Extreme Substrate Promiscuity of the Neisseria Oligosaccharyl Transferase Involved in Protein O-Glycosylation

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Neisseria meningitidis PglL belongs to a novel family of bacterial oligosaccharyltransferases (OTases) responsible for O-glycosylation of type IV pilins. Although members of this family are widespread among pathogenic bacteria, there is little known about their mechanism. Understanding the O-glycosylation process may uncover potential targets for therapeutic intervention, and can open new avenues for the exploitation of these pathways for biotechnological purposes. In this work, we demonstrate that PglL is able to transfer virtually any glycan from the undecaprenyl pyrophosphate (UndPP) carrier to pilin in engineered Escherichia coli and Salmonella cells. Surprisingly, PglL was also able to interfere with the peptidoglycan biosynthetic machinery and transfer peptidoglycan subunits to pilin. This represents a previously unknown post-translational modification in bacteria. Given the wide range of glycans transferred by PglL, we reasoned that substrate specificity of PglL lies in the lipid carrier. To test this hypothesis we developed an in vitro glycosylation system that employed purified PglL, pilin, and the lipid farnesyl pyrophosphate (FarPP) carrying a pentasaccharide that had been synthesized by successive chemical and enzymatic steps. Although FarPP has different stereochemistry and a significantly shorter aliphatic chain than the natural lipid substrate, the pentasaccharide was still transferred to pilin in our system. We propose that the primary roles of the lipid carrier during O-glycosylation are the translocation of the glycan into the periplasm, and the positioning of the pyrophosphate linker and glycan adjacent to PglL. The unique characteristics of PglL make this enzyme a promising tool for glycoengineering novel glycan-based vaccines and therapeutics.

Bacterial surface components are frequently composed of, or decorated with, carbohydrates. Among these glycosylated components are the type IV pili, hair-like structures protruding from the bacterial surface, mainly formed by a single protein generically named pilin (1). Type IV pili are important for host cell adhesion and virulence. Furthermore, pilins are O-glycosylated in diverse pathogenic bacteria, including Neisseria meningitidis and N. gonorrhoea. The glycan moieties in both species consist of short oligosaccharides, up to three sugar residues in length. Several pilin glycosylation (pgl) genes have been identified in N. meningitidis encoding for glycosyltransferases and sugar-modifying enzymes that are required for the biosynthesis of the oligosaccharides (2). However, how the glycans are transferred to pilin has been just recently described. Power et al. identified in a gene in N. meningitidis containing the Wzy_C PFAM domain (PF04932), a signature of enzymes that participate in O antigen biosynthesis, and which is also present in the PioO oligosaccharyltransferase (OTase) involved in pilin glycosylation in Pseudomonas aeruginosa 1244 (3). Inactivation of this gene, named pglL, resulted in an increase in the electrophoretic mobility of pilin, compatible with loss of the pilin glycan. Based on this, it was suggested that PglL was responsible for the attachment of the glycan to pilin (4). Aas et al. (5) showed that mutagenesis of the PglL ortholog in N. gonorrhoea (PglO) abolished pilin glycosylation. The final demonstration that PglL is the OTase responsible for the transfer of the glycan to pilin was provided by Faridmoayer et al. (6) who showed PglL-dependent pilin glycosylation by reconstituting the process in E. coli.

Pilin glycosylation is initiated in the cytoplasm with the assembly of oligosaccharides by the sequential action of glycosyltransferases on undecaprenyl pyrophosphate (UndPP),2 which is also the carrier for the synthesis of other important bacterial surface components, such as lipopolysaccharide (LPS), peptidoglycan (PG), and capsule. The lipid-linked oligosaccharide (LLO) is then flipped into the periplasm, where the oligosaccharide is attached to pilin (4–6).

To date, only a few bacterial OTases have been described, including PglL, PioO, and Campylobacter jejuni PglB. These OTases are integral inner membrane proteins containing mul-

*2 The abbreviations used are: UndPP, undecaprenyl pyrophosphate; DATDH, 2,4-diacetamido-2,6-trideoxyhexose; FarPP, farnesyl pyrophosphate; LLO, lipid-linked oligosaccharide; OTase, oligosaccharyltransferase; PG, peptidoglycan; ProK, proteinase K; LPS, lipopolysaccharide; IPTG, isopropyl-1-thio-β-D-galactopyranoside; Amp, ampicillin; Cm, chloramphenicol; Gm, gentamycin; Km, kanamycin; Sp, spectinomycin; Strep, streptomycin; Tet, tetracycline; Tp, trimethoprim.

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tiple membrane-spanning regions (4, 7, 8). PglB is homologous to the Stt3 component of the eukaryotic OTase complex, but not the OTases involved in O-linked protein glycosylation. PglB is responsible for the N-glycosylation of more than 30 proteins, and is thus referred to as an N-OTase. PglB has been also functionally expressed in E. coli, and it has been shown that it can glycosylate folded proteins in an in vitro assay (7, 9). In contrast, PglL and PilO are both involved in O-glycosylation, and therefore are called O-OTases. Analogous enzymes have not been described in eukaryotes. Similarity between PglL and PilO is restricted to a single domain also present in the O antigen ligases involved LPS biosynthesis. In P. aeruginosa 1244, the pilin glycan is a single O antigen-repeating unit (10). In contrast, C. jejuni and N. meningitidis do not possess O antigens, and instead, there is machinery dedicated to the synthesis of the LLO that will act as the sugar donor for glycosylation.

In this work, we have analyzed the substrate specificity of PglL. We took advantage of the availability of well-defined UndPP-linked glycans naturally used in LPS and capsule biosynthesis, which can be produced in E. coli, to demonstrate that PglL can transfer virtually any glycan to pilin in vivo. This finding prompted us to hypothesize that PglL substrate specificity is located in the lipid carrier. To test this hypothesis, we established an in vitro pilin O-glycosylation assay using a synthetic lipid-linked glycan obtained through successive chemical and enzymatic steps. Our results allowed us to map the region of the substrate recognized by PglL to a very short part of the molecule located at the interface between the glycan and lipid moieties.

**EXPERIMENTAL PROCEDURES**

**Plasmids Construction and Bacteria Growth**—The details of the construction of plasmids pAMF10, expressing PglL C-His10, and pAMF15 and pAMF16 expressing C-His10-tagged N. meningitidis PilE (pilin) under IPTG or arabinose-inducible promoters, as well as the bacteria growth conditions are given in supplemental data.

**Purification of Recombinant Pilin and PglL**—Biosynthesis of recombinant N. meningitidis MC58 pilin glycosylated with different glycans (e.g., O antigens) was achieved by expressing PglL and N. meningitidis MC58 pilin in different E. coli and S. enterica strains (Table 1). pAMF8 (expressing PglL) and pAMF16 (expressing C-His10-tagged pilin) were co-transformed into a target bacterium synthesizing UndPP-linked glycans (Table 1). Cells were cultured at 37 °C in the shaker incubator (200 rpm) until an A600 of 0.4–0.6 was reached, after which the cells were induced by IPTG (0.1 mM) and l-arabinose (0.2% w/v). After induction, the temperature was reduced to 30 °C, and l-arabinose (0.2% w/v) was added again to the culture 6 h after the first induction. Cells were harvested after overnight incubation at 30 °C. Pellets were washed with 20 mM phosphate buffer (pH 7.2) containing 0.3 M NaCl (buffer 1) and resuspended in the same buffer containing complete EDTA-free protease inhibitor mixture (Roche Applied Science). Cells were disrupted and centrifuged at 10,000 × g for 10 min to remove cell debris. Membrane was separated by ultracentrifugation (200,000 × g for 1 h) of the supernatant from the previous step and resuspended in buffer 1 containing 2% Elugent (Calbiochem) and 25 mM imidazole (buffer 2). Proteins were solubilized by mild tumbling overnight at 4 °C. The suspension was centrifuged (200,000 × g for 1 h), supernatant removed, and the concentration of Elugent reduced to 1% by adding an equal volume of buffer 2 without the detergent. The solution was applied to a Ni-nitrolotriacetic acid (Ni-NTA) agarose column (Qiagen) equilibrated with buffer 2 (containing 1% Elugent) and washed with the same buffer containing 50 mM imidazole to remove unbound proteins. Proteins were eluted from the column with buffer 2 containing 250 mM imidazole. Similarly, unglycosylated pilin was purified from DH5α transformed with pAMF15 (expressing pilin-His10) and used for the in vitro O-glycosylation assay.

E. coli CLM24 transformed with pAMF10 was used for expression and purification of PglL-His10 (Table 1). The same protocol described for purification of pilin was employed here by replacing detergent from Elugent (%1 w/v) with n-dodecyl-β-D-maltopyranoside (0.5% w/v) in the wash and elution steps.

**Western Blot Analysis**—Western blot analyses were carried out using standard methods. Anti-E. coli O2, O16 and Salmodyella serogroup B sera were purchased from Statens Serum Institut, Denmark. Anti-abequose monoclonal antibody, SE155-4 (11) was courteously provided by Dr. David Bundle, Department of Chemistry, University of Alberta. E. coli O9 polyclonal antibody was kindly provided by Dr. Chris Whitefield, Department of Molecular and Cellular Biology, University of Guelph. SM1 monoclonal anti-pilin antibody was a kind gift from Dr. Muntaaz Virji, Department of Cellular and Molecular Medicine, University of Bristol.

**Synthesis of FarPP-linked E. coli O86 Pentasaccharide Subunit**—The strategy used for the synthesis of the E. coli O86 O antigen subunit linked to the FarPP carrier as well as the characterization of the compound was based on previous work by Yi et al. (12) and is presented in supplemental data.

**In Vitro Protein Glycosylation**—E. coli CLM24 (waalmut) transformed with pACYCpglBmut were used for extraction of LLO. The LLO was extracted from CLM24, accumulating undecaprenyl-linked heptasaccharide of C. jejuni as described elsewhere (13). Alternatively, the synthetic substrate FarPP coupled to the O86 O antigen subunit (FarPP-O86) was used as a sugar donor for the in vitro glycosylation assay. This compound was synthesized using a combination of chemical and enzymatic steps starting from commercially available trans-trans farnesol (Sigma). The details of the synthesis and the characterization of the synthetic glycolipid by NMR and MS are provided in the supplemental data. For the in vitro assay, purified unglycosylated pilin (180 ng), PglL (1.2 μg), FarPP-O86 (150 μM), and 5 μl of 10× reaction buffer (500 mM Tris-HCl containing 1 M sucrose and 10 mM MnCl2, pH 7.5) were mixed. The volume was adjusted to 50 μl with water, and the mixture was incubated overnight at 30 °C. The concentration of FarPP-linked glycan was about 30–50 times higher than the Km determined for the C. jejuni N-OTase PglB (14).

**Nano LC-ESI MS and MS/MS Analyses**—In-gel digestion of purified pilin was carried out using proK or thermolysin. A hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer, Q-TOF Premier (Waters), equipped with a
NanoACQUITY Ultra performance liquid chromatography system (Waters) was used for MS and MS/MS analyses of the peptides as described elsewhere (6). Digestion of pilin with proK and thermolysin reproducibly release $^{63}$SAGVA$^{67}$ ($m/z$ 404.2$^+$) and $^{59}$LNHGEPGNTSAG$^{72}$ ($m/z$ 1453.5$^+$), respectively. Both peptides carry Ser-63, the O-glycosylation site (6).

### TABLE 1

| Strain      | Description                          | Ref./source                          |
|-------------|--------------------------------------|--------------------------------------|
| **E. coli** |                                      |                                      |
| W3110       | rpsL-1 N(rrnD-rrnE1)                  | Miguel Valvano (University of Western Ontario) |
| DH5a        | F-@lacZAM15 Δ(lacZYA-argF) U169 deoR recA1 endA1 hisD17 (r€, m€) gal- phoA supE44 l-thi 1 gyrA96 relA1 | Invitrogen                           |
| CLM24       | W3110, ΔLac, ligase                   | (17)                                 |
| JM109       | recA1, endA1, gryA96, thi-1, hisD17(K$^€$, m$^€$), supE44, relA1, Δ(lac-proAE)/F' | Promega                              |
| O2:NM       | Wild type                            | Carlton Gyles (U. Guelph), unpublished |
| E69         | O9a:K30:1H12                         | (30)                                 |
| CWG281      | E69 wzac30::aacC1, wzac2::aadA; Gm$^+$, Sp$^+$ | (30)                                 |
| **S. enterica** | Serovar Typhimium LT2, Δwzy, Strep$^+$ | Salmonella genetic stock centre, University of Calgary, Canada |
| M1224       | Serovar Typhi Ty2, Δwzy               | (31)                                 |
| **Plasmids** |                                      |                                      |
| pMLBAD      | Cloning and expression vector, arabinose-inducible, Tp$^+$ | (32)                                 |
| pEXT20      | Cloning and expression vector, IPTG-inducible, Amp$^R$ | (33)                                 |
| pEXT21      | Cloning and expression vector, IPTG-inducible, Sp$^R$ | (33)                                 |
| pMF19       | 0.9-kb PCR ampiclone containing the wbbL (rhamnosyltransferase) gene cloned in pEXT21, Sp$^R$ | (16)                                 |
| pACYCpgbmut | Carries C. jejuni protein glycosylation locus (pgb1) containing mutations W458A and D459A in PgbL, Cm$^R$ | (7)                                  |
| pAMF8       | Pgpl cloned in pEXT20, Amp$^R$       | This study                           |
| pAMF10      | C-10×His-tagged PglL clonned in pEXT20, Amp$^R$ | This study                           |
| pAMF15      | P1E$r^€$, cloned in pEXT20, Amp$^R$  | This study                           |
| pAMF16      | P1E$s^€$, cloned in pMLBAD, Tp$^+$   | This study                           |
| pPR1347     | pSC101-based cosmids expressing O antigen of Salmonella typhimurium, Km$^R$ | (21)                                 |
| pLPS2       | pLAF1-based cosmids carrying Pseudomonas aeruginosa PA103-expressing O11 O-antigen, Tet$^R$ | (20)                                 |

FIGURE 1. Structure of sugars transferred by PglL. Abe (abequose), 3-deoxy-o-fucose; DATDH, 2,4-diacetamido-2,4,6-trideoxyhexose; FucNAc, 4-acetamido-4,6-dideoxy-D-galactose; VioNAc, (N-acetylviosamine), 4-acetamido-4,6-dideoxyglucose.

**Neisseria O-OTase Substrate Specificity**
RESULTS

PglL Can Transfer Polysaccharides Containing β-(1→4) and α-(1→6) Sugars Linked to the Residue at Position 1—We have previously shown that PglL is able to transfer the C. jejuni hexasaccharide and E. coli O17 antigen from the UndP carrier to pilin in engineered E. coli cells (6). In these glycans, the two sugars proximal to the lipid carrier are attached via an α-(1→3)- or a β-(1→3)-linkage, respectively (Fig. 1). E. coli O2 and O16 antigens have a rhamnose β-(1→4)-linked and a Glc residue α-(1→6) linked to the GlcNAc at the reducing end, respectively. To further characterize PglL glycans specificity, transfer of these polysaccharides to pilin was tested (Fig. 1) (15, 16).

Plasmids encoding PglL and a His10-tagged version of N. meningitidis pilin were introduced into E. coli NM cells, which belong to the serotype O2 (Table 1). Pilin was purified from these cells by affinity chromatography, and analyzed by SDS-PAGE and Western blot (Fig. 2). The typical pattern of bands corresponding to pilin attached to O antigen was detected with Coomassie Blue staining (Fig. 2A, lane 1), a monoclonal anti-pilin antibody, and anti-O2-specific antiserum (Fig. 2B, lanes 3 and 5, respectively). All the bands disappeared after treatment with proteinase K (proK), indicating that they corresponded to pilin glycosylated with O2 polysaccharide with different degrees of polymerization, and not to contaminating LPS (Fig. 2B). For further analysis of the glycoprotein, a band with slightly reduced electrophoretic mobility compared with unglycosylated pilin was excised from the gel and digested with proK (Fig. 2A, lane 1). The peptides and glycopeptides resulting from the digestion were analyzed by nano liquid chromatography (LC) electrospray ionization (ESI) mass spectrometry (MS). This analysis confirmed the attachment of a single O2 unit bound to Ser-63 of pilin (Fig. 2C).

Similarly, PglL and pilin were expressed in the E. coli strain CLM24, which carries an insertion in the wbbL gene encoding for the rhamnosyltransferase required for O16 antigen biosynthesis. Synthesis of the O16 polysaccharide can be restored in CLM24 by expressing wbbL from plasmid pMF19 (16, 17). SDS-PAGE followed by Coomassie Blue staining (Fig. 2A, lane 2) or Western blot of pilin purified from CLM24 carrying pMF19 indicated that the O16 antigen was transferred to pilin by PglL (Fig. 2B, lanes 7 and 9). To determine the composition of the O16 subunit attached to pilin, the strain EVV11 was used. This strain lacks the Wzy polymerase, and therefore produces only a O16 subunit attached to pilin, the strain EVV11 was used. This strain lacks the Wzy polymerase, and therefore produces only a single O2 unit bound to Ser-63 of pilin (Fig. 2C).

MS/MS data across the elution profile were manually screened for the presence of the peptides resulted from the fragmentation of glycopeptides. The data analysis was done using MassLynx V4.1 (Waters).
The sugars previously transferred by PglL all have an acetamido group at position C-2. To analyze if the presence of this group is essential for PglL activity, we tested the capability of PglL for transferring glycans containing 2,4-diacetamido-2,4,6-trideoxyhexose (DATDH), glyceraldehyde, or HexNAc at the reducing end are substrates for PglL. 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of high molecular weight K30 polysaccharide, and shorter K30 oligosaccharides linked to lipid A-core in a LPS molecule (K30 polysaccharide, and shorter K30 oligosaccharides linked to lipid A-core in a LPS molecule) (23). Western blot analysis showed pilins glycosylated with O9 antigen (Fig. 5 (23)). Therefore both glycans in the absence of PglL did not show attachment of PG. Therefore, we concluded that under our experimental conditions, PglL is able to interfere with the PG biosynthetic machinery and transfer PG subunits, which are also assembled onto the UndPP carrier, to the pilin acceptor.

PglL Can Transfer a Peptidoglycan Subunit to Pilin—SDS-PAGE analysis of N. meningitidis pilin purified from the O16 strain expressing PglL showed a band with slightly lower electrophoretic mobility than unglycosylated pilin. This band (indicated with an asterisk in Fig. 2A, lane 2) was extracted from the gel, digested with proK, and analyzed by nano LC-ESI-MS and MS/MS. From MS/MS data analysis, two ions carrying the peptide $^{63}$SAGVA$^{67}$ were identified. The MS/MS spectra exhibited the characteristic peaks derived from the fragmentation of a PG subunit consisting of MurNAc, GlcNAc, and the tripeptide formed by Glu, diaminopimelic acid (DAP), and one (Fig. 6D) or two Ala residues (Fig. 6B) (24). We did not detect pilin modified with a complete PG pentapeptide subunit. PglL-dependent attachment of PG subunits to pilin was also identified by Western blot and MS analysis in N. meningitidis pilin purified from both wild type and a mutant Salmonella strain that does not synthesize O antigen (data not shown). Furthermore, MS analysis of purified pilin in absence of PglL did not show attachment of PG. Therefore, we reasoned that the substrate specificity of PglL lies in the lipid carrier. To test this hypothesis, we developed an in vitro glycosylation assay. Purified PglL and pilin were incubated in the presence of an extract containing the UndPP-linked C. jejuni heptasaccharide (Fig. 1), which was prepared as previously described (13). Western blot analysis using monoclonal anti-pilin antibody revealed an extra band only in the sample containing the three components of the mixture (Fig. 7B, left panel). This experiment suggested that the C. jejuni heptasaccharide was transferred from UndPP to pilin. We then tested if the structure of the polyenyl lipid carrier was important for PglL activity. For this, we used a combination of chemical and enzymatic steps to synthesize a glycolipid consisting of an O antigen subunit of E. coli O86 (Fig. 1) attached to farnesyl pyrophosphate (FarPP) (Fig. 7A). The strategy used for the synthesis and the characterization of the FarPP-O86 glycan by MS and NMR techniques is presented in supplemental data. FarPP contains only three isoprene units, compared with the eleven isoprene repeats of UndPP and therefore differs in olefin double bonds near the OH group, followed by three trans double bonds and a terminal double bond (Fig. 7A). Purified pilin and PglL were incubated with FarPP-linked E. coli O86 subunit, and glycosylation was analyzed by Western blot. A shift in the electro-
phoretic mobility of pilin was observed provided PglL and the FarPP-linked glycan were added to the mixture (Fig. 7B, right panel). The assay was carried out on a larger scale and the mixture was separated by SDS-PAGE and stained with Coomassie Blue. The band presumably corresponding to glycosylated pilin was excised from the gel, digested with thermolysin and analyzed by MS, which confirmed the presence of the *E. coli* O86 antigen repeating unit attached to pilin (Fig. 7C). Thus, in vitro, PglL can transfer the pentasaccharide from the FarPP carrier to pilin. Therefore, in addition to being able to transfer virtually any glycan, PglL seems remarkably unspecific regarding the nature of the lipid carrier.

**DISCUSSION**

In this work we have exploited the “natural library” of UndPP-bound glycans used for the synthesis of various bacterial glycoconjugates to investigate the glycan specificity of the *N. meningitidis* O-OTase PglL. These glycans contain diverse monosaccharides at the reducing end, including DATDH, GlcNAc, FucNAc, GalNAc, and Gal and the second sugar is attached to these via an $\alpha$-(1→3), $\beta$-(1→3), $\beta$-(1→4) or $\alpha$-(1→6) linkage (Fig. 1). We found that all of them are substrates for PglL (Fig. 1). Therefore, from this analysis, we could not determine any structural requirement for the transfer of the glycans from the UndPP carrier to pilin by PglL, suggesting that the glycan structure is irrelevant for PglL activity. Relaxed glycan specificity has been previously demonstrated for other bacterial O-Tases (17, 18, 25). However, the extent of glycan substrate promiscuity determined for PglL is unprecedented. For example, *C. jejuni* PglB requires an acetamido sugar at the reducing end and cannot transfer glycans containing $\beta$-(1→4) linkages between the first two sugars (18, 26). Interestingly, PglL was able to link a capsule-repeating unit to pilin. Transfer of type I capsule was found in a strain deficient in the capsule export machinery, which produces short K30 capsule chains, but does not assemble any high molecular weight capsule on the cell surface (23). We hypothesize that the export apparatus prevents access of PglL to the UndPP-bound capsule intermediates in the *E. coli* E69 strain (Table 1).

O-Tases and O antigen ligases share a conserved periplasmic domain. Although not studied in detail, the ligases are also able to transfer a variety of O antigens from the UndPP carrier to the lipid A core to form the LPS. Lateral gene transfer plays a key role in generating the diversity of the O-polysaccharides, which are commonly encoded in a single gene cluster (27). Ligases and O-Tases are usually not encoded in these clusters. It is tempting to speculate that the relaxed glycan specificity of
As PgL activity was independent of the carbohydrate moiety of its lipid-linked glycan substrate, we reasoned that the lipid component would be crucial for this enzyme. Surprisingly, in the in vitro assay, PgL was able to efficiently transfer a pentasaccharide from the FarPP carrier, which is composed only of three isoprene units. The different stereochemistry of both lipid carriers in the region near the pyrophosphate group might be expected to have a significant impact on the conformation of the substrate. This experiment demonstrated that the relaxed specificity of PgL applies not only to the glycan, but also to the lipid moiety. Thus, the experiments presented here delineate the region of the substrate recognized by PgL to the C1 and O1 of the monosaccharide at the reducing end of the glycanchain, the pyrophosphate linker, and the first few carbon atoms of the lipid. Thus, this region of the glycolipid must contain all the structural determinants for binding and catalysis. We hypothesize that the roles of the lipid carrier are the translocation of the oligosaccharide into the periplasm and the positioning of the pyrophosphate group and glycan in the periplasm, adjacent to PgL catalytic site. If this model can be also applied to other enzymes using UndPP-linked glycans as substrates, like the eukaryotic O'Tase complex, the O antigen ligases, and enzymes involved in PG synthesis remains to be tested. PgLB, the C. jejuni N-O'Tase seems to have more specific lipid requirements for activity (14). In the future, the in vitro glycosylation assay established here will be employed for the complete enzymatic characterization of PgL with a variety of substrates.

The low glycane and lipid specificity of PgL may be important for the application of this enzyme in biotechnology. It has been suggested that bacterial glycoproteins could constitute a new generation of conjugate vaccines (17, 29). PgL is able to transfer polysaccharides containing a hexose at the reducing end, as well as capsule-derived glycans. These features could be valuable for the generation of conjugate vaccines based on polysaccharides that contain a hexose at the reducing end (i.e. Streptococcus pneumoniae capsule). Moreover, several antigens are common to human and bacterial cells. The E. coli O86 antigen contains the human blood group B trisaccharide epitope (12). The glycoprotein obtained in our in vitro assay therefore contains a human epitope. Similarly, PgL could be exploited in vivo in engineered strains or in vitro using semi-synthetic LLOs for the production of O-glycosylated proteins and peptides containing other human epitopes that could be used as therapeutics against cancer and other diseases.

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O-OTases and ligases confer a selective advantage to bacteria when new genes that allow the generation of novel carbohydrates are horizontally acquired. This enables bacteria to readily modify their LPS and glycoproteins, facilitating the evasion of host immune defenses.

Unexpectedly, PgL transferred PG subunits to pilin. The PG structures attached to pilin contain a GlcNAc β-(1→4)-linked to a MurNAc at the reducing end, and the tetrapeptide Ala-Glu-DAP-Ala or the tripeptide Ala-Glu-DAP linked to the carboxylic acid group of MurNAc. We did not detect units containing the complete pentapeptide attached to pilin. Two types of enzymes could account for the structures containing tetra- or tri-peptides found linked to pilin. These are the D,D-carboxypeptidases that modulate PG rigidity by removing some of the terminal D-Ala residues from the pentapeptide, and the cytoplasmic L,D-carboxypeptidases that release the D-alanine from Ala-Glu-DAP-Ala (28). The finding that PG intermediates can be intercepted and transferred to proteins further emphasizes the substrate promiscuity of the PgL O'Tase. The ability of PgL to transfer PG repeating units to pilin could be exploited to study some of the still obscure aspects of PG biosynthesis. If this novel post-translational modification also occurs in N. meningitidis or in other bacteria known to carry PgL orthologs remains to be determined.
Neisseria O-OTase Substrate Specificity

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