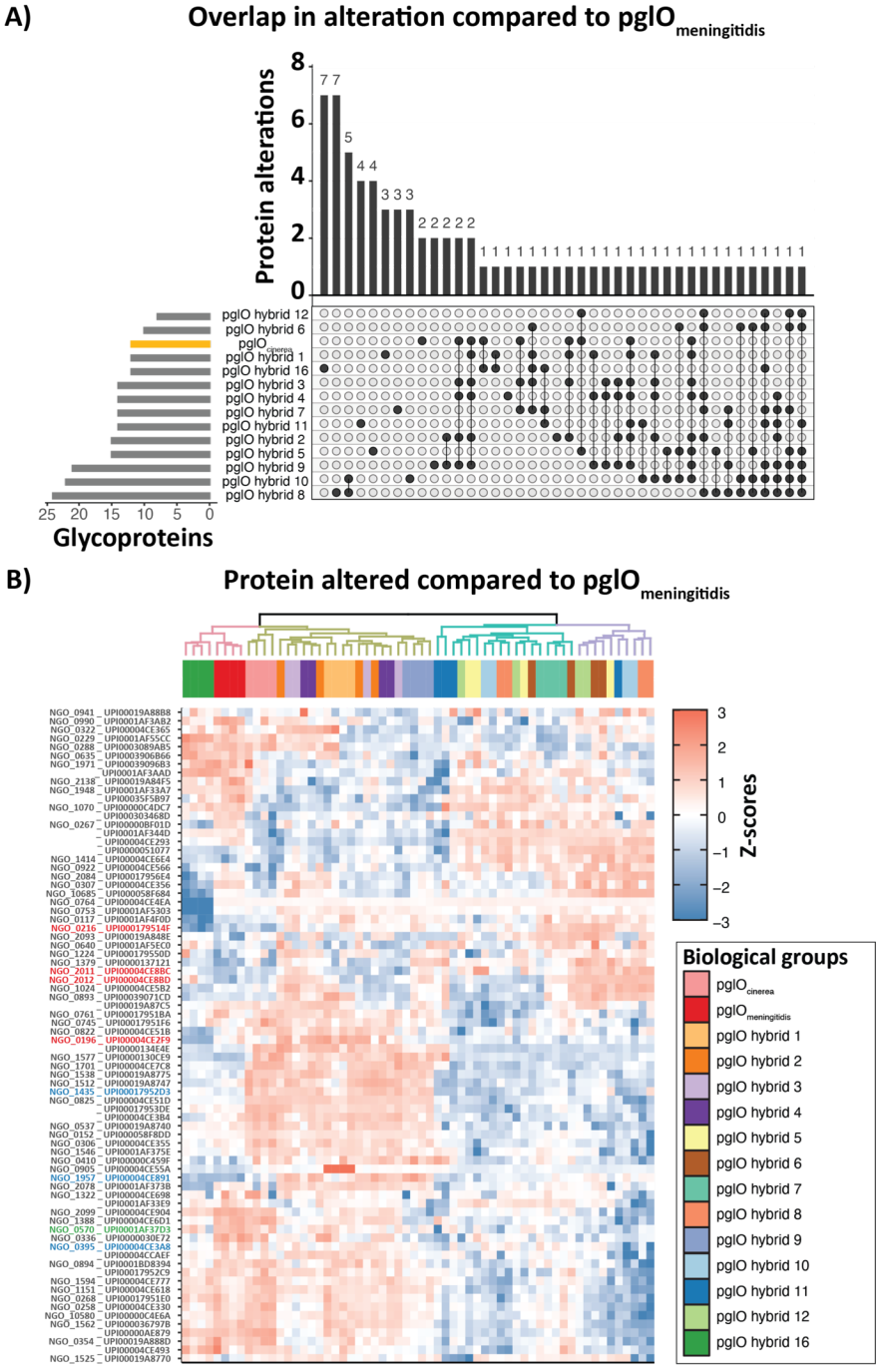

Figure 4. Proteomic alterations observed across *N. gonorrhoeae* strains expressing different *pglO* alleles compared to $pglO_{meningitidis}$. **A)** Upset plot of proteome alterations compared to $pglO_{meningitidis}$reveal 76 protein alterations between different alleles. **B)**Heatmap of unsupervised clustered z-scored alterations support different*pglO* alleles lead to different proteomic effects with changes in membrane proteins highlighted in blue, transporters, highlighted in red and the single glycoprotein differentially impacted (NGO_1577) highlighted in green. Unsupervised clustering of biological replicates denoted by coloring of the dendrogram branches.

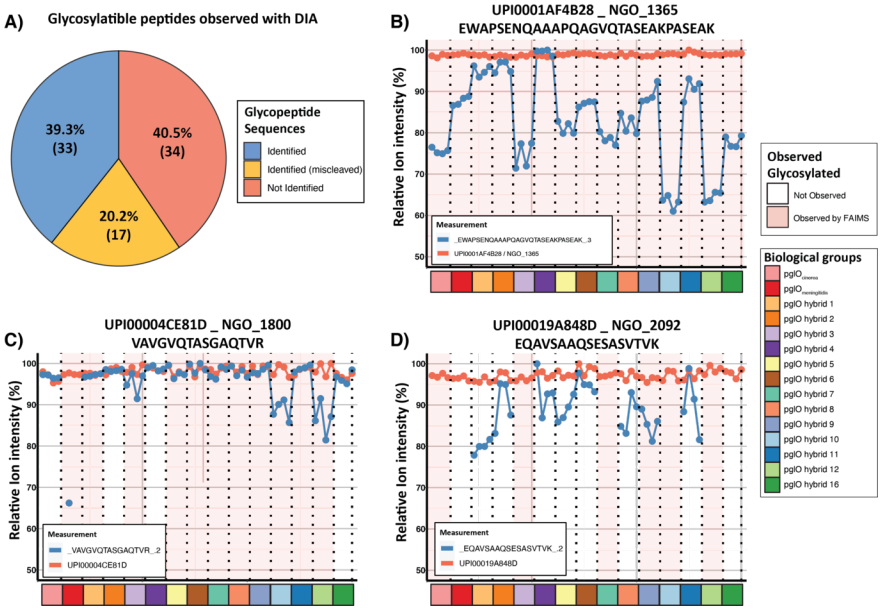

Figure 5. Peptide-centric analysis of non-glycosylated peptides within the *N. gonorrhoeae* proteome expressing different*pglO* alleles. **A)** Pie chart of peptides which can be glycosylated identifiable with DIA analysis. **B)**Glycoprotein/Peptide analysis of NGO_1365 reveals no alterations in the protein levels yet the peptide^{384}EWAPSENQAAAPQAGVQTASEAKPASEAK^{412}demonstrates variations in relative abundance across all strains.**C & D)** Glycoprotein/Peptide analysis of NGO_1800 and NGO_2092 reveals no alterations in protein levels yet the lack of peptides ^{173}VAVGVQTASGAQTVR^{187} and^{31}EQAVSAAQSESASVTVK^{50} within strains observed to glycosylate these peptides support changes in glycosylation occupancy across strains. Glycoprotein levels are denoted in red with peptide levels denoted in blue within B to D.

Supplementary tables

Supplementary Table 1. FAIMS DDA glycopeptide identifications of*N. gonorrhoeae* MS11. Combined MSFragger PSM peptide level searches of all potential glycopeptides identified across biological replicates (n=3) are provided. For all identified glycopeptides the spectrum, data files, peptide sequence, peptide length, charge, retention times, observed m/z, calibrated m/z, observed mass, calibrated mass, delta mass, scores, peptide position within protein, assigned modifications, glycan assignment quality, compensation voltage, protein, protein.ID and replicate number are provided. The manually curated glycopeptides spectra used for the creation of the Supplementary Data 1 as well as the localized glycosylation sites and list of all PSM events matching identified curated glycopeptides used for the generation of figures are also provided.

Supplementary Table 2. FAIMS DDA glycopeptide identifications of*N. gonorrhoeae* expressing different *pglO* alleles.Combined MSFragger PSM peptide level searches of all potential glycopeptides

identified across pooled references (n=15) are provided. For all identified glycopeptides the spectrum, data files, peptide sequence, peptide length, charge, retention times, observed m/z, calibrated m/z, observed mass, calibrated mass, delta mass, scores, peptide position within protein, assigned modifications, glycan assignment quality, compensation voltage, protein, protein.ID and genotype are provided. The manually curated glycopeptides spectra used for the creation of the Supplementary Data 2 including the localized glycosylation sites and list of all PSM events matching identified curated glycopeptides used for the generation of figures are also provided.

Supplementary table 3. DIA Protein level analysis of *N. gonorrhoeae* expressing different *pglO* alleles. The Spectronaut search results for protein level analysis across all biological replicates (n=4), the MaxLFQ Perseus processed data and Z-score Perseus processed data are provided. For each identified protein, the $\log_2$ LFQ protein values, T–test information including the -$\log_{10}(p$ -value), difference in the mean between the groups and if the resulting p -values are below 0.05, the multiple hypothesis corrected p -values (permutation-based false discovery rate of 0.05) are provided. Categorical information associated with protein accessions, gene name, and GO terms are provided in addition to if proteins were identified by a single PSM as well as the total number of precursors assigned for each protein within each replicate.

Supplementary table 4. Glycoproteins identified across FAIMS experiments. All proteins identified to be glycosylated within this study and their corresponding NGO assignments are provided.

Supplementary Table 5. Enrichment analysis of global proteome changes in response to *N. gonorrhoeae* expressing different *pglO* alleles . Fisher exact tests of proteins determined to undergo statistically significant changes were assessed for co-occurrence of statistically significant changes across strains as well as enrichment of GO terms.

Supplementary table 6. DIA peptide level information of non-glycosylated peptides targeted for glycosylation within the *N. gonorrhoeae* proteome. The Spectronaut search results for peptides of interest observed across *N. gonorrhoeae* expressing different*pglO* alleles biological replicates (n=4) are provided. For each peptide the protein group, UniProt ID, protein group p-value, Q-value, C-score, Protein Molecular Weight, Proteotypic status of the peptide, Peptide Position, Precursor ID, Precursors Qvalue, Signal To Noise and Total Quantity (ion intensity) are provided.

Supplementary Data 1: Manually curated *N. gonorrhoeae*MS11 glycopeptides (Best scoring unique glycopeptides). For each of the best scoring unique glycopeptides, the MSfragger assigned spectra is provided in addition to the assigned Ngo assignment, site, peptide assignment, assignment associated metrics (m/z; observed m/z, score, position in protein) and page within the pdf the HCD and EThcD spectra can be found are provided.

Supplementary Data 2: Manually curated glycopeptides in *N. gonorrhoeae* expressing different *pglO* alleles (Best scoring unique glycopeptides). For each of the best scoring unique glycopeptides, the MSfragger assigned spectra is provided in addition to the assigned Ngo assignment, site, peptide assignment, assignment associated metrics (m/z; observed m/z, score, position in protein) and page within the pdf the HCD and EThcD spectra can be found are provided.

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