

Biosynthesis of UDP-*N*-acetyl-L-fucosamine, a Precursor to the Biosynthesis of Lipopolysaccharide in *Pseudomonas aeruginosa* Serotype O11*

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UDP-*N*-acetyl-L-fucosamine is a precursor to L-fucosamine in the lipopolysaccharide of *Pseudomonas aeruginosa* serotype O11 and the capsule of *Staphylococcus aureus* type 5. We have demonstrated previously the involvement of three enzymes, WbjB, WbjC, and WbjD, in the biosynthesis of UDP-2-acetamido-2,6-dideoxy-L-galactose or UDP-*N*-acetyl-L-fucosamine (UDP-L-FucNAc). An intermediate compound from the coupled-reaction of WbjB-WbjC with the initial substrate UDP-2-acetamido-2-deoxy- α -D-glucose or UDP-*N*-acetyl-D-glucosamine (UDP-GlcNAc) was purified, and the structure was determined by NMR spectroscopy to be UDP-2-acetamido-2,6-dideoxy-L-talose (UDP-L-PneNAc). WbjD could then convert this intermediate into a new product with the same mass, consistent with a C-2 epimerization reaction. Those results led us to propose a pathway for the biosynthesis of UDP-L-FucNAc; however, the exact enzymatic activity of each of these proteins has not been defined. Here, we describe a fast protein liquid chromatography (FPLC)-based anion-exchange procedure, which allowed the separation and purification of the products of C-2 epimerization due to WbjD. Also, the application of a cryogenically cooled probe in NMR spectrometry offers the greatest sensitivity for determining the structures of minute quantities of materials, allowing the identification of the final product of the pathway. Our results showed that WbjB is bifunctional, catalyzing firstly C-4, C-6 dehydration and secondly C-5 epimerization in the reaction with the substrate UDP-D-GlcNAc, producing two intermediates. WbjC is also bifunctional, catalyzing C-3 epimerization of the second intermediate followed by reduction at C-4. The FPLC-based procedure provided good resolution of the final product of WbjD reaction from its epimer/substrate UDP-L-PneNAc, and the use of the cryogenically cooled probe in NMR revealed unequivocally that the final product is UDP-L-FucNAc.

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The biosynthesis of lipopolysaccharide (LPS)¹ O antigen involves synthesizing di- to hexasaccharide repeating units from sugar nucleotide precursors and further polymerizing these units into long chain polysaccharides (1). *Pseudomonas aeruginosa* is known to co-produce two forms of LPS simultaneously, namely, A band, which is homopolymeric, and B band, which is heteropolymeric (2). Differences in the constituent sugars and linkages within the heteropolymeric repeating structure distinguish *P. aeruginosa* into 20 different serotypes (3, 4). *P. aeruginosa* LPS is involved in protecting the bacterium from phagocytosis (5) as well as serum-mediated killing (6). LPS mutants lacking the O antigen are 1000 times less virulent than a wild-type bacteria in an animal model study (7).

The B-band O antigen of *P. aeruginosa* serotype O11 is a trisaccharide repeating unit composed of L-FucNAc, D-FucNAc, and D-glucose (8). L-FucNAc is exclusively a component of bacterial polysaccharides and has not been found elsewhere in nature. It is a constituent of the LPS of *P. aeruginosa* serotype O4, O11, and O12 as well as *Escherichia coli* O26 (8, 9). It is also a component of the capsule of *Staphylococcus aureus* type 5 and 8 and *Streptococcus pneumoniae* type 4 (10–12). Importantly, *P. aeruginosa* O4 and O11 and *S. aureus* type 5 and 8 are among the most commonly isolated bacteria from clinical sources (13, 14). Therefore, a better understanding of the enzymes involved in the biosynthesis of the sugar nucleotide precursor, UDP-L-FucNAc, would be an important step toward the development of novel antimicrobial agents.

Recently, our group demonstrated the involvement of three enzymes (namely, WbjB, WbjC, and WbjD in *P. aeruginosa* O11 and homologous proteins Cap5E, Cap5F, and Cap5G in *S. aureus* type 5) in the biosynthesis of UDP-L-FucNAc (15). A knock-out mutation in the gene encoding the first enzyme of the pathway (WbjB) caused the bacterium to produce LPS that was completely devoid of B band. Subsequent biochemical characterization led us to propose the involvement of WbjB in catalyzing the first three reactions from UDP-D-GlcNAc, which are C-4, C-6 dehydration; C-5 epimerization; and C-3 epimerization. Accordingly, this enzyme would produce three interme-

¹ The abbreviations used are: LPS, lipopolysaccharide; CE, capillary electrophoresis; HMQC, heteronuclear multiple quantum coherence; HSQC, heteronuclear single quantum coherence; NOESY, nuclear Overhauser enhancement spectroscopy; TOCSY, total correlation spectroscopy; FPLC, fast protein liquid chromatography; MES, 4-morpholineethanesulfonic acid; D-FucNAc, 2-acetamido-2,6-dideoxy-D-galactose; UDP-D-GlcNAc, UDP-2-acetamido-2-deoxy- α -D-glucose or UDP-*N*-acetyl-D-glucosamine; UDP-L-FucNAc, UDP-2-acetamido-2,6-dideoxy-L-galactose or UDP-*N*-acetyl-L-fucosamine; UDP-L-PneNAc, UDP-2-acetamido-2,6-dideoxy-L-talose; UDP-L-QuiNAc, UDP-2-acetamido-2,6-dideoxy-L-glucose; UDP-L-RhaNAc, UDP-2-acetamido-2,6-dideoxy-L-mannose; UDP-D-ManNAc, UDP-D-mannosamine.

diates; however, only two product peaks were observed when the WbjB reactions with the substrate (UDP- α -D-GlcNAc) were analyzed by capillary electrophoresis (CE). Using CE coupled to mass spectrometry (CE-MS, CE-tandem MS) and NMR, the first two keto-intermediates were determined to be 2-acetamido-2,6-dideoxy-4-hexuloses (15). The possibility of WbjB catalyzing the first and second reactions only and WbjC catalyzing the third and fourth reactions was suggested but not explored in the previous work.

CE analysis of the coupled WbjBC reaction with UDP-D-GlcNAc, NADP⁺, and NADPH showed a reduction in the two intermediate peaks as well as the formation of a new peak that migrated at the same position as UDP-D-GlcNAc (15). This new peak was identified as UDP-2-acetamido-2,6-dideoxy- β -L-talose (UDP- β -L-PneNAc) (15). Coupling the reactions of all three enzymes (WbjBCD) yielded a new, unidentified peak shown by CE. CE-tandem mass spectrometry analysis showed that the product of this reaction had a mass identical to that of UDP- β -L-PneNAc; however, attempts to determine the structure of this new product by NMR were unsuccessful due to its inherent instability (15).

In this study we have optimized conditions for the production and purification of the final product of this pathway and conclusively showed the structure to be UDP-L-FucNAc. Moreover, we have refined the CE-based analysis of the enzyme-substrate reactions of WbjB, WbjC, and WbjD, respectively, and elucidated the role of these three enzymes in the pathway. WbjC acts on the second intermediate and most likely retains this intermediate in the active site until it can be reduced to UDP- β -L-PneNAc. These results demonstrate a novel pathway in which separate C-5 and C-3 epimerase enzymes act on nucleotide-activated carbohydrates.

EXPERIMENTAL PROCEDURES

Materials—All reagents and antibiotics were obtained from Sigma-Aldrich Canada (Oakville, Ontario, Canada) unless otherwise stated. Difco Bacto™ Tryptone and Difco Bacto™ Yeast Extract were obtained from Fisher Scientific Ltd. (Nepean, Ontario, Canada). All aqueous solutions were prepared using ultrapure water obtained from a Super-Q™ water system (Millipore, Nepean, Ontario, Canada). Econo-Pac High Q columns were used in anion-exchange chromatography for the purification of nucleotide-activated sugars (Bio-Rad).

Overexpression of WbjB, WbjC, and WbjD—pFuc11, pFuc12, and pFuc13 encoding the N-terminal histidine-tagged fusion proteins WbjB, WbjC, and WbjD, respectively, from *P. aeruginosa* serotype O11 were used to produce these proteins as described previously (15). These constructs were transformed into *E. coli* Rosetta™(DE3) (Novagen-EMD Biosciences, San Diego, CA) using 34 μ g/ml chloramphenicol and either 50 μ g/ml kanamycin (WbjB and WbjC) or 100 μ g/ml ampicillin (WbjD) for selection. For expression, 250 ml of Terrific broth (16), containing the appropriate antibiotics, was inoculated with 5 ml of overnight culture and grown at 37 °C. When A₆₀₀ reached 0.6, expression was induced by adding isopropyl 1-thio- β -D-galactopyranoside (Invitrogen) to a final concentration of 0.4 mM. Expression was carried out for 4 h at 37 °C. Cells were harvested by centrifugation at 5000 \times g, and the pellets were stored at -20 °C.

Purification of WbjB, WbjC, and WbjD—Following induction of protein expression, cell pellets were resuspended in 10 ml of binding buffer (50 mM sodium phosphate, pH 8.0, 300 mM sodium chloride) and disrupted on ice by ultrasonication. Cell debris and membrane fractions were removed by ultracentrifugation at 175,000 \times g for 1 h. Purifications were carried out using either HiTrap Chelating columns (Amersham Biosciences) according to the method of Kneidinger *et al.* (15) or His-Bind® Quick 900 Cartridges (Novagen-EMD Biosciences) according to the following procedure: after equilibration with 10 ml of binding buffer, soluble cell extracts were loaded onto the cartridge. The cartridge was rinsed with 20 ml of binding buffer followed by a wash with 20 ml of binding buffer containing 60 mM imidazole. The protein was eluted using 10 ml of binding buffer containing 300 mM imidazole. Purified protein was stored for up to 2 weeks at -20 °C in elution buffer containing 40% glycerol and 1 mM dithiothreitol. The purity of the enzymes was verified by SDS-PAGE performed according to the proce-

dures of Laemmli (17) on a 12.5% running gel. Gels were stained with Coomassie Brilliant Blue R-250, and protein concentrations were determined by the method of Bradford (18).

Analysis of UDP-L-FucNAc Biosynthesis by CE—Standard reactions were performed and analyzed by CE as described previously (15), with some modifications. For determination of the pH optima of the coupled reaction, the following buffers were used at a final concentration of 20 mM: sodium citrate (pH 5.0), MES (pH 6.0), HEPES (pH 7.0), Tris-HCl (pH 8.0), and Bis-Tris-propane (pH 9.0 and 10.0). To determine the function of WbjB and WbjC, reactions containing only the first enzyme (WbjB) were incubated for 1 h at 37 °C followed by the removal of the enzyme by ultrafiltration through a Microcon YM-10 cartridge with a molecular weight cut off of 10,000 (Millipore, Nepean, Ontario, Canada). Either ~1 μ g of WbjC or ~1 μ g each of WbjC and WbjD plus an excess of the hydride donor, 0.75 mM NADPH, was added to 60 μ l of the filtrate. The reaction was incubated for an additional 1 h at 37 °C, and the reaction products were analyzed by CE. For structure elucidation, reactions were performed containing 1.5 mM of the substrate UDP-D-GlcNAc.

Purification of the WbjBCD Reaction Products—Nine milliliters of the reaction mix, prepared by combining 15–600 μ l of standard reactions, was purified by anion-exchange FPLC using an Econo-Pac High Q column with a bed volume of 5 ml. For separation of the epimers, the purification was performed according to the method described by Miller *et al.* (19), with some modifications. The column was first equilibrated with 240 mM triethylammonium bicarbonate (pH 8.0) for 5 column volumes immediately prior to elution with a linear gradient of 240–400 mM over 25 column volumes using a flow rate of 1 ml/min. One-milliliter fractions were collected and analyzed by CE. For structural studies of the reaction intermediates and products, the purification of these compounds was performed with a linear gradient of 0–250 mM ammonium bicarbonate (pH 7.7). Fractions containing sugar nucleotides were pooled and lyophilized to dryness for analysis by NMR spectroscopy. At present, quantitative analysis of the sugar nucleotides that were purified in this study could not be achieved due to a lack of available standards for comparison.

Sample Preparation and NMR Analysis of the WbjBCD Reaction Products—The lyophilized sample containing UDP-L-PneNAc and UDP-L-FucNAc was resuspended in 150 μ l of 98% D₂O buffered with NH₄HCO₃ (54 mM, pD 8.6) and analyzed by NMR spectroscopy. Proton, ¹³C HSQC, HMBC, and selective one-dimensional TOCSY and NOESY NMR experiments were performed at 600 MHz (¹H) using a 5 mm Z gradient triple resonance cryogenically cooled probe (Varian). The methyl resonance of acetone was used as an internal reference at δ_{H} 2.225 ppm and δ_{C} 31.07 ppm. The ³¹P HMQC experiment was performed using a Varian Inova 500 MHz spectrometer equipped with a Z-gradient 3 mm triple resonance (¹H, ¹³C, and ³¹P) probe. The ³¹P spectrum was acquired on a 200 MHz (¹H) spectrometer. External 85% phosphoric acid was used as a reference (δ_{P} 0 ppm). Experiments were performed at 25 °C. Proton detected experiments were acquired with suppression of the deuterated H₂O resonance at 4.78 ppm. Standard homo- and heteronuclear correlated two-dimensional pulse sequences from Varian, correlation spectroscopy, HSQC, HMBC, and ³¹P HMQC were used for general assignments. Selective one-dimensional TOCSY experiments with a Z-filter and one-dimensional NOESY experiments were performed for complete residue assignment and for the determination of $J_{(\text{H-H})}$ coupling constants obtained by spin simulation of the spectra. Mixing times of 50–80 ms were used for one-dimensional TOCSY and TOCSY-TOCSY experiments, and a mixing time of 800 ms was used for the one-dimensional NOESY experiments (15, 20, 21).

RESULTS

Expression and Purification of WbjB, WbjC, and WbjD—Following overexpression, the majority of the protein was found in the soluble fraction. The proteins were purified to near homogeneity, and the yield was ~5 mg of each protein from 250 ml of bacterial culture.

pH Optima for the WbjBCD-coupled Reaction—To maximize the yield of the final product (UDP- β -L-FucNAc) and facilitate NMR analysis, it was necessary to determine the optimum pH for the coupled reactions involving all three proteins, WbjB, WbjC, and WbjD. When coupled reactions were performed, less degradation of the keto-intermediates was observed than in the sequential reactions, as evidenced by the amount of UDP present in the reaction visualized by CE (data not shown). At pH

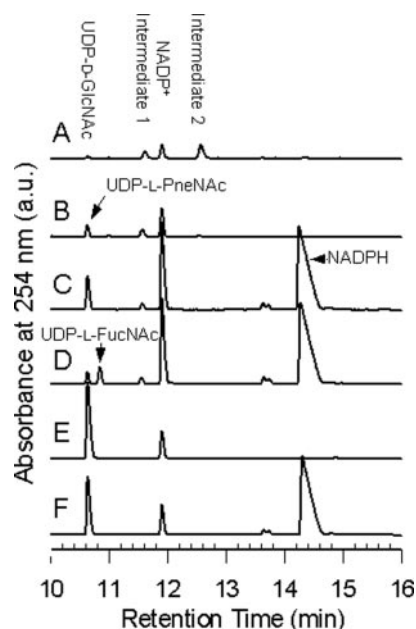


FIG. 1. Capillary electrophoresis analysis of the reaction mechanisms of WbjB and WbjC. Note that WbjB was completely removed from all the reaction mixtures before any analysis or further reactions were performed. A, UDP-D-GlcNAc converted with WbjB and NADP⁺; B, products from the reaction in A were incubated with WbjC without the addition of NADPH. C, products of the reaction in A were incubated with WbjC and NADPH. D, products of the reaction in A were converted with WbjC, WbjD, and NADPH. E, WbjB reaction mixture without the substrate UDP-D-GlcNAc. The mixture was spiked with UDP-D-GlcNAc after removal of WbjB. F, products of the reaction in E spiked with WbjC, WbjD, and NADPH. Intermediate 1, UDP-2-acetamido-2,6-dideoxy- α -D-xylo-4-hexulose. Intermediate 2, UDP-2-acetamido-2,6-dideoxy- β -L-arabino-4-hexulose.

9.0, the C-2 epimerization reaction catalyzed by WbjD favored the production of UDP-L-FucNAc over UDP-L-PneNAc as observed in the electropherogram (data not shown).

Analysis of the Enzymatic Activities of WbjB and WbjC—WbjB-catalyzed reactions were incubated for 1 h, and the enzyme was then removed by centrifugation through a Microcon YM-10 filter cartridge. The two keto-intermediates present in the filtrate were then used as the substrate for the WbjC reaction (in both the presence and absence of NADPH) and the WbjCD-catalyzed reactions, respectively (Fig. 1).

When WbjC was incubated with the WbjB reaction filtrate without addition of NADPH (Fig. 1A), the peak corresponding to intermediate 2 (UDP-2-acetamido-2,6-dideoxy- β -L-arabino-4-hexulose) was completely utilized, and only a small amount of UDP- β -L-PneNAc was produced (Fig. 1B). Upon addition of WbjC and NADPH to the keto-intermediates, an increase in the peak corresponding to UDP-L-PneNAc, which migrates at the same position as UDP-D-GlcNAc, could be discerned (Fig. 1C). When both WbjC and WbjD were added to the filtrate plus NADPH, a new peak appeared that migrated at the same location as the previously reported UDP-L-FucNAc peak (Fig. 1D).

To ascertain that the enzyme was effectively removed in our procedures, control reactions were performed involving all the components of the WbjB reaction mixture without the substrate, UDP-D-GlcNAc. The enzyme was then removed by the method mentioned above, and UDP-D-GlcNAc was added to the filtrate. The conversion of UDP-D-GlcNAc to intermediates or to products in the presence of UDP-D-GlcNAc alone (Fig. 1E) or UDP-D-GlcNAc, WbjC, WbjD, and NADPH (Fig. 1F) was not observed. Based on these results, the method for removal of the enzyme was deemed effective; therefore, con-

version of intermediate 2 was clearly attributed to WbjC and was not due to the presence of residual WbjB in the filtrate.

Purification of UDP-L-PneNAc and UDP-L-FucNAc by Anion-exchange Chromatography—Separation of the epimers was achieved by anion-exchange chromatography to yield two major peaks (Fig. 2A). The peak that eluted first contained a shoulder region and was determined to be the sugar nucleotide mixture by subsequent analysis of the individual fractions by capillary electrophoresis (Fig. 2B). The last two fractions of the shoulder region contained only UDP- β -L-FucNAc. Therefore, the two peaks were successfully resolved. The second major peak, which eluted later, was determined to be NADP⁺ by comparison with a NADP⁺ standard using CE (data not shown).

Identification of Reaction Product by NMR—One- and two-dimensional NMR experiments provided unequivocal evidence of the identity of the intermediate and reaction product. ¹H-NMR analysis confirmed the presence of UDP-L-PneNAc (15) and UDP-L-FucNAc (Figs. 3 and 4A) in the sample. Despite the labile nature of sugar nucleotides, ¹H-¹³C HSQC and HMBC experiments permitted the assignment of ¹³C resonances, and TOCSY, correlation spectroscopy, and selective one-dimensional TOCSY and NOESY experiments were used to assign proton resonances and accurately determine $J_{(H-H)}$ coupling constants for UDP-L-PneNAc and UDP-L-FucNAc that were later confirmed by spin simulations of the spectra (Table I).

Selective one-dimensional TOCSY-TOCSY analysis of UDP-L-PneNAc H-1 and H-2 resonances at 5.26 and 4.41 ppm, respectively, revealed spin-coupled resonances at 3.97 and 3.74 ppm belonging to UDP-L-PneNAc H-3 and H-4, respectively (Fig. 4B) (15). A scalar coupling $J_{1,2}$ of 1.5 Hz indicated that H-1 was *gauche* to H-2 and was consistent with previously reported data for UDP-L-PneNAc (15). One-dimensional NOESY analysis of the UDP-L-PneNAc H-1 proton at 5.26 ppm revealed nuclear Overhauser effects for resonances at 4.41, 3.97, and 3.79 ppm belonging to UDP-L-PneNAc H-2, H-3, and H-5, respectively (15), and confirmed that this sugar had a β -configuration (Fig. 4C). Of importance, the strong nuclear Overhauser effect between H-1 and H-2 for UDP-L-PneNAc indicated that these protons exhibit a *cis* relationship.

One-dimensional TOCSY analysis of UDP-L-FucNAc H-1 proton at 5.01 ppm permitted the assignment of H-2, H-3, and H-4 for this reaction product at 3.94, 3.77, and 3.79 ppm, respectively (Fig. 4D). A $J_{1,2}$ of 8.6 Hz indicated that H-1 was *anti* to H-2 and pivotal in distinguishing the final reaction product, UDP-L-FucNAc, from its precursor UDP-L-PneNAc. One-dimensional NOESY analysis of UDP-L-FucNAc H-1 at 5.01 ppm demonstrated enhancements for UDP-L-FucNAc H-3 and H-5 protons at 3.77 and 3.84 ppm, respectively, and confirmed that this sugar had a β -configuration. Of particular importance, in contrast to UDP-L-PneNAc, the absence of a nuclear Overhauser effect between H-1 and H-2 for UDP-L-FucNAc confirmed that these protons were in fact axial. ³¹P HMQC analysis showed phosphorus correlations at $\delta_{P(\beta)}$ -12.8 ppm for UDP-L-PneNAc and $\delta_{P(\beta)}$ -12.4 ppm for UDP-L-FucNAc, with their respective anomeric resonances at 5.26 and 5.01 ppm (Fig. 4F). Multiple correlations in the ³¹P HMQC spectrum at $\delta_{P(\alpha)}$ -10.7 (UDP-L-PneNAc) and -10.6 ppm (UDP-L-FucNAc) with the H-5 resonances of ribose at 4.22 ppm (data not shown) were also observed (15). The ³¹P chemical shifts and $J_{P(\alpha),P(\beta)}$ of 18 Hz were characteristic of a pyrophosphate linkage. Clear carbon-proton correlations observed using a ¹H-¹³C HSQC experiment permitted complete carbon assignments for both for UDP-L-PneNAc and UDP-L-FucNAc

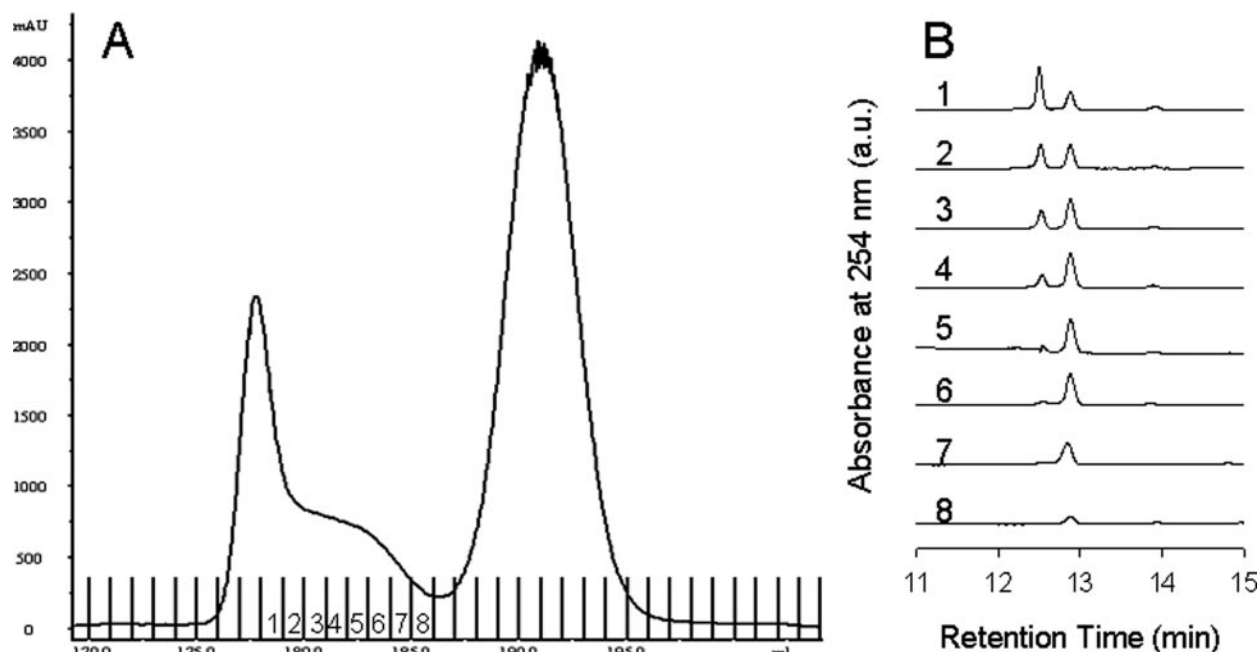


FIG. 2. Purification of UDP-L-PneNAc and UDP-L-FucNAc. A, chromatogram of the separation by FPLC anion-exchange chromatography. B, analysis of the individual fractions by CE.

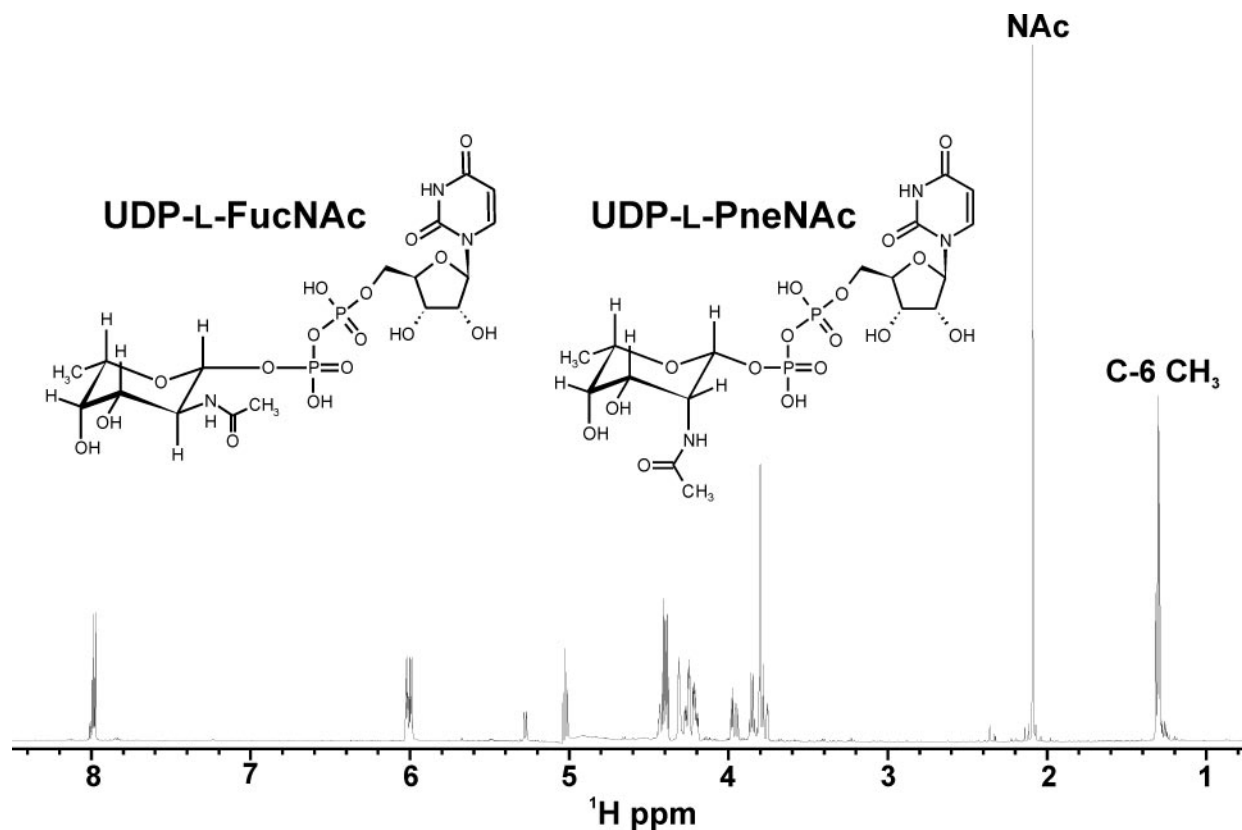


FIG. 3. ^1H NMR spectrum of a sample containing UDP-L-PneNAc and UDP-L-FucNAc. The spectrum acquired with eight transients was obtained for a sample dissolved in 98% D_2O (54 mM NH_4HCO_3 buffer, pH 8.6) at 600 MHz and 25 °C using a cryogenically cooled probe.

(Fig. 4G). Two- and three-bond proton-carbon correlations observed using an HMBC experiment permitted carbon assignments for the quaternary carbonyl NAc resonances of both compounds (data not shown).

DISCUSSION

In this report, we successfully elucidated the role of three enzymes, WbjB, WbjC, and WbjD, in the biosynthesis of the

sugar nucleotide UDP-L-FucNAc and developed optimized conditions for the production and identification of this product. Both WbjB and WbjC are members of the short chain dehydrogenase/reductase family (22) that contain either a Ser, Met, Lys (SMK in the case of WbjB) or Ser, Tyr, Lys (SYK in the case of WbjC) catalytic triad (15). The difference in enzyme activity conferred by the SMK catalytic triad compared with the SYK triad has been examined previously for

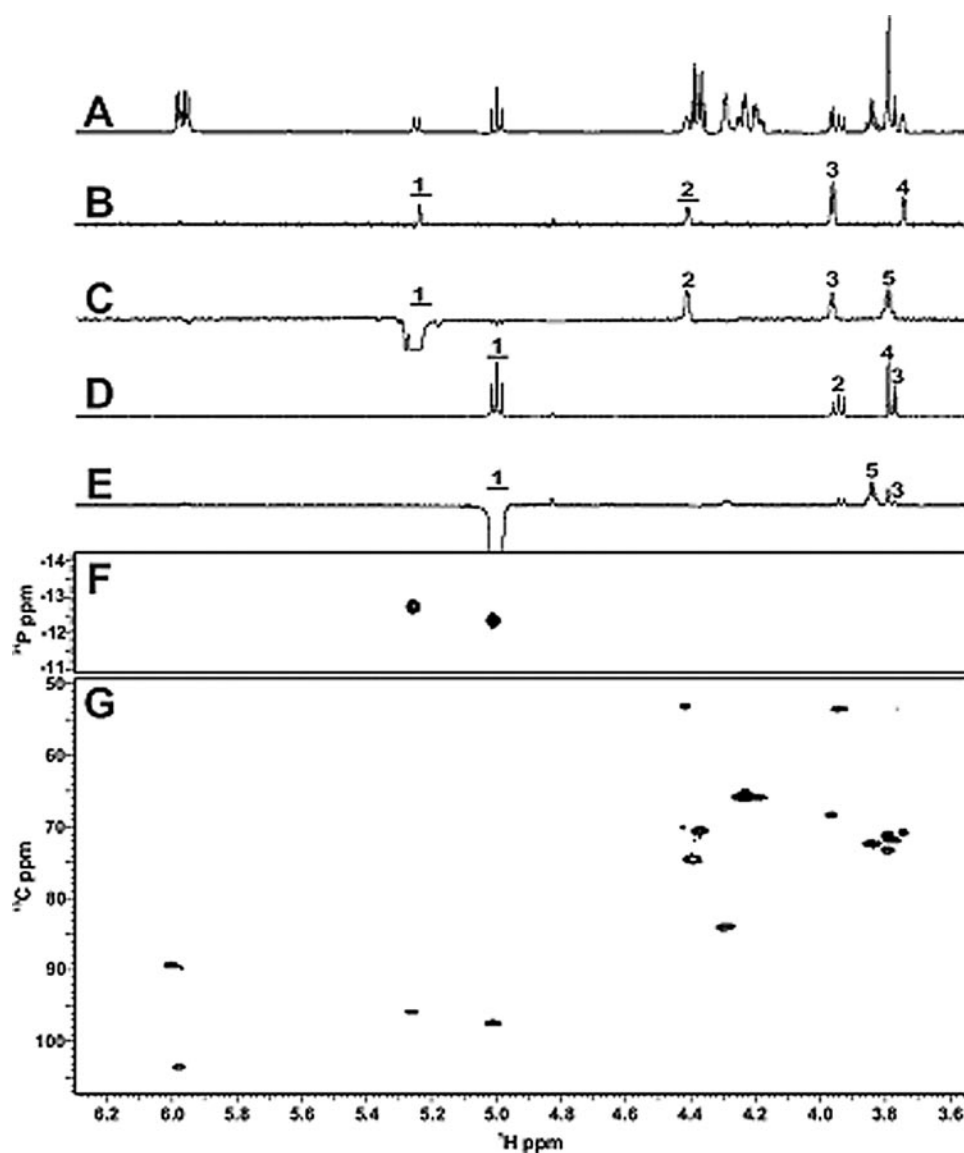


FIG. 4. NMR spectra of a sample containing UDP-L-PneNAc and UDP-L-FucNAc. A, ^1H NMR spectrum. B, TOCSY-TOCSY with mixing times of 80 ms for selective excitation of the UDP-L-PneNAc H-1 and H-2 resonances at 5.26 and 4.41 ppm, respectively (underlined). C, NOESY with a mixing time of 800 ms for selective excitation of the UDP-L-PneNAc H-1 resonance at 5.26 ppm (underlined). D, TOCSY with a mixing time of 50 ms for selective excitation of the UDP-L-FucNAc H-1 resonance at 5.01 ppm (underlined). E, NOESY with a mixing time of 800 ms for selective excitation of the UDP-L-FucNAc H-1 resonance at 5.01 ppm (underlined). F, ^{31}P HMQC spectrum using a $J_{\text{H,P}}$ coupling constant of 11 Hz. G, ^{13}C HSQC spectrum using a $^1J_{\text{H,C}}$ of 140 Hz.

TABLE I
NMR analysis of compounds UDP- β -L-PneNAc and UDP- β -L-FucNAc

δ is shown in ppm, and proton coupling constants ($J_{\text{H,H}}$) are shown in Hz.

		^1H and ^{13}C chemical shifts, δ , and proton coupling constants ($J_{\text{H,H}}$)							
Compound		H-1 ^a	H-2	H-3	H-4	H-5	H-6	NAc-CH ₃	NAc-CO
		C-1	C-2	C-3	C-4	C-5	C-6		
UDP-L-PneNAc	δ_{H}	5.26	4.41	3.97	3.74	3.79	1.30	2.08	175.7
	δ_{C}	95.8	53.1	68.4	70.8	73.3	16.3	23.3	
	$J_{\text{H,H}}$	1.5	4.2	3.5	<1	6.5			
UDP-L-FucNAc	δ_{H}	5.01	3.94	3.77	3.79	3.84	1.29	2.08	175.7
	δ_{C}	97.4	53.6	71.8	71.4	72.4	16.4	23.3	
	$J_{\text{H,H}}$	8.6	9.9	3.5	<1	6.5			

^a $J_{\text{H,P}(\beta)}$ was 8.6 Hz for both UDP-L-PneNAc and UDP-L-FucNAc.

other members of the short chain dehydrogenase/reductase family. WbpM from *P. aeruginosa* and FlaA1 from *Helicobacter pylori* for example, are both UDP-D-GlcNAc C-6 dehydratases/C-4 reductases; however, WbpM contains a SMK catalytic triad, whereas FlaA1 contains a SYK triad (23). It was found that the methionine residue in the catalytic triad

of WbpM allowed the enzyme to have an optimum pH in the 9–10 range (23). The optimal pH of FlaA1 was shown to be in the 7–8 range when the wild-type triad (SYK) was present, but it was significantly higher (pH 9–10) when the triad was changed to SMK by site-directed mutagenesis (23). It is possible then that the first two steps of this pathway, catalyzed

by WbjB, control the optimal pH of the overall reaction because it is WbjB that contains the SMK catalytic triad.

WbjB was previously proposed to be trifunctional, catalyzing C-4, C-6 dehydration; C-5 epimerization; and C-3 epimerization (15). However, the product of the C-3 epimerization reaction (UDP-2-acetamido-2,6-dideoxy- β -L-lyxo-4-hexulose, intermediate 3) was never seen in either capillary electrophoresis or NMR analysis of the whole reaction mixture. This prompted the investigation of the exact enzymatic role of each of these enzymes. Our results show that the WbjB reaction produces two visible intermediates as seen by CE, confirming the data published previously (15). When the enzyme was removed and WbjC was added without NADPH, intermediate 2 completely disappeared, with only a small peak corresponding to UDP-L-PneNAc produced. This is probably due to the fact that the C-3 epimerization step does not require NADPH, but the product of this reaction is retained in the active site of WbjC until it is subsequently reduced to UDP-L-PneNAc. This would also account for the fact that the product of the C-3 epimerization was not observed by CE or NMR in the previous study by our group (15). The small amount of conversion of intermediate 2 to UDP-L-PneNAc observed was most likely due to the presence of NADPH that co-purified with the enzyme. When an excess amount of the hydride donor is present, there is a marked increase in the peak corresponding to UDP-L-PneNAc. This conversion in the absence of WbjB supports our hypothesis that WbjC is a bifunctional enzyme catalyzing the C-3 epimerization and the C-4 reduction steps. This result is also consistent with the data from a basic local alignment search tool (BLAST) (24) analysis of WbjC showing that it shares homology with both epimerase and reductase protein families.

Using an anion-exchange FPLC-based approach, we were able to resolve small amounts of the slower moving epimer (UDP-L-FucNAc) from the faster moving epimer (UDP-L-PneNAc). The subtle difference between the positioning of the *N*-acetyl group at the C-2 position makes resolving these compounds very difficult. A unique method involving triethylammonium bicarbonate was employed to accomplish this. For NMR analysis it was not necessary to resolve the two sugar nucleotides because they can be easily distinguished in the spectra; however, the compounds needed to be stabilized in a buffer compatible with NMR spectroscopy. For this reason, an FPLC-based anion-exchange approach was used that involved a linear elution with ammonium bicarbonate. Because of the volatility of this buffer, the pooled fractions could then be lyophilized to dryness without any further treatment for removal of the buffer. This approach has proven to be very effective because the majority of the sugar nucleotide sample was intact, and the spectra were much sharper than previously seen (data not shown). There is also the advantage of using a cryogenically cooled probe for NMR analysis, which allows for much greater sensitivity, so that minute quantities of sample can be analyzed. As seen in Fig. 3, a proton spectrum with only eight transients had a very good signal to noise ratio. This permitted the proton experiments shown in Fig. 4 to be done in a short time (<1 h). The HSQC experiments could also be done in a matter of hours (Fig. 4G). The small acquisition time necessary to acquire these experiments proved useful because the sugar nucleotide sample degraded overnight.

In this study we have provided unequivocal evidence to show that both WbjB and WbjC are bifunctional enzymes, with the former being a C-4, C-6 dehydratase/C-5 epimerase and the latter being a C-3 epimerase/C-4 reductase. Epimerization at the C-5 and C-3 positions of a particular nucleotide-activated sugar can be catalyzed by either the same enzyme or separate

enzymes. For instance, RmlC, which is the one of the four enzymes involved in the pathway for biosynthesis of dTDP-L-rhamnose, has been shown to catalyze both C-5 and C-3 epimerization of its substrate, dTDP-6-deoxy-D-xylo-4-hexulose. These reactions apparently occur via a keto-intermediate that can form the C-3-C-4 and C-4-C-5 enolates and involve re-orientation of the substrate in the active site between the epimerization reactions (25, 26). In contrast, EvaD is a C-5 epimerase involved in the biosynthesis of dTDP-L-epivanco-samine (27, 28). The crystal structure of EvaD has been solved recently, and comparisons of EvaD to the structure of RmlC (25) revealed that this reaction probably occurs by a very similar mechanism to the C-5 epimerization reaction of RmlC (29). There are other examples of C-5 epimerase enzymes that act on nucleotide-activated uronic acid sugars. However, the mechanism proposed for this epimerization is different than that proposed for non-uronic acids. A microsomal enzyme known as heparosan-*N*-sulfate D-glucuronosyl 5-epimerase involved in the biosynthesis of heparin has been studied in detail, and the reaction was proposed to proceed via a carbanion intermediate at C-5 (30, 31). Using tritiated substrates, the possibility of a mechanism involving a hexos-4-eneuronosyl intermediate or a hexos-3-eneuronosyl intermediate was eliminated because the label was only lost from the C-5 position (30). Since the product of the WbjB reaction (UDP-2-acetamido-2,6-dideoxy- β -L-arabino-4-hexulose), which is the substrate for the WbjC reaction contains a keto group, both the C-5 and C-3 epimerization reactions most likely occur via a mechanism similar to that of RmlC.

By elucidating the structure of the final product of the WbjB/WbjC/WbjD reaction, we now have the evidence to name WbjD as a UDP-L-PneNAc C-2 epimerase. Epimerization of the *N*-acetyl group on the carbon adjacent to the anomeric center may follow a mechanism distinct from that of RmlC discussed above. WbjD shows homology to a number of other UDP-D-GlcNAc C-2 epimerases, which catalyze the conversion of UDP-D-GlcNAc to UDP-D-ManNAc. Examples include Cap5P (47% similarity) from *S. aureus* and WecB from *E. coli* (45% similarity; formerly known as RffE). Initial studies of UDP-D-GlcNAc C-2 epimerase from *E. coli* proposed that epimerization occurs via a ketone at the C-3 position (32). However, recent work on RffE led to the identification of a novel mechanism involving the *anti* elimination and further *syn* addition of the O-UDP moiety to the anomeric carbon (26, 33–36). This mechanism has also been demonstrated in a hydrolyzing UDP-D-GlcNAc C-2 epimerase, NeuC, from *Neisseria meningitidis* (37, 38). For hydrolysis of UDP-D-ManNAc to occur, the *syn* addition of a water molecule replaces the *syn* addition of O-UDP, and subsequently, UDP and D-ManNAc are released from the enzyme (37, 38). We propose that the epimerization of UDP-L-PneNAc to UDP-L-FucNAc catalyzed by WbjD most likely occurs through the *anti* elimination and *syn* addition of the O-UDP moiety as seen in UDP-D-GlcNAc C-2 epimerases such as RffE.

Intermediate 3 of the current pathway, UDP-2-acetamido-2,6-dideoxy-L-lyxo-4-hexulose, has been proposed to be a precursor in the biosynthesis of UDP-L-RhaNAc and UDP-L-QuiNAc in *Vibrio cholerae* O37 (39). This pathway in *V. cholerae* is catalyzed by three enzymes (WbvB, WbvR, and WbvD), and two of these enzymes (WbvB and WbvD) are highly homologous to WbjB and WbjD, respectively, of *P. aeruginosa* serotype O11 (39). As in the UDP-L-FucNAc pathway, the formation of this intermediate has been proposed to be catalyzed by the WbjB homologue, WbvB; however, such an intermediate was not detected using CE or NMR methods (39). This intermediate was then proposed to be reduced at C-4 by a reductase

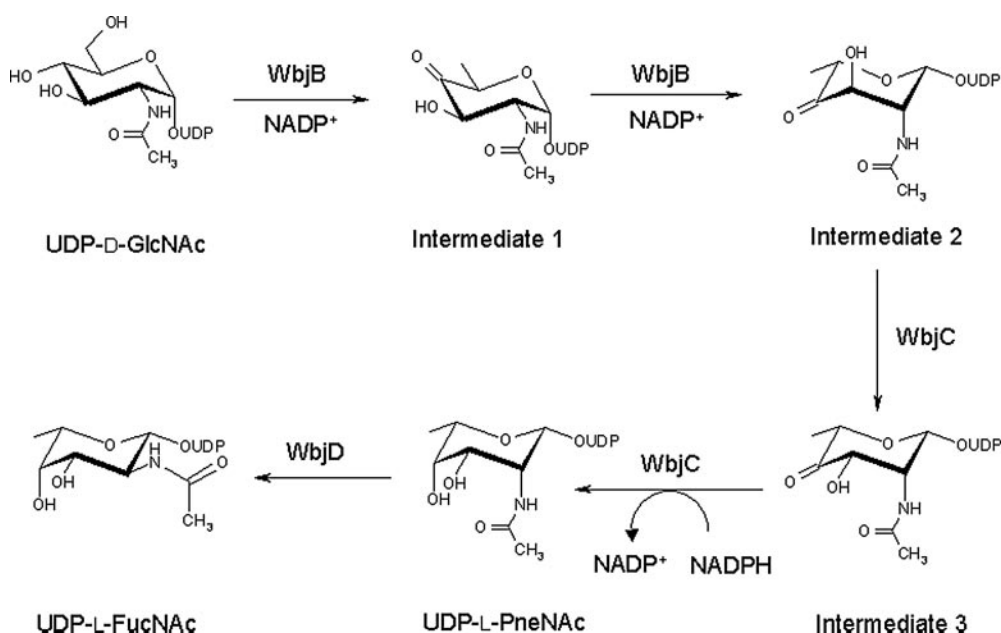


FIG. 5. Revised pathway for the biosynthesis of UDP-L-FucNAc. *Intermediate 1*, UDP-2-acetamido-2,6-dideoxy- α -D-xylo-4-hexulose. *Intermediate 2*, UDP-2-acetamido-2,6-dideoxy- β -L-arabino-4-hexulose. *Intermediate 3*, UDP-2-acetamido-2,6-dideoxy- β -L-lyxo-4-hexulose.

known as WbvR. The product of this reaction is UDP-L-RhaNAc, which is a C-4 epimer of the WbjC product UDP-L-PneNAc. However, WbjC and WbvR do not share significant homology (39). Based on our data showing that the product of the WbjB reaction with the substrate UDP-D-GlcNAc is actually intermediate 2, with the structure of UDP-2-acetamido-2,6-dideoxy- β -L-arabino-4-hexulose, this opens the possibility that WbvR is only performing the C-4, C-6 dehydration and the C-5 epimerization, which would be consistent with the activities observed in its homologue, WbjB. All together, these enzymes, WbjB/WbjC/WbjD of *P. aeruginosa* and WbvB/WbvR/WbvD of *V. cholerae*, are involved in the biosynthesis of four types of nucleotide-activated L-HexNAc sugars (L-PneNAc, L-FucNAc, L-RhaNAc, and L-QuiNAc). The present study has contributed toward improved understanding of the reactions catalyzed by these enzymes, which is crucial for future development of novel antimicrobial agents.

Having determined the identity of the final product as well as more clearly defining the enzymatic activities of the first two enzymes (WbjB and WbjC) in this pathway, we are now able to present a revised pathway for the biosynthesis of UDP-L-FucNAc (Fig. 5).

In conclusion, we have shown that WbjB, the first enzyme in the pathway, is not trifunctional but actually possesses bifunctional activities, firstly catalyzing C-4, C-6 dehydration and secondly catalyzing C-5 epimerization. We presented new evidence to show that the second enzyme, WbjC, is bifunctional, catalyzing C-3 epimerization, followed by a C-4 reduction step. This is the first report of separate C-5 and C-3 epimerases acting on non-uronic acid nucleotide-activated sugars. In addition, by purifying the coupled reaction products and subjecting these compounds to NMR analysis, we have shown unequivocally that the final product of this pathway is UDP-L-FucNAc, a precursor to L-FucNAc found in LPS of *P. aeruginosa*. By virtue of the evidence in a previous study (15) that Cap5E, Cap5F, and Cap5G possess the same activities as their orthologues, WbjBCD proteins, it can also be concluded that the same final product, UDP-L-FucNAc, is produced by the catalytic activities of coupled reactions of Cap5EFG for capsule biosynthesis in the Gram-positive and Gram-negative bacterium *S. aureus*.

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Biosynthesis of UDP-N-acetyl-1-fucosamine, a Precursor to the Biosynthesis of Lipopolysaccharide in *Pseudomonas aeruginosa* Serotype O11
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