In Vitro Biosynthesis of UDP-*N*,*N*′-Diacetylbacillosamine by Enzymes of the *Campylobacter jejuni* General Protein Glycosylation System†

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ABSTRACT: In *Campylobacter jejuni* 2,4-diacetamido-2,4,6-trideoxy-α-D-glucopyranose, termed *N*,*N*′-diacetylbacillosamine (Bac2,4diNAc), is the first carbohydrate in the glycoprotein N-linked heptasaccharide. With uridine diphosphate-*N*-acetylglucosamine (UDP-GlcNAc) as a starting point, two enzymes of the general protein glycosylation (Pgl) pathway in *C. jejuni* (PglF and PglE) have recently been shown to modify this sugar nucleotide to form UDP-2-acetamido-4-amino-2,4,6-trideoxy-α-D-glycopyranose (UDP-4-amino-sugar) [Schoenhofen, I. C., et al. (2006) *J. Biol. Chem. 281*, 723−732]. PglD has been proposed to catalyze the final step in *N*,*N*′-diacetylbacillosamine synthesis by N-acetylation of the UDP-4-amino-sugar at the C4 position. We have cloned, overexpressed, and purified PglD from the *pgl* locus of *C. jejuni* NCTC 11168 and identified it as the acetyltransferase that modifies the UDP-4-amino-sugar to form UDP-*N*,*N*′-diacetylbacillosamine, utilizing acetyl-coenzyme A as the acetyl group donor. The UDP-*N*,*N*′-diacetylbacillosamine product was purified from the reaction by reverse phase C18 HPLC and the structure determined by NMR analysis. Additionally, the full-length PglF was overexpressed and purified in the presence of detergent as a GST fusion protein, allowing for derivation of kinetic parameters. We found that the UDP-4-amino-sugar was readily synthesized from UDP-GlcNAc in a coupled reaction using PglF and PglE. We also demonstrate the in vitro biosynthesis of the complete heptasaccharide lipid-linked donor by coupling the action of eight enzymes (PglF, PglE, PglD, PglC, PglA, PglJ, PglH, and PglI) in the Pgl pathway in a single reaction vessel.

Campylobacter jejuni is the Gram-negative enteropathogen identified as the primary cause of gastroenteritis in humans and the most frequent infection to precede the peripheral neuropathy Guillain-Barré syndrome (*1*−*3*). Infection of humans generally occurs by ingestion of contaminated livestock or water. Although the mechanism of infection is not clearly understood, production of glycolipids and glycoproteins by the pathogen has been found to influence cell motility, host cell interactions, and competence for DNA uptake (*4*−*6*). As resistance to antimicrobial agents grows, the potential for development of novel therapeutics against enzymes that produce glycoconjugates has intensified efforts targeted at the characterization of their biosynthetic pathways. In *C. jejuni*, four major glycan structures have been identified: lipooligosaccharide (LOS),[1] capsule, O-linked glycan, and N-linked glycan (*7*−*10*). The genes encoding enzymes that synthesize these glycan moieties have been found in clusters throughout the *C. jejuni* genome. The respective biochemical functions of the gene products have been assigned on the basis of biochemical data or sequence homology to glycosyltransferases and other enzymes known to modify sugars (*11*−*13*).

A common N-linked glycan has been detected in at least 22 periplasmic and cell-surface proteins (*10*, *14*) in *C. jejuni*, and the Pgl pathway has been identified as the source of its biosynthesis. Genes of the *pgl* locus in the pathogen have been designated *cj*1119c−*cj*1130c, and the structure of the N-linked glycan has been shown to be the heptasaccharide GalNAc-α1,4-GalNAc-α1,4-(Glcβ1,3)-GalNAc-α1,4-GalNAc-α1,4-GalNAc-α1,3-Bac2,4diNAc-β1-Asn, where GalNAc, GlcNAc, and Glc represent *N*-acetylgalactosamine, *N*-acetyl-

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[1] Abbreviations: 2AB, 2-aminobenzamide; AcCoA, acetyl-coenzyme A; Bac2,4diNAc, 2,4-diacetamido-2,4,6-trideoxy-α-D-glucopyranose (also called *N*,*N*′-diacetylbacillosamine in this report); BCA, bicinchoninic acid; CE, capillary electrophoresis; CoASH, coenzyme A; cv, column volume; ESI, electrospray ionization; GS, glutathione−Sepharose 4 Fast Flow resin; GST, glutathione *S*-transferase; IPTG, isopropyl β-D-1-thiogalactopyranoside; LB, Luria-Bertani; LOS, lipooligosaccharide; MALDI, matrix-assisted laser desorption ionization; MWC, molecular weight cutoff; NAD^+, nicotinamide adenine dinucleotide; $NADP^+$, nicotinamide adenine dinucleotide phosphate; Ni−NTA, nickel−nitrilotriacetic acid; NMR, nuclear magnetic resonance; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; Pgl, protein glycosylation; PLP, pyridoxal 5′-phosphate; RP-HPLC, reverse phase high-performance liquid chromatography; SDS, sodium dodecyl sulfate; UDP, uridine 5′-diphosphate; UDP-4-amino, UDP-2-acetamido-4-amino-2,4,6-trideoxy-α-D-glucopyranose; UDP-4-keto, UDP-2-acetamido-2,6-dideoxy-α-D-*xylo*-4-hexulose; UDP-GlcNAc, UDP-2-acetamido-α-D-glucopyranose; UDP-GalNAc, UDP-2-acetamido-α-D-galactopyranose; Und-P, undecaprenyl phosphate; Glc, glucose; Gal, galactose.

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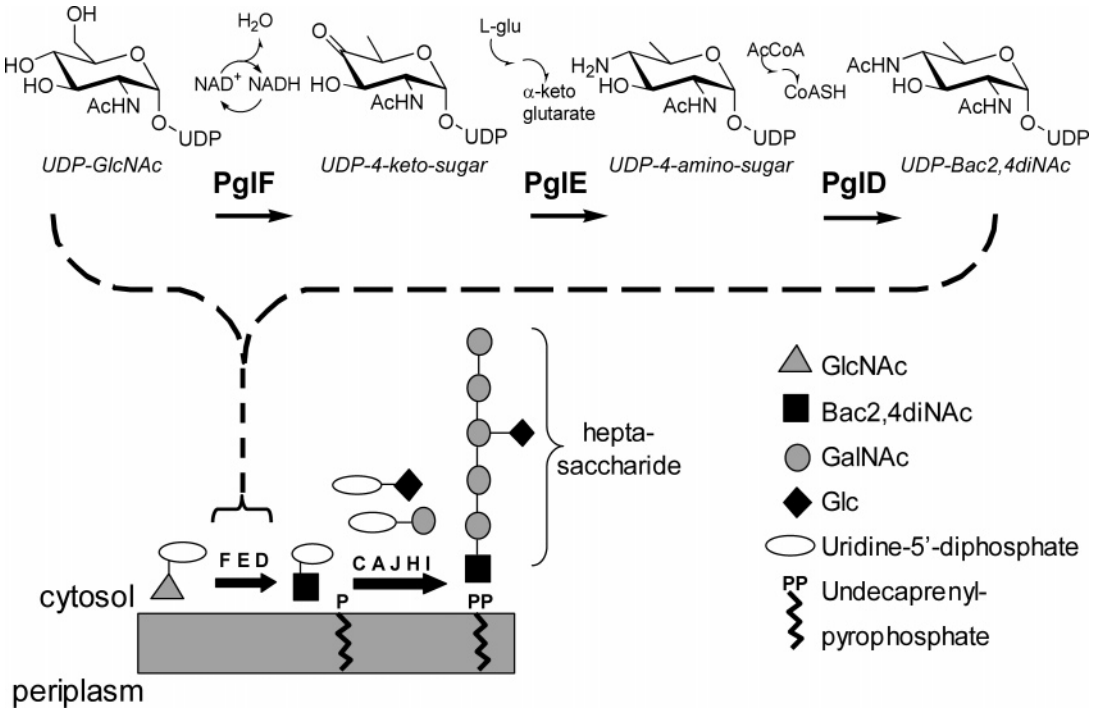

FIGURE 1: Pathway of the enzymatic synthesis of the bacterial glycoprotein heptasaccharide from UDP-GlcNAc by enzymes of the general protein glycosylation locus in *C. jejuni*. Enlarged is a detailed view of the pathway for UDP-Bac2,4diNAc synthesis.

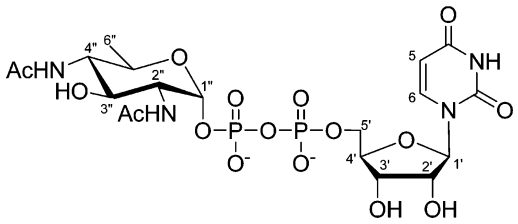

FIGURE 2: Structure of UDP-2,4-diacetamido-2,4,6-trideoxy-α-D-glycopyranose.

glucosamine, and glucose, respectively (Figure 1) (*10*). Assembly of this bacterial glycan begins with formation of a pyrophosphate between the Bac2,4diNAc phosphate and undecaprenylphosphate (Und-P) by Cj1124c (PglC) which forms Bac2,4diNAc-α1-PP-Und (*15*). One *N*-acetylgalactosamine (GalNAc) is linked to the polyisoprenepyrophosphate-bound *N*,*N*′-diacetylbacillosamine by Cj1125c (PglA) to form GalNAc-α1,3-Bac2,4diNAc-α1-PP-Und (*16*, *17*). Next, four additional GalNAc molecules and one branching glucose are added sequentially to the isoprenepyrophosphate-linked disaccharide by PglJ, PglH, and PglI, to form the heptasaccharide (*17*). In N-linked glycoproteins, the heptasaccharide is covalently attached at the *N*,*N*′-diacetylbacillosamine through a β-linkage to the amide nitrogen (N-linked) of an asparagine side chain in the canonical sequence motif Asn-Xaa-Ser/Thr, where Xaa can be any amino acid except proline (4).

Nearly all enzymes encoded in the *pgl* locus from *C. jejuni* strain NCTC 11168 responsible for synthesis of the heptasaccharide have been biochemically characterized (*15*−*18*). Recently, our laboratory reported biochemical evidence of enzyme-mediated glycosyltransferase activity, which strongly suggested that *N*,*N*′-diacetylbacillosamine was initially formed in *C. jejuni* as a uridine diphosphate derivative (Figure 2) (*15*). In these experiments, a lengthy chemical synthesis was undertaken to produce UDP-Bac2,4diNAc and PglC was found to transfer Bac2,4diNAc phosphate to the undecaprenylphosphate lipid. The original bacillosamine, or 4-acetamido-2-amino-2,4,6-trideoxy-D-glucose, was isolated and identified more than 30 years ago from *Bacillus licheniformis* (*19*, *20*). The difference between that bacillosamine and the derivative found in the *C. jejuni* N-linked heptasaccharide is an *N*-acetyl group at the C2 position. Figure 1 shows a scheme for the biosynthesis of UDP-*N*,*N*′-diacetylbacillosamine in *C. jejuni* beginning with UDP-GlcNAc. In this model, Cj1120c (PglF) performs a cofactor-dependent hydride transfer from the C4 position of UDP-GlcNAc to C6, in conjunction with the elimination of water across the glucosyl C5−C6 bond. The mechanism for the reaction is believed to follow a process similar to those of other C6 dehydratases such as dTDP-D-glucose-4,6-dehydratase (RmlB) from *Salmonella enterica* (*21*), resulting in the production of UDP-2-acetamido-2,6-dideoxy-α-D-4-ketohexulose (UDP-4-keto-sugar) (*18*). Cj1121c (PglE) catalyzes the pyridoxal-dependent transfer of an amino group from L-glutamate to the C4 position of the UDP-4-keto-sugar to form the UDP-4-amino-sugar (*18*). The final step in the enzymatic synthesis of UDP-Bac2,4diNAc is the proposed N-acetylation at the C4 position of the UDP-4-amino-sugar by Cj1123c (PglD).

The native dehydratase PglF has three domains: an N-terminal transmembrane domain, a C-terminal catalytic domain, and a linker between the transmembrane and catalytic domains. Recently, Logan and co-workers identified the biochemical activity of PglF by studying a construct that lacked the transmembrane domain (*18*). These investigators further reported that enzyme activity could not be detected in a coupled reaction between the truncated form of PglF and full-length PglE, suggesting that the transmembrane domain plays an important role in the coupled enzyme reaction. The truncation approach has also proven to be advantageous in the functional analysis of WbpM, a homologue of PglF, which is involved in pseudaminic acid

Table 1: Constructs and Oligonucleotides Used for This Report

construct	vector	primer (5′ → 3′)
GST−PglF	pGEX4T-2	forward, CGCGGATCCATGATTTTTTATAAAAGCAAAAGATTAGC reverse, CGGCTCGAGTCAGTGATGATGATGATGATGATGGTGTACACCTTCTTTATTGTGTTTAAATTC
GST−PglF$_{130}$	pGEX4T-2	forward, CGCGGATCCATGCTTGTGGATTTTAAACCTTC reverse, CGGCTCGAGTCAGTGATGATGATGATGATGATGGTGTACACCTTCTTTATTGTGTTTAAATTC
PglE	pET24a(+)	forward, CGCGGATCCATGAGATTTTTTCTTTCTCC reverse, CGGCTCGAGAGCCTTTATGCTCTTTAAG
PglD	pET24a(+)	forward, CGCGGATCCATGGCAAGAACTGAAAAAATTTATATTTATGGTGC reverse, CGGCTCGAGCATCCTTTTTGCAGGTAC

biosynthesis in *Pseudomonas aeruginosa*. In their experiments, Creuzenet and Lam found that the full-length WbpM retained activity, but only when isolated in the membrane fraction of the host expression system (*22*).

In this report, we detail the functional characterization of PglD in addition to the biosynthesis and purification of UDP-Bac-2,4-diNAc. By harnessing the UDP-Bac2,4diNAc biosynthetic process in vitro, we provide a rapid and cost-effective means of producing this rare sugar in milligram quantities. We also characterized the full-length PglF and demonstrate the in vitro enzymatic synthesis of the undecaprenylpyrophosphate-linked heptasaccharide in a coupled reaction that combined PglF, -E, -D, -C, -A, -J, -H, and -I in a single reaction vessel. The results presented herein complete the functional identification of enzymes involved in the biosynthesis of the N-linked heptasaccharide in *C. jejuni*.

EXPERIMENTAL PROCEDURES

Molecular Biology. Amplification of *pgl* gene sequences from *C. jejuni* 11168 genomic DNA (ATCC 700819, designation NCTC 11168) was performed with oligonucleotides described in Table 1. BamHI and XhoI restriction sites were engineered to facilitate cloning of all amplicons into either pGEX4T-2 (GE Healthcare) to incorporate an N-terminal GST tag or pET-24a(+) (Novagen) to incorporate an N-terminal T7 and a C-terminal hexahistidine tag. Full-length *pgl*D and *pgl*E were subcloned into the pET vector. Full-length *pgl*F and a truncated form of *pgl*F encoding residues M130−V590 were subcloned into the pGEX vector to express GST−PglF and GST−PglF$_{130}$ fusion proteins, respectively. The full-length GST fusion PglF construct also included a C-terminal octahistidine tag. Amplifications were accomplished with the Stratagene Cloned Pfu Polymerase system as described by the manufacturer. Amplicons were purified and double-digested with BamHI and XhoI. Digested inserts and linearized vectors were fractionated by agarose gel electrophoresis and purified with the QIAquick gel extraction kit (Qiagen). Ligations were conducted with the T4 DNA ligase kit (Promega) using an overnight incubation at 16 °C. The chemically competent DH5α strain of *Escherichia coli* (Invitrogen) was transformed with the ligation reactions and colonies selected on Luria-Bertani (LB) agar plates supplemented with either kanamycin or carbanicillin. Plasmids from colonies representing each construct were isolated and sequenced at the MIT CCR HHMI Biopolymers Laboratory.

PglD and PglE Expression and Purification. The chemically competent BL21(DE3) pLysS strain of *E. coli* (Invitrogen) was transformed for expression. For each construct, 10 mL of an overnight culture was used to inoculate 1 L of LB broth supplemented with 30 μg/mL kanamycin and 50 μg/mL chloramphenicol for selection and incubated at 37 °C. At an OD_{600} of 0.6−1.0, the cultures were cooled to 16 °C prior to induction with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). After incubation for 22 h at 16 °C, the cells were harvested by centrifugation, resuspended in a solution of 0.9% NaCl, and pelleted for storage at −80 °C. Pellets of cells that expressed PglD or PglE were resuspended in 50 mL of ice-cold lysis buffer [50 mM Tris-acetate and 100 mM NaCl (pH 7.5)] supplemented with 30 mM imidazole and lysed by sonication, and the lysate was cleared by centrifugation at 145000*g* for 45 min at 4 °C. Further handling of the protein occurred at 4 °C unless otherwise stated. Cleared lysate was mixed with 4 mL of Ni−NTA (Qiagen) resin per liter of original culture and then tumbled for 4 h. The Ni−NTA resin/protein mixture was packed into a K 9/15 column (GE Healthcare) and washed with 10 column volumes (cv) of lysis buffer supplemented with 30 mM imidazole at a flow rate of 3 mL/min. The resin-bound protein was further washed with 20 cv of lysis buffer containing 40 mM imidazole and then 10 cv of lysis buffer containing 50 mM imidazole. To elute the protein of interest, a 30 cv gradient of imidazole and lysis buffer from 50 to 500 mM was applied to the column. Protein concentrations were determined using the Micro BCA kit (Pierce).

GST−PglF$_{130}$ Expression and Purification. Chemically competent BL21(DE3) pLysS cells were transformed for expression of the GST−PglF$_{130}$ construct. A 10 mL volume of an overnight culture was used to inoculate 1 L of LB broth and incubated at 37 °C with 50 μg/mL carbanicillin and 50 μg/mL chloramphenicol for selection. At an OD_{600} of 0.6−1.0, the culture was cooled to 30 °C, then induced with 1 mM IPTG and incubated at 30 °C for 3 h, then harvested, and stored at −80 °C. Frozen cell pellets were resuspended in 50 mL of ice-cold PBS buffer [140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4 (pH 7.3)] and lysed by sonication, and the lysate was cleared by centrifugation at 145000*g* for 45 min. To purify, cleared lysate was mixed with 4 mL of glutathione−Sepharose 4 Fast Flow resin (GS) (GE Healthcare) per liter of original culture and tumbled to mix for 4 h. The GS resin/protein mixture was packed into a K 9/15 column and washed with 25 cv of PBS at a flow rate of 3 mL/min. Protein was eluted with an isocratic flow of GS elution buffer [50 mM Tris and 10 mM L-glutathione (pH 8.0)]. Fractions containing purified material were identified by SDS−PAGE and subjected to Coomassie staining or Western blot analysis probing for the affinity tag. Target fractions were pooled and exhaustively dialyzed against PBS. Protein concentrations were determined using the Micro BCA kit.

Full-Length PglF Expression and Purification. A 20 mL volume of an overnight culture of BL21(DE3) pLysS cells transformed with the GST−PglF construct was used to inoculate 2 L of LB broth supplemented with 50 μg/mL carbenicillin and 50 μg/mL chloramphenicol and cultured at 37 °C. At an OD_{600} of 0.6−1.0, the culture was cooled to 16 °C, induced with 0.5 mM IPTG, and incubated at 16 °C for 22 h. Cells were harvested and frozen at −80 °C until they were required. Frozen cell pellets were thawed in 100 mL of ice-cold PBS, lysed by sonication, and then supplemented with 200 μM NAD^+. Cellular debris was cleared by low-speed (8000*g*) centrifugation for 45 min. The resulting supernatant was transferred to a clean centrifuge tube and subjected to high-speed centrifugation (145000*g*) for 45 min to pellet the cell envelope fraction. The supernatant was discarded and the resulting pellet homogenized in 10 mL of PBS supplemented with 1.0% (v/v) Triton X-100 and 200 μM NAD^+ (PBS-T). The detergent solution was tumbled at 4 °C for 2 h and then centrifuged at 145000*g* for 1 h. The supernatant containing the detergent-solubilized GST−PglF construct was carefully removed so that the pellet would not be disturbed and then combined with 2 mL of GS resin preequilibrated in PBS-T. The GS resin/protein solution was tumbled for 4 h and then poured into a column, and the resin was allowed to settle. Using gravity flow, the resin-bound protein was washed with 25 cv of PBS-T containing 0.1% Triton X-100 and then eluted with the GS elution buffer supplemented with 0.1% Triton X-100 and 200 μM NAD^+. Fractions containing purified material were assessed by SDS−PAGE (10%) and subjected to Coomassie staining or Western blot analysis probing for the His tag. To determine protein concentrations, a sample of the pooled fractions was exhaustively dialyzed against 0.1% Triton X-100 and PBS-T and then assayed with the Micro-BCA kit.

Enzymatic Reactions. To synthesize UDP-4-keto-sugar with the purified GST−PglF construct, 1.5 mg (16.1 nmol) of enzyme in a 15 mL solution containing 5 mM L-glutathione, 50 mM Tris-acetate, 0.1% Triton X-100, 200 μM NAD^+, 50 mM NaCl, and 1 mM (15 μmol) UDP-GlcNAc (pH 8) was incubated for 10 h at 28 °C. A sample of the reaction mixture was filtered using a Microcon filtration unit [10K molecular weight cutoff (MWC), Millipore] for capillary electrophoresis (CE) analysis. A sample of the filtrate was equally mixed with methanol and injected into a Mariner Biospectrometry Workstation under a constant flow of 10 μL/min with data acquisition conducted in negative mode. To synthesize the UDP-4-amino-sugar by coupling the reactions of the GST−PglF construct and PglE, the following components were added to a reaction mixture as described above: 5 mg (108.5 nmol) of purified PglE and L-glutamate and pyridoxal 5′-phosphate (PLP) to final concentrations of 15 mM and 100 μM, respectively; the mixture was then incubated for 24 h at 28 °C. The reaction volume was passed through a 10K MWC filter, then diluted 100-fold with H_2O, and loaded onto a 5 mL HiTrap Q FF anion exchange column (GE Healthcare) at a flow rate of 3 mL/min. The bound sugar was washed with 100 mL of H_2O and eluted over a 120 mL gradient from 0 to 220 mM triethylammonium bicarbonate (TEAB) (pH 8.6). Fractions containing purified UDP-4-amino-sugar were identified by CE and then pooled, lyophilized, and analyzed by ESI-MS as described above. The UDP-4-amino-sugar product was quantified spectrophotometrically using the molar extinction coefficient of UDP [$\epsilon_{262} = 10\,000\ M^{-1}\ cm^{-1}$ (*23*)]. Approximately 2.3 mg (3.9 μmol) of UDP-4-amino-sugar was recovered from the coupled enzyme reaction. As an alternative for synthesizing UDP-4-amino-sugar, approximately 15 mg (190.5 nmol) of the GST−$PglF_{130}$ construct was bound to 4 mL of GS resin. PBS was added to the slurry such that the total volume measured 15 mL; 20 mg (33 μmol) of UDP-GlcNAc was added and the reaction mixture incubated at 28 °C overnight. The reaction mixture was resuspended in a slurry and poured into a column, and the flow-through was collected and then filtered with a 10K MWC membrane. In a total volume of 25 mL, the filtrate was combined with 15 mg (325.5 nmol) of purified PglE. L-Glutamate and PLP were added to final concentrations of 20 mM and 100 μM, respectively. The reaction mixture was incubated for 48 h at 28 °C and the product purified as described for the coupled reaction. Recovery of UDP-4-amino-sugar by this method was approximately 9.5 mg (16 μmol). NMR chemical shifts and coupling constants of this material were essentially identical to published data (*18*). To synthesize UDP-Bac2,4diNAc enzymatically, in a total volume of 5 mL, 5 mg (212 nmol) of PglD was combined with 10 mg (17.0 μmol) of UDP-4-amino-sugar and 14 mg (17.3 μmol) of AcCoA (Sigma-Aldrich). The reaction mixture was incubated at 37 °C for 3 h and then filtered with a 10K MWC membrane. The entire filtrate was loaded onto a preparative scale reverse phase C-18 column [Waters YMC Pack-Pro C18, 250 mm × 20 mm (inside diameter), S-5 μm particle size, 12 nm pore size] equilibrated with 50 mM TEAB (pH 7.1) and eluted with a linear gradient from 0 to 25% acetonitrile over 300 mL. Eluting material was monitored for absorbance at 262 nm, and fractions containing pure UDP-Bac2,4diNAc were identified by CE. Target fractions were pooled, and the solvent was concentrated under reduced pressure to yield 6 mg of UDP-Bac2,4diNAc.

NMR Spectroscopy. A 4 mg sample of purified UDP-Bac2,4diNAc was resuspended in 600 μL of 99% D_2O (Cambridge Isotopes) and placed in a 5 mm tube. All data were collected at 25 °C. A solution of 85% phosphoric acid in D_2O was used as an external standard for ^{31}P NMR and D_2O (4.8 ppm) as the internal standard for ^1H NMR spectrum calibration. The NMR data were processed with the MestRe-C (Mestrelab Research) software package. ^1H and COSY spectra were obtained on a Bruker Avance-600 MHz NMR spectrometer equipped with a pulse-field gradient triple-resonance probe using standard pulse sequences from Bruker NMR. Homonuclear decoupling spectra were recorded on a Varian Inova-500 MHz NMR spectrometer equipped with a pulse-field gradient triple-resonance probe using standard pulse sequences from Varian. ^{31}P spectra were measured with a Varian Mercury 300 NMR spectrometer with a pulse-field autoswitchable probe.

CE Analysis. CE was performed using a Hewlett-Packard 3D CE system with UV detection and manual integration with the HP 3D CE software package. The running buffer was composed of 25 mM sodium tetraborate (pH 9.4) using a bare silica capillary (75 μm × 80 cm) with a detector distance of 72 cm. The capillary was conditioned before each run by being washed with 0.4 M NaOH for 2 min, water for 2 min, and running buffer for 2 min. In general, each sample was prepared by filtration with a 10K MWC membrane and

then diluted with water by a ratio of 1:4. Samples were introduced by pressure injection for 16 s at 30 mbar, and the separation was performed at 22 kV.

Kinetic Measurements. For the GST−PglF construct, 1.3 μg of enzyme was incubated in a reaction volume of 50 μL with 200 μM NAD^+, 0.1% Triton X-100, 50 mM Tris-acetate, 50 mM NaCl, and 5 mM L-glutathione (pH 7.7) with UDP-GlcNAc at six different concentrations varying from 0.8 to 31 mM. The reaction mixtures were incubated at 37 °C for 200 min, boiled for 2 min, and then filtered through a 10K MWC membrane and analyzed by CE as described above. UDP-4-amino-sugar substrate for the PglD assays was enzymatically synthesized and quantified as described above. For each reaction, 0.025 ng of enzyme was incubated in a total volume of 20 μL containing 50 mM Tris-acetate, 50 mM NaCl, 2 mM AcCoA (pH 7.7), and 5 μg of bovine serum albumin to serve as a carrier protein with UDP-4-amino-sugar at six different concentrations varying from 0.1 to 2 mM. The reaction mixtures were incubated at 37 °C for 90 min, boiled, and filtered which was done for the GST−PglF kinetic reactions.

Radioactive Assay Coupling PglD, -C, and -A Using Labeled AcCoA. Und-P was synthesized as described elsewhere (*24*, *25*). A 3 μL volume of DMSO and a 7 μL volume of 14.3% (v/v) Triton X-100 were added to a tube containing 5 nmol of Und-P. After the sample had been vortexed and sonicated in a water bath, 1 μL of 1 M $MgCl_2$, 10 μL of 10 mM UDP-GalNAc, 15 μL of 7 mM UDP-4-amino-sugar, 10 μL of PglC at 0.25 mg/mL, 10 μL of PglA at 0.5 mg/mL, 1.5 μL of unlabeled 14 mM AcCoA, and 1 μL of 20 μM [^{3}H]AcCoA (American Radiolabeled Chemicals) were added, giving the reaction mixture a specific activity of 48 nCi/nmol. The reaction was initiated by adding 10 μL of PglD at 0.5 mg/mL and the mixture incubated at room temperature. Aliquots of 12 μL were taken at 2, 4, 8, 30, and 60 min. Reactions were quenched and mixtures prepared for liquid scintillation counting as described elsewhere (*15*).

Heptasaccharide Biosynthesis. Components of the reaction were assembled in two tubes. Tube 1 contained Und-P (5 nmol), UDP-GlcNAc, UDP-GalNAc, and UDP-Glc (final concentrations of 1, 1, and 0.2 mM, respectively) suspended with 3 μL of DMSO and 7 μL of 14.3% Triton X-100 by vigorous vortexing and water bath sonication. Next, 4 μL of purified PglC, PglA, PglJ, and PglH (the latter two at a stock concentration of 0.5 mg/mL) was added, along with 10 μL of a PglI cell envelope fraction. Each was prepared as described previously (*17*). Combined in tube two were 3 μL of 15 mM NAD^+, 10 μL of 200 mM L-Glu, 10 μL of 2.7 mM PLP, 5 μL of 20 mM AcCoA, 10 μL of PglD, 25 μL of PglE, 37 μL of the GST−PglF construct (protein stock concentrations of 0.5, 0.5, and 0.15 mg/mL, respectively). The reaction was initiated by adding 64 μL of tube 2 to tube 1, and then the mixture was incubated at room temperature for 7 h. The heptasaccharide was isolated and analyzed by HPLC and MALDI-MS as described previously (*15*).

RESULTS

Cloning, Expression, and Purification of PglD, PglE, and PglF. Full-length *pglD*, *pglE*, and *pglF* were cloned from *C. jejuni* strain NCTC 11168 genomic DNA and expressed in *E. coli*. Clones encoding PglE and PglD were expressed with an N-terminal T7 tag and a C-terminal His_6 tag (Table 1). Both constructs overexpressed robustly in *E. coli* and were purified by nickel affinity chromatography with protein yields near 25 mg/L of culture. Full-length PglF was expressed with an N-terminal glutathione *S*-transferase (GST) domain concurrently with a C-terminal octahistidine tag (GST−PglF). GST−PglF was purified in the presence of detergent and NAD^+ by affinity chromatography with a yield of 1.5 mg/L of culture. Similar to the construct reported by Logan and co-workers (*18*), GST−$PglF_{130}$ (M130−V590) which omitted the putative transmembrane domain of full-length PglF was expressed in this study. This truncated construct expressed well in *E. coli* yielding approximately 20 mg/L of culture. Confirmation that purified material represented the target proteins was accomplished by SDS−PAGE and Western blot analysis for detection of the affinity tags (Figure 3).

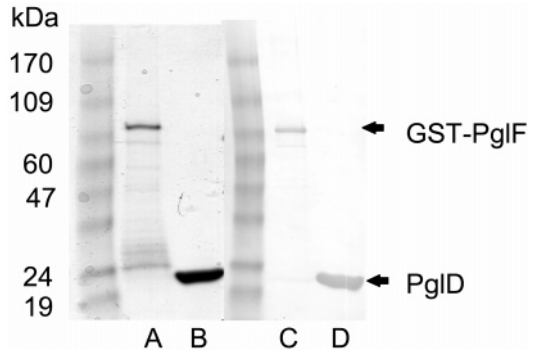

FIGURE 3: Coomassie-stained 4 to 15% gradient SDS−PAGE gel of glutathione−Sepharose-purified GST−PglF and Ni−NTA-purified PglD (lanes A and B, respectively). Calculated molecular masses for GST−PglF and PglD-His_6 are 93 and 23.5 kDa, respectively. Corresponding Western blot probing for the C-terminal His tag (lanes C and D). Arrows indicate band representing the respective construct.

Dehydratase Activity of GST−PglF. The reaction of GST−PglF was analyzed by CE using UV absorbance (262 nm) to detect the eluting species (Figure 4A). Although consumption of the cofactor was not apparent, the catalytic activity of GST−PglF was enhanced in the presence of additional NAD^+ but not $NADP^+$ (data not shown). Electrospray ionization mass spectrometry (ESI-MS) analysis of the GST−PglF reaction indicated a mass of 587.5 Da, consistent with the formation of UDP-4-keto-sugar. CE analysis of the same reaction indicated the presence of starting material (data not shown), consistent with the ESI-MS observation of a species having a mass of 605.5 Da (Figure 5A). Alternatively, it is possible for the keto-sugar to exist in equilibrium with the corresponding hydrate form (*26*). The hydrate form of the catalytic product of PglF would correspond to the addition of 18 atomic mass units (water) to the UDP-4-keto-sugar. Therefore, the mass of 605.5 Da may be attributed, in part, to the hydrate form of the PglF product.

Acetyltransferase Activity of PglD. PglD is a putative acetyltransferase (*6*). Amino acid sequence analysis shows the C-terminal domain to be consistent with a hexapeptide repeat motif. Members from a subclass of the hexapeptide repeat enzymes, known as xenobiotic acetyltransferases, have been shown to use acetyl-coenzyme A (AcCoA) for acetylation of a variety of substrates (*27*, *28*). Purified PglD was incubated with the purified UDP-4-amino-sugar and AcCoA

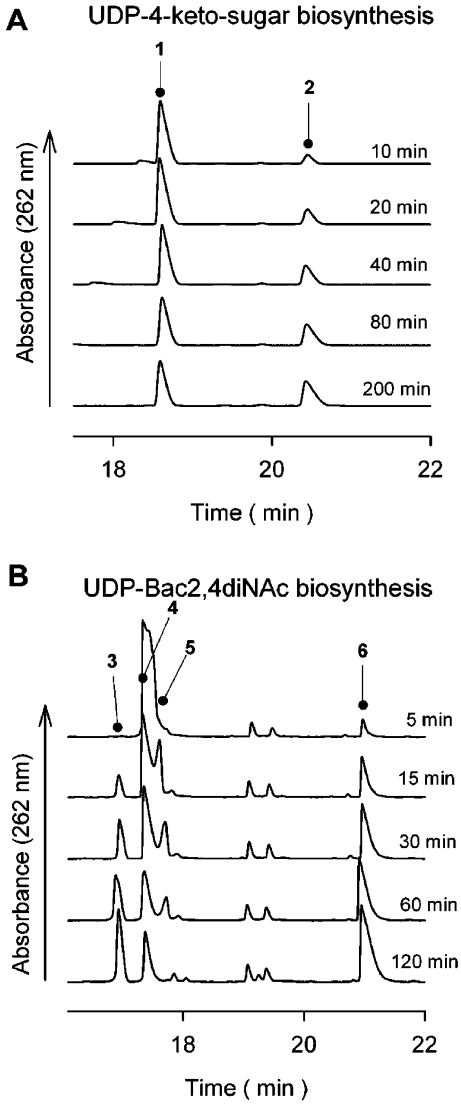

FIGURE 4: CE time course analysis of (A) GST−PglF using 10 μg of enzyme with UDP-GlcNAc at a final concentration of 1 mM and (B) PglD using 1.3 ng of enzyme with UDP-4-amino and AcCoA at final concentrations of 1.2 and 1.0 mM, respectively. Both reactions were conducted at 37 °C and pH 7.7 with aliquots taken at the indicated times. Numbered peaks are (1) UDP-GlcNAc, (2) UDP-4-keto, (3) UDP-Bac, (4) UDP-4-amino, (5) AcCoA, and (6) CoASH.

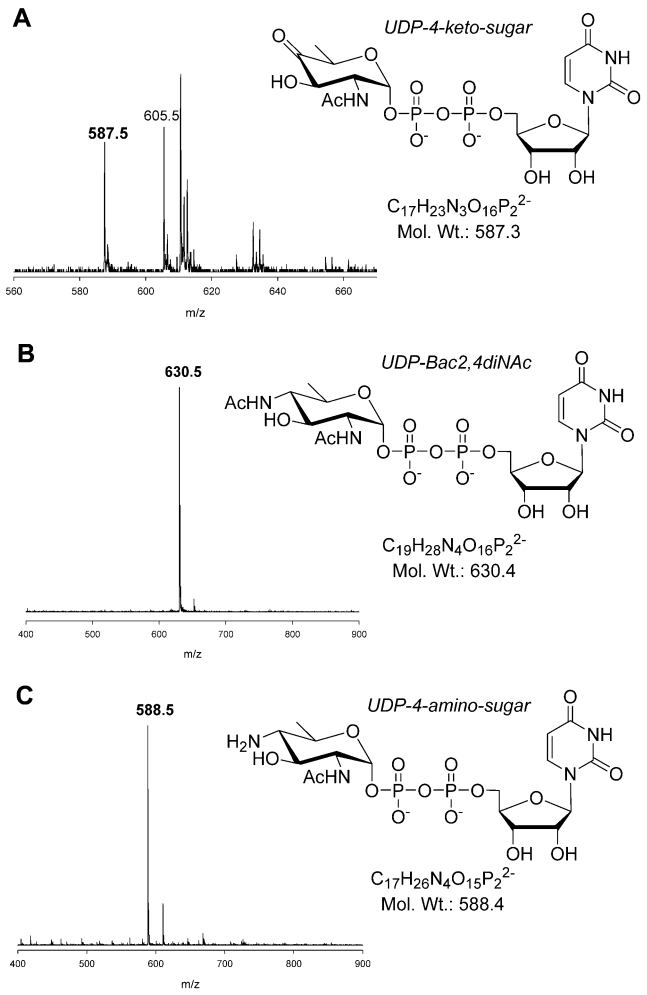

FIGURE 5: ESI-MS of (A) the GST−PglF reaction mixture, (B) HPLC-purified UDP-Bac, and (C) ion exchange purification of UDP-4-amino from the GST−PglF and PglE coupled reaction.

in excess. The resultant CE elution profile exhibited two additional peaks that flanked the substrate and cofactor signals (Figure 4B). To confirm that an acetyl group was transferred to UDP-4-amino-sugar, the PglD reaction products were purified by reverse phase high-performance liquid chromatography (RP-HPLC) and analyzed by ESI-MS and CE. Consistent with the loss of a proton and addition of an acetyl group to form UDP-Bac2,4diNAc, ESI-MS revealed a mass peak of 630.5 (Figure 5B). The RP-HPLC purification step also separated a species with an atomic mass of 767, consistent with the coenzyme A (CoASH) (data not shown).

To further demonstrate transfer of an acetyl group by PglD to the UDP-4-amino-sugar, a coupled enzyme assay was employed using [^{3}H]AcCoA. Isolation of the labeled UDP-Bac2,4diNAc was simplified by incorporating purified PglC, an integral membrane glycosyltransferase from *C. jejuni* responsible for transferring Bac2,4diNAc phosphate to the isoprene lipid carrier Und-P (*15*), into the assay. PlgA, which transfers the first GalNAc onto Bac2,4diNAc-PP-Und (*17*), was also included to consume the product of PglC, thus driving the reaction forward and facilitating isolation of the labeled product. In addition to cofactors and salts, reactants include the purified UDP-4-amino-sugar, Und-P, and UDP-GalNAc (Figure 6A). The organic phase was extracted and assayed for radioactivity. Because AcCoA was expected to remain in the aqueous layer, counts in the organic layer were attributed to incorporation of a radiolabled acetyl group into the polyisoprenepyrophosphate-linked substrate (Figure 6B). For control reactions, the Und-P or the UDP-4-amino-sugar was omitted. Both control reactions resulted in background levels of counts, strongly suggesting acetyl group transfer to UDP-4-amino by PglD (Figure 6B).

NMR Analysis of the PglD Product. To further establish the identity of the enzymatic product of PglD, the proton NMR spectra of the purified PglD reaction product were analyzed. A series of homonuclear decoupling experiments was conducted to identify the chemical shifts for protons from the pyranose ring (Figure S-1 of the Supporting Information). In the proton spectrum, two peaks centered at 2.05 ppm with a total peak area of six protons suggested the presence of two acetyl groups in the compound (Figure 7). In comparison to the observed chemical shift of the H4 proton on the UDP-4-amino-sugar (*18*), the H4 apparent triplet shifted to a more deshielded position of 3.69 ppm from 3.11 ppm (Figure 7,

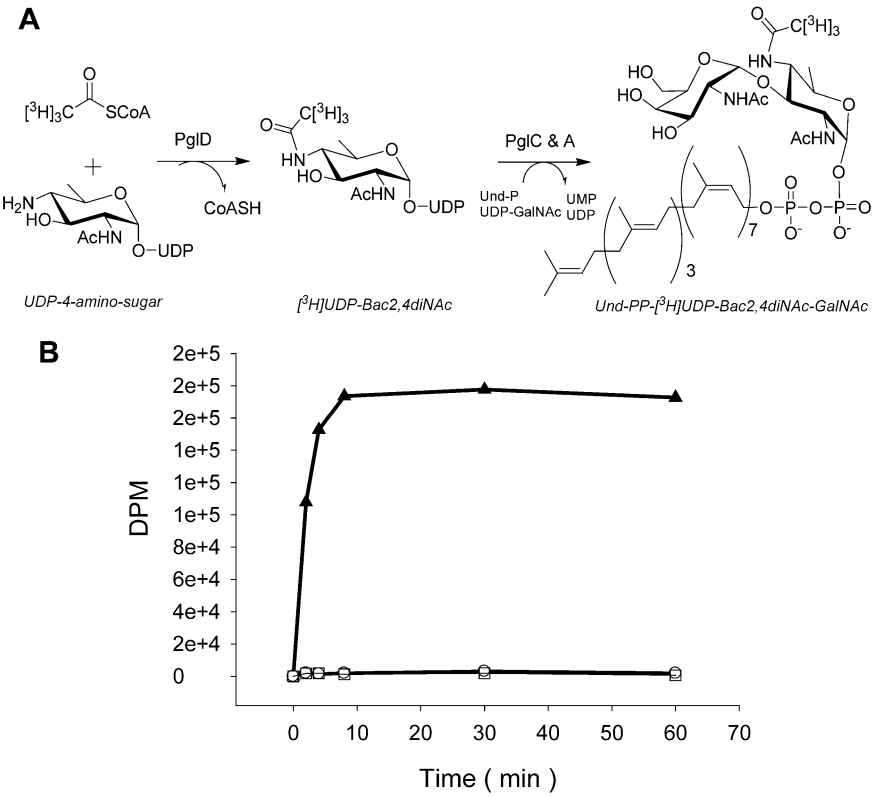

FIGURE 6: (A) Reaction scheme for the transfer of a ^{3}H-labeled acetyl group to UDP-4-amino and onto Und-P by the enzymes PglD and PglC. PglA adds GalNAc to Und-PP-[^{3}H]Bac. Aliquots of the reaction were taken over time, and the organic phase was subjected to liquid scintillation counting. (B) Plot of the liquid scintillation counts. Reactants included UDP-GalNAc, [^{3}H]AcCoA, Und-P, and UDP-4-amino (▲). Control reactions were without Und-P (○) or UDP-4-amino (□).

inset). Using this spectrum, the $J_{1''2''}$, $J_{2''3''}$, $J_{3''4''}$, $J_{4''5''}$, and $J_{5''6''}$ coupling constants were measured and are listed in Table 2. The coupling constant values were essentially identical to published values for the UDP-4-amino-sugar; hence, the UDP-Bac2,4diNAc glucopyranose ring has the same stereochemistry. The ^{31}P spectrum (Figures S-2 and S-3 of the Supporting Information) and chemical shift assignments for uracil and ribose moiety protons (Table 2) matched published values for UDP-sugars (*18*, *29*). On the basis of the results presented here, the product of PglD was identified as UDP-2,4-diacetamido-2,4,6-trideoxy-α-D-glucopyranose.

Kinetic Analysis of PglF and PglD. GST−PglF and PglD appeared to be suitable for analysis by Michaelis−Menten kinetics since the reaction velocities were observed to plateau at substrate concentrations below 20 and 2 mM, respectively, in the presence of 200 μM NAD$^+$ for GST−PglF and 2 mM AcCoA for PglD. Product formation velocities were averaged from two reactions run in parallel for each substrate concentration. Observed values were plotted and kinetic parameters derived with SigmaPlot (Systat Software, Inc.). Apparent kinetic parameters for GST−PglF were as follows: $K_m = 7.0 \pm 1.4$ mM, and $k_{cat} = 7.1 \pm 0.53$ min^{-1}. Apparent kinetic parameters for PglD were as follows: $K_m = 0.41 \pm 0.078$ mM, and $k_{cat} = (4.83 \pm 0.30) \times 10^5$ min^{-1}.

Coupled Reaction with PglF, -E, -D, -C, -A, -J, -H, and -I. We noted inhibition of GST−PglF$_{130}$ dehydratase activity on UDP-GlcNAc in the presence of PglE (data not shown). This observation suggested a physical interaction between the distinct enzymes, as reported by Logan and co-workers using a PglF$_{130}$ construct with a C-terminal His tag only (18). We coupled the reaction of GST−PglF with PglE and analyzed the products by CE. A peak eluting ahead of the UDP-GlcNAc peak was observed, consistent with the CE migration of the UDP-4-amino sugar (Figure S-4 of the Supporting Information). To confirm the UDP-4-amino sugar was indeed formed in the reaction, GST−PglF and PglE were combined with UDP-GlcNAc, PLP, and L-Glu. The reaction mixture was incubated overnight, and the reaction product was purified by anion exchange chromatography. ESI-MS analysis of the purified material showed a prominent mass peak of 588.5 (Figure 5c). Because PglE specifically uses the UDP-4-keto-sugar as its primary substrate, we conclude the product of GST−PglF is indeed the UDP-4-keto-sugar.

Using purified Pgl enzymes from *C. jejuni* NCTC 11168, our lab has investigated the in vitro transfer of *N*,*N*′-diacetylbacillosamine phosphate onto Und-P and the in vitro enzymatic synthesis of the heptasacccharide using a chemically synthesized undecaprenyl pyrophosphate-linked *N*,*N*′-diacetylbacillosamine (*15*, *17*). With UDP-GalNAc, UDP-Glc, Und-P, and chemically synthesized UDP-Bac-2,4-NAc at the start, the reactions of PglC, PglA, PglH, PglJ, and PglI have been coupled in a single reaction vessel to synthesize the bacterial glycan heptasaccharide (*15*). We wanted to confirm the presence of GST−PglF, PglE, and PglD in the same reaction vessel did not inhibit the downstream reactions. In the absence of chemically synthesized UDP-Bac-2,4-NAc, we combined the required substrates and cofactors for the reaction of each specific enzyme to proceed. The polyisoprenepyrophosphate-linked glycan was extracted from the organic phase and subsequently released from the polyisoprene by trifluoroacetic acid treatment and labeled with the fluorophore 2-aminobenzamide

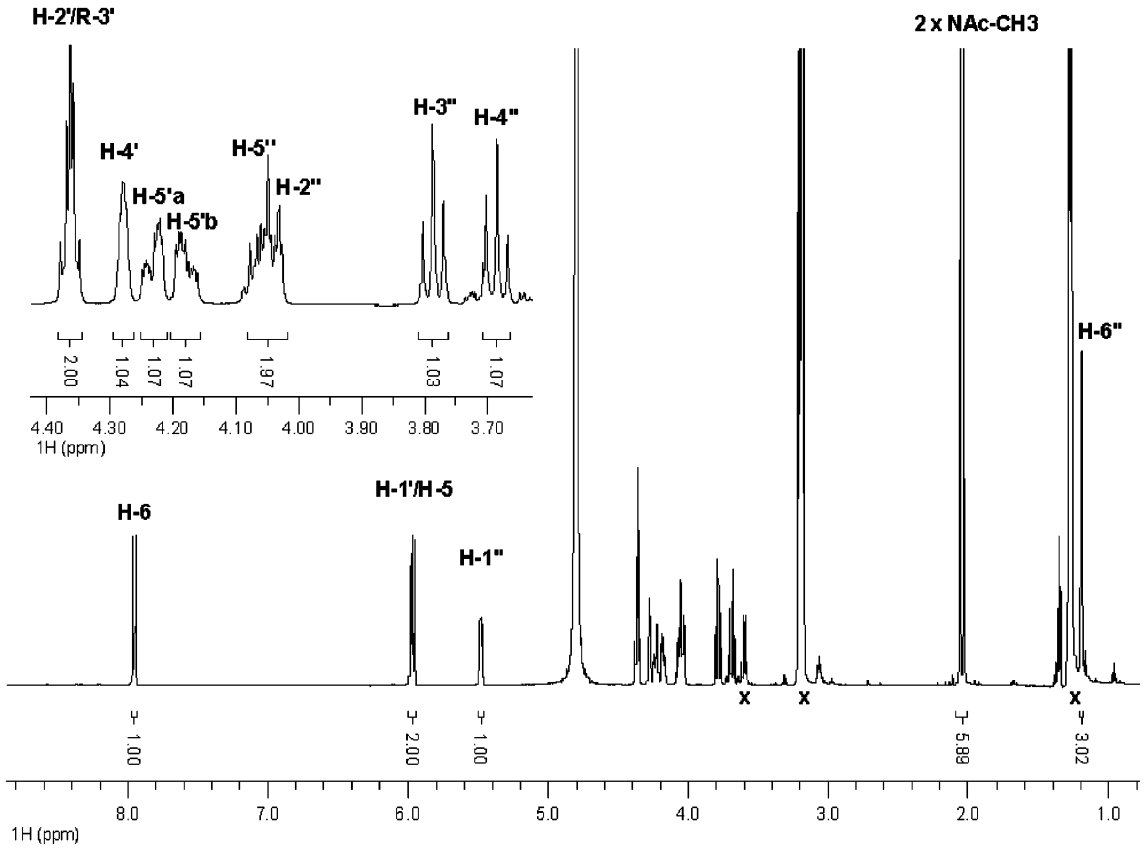

FIGURE 7: ^{1}H NMR spectrum of enzymatically synthesized UDP-Bac. Proton chemical shift labels follow the scheme represented in Figure 2 and values in Table 2. Peaks marked with an x indicate signals from impurities and TEAB buffer. Peak surface and integration values are also indicated. The inset enlarges the view of proximally occurring chemical shifts from ribose and pyranose ring moieties.

(2AB) at the C1 position of the diacetamido bacillosamine residue through reductive amination. HPLC analysis yielded a peak consistent with the known elution profile of the heptasaccharide, and the identity of the eluted material was confirmed by MALDI-MS (Figure 8).

DISCUSSION

UDP-GlcNAc is modified by PglF to form the UDP-4-keto-sugar substrate of PglE in the prokaryotic glycoprotein N-linked heptasaccharide biosynthesis pathway (*18*). In *C. jejuni*, there are distinct metabolic pathways that share UDP-GlcNAc as a substrate, thus suggesting a rationale for the reaction kinetics observed in vitro. Pseudaminic acid, the major modification of flagellin, is synthesized via the UDP-GlcNAc dehydratase PseB pathway (*18*, *30*). GalNAc, a major component in the biosynthesis of glycoprotein N-linked heptasaccharide, lipooligosaccharide (LOS), and capsule (*7*, *8*, *10*), is synthesized intracellularly by the C4 epimerase Gne (*31*). Table 3 lists the kinetic parameters of PseB and Gne derived from other studies, as well as those for GST−PglF from this study. Clearly, Gne is the most efficient of all three enzymes with a k_{cat}/K_m greater by 2 orders of magnitude than those of PseB and GST−PglF. This is to be expected since one or more copies of GalNAc/Gal or subsequent derivatives occur more frequently on cell surface structures (heptasaccharide, LOS, capsule) than GlcNAc/Glc-related glycosides. The k_{cat}/K_m value of PseB indicates this enzyme is a moderately effective dehydratase, more so than GST−PglF, by a factor of 30. However, *pgl*

Table 2: ^{1}H and ^{31}P NMR Chemical Shift and Coupling Constant Assignments for UDP-Bac[a]

moiety	δ_H (ppm)	J
uracil		
H5	5.97 (d)	$J_{5',6'} = 8.1$ Hz
H6	7.95 (d)	
ribose		
H1′	5.97 (d)	$J_{1',2'} = 4.5$ Hz
H2′	∼4.37	
H3′	∼4.37	
H4′	4.28 (m)	
H5′a	4.18 (ddd)	$J_{5'a,P} = 5.5$ Hz
		$J_{5'a,4'} = 3.0$ Hz
		$J_{5'a,5'b} = 11.7$ Hz
H5′b	4.23 (ddd)	$J_{5'b,P} = 4.3$ Hz
		$J_{5'b,4'} = 2.6$ Hz
pyranose		
H1″	5.48 (dd)	$J_{1'',2''} = 3.2$ Hz
		$J_{1'',P} = 6.9$ Hz
H2″	4.02 (m)	
H3″	3.79 (at)	$J_{2'',3''} = 10.2$ Hz
		$J_{3'',4''} = 10.2$ Hz
H4″	3.69 (at)	$J_{4'',5''} = 10.2$ Hz
H5″	4.05 (m)	
H6″	1.19 (d)	$J_{5'',6''} = 6.2$ Hz
acetyl		
CH_3	2.06 (s)	
CH_3	2.04 (s)	
pyrophosphate		
P_a	−10.84 (d)	
P_b	−12.66 (d)	

[a] Data collected at 25 °C in D_2O, using 4.8 ppm for the solvent chemical shift: d, doublet; dd, doublet of doublets; ddd, doublet of doublets of doublets; m, multiplet; at, apparent triplet; s, singlet.

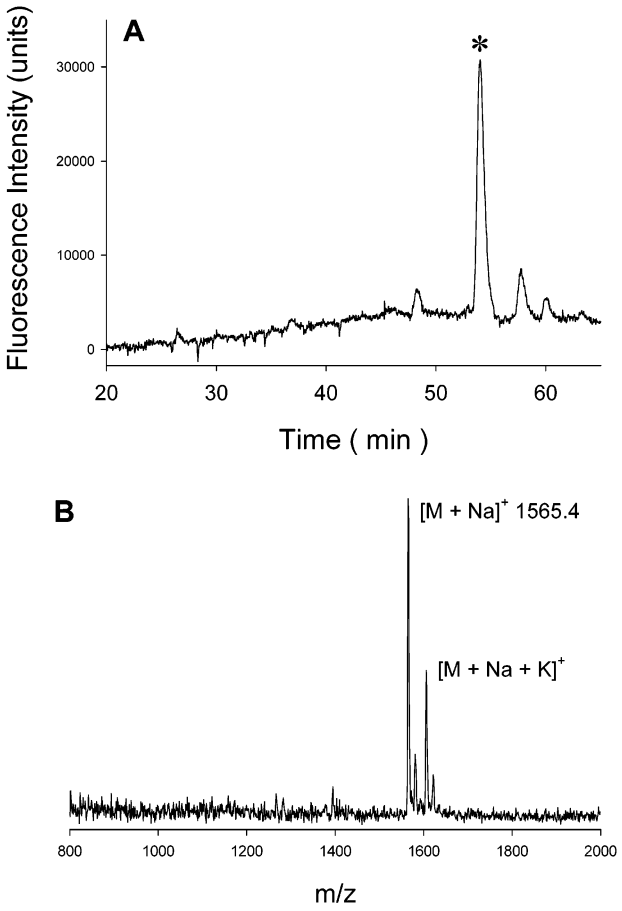

FIGURE 8: (A) Fluorescence trace from HPLC analysis of 2AB-labeled heptasaccharide isolated from a reaction that coupled PglF, -E, -D, -C, -A, -J, and -H and the cell envelope fraction of PglI in one reaction vessel. The asterisk denotes the major saccharide peak. (B) MALDI-MS of eluted material.

gene knockout studies on *C. jejuni* NCTC 11168 showed a significant disparity between wild-type and mutant strain signatures of the high-resolution magic angle spinning NMR spectra and whole-cell lysate blotting with lectins (*32*). These results indicate the substrate turnover rate of PseB is insufficient to complement the wild-type consumption levelsof the UDP-4-keto-sugar by the Pgl pathway in the absence of PglF. Comparison of the kinetics data, together with the knockout study results, supports the hypothesis of a channeling mechanism where a sequential transfer of products between enzymes of the same biosynthetic pathway exists (*33*). Of the two dehydratases that are compared, PseB has the lowest K_m, indicating a higher affinity for substrate. Studies on PseB knockouts in *C. jejuni* strain 81-176 showed unglycosylated flagella accumulated in cells intracellularly, thus explaining a lack of motility (*30*). Arguably, when UDP-GlcNAc is in low abundance, the pathogen would likely channel available stores toward the function of motility over host cell interactions.

Using a combination of radiolabel transfer, NMR, and ESI-MS, we have shown that PglD catalyzes the acetyl group transfer from AcCoA to the UDP-4-amino-sugar, resulting in the formation of UDP-*N*,*N*′-diacetylbacillosamine (Figure 2). The proton NMR spectrum of UDP-Bac2,4diNAc shows a significant chemical shift of the H4 proton to a more deshielded position, consistent with the conversion of a primary amine at the C4 position of the pyranose ring to an amide (Figure 7). The k_{cat}/K_m of PglD is 1.18×10^6 min^{-1} mM^{-1}, indicating the enzyme is very efficient in catalyzing the acetyl group transfer to the UDP-4-amino sugar. An advantage to such a high degree of activity results in the rapid consumption of the UDP-4-amino-sugar, thereby driving the enzymatic conversion of UDP-GlcNAc by PglF to UDP-4-keto-sugar, the substrate of PglE. Comparing kinetic data of the Pgl enzymes in Table 3 indicates the rate-limiting step in UDP-Bac2,4diNAc biosynthesis is the formation of the UDP-4-keto-sugar.

In vitro biosynthesis of *N*,*N*′-diacetylbacillosamine and its precursors by enzymes of the Pgl pathway broadens the scope for potential applications. The *C. jejuni* oligosaccharyl transferase, PglB, was shown to prefer a diacetylated sugar linked to the polysioprenepyrophosphate for glycosylation of peptides when the transferred sugar was a disaccharide (*34*). As the structure−function relationship between Pgl enzymes and respective substrates becomes more defined, the potential to derive novel substrate analogues arises. Additionally, the capacity to synthesize milligram quantities of UDP-Bac2,4diNAc relatively inexpensively brings forth a new biologically relevant molecule that is suitable for modification by chemical means for probing biological processes. Because enzyme quantities were used in excess to ensure complete substrate turnover, the in vitro reaction conditions may be optimized further to improve enzyme efficiency on a preparative scale. A more specific application is the use of purified UDP-Bac2,4diNAc to study protein modification pathways in other pathogens such as the *Neisseria* species, known to synthesize a diacetylated sugar for pilin glycosylation (*35*).

Successful coupling of reactions by PglE and GST−PglF suggests the involvement of the PglF transmembrane domain in preventing the inhibitory reaction observed between PglE and the soluble catalytic domain of PglF (*18*). Addition of the GST tag to the full-length PglF was critical for enhancing expression levels and simplifying the purification process. Reconstitution of heptasaccharide synthesis in vitro with full-

Table 3: Kinetic Parameters for GST−PglF and PglD Compared with Published Values of PglE and Gne

enzyme	substrate	protein (pmol/assay)	$K_{m(app)}$ (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})
GST−PglF[a]	UDP-GlcNAc	20.7	7.00	7.1	1.0
PglD[a]	UDP-4-amino	0.011	0.41	4.83×10^5	1.18×10^6
PglE[b]	UDP-4-keto	3.32	0.048	144	3000
MalE−Gne[c]	UDP-GlcNAc	0.083	1.087	4938	4543
PseB[d]	UDP-GlcNAc	25.8	0.05	1.5	30.6

[a] In this study, values are the average of two experiments and assays were conducted at 37 °C and pH 7.7. [b] From *C. jejuni* NCTC 11168, assays conducted at 37 °C and pH 7.2 (*18*). [c] From *C. jejuni* NCTC 11168, assays conducted at 37 °C and pH 8.0 (*31*). [d] From *C. jejuni* subspecies *doylei* ATCC49349, assays conducted at 42 °C and pH 7.0 (*33*).

length isolated proteins clearly demonstrates all eight *pgl* enzymes are able to perform their respective functions in a single reaction vessel. Because substrate turnover was not observed in a reaction coupling GST−$PglF_{130}$ and PglE, we believe the presence of the GST tag fused to PglF was not responsible for disruption of inhibition in the coupled assay with full-length PglF and PglE. Experiments currently underway in our lab focus on the characterization of the presumed physical interaction between PglF and other enzymes of the Pgl pathway.

In summary, the evidence presented in this report identifies PglD as an acetyltransferase that catalyzes the last step in a three-step biosynthetic reaction of UDP-Bac2,4diNAc from UDP-GlcNAc. This enzyme proved to be very efficient and may act as the engine that drives the metabolic conversion of UDP-GlcNAc to UDP-Bac2,4diNAc. The in vitro enzymatic synthesis of UDP-Bac2,4diNAc provides a straightforward method for producing milligram quantities of this material. In turn, this allows for a shift of attention toward investigating the mechanism of N-linked protein glycosylation and further the development of a bacterial protein glycosylation system for applications in biotechnology. We also report that as a GST fusion protein, the dehydratase PglF, functioned in a coupled reaction with enzymes of the *pgl* locus to synthesize the undecaprenylpyrophosphate heptasaccharide in vitro. This result suggests a greater role for the PglF transmembrane domain in the bacterial glycan biosynthesis other than tethering of the catalytic domain of PglF to the lipid bilayer.

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SUPPORTING INFORMATION AVAILABLE

Homonuclear decoupling spectra of UDP-Bac (Figure S-1), ^{31}P NMR spectrum of UDP-Bac2,4diNAc (Figure S-2), ^{1}H COSY spectrum of UDP-Bac2,4diNAc (Figure S-3), and CE A_{262} elution profile of the coupled reaction between PglF and PglE (Figure S-4). This material is available free of charge via the Internet at http://pubs.acs.org.

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