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†

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ABSTRACT: *Campylobacter jejuni* has a general N-linked glycosylation pathway, encoded by the *pgl* gene cluster. In *C. jejuni*, a heptasaccharide is transferred from an undecaprenyl pyrophosphate donor [GalNAc-α1,4-GalNAc-α1,4-(Glcβ1,3)-GalNAc-α1,4-GalNAc-α1,4-GalNAc-α1,3-Bac-α1-PP-undecaprenyl, where Bac is bacillosamine (2,4-diacetamido-2,4,6-trideoxyglucose)] to the asparagine side chain of target proteins at the Asn-X-Ser/Thr motif. In this study, we have cloned, overexpressed in *Escherichia coli*, and purified PglC, the glycosyl-1-phosphate transferase responsible for the first step in the biosynthesis of the undecaprenyl-linked heptasaccharide donor. In addition, we report the first synthetic route to uridine 5′-diphosphobacillosamine. Using the uridine 5′-diphosphobacillosamine and undecaprenyl phosphate, we demonstrate the ability of PglC to produce undecaprenyl pyrophosphate bacillosamine using radiolabeled HPLC and mass spectral analysis. In addition, we revealed that PglC does not accept uridine 5′-diphospho-*N*-acetylglucosamine or uridine 5′-diphospho-*N*-acetylgalactosamine as substrates but will accept uridine 5′-diphospho-6-hydroxybacillosamine, an analogue of bacillosamine that retains the C-6 hydroxyl functionality from the biosynthetic precursor. The in vitro characterization of PglC as a bacillosamine 1-phosphoryl transferase provides direct evidence for the early steps in the *C. jejuni* N-linked glycosylation pathway, and the coupling of PglC with the latter glycosyltransferases (PglA, PglJ, PglH, and PglI) allows for the "one-pot" chemoenzymatic synthesis of the undecaprenyl pyrophosphate heptasaccharide donor.

Recent studies have revealed the presence of N-linked glycosylation in the bacterial domain. Specifically, the Gram-negative bacterium *Campylobacter jejuni* has a general N-linked glycosylation pathway encoded by the *pgl* gene locus (*1*−*4*). *C. jejuni*[1] has been implicated in a variety of gastroenterological disorders, and evidence suggests that these N-linked glycans play a major role in host adherence, invasion, and colonization (*5*). Similar to the analogous process in eukaryotes, N-linked glycosylation involves the transfer of a preassembled glycan from a polyisoprenyl pyrophosphate-linked donor onto the asparagine side chain at an Asn-Xaa-Ser/Thr motif. In *C. jejuni*, the glycan donor is an undecaprenyl pyrophosphate (Und-PP) linked heptasaccharide, GalNAc-α1,4-GalNAc-α1,4-(Glcβ1,3)-GalNAc-α1,4-GalNAc-α1,4-GalNAc-α1,3-Bac-α1-PP-Und, where Bac is the unusual sugar, bacillosamine (2,4-diacetamido-2,4,6-trideoxyglucose) (*6*) (Figure 1).

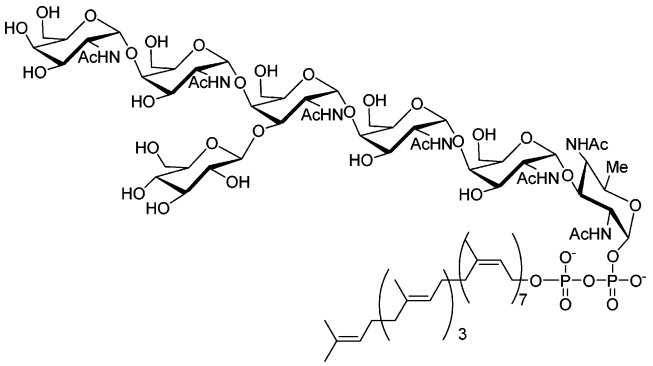

FIGURE 1: Chemical structure of the undecaprenyl pyrophosphate-linked heptasaccharide.

In the *pgl* gene locus, bioinformatic analysis has identified five glycosyltransferases (PglC, PglA, PglJ, PglH, and PglI) (*3*, *7*). Mutational and in vitro analyses of these glycosyltransferases have established their involvement in the assembly of the undecaprenyl pyrophosphate-linked heptasaccharide. PglA adds a GalNAc residue to undecaprenyl pyrophosphate bacillosamine (Und-PP-Bac) to form the disaccharide, PglJ adds the second GalNAc residue to form the trisaccharide, PglH adds the next three GalNAc residues to afford the hexasaccharide, and finally PglI adds the single branching glucose residue (*8*, *9*). However, the role of PglC, a putative glycosyl-1-phosphate transferase, which is proposed to be involved in the synthesis of the first Und-PP-glycan intermediate, has not been fully determined.

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[1] Abbreviations: *C. jejuni*, *Campylobacter jejuni*; UDP-Bac, uridine 5′-diphosphobacillosamine; UDP-GlcNAc, uridine 5′-diphospho-*N*-acetylglucosamine; UDP-GalNAc, uridine 5′-diphospho-*N*-acetylgalactosamine; Und-P, undecaprenyl phosphate; Glc, glucose; HexNAc, *N*-acetylhexosamine; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

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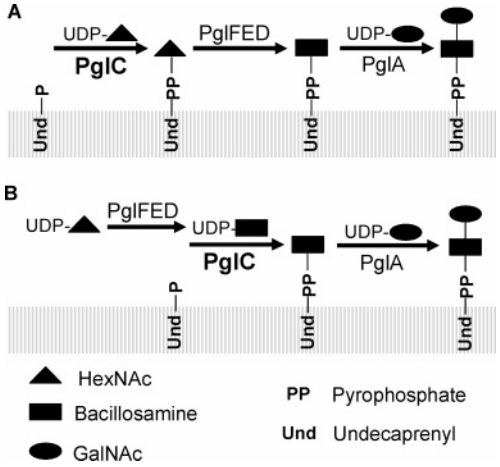


FIGURE 2: Two proposed pathways for the early steps in the Pgl pathway. (A) PglC accepts UDP-Bac. (B) PglC accepts UDP-GlcNAc/GalNAc.

In a series of key experiments, Aebi and co-workers were able to observe N-linked glycosylation in *Escherichia coli* when they introduced the *pgl* locus. Interestingly, they observed two distinct heptasaccharide structures on target glycoproteins by mass spectral analysis. One saccharide had bacillosamine as the first sugar while the other had GalNAc/GlcNAc (*N*-acetylgalactosamine/*N*-acetylglucosamine) as the first sugar (*2*). The observation of the GalNAc- or GlcNAc-containing heptasaccharide led to the hypothesis that an isoprenyl-linked GalNAc or GlcNAc represented a precursor to Und-PP-Bac (Figure 2A). Therefore, a model of the *pgl* pathway in which PglC utilized UDP-GlcNAc or UDP-GalNAc to form the membrane-associated intermediate, Und-PP-GlcNAc or GalNAc, was generated. The Und-PP-GlcNAc- or GalNAc-containing precursor would then be modified sequentially on the isoprenoid carrier by a dehydratase (PglF), an aminotransferase (PglE), and a *N*-acetyltransferase (PglD) to afford Und-PP-Bac, which can be elaborated by PglA to form Und-PP-Bac-GalNAc (*2*, *3*, *7*). The remaining glycosyltranferases (PglJ, PglH, and PglI) would then complete the heptasaccharide assembly.

Recent studies using the glycosylation-competent *E. coli* system revealed that when the native *E. coli* enzyme WecA, a GlcNAc-1-P-transferase, was deleted, only the bacillosamine heptasaccharide was observed on target proteins, suggesting that Und-PP-GlcNAc/GalNAc was not a direct precursor of Und-PP-Bac (*9*). It was then postulated that PglC directly transfers a bacillosamine to the Und-P carrier (*9*). This resulted in a revised model of the early steps in the *pgl* pathway (Figure 2B): UDP-GlcNAc is converted sequentially by PglF, PglE, and PglD to afford uridine 5′-diphosphobacillosamine (UDP-Bac). UDP-Bac is then utilized by PglC to afford Und-PP-Bac, which can be elaborated by PglA, PglJ, PglH, and PglI (*4*, *9*, *10*). Although these experiments inferred that PglC utilizes UDP-Bac, direct biochemical evidence for PglC function has yet to be provided. This can be best achieved by observing the function of PglC in vitro using purified PglC and the putative UDP-Bac, UDP-GlcNAc, or UDP-GalNAc substrates. PglC is a 23 kDa protein, which is predicted to have a single transmembrane-spanning domain at the amino terminus (TMHMM, ExPASY). Similar to many of the enzymes from the *pgl* gene cluster, PglC shares significant homology with proteins involved in O-antigen synthesis (*11*). There are two distinct classes of bacterial glycophosphoryltransferases. One class encodes a protein that is highly hydrophobic with no significant soluble domains (e.g., WecA, WbpL, and MraY) and is generally associated with the utilization of nucleoside 5′-diphosphate-linked HexNAc substrates. The other class encodes a protein that has a single transmembrane domain and a significant soluble domain. PglC is homologous to the latter class that includes Rfbp, a galactosyl-1-phosphoryltransferase involved in the first step of O-antigen synthesis in *Salmonella enterica* (*12*). In contrast, PglC bears no significant homology to yeast Alg7p that catalyzes the analogous process in the eukaryotic system which utilizes dolichyl phosphate and UDP-GlcNAc as substrates.

Our interest lies in defining the precise role of PglC in order to complete the picture of the *pgl* pathway. In this work we have overexpressed and purified PglC in *E. coli* and demonstrated its action on chemically synthesized UDP-Bac using a coupled radioactive assay in tandem with HPLC and mass spectral analysis. Furthermore, we have shown that PglC does not accept UDP-GalNAc or UDP-GlcNAc, rendering the first model for the pathway unlikely (Figure 2A). Additionally, we have demonstrated that PglC will accept UDP-6-hydroxybacillosamine, suggesting that C-6 deoxygenation in bacillosamine is not a major determinant for PglC. The enzyme is compatible with a variety of detergents including Triton X-100, NP-40, dihexanoylphosphatidylcholine, CHAPS, and CHAPSO. Lastly, a single reaction containing all five glycosyltransferases (PglC, PglA, PglJ, PglH, and PglI) resulted in the efficient formation of Und-PP-Bac-$(GalNAc)_6$-Glc from Und-P and UDP sugar donors. This demonstrates the in vitro reconstitution of sequential steps in the Pgl pathway.

The biochemical validation of PglC function adds significantly to our understanding of the heptasaccharide assembly process and sets the stage for the use of PglC to create native and novel substrates for the bacterial N-linked glycosylation machinery.

MATERIALS AND METHODS

Cloning. PglC was amplified by PCR from *C. jejuni* genomic DNA (ATCC 700819D, designation NCTC 11168) with the oligonucleotides PglC *Bam*HI (CGCGGATCCATGTATGAAAAAGTTTTTAAAAGAATTTTTG) and PglC *Xho*I (CCGCTCGAGGTTCTTGCCATTAAATTTCTCTGTTGTAAC) using Pfu Turbo (Stratagene) polymerase. The PCR product was digested with *Bam*HI and *Xho*I and cloned into the same sites of vector pET-24a (Novagen). The final gene construct encoded a protein product with an N-terminal T7•Tag and a C-terminal His tag.

Expression. pET(PglC)-24a was transformed into BL21-CodonPlus (DE3)-RIL *E. coli* cells (Stratagene). Starting from a 5 mL overnight culture, PglC was grown at 37 °C in LB broth to an OD_{600} of 0.6−0.8. At that point, the temperature was reduced to 16 °C, and protein production was induced by the addition of IPTG (1 mM). After 24 h, the cells were harvested by centrifugation (5000*g*), washed with 0.9% NaCl, recentrifuged (5000*g*), and frozen at −80 °C until needed.

Preparation of the PglC Membrane Fraction. All steps were performed at 4 °C. The cell pellet of PglC (from 1 L

of original culture volume) was thawed and resuspended in 5% of the original culture volume in buffer M [50 mM Tris−acetate (pH 8.0), 1 mM EDTA]. The cells were then subjected to sonication, unbroken cells were removed by centrifugation at 5697*g* for 15 min, and the membrane fraction was collected by centrifugation at 142414*g* for 60 min. The pellet was washed once with buffer M, centrifuged again, and resuspended in 0.25% of the original culture volume in buffer M. The final suspension was aliquoted and stored at −80 °C.

Purification of PglC by Ni^{2+} Affinity Chromatography. All steps were performed at 4 °C. Bacterial membranes prepared using the above protocol were resuspended in 0.5% of the original culture volume in buffer L [50 mM Tris−acetate (pH 8.0), 20 mM imidazole, 2% Triton X-100]. After being gently mixed for 10 min, the mixture was centrifuged at 142414*g* for 60 min, and the supernatant was loaded onto a column containing Ni-NTA agarose equilibrated with buffer L. After being washed with 10 column volumes of buffer W [50 mM Tris−acetate (pH 8.0), 45 mM imidazole], the purified protein was eluted with buffer E [50 mM Tris−acetate (pH 8.0), 250 mM imidazole]. Fractions containing at least 500 μg/mL protein were used for enzyme assays, and no further purification of the proteins was undertaken.

*Synthesis of UDP-Bac (Uridin-5′-yl 2,4-Diacetamido-2,4,6-trideoxy-α-D-glucopyranosyl Diphosphate) (**2**).* The benzoyl-protected bacillosamine phosphate derivative **1** (10.0 mg, 0.02 mmol, 1.0 equiv) was coevaporated several times with dry pyridine. To this dried compound was added 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidinium uridine 5′-monophosphomorpholidate (17.9 mg, 0.03 mmol, 1.3 equiv), and the mixture was coevaporated twice with dry pyridine and dried under vacuum. The solid was then dissolved in 0.5 mL of dry pyridine, 1*H*-tetrazole (0.04 mmol, 2.0 equiv, 3.0 mg) was added, and the solution was stirred at room temperature for 3 days. The solvent was removed under vacuum and the residue dissolved in water and purified by RP-HPLC (C18) [0.05 M triethylammonium bicarbonate (TEAB) buffer; gradient 4−18% CH_3CN over 30 min; flow rate, 1 mL/min; t_R = 28.7 min]. Benzoyl-protected UDP-Bac (9.0 mg, 50%) was obtained as a white solid. ^{1}H NMR (400 MHz, CD_3OD): δ 1.25 (d, J = 6.8 Hz, 3H), 1.82 (s, 3H), 1.94 (s, 3H), 4.03 (t_{app}, J = 10.3 Hz, 1H), 4.22 (m, 1H), 4.28 (m, 2H), 4.38 (m, 1H), 4.41 (m, 2H), 4.58 (t_{app}, J = 4.2 Hz, 1H), 5.40 (t_{app}, J = 10.4 Hz, 1H), 5.75 (dd, J = 7.3, 3.1 Hz, 1H), 5.85 (d, J = 8.1 Hz, 1H), 6.05 (d, J = 5.3 Hz, 1H), 7.45 (t, J = 7.7 Hz, 2H), 7.58 (t, J = 7.6 Hz, 1H), 7.95 (d, J = 7.1 Hz, 2H), 8.18 (d, J = 8.1 Hz, 1H). ^{31}P NMR (162 MHz, CD_3OD): δ −10.8, −13.0. LRMS calculated for $[C_{26}H_{33}N_4O_{17}P_2 - H]^-$ requires *m*/*z* 734.5. Found 734.4 (ESI^-). The protected UDP-Bac analogue from above (5.0 mg, 0.007 mmol, 1.0 equiv) was dissolved in MeOH (0.5 mL). Sodium methoxide (1.0 μL) (25 wt % in MeOH) was added and the mixture stirred at room temperature for 30 min. The solvent was removed under vacuum and the residue dissolved in water and purified by RP-HPLC (C18) [0.05 M triethylammonium bicarbonate (TEAB) buffer; 1.0% CH_3CN; flow rate, 1.0 mL/min; t_R = 15.0 min]. Compound **2** was obtained as a white solid (3.0 mg, 68%). LRMS calculated for $[C_{19}H_{29}N_4O_{16}P_2 - H]^-$ requires *m*/*z* 630.4. Found 630.5 (ESI^-).

Synthesis of UDP-6-hydroxybacillosamine (Uridin-5′-yl 2,4-Diacetamido-2,4-dideoxy-α-D-glucopyranosyl Diphosphate). The procedure used for the synthesis of UDP-Bac (**2**) was followed using dibenzoyl-protected 6-hydroxybacillosamine phosphate (12.6 mg, 0.02 mmol, 1.0 equiv), 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidinium uridine 5′-monophosphomorpholidate (17.9 mg, 0.03 mmol, 1.3 equiv), and 1*H*-tetrazole (0.04 mmol, 2.0 equiv, 3.0 mg) in pyridine (0.5 mL) to afford dibenzoyl-protected UDP-6-hydroxybacillosamine (11.6 mg, 50%, t_R = 27.5 min) as a white solid. ^{1}H NMR (400 MHz, CD_3OD): δ 1.73 (s, 3H), 1.88 (s, 3H), 4.17 (m, 1H), 4.25 (m, 1H), 4.36 (m, 3H), 4.55 (m, 9H), 5.46 (t_{app}, J = 1.0 Hz, 1H), 5.70 (dd, J = 3.2, 7.3, 1H), 5.80 (d, J = 8.1 Hz, 1H), 5.99 (d, J = 5.2 Hz, 1H), 7.39 (t, J = 7.7 Hz, 2H), 7.47 (t, J = 7.6 Hz, 2H), 7.55 (m, 2H), 7.91 (d, J = 7.8 Hz, 1H), 8.08 (d, J = 7.8 Hz, 1H), 8.12 (d, J = 8.2 Hz, 1H). ^{31}P NMR (162 MHz, CD_3OD): δ −10.9, −13.2. LRMS calculated for $[C_{33}H_{37}N_4O_{19}P_2 - H]^-$ requires *m*/*z* 854.2. Found 854.3 (ESI^-). The protected UDP-6-hydroxybacillosamine analogue from above (5.0 mg, 0.005 mmol, 1.0 equiv) was deprotected with sodium methoxide following a procedure identical to the deprotection of UDP-Bac. The final deprotected UDP-6-hydroxybacillosamine was obtained as a white solid (3.1 mg, 68%, t_R = 17.0 min). LRMS calculated for $[C_{19}H_2N_4O_{17}P_2 - H]^-$ requires *m*/*z* 646.1. Found 646.5 (ESI^-).

Radioactive Enzyme Assay Using UDP-Bac or UDP-6-hydroxybacillosamine. To a tube containing 0.06 mg of dried Und-P were added 3 μL of DMSO and 7 μL of 14.3% (v/v) Triton X-100. After vortexing and sonication (water bath), 70 μL of H_2O, 4.5 μL of 1 M Tris−acetate, pH 8.0, 1 μL of 1 M $MgCl_2$, 5 μL of PglC, and 5 μL of PglA were added. For the two control reactions (Figure 4), the PglA or PglC samples were replaced with buffer E. The reaction was initiated by the addition of 4.5 μL of a 53 μM solution of UDP-Bac or UDP-6-hydroxybacillosamine and 22 μM uridine disphosphate *N*-acetyl-D-galactosamine-[6-^{3}H] (UDP-[^{3}H]GalNAc) (2.02 nCi/nmol). Reactions were run at room temperature, and 20 μL aliquots were taken at 1.5, 3, 4.5, and 6 min. Reactions were quenched by addition to a tube containing 400 μL of 2:1 chloroform:methanol and 200 μL of pure solvent upper phase [PSUP (15 mL of chloroform/240 mL of methanol/1.83 g of potassium chloride in 235 mL of water)]. After being vortexed briefly the layers were allowed to separate, and the aqueous layer was removed. The organic layer was washed two times with 400 μL of PSUP and dried under a stream of nitrogen. The residue was redissolved in 200 μL of Solvable (Perkin-Elmer) by vigorous vortexing followed by the addition of 5 mL of Formula 989 scitillation fluid (Perkin-Elmer). The tubes were allowed to rest for 1 h and counted in a scintillation counter (5 min per sample).

Radioactive Enzyme Assay Using UDP-GlcNAc or UDP-GalNAc. To a tube containing 0.06 mg of dried Und-P were added 3 μL of DMSO and 7 μL of 14.3% (v/v) Triton X-100. After vortexing and sonication (water bath), 70 μL of H_2O, 4.5 μL of 1 M Tris−acetate, pH 8.0, 1 μL of 1 M $MgCl_2$, and 5 μL of PglC were added. The reaction was initiated by the addition of 4.5 μL of a 53 μM solution of UDP-[^{3}H]-GalNAc or UDP-[^{3}H]GlcNAc (2.02 nCi/nmol). Reactions were processed and analyzed as described above.

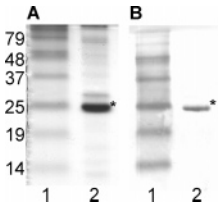


FIGURE 3: Ni-NTA-purified PglC. (A) Coomassie-stained polyacrylamide gel. (B) Anti-T7·Tag Western blot analysis: molecular weight markers (lane 1); PglC (lane 2).

Enzyme Assay for HPLC and MALDI-MS Analysis. To a tube containing 0.06 mg of dried Und-P were added 3 μL of DMSO and 7 μL of 14.3% (v/v) Triton X-100. After vortexing and sonication (water bath), 15 μL of H_2O, 4 μL of 1 M Tris−acetate, pH 8.0, 1 μL of 1 M $MgCl_2$, and 50 μL of the PglC membrane fraction were added. The reaction was run at room temperature for 120 min and quenched by addition to a tube containing 800 μL of 2:1 chloroform:methanol and 160 μL of PSUP. After vortexing for 20 s, the tubes were centrifuged briefly, and the organic layer (bottom) containing product was removed and dried.

HPLC and MALDI-MS Analysis. The saccharide sample was hydrolyzed by dissolving the dried sample in 1-propanol/2 M TFA (1:1), heating to 50 °C for 15 min, and then evaporating to dryness. The hydrolyzed sugars were then labeled with 2-aminobenzamide. The labeling reagent includes a solution of 2-aminobenzamide (5 mg) in 100 μL of acetic acid/DMSO (1:2.3). This solution was added to 6 mg of sodium cyanoborohydride, and aliquots of 5 μL of the reagent were added to dried samples of hydrolyzed, desalted glycans and heated to 60 °C for 2−4 h. Postlabeling cleanup was accomplished using GlykoClean S cartridges (ProZyme Inc.) according to the manufacturer's instructions. The labeled glycans were separated on a normal phase analytical HPLC column (GlykoSepN; ProZyme, Inc.) using 50 mM ammonium formate, pH 4.4 (solvent A), and acetonitrile (solvent B) as eluents. A gradient of 20−52% solvent A over 80 min was used at a flow rate of 0.4 mL/min. The peaks were detected using a fluorescence detector (λ_{ex} = 330 nm, λ_{em} = 420 nm) and collected and characterized with MALDI-MS using a matrix composed of DHB (2,5-dihydrobenzoic acid) in acetonitrile/water with Nafion perfluorinated resin (Aldrich) and trifluoroacetic acid as additives.

Detergent Screen of PglC. Solutions [5% (w/v)] of the following detergents were prepared: CHAPS, dihexanoylphosphatidylcholine, NP-40, CHAPSO, dodecyl maltoside, Triton X-100, and LDAO. To a tube containing 0.06 mg of dried Und-P was added 10 μL of a 5% detergent solution. After vortexing and sonication (water bath), 41 μL of H_2O, 3 μL of 1 M Tris−acetate, pH 8.0, 1 μL of 1 M $MgCl_2$, and 40 μL of PglC membrane fraction were added. The reaction was initiated by the addition of 5 μL of a solution containing 24 μM UDP-Bac and 20 μM UDP-[^{3}H]GalNAc (0.27 nCi/nmol). Reactions were quenched by addition to a tube containing 1.6 mL of 2:1 chloroform:methanol and 160 μL of PSUP. After being vortexed briefly the layers were allowed to separate, and the aqueous layer was removed. The organic layer was washed two more times with 160 μL of PSUP and dried over a stream of nitrogen. The residue was redissolved in 200 μL of Solvable (Perkin-Elmer) by vigorous vortexing followed by the addition of 5 mL of Formula 989 scitillation fluid (Perkin-Elmer). The tubes were allowed to rest for 1 h and counted in a scintillation counter (5 min per sample).

Synthesis of Und-PP-heptasaccharide. A tube containing dried Und-P (0.02 mg), UDP-Bac (100 μM final concentration), UDP-GalNAc (1 mM final concentration), and UDP-glucose (100 μM final concentration) was suspended with 3 μL of DMSO and 7 μL of 14.3% Triton X-100 through vigorous vortexing and sonication (water bath). Buffer was added to bring the total volume to 55 μL with final concentrations of 50 mM Tris−acetate, pH 8.5, and 10 μM $MgCl_2$. Next, 5 μL of purified PglC, PglA, PglJ, and PglH was added, along with 25 μL of the PglI cell envelope fraction. Reactions were carried out at room temperature for 120 min and quenched by addition of 800 μL of 2:1 chloroform:methanol and 160 μL of pure solvent upper phase (PSUP). After vortexing for 20 s, the tubes were centrifuged briefly, and the organic layer (bottom) containing product was removed and dried.

RESULTS

Cloning, Expression, and Purification of PglC. PglC was cloned from *C. jejuni* genomic DNA and inserted into a pET vector (Novagen) that encoded a final construct containing an N-terminal T7·Tag and a C-terminal hexahistidine tag. PglC was overexpressed in *E. coli*, purified using Ni^{2+} affinity chromatography, and confirmed by SDS−PAGE (Coomassie staining) and T7·Tag Western blot analysis (Figure 3).

Synthesis of UDP-Bac. UDP-Bac was chemically synthesized via the coupling of a 3-*O*-benzoylbacillosamine phosphate (**1**) (*13*) with UMP-morpholidate (Figure 4). This coupling was performed in dry pyridine in the presence of 1*H*-tetrazole to yield the 3-*O*-benzoyl-protected UDP-Bac derivative (**3**). The 3-*O*-benzoyl protection on this intermedi-

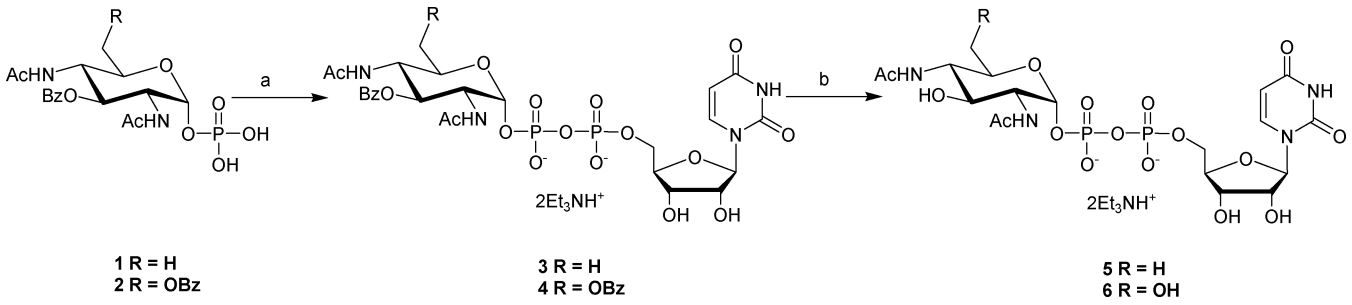


FIGURE 4: Synthesis of UDP-Bac and UDP-6-hydroxybacillosamine. Reagents and conditions: (a) 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidinium uridine 5′-monophosphomorpholidate, 1*H*-tetrazole, pyridine, room temperature, 3 days, 50%; (b) NaOMe, MeOH, room temperature, 68%.

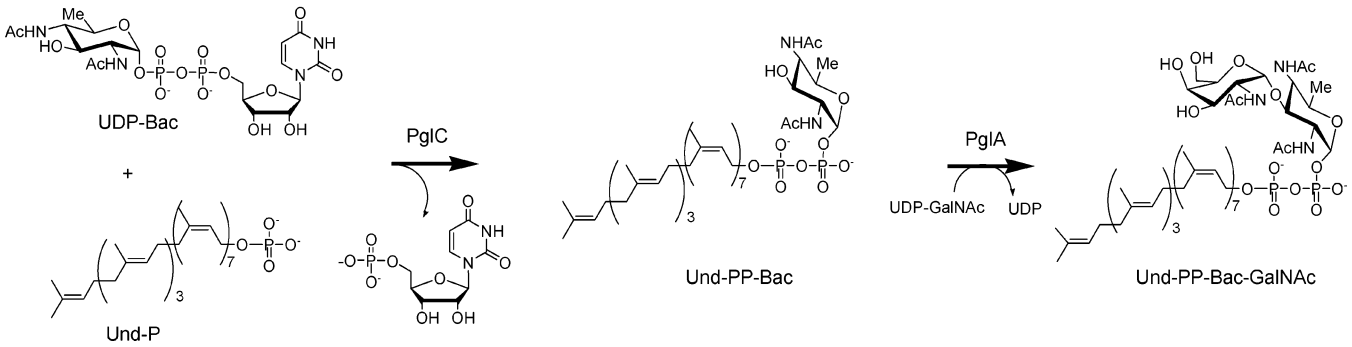


FIGURE 5: (A) Reaction catalyzed by PglC and PglA.

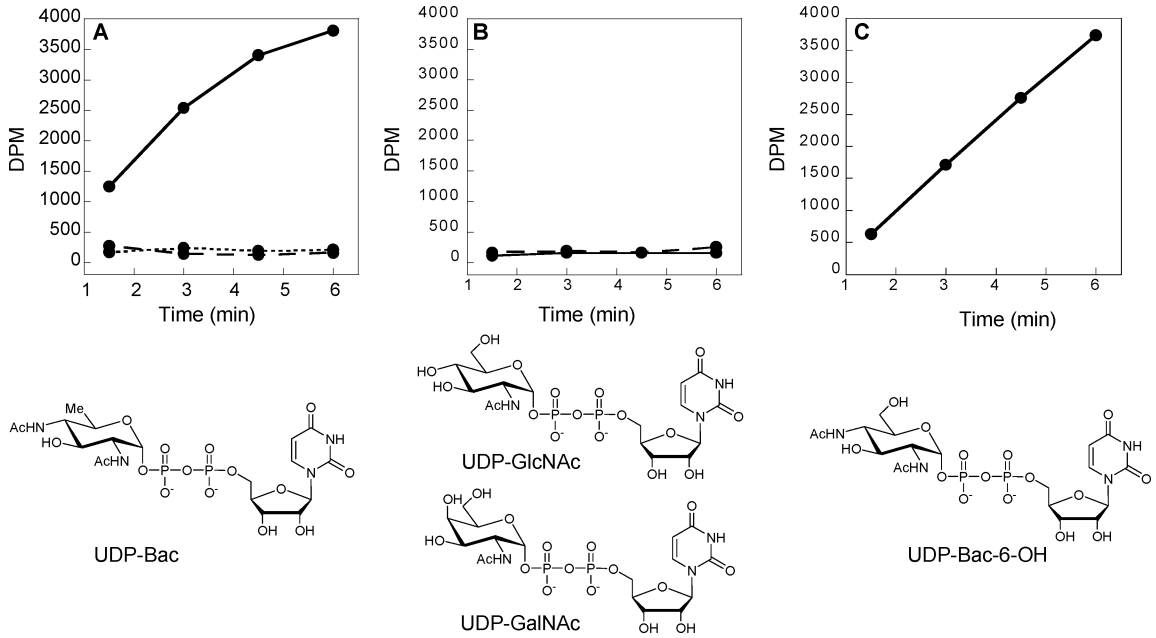


FIGURE 6: (A) Plot of Und-PP-Bac-GalNAc product formation over time using UDP-Bac and Und-P: solid line, PglC and PglA; dotted line, PglA only; dashed line, PglC only. (B) Plot of product formation over time with radiolabeled UDP-sugars, Und-P, and PglC: solid line, UDP-[^{3}H-]-GlcNAc; dashed line, UDP-[^{3}H-]-GalNAc. (C) Plot of Und-PP-6-hydroxybacillosamine-GalNAc product formation over time using UDP-6-hydroxybacillosamine, Und-P, PglC, and PglA.

ate was then removed with sodium methoxide to afford the fully deprotected UDP-Bac (**5**). This compound was purified by reverse-phase HPLC to afford milligram quantities of highly pure UDP-Bac. An analogue of UDP-Bac, UDP-6-hydroxybacillosamine (**6**), was also synthesized under similar coupling conditions using 3,6-di-*O*-benzoyl-2,4-diacetamidoglucose α-1-phosphate (**2**) and UMP-morpholidate. This intermediate is useful in determining the glycan-binding determinants of PglC and is synthetically easier to access that UDP-Bac. The Und-P substrate for PglC was prepared from undecaprenol using phosphoramidite chemistry (*14*).

PglC Activity with UDP-Bac. Our first investigation involved observing the activity of PglC in the presence of UDP-Bac. Typically, glycosyltransferase activity is monitored by the transfer of radiolabeled glycan from the aqueous-soluble UDP derivative to the organic-soluble Und-PP derivative. Since radiolabeled UDP-Bac is not readily available through the current synthetic route, the formation of Und-PP-Bac by PglC cannot be observed directly. To circumvent this problem, PglC activity is monitored indirectly by adding PglA to the reaction mixture, which will transfer a radiolabeled GalNAc residue from UDP-[^{3}H]GalNAc to Und-PP-Bac. (Figure 5) (*8*).

The data in Figure 6A reveal that when UDP-Bac, UDP-GalNAc, and Und-P are present with PglC and PglA, a significant amount of radioactivity is transferred to products that can be extracted into the organic layer, which would correspond to the formation of a radiolabeled Und-PP-glycan product. Conversely, reactions in which either PglA or PglC is omitted showed no transfer of radiolabeled glycan into the organic layer, confirming that the formation of the radiolabeled Und-PP-glycan is dependent on the presence of both PglC and PglA. Next, the product from a 120 min reaction was isolated from the reaction mixture by extraction into organic solvent, and the extract was hydrolyzed to remove the undecaprenyl pyrophosphate moiety. The free saccharide was then fluorescently labeled by reductive amination (2-aminobenzamide/sodium cyanoborohydride, Figure 7A) and analyzed by HPLC using a normal-phase GlykoSepN column (ProZyme), which shows characteristic retention times for saccharides of varying length. When the labeled product of the PglC reaction was injected, a peak with a retention time of 22 min (Figure 7B) was observed, which is the expected retention time for a disaccharide product based on glycan standards. When this peak was collected and subjected to MALDI-MS analysis, a mass of

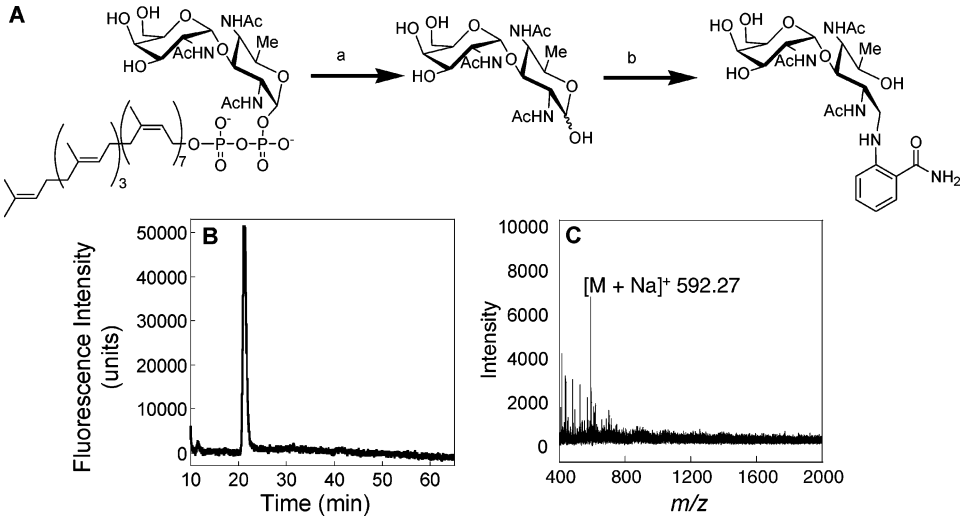
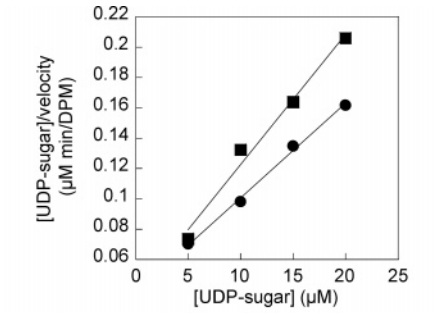


FIGURE 7: (A) Hydrolysis and 2-aminobenzamide labeling of saccharides. Reagents: (a) TFA; (b) 2-aminobenzamide, sodium cyanoborohydride. (B) HPLC trace of 2-aminobenzamide-labeled disaccharide. (C) MALDI-MS of 2-aminobenzamide-labeled disaccharide.



UDP-sugar	K_M (apparent)	Relative V_{max}
UDP-Bac	6.2 μM	100%
UDP-6-hydroxybac	4.3 μM	73%

FIGURE 8: Hanes plot of PglC with UDP-bacillosamine (circles) and UDP-6-hydroxylbacillosamine (squares).

$[M + Na]^+$ *m/z* of 592.27 was observed (Figure 7C). This mass was consistent with a 2-aminobenzamide-labeled Bac-GalNAc disaccharide and was identical to the product from a reaction of synthetic Und-PP-Bac with PglA (*13*).

PglC Activity with UDP-GlcNAc/GalNAc. Next, the activity of PglC with UDP-GlcNAc and UDP-GalNAc was investigated. Since both of these UDP donors are commercially available in radiolabeled form, their activity was measured directly without coupling to PglA. Clearly, from Figure 6B, there is negligible radioactivity in the organic layer extract in the presence of either tritiated UDP-GlcNAc or UDP-GalNAc. This indicates that PglC does not accept either of these UDP-sugar donors.

PglC Activity with UDP-6-hydroxybacillosamine. To investigate the UDP-glycan specificity of PglC, we used an analogue of bacillosamine, UDP-6-hydroxybacillosamine. UDP-6-hydroxybacillosamine is very similar to bacillosamine, except that it retains the C-6 hydroxyl functionality of the bacillosamine precursor. Therefore, studies using this compound can reveal the importance of C-6 deoxygenation for substrate recognition and enzymatic turnover. Analogously, the PglC reaction was coupled with PglA since UDP-6-hydroxybacillosamine was not available in radiolabeled form. Previous studies have shown that PglA accepts the Und-PP-6-hydroxybacillosamine substrate very efficiently, precluding the possibility that PglA would not act upon the 6-hydroxybacillosamine (*8*). As shown in Figure 6C, there is a significant transfer of radioactivity to the organic layer over time for the reaction utilizing UDP-6-hydroxybacillosamine, suggesting that PglC accepts this non-native UDP-linked glycosyl donor. Kinetic analysis of PglC activity in the presence of UDP-Bac and UDP-6-hydroxybacillosamine revealed apparent K_M values for the two substrates of 6.2 and 4.3 μM, respectively (Figure 8). Both substrates were utilized by PglC with comparable efficiency, suggesting that 6-hydroxybacillosamine is a suitable substitute for bacillosamine in future studies.

Properties of PglC. Glycosyltransferases are typically dependent on divalent magnesium for catalysis. The magnesium is thought to coordinate to the pyrophosphate group of the nucleotide-activated sugar donors. Using a membrane fraction of PglC, which had been pretreated with EDTA, we

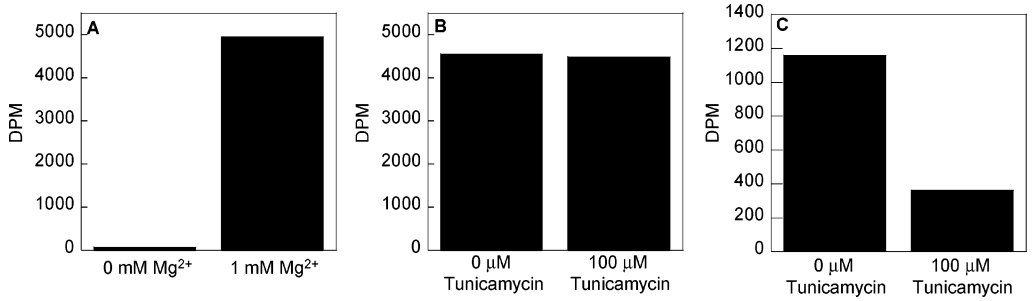


FIGURE 9: (A) PglC activity in the presence and absence of divalent magnesium. (B) PglC activity in the presence and absence of tunicamycin. (C) WecA activity in the presence and absence of tunicamycin.

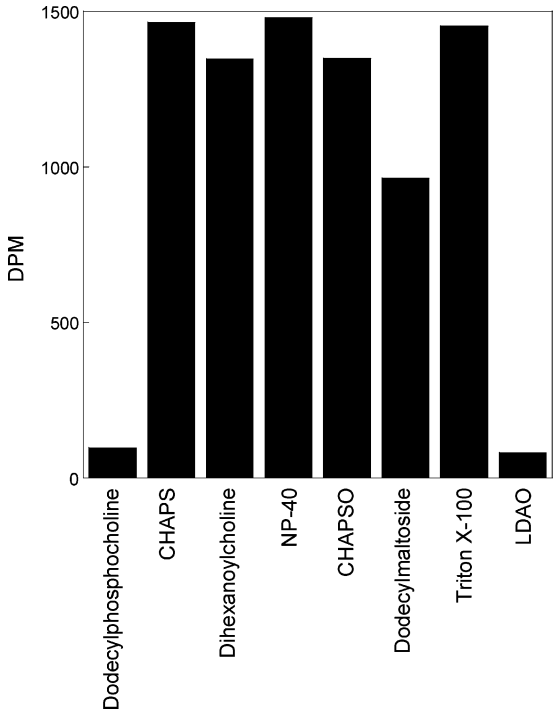


FIGURE 10: Activity of PglC with various detergents.

did not observe any detectable transferase activity, unless exogenous magnesium was added (Figure 9A). Also, this enzyme is not inhibited by tunicamycin, a microbial natural product that has been shown to inhibit members of the HexNAc-1-P transferase family (Figure 9B). For comparison purposes, we used WecA, an *E. coli* GlcNAc-1-P transferase which is known to be inhibited by tunicamycin. As shown in Figure 9C, in the presence of the same amount of tunicamycin, a significant decrease in WecA activity was observed.

Detergent Screen of PglC. Since PglC is an integral membrane protein, a detergent screen was carried out to establish the optimal detergent for PglC activity. For these studies, various detergents were added to a membrane fraction preparation of PglC, and the effect on activity was observed. It is evident that most of the detergents tested (Figure 10) gave comparable activities for PglC with the exception of dodecylphosphocholine and lauryldimethylamine *N*-oxide (LDAO).

Heptasaccharide Assembly Using PglC. Purified PglC, PglA, PglJ, and PglH and the membrane fraction of PglI were mixed together in the presence of Und-P, UDP-Bac, UDP-GalNAc, and UDP-Glc. Extraction into chloroform revealed that the product was indeed polyisoprene-linked, and hydrolysis, labeling, and HPLC analysis yielded a peak that corresponded to heptasaccharide by MALDI-MS (Figure 11).

DISCUSSION

The processes by which nature assembles complex saccharides is of fundamental biological importance. In the *C. jejuni* system, a heptasaccharide is assembled in a stepwise fashion on an undecaprenyl pyrophosphate carrier by a process that has many parallels with the dolichol pathway in eukaryotes and the O-antigen pathway in bacteria (*15*, *16*). PglC is the first to act in the biosynthesis of the isoprenyl pyrophosphate-linked heptasaccharide, catalyzing the transfer of bacillosamine phosphate to undecaprenyl phosphate to form Und-PP-Bac.

To study the activity of PglC in vitro, significant quantities of UDP-Bac are necessary. Purification of the metabolite from the natural source is not viable due to the low cellular levels of UDP-Bac and the extreme pathogenicity of *C. jejuni*, which calls for specialized handling procedures. In contrast, using the chemical synthesis route shown in Figure 4, access to the desired compound in milligram quantities is possible.

The in vitro analysis of PglC has revealed that UDP-Bac is the preferred substrate, while neither UDP-GlcNAc nor UDP-GalNAc is utilized. This evidence strongly suggests that the model (Figure 2A) in which a UDP-HexNAc is converted to bacillosamine on the undecaprenyl carrier is not viable; instead, the second model (Figure 2B) is more likely. Favorably, PglC also accepts the 6-hydroxybacillosamine derivative, suggesting that the C-6 deoxygenation of bacillosamine is not a major factor in substrate recognition as evidenced by the similar apparent K_M and V_{max} values for the two substrates. Since the synthesis of 6-hydroxybacillosamine is considerably more facile than that of bacillosamine, this compound represents a suitable alternative for UDP-Bac for future investigations of this process.

Interestingly, PglC is compatible with a variety of detergents, in contrast to other glycosylphosphotransferases such as WecA which show a strong preference for a single class of detergents, namely, CHAPS or CHAPSO (*17*). Only dodecylphosphocholine and LDAO did not support PglC activity. This analysis may prove useful in future biophysical or structural studies where detergent flexibility is necessary.

In retrospective analysis, the second model (Figure 2B) may be the more likely alternative. If the pathway were to proceed via the first route (Figure 2A), specialized sugar-modifying enzymes, that function on membrane-associated polyisoprene-linked substrates,would have had to evolve in

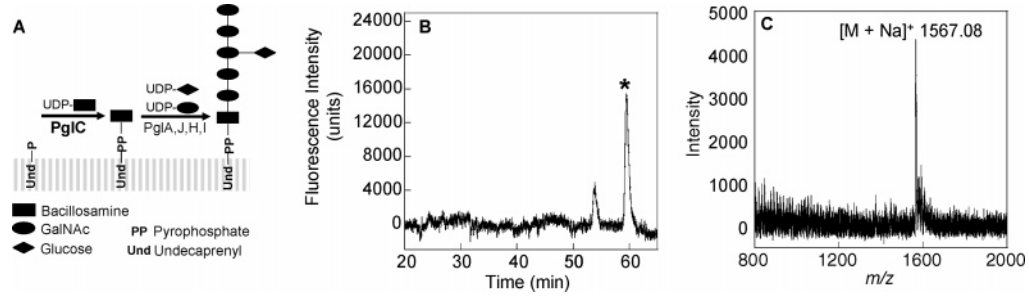


FIGURE 11: (A) Outline of heptasaccharide assembly by the *pgl* glycosyltransferases. (B) HPLC trace of 2-aminobenzamide-labeled heptasaccharide (asterisk denotes major saccharide product). (C) MALDI-MS of 2-aminobenzamide-labeled heptasaccharide.

C. jejuni. In contrast, in the first model the process of bacillosamine biosynthesis could potentially exploit enzymes that are already present in the organism to act on the soluble carbohydrate substrates. Furthermore, it is noteworthy that PglC is not inhibited by tunicamycin, which inhibits most members of the HexNAc-1-phosphate transferase family. Hence, although PglC also utilizes an *N*-acetyl sugar, bacillosamine, it shows markedly different properties from the HexNAc-1-phosphate transferase family. This suggests that there are fundamental differences between these two enzyme classes.

One of the distinguishing features of the N-linked glycosylation pathways in both eukaryotes and bacteria is the assembly of the sugar donor on a polyisoprenyl carrier at the membrane interface. It has been postulated that this membrane localization facilitates the rapid and efficient synthesis of the sugar in vivo. We have been able to reconstitute this process in vitro and have observed the efficient assembly of the heptasaccharide with negligible amounts of intermediate saccharides formed. Perhaps these enzymes associate with each other in some manner to maintain the fidelity of this process. Studies are currently underway to probe for evidence of complex formation between enzymes in the Pgl pathway.

In conclusion, the confirmation of PglC activity in vitro adds crucial clarification to the details of the early steps of heptasaccharide assembly and is an important milestone in the validation of the *pgl* pathway. As more genomic information becomes available and N-linked glycosylation pathways are revealed in new organisms, a clear understanding of the Pgl pathway will undoubtedly serve as a valuable resource in elucidating these new pathways.

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