Polyisoprenol Specificity in the *Campylobacter jejuni* N-Linked Glycosylation Pathway†

Mark M. Chen,‡ Eranthie Weerapana,‡ Ewa Ciepichal,§ Jacek Stupak,|| Christopher W. Reid,|| Ewa Swiezewska,§ and Barbara Imperiali*,‡

Departments of Chemistry and Biology, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Pawinskiego 5a, 02-106 Warsaw, Poland, and Institute for Biological Sciences, National Research Council of Canada, 100 Sussex Drive, Ottawa, Ontario K1A 0R6, Canada

Received September 28, 2007; Revised Manuscript Received November 7, 2007

ABSTRACT: *Campylobacter jejuni* contains a general N-linked glycosylation pathway in which a heptasaccharide is sequentially assembled onto a polyisoprenyl diphosphate carrier and subsequently transferred to the asparagine side chain of an acceptor protein. The enzymes in the pathway function at a membrane interface and have in common amphiphilic membrane-bound polyisoprenyl-linked substrates. Herein, we examine the potential role of the polyisoprene component of the substrates by investigating the relative substrate efficiencies of polyisoprene-modified analogues in individual steps of the pathway. Chemically defined substrates for PglC, PglJ, and PglB are prepared via semisynthetic approaches. The substrates included polyisoprenols of varying length, double bond geometry, and degree of saturation for probing the role of the hydrophobic polyisoprene in substrate specificity. Kinetic analysis reveals that all three enzymes exhibit distinct preferences for the polyisoprenyl carrier whereby *cis*-double bond geometry and α-unsaturation of the native substrate are important features, while the precise polyisoprene length may be less critical. These findings suggest that the polyisoprenyl carrier plays a specific role in the function of these enzymes beyond a purely physical role as a membrane anchor. These studies underscore the potential of the *C. jejuni* N-linked glycosylation pathway as a system for investigating the biochemical and biophysical roles of polyisoprenyl carriers common to prokaryotic and eukaryotic glycosylation.

The Gram-negative bacterium *Campylobacter jejuni* contains a general N-linked protein glycosylation (pgl)[1] pathway (*1*) with significant similarity to the corresponding eukaryotic pathway (*2*). In this pathway, a phosphoglycosyltransferase (PglC) and a series of glycosyltransferases (PglA, PglJ, PglH, and PglI) act sequentially to assemble a heptasaccharide on an undecaprenyl diphosphate (Und-PP) carrier anchored at the cytoplasmic face of the inner membrane (Figure 1) (*3*, *4*). The polyisoprenyl-linked heptasaccharide, GalNAc-α1,4-GalNAc-α1,4-(Glcβ1,3)-GalNAc-α1,4-GalNAc-α1,4-GalNAc-α1,3-Bac2,4diNAc (*5*), where Bac2,4diNAc is 2,4-diacetamido-2,4,6-trideoxyglucose, is then flipped by PglK to the periplasmic face of the membrane (*6*). In the periplasm, PglB, the bacterial oligosaccharyl transferase (OT), transfers the heptasaccharide to the asparagine side chain of acceptor proteins within a D/E-X_1-N-X_2-S/T consensus sequence, where X can be any amino acid except proline (*7*, *8*). This pathway parallels the N-linked glycosylation process in eukaryotes, where a tetradecasaccharide is sequentially assembled on a dolichyl diphosphate carrier prior to being transferred to protein (*9*). In contrast to eukaryotic N-linked glycosylation, the enzymes from *C. jejuni* can be readily overexpressed in functional form in *Escherichia coli* (*10*), which makes them more amenable to biochemical studies. Furthermore, each substrate in the pathway can be prepared using chemoenzymatic strategies (*11*) and purified to homogeneity, thereby enabling comparative kinetic studies of the function of the *C. jejuni* enzymes in vitro. The studies presented illustrate the use of *C. jejuni* as an ideal model system for investigation of the complex process of N-linked protein glycosylation and suggest the opportunity for understanding the fundamental roles played by the polyisoprene component of the substrates in the membrane-associated pathway.

The goal of this study is to address whether the polyisoprenyl carrier that is common to both eukaryotic and prokaryotic protein glycosylation plays a simple physical role as a hydrophobic membrane anchor or a more specific chemical role in substrate−enzyme interactions. The *C. jejuni* pathway integrates the functions of a series of enzymes that demonstrate a spectrum of membrane association properties. The current efforts focus on three enzymes from this pathway (Figure 2), PglC, PglJ, and PglB, which act on undecaprenyl-linked substrates at various stages of the N-linked glycosylation process and differ significantly in their degree of

† This work was supported by National Institutes of Health Grants GM68692 and GM39334 to B.I.

* To whom correspondence should be addressed. E-mail: imper@mit.edu. Phone: (617) 253-1838. Fax: (617) 452-2419.

‡ Massachusetts Institute of Technology.

§ Polish Academy of Sciences.

|| National Research Council of Canada.

[1] Abbreviations: pgl, protein glycosylation; Und, undecaprenol; PP, diphosphate; GalNAc, *N*-acetylgalactosamine; Glc, glucose; Bac2,4diNAc, 2,4-diacetamido bacillosamine; UDP, uridine diphosphate; OT, oligosaccharyl transferase; PSUP, pure solvent upper phase.

10.1021/bi701956x CCC: $37.00
Published on Web 11/23/2007

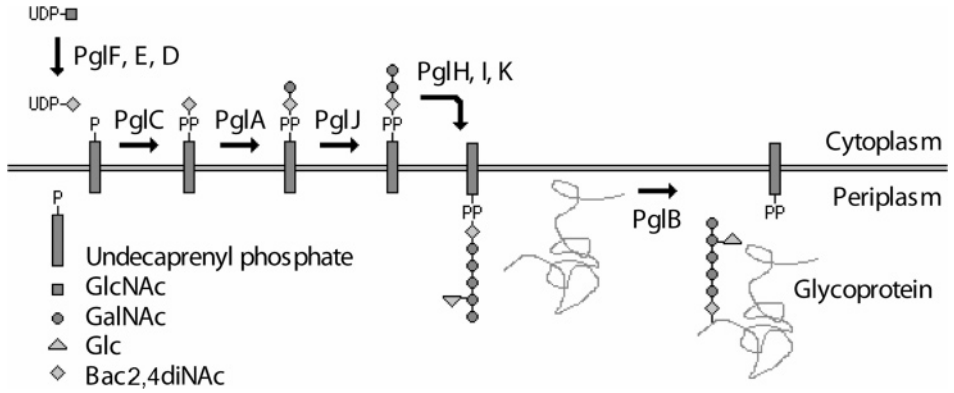

FIGURE 1: *C. jejuni* N-linked glycosylation pathway.

Undecaprenol	l = 3, m = 7, n = 0	Dolichol 17-19	l = 2, m = 13-15, n = 1
Prenol-9	l = 3, m = 5, n = 0	Solanesol	l = 8, m = 0, n = 0
Prenol-8	l = 2, m = 5, n = 0	Geranylgeraniol	l = 3, m = 0, n = 0
Dihydroprenol-11	l = 3, m = 6, n = 1		

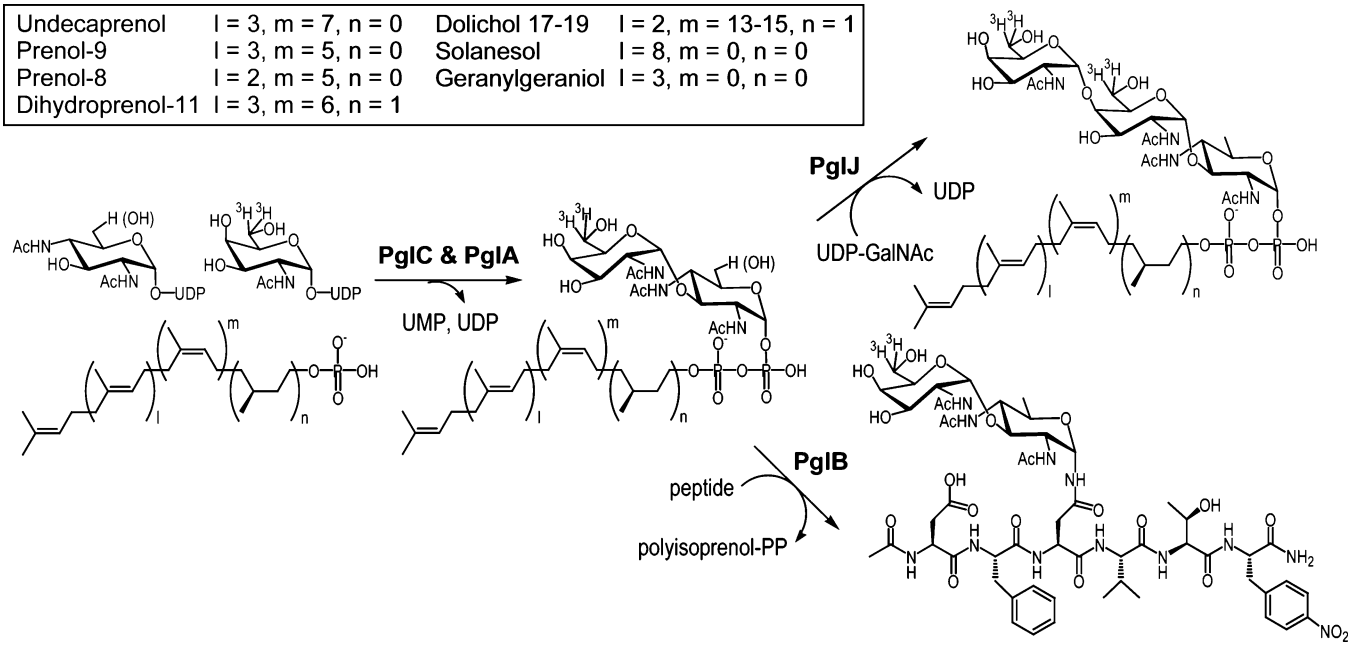

FIGURE 2: PglC and PglA coupled reaction for the synthesis of polyisoprenyl-PP-Bac2,4diNAc-GalNAc, followed by PglB transfer of the glycan to the peptide acceptor, and PgIJ reaction for the synthesis of polyisoprenyl-PP-Bac2,4diNAc-GalNAc-GalNAc. At position 6 of bacillosamine, H = Bac2,4diNAc and OH = 6-hydroxylBac2,4diNAc.

membrane association (Figure S4 of the Supporting Information). The phosphoglycosyltransferase PglC transfers a Bac2,4diNAc phosphate to undecaprenyl phosphate to afford the first membrane-associated intermediate in the pathway (*4*). PglC includes a single N-terminal transmembrane domain of 22 amino acids (TMHMM, ExPASY) and a large cytoplasmic domain. An intermediate step in the pathway is catalyzed by PglJ, a glycosyltransferase, which transfers a GalNAc to the Und-PP-Bac2,4diNAc-GalNAc disaccharide (*3*). PglJ is not predicted to include any transmembrane domains but is postulated to include an N-terminal hydrophobic domain which interacts with the bacterial membrane (Figure S4). The final step in the pathway is catalyzed by PglB, which transfers the completed heptasaccharide from the undecaprenyl diphosphate carrier to the protein acceptor (*7*). PglB comprises 10−12 predicted transmembrane domains (TMHMM, ExPASY) and a small C-terminal periplasmic domain. These three enzymes provide a panel of proteins that span the spectrum of membrane association, ranging from no transmembrane domains to multiple integral membrane regions. Additionally, the site of action of these enzymes varies, with PglC and PglB acting near the polyisoprenyl phosphate group proximal to the membrane and PglJ catalyzing glycosidic bond formation at a site away from the membrane at a distal saccharide unit. Using this system, the goal was to explore the extent of interaction of these enzymes with the polyisoprenyl moiety. If the native undecaprenyl carrier fulfills a specific role in substrate recognition, we would anticipate that modifying the structure of the undecaprenyl moiety to polyisoprenols differing in length and saturation would influence enzyme activity. In contrast, if the undecaprenyl moiety serves only a physical role as a hydrophobic anchor, then the introduction of structurally altered polyisoprenols of comparable hydrophobicity should have a negligible effect on activity. The diverse physical properties of the enzymes in the system also allow us to probe how the membrane association properties of each enzyme dictate the degree of interaction with the polyisoprenyl group in the substrates.

To address these questions, the native undecaprenyl-linked substrates for PglC, PglJ, and PglB were synthesized using chemoenzymatic approaches and purified to homogeneity. Additionally, a panel of "nonnatural" substrates for each enzyme was obtained by analogous approaches that incorporated polyisoprenols that differed in polyisoprene length, degree of saturation, and double bond geometry (Figure 3).

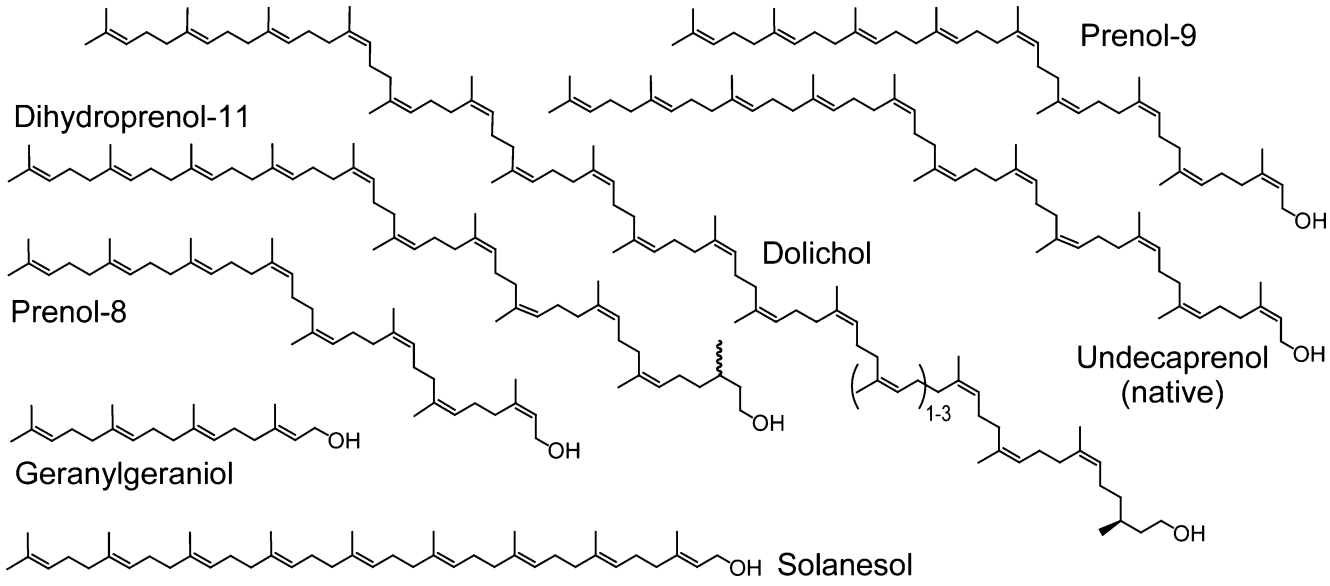

FIGURE 3: Polyisoprenols used in this study.

Kinetic analyses were performed using the various polyisoprenyl-linked substrates for each of the three enzymes (PglC, PglJ, and PglB). The studies revealed that the activity of each of the enzymes was significantly influenced by the structure of the polyisoprenol. Additionally, the results show remarkably common trends in the specificity for each of the enzymes studied regardless of the degree of membrane association of each protein.

Understanding the polyisoprenol recognition of these enzymes will afford valuable general insight into the determinants that mediate the interaction of membrane-bound enzymes with their lipophilic substrates. Furthermore, *C. jejuni* is known to be involved in human gastroenterological disorders (*12*, *13*), and evidence suggests that the N-linked glycans of this microorganism play a critical role in host adherence, invasion, and colonization which ultimately profoundly impact pathogenicity (*14*, *15*). Therefore, this understanding of substrate specificity may facilitate the development of therapeutics toward gastroenterological disorders caused by *C. jejuni* while also providing clues about manipulation of this pathway for future glycoengineering applications (*10*, *16*).

MATERIALS AND METHODS

UDP-Sugar Donors. UDP-Bac2,4diNAc was synthesized enzymatically from UDP-GlcNAc using the PglF, PglE, and PglD enzymes from the *C. jejuni* pathway as described previously (*17*). The preparation of UDP-6-hydroxylBac2,-4diNAc, which is a synthetic intermediate in the chemical synthesis of UDP-Bac2,4diNAc, was carried out as reported previously (*4*, *11*). Radiolabeled UDP-[^{3}H]GalNAc was purchased from American Radiolabeled Chemicals Inc.

Polyisoprenol Isolation and Phosphorylation. Solanesol and geranylgeraniol were purchased from Sigma-Aldrich. All remaining polyisoprenols were isolated from diverse plant sources (*18*) and purified from the natural extracts by chromatographic methods (*19*). Undecaprenol was from *Magnolia kobus* leaves, prenol-9 from the *Tilia cordata* leaves, and prenol-8 from the wood of *Betula veruccosa*, and prenols-17−19 were from *Ginkgo biloba* leaves. Hydrogenation of undecaprenol resulted in a racemic mixture of dihydroprenol-11 (*20*). Dolichols-17−19 were synthesized via asymmetric hydrogenation of prenols-17−19 (*21*). Undecaprenol, dolichols, solanesol, and geranylgeraniol were chemically phosphorylated using phosphoramidite chemistry (*22*, *23*). Prenol-9 and prenol-8 were phosphorylated with phosphoric acid and trichloroacetonitrile (*24*). Dihydroprenol-11 was phosphorylated with phosphorus oxychloride (*25*). The purity of the polyisoprenyl phosphates was evaluated by thin layer chromatography and determined to be >95% in all cases.

Enzyme Purification. The expression and purification of PglA (*3*), PglJ (*3*), PglC (*4*), and PglB (*7*) enzymes have been previously described in detail. For this study, both PglA and PglJ were further subjected to an additional chromatographic purification on a Sephacryl 300 gel filtration column (GE Healthcare). PglA and PglJ were evaluated to be 95% pure by SDS−PAGE, while PglC and PglB were used as a semipure membrane fraction necessary for preserving enzyme stability.

Enzymatic Synthesis of Radiolabeled Polyisoprenyl Diphosphate Disaccharide Donors. To an Eppendorf tube containing 50 nmol of the polyisoprenyl phosphate were added 6 μL of DMSO and 14 μL of 14.3% (v/v) Triton X-100. After vortexing and sonication (water bath) had been carried out, 42.5 μL of H_2O, 5.5 μL of 1 M Tris-acetate (pH 8.0), 2 μL of 1 M $MgCl_2$, 20 μL of 5 mM UDP-Bac2,4diNAc, 20 μL of 2.5 μM UDP-GalNAc (specific activity of 152 nCi nmol^{-1}), 70 μL of PglA, and 40 μL of PglC were added. The concentrations of enzyme stock solutions used were approximately 500 and 250 μg/mL for PglA (*3*) and PglC (*4*), respectively. Reaction mixtures were left shaking at room temperature for 3 h and then reactions quenched by the addition of 800 μL of a 2:1 chloroform/methanol mixture and 100 μL of pure solvent upper phase (PSUP; 3% chloroform, 49% methanol, and 48% water with 100 mM KCl). After being briefly vortexed, the layers were allowed to separate and the aqueous layer was removed. The organic layer was washed three times with 200 μL of PSUP, dried under a stream of nitrogen, and then redissolved in 100 μL

of a 4:1 chloroform/methanol mixture. The crude product was then purified by HPLC on a Microsorb 60 Si normal phase column (Varian), eluting with a linear gradient of 100% solvent C (4:1 chloroform/methanol mixture) to 100% solvent D [10:10:3 chloform/methanol/2 M ammonium acetate mixture (pH 7.2)] over 50 min at a flow rate of 1 mL/min. Fractions containing a significant amount of radiolabeled product were combined and immediately aliquoted, dried, and stored at −80 °C until they were needed.

PglJ Radioactivity Assay. To a tube containing a specified amount of dried radiolabeled polyisoprenyl diphosphate Bac2,4diNAc-[^{3}H]GalNAc were added 10 μL of DMSO and 7 μL of 1.43% (v/v) Triton X-100. After vortexing and sonication (water bath) had been carried out, 5 μL of 1 M Tris-acetate (pH 8.0), 1 μL of 1 M $MgCl_2$, 5 μL of 50 mM DTT, 62 μL of H_2O, and 5 μL of 500 μg/mL PglJ were added. For negative control reactions (not shown), the PglJ samples were replaced with 50 mM Tris-acetate buffer (pH 8.0). Four reactions containing different amounts of polyisoprenyl diphosphate disaccharides were carried out in parallel. The reactions were initiated by the addition of 5 μL of 1 μM radiolabeled UDP-GalNAc [specific activity of 19.8 μCi nmol^{-1}; K_M(UDP-GalNAc) $\sim$ 1 μM]. All assays were carried out at 22 $\pm$ 1 °C, and 17 μL aliquots were taken at 2, 4, 6, and 8 min. Reactions were quenched by addition to an Eppendorf tube containing 800 μL of a 2:1 chloroform/methanol mixture and 200 μL of PSUP (*26*). After being briefly vortexed, the layers were allowed to separate and the aqueous layer was removed. The organic layer was washed two times with 100 μL of PSUP and dried under a stream of nitrogen. The residue was then redissolved in 200 μL of DMSO by vigorous vortexing followed by the addition of 5 mL of EcoLite scintillation fluid (MP Biomedicals) and subjected to scintillation counting (5 min per sample). All assays were carried out in duplicate or better.

PglB and PglC/PglA Coupled Radioactivity Assay. Procedures for these two assays are based on previous reports (*4*, *27*) and are described in detail in the Supporting Information.

Mass Spectral Characterization of Unnatural Polyisoprenyl Diphosphate Disaccharide Substrates. Mass spectra were acquired using a 4000 QTrap mass spectrometer (Applied Biosystems/Sciex, Concord, ON). Capillary electrophoresis was performed using a Prince CE system (Prince Technologies, The Netherlands). The separation was obtained on a 90 cm length bare fused-silica capillary [365 μm (outside diameter) $\times$ 50 μm (inside diameter)] with CE-MS coupling using a liquid sheath-flow interface and a 2-propanol/methanol mixture (2:1) as the sheath liquid. Precursor ion scanning (*m*/*z* 79, PO_4) for the dolichol sample was conducted in the negative-ion mode, with a collision energy of 70 V. Samples were dissolved in a 3:1 chloroform/methanol mixture, and a CE buffer consisting of a 3:1 chloroform/methanol mixture with 30 mM ammonium acetate was used for all CE-MS experiments. Results are shown in the Supporting Information (Table 2).

RESULTS

The N-linked glycosylation pathway in *C. jejuni* comprises a series of enzymes that act at the surface of the plasma membrane on substrates anchored to the membrane via an undecaprenyl diphosphate moiety (*1*, *28*). To explore the determinants mediating interaction of enzymes with these membrane-associated substrates, the polyisoprenol specificity of these enzymes was investigated. Chemoenzymatic synthesis was applied to incorporate a series of polyisoprenols varying in length, degree of saturation, and double bond geometry into selected substrates involved in the *C. jejuni* N-linked glycosylation pathway. These polyisoprenols were isolated from natural sources and can be grouped into three structural classes (Figure 3). The first class includes the polyisoprenol that is naturally found in the *C. jejuni* substrates, undecaprenol, containing 11 unsaturated isoprene units, with *cis* (*Z*) double bond geometry at the α-isoprene unit and an array of *E* and *Z* isoprene units in the remainder of the structure. In addition to undecaprenol, this class of polyisoprenols included prenol-9 and prenol-8, which are very similar in saturation and geometry but contain fewer isoprene units. This class of polyisoprenols allows investigation of the effect of isoprene length on enzyme activity. In the second class are solanesol and geranylgeraniol, which are shorter in length than undecaprenol, including nine and four isoprene units, respectively. Importantly, these two polyisoprenols are exclusively *trans* (*E*) in their double bond geometry. These polyisoprenols enable investigation of the effect of double bond geometry on enzyme activity. In the final class are dihydroprenol-11 and the dolichols, which are the same length as undecaprenol and longer, respectively, but are both saturated at the α-isoprene unit. This final class of polyisoprenols allows the exploration of the effect of α-isoprene saturation on enzyme activity. The dolichols are particularly interesting because these constitute part of the native substrates in the eukaryotic N-linked glycosylation pathways (*9*).

The polyisoprenols were incorporated into substrates for enzymes PglC, PglJ, and PglB (Figure S4) using a chemoenzymatic approach. First, the polyisoprenyl alcohols were chemically phosphorylated (*22*, *23*) to afford the polyisoprenyl phosphates. These polyisoprenyl phosphates all proved to be substrates for PglC, the enzyme which transfers a Bac2,4diNAc phosphate to the polyisoprenol phosphate, providing the first membrane-associated intermediate in the pathway. Using PglC and the glycosyltransferase PglA from the *C. jejuni* pathway, radiolabeled polyisoprenyl diphosphate Bac2,4diNAc-GalNAc acceptors were enzymatically synthesized. These polyisoprenyl diphosphate-linked disaccharides are substrates for PglJ, which then transfers an additional GalNAc to afford polyisoprenyl diphosphate Bac2,4diNAc-$GalNAc_2$. Additionally, the polyisoprenyl phosphate-linked disaccharide can also act as a substrate for PglB. Although the native substrate for PglB is a polyisoprenyl diphosphate-linked heptasaccharide, previous studies have shown that PglB efficiently accepts a disaccharide substrate and facilitates the transfer to a peptide substrate in vitro (*7*). The use of the disaccharide donor together with a truncated peptide substrate provides a reliable assay for studying PglB activity.

The efficiencies of the enzymatic syntheses of the polyisoprenyl diphosphate-linked disaccharides, using a combination of PglC and PglA, varied among the polyisoprenyl phosphates and were optimized by the use of excess UDP-sugars. Typical yields were between 20 and 70% for a 50 nmol scale reaction, and the disaccharide products were

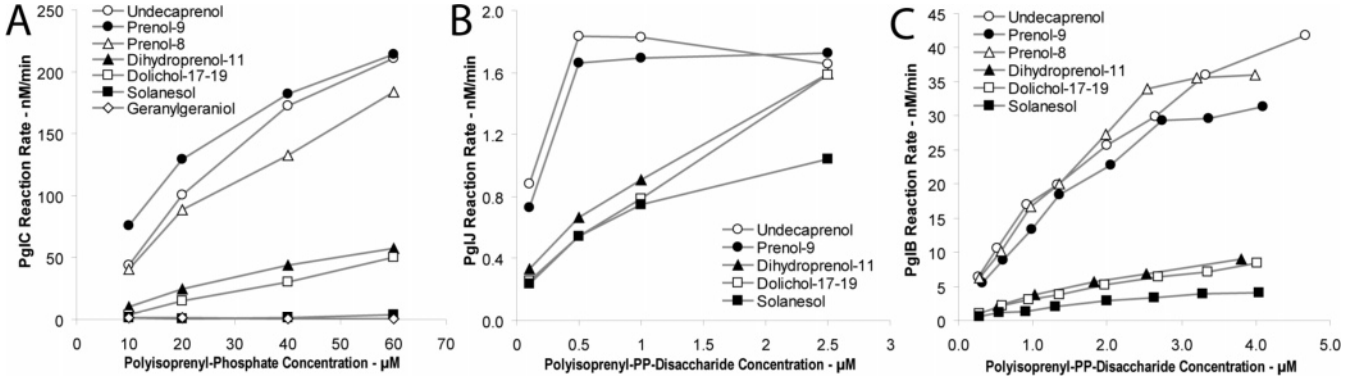

FIGURE 4: Comparison of enzyme reaction rates: (A) phosphoglycosyltransferases PglC and PglA coupled, (B) glycosyltransferase PglJ, and (C) oligosaccharyltransferase PglB. Radiolabeled disaccharide donor for PglB and acceptor for PglJ refers to polyisoprenyl-PP-Bac2,-4diNAc-[^{3}H]GalNAc. All assays were carried out in duplicate or better with a representative data set shown.

Table 1: Comparison of Polyisoprenyl Substrate Kinetic Parameters with Pgl Enzymes[a]

polyisoprene	common name	geometry	PglC initial rate (nM/min)	PglJ initial rate (nM/min)	PglB V_{max} (nM/min)	PglB K_M (μM)	PglB V_{max}/K_M ($\times 10^3$ min^{-1})
prenol-11	undecaprenol	*trans* 3, *cis* 7	210.6 ± 18.1	1.83 ± 0.20	63.2 ± 4.6	2.63 ± 0.36	24.0 ± 5.0
prenol-9		*trans* 3, *cis* 5	214.4 ± 24.4	1.66 ± 0.11	56.0 ± 5.0	2.97 ± 0.50	18.9 ± 4.8
prenol-8		*trans* 2, *cis* 5	183.8 ± 6.2	nd[b]	64.4 ± 6.9	2.77 ± 0.56	23.2 ± 7.2
dihydroprenol-11		*trans* 3, *cis* 6	57.5 ± 5.6	0.67 ± 0.05	24.4 ± 2.0	5.87 ± 0.82	4.2 ± 0.9
dihydroprenol-17−19	dolichol	*trans* 2, *cis* 13−15	50.6 ± 5.1	0.54 ± 0.03	16.6 ± 1.3	4.23 ± 0.53	3.9 ± 0.8
all-*trans* prenol-9	solanesol	*trans* 8	3.8 ± 2.0	0.54 ± 0.07	8.5 ± 0.8	4.06 ± 0.68	2.1 ± 0.6
all-*trans* prenol-4	geranylgeraniol	*trans* 3	0.6 ± 1.0	nd[b]	nd[b]	nd[b]	nd[b]

[a] PglC and PglJ initial rate comparisons are measured at subsaturating substrate concentrations of 60 μM polyisoprenyl phosphate and 0.5 μM polyisoprenyl PP-Bac2,4diNAc-GalNAc. PglB K_M values refer to those of the polyisoprenyl substrate at a saturating peptide acceptor concentration of 100 μM. $K_{M(peptide)} = 1.21$ μM. [b] Not determined.

separated from the unreacted polyisoprenyl phosphates using normal phase HPLC and characterized by ESI-MS (Table S2 in the Supporting Information). The ^{3}H radiolabel offers a convenient means of accurately quantifying both substrates and products in the low micromolar concentration range. These studies demonstrate the power of chemoenzymatic synthesis to yield complex polyisoprenyl diphosphate-linked glycan substrates for biochemical studies.

With the purified substrates in hand, enzymatic assays were performed to determine the specificity of PglC, PglJ, and PglB for each of the different polyisoprenyl phosphate-containing intermediates. To study PglB activity, a membrane fraction from *E. coli* in which PglB has been overexpressed (*7*) was used. The assay involved monitoring the transfer of radiolabeled glycan from the organic soluble polyisoprenyl diphosphate donor to the aqueous soluble peptide acceptor. The peptide acceptor used in this study was the hexapeptide Ac-DFNVT-(*p*NF)-NH_2, where *p*NF is *p*-nitrophenylalanine (*27*). The peptide was used at saturating concentrations, and the amount of polyisoprenyl diphosphate-linked glycan substrates was varied to afford the results shown in Figure 4C. The studies demonstrate that the transfer efficiency of disaccharide to peptide from the unnatural polyisoprenols, prenol-9 and prenol-8, is very similar to that of the native undecaprenol. These data suggest that PglB is not affected by the presence of a slightly truncated form of undecaprenol. The presence of dihydroprenol-11 and dolichols, both of which include a saturated terminal α-isoprene unit, results in a significant decrease in PglB activity, suggesting that PglB is highly sensitive to the degree of saturation proximal to the site of enzymatic action. Finally, the solanesol-linked substrate demonstrates very low activity with PglB, suggesting that the all-*trans* geometry disrupts interactions between PglB and the polyisoprenol moiety. Both K_M and V_{max} seem to be affected in the case of these nonnatural substrates (Table 1).

Similar to PglB, PglC was also used as a membrane fraction that yielded greater stability and activity relative to that of the purified form of the enzyme due to the stabilizing effect of components of the cell envelope fraction (*4*). UDP-6-hydroxylBac2,4diNAc was used as the glycosyl phosphate donor since it is known to be accepted by PglC with an efficiency similar to that of the native UDP-Bac2,4diNAc (*4*) and has the advantage of being more accessible by chemical synthesis. To assay for PglC activity, a previously described coupled assay was applied, which combined the activity of PglC with PglA, the next glycosyltransferase in the pathway. This coupled assay (*4*) was used due to the lack of radiolabeled UDP-Bac2,4diNAc to directly assay PglC activity prior to the report of UDP-Bac2,4diNAc biosynthesis using PglF, PglE, and PglD (*17*). A large excess of PglA was used to ensure that reaction rates reflect the rate-limiting PglC step (data not shown). The assay for PglC activity monitored the transfer of radioactivity from the aqueous soluble UDP-[^{3}H]GalNAc to the organic soluble polyisoprenyl diphosphate substrate. Figure 4A illustrates the efficiency of product formation using each of the different polyisoprenyl phosphates as substrates for PglC. Similar to the substrate selectivity demonstrated by PglB, PglC accepts prenol-8 and prenol-9 with efficiencies equal to that of the native undecaprenol-based substrate. Dolichols- and dihydroprenol-11-based substrates were accepted with much lower efficiency, suggesting that PglC is sensitive to the saturation of the α-isoprene unit. Finally, the all-*trans* solanesol- and geranylgeraniol-containing intermediates were extremely poor substrates for PglC, suggesting that the isoprene

geometry plays a critical role in substrate recognition.

PglJ is the peripheral membrane protein that transfers the third sugar in the heptasaccharide biosynthesis. The PglJ enzyme samples used in this study were solubilized using Triton X-100 detergent and purified to homogeneity using Ni-NTA affinity chromatography as previously described (*3*). The purified radiolabeled disaccharide substrates used in the PglB assay above were also used to assay PglJ activity. In this case, since the disaccharide acceptor is already radiolabeled, we measured the increase in radioactivity resulting from the transfer of an additional GalNAc residue from UDP-[^{3}H]GalNAc. Similar to PglB and PglC, PglJ accepts the prenol-9 substrate with efficiencies equal to that of the native undecaprenol-based substrate (Figure 4B). The nonnatural dihydroprenol-11, dolichols, and solanesol-based substrates are also accepted by PglJ but with efficiencies lower than those of the corresponding undecaprenol and prenol-9 substrates.

Our studies reveal that the three enzymes studied exhibit distinct preferences for the structural features of the polyisoprenols within their target substrates. The native undecaprenol-based substrates were well accepted in all cases. Additionally, polyisoprenyl carriers of shorter length, such as prenol-9 and prenol-8, displayed turnover similar to that of the native substrate, indicating that these enzymes do not have stringent specificity for the number of isoprene units; the C40 substrate is virtually identical to the C55 native substrate. Although solanesol is of the same length as prenol-9, its incorporation into substrates for each of the enzymes proved highly deleterious, most likely due to the unfavorable all-*trans* double bond geometry. Furthermore, dihydroprenols such as dolichols, being saturated at the α-isoprene position, were also poorly accepted in comparison to their unsaturated counterparts. This clear specificity with respect to the polyisoprenyl carrier, especially toward the features at α-isoprene units, is consistent with the hypothesis that membrane-associated hydrophobic proteins interact extensively with their lipophilic substrates. Intriguingly, there was no clear correlation between the stringency of the substrate specificity and the number of predicted transmembrane domains possessed by each enzyme.

We considered the possibility that the differences observed in V_{max} and K_M do not actually reflect differences in catalytic efficiencies or their binding affinities for the enzymes, but rather a function of substrate solubility as well as, in the case of PglB and PglC, enzyme accessibility which is limited by the permeability of the cell membrane. However, the concentration of detergent used under the assay conditions was sufficient for fully dissolving the cell membrane, and therefore, the interaction of the polyisoprenyl-linked substrates should not be influenced or limited by the membrane structure (*29*). Furthermore, the detergent is known to form mixed micelles with the substrate such that each polyisoprenyl-linked substrate should be effectively solubilized (*30*).

DISCUSSION

The recognition of membrane-bound substrates by membrane-associated enzymes is a poorly understood phenomenon due to the difficulties associated with studying these enzymes in vitro with discrete substrate analogues. In *C. jejuni*, the topological diversity of enzymes from the N-linked glycosylation pathway, and our ability to chemoenzymatically synthesize and purify selected substrates that varied in the identity of the polyisoprenyl carrier, provided a unique opportunity for addressing this question.

The three enzymes for the study differed in their degree of membrane association, with PglB having 10−12 predicted transmembrane domains, PglC having one, and PglJ being only a peripheral membrane protein (Figure S4). Despite this variation in structure, we have demonstrated that the three enzymes display similar specificity toward their respective polyisoprenol-based substrates. Detailed analysis of the substrate preferences of these enzymes reveals that the double bond geometry and α-unsaturation at the alcohol terminus of the polyisoprenols play a more important role in substrate recognition than the overall number of isoprene units (Figure 3). Even though the enzymes catalyze reactions that occur at the membrane surface, there appear to be important interactions within the hydrophobic component of the substrate that contribute to specificity.

These studies concur with a previous report on the N-linked glycosylation pathway of *Saccharomyces cerevisiae*, whereby polyisoprenyl specificity was found to be maintained by both Alg7 and the oligosaccharyl transferase (OT), the two eukaryotic enzymes that catalyze the analogous reactions to PglC and PglB (*31*). However, since Alg7 did not accept any α-unsaturated polyisoprenyl phosphates or the all-*trans* solanesyl phosphate, the corresponding OT substrates could not be accessed, and therefore, the study could not be pursued to the level of detail presented herein for the *C. jejuni* enzymes. To our knowledge, the specificity of OT toward α-unsaturated and all-*trans* polyisoprenols has not been previously tested in any other systems. A simplified generalization has been that α-unsaturated polyisoprenols are primarily found in bacteria and plants, while dihydropolyisoprenols are present in mammalian and yeast cells (*32*). Therefore, many examples of glycosyltransferases from eukaryotic and prokaryotic sources have been found to reflect this specificity toward their native type of polyisoprenyl carrier (*33*−*35*). In this case, the in vitro radioactivity-based assay allowed us to take advantage of the versatility of a chemoenzymatic synthesis to evaluate unnatural substrates for the bacterial OT. Interestingly, all three enzymes appear to be capable of accepting the eukaryotic dolichol-based carrier in vitro, albeit with lower efficiency.

In conclusion, the availability of purified substrates and purified enzymes of varying membrane affinity makes *C. jejuni* a viable model system for studying how membrane-associated enzymes interact with their lipophilic substrates. Our studies suggest that the polyisoprenyl carrier common to both eukaryotic and prokaryotic protein glycosylation plays a more specific chemical role in substrate−enzyme interactions rather than a simple physical role as a hydrophobic membrane anchor. The establishment of experimental approaches for investigating the role of the polyisoprenyl moiety in individual steps in the pgl pathway now sets the stages for studies aimed at evaluating the role of these essential biochemical components in the integrated action of the series of enzymes that ultimately results in the efficient biosynthesis of N-linked glycoproteins in prokaryotes.

ACKNOWLEDGMENT

We thank Dr. Nelson Olivier and Dr. Jerry Troutman for critical reading of the manuscript, Dr. Jerry Troutman for optimization of PglJ assay conditions, and Dr. Kerney Jebrell Glover for helpful discussion.

SUPPORTING INFORMATION AVAILABLE

Detailed assay protocols for PglC, PglA, and PglB; polyisoprenyl disaccharide mass spectral characterization; TMHMM, ExPASY transmembrane domain predictions; and raw assay data. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Szymanski, C. M., Yao, R., Ewing, C. P., Trust, T. J., and Guerry, P. (1999) Evidence for a system of general protein glycosylation in *Campylobacter jejuni*, *Mol. Microbiol. 32*, 1022−1030.
2. Weerapana, E., and Imperiali, B. (2006) Asparagine-linked protein glycosylation: From eukaryotic to prokaryotic systems, *Glycobiology 16*, 91−101.
3. Glover, K. J., Weerapana, E., and Imperiali, B. (2005) In vitro assembly of the undecaprenylpyrophosphate-linked heptasaccharide for prokaryotic N-linked glycosylation, *Proc. Natl. Acad. Sci. U.S.A. 102*, 14255−14259.
4. Glover, K. J., Weerapana, E., Chen, M. M., and Imperiali, B. (2006) Direct biochemical evidence for the utilization of UDP-bacillosamine by PglC, an essential glycosyl-1-phosphate transferase in the *Campylobacter jejuni* N-linked glycosylation pathway, *Biochemistry 45*, 5343−5350.
5. Young, N. M., Brisson, J. R., Kelly, J., Watson, D. C., Tessier, L., Lanthier, P. H., Jarrell, H. C., Cadotte, N., St Michael, F., Aberg, E., and Szymanski, C. M. (2002) Structure of the N-linked glycan present on multiple glycoproteins in the Gram-negative bacterium *Campylobacter jejuni*, *J. Biol. Chem. 277*, 42530−42539.
6. Alaimo, C., Catrein, I., Morf, L., Marolda, C. L., Callewaert, N., Valvano, M. A., Feldman, M. F., and Aebi, M. (2006) Two distinct but interchangeable mechanisms for flipping of lipid-linked oligosaccharides, *EMBO J. 25*, 967−976.
7. Glover, K. J., Weerapana, E., Numao, S., and Imperiali, B. (2005) Chemoenzymatic synthesis of glycopeptides with PglB, a bacterial oligosaccharyl transferase from *Campylobacter jejuni*, *Chem. Biol. 12*, 1311−1315.
8. Kowarik, M., Young, N. M., Numao, S., Schulz, B. L., Hug, I., Callewaert, N., Mills, D. C., Watson, D. C., Hernandez, M., Kelly, J. F., Wacker, M., and Aebi, M. (2006) Definition of the bacterial N-glycosylation site consensus sequence, *EMBO J. 25*, 1957−1966.
9. Lennarz, W. J. (1975) Lipid linked sugars in glycoprotein synthesis, *Science 188*, 986−991.
10. Wacker, M., Linton, D., Hitchen, P. G., Nita-Lazar, M., Haslam, S. M., North, S. J., Panico, M., Morris, H. R., Dell, A., Wren, B. W., and Aebi, M. (2002) N-Linked glycosylation in *Campylobacter jejuni* and its functional transfer into *E. coli*, *Science 298*, 1790−1793.
11. Weerapana, E., Glover, K. J., Chen, M. M., and Imperiali, B. (2005) Investigating bacterial N-linked glycosylation: Synthesis and glycosyl acceptor activity of the undecaprenyl pyrophosphate-linked bacillosamine, *J. Am. Chem. Soc. 127*, 13766−13767.
12. Wassenaar, T. M., and Blaser, M. J. (1999) Pathophysiology of *Campylobacter jejuni* infections of humans, *Microbes Infect. 1*, 1023−1033.
13. Nachamkin, I. (2002) Chronic effects of *Campylobacter* infection, *Microbes Infect. 4*, 399−403.
14. Szymanski, C. M., Burr, D. H., and Guerry, P. (2002) *Campylobacter* protein glycosylation affects host cell interactions, *Infect. Immun. 70*, 2242−2244.
15. Kelly, J., Jarrell, H., Millar, L., Tessier, L., Fiori, L. M., Lau, P. C., Allan, B., and Szymanski, C. M. (2006) Biosynthesis of the N-linked glycan in *Campylobacter jejuni* and addition onto protein through block transfer, *J. Bacteriol. 188*, 2427−2434.
16. Wacker, M., Feldman, M. F., Callewaert, N., Kowarik, M., Clarke, B. R., Pohl, N. L., Hernandez, M., Vines, E. D., Valvano, M. A., Whitfield, C., and Aebi, M. (2006) Substrate specificity of bacterial oligosaccharyltransferase suggests a common transfer mechanism for the bacterial and eukaryotic systems, *Proc. Natl. Acad. Sci. U.S.A. 103*, 7088−7093.
17. Olivier, N. B., Chen, M. M., Behr, J. R., and Imperiali, B. (2006) *In Vitro* Biosynthesis of UDP-N,N′-Diacetylbacillosamine by Enzymes of the *Campylobacter jejuni* General Protein Glycosylation System, *Biochemistry 45*, 13659−13669.
18. Wellburn, A. R., and Hemming, F. W. (1966) The occurrence and seasonal distribution of higher isoprenoid alcohols in the plant kingdom, *Phytochemistry 5*, 969−975.
19. Chojnacki, T., Jankowski, W., Mankowski, T., and Sasak, W. (1975) Preparative separation of naturally occurring mixtures of polyprenols on hydroxyalkoxypropyl-Sephadex, *Anal. Biochem. 69*, 114−119.
20. Mankowski, T., Jankowski, W., Chojnacki, T., and Franke, P. (1976) C55-Dolichol: Occurrence in pig liver and preparation by hydrogenation of plant undecaprenol, *Biochemistry 15*, 2125−2130.
21. Imperiali, B., and Zimmerman, J. W. (1988) Synthesis of Dolichols Via Asymmetric Hydrogenation of Plant Polyprenols, *Tetrahedron Lett. 29*, 5343−5344.
22. Branch, C. L., Burton, G., and Moss, S. F. (1999) An expedient synthesis of allylic polyprenyl phosphates, *Synth. Commun. 29*, 2639−2644.
23. Ye, X. Y., Lo, M. C., Brunner, L., Walker, D., Kahne, D., and Walker, S. (2001) Better substrates for bacterial transglycosylases, *J. Am. Chem. Soc. 123*, 3155−3156.
24. Danilov, L. L., Druzhinina, T. N., Kalinchuk, N. A., Maltsev, S. D., and Shibaev, V. N. (1989) Polyprenyl phosphates: Synthesis and structure-activity relationship for a biosynthetic system of *Salmonella anatum* O-specific polysaccharide, *Chem. Phys. Lipids 51*, 191−203.
25. Danilov, L. L., and Chojnacki, T. (1981) A Simple Procedure for Preparing Dolichyl Monophosphate by the Use of Poc13, *FEBS Lett. 131*, 310−312.
26. Osborn, J. M., Cynkin, M. A., Gilber, J. M., Muller, L., and Singh, M. (1972) Synthesis of bacterial O-antigens, *Methods Enzymol. 28*, 583−601.
27. Chen, M. M., Glover, K. J., and Imperiali, B. (2007) From Peptide to Protein: Comparative Analysis of the Substrate Specificity of N-Linked Glycosylation in *C. jejuni*, *Biochemistry 46*, 5579−5585.
28. Linton, D., Dorrell, N., Hitchen, P. G., Amber, S., Karlyshev, A. V., Morris, H. R., Dell, A., Valvano, M. A., Aebi, M., and Wren, B. W. (2005) Functional analysis of the *Campylobacter jejuni* N-linked protein glycosylation pathway, *Mol. Microbiol. 55*, 1695−1703.
29. Bergman, A., Mankowski, T., Chojnacki, T., De Luca, L. M., Peterson, E., and Dallner, G. (1978) Glycosyl transfer from nucleotide sugars to C85- and C55-polyprenyl and retinyl phosphates by microsomal subfractions and Golgi membranes of rat liver, *Biochem. J. 172*, 123−127.
30. Low, P., Peterson, E., Mizuno, M., Takigawa, T., Chojnacki, T., and Dallner, G. (1986) Effectivity of dolichyl phosphates with different chain lengths as acceptors of nucleotide activated sugars, *Biosci. Rep. 6*, 677−683.
31. Palamarczyk, G., Lehle, L., Mankowski, T., Chojnacki, T., and Tanner, W. (1980) Specificity of solubilized yeast glycosyl transferases for polyprenyl derivatives, *Eur. J. Biochem. 105*, 517−523.
32. Swiezewska, E., and Danikiewicz, W. (2005) Polyisoprenoids: Structure, biosynthesis and function, *Prog. Lipid Res. 44*, 235−258.
33. Rush, J. S., Shelling, J. G., Zingg, N. S., Ray, P. H., and Waechter, C. J. (1993) Mannosylphosphoryldolichol-mediated reactions in oligosaccharide-P-P-dolichol biosynthesis. Recognition of the saturated α-isoprene unit of the mannosyl donor by pig brain mannosyltransferases, *J. Biol. Chem. 268*, 13110−13117.
34. Mankowski, T., Sasak, W., Janczura, E., and Chojnacki, T. (1977) Specificity of polyprenyl phosphates in the in vitro formation of lipid-linked sugars, *Arch. Biochem. Biophys. 181*, 393−401.
35. Mankowski, T., Sasak, W., and Chojnacki, T. (1975) Hydrogenated polyprenol phosphates: Exogenous lipid acceptors of glucose from UDP glucose in rat liver microsomes, *Biochem. Biophys. Res. Commun. 65*, 1292−1297.

BI701956X