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Biochemical Characterization of the O-Linked Glycosylation Pathway in *Neisseria gonorrhoeae* Responsible for Biosynthesis of Protein Glycans Containing *N,N′*-Diacetylbacillosamine

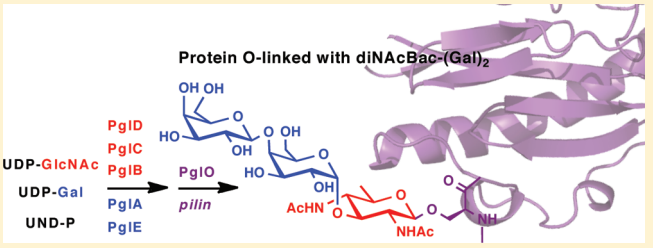
Meredith D. Hartley,[†] Michael J. Morrison,[†] Finn Erik Aas,[‡] Bente Børud,[‡] Michael Koomey,[‡] and Barbara Imperiali*[,†]

[†]Departments of Chemistry and Biology, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139, United States

[‡]Center for Molecular Biology and Neuroscience and Department of Molecular Biosciences, University of Oslo, 0216 Oslo, Norway

Supporting Information

ABSTRACT: The O-linked protein glycosylation pathway in *Neisseria gonorrhoeae* is responsible for the synthesis of a complex oligosaccharide on undecaprenyl diphosphate and subsequent *en bloc* transfer of the glycan to serine residues of select periplasmic proteins. Protein glycosylation (*pgl*) genes have been annotated on the basis of bioinformatics and top-down mass spectrometry analysis of protein modifications in *pgl*-null strains [Aas, F. E., et al. (2007) *Mol. Microbiol. 65*, 607−624; Vik, A., et al. (2009) *Proc. Natl. Acad. Sci. U.S.A. 106*, 4447−4452], but relatively little biochemical analysis has been performed to date. In this report, we present the expression, purification, and functional characterization of seven Pgl enzymes. Specifically, the enzymes studied are responsible for synthesis of an uncommon uridine diphosphate (UDP)-sugar (PglD, PglC, and PglB-acetyltransferase domain), glycan assembly (PglB-phospho-glycosyltransferase domain, PglA, PglE, and PglH), and final oligosaccharide transfer (PglO). UDP-2,4-diacetamido-2,4,6-trideoxy-α-D-hexose (DATDH), which is the first sugar in glycan biosynthesis, was produced enzymatically, and the stereochemistry was assigned as uridine diphosphate *N′*-diacetylbacillosamine (UDP-diNAcBac) by nuclear magnetic resonance characterization. In addition, the substrate specificities of the phospho-glycosyltransferase, glycosyltransferases, and oligosaccharyltransferase (OTase) were analyzed in vitro, and in most cases, these enzymes exhibited strong preferences for the native substrates relative to closely related glycans. In particular, PglO, the O-linked OTase, and PglB(*Cj*), the N-linked OTase from *Campylobacter jejuni*, preferred the native *N. gonorrhoeae* and *C. jejuni* substrates, respectively. This study represents the first comprehensive biochemical characterization of this important O-linked glycosylation pathway and provides the basis for further investigations of these enzymes as antibacterial targets.



In *Neisseria gonorrhoeae*, individual pilin subunits rapidly assemble and disassemble to form the flagellar-like type IV pili, which mediate essential interactions with host cells and affect many aspects of pathogenicity, including surface motility, bacteria−host communication, cell signaling, bacterial dissemination, and biofilm formation.[3−6] Recently, the gonococcal pilin glycosylation system was shown to be a general O-linked system in which many structurally distinct periplasmic proteins undergo glycosylation.[2] Glycan modifications on pili, flagella, and other extracellular proteins have been implicated in bacterial pathogenicity, which has led to increased interest in bacterial glycosylation pathways as potential antibacterial targets.[4,7−11] The focus of this study is the protein glycosylation (*pgl*) locus identified in *N. gonorrhoeae*, which is responsible for the addition of glycan to distinct serine residues.[1,12]

The protein glycan modifications present in *N. gonorrhoeae pgl*-null strains have been analyzed by top-down mass spectrometry (MS),[1,2] and the following model of the protein glycosylation pathway has been developed (Figure 1). The core *pgl* locus contains four genes, three of which [*pglD*, *pglC*, and *pglB* (accession numbers for the genes are available in the Supporting Information)] are required for the synthesis of an undecaprenyl diphosphate 2,4-diacetamido-2,4,6-trideoxy-α-D-hexose (Und-PP-DATDH).[13] The term DATDH indicates that the stereochemistry of this sugar has not been previously determined. PglD and PglC perform NAD^+-dependent dehydratase and aminotransferase reactions, respectively, to convert UDP-HexNAc to UDP-2-acetamido-4-amino-2,4,6-trideoxy-α-D-hexose (UDP-4-amino), but it was not known if the initial substrate is UDP-GlcNAc or UDP-GalNAc. PglB is a bifunctional enzyme, which catalyzes the amino acetylation of UDP-4-amino to form UDP-DATDH and the transfer of the phospho-DATDH to

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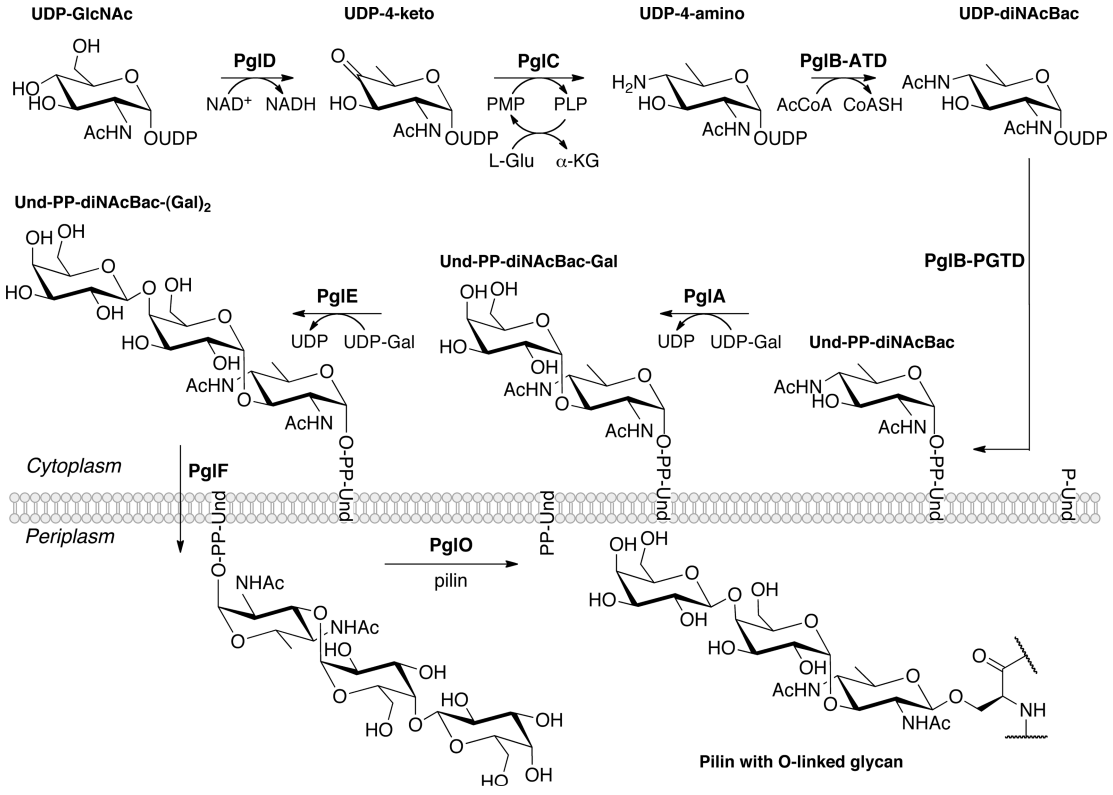

Figure 1. Biosynthetic pathway of the pilin glycan in *N. gonorrhoeae*.

undecaprenyl phosphate (Und-P). The fourth gene, *pglF*, shares homology with ABC transporter-type flippases and is putatively involved in the translocation of the undecaprenyl diphosphate-linked glycan across the periplasmic membrane. Although the function of this gene has not been demonstrated, the *pglF*-null strain does exhibit a diminished level of glycosylation.[1,12]

Interestingly, the other genes involved in pilin glycosylation are not linked to the core *pgl* locus. The products of the *pglA* and *pglE* genes further elaborate the polyprenyl-linked DATDH with the transfer of two sequential galactose units (Figure 1).[1] The *pglA* and *pglE* genes can undergo phase variation in which the genes are alternately turned on and off. Phase-variant *pglA* alleles have been proposed to be associated with more virulent strains of *N. gonorrhoeae*, although these studies have been disputed.[1,8,9] In addition to PglA and PglE, an alternate glycosyltransferase PglH adds a Glc unit instead of Gal to Und-PP-DATDH (Figure 2).[14] Finally, a gene has been identified, *pglO*, that shares homology with the O-antigen ligase (WaaL) family and is required for formation of the protein−glycan linkage (Figure 1).[2]

Considerable recent studies have focused on the highly homologous pilin glycosylation pathway in the related species *N. meningitidis*. The proposed model of the *N. meningitidis* pathway is similar to the *N. gonorrhoeae* pathway (Figure 2) and was also developed by bioinformatic analysis and experiments with *pgl*-null strains.[15−20] The oligosaccharyltransferase from *N. meningitidis*, PglL(*Nm*), has been purified to homogeneity and shown to glycosylate pilin using a farnesyl substrate analogue.[21] In addition, upon heterologous expression in *Escherichia coli*, PglL(*Nm*) was shown to transfer a variety of complex glycan substrates to pilin.[21,22]

The Pgl pathways from *N. gonorrhoeae* and *N. meningitidis* represent the first examples of O-linked protein glycans derived from polyprenyl-linked intermediates; all other identified O-linked pathways glycosylate protein substrates by sequential transfer of individual saccharide units from nucleotide or polyprenyl phosphate-activated glycan donors. Another intriguing facet of the *N. gonorrhoeae* O-linked glycosylation pathway is the fact that the first three enzymes (PglD, PglC, and PglB) share homology with the first four enzymes in the N-linked protein glycosylation (also designated Pgl) pathway in *Campylobacter jejuni*,[23] the exception being that the *C. jejuni* locus encodes separate enzymes for the sequential acetyltransferase and phospho-glycosyltransferase reactions. Both the *N. gonorrhoeae* and *C. jejuni* pathways produce an initial Und-PP-DATDH intermediate, but this intermediate is elaborated in distinct ways (Figure 2). The *N. gonorrhoeae* pathway produces a serine-linked mono-, di-, or trisaccharide,[13] and the *C. jejuni* pathway generates an asparagine-linked heptasaccharide.[24] The *C. jejuni* glycosylation pathway serves as an important model for the *N. gonorrhoeae* system, and previous work has resulted in the complete biochemical characterization of the *C. jejuni* Pgl pathway enzymes except for the flippase (PglK).[25−28]

In this study, the biochemical functions of proteins PglD, PglC, PglB, PglA, PglE, and PglO from *N. gonorrhoeae* are characterized for the first time through in vitro biochemical analysis. Importantly, the previously undefined stereochemical assignment of the UDP-DATDH produced by PglD, PglC, and PglB is unequivocally shown to be UDP-diNAcBAc, which is also the identity of the first sugar added in the *C. jejuni* N-linked glycosylation pathway. In vitro assays demonstrate that the phospho-glycosyltransferase (PglB) and two glycosyltransferases (PglA and PglE) build the glycan on an undecaprenyl diphosphate linker prior to *en bloc* transfer to protein and that these enzymes display strict specificity for the UDP-saccharide donor.

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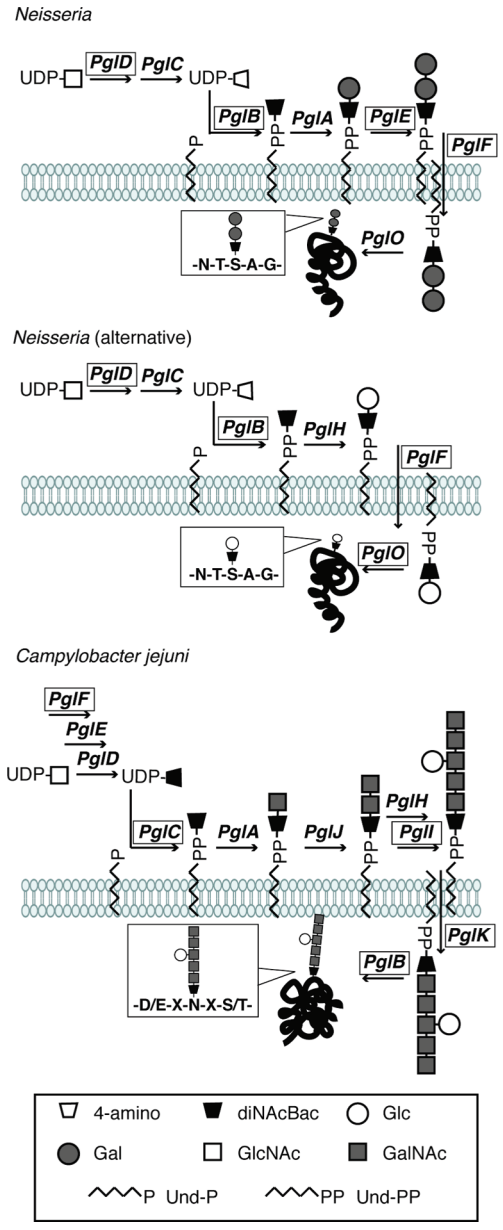


Figure 2. Schematic representations of bacterial protein glycosylation pathways: (top) O-linked pathway in *N. gonorrhoeae* and *N. meningitidis*; (middle) alternative O-linked pathway in *Neisseria* species, and (bottom) N-linked pathway in *C. jejuni*. The proteins predicted to contain transmembrane domains by TMHMM are denoted with a black box.

Finally, glycan substrate specificity analyses suggest that the O-linked OTase is highly selective for native *N. gonorrhoeae* glycan substrates in vitro.

EXPERIMENTAL PROCEDURES

Common Materials. All radioactive materials and undecaprenol were obtained from American Radiolabeled Chemicals. UDP-4-amino and UDP-diNAcBac were prepared as previously described[28] with enzymes from *C. jejuni* or as described herein with PglD, PglC, and PglB. All other chemicals were obtained from Sigma-Aldrich unless stated otherwise. Radioactivity was determined using a LS6500 Beckman scintillation counter; organic samples were dried and resuspended in 200 μL of Solvable (Perkin-Elmer) and 5 mL of scintillation fluid (Opti-Fluor, Perkin-Elmer). Aqueous samples were mixed with 5 mL of Ecolite(+) (MP Biomedicals) prior to scintillation counting.

Preparation of Genetic Constructs. The *pglD*, *pglC*, *pglB*, *pglA*, *pglO*, and *pilE* genes were amplified via polymerase chain reaction (PCR) from *N. gonorrhoeae* strain MS11,[1,8,12] while *pglE* was amplified from *N. gonorrhoeae* strain FA 1090 and *pglH* from *N. meningitidis* strain Z2491. The PCR products of *pglD*, *pglC*, *pglB*, *pglA*, *pglO*, and *pglE* were cloned into *Bam*HI and *Xho*I sites in the pET-24a(+) vector (Novagen). The *pilE* and *pglH* genes were cloned into the *NDe*I and *Xho*I sites in the pET-24a(+) vector (Novagen). The *Xho*I site was inserted prior to the stop codon to encode a His_6 tag at the C-terminal end of each protein.

The acetyltransferase domain of PglB (PglB-ATD) was identified through sequence homology with the related *C. jejuni* protein, PglD(*Cj*). The gene encoding the domain was amplified from the full-length gene using the forward primer 5′-CGCGGATCCATGGCGGGGAATCGCAAACTCG-3′ and the reverse primer 5′-GCAACCCGGCAAAGCCCCTTTAGCTCGAGCGG-3′ to generate a gene encoding the acetyltransferase domain. The gene was inserted into the *Bam*HI and *Xho*I sites in a modified pET-30b(+) vector that contains an N-terminal His_8 tag followed by a tobacco etch virus (TEV) protease site prior to the *Bam*HI site. Also, *pglH* was amplified by PCR and inserted into the *Bam*HI and *Xho*I sites in the pMAL-c2X vector. This construct encoded the addition of an N-terminal maltose binding protein (MBP).

Expression of Proteins. In general, all proteins [PglD, PglC, PglB, PglB-ATD, PglA, PglE, PglO, PglH, PilE (pilin), PglB-(*Cj*),[27] and *Streptococcus mutans* undecaprenol kinase[29]] were expressed heterologously in *E. coli* BL21 cells (Agilent). PglD, PglC, and PglB-ATD were expressed in the BL21(DE3) pLysS strain; all other proteins were expressed in the BL21-Gold(DE3) strain. A typical expression protocol involved preparation of an overnight culture of cells (5 mL), which was used to inoculate 1 L of LB medium with shaking at 37 °C. After the cells had reached an optical density of ~0.8 absorbance units, the temperature was lowered to 16 °C and the cells were induced with 0.5 mM iso-β-D-thiogalactosylpyranoside (IPTG). After being incubated for 16−18 h, the cells were harvested, and the pellets were stored at −80 °C.

Protein Purification. In general, all steps of protein purification were conducted at 4 °C. Protein concentrations were determined with the appropriate extinction coefficients at a UV absorbance of 280 nm, with the exception of PglO and pilin, which were quantified with the MicroBCA Assay (Pierce) because of the presence of the UV-active detergent Triton X-100.

The cell pellets generated from the expression of the soluble proteins, PglC and PglB-ATD, were resuspended in 50 mL of ice-cold 50 mM HEPES (pH 7.4) and 100 mM NaCl (buffer A), supplemented with 30 mM imidazole, and lysed by sonication. In the case of PglC, 200 μM pyridoxal 5′-phosphate was also added to the buffer. The lysate was cleared by centrifugation (145000*g*) for 45 min. Cleared lysate was mixed with 2 mL of Ni-nitrilotriacetic acid (Ni-NTA) resin (Qiagen), tumbled for 4 h, and then packed into a K 9/15 column (GE Healthcare). Using gravity flow, the resin-bound protein was washed with 10 column volumes of buffer A containing 30 mM imidazole. The resin was further washed with 20 column volumes of buffer A supplemented with 40 mM imidazole and then 10 column volumes of buffer A containing 60 mM imidazole. The protein was eluted in buffer

A supplemented with 250 mM imidazole, and 1 mL fractions were collected. Fractions containing purified material were assessed via sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) (12%) and Western blot analysis probing for the His_6 tag. Pooled fractions of PglC and PglB-ATD were dialyzed against buffer A, concentrated, supplemented with a final glycerol concentration of 15%, and frozen at −80 °C (Figure S1 of the Supporting Information, lanes 2 and 3).

Purification of the glycosyltransferase PglA was similar to that of PglC and PglB-ATD with a few exceptions. A buffer containing 50 mM Tris (pH 8.0) and 150 mM NaCl (buffer B) was used instead of buffer A, and the cells were incubated with 1% Triton X-100 for 20 min immediately following lysis and prior to centrifugation. In addition, 5% glycerol was added to all buffers. Following elution, the most concentrated 1.5 mL fraction as determined by SDS−PAGE was desalted using a Hi-Trap desalting cartridge (GE Healthcare) with buffer B and stored at −20 °C in 30% glycerol (Figure S1 of the Supporting Information, lane 5).

To purify the membrane-associated proteins (PglD, PglB, PglE, PglO, and pilin), cell envelope fractions (CEF) were prepared. To do this, the cells were thawed in 40 mL of buffer/L of cell culture and lysed by sonication. PBS supplemented with 200 μM NAD^+ was used for PglD, and buffer B with 1 mg/mL lysozyme was used for PglB, PglE, PglO, and pilin. Cellular debris was cleared by centrifugation at 9000*g* for 45 min. The resulting supernatant was transferred to a clean centrifuge tube and subjected to centrifugation at 145000*g* for 65 min to pellet the CEF. For PglB and PglE, the CEFs were resuspended in half the volume of the unlysed cell pellet weight (i.e., 1.5 mL was used for a 3 g cell pellet). The CEF was aliquoted and stored at −80 °C (Figure S1 of the Supporting Information, lanes 4 and 6). The CEFs of PglB and PglE were used in all glycosyltransferase assays.

PglD, PglO, and pilin were further purified from the CEF. The CEF was homogenized in 10 mL of buffer containing 1% Triton X-100 (PBS with 200 μM NAD^+ for PglD and buffer B for PglO and pilin) per liter of cell culture. Each CEF was incubated with detergent for several hours and then centrifuged again (145000*g*) to remove insoluble material. The resultant supernatants were incubated with 0.5−2 mL of Ni-NTA resin for 1−2 h; the resins were washed as previously described with the addition of 0.1% Triton X-100 to the wash and elution buffers. The proteins were eluted from the resin in 1 mL fractions. Pooled fractions of PglD were dialyzed against PBS containing 200 μM NAD^+ and 0.1% Triton X-100, supplemented with a final glycerol concentration of 30%, and frozen at −80 °C (Figure S1 of the Supporting Information, lane 1). The most concentrated fractions of PglO and pilin were desalted as described above for PglA and stored at −80 °C (Figure S1 of the Supporting Information, lanes 7 and 9). For the in vitro biosynthesis of UDP-diNAcBac, PglB was purified in a manner similar to that of PglD, except that NAD^+ was not added to the purification buffers.

PglH was expressed as an MBP fusion protein and purified as described in ref 14 (Figure S1 of the Supporting Information, lane 8). In addition, PglB(*Cj*) and undecaprenol kinase from *S. mutans* were expressed and purified as cell envelope fractions as described previously.[27,29]

Acetyltransferase (PglB-ATD) Activity Assay. The kinetic constants for PglB-ATD were determined using Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoic acid), in a continuous fashion. Ellman's reagent was utilized to quantify substrate turnover as monitored by measuring conversion of acetyl-coenzyme A (AcCoA) to CoASH using the released TNB chromophore (λ_{max} = 412 nm; ε_{max} = 14150 M^{-1} cm^{-1}). The in vitro assay contained 50 mM HEPES (pH 7.4), 1 mM EDTA, 1 mM DTNB, and 25 nM PglB-ATD in a quartz cuvette. The substrate concentrations of AcCoA and UDP-4-amino were varied separately to determine kinetic parameters using initial velocity measurements while keeping the other substrate at a saturating level. The reaction was initiated with the UDP-4-amino substrate and took place at room temperature over a 200 s time period. The absorbance change at 412 nm was measured. A blank reaction mixture lacking UDP-4-amino was prepared as a background control. Steady-state rate parameters were calculated from eq 1 using GraFit 6.0.12 (Erithacus Software).

$$v = V_{max}[S]/K_m + [S] \quad (1)$$

Aminotransferase (PglC) Activity Assay. The aminotransferase reaction was assayed by coupling generation of UDP-4-amino from the PglC reaction to the acetyltransferase activity of PglD from *C. jejuni* producing CoASH, which was detected by Ellman's reagent in a fashion similar to the PglB-ATD assay. In a flat bottom 96-well plate (Nunc), 50 mM HEPES (pH 7.4), 1 μM PglD(*Cj*), 400 μM AcCoA, and 400 nM PglC were added. Because PglC activity was coupled to the turnover of the acetyltransferase PglD(*Cj*), addition of excess PglD(*Cj*) ensured that the initial velocity measurements were dependent only upon PglC activity. The concentrations of L-glutamate and UDP-4-keto were varied separately to determine kinetic parameters using initial velocity measurements while keeping the other substrate at a saturating level. Interference of Ellman's reagent with PglC activity required the implementation of a discontinuous assay in which reactions were initiated with L-glutamate and quenched over a 30 min time period with 20% *n*-propanol, 2 mM DTNB, and 1 mM EDTA. The absorbance at 415 nm was followed on an Ultramark EX microplate imaging system (Bio-Rad). A blank reaction mixture without L-glutamate was set up as a background control.

Biosynthesis and Stereochemical Assignment of UDP-DATDH. To biosynthesize UDP-DATDH, 0.3 mg of PglD (bound to Ni-NTA resin) was added to 15 mL of buffer containing 50 mM HEPES (pH 7.4), 100 mM NaCl, 200 μM NAD^+, and 30 mg of UDP-GlcNAc. The reaction was conducted at room temperature for 12 h with gentle rocking. Once conversion to the UDP-4-keto sugar was complete, as verified by capillary electrophoresis as described previously,[28] the reaction mixture was filtered and the flow-through collected. The filtrate containing the UDP-4-keto sugar was supplemented with 15 mg of PglC (bound to Ni-NTA resin), 20 mM L-glutamate, and 200 μM pyridoxal 5′-phosphate. This reaction mixture was filtered after being rocked for 18 h at room temperature and reaching 80% conversion to the UDP-4-amino sugar. The filtrate was supplemented with 0.2 mg of purified, full-length PglB (bound to Ni-NTA resin) and 1.2 mM AcCoA and allowed to react at room temperature while being rocked for 12 h. The slurry was filtered, and the flow-through containing the UDP-DATDH sugar was collected. Purification and nuclear magnetic resonance (NMR) characterization of the final UDP-DATDH product were completed as previously described.[28]

Preparation of Radiolabeled Und-PP-Linked Substrates. In general, radiolabeled Und-PP-linked substrates were prepared at two different specific activity levels. A higher specific activity was used for the OTase assay and analysis by normal phase

high-performance liquid chromatography (NP-HPLC), and a lower specific activity was appropriate for the glycosyltransferase assays.

Und-PP-[^{3}H]diNAcBac was enzymatically synthesized using *S. mutans* undecaprenol kinase[30] and PglB. An undecaprenol kinase from *N. gonorrhoeae* has not been characterized, and thus, the undecaprenol kinase from *S. mutans*[29] was used a tool to effect the undecaprenol phosphorylation in situ. A typical reaction mixture contained 3% DMSO, 1% Triton X-100, 50 mM $MgCl_2$, 30 mM Tris-acetate (pH 8.0), 500 μM undecaprenol, 1 mM ATP, 500 μM UDP-4-amino, 500 μM [^{3}H]AcCoA (20 mCi/mmol), 15−20 μL of undecaprenol kinase CEF, 15−20 μL of PglB CEF, and water in a final volume of 100 μL. The reaction mixture was modified to prepare Und-PP-[^{3}H]diNAcBac with high specific activity by adjusting the undecaprenol and UDP-4-amino concentrations to 100 μM and the [^{3}H]AcCoA concentration to 4.5 μM (20 Ci/mmol). After incubation at room temperature for 2 h, the reactions were quenched into 1 mL of a 2:1 $CHCl_3$/MeOH mixture and the mixtures were extracted three times with 400 μL of an aqueous extract prepared by dissolving 1.83 g of potassium chloride in 235 mL of water, 240 mL of chloroform, and 15 mL of methanol. The organic layer containing the Und-PP-[^{3}H]diNAcBac product was dried and purified using NP-HPLC as described below.

Und-PP-diNAcBac-[^{3}H]Gal was prepared in a manner similar to that of Und-PP-diNAcBac. The reaction components were as described above for Und-PP-diNAcBac with the following exceptions; 500 μM UDP-diNAcBac was added instead of UDP-4-amino and AcCoA, and 2 μM PglA and 500 μM UDP-[^{3}H]Gal (20 mCi/mmol) were added to effect the transfer of the galactosyl unit, which is the second sugar in the glycan. To prepare Und-PP-diNAcBac-[^{3}H]Gal with higher specific activity, undecaprenol and UDP-diNAcBac concentrations were lowered to 100 μM and the UDP-[^{3}H]Gal concentration was adjusted to 4.5 μM (20 Ci/mmol). The reactions were quenched after 2 h and the mixtures extracted as described above.

The synthesis of Und-PP-diNAcBac-Gal-[^{3}H]Gal utilized unlabeled Und-PP-diNAcBac-Gal, which was prepared as described above with the exception that UDP-Gal was not radioactive. A typical biosynthesis reaction mixture contained 3% DMSO, 0.05% Triton X-100, 50 mM $MnCl_2$, 50 mM HEPES (pH 7.5), 20 μM Und-PP-diNAcBac-Gal, 20 μM UDP-[^{3}H]Gal (20 mCi/mmol), 20 μL of PglE CEF, and water in a final volume of 100 μL. To prepare the substrate with higher specific activity, the UDP-[^{3}H]Gal concentration was lowered to 4.5 μM (20 Ci/mmol). The reactions were quenched after 2 h and the mixtures extracted as described above.

Und-PP-diNAcBac-[^{3}H]Glc was prepared from unlabeled Und-PP-diNAcBac. The reaction mixture contained 3% DMSO, 0.1% DDM, 50 mM $MgCl_2$, 30 mM Tris (pH 8.0), 20 μM Und-PP-diNAcBac, 20 μM UDP-[^{3}H]Glc (20 mCi/mmol), 10 μM PglH, and water in a final volume of 100 μL. The substrate was also prepared with a higher specific activity by lowering the UDP-[^{3}H]Glc concentration to 4.5 μM (20 Ci/mmol).

The *C. jejuni* substrates (Und-PP-diNAcBac-[^{3}H]GalNAc and Und-PP-diNAcBac-GalNAc-[^{3}H]GalNAc) for the OTase reactions were prepared as previously described[25−27,29,31] with specific activities (20 Ci/mmol) similar to those of the *N. gonorrhoeae* OTase substrates.

NP-HPLC Purification of Und-PP-Linked Substrates. The dried Und-PP-linked substrates were purified via NP-HPLC with a Varian Microsorb column using the previously described gradient.[31] The substrates were resuspended in 100 μL of a 4:1 $CHCl_3$/MeOH mixture for injection onto the column. Fractions of 1 mL were collected, and 10 μL of each fraction was solubilized in 200 μL of Solvable for the detection of radioactivity. The fractions containing substrate were combined, aliquoted, and stored at −20 °C.

To obtain the NP-HPLC analytical traces, Und-PP-linked glycan fractions were resolubilized in a 4:1 $CHCl_3$/MeOH mixture and 100 μL of the appropriate sample was injected onto the column. The 1 mL elution fractions were dried completely and resuspended in 200 μL of Solvable for scintillation counting.

Preparation and Analysis of 2-aminobenzamide-Labeled Oligosaccharides. Unlabeled versions of Und-PP-diNAcBac-Gal and Und-PP-diNAcBac-$(Gal)_2$ were prepared in a manner identical to that of the radiolabeled substrates, except that unlabeled substrates were used in all reactions. The oligosaccharides were labeled with 2-aminobenzamide as previously described[26,32] and purified using a GlykoNSep column (Prozyme). The appropriate peaks were collected, and matrix-assisted laser desorption ionization mass spectrometry (MALDI MS) was used to determine the mass of the 2-AB-labeled glycans.

Glycosyltransferase Substrate Specificity Assays. To determine the UDP-sugar specificity of PglB, PglA, and PglE, we performed radioactivity-based assays with a variety of UDP-linked sugar substrates.

The ability of PglB to transfer UDP-diNAcBac, UDP-Glc, UDP-Gal, UDP-GlcNAc, and UDP-GalNAc was analyzed. The activity of PglB was coupled to the action of *S. mutans* undecaprenol kinase to provide the undecaprenyl phosphate in situ.[29] The specificity assays included 3% DMSO, 1% Triton X-100, 50 mM $MgCl_2$, 30 mM Tris-acetate (pH 8.0), 20 μM undecaprenol, 1 mM ATP, 2 μM UDP-[^{3}H]sugar (20 mCi/mmol), 20 μL of undecaprenol kinase CEF, 5 μL of PglB CEF, and water in a final volume of 100 μL. In the case of UDP-diNAcBac transfer, 2 μM UDP-4-amino and 2 μM [^{3}H]AcCoA (20 mCi/mmol) were included to assay both activities of the bifunctional PglB, which conducts the transfer of the acetyl group to UDP-4-amino and the transfer of phospho-diNAcBac to undecaprenyl phosphate. The reactions were initiated with a mixture of ATP and PglB and were monitored by quenching 15 μL aliquots at 20, 40, 60, 80, and 100 s. The radioactivity present in the organic and aqueous layers was determined as described above.

The ability of PglA and PglE to transfer UDP-Glc, UDP-Gal, UDP-GlcNAc, and UDP-GalNAc was also analyzed. In the case of PglA, the assays contained 3% DMSO, 0.05% Triton X-100, 50 mM $MgCl_2$, 30 mM Tris-acetate (pH 8.0), 10 μM Und-PP-diNAcBac, 2 μM UDP-sugar (20 mCi/mmol), 0.1 μM PglA, and water in a final volume of 100 μL. The reaction was monitored by quenching 15 μL aliquots at 1, 2, 3, 4, and 5 min. For PglE, the assays contained 3% DMSO, 0.05% Triton X-100, 50 mM $MnCl_2$, 50 mM HEPES (pH 7.5), 2 μM Und-PP-diNAcBac-Gal, 5 μM UDP-sugar (20 mCi/mmol), 5 μL of PglE CEF, and water in a final volume of 100 μL. The reaction was monitored by quenching 15 μL aliquots at 2, 4, 6, 8, and 10 min. For both assays, the reactions were initiated by addition of enzyme and the radioactivity present in the organic and aqueous layers was determined as described above.

In addition, a coupled reaction was performed to test the ability of PglB and PglA to distinguish between UDP-4-amino and UDP-diNAcBac. The reaction components contained 3% DMSO, 1% Triton X-100, 40 mM $MgCl_2$, 30 mM Tris-acetate (pH 8.0), 50 μM undecaprenol, 1 mM ATP, 500 μM

UDP-4-amino, 500 μM AcCoA, 90 nM UDP-Gal (20 Ci/mmol), 5 μL of undecaprenol kinase CEF, 10 μL of PglB CEF, and 1 μM PglA in a final volume of 100 μL. The reaction was initiated with a mixture of ATP and PglB. The extent of the reaction was monitored by quenching 15 μL aliquots at 1, 3, 5, 7, and 9 min. To test if PglB and PglA could recognize UDP-4-amino, a second reaction mixture was prepared without AcCoA. The quenched aliquots were extracted as described above, and the radioactivity present in the organic fraction was determined by scintillation counting.

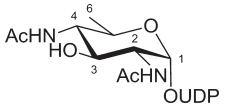
Oligosaccharyltransferase Assays. The OTase reactions were performed with a variety of Und-PP-linked glycosyl donors. In general, the reaction mixtures contained 5% DMSO, 0.7% Triton X-100, 50 mM $MnCl_2$, 25 mM HEPES (pH 7.5), 70 mM sucrose, 10−20 nM Und-PP-substrate (20 Ci/mmol), 8 μM pilin, and 1 μM PglO in a reaction volume of 100 μL. The reaction mixtures were incubated overnight at room temperature with shaking. The glycosylated pilin protein was isolated via Ni-NTA purification. Briefly, the reaction mixture was incubated with 15 μL of Ni-NTA resin for several hours in a 1.5 mL Eppendorf tube. The tube was briefly centrifuged, and the supernatant was removed. The resin was then washed five times with 500 μL of buffer A containing 30 mM imidazole and 0.1% Triton X-100. For each wash, the buffer was added to the Eppendorf tube, the resin was mixed thoroughly with buffer, and the supernatant was removed following a brief centrifugation. The protein was eluted in three fractions of 500 μL of buffer A containing 300 mM imidazole and 0.1% Triton X-100. Scintillation fluid [Ecolite(+), MP Biomedicals] was added to all flow-through, wash, and elution fractions, and the radioactivity of each sample was determined.

Glycosylated protein samples for Western blot analysis were prepared and purified in the same manner, except that unlabeled versions of the Und-PP-diNAcBac substrate were used at concentrations of 10−24 μM. Parallel reactions with radioactive substrates were performed at identical concentrations to determine reaction yields. The Western blot analysis was performed following standard protocols. An antibody specific for His_4 (Qiagen) was used as a positive control for the purified proteins, and a diNAcBac epitope monoclonal antibody termed npg1, which was previously described,[33] was used to detect diNAcBac-modified protein.

RESULTS

Determination of UDP-DATDH Stereochemistry by NMR. The biosynthesis of UDP-DATDH from UDP-GlcNAc was conducted in the presence of purified dehydratase (PglD), aminotransferase (PglC), and acetyltransferase/phosphoglycosyl transferase (PglB). PglC, a soluble protein, was purified to homogeneity (Figure S1 of the Supporting Information, lane 2). TMHMM, a transmembrane prediction program,[34] predicts that PglD and PglB contain four transmembrane helices and one transmembrane helix, respectively. SDS−PAGE analysis of purified PglD demonstrated that the desired protein product is the dominant component (Figure S1 of the Supporting Information, lane 1). PglB was purified for this experiment, but the enzyme was used as a partially purified CEF (Figure S1 of the Supporting Information, lane 4) in all other assays to prevent problems with protein stability. The anti-His_4 Western blot analysis revealed that both purified PglD and PglB CEF contained His_6-tagged truncation products that formed during protein expression (Figure S1 of the Supporting Information, lanes 1 and 4).

Table 1. Comparison of *C. jejuni* and *N. gonorrhoeae* UDP-diNAcBac ^{1}H Chemical Shift and Coupling Constant Assignments



	C. jejuni[28]		*N. gonorrhoeae*	
moiety	δ_H (ppm)	J	δ_H (ppm)	J
H1	5.48 (dd)	$J_{1,2}$ = 3.2 Hz	5.46 (dd)	$J_{1,2}$ = 3.2 Hz
		$J_{1,P}$ = 6.9 Hz		$J_{1,P}$ = 6.9 Hz
H2	4.02 (m)		4.03 (m)	
H3	3.79 (at)	$J_{2,3}$ = 10.2 Hz	3.76 (at)	$J_{2,3}$ = 10.1 Hz
		$J_{3,4}$ = 10.2 Hz		$J_{3,4}$ = 10.2 Hz
H4	3.69 (at)	$J_{4,5}$ = 10.2 Hz	3.67 (at)	$J_{4,5}$ = 10.1 Hz
H5	4.05 (m)		4.03 (m)	
H6	1.19 (d)	$J_{5,6}$ = 6.2 Hz	1.17 (d)	$J_{5,6}$ = 6.2 Hz

The biosynthesis of UDP-diNAcBac by the action of PglD, PglC, and PglB was followed by capillary electrophoresis to ensure complete turnover of the substrates. This method also verified that the HexNAc substrate of PglD is UDP-GlcNAc, and not UDP-GalNAc (data not shown). Purification by RP-HPLC removed unreacted substrates and cofactors leading to a final UDP-DATDH purity of >95% as determined by capillary electrophoresis. To determine the final stereochemistry of the sugar, ^{1}H NMR (Figure S2 of the Supporting Information) was employed to compare the chemical shifts and coupling constants with those of UDP-diNAcBac from the *C. jejuni* pathway (Table 1). The values for the UDP-DATDH sugar from *N. gonorrhoeae* exactly match the values of UDP-diNAcBac from *C. jejuni*.[28] Further confirmation was provided by the ^{31}P, ^{13}C, and ^{1}H−^{1}H COSY NMR spectra (Figures S3−S5 of the Supporting Information). Therefore, the stereochemistry of the DATDH sugar in the *N. gonorrhoeae* pathway is confirmed as diNAcBac.

Functional Characterization of PglB-ATD. The similarity of the *N. gonorrhoeae* protein glycosylation pathway to the pathway in *C. jejuni* suggests that the acetyltransferase domain of PglB acts first on UDP-4-amino to generate UDP-diNAcBac, which is then utilized as a substrate by the phospho-glycosyltransferase domain of PglB [PglB-PGTD (Figure 1)]. The C-terminal acetyltransferase domain of full-length PglB [PglB-ATD, based upon a ClustalW alignment with PglD(*Cj*)] was purified (Figure S1 of the Supporting Information, lane 3). This provided a suitable amount of well-behaved, soluble protein in the absence of the N-terminal phospho-glycosyltransferase domain, which is predicted by TMHMM to contain a single transmembrane domain.[34] Functional analysis of PglB-ATD described below confirmed definitively that this domain acetylates UDP-4-amino to produce UDP-diNAcBac, which is a substrate for PglB-PGTD-mediated transfer of P-diNAcBac to Und-P.

Kinetic Characterization of PglC and PglB-ATD. Both the aminotransferase (PglC) and acetyltransferase (PglB-ATD) reactions exhibited typical Michaelis−Menten kinetics over a wide range of substrate concentrations (Figure S6 of the Supporting Information). Initial velocity data were used to calculate kinetic

Table 2. Steady-State Parameters for PglC and PglB-ATD

enzyme	substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} M^{-1})
PglC	L-glutamate	0.025 ± 0.001	4900 ± 900	5.1
PglC	UDP-4-keto	0.039 ± 0.002	233 ± 35	167
PglB-ATD	AcCoA	0.928 ± 0.032	456 ± 34	2035
PglB-ATD	UDP-4-amino	0.416 ± 0.016	122 ± 17	3410

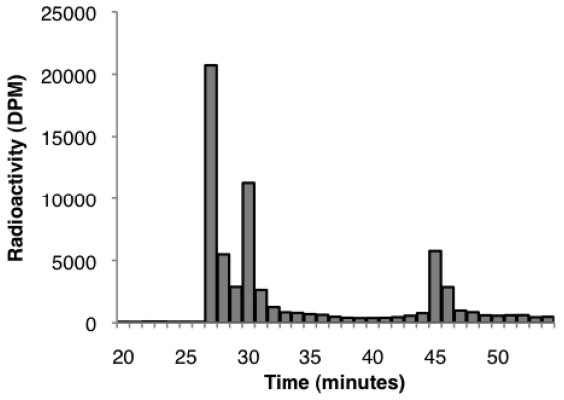


Figure 3. NP-HPLC separation of radiolabeled Und-PP-[^{3}H]diNAcBac (27 min), Und-PP-diNAcBac-[^{3}H]Gal (30 min), and Und-PP-diNAcBac-Gal-[^{3}H]Gal (45 min). Fractions were eluted at 1 mL/min, and radioactivity (disintegrations per minute) was determined by scintillation counting.

parameters of L-Glu and UDP-4-keto for PglC and AcCoA and UDP-4-amino for PglB-ATD. Each reaction was run in duplicate, and the initial velocities were fit to eq 1 to yield the kinetic parameters listed in Table 2.

Functional Characterization of the Glycosyltransferases. As mentioned above, TMHMM[34] predicts that PglB has a single N-terminal transmembrane helix. In addition, PglE is predicted to contain two C-terminal transmembrane helices. Purification of these proteins by detergent solubilization and extraction resulted in low yields and loss of activity; to avoid these problems, both PglB and PglE were purified as crude CEFs for the glycosyltransferase assays. SDS−PAGE and Western blot analysis showed that PglB and PglE are the predominant bands present in the respective CEFs (Figure S1 of the Supporting Information, lanes 4 and 6). In all assays involving PglB and PglE, negative controls with CEFs lacking overexpressed PglB or PglE showed no glycosyltransferase activity (data not shown). PglA is predicted to be soluble and was purified to homogeneity (Figure S1 of the Supporting Information, lane 5).

Tritium-labeled products of PglB, PglA, and PglE were analyzed by NP-HPLC. Und-PP-[^{3}H]diNAcBac, Und-PP-diNAcBac-[^{3}H]Gal, and Und-PP-diNAcBac-Gal-[^{3}H]Gal had retention times consistent with glycan size (Figure 3). Each product was analyzed separately to confirm the identity of the peaks (Figure S7 of the Supporting Information). In addition, the glycosyltransferase products were characterized by a standard 2-AB fluorescence labeling protocol as previously described.[35] The 2-AB-labeled disaccharide and trisaccharide were purified, and MALDI MS was used to verify the masses of the products (Figure S8 of the Supporting Information). These studies definitively annotate the biochemical functions of PglB, PglA, and PglE as the phospho-glycosyltransferase and the two

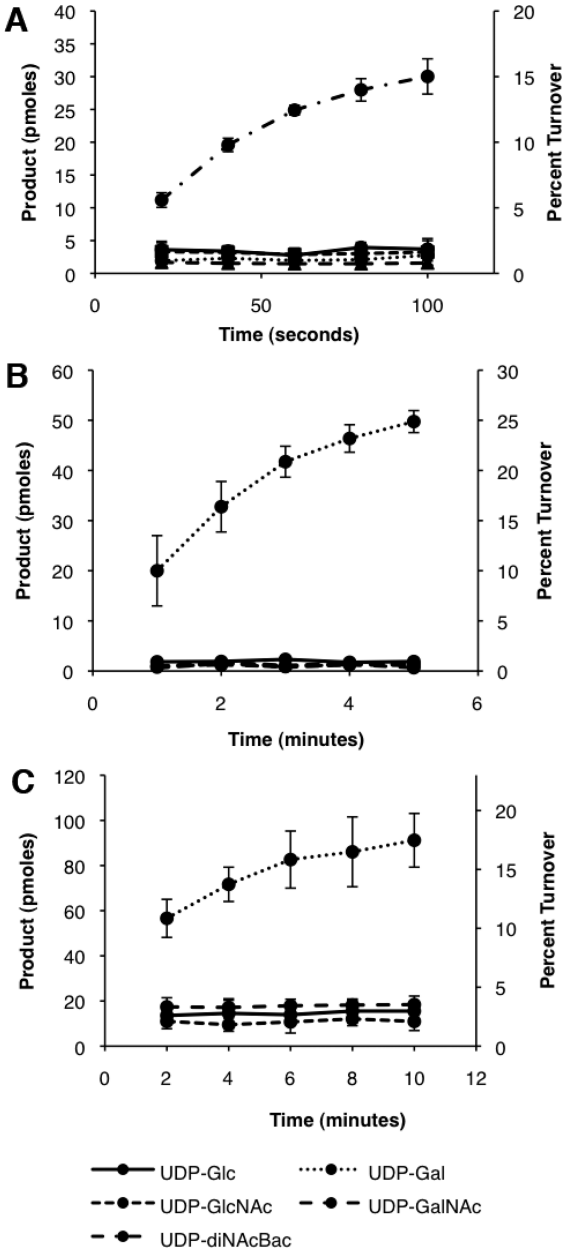


Figure 4. Specificity analyses of PglB (A), the phospho-glycosyltransferase, and PglA (B) and PglE (C), the galactosyltransferases, in the presence of a panel of UDP-sugar substrates. The reactions were conducted in a volume of 100 μL. The assays were performed in triplicate, and the error bars indicate standard deviation.

glycosyltransferases that produce Und-PP-linked mono-, di-, and trisaccharides, respectively.

UDP-Saccharide Specificity of Glycosyltransferases. The substrate specificities of PglB, PglA, and PglE were explored through the use of radioactivity-based assays (Figure 4). Organic extraction of the hydrophobic undecaprenyl-linked product allowed for quantification of the amount of radiolabeled sugar transferred to the undecaprenyl substrate similar to previously described assays.[26,31] The isoprenyl-linked substrates for the assays (Und-PP-diNAcBac for PglA and Und-PP-diNAcBac-Gal for PglE) were produced enzymatically and purified by NP-HPLC. The undecaprenyl phosphate required for the PglB

reaction was generated in situ from undecaprenol and ATP with *S. mutans* undecaprenol kinase as previously described.[29] The activities of the three enzymes were screened with UDP-Glc, UDP-Gal, UDP-GlcNAc, UDP-GalNAc, and, in the case of PglB, UDP-diNAcBac (Figure 4). In addition, the ability of PglB and PglA to distinguish between UDP-4-amino and UDP-diNAcBac was evaluated through a coupled assay (Figure S9 of the Supporting Information). In all cases, the enzymes were highly specific for the corresponding predicted sugar substrate; PglB exclusively transferred phospho-diNAcBac, while PglA and PglE transferred only Gal (Figure 4).

Undecaprenyl Diphosphate Disaccharide Specificity of PglE. Facile enzymatic synthesis of Und-PP-diNAcBac-Gal and Und-PP-diNAcBac-GalNAc produced by the *Neisseria* and *C. jejuni* pathways, respectively, allowed for examination of the substrate specificity of PglE for the acceptor oligosaccharide. Somewhat surprisingly, PglE is able to add a Gal residue to both the native substrate, Und-PP-diNAcBac-Gal, and the *C. jejuni* substrate, Und-PP-diNAcBac-GalNAc (Figure 5). This confirms in vivo studies in which the *C. jejuni* PglA was expressed in *N. gonorrhoeae* and the resultant trisaccharide (diNAcBac-GalNAc-Gal) was observed as a covalent pilin modification.[1]

Characterization of PglH, an Alternative Glycosyltransferase. Recently, an alternative glycosyltransferase in *N. gonorrhoeae*, PglH, was identified and shown to transfer a Glc unit to Und-PP-diNAcBac[14] (Figure 2). PglH was shown to be responsible for the specific addition of Glc to Und-PP-diNAcBac by a variety of in vivo and in vitro methods. In vitro radiolabeled assays demonstrated that PglH transfers Glc from UDP-Glc to Und-PP-diNAcBac and does not transfer Man, Gal, GlcNAc, or GalNAc; MALDI MS of the 2-AB-labeled diNAcBac-Glc confirmed the identity of the PglH product.[14]

Herein, we further characterize PglH and compare its function to those of the other *N. gonorrhoeae* glycosyltransferases (Figure S1 of the Supporting Information, lane 8). Analysis of the radiolabeled PglH product, Und-PP-diNAcBac-[^{3}H]Glc, by NP-HPLC revealed that the retention time (30 min) was very similar to the retention time of Und-PP-diNAcBac-[^{3}H]Gal (29−30 min) (Figure 6). In addition, prior in vivo evidence suggested that unlike the *C. jejuni* disaccharide, the PglH product was not further modified by the third glycosyltransferase, PglE.[14] This result was validated by the in vitro specificity assay described above for PglE and established that PglE was unable to transfer Gal to Und-PP-diNAcBac-Glc (Figure 5).

Functional Characterization of Oligosaccharyltransferase, PglO. PglO and pilin are integral membrane proteins and were expressed in *E. coli*, extracted from the CEF with Triton X-100, and purified to homogeneity (Figure S1 of the Supporting Information, lanes 7 and 9). To assay for OTase activity, purified PglO was incubated with pilin and the radiolabeled Und-PP-diNAcBac-[^{3}H]Gal glycan donor. After overnight incubation, the reaction mixture was bound to Ni-NTA resin and washed thoroughly to remove most (>99%) of the unreacted Und-PP-diNAcBac-[^{3}H]Gal donor. The pilin protein was then eluted with imidazole, and the radioactivity associated with the wash and elution fractions was determined by scintillation counting (Figure S10 of the Supporting Information). Under these assay conditions, in which pilin protein is in excess over Und-PP-diNAcBac-[^{3}H]Gal, PglO transferred ~60% of the sugar substrate to pilin (Figure 7). Interestingly, unlike OTases in N-linked glycosylation pathways,[27,36] PglO does not readily glycosylate a short peptide based on the pilin glycosylation sequence (Figure S11 of the Supporting Information).

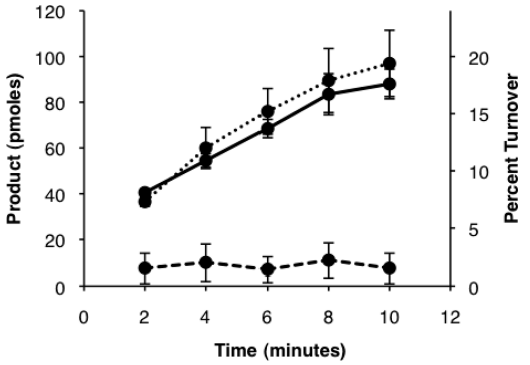


Figure 5. Determination of the isoprenyl-linked substrate preferences of PglE in the presence of Und-PP-diNAcBac-Glc (---), Und-PP-diNAcBac-Gal (—), or Und-PP-diNAcBac-GalNAc (···). The reactions were conducted in a volume of 100 μL. The assays were performed in triplicate, and the error bars indicate the standard deviation.

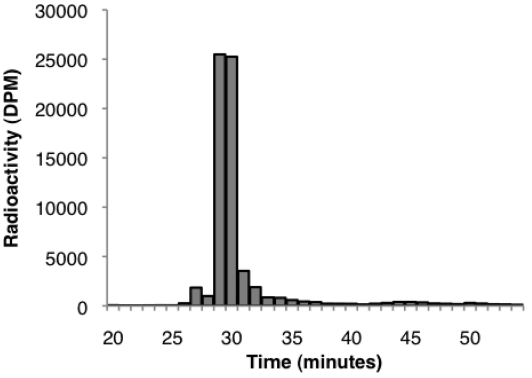


Figure 6. NP-HPLC separation of radiolabeled Und-PP-diNAcBac-[^{3}H]Glc, the product of PglH (30 min). Fractions were eluted at 1 mL/min, and radioactivity (disintegrations per minute) was determined by scintillation counting.

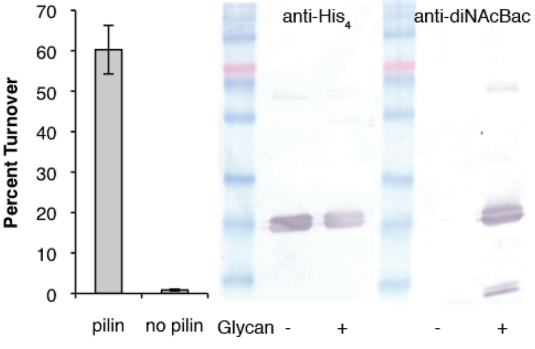


Figure 7. PglO reaction turnover after overnight incubation with Und-PP-diNAcBac-[^{3}H]Gal in the presence of pilin or a negative control with no protein substrate. Western blot analysis of unmodified pilin and PglO-glycosylated pilin using a His_4 antibody (left blot, positive control) and a diNAcBac epitope-recognizing monoclonal antibody termed npg1 (right blot, specific for glycan).

These results were further verified via Western blot analysis utilizing a monoclonal antibody recognizing a diNAcBac-associated epitope (Figure 7). For the Western blot analysis, the pilin

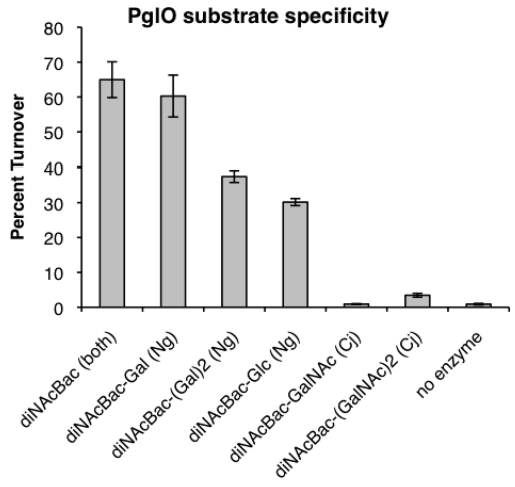


Figure 8. PglO reaction turnover after overnight incubation with pilin protein in the presence of a panel of Und-PP-linked substrates from *N. gonorrhoeae* [diNAcBac-Gal, diNAcBac-$(Gal)_2$, and diNAcBac-Glc], *C. jejuni* [diNAcBac-GalNAc and diNAcBac-$(GalNAc)_2$], or both (diNAcBac). A negative control is shown in which the assay was performed in the presence of Und-PP-diNAcBac-Gal and in the absence of PglO. The assays were performed in triplicate, and the error bars indicate the standard deviation.

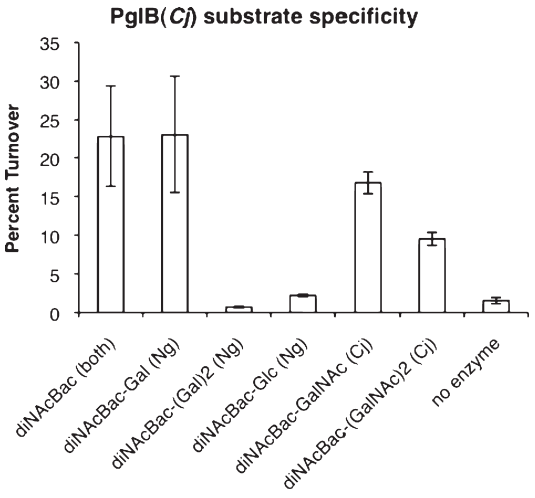


Figure 9. PglB(*Cj*) reaction turnover after overnight incubation with pilin protein in the presence of a panel of Und-PP-linked substrates from *N. gonorrhoeae* [diNAcBac-Gal, diNAcBac-$(Gal)_2$, and diNAcBac-Glc], *C. jejuni* [diNAcBac-GalNAc and diNAcBac-$(GalNAc)_2$], or both (diNAcBac). A negative control is shown in which the assay was performed in the presence of Und-PP-diNAcBac-GalNAc and in the absence of PglB(*Cj*). The assays were performed in triplicate, and the error bars indicate the standard deviation.

glycosylation reaction was performed with equimolar amounts of protein substrate and Und-PP-diNAcBac donor, and under these conditions, ~13% of the pilin protein was associated with glycan. The antibody raised against diNAcBac showed strong staining with the glycosylated pilin and was unreactive with the unmodified pilin.[33]

Glycan Donor Specificity of PglO. To further characterize PglO, we performed a screen of various oligosaccharyl donors. Previous studies by Feldman and co-workers[21,22] on PglL, the homologous oligosaccharyltransferase found in *N. meningitidis*, have suggested that these enzymes exhibit relaxed substrate specificity in vivo and can transfer oligosaccharides composed of different sugars, linkages, and lengths. Thus, PglO was assayed with the four native substrates (the products of PglB, PglA, PglE, and PglH) and with two substrates from the *C. jejuni* pathway, Und-PP-diNAcBac modified with one or two GalNAc residues. (For structures of the six glycans tested and further discussion of OTase specificity, see Figure S12 of the Supporting Information.) All glycosyl donors were prepared with high specific activity and purified via NP-HPLC. Surprisingly, and in contrast to the in vivo studies with PglL(*Nm*),[21,22] PglO was able to transfer only the four native substrates; the two *C. jejuni* substrates exhibited <3% turnover (Figure 8).

To verify that the *C. jejuni* substrates were functional as glycan donors, we assayed all six substrates with PglB(*Cj*). Pilin was used as the protein substrate in these assays as well, because it contains an N-linked glycosylation sequon (^{59}EN**N**T**S**^{63}) adjacent to the site of O-linked glycosylation.[37,38] As seen in Figure 9, PglB(*Cj*) transferred the native *C. jejuni* substrates in addition to the diNAcBac-Gal disaccharide from *N. gonorrhoeae*; it showed low reactivity with diNAcBac-Glc and diNAcBac-$(Gal)_2$.

Because pilin is glycosylated by both PglB(*Cj*) and PglO(Ng), it was important to confirm the identity of the glycosylated residues. Pilin variants were prepared with alanine mutations at the expected sites of glycosylation for PglB(*Cj*) and PglO, Asn 61 and Ser 63, respectively. PglB(*Cj*) was unable to glycosylate pilin-N61A, validating this residue as the *N*-glycan acceptor site. However, PglO showed ~85% of normal activity with the pilin S63A mutant, suggesting that another site, potentially Thr 62, is a glycosyl acceptor site in the absence of Ser 63 (Figure S13 of the Supporting Information). Further mutational analysis confirmed this hypothesis; pilin-T62A exhibited normal glycosylation, whereas the level of glycosylation was greatly reduced in the pilin double mutant (T62A/S63A) (Figure S13 of the Supporting Information).

DISCUSSION

Stereochemistry of DATDH Defined as diNAcBac. The extreme diversity of bacterial glycans is highly significant because these glycans typically decorate the bacterial cell surface, facilitating interactions with host cells[7,10] and potentially confounding the immune response.[8,39−42] Bacillosamine was originally identified as an unusual 2,4-diamino-2,4,6-trideoxy-α-D-glucose in *Bacillus subtilis*,[43] but it also appears in the mono- and diaminoacetylated forms in a large variety of bacterial glycoconjugates.[44] It has been found frequently in the O-antigen and capsular polysaccharide of Gram-negative bacteria but has also been identified in the S-layer of Gram-positive bacteria and as the UDP-activated donor in cellular extracts.[45] DiNAcBac was initially discovered in the N-linked glycosylation pathway of *C. jejuni*,[24] but more recently, a second route to diNAcBac was biochemically characterized in *C. jejuni*, in which GDP-diNAcBac is an intermediate in the CMP-legionaminic acid biosynthetic pathway.[46] The pilin oligosaccharide in *N. gonorrhoeae* was thought originally to comprise Gal-α-(1,3)-GlcNAc-β-Ser(63).[47] However, mass spectrometry and bioinformatic analysis suggested that the linking sugar unit was DATDH instead of GlcNAc.[1] Herein, we confirm the stereochemical assignment of this sugar for the first time showing that the DATDH sugar in *N. gonorrhoeae* is diNAcBac (Table 1). This adds to the growing number of oligosaccharides identified in bacteria that contain forms of bacillosamine.

Table 3. Percent Sequence Identity between *N. gonorrhoeae* (*Ng*) and *C. jejuni* (*Cj*) and between *N. gonorrhoeae* (*Ng*) and *N. meningitidis* (*Nm*) Proteins

function	*N. gonorrhoeae*	*C. jejuni*	% *Ng* and *Cj*	*N. meningitidis*	% *Ng* and *Nm*
dehydratase	PglD	PglF	29.8	PglD	92.5
aminotransferase	PglC	PglE	21.2	PglC	92.8
acetyltransferase	PglB-ATD	PglD	29.7	PglB-ATD	84.9
phospho-glycosyltransferase	PglB-PGTD	PglC	52.3	PglB-PGTD	90.3
glycosyltransferase	PglA	PglA	21.3	PglA	95.5
glycosyltransferase	PglE	PglJ	12.0	PglE	93.0
OTase	PglO	PglB	11.8	PglL	95.0

PglD, PglC, and PglB produce Und-PP-diNAcBac in *N. gonorrhoeae*; these three enzymes are functionally homologous to PglF(*Cj*), PglE(*Cj*), PglD(*Cj*), and PglC(*Cj*) in *C. jejuni*, which produce the same polyprenyl-linked intermediate (Figure 2). Even though the early enzymes in these two pathways conduct identical reactions, the level of sequence identity is relatively low (25−30%), except for the phospho-glycosyltransferase domain of PglB, which is 52% identical in sequence to PglC(*Cj*) (Table 3). These numbers starkly contrast the levels of sequence identity observed between *N. gonorrhoeae* and *N. meningitidis*, which indicate much closer homologies [>84% (Table 3)]. These numbers imply that the *C. jejuni* and *N. gonorrhoeae* pathways are only distantly related from an evolutionary standpoint.

Work to understand the low level of homology between the *C. jejuni* and *N. gonorrhoeae* diNAcBac biosynthetic enzymes is ongoing. As a first step, this study describes the kinetic parameters of both the aminotransferase (PglC) and the acetyltransferase (PglB-ATD) (Table 2). The apparent K_m of the UDP-4-keto sugar for PglC (233 μM) was comparable to that of the PglE(*Cj*) homologue (48 μM[48] and 610 μM[49]) and well within the range of typical binding efficiencies for this type of substrate. Likewise, K_m values of the UDP-4-amino substrate for the acetyltransferases PglB-ATD (122 μM) and PglD(*Cj*) (410 μM[28]) lead to a similar conclusion. However, the *N. gonorrhoeae* enzymes presented here are catalytically much less efficient (k_{cat} 10−100-fold lower for PglC and 1000-fold lower for PglB-ATD) than their *C. jejuni* counterparts with respect to the UDP-sugar. This observation is reflected in the differences between their specificity constants (k_{cat}/K_m) (Table 2). The high acetyltransferase activity in the *C. jejuni* pathway is used to drive the biosynthesis of the UDP-diNAcBac sugar.[28] A similar phenomenon is observed in the *N. gonorrhoeae* pathway, with a 20-fold enhancement in the k_{cat}/K_m of PglB-ATD with respect to aminotransferase activity.

For PglB-ATD, one cannot rule out interplay between the acetyltransferase domain and the missing C-terminal phospho-glycosyltransferase domain. Therefore, care must be taken in interpreting the reduced *N. gonorrhoeae* acetyltransferase efficiency compared to that of PglD(*Cj*). Further work will be necessary to clarify how domain interactions affect kinetic parameters. In addition, the low level of sequence homology between the *C. jejuni* and *N. gonorrhoeae* UDP-diNAcBac pathway enzymes (Table 3) could contribute to the differences in catalytic efficiency observed here.

Significant Glycan Diversity in *N. gonorrhoeae* Protein Glycans. The *C. jejuni* and *N. gonorrhoeae* pathways diverge after the synthesis of Und-PP-diNAcBac. The *C. jejuni* pathway continues to N-linked glycan assembly with the successive addition of five α-(1,4)-linked GalNAc units and a branching Glc unit. However, while the *C. jejuni* N-linked heptasaccharide is highly conserved, *N. gonorrhoeae* strains display high O-linked glycan diversity. Strains that contain not only O-linked disaccharide [Gal-α-(1,3)-diNAcBac] and trisaccharide [Gal-β-(1,4)-Gal-α-(1,3)-diNAcBac] produced by PglA and PglE, respectively, but also an alternate disaccharide [Glc-α-(1,3)-diNAcBac] produced by PglH have been identified.[14] Further glycan modification occurs from the addition of *O*-acetyl groups by PglI.[1] In addition, an alternate allele (*pglB2*) has been identified in *N. meningitidis* that contains a domain proposed to transfer a glyceroyl moiety instead of an acetyl group to produce 4-glyceramido-2-acetamido-2,4,6-trideoxy-α-D-hexose (GATDH).[20] This combination of biosynthetic enzymes allows neisserial strains to display a glycan repertoire with at least 13 identified glycan permutations.[14] Additional glycan variation can occur within a single strain as phase variation of the genes encoding PglI, PglA, PglE, and PglH acts as another mode of glycan regulation.[1,14]

In light of the significant amount of protein glycan variation present within strains of *N. gonorrhoeae*, it is surprising that the glycosyltransferases display such strict specificity (Figure 4 and Figure S9 of the Supporting Information). We have demonstrated that PglB, PglA, and PglE are specific for the native substrate (UDP-diNAcBac or UDP-Gal) and will not accept any other form of the nucleotide-activated sugars commonly found in vivo, even though one of the alternate substrates contains only a single stereochemical change (UDP-Glc vs UDP-Gal) and another contains only an additional acetamido group (UDP-GalNAc vs UDP-Gal). Parallel work has shown similar strict specificity of the fourth glycosyltransferase, PglH.[14] These results suggest that glycan identity is regulated at the level of biosynthesis and that these enzymes have evolved to selectively catalyze reactions in the milieu of intracellular NDP-sugars.

Along the same lines, PglE transferred a Gal unit onto Und-PP-diNAcBac-Gal, but it showed little activity with Und-PP-diNAcBac-Glc, the alternate disaccharide produced by PglH (Figure 5). PglE has evolved to detect the stereochemical difference between Glc and Gal, which is consistent with the model in which PglA and PglE have evolved in tandem to produce a trisaccharide that is structurally distinct from the disaccharide produced by PglH.[14] In contrast, it is surprising that PglE would recognize the *C. jejuni* substrate Und-PP-diNAcBac-GalNAc (Figure 5), but it is consistent with the hypothesis that the glycosyltransferases exhibit specificity relative to other substrates present in the organism. PglE may not have developed selectivity against the additional acetamido group in the *C. jejuni* disaccharide, because it is not found in the native *N. gonorrhoeae* glycome.

The diverse O-linked glycans produced by *N. gonorrhoeae* decorate a number of extracellular proteins but were first discovered

as covalent modifications of Ser 63 in pilin. This residue is found within the α−β loop, which is surface-exposed in the assembled pilin structure[50] and highly modified by other post-translational modifications, including addition of phosphoethanolamine and phosphocholine.[12] This modified loop also contains a bacterial N-linked sequon that overlaps the O-linked glycosylation site, ^{59}EN**N**T**S**^{63}, which can act as a protein acceptor substrate for the N-linked OTase PglB(*Cj*).[38,51] The presence of the N-linked sequon in the pilin structure is interesting from an evolutionary standpoint, suggesting that post-translational modification of this conserved loop may be important for pilin structure and function and that in other bacteria, this loop could contain N-linked glycans. A survey of sequenced bacterial genomes that contain a PglB(*Cj*) OTase homologue revealed at least one bacterium, *Nitrosococcus halophilus*, with a predicted pilin homologue containing the requisite Asn consensus sequence.

***En Bloc* Transfer of Glycan from the Polyprenyl-Linked Intermediate to the Ser Residue in Pilin.** The *C. jejuni* and *N. gonorrhoeae* pathways culminate in transfer of the oligosaccharide to protein; in the bacterial N-linked glycosylation pathway, PglB(*Cj*) transfers a heptasaccharide *en bloc* to the amide side chain of asparagine residues. Herein, we demonstrate that PglO acts in a similar *en bloc* manner to transfer mono-, di-, and trisaccharides to hydroxyl side chains of serine or threonine residues (Figures 7 and 8).

PglO was originally identified as the enzyme responsible for glycosylation of *N. gonorrhoeae* type IV pili but has since been shown to glycosylate a wide variety of periplasmic and extracellular lipoproteins. In all examples of non-pilin glycosylation substrates, the acceptor serine or threonine residues are present in loop regions predicted to have undefined structures rich in Ala, Ser, and Pro residues.[2] Interestingly, PglO was not active in the presence of a peptide substrate modeled on the pilin sequence (Figure S10 of the Supporting Information). The inactivity of PglO contrasts the behavior of the bacterial N-linked OTase, PglB(*Cj*), which under comparable conditions is capable of glycosylating a peptide substrate (Figure S10 of the Supporting Information). PglO glycosylates a wide range of periplasmic proteins containing serine and threonine residues in vivo, but it is unclear what binding determinants affect this reaction. Further biochemical and structural analyses are needed to understand how PglO recognizes pilin and non-pilin protein substrates.

PglO in Vitro Assays Demonstrate Specificity for Native Glycans. Recent in vivo analyses revealed that both PglL(*Nm*), which is 95% identical in sequence to PglO, and the N-linked OTase PglB(*Cj*) were promiscuous enzymes that transferred a variety of Und-PP-linked O-antigen substrates to serine and asparagine residues in proteins, respectively.[21,22] These experiments were performed by coexpressing PglL(*Nm*) or PglB(*Cj*) heterologously in *E. coli* with the glycan acceptor protein and a locus encoding the biosynthesis of an Und-PP-linked substrate. In addition, previous studies showed that *N. gonorrhoeae* strains with heterologously expressed PglA(*Cj*) contained proteins modified by the *C. jejuni* disaccharide, diNAcBac-GalNAc, implying that PglO can recognize this glycan in vivo.[1] However, in this study, we have found that the OTases do not show a comparable substrate promiscuity in vitro; in fact, it appears that PglO and PglB(*Cj*) are both specific for their native substrates in vitro (Figures 8 and 9 and Figure S12 of the Supporting Information).

The previous in vivo studies of PglO, PglL(*Nm*), and PglB(*Cj*) were performed in the absence of native substrate and under conditions in which the non-native undecaprenyl-linked glycan accumulated in the membrane.[21,22] Thus, the local concentration of the substrate within the two-dimensional plane of the membrane was likely to be much higher than in the in vitro assay, which would promote reaction with PglO. Additionally, in the context of a lipid bilayer, the membrane-bound undecaprenyl moiety may play a greater role in enzyme recognition of the substrate. In our assay, the concentration of undecaprenyl substrate (10−20 nM) was well below 2.7 μM, the apparent K_m of PglB(*Cj*) for Und-PP-disaccharide,[37] and thus, specificity differences between native and non-native substrates were easily distinguished. In addition, it should be noted that the OTases exhibit substrate specificity in native cellular contexts. In the native bacteria, PglO, PglL(*Nm*), and PglB(*Cj*) selectively transfer the correct oligosaccharide to pilin in the presence of other undecaprenyl-linked substrates, including those involved in capsular polysaccharide biosynthesis in *N. meningitidis*[52] and *C. jejuni*[53] and the peptidoglycan subunits in the cellular membranes of all three species.[54]

In conclusion, this work represents the first complete biochemical characterization of the unusual O-linked glycosylation pathway in *N. gonorrhoeae*. The stereochemistry of the DATDH sugar has been identified as diNAcBac. In addition, the substrate preferences of the glycosyltransferases have been characterized, and in general, these enzymes are shown to be specific for their native substrates. Finally, in vitro characterization of the OTases from *N. gonorrhoeae* and *C. jejuni* has suggested that these enzymes prefer their respective native glycans to closely related oligosaccharides. The O-linked pathways found in *N. gonorrhoeae* and *N. meningitidis* are interesting hybrids of O-linked and N-linked glycosylation pathways. While the role of the O-linked glycans in *Neisseria* pathogenicity is not yet understood, the Pgl enzymes may represent unique virulence targets, and this initial study provides the foundation for further investigations into the biochemistry of the enzymes.

■ ASSOCIATED CONTENT

Supporting Information. *N. gonorrhoeae* gene accession numbers, NMR spectra of UDP-diNAcBac, kinetic analyses of PglC and PglB-ATD, SDS−PAGE and Western blot analyses of the purified Pgl proteins, HPLC traces of purified Und-PP-glycans and 2-AB-labeled glycans, and several control experiments for the OTase assays. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: (617) 253-1838. Fax: (617) 452-2419. E-mail: imper@mit.edu.

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■ ABBREVIATIONS

2-AB, 2-aminobenzamide; AcCoA, acetyl-coenzyme A; *Cj*, *C. jejuni*; CEF, cell envelope fraction; DDM, *n*-dodecyl β-D-maltopyranoside; Gal, galactose; GalNAc, *N*-acetylgalactosamine; Glc, glucose; GlcNAc, *N*-acetylglucosamine; NP-HPLC, normal phase high-performance liquid chromatography; MALDI MS, matrix-assisted laser desorption ionization mass spectrometry; Man, mannose; MBP, maltose binding protein; *Ng*, *N. gonorrhoeae*; Ni-NTA, Ni-nitrilotriacetic acid; *Nm*, *N. meningitidis*; Pgl, protein glycosylation; PglB-ATD, acetyltransferase domain of PglB; PglB-PGTD, phospho-glycosyltransferase domain of PglB; TMHMM, Tied Mixture Hidden Markov Model (a transmembrane prediction model); UDP, uridine diphosphate; UDP-4-amino, UDP-2-acetamido-4-amino-2,4,6-trideoxy-α-D-glucose or UDP-2-acetamido-4-amino-α-D-quinovose; UDP-DATDH, 2,4-diacetamido-2,4,6-trideoxy-α-D-hexose; UDP-diNAcBac, UDP-*N*,*N*′-diacetylbacillosamine or UDP-2,4-diacetamido-2,4,6-trideoxy-α-D-glucose; UDP-4-keto, UDP-2-acetamido-4-keto-2,4,6-trideoxy-α-D-glucose or UDP-2-acetamido-4-keto-α-D-quinovose; Und-P, undecaprenyl phosphate; Und-PP, undecaprenyl diphosphate.

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