

# Broad spectrum O-linked protein glycosylation in the human pathogen *Neisseria gonorrhoeae*

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Protein glycosylation is an important element of biologic systems because of its significant effects on protein properties and functions. Although prominent within all domains of life, O-linked glycosylation systems modifying serine and threonine residues within bacteria and eukaryotes differ substantially in target protein selectivity. In particular, well-characterized bacterial systems have been invariably dedicated to modification of individual proteins or related subsets thereof. Here we characterize a general O-linked glycosylation system that targets structurally and functionally diverse groups of membrane-associated proteins in the Gram-negative bacterium *Neisseria gonorrhoeae*, the etiologic agent of the human disease gonorrhea. The 11 glycoproteins identified here are implicated in activities as varied as protein folding, disulfide bond formation, and solute uptake, as well as both aerobic and anaerobic respiration. Along with their common trafficking within the periplasmic compartment, the protein substrates share quasi-related domains bearing signatures of low complexity that were demonstrated to encompass sites of glycan occupancy. Thus, as in eukaryotes, the broad scope of this system is dictated by the relaxed specificity of the glycan transferase as well as the bulk properties and context of the protein-targeting signal rather than by a strict amino acid consensus sequence. Together, these findings reveal previously unrecognized commonalities linking O-linked protein glycosylation in distantly related life forms.

bacteria | glycoprotein | pilin | post-translational modification | *pgl*

Targeting of a broad array of heterogeneous protein substrates is a fundamental feature of O-linked glycosylation in eukaryotes and N-linked glycosylation systems in all domains of life (1). The covalent addition of uniform glycan tags enables diverse proteins to be recognized and modified by conserved core processes influencing protein trafficking, folding, and turnover. Glycosylation substrate selection involves not only colocalization with the glycosylation machinery but also the presence and availability of occupancy sites. In bacterial, archaeal, and eukaryotic N-linked systems, glycosylation sites are characterized by the N-X-S/T tripeptide sequon that is necessary but not sufficient for modification (2). In contrast, structural determinants intrinsic to O-linked protein glycosylation have been more difficult to characterize (3). In higher eukaryotes, the seemingly most prevalent (and arguably the most thoroughly studied) form of O-linked glycosylation is termed mucin-type glycosylation, in which cell-surface proteins are modified with N-acetylgalactosamine through the action of polypeptide N- $\alpha$ -acetylgalactosaminyltransferases (4). Glycosylation acceptor sites in this system are associated with domains rich in serine, threonine, and proline that are localized to coiled or turn regions and that are often tandemly repeated (5). In the case of the eukaryotic O-linked N-acetylglucosamine modification mediated by the O-GlcNAc transferase, few clear patterns have emerged although systematic peptide-based assays have begun to reveal some sequence preferences (6, 7).

Bacterial protein glycosylation systems have come under enhanced scrutiny because of the increasing frequencies with which they are seen in pathogenic and symbiotic species, as well as their

potential for exploitation in recombinant glycoprotein engineering strategies. Studies of these systems also have the potential to provide insight into the evolution of glycan biosynthesis, glycosyltransferases, and structural determinants of substrate recognition. Indeed, the Gram-negative species *Campylobacter jejuni* targets multiple proteins at asparagine residues like its eukaryotic counterparts and uses an oligosaccharyltransferase (OTase) originally recognized on the basis of its sequence identity with the STT3, the catalytic subunit of eukaryotic OTases (8). An STT3-like OTase also acts in N-linked glycosylation in the thermophilic archaeon *Pyrococcus furiosus* (9). In contrast, no clear relationships have been delineated for conventional O-linked systems that predominate in eukaryotes and bacteria. Most conspicuously, bacterial O-linked systems are uniformly dedicated to the modification of individual proteins or sets of highly related proteins. Prime examples include the subunits of S-layers, flagella, and Type IV pili, as well as non-pilus adhesins, such as autotransporters and 2-partner secretion pathway exoproteins (10). Evidence for glycosylation of multiple, structurally related protein substrates has been reported in *Bacteroides fragilis*, although the nature of the glycan linkage involved remains unknown (11). Two surface lipoproteins of *Mycobacterium tuberculosis* have been proven to be O-glycosylated, and an implicated transferase shares some structural similarity to eukaryotic protein mannosyltransferases (12, 13). However, whether this system is representative of bona fide general protein glycosylation has not been established.

The PilE pilin protein subunit of the Type IV pilus colonization factor expressed by the Gram-negative human pathogen *Neisseria gonorrhoeae* (*Ngo*) undergoes O-glycosylation at a single, defined serine residue (Ser-63) (14). Genetic analyses including interspecies complementation have revealed that this system (termed *pgl* for pilin glycosylation) is remarkably similar to the N-linked system of *C. jejuni* with regard to the use of a peptide-proximal 2,4 diacetyl-amido-2,4,6 trideoxyhexose (DATDH) sugar and related biosynthetic pathways for generating lipid-linked glycan substrates (15). These systems then diverge at the step of oligosaccharide transfer with the Ser-targeting PglO OTase acting in *Ngo* and the sequon targeted PglB OTase in *C. jejuni* (supporting information Fig. S1). Thus, in contrast to eukaryotic O-linked glycosylation systems, the gonococcal O-linked system uses *en bloc* transfer of oligosaccharide from a lipid-linked donor rather than single UDP-monosaccharide donors and downstream glycan elaboration.

## Results

**Evidence for Multiple Glycoproteins in *N. gonorrhoeae*.** We made the observation that polyclonal rabbit antiserum raised against glyco-

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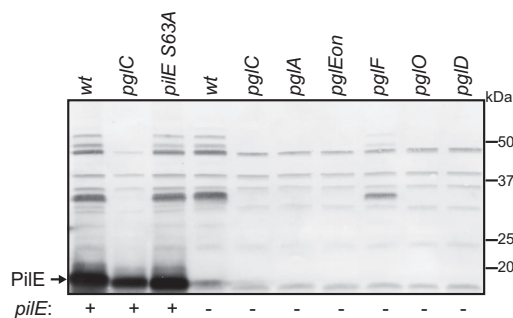
The authors declare no conflict of interest.

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**Fig. 1.** Evidence for multiple *Ngo* glycoproteins revealed via detection of glycan-associated epitopes. Immunoblotting of whole-cell lysates with rabbit serum (termed antiglycan) raised against purified Pile bearing the O-AcHexDATDH glycan reacts with multiple proteins in a *pgl* pathway-dependent fashion (see Fig. S1 for an overview of the *pgl* pathway). Note that the lack of immunoreactivity observed in the *pglA* and *pglE<sub>on</sub>* backgrounds is not associated with a lack of glycosylation but rather the presence of monosaccharide DATDH and trisaccharide O-AcHexHexDATDH glycans, respectively.

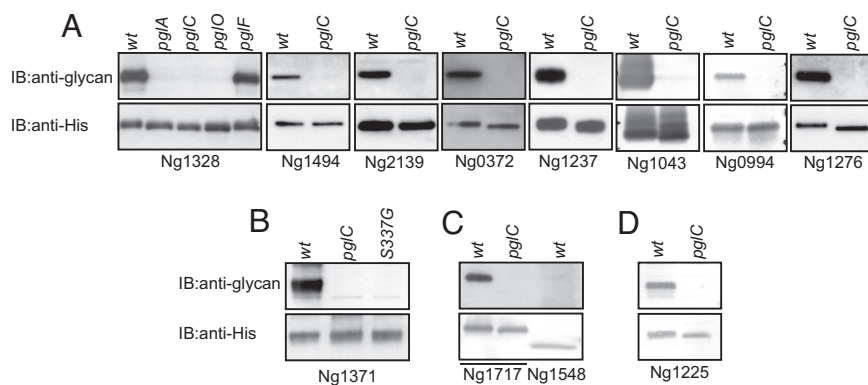
sylated Pile protein (in purified Type IV pili) reacted with multiple gonococcal proteins when examined by immunoblotting (Fig. 1). This pattern of immunoreactivity occurred independently of Pile expression and Pile glycosylation (as seen in a Pile S63A background) but was abolished in all *pgl* null mutants except that lacking PglF (encoding a putative flippase), in which the case the signal intensities were diminished. PglF has been modeled as a membrane translocase for the lipid-linked oligosaccharide, and we previously showed that Pile is hypoglycosylated in a *pglF* null mutant [residual glycosylation presumably being due to the presence of partially compensatory translocases in this background (14)]. In addition, specific immunoreactivity was absent in backgrounds expressing either the DATDH monosaccharide (*pglA*) or the O-acetylated (O-Ac) HexHexDATDH trisaccharide (*pglE<sub>on</sub>*) glycan precursor forms. These findings suggested that the immunoreactive proteins shared specific glycan-associated epitopes generated by the same glycosylation pathway that acts on Pile.

**Identification of a Major Subset of *N. gonorrhoeae* Glycoproteins.** To examine these possibilities in more detail, a cell fraction enriched for the immunoreactive proteins was subjected to 2D gel electrophoresis and probed by immunoblotting with antiglycan antibodies (Fig. S2). Of the more than 15 immunoreactive proteins detected, 10 candidates were identified using MS analyses of corresponding spots excised from preparative gels. However, insufficient material was available by this method to unambiguously characterize glyco-

sylation status. Therefore, gonococcal strains were individually engineered so that each protein carried a hexahistidine (6xHis) extension at its carboxy-terminus while remaining expressed from its endogenous locus and promoter. After affinity purification, it was confirmed that each of the 10 proteins retained *pglC*-dependent reactivity with glycan-specific antibodies, and in the case of Ng1328 this property was also dependent on *pglA* and *pglO* (but not *pglF*) (Fig. 2 A–C).

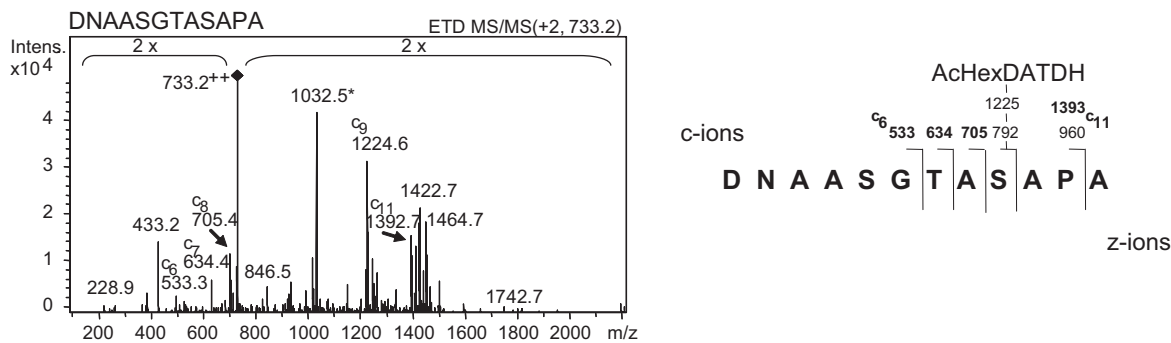
**Mass Spectrometric Confirmation of Glycosylation Status via Glycopeptide Identification.** We next identified glycosylated peptides derived by proteolysis from these purified proteins using liquid chromatography-electrospray ionization–tandem mass spectrometry and collision-induced dissociation (CID). Data indicative of glycopeptides carrying the O-AcHexDATDH glycan moiety [as diagnostic oxonium ion fragments appearing at mass to charge ratio ( $m/z$ ) 433.2 due to CID] were obtained from 7 of the 10 proteins (Fig. 3). In the case of Ng1043, 2 non-overlapping glycopeptides were detected (Fig. 3 G and H), and the signals seen for the larger indicated the presence of 2 glycans. Signals indicating the presence of 2 glycans were also detected on the glycopeptide found for Ng1276 (Fig. 3C).

**Evidence for Glycan Linkage at Serine Residues.** Consistent with the *pgl*-dependent glycosylation of Ser-63 of Pile, we noted that serine residues were present in all 8 glycopeptides, whereas threonine residues were represented in only 5 instances (see below). Because the Ng1371 glycopeptide only contained 2 serines, missense mutants bearing a substitution individually at each site were constructed and examined. Whereas substitution at Ser-327 with a non-hydroxyl-bearing amino acid had no effect on glycan antibody reactivity (data not shown), that at Ser-337 abolished reactivity (Fig. 2B). Directly locating the exact sites of glycosylation using conventional MS/MS CID techniques is difficult owing to the relative lability of glycosidic bonds. We therefore used ion trap MS with electron transfer dissociation (ETD). As shown for the 165-DNAASGTASAPA-176 glycopeptide from Ng1328, the ETD spectrum provided the necessary peptide backbone fragmentation to assign Ser-173 as bearing the glycan (Fig. 4). Its location to Ser-173 is derived by detection of fragment ions  $c_9^+$  (DNAASGTAS) and  $c_{11}^+$  (DNAASGTASAP) at  $m/z$  1224.6 and 1392.7, respectively, representing the only c-ions of the c-ion series  $c_6^+$  to  $c_{11}^+$  with a mass increase of 432 Da, which corresponds to the glycan residue. Nonetheless, a missense form of Ng1328 with a non-hydroxyl-bearing amino acid replacement at this residue retained reactivity with the glycan antibodies (data not shown), showing that this protein carries an additional acceptor site. Taken together with



**Fig. 2.** Affinity purification confirms the identities of candidate *Ngo* glycoproteins. (A–D) C-terminally 6xHis-tagged candidate proteins were affinity purified from wild-type (*wt*) and *pgl* backgrounds and tested for glycan-associated antigenicity by immunoblotting. (B) For Ng1371, a mutant form of the protein carrying a glycine substitution at Ser-337 was also purified and tested, whereas for Ng1717 (C), the Ng1548 paralogue (Fig. S3) was examined in parallel. (D) Ng1225 was identified as a potential glycoprotein on the basis of its lipoprotein processing site and proximal ASP-rich LCR (Table S1).





**Fig. 4.** Identification of Ser-173 of Ng1328 as a glycan acceptor site using ETD. Fragmentation of the doubly charged peptide at  $m/z$  733.2 shows the site of O-AcHexDATDH modification by detection of fragment ions  $c_9^+$  and  $c_{11}^+$  at  $m/z$  1224.6 and 1392.7, respectively. These represent the only c-ions with a mass increase of 432 Da, which corresponds to the glycan moiety.

the observations that serine is the only hydroxyl-bearing residue in Ng1043 and Ng2139 glycopeptides, these data strongly suggest that *Ng*o general protein glycosylation is primarily attributable to linkage at serine residues. However, we cannot formally rule out that threonine or other hydroxyl-bearing residues might also be targeted.

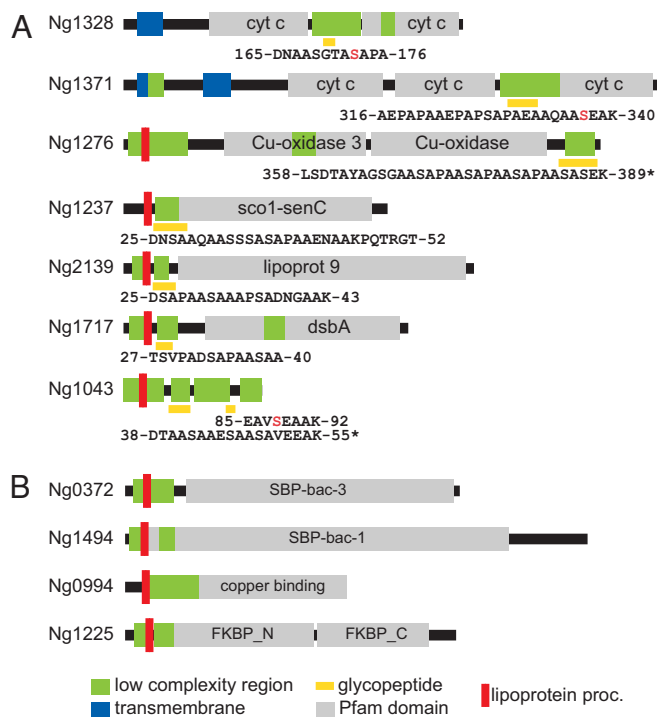
#### Glycan Occupancy Sites Are Associated with Low-Complexity Regions.

Examination of the glycopeptides revealed a remarkably conserved set of features, including an overabundance of alanine, serine, and proline residues (Fig. 5A). At the same time, the relative positions

of the glycopeptides within the proteins were quite diverse, being found in the N-terminus in 4 cases, in the middle in 2 others, and at the C-terminus in another. To confirm the biased composition of the glycopeptides, glycoproteins were examined using the SEG algorithm to identify low-complexity regions (LCRs) (16). All 8 glycopeptides were predicted to be encompassed within LCRs (Fig. 5A), and although specific glycopeptides have yet to be identified from the 4 remaining substrates, each of these glycoproteins carries an alanine, serine, and proline (ASP)-rich LCR near their mature N-termini similar to those seen in the other glycoproteins (Fig. 5B and Table S1). To gain further evidence for the association between glycosylation and these ASP-rich LCR elements, the Ng1548 parologue of Ng1717, which lacks the corresponding ASP-rich LCR domain (Fig. S3), was affinity tagged, purified, and found to lack reactivity with glycan antibodies (Fig. 2C). Finally, an *in silico* approach predicated on the presence of the ASP-rich LCR together with periplasmic targeting identified a number of candidate glycoproteins, and among these we were able to successfully affinity tag and purify Ng1225 (Fig. 5B and Table S1). Glycan antibodies reacted with affinity-purified Ng1225, and this recognition was abolished when the protein was derived from a *pglC* background (Fig. 2D).

#### General Characteristics and Features of *N. gonorrhoeae* Glycoproteins.

*Ng*o glycoproteins share a number of intriguing features including their membrane tethering and translocation to or through the periplasm (Table 1). Trafficking to this compartment is likely a prerequisite for glycosylation because the PglO OTase is predicted to act in the periplasm (15, 17). Potential functional connections can also be discerned among them. For example, Ng1276 is a copper-containing nitrite reductase essential for growth under oxygen-limiting conditions that has been proposed to acquire electrons via Ng0994, a lipid-linked azurin (18). Ng1371 is a multiheme, c-type cytochrome of the cytochrome *cbb3* oxidase complex (the sole predicted terminal respiratory oxidase in *Ng*o) and is believed to transfer electrons to the catalytic subunit (19), whereas Ng1328 is an integral-membrane, diheme c-type cytochrome that potentially channels electrons to both Ng1276 and Ng1371 (20). On the basis of their relative signal intensities in immunoblotting, Ng1276, Ng1328, and Ng1371 represent the most abundant glycoproteins after PilE (Fig. S4). Ng1717 is an isoform of the disulfide oxidoreductase DsbA, whose reoxidation is linked via DsbB and quinones to the terminal oxidase of electron transport (21), which in this case is the cytochrome *cbb3* oxidase. Finally, Ng1237 is a lipoprotein member of the Sco protein family whose constituents are implicated in copper binding and cytochrome oxidase biogenesis (22). The presence of a thioredoxin fold in these proteins suggests that they may have thiol/disulfide-based oxidoreductase activity and metal ion homeostasis functions, and an Ng1237 null mutant was shown



**Fig. 5.** Domain organization and structural architecture of *Ng*o glycoproteins. (A) Glycoproteins for which glycopeptides have been identified by MS/MS. Regions encompassed by glycopeptides are indicated by yellow rectangles, and specific residues are numbered according to those of unprocessed polypeptides. Serines identified as acceptor sites (via ETD, substitution mutation, or by virtue of being the sole hydroxyl-bearing residue) are labeled in red. Asterisks denote glycopeptides bearing multiple glycan moieties. (B) Glycoproteins for which glycopeptides have yet to be identified. Sequences of the LCRs shown for these proteins are found in Table S1. Further details and specific Pfam family identifiers are found in Table 1.



**Table 1. Features and properties of *Ngo* glycoproteins**

ORF	Protein name	Pfam domain(s)	Predicted mass (kDa)	Function	Target site associated with tandem repeat elements	Membrane association	Predicted localization
—	PilE	PF07963 PF00114		Type IV pilus subunit	—	Transmembrane domain	Periplasm/cell surface
Ng0372	—	PF00497	29	ABC transporter, periplasmic binding protein (amino acid)	ND	Lipoprotein	Periplasm
Ng0994	Laz	PF00127	19	Lipid-modified azurin Cu binding resistance to oxidative stress	ND	Lipoprotein	Periplasm
Ng1043	—	—	11	—	+	Lipoprotein	Periplasm
Ng1225	Mip	PF00254 PF01346	29	Peptidyl-prolyl isomerase	ND	Lipoprotein	Periplasm/cell surface
Ng1237	Sco	PF02630	23	Cu homeostasis, resistance to oxidative stress	—	Lipoprotein	Periplasm
Ng1276	AniA	PF00394 PF07732	41	Cu containing nitrite reductase	+	Lipoprotein	Periplasm
Ng1328	CycB	PF00034	29	Cytochrome c5	—	Transmembrane domain	Periplasm
Ng1371	CcoP	PF00034	48	Cytochrome cbb3 oxidase subunit	+	Transmembrane domain	Periplasm
Ng1494	PotF	PF01547	41	ABC transporter, periplasmic binding protein (polyamine)	ND	Lipoprotein	Periplasm
Ng1717	DsbA	PF01323	25	Thiol-disulfide isomerase	—	Lipoprotein	Periplasm
Ng2139	Gna1946	PF03180	31	ABC transporter, periplasmic binding protein (metal)	—	Lipoprotein	Periplasm/cell surface

ND, acceptor sites not yet defined.

to be hypersensitive to oxidative stress (23). Together, these findings establish a link between protein glycosylation, electron transport systems, and redox components. The remaining glycoproteins include a peptidyl prolyl-isomerase (Ng1225), 2 solute-binding proteins associated with ABC transport systems (Ng0372 and Ng1494), and 2 lipoproteins of unknown function (Ng1043 and Ng2139). We further note that with the exceptions of PilE as well as Ng1225 and Ng2139 [for which evidence for surface exposure exists (24, 25)], the glycoproteins are predicted to function in the periplasmic compartment, although their precise sites of localization await confirmation.

## Discussion

In this study, we discovered and characterized a general O-linked glycosylation system in bacteria. Additionally, our findings document an instance in which bacterial proteins carrying O-linked glycans are destined to carry out their roles within the cell rather than at the cell surface or beyond. Although we remain ignorant as to what properties might be imparted or modified by glycosylation, these findings imply that *Ngo* O-linked protein glycosylation acts in both intracellular and extracellular capacities and contributes at a system level to the biology of the organism. It seems unlikely, however, that *Ngo* will be the only bacterial species with a complex repertoire of O-linked glycoproteins. The closely related species *Neisseria meningitidis* expresses a very similar pilin glycosylation system, and immunoblotting with the glycan-recognizing antibodies used here reveals analogous patterns of reactive protein forms in some meningococcal backgrounds (B. Børud, unpublished data). In addition, LCRs similar in composition and localization to those seen in *Ngo* glycoproteins are discernible among orthologous counterparts in some proteobacterial species. It is also perhaps relevant to note that the ASP-rich LCRs defined here are reminiscent of those sites of glycan occupancy documented in *M. tuberculosis* lipoglycoproteins (12, 13).

Although further studies are required to delineate the specific targeting signals associated with the ASP-rich LCRs, it seems clear that there is no consensus amino acid sequence or obvious sequon. A tetrapeptide serine–alanine–proline–alanine motif is notably over-

represented within both the defined glycopeptides (in 6 of 8 instances and tandemly triplicated in Ng1276; Fig. 5A) and the LCRs of those proteins for which glycopeptides have yet to be identified (in 2 of 4 cases; Table S1). However, it is neither sufficient (as seen in the nonglycosylated Ng1371 mutant expressing a substitution at Ser-337) nor necessary (given that it is absent in PilE and Ng1043). Rather, the LCRs are themselves evocative of the structural features associated with acceptor sites in mucin-type glycoproteins. Commonalities include the biased overrepresentation of alanine, serine, and proline residues, the structural contexts of acceptor sites confined to interdomain regions, the clustering of multiple acceptor sites, and an association with tandem repeat elements (26). Relative surface exposure and accessibility of the acceptor sites are other likely shared determinants of these elements. It is important to note in this context that although PilE (the most abundant glycoprotein) lacks any discernible LCR, its single acceptor site maps on the protein surface as defined by x-ray diffraction (27). Taken together with findings suggesting that PglO exhibits relaxed specificity for lipid-linked oligosaccharide donors (17, 28), the promiscuity of PglO with regard to protein substrates demonstrated here significantly augments the potential of this system for exploitation in novel glycoprotein engineering strategies.

From a larger perspective, our findings reveal remarkable similarities in both the global contexts and structural signals dictating glycan acceptor sites of bacterial and eukaryotic O-linked glycosylation systems. They also extend the ways in which the *Ngo* O-linked system emulates the *C. jejuni* N-linked systems to include the targeting of multiple periplasmic substrates. Like in the *C. jejuni* system, these findings raise the obvious questions as to what biologic significance global protein glycosylation may have and what forces and processes have shaped glycoproteome content.

## Materials and Methods

**Bacterial Strains, Media, and Culture Conditions.** The bacterial strains used in this study are described in Table S2 and were grown on conventional GC medium as described previously (15). Carboxyterminal 6xHis-tagged alleles as well as missense alleles of Ng1328 and Ng1371 were generated using PCR-based splicing by overlap extension reactions (29). Purified PCR products were used to genetically transform *Ngo* strains (30), and correct transformants were screened for by PCR using diagnostic primer sets. Protein glycosylation null mutations were intro-

duced into various strain backgrounds using transformation as previously described (15).

**Purification of 6xHis-Tagged Proteins.** 6xHis-tagged proteins were purified from *Ngo* using magnetic Ni-NTA beads (Qiagen). Frozen cell pellets were lysed by sonication in buffer containing 100 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM Tris, 8 M urea, 0.05% Tween, 100 mM NaCl, 15 mM imidazole, 1× complete protease inhibitor mixture, EDTA-free (Roche), 1 mM PMSF, and 1% detergent (Triton X-100 for Ng1371, Ng0372, and Ng1494 and Sarkosyl for Ng1328, Ng1237, Ng1276, Ng2139, Ng1548, Ng1717, Ng1043, Ng0994, and Ng1225), pH 8.0, as described in the manufacturer's instructions. Proteins were eluted directly into SDS-sample loading buffer.

**SDS-PAGE/2D Gel Electrophoresis and Immunoblotting.** For SDS-PAGE, whole-cell lysates were prepared from equivalent numbers of cells by heating cell suspensions to 65 °C in SDS-sample loading buffer. Procedures for SDS-PAGE and immunoblotting using alkaline phosphatase-coupled goat antirabbit and goat antimouse antibodies (Tago) have been described previously (31). For 2D gel analyses, cells were harvested from plates incubated overnight, lysed by sonication in 62.5 mM Tris-HCl (pH 6.8) with protease inhibitors (described above), and centrifuged at 200,000 × g for 2 h at 4 °C. The pellet from this step was resuspended in Tris buffer with 1% Triton X-100 and then centrifuged again. The supernatant from the second centrifugation was then fractionated by ammonium sulfate precipitation (a 20%–30% saturation cut) and concentrated using Trichloroacetic acid precipitation and acetone washes. Samples containing 80 μg protein dissolved in isoelectric focusing (IEF) buffer were subjected to IEF in a Protean IEF cell (Bio-Rad) using 11-cm ReadyStrip IPG strips (Bio-Rad) with non-linear gradients of pH 3–10, as follows: 250 V for 15 min, 8,000 V for 2.5 h, and 8,000 V until 35,000 Vh were reached. After IEF, the strips were subjected to standard disulfide reduction with DTT and cysteine alkylation with iodoacetamide, followed by second-dimension electrophoresis using 12% ReadyGels (Bio-Rad). Gels were treated with MS-compatible silver stain (32) or immunoblotted as detailed above. For glycan detection, polyclonal antiserum no. 904 [ref. 33; termed antiglycan antibodies in this text] raised against purified Type IV pili bearing the O-AcHexDATDH glycan was used at a 1:1,000 dilution. For 6xHis-

tagged protein detection, a penta-His-recognizing mouse monoclonal antibody (Qiagen) was used at a 1:1,000 dilution.

**Bioinformatic Analysis.** *Ngo* gene and protein data were obtained through the DBGET web site (<http://www.genome.jp/dbget/>), an integrated database retrieval system operated by the Kyoto University Bioinformatics Center. Because the *Ngo* genome data archived there are derived from a strain different from that used here, all relevant gene sequences examined here were confirmed by DNA sequencing. Pfam protein domain designations were derived through the UniProt Knowledgebase (UniProtKB) web site (<http://www.uniprot.org/help/uniprotkb>). LCRs were identified using the SEG program (16).

**MS/MS CID and ETD Analysis.** Each gel spot/slice was destained, and the encompassed proteins were subjected to reduction, alkylation, and digestion with either trypsin, AspN, or proteinase K (Roche/Sigma) at 37 °C overnight. The resulting peptides were extracted with 5% formic acid and acetonitrile, evaporated, and then redissolved in 0.1% formic acid. Samples were subjected immediately to mass spectrometric analyses or frozen at –80 °C. Conditions for these methods have been previously described (14). Reverse-phase (0.75 × 43-mm Zorbax 300SB-C18, 5-μm analytical column; Agilent Technologies) chip-based nanoflow liquid chromatographic MS and MS/MS (CID and ETD mode) analyses (nano-LC-chip MS/MS) of proteolytic peptides were performed using an Agilent 1100 LC/MSD Trap XCT Ultra equipped with a Chip/MS Cube interface. Alternatively, proteolytic peptides were directly applied onto a stainless steel target (Bruker Daltonics) as a mixture of 1:1 (vol/vol) with saturated α-cyano-4-hydroxycinnamic acid (Bruker Daltonics) solution (30% acetonitrile/70% 0.1% TFA). Analyses were performed using an Ultraflex TOF/TOF (time of flight) mass spectrometer equipped with a nitrogen laser (337 nm, 5 Hz) in the reflector mode for matrix-assisted laser desorption/ionization (MALDI)-TOF peptide mass fingerprint (PMF) or LIFT mode for MALDI-TOF/TOF analyses. MS/MS or PMF data were searched using the Mascot program (Matrix Sciences). All searches were performed against the *Neisseria* protein sequence database.

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