Three Highly Conserved Proteins Catalyze the Conversion of UDP-N-acetyl-b-glucosamine to Precursors for the Biosynthesis of O Antigen in Pseudomonas aeruginosa O11 and Capsule in Staphylococcus aureus Type 5

IMPLICATIONS FOR THE UDP-N-ACETYL-L-FUCOSAMINE BIOSYNTHETIC PATHWAY*

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N-Acetyl-l-fucosamine is a constituent of surface polysaccharide structures of Pseudomonas aeruginosa and Staphylococcus aureus. The three P. aeruginosa enzymes WbjB, WbjC, and WbjD, as well as the S. aureus homologs Cap5E, Cap5F, and Cap5G, involved in the biosynthesis of N-acetyl-l-fucosamine have been overexpressed and purified to near homogeneity. Capillary electrophoresis (CE), mass spectroscopy (MS), and nuclear magnetic resonance spectroscopy have been used to elucidate the biosynthesis pathway, which proceeds in five reaction steps. WbjB/Cap5E catalyzed 4,6-dehydration of UDP-N-acetyl-b-glucosamine and 3- and 5-epimerization to yield a mixture of three keto-deoxy-sugars. The third intermediate compound was subsequently reduced at C-4 to UDP-2-acetamido-2,6-dideoxy-b-talose by WbjC/Cap5F. Incubation of UDP-2-acetamido-2,6-dideoxy-b-talose (UDP-TalNAc) with WbjD/Cap5G resulted in a new peak separable by CE that demonstrated identical mass and fragmentation patterns by CE-MS/MS to UDP-TalNAc. These results are consistent with WbjD/Cap5G-mediated 2-epimerization of UDP-TalNAc to UDP-FucNAc. A nonpolar gene knockout of wbjB, the first of the genes associated with this pathway, was constructed in P. aeruginosa serotype O11 strain PA103. The corresponding mutant produced rough lipopolysaccharide devoid of B-band O antigen. This lipopolysaccharide deficiency could be complemented with P. aeruginosa wbjB or with the S. aureus homolog cap5E. Insertional inactivation of either the cap5G or cap5F genes abolished capsule polysaccharide production in the S. aureus strain Newman. Providing the appropriate gene in trans, thereby complementing these mutants, fully restored the capsular polysaccharide phenotype.

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resistance to antibiotics; the first documented case of a vancomycin-resistant *S. aureus* infection in a United States patient was recently reported by the Centers for Disease Control (60).

I-FucNAc has thus far been described exclusively as a constituent of bacterial polysaccharide structures. It is part of the O antigen of *P. aeruginosa* serotypes O4, O11, and O12, of the CP of *S. aureus* serotypes 5 and 8, and of *Streptococcus pneumoniae* CP type 4 (9–12). LPSs of *Escherichia coli* O26 and CP of *Bacteroides fragilis* also contain i-FucNAc (13, 14). It should be noted that not all i-FucNAc-containing bacteria are listed above; for the scope of this study, only those bacteria for which sequence data of the corresponding polysaccharide biosynthesis genes are available have been taken into consideration (*E. coli* O26 polysaccharide cluster data are referred to in Ref. 15). With respect to *P. aeruginosa*, the i-FucNAc-containing strains, particularly International Antigenic Typing System O4 and O11, belong to the most clinically prevalent strains besides serotypes O3 and O6 (16). The O11 strain PA103 is a high level exotoxin A producer (17). *S. aureus* CP serotypes 5 and 8 make up ~80% of clinical isolates (18), and i-FucNAc is a component of both CP structures. Moreover, both *P. aeruginosa* and *S. aureus* are associated with nosocomial infections, as well as being known for resistance against antibiotics. *S. pneumoniae* type 4 capsule is a component of a heptavalent streptococcal vaccine, which is available commercially (19). These data implicate the importance of the i-FucNAc residue in pathogenicity. Targeting its biosynthesis could lead to the development of therapeutic agents that affect important virulence factors of Gram-positive and Gram-negative bacteria.

To date, the biosynthesis pathway leading to the nucleotide-activated precursor of i-FucNAc is not clearly defined. Two putative pathways have been proposed, both lacking any experimental evidence. Lee and Lee (20) suggested a three-step route starting from UDP-α-ManNAc and involving (i) 3-epimerization, (ii) 6-epimerization, and (iii) 6-dehydration leading to UDP-α-FucNAc. Jiang et al. (15) proposed a reaction scheme analogous to the biosynthesis of GDP-L-fucose from GDP-α-mannose. This group suggested that UDP-α-GlcNAc is first converted to UDP-α-ManNAc to get the same precursor as in the above pathway. The next reaction step is 4,6-dehydration to get UDP-α-acetamido-2,6-dideoxy-D-lyxo-hexose, and finally a bifunctional enzyme would catalyze 5,6-epimerization and 4-reduction to yield UDP-β-FucNAc. This *S. aureus* genes cap5I8E, cap5I8F, and cap5I8G or the homologous *S. pneumoniae* genes cps4M(J), cps4N(K), and cps4L have been proposed to be involved in this biosynthesis (15, 20).

We present for the first time a proposed biosynthetic pathway for nucleotide-activated i-FucNAc, namely UDP-α-i-FucNAc from its precursor UDP-α-GlcNAc. We provide evidence that three *P. aeruginosa* enzymes, WbjB, WbjC, and WbjD (and their *S. aureus* homologs Cap5E, Cap5F, and Cap5G) catalyze a five-step reaction cascade.

**EXPERIMENTAL PROCEDURES**

**Materials**—UDP-N-acetyl-β-D-glucosamine, UDP-N-acetyl-β-D-galactosamine, UDP-β-glucose, NAD⁺, NADH, NADP⁺, NADPH, and the antibiotics used in this study were obtained from Sigma-Aldrich. HitTrap Chelating columns were purchased from Amersham Biosciences, Econo-Pac High Q columns were from Bio-Rad, and pET28a, pET24a+ and pET24c+ were from Novagen (Madison, WI).

**Bacterial Strains, Cloning Vectors, and Growth Conditions**—The bacterial strains and plasmids used in this study are listed in Table I. *P. aeruginosa* strains were grown in Luria broth (Invitrogen) or on *Pseudomonas* isolation Agar (Difco Laboratories, Detroit, MI). *S. aureus* was grown in tryptic soy broth (Difco Laboratories). *E. coli* strains Top10 (Invitrogen) and JM109 (21) were used for plasmid propagation. *E. coli* strain SM10 (22) was used as donor in bacterial conjugation. For protein overexpression *E. coli* strain BL21(DE3) was used (Novagen). Overexpression was induced by the addition of isopropyl-1-thio-β-β-galactopyranoside (Invitrogen) at a final concentration of 1 mM. The media were supplemented with ampicillin (100–250 μg/ml), kanamycin (25 μg/ml or 50 μg/ml), carbenicillin (250 μg/ml for *E. coli* and 300 μg/ml for *P. aeruginosa*), gentamicin (15 μg/ml for *E. coli* and 300 μg/ml for *P. aeruginosa*), tetracycline (15 μg/ml for *E. coli* and 100 μg/ml for *P. aeruginosa*), chloramphenicol (10 μg/ml), or erythromycin (10 μg/ml) when necessary.

**Analytical Techniques—SDS-PAGE was done according to the method of Laemmli (23) with slight modifications. Gels were stained with Coomassie Brilliant Blue R-250 (Sigma-Aldrich).** The protein concentrations were determined as described by Bradford (24). LPS was prepared according to the method of Hitchcock and Brown (25). The samples were run on a 12% SDS-PAGE gel, and the LPS was either silver-stained or to a nitrocellulose membrane or visualized by a rapid silver staining procedure (26). Antibodies MF55-1 (mAb to B-band antigen of serotype O11) (27), grouping antisera O11 (polyclonal antibody to B-band O antigen of serotype O11; Kendu Institute of Biological Products, Chengdu, China), and NIP10 (mAb to B-band antigen) (28) were used for Western immunoblotting analysis. The blots were developed with anti-mouse immunoglobulin G alkaline phosphatase (Jackson Immunoresearch, West Grove, PA) for mAbs, and anti-rabbit immunoglobulin G alkaline phosphatase (Bio-Rad) for polyclonal rabbit antisera. The blots were developed using protein A-horseradish peroxidase conjugate (Zymed Laboratories Inc., South San Francisco, CA) and the Bio-Rad horseradish peroxidase conjugate substrate kit.

**Sequence Analysis—**BLAST (30) and Multalin (31) were used for analysis of nucleotide and protein sequences.

**DNA Manipulations, PCR, DNA Sequencing, and Protein Sequencing**—All of the standard DNA recombinant procedures were performed according to the methods described by Sambrook et al. (32) or as recommended by the corresponding manufacturer. PCR was carried out with a GeneAmp PCR System 2400 (PerkinElmer Life Sciences). DNA sequencing was performed with primers CpE1F and CpE2R, obtained from the Beth Israel Deacossen Molecular Medicine Unit (Boston, MA). N-terminal protein sequencing was performed by the Dana Farber Molecular Biology Core Facility (Boston, MA).

**Plasmid Construction—**The oligonucleotide primer sequences are given in Table II. Chromosomal DNA of *P. aeruginos* iso 2540 (International Antigenic Typing System strain O11 or *S. aureus* cap5E type 5 was used for amplification of the genes. Two polymerase (Roche Molecular Biochemicals) was used for PCR amplification. Primer pairs wbjB1F/wbjB2R, wbjC1F/wbjC2R, wbjD1F/wbjD2R, and capE1F/capE2R were used for amplification of the wbjB, wbjC, wbjD, and cap5E (clinical isolate provided by A. Chow, Vancouver, Canada) genes, respectively. The PCR products of genes cap5E and cap5G were digested with *NotI* and *BamHI* and ligated into the modified pET23 derivative (33), cut with the same enzymes. The PCR product wjbC was cut with NdeI and HindIII and ligated into appropriately digested pET28a. Finally, PCR product wjbD was digested with NdeI and EcoRI and ligated into NdeI/EcoRI-cut pET28a. The corresponding plasmids pFuc11 (wjbD), pFuc12 (wjbC), pFuc13 (wjbD), and pFuc21 (cap5E) were confirmed by DNA sequencing and used for amplification of PCR products for complementation studies and/or overexpression of N-terminal histidine-tagged fusion proteins.

**Materials**—UDP-N-acetyl-β-D-glucosamine, UDP-N-acetyl-β-D-galactosamine, UDP-β-glucose, NAD⁺, NADH, NADP⁺, NADPH, and the antibiotics used in this study were obtained from Sigma-Aldrich. HitTrap Chelating columns were purchased from Amersham Biosciences, Econo-Pac High Q columns were from Bio-Rad, and pET28a, pET24a+, and pET24c+ were from Novagen (Madison, WI).
To create a plasmid for \textit{S. aureus} cap5G gene disruption, subclone pJC69 (containing the end of cap5E gene) was PCR-amplified using primers 12402F1 and 12402R1 for cap5G, with a 294 bp fragment 50 bp downstream of the ATG start site of cap5G yielding pJC69-2. The ermB gene from pERMB was ligated into the BclI site of pJC69-2 to create pJC69-2ermB. A 4002-bp XbaI/BamHI fragment from pJC69-2ermB was ligated to digested pCL10 to yield pKOR1. To create a knockout plasmid for use in cap5E allele replacement, a modified pCL10 plasmid containing ermB in the BamHI site was constructed by digesting pCL10 with BamHI and ligating the 1.6-kb region of the cap5E gene locus (containing 450 bp of cap5E gene and 1124 bp of the 5' upstream region) into pCL10 containing ermB cassette. A 1.6-kb amplicon (cap5G and 450 bp of cap5F) in pPAP1.2EX (XbaI) and a 1.9-kb amplicon (385 bp of cap5D, cap5E and 530 bp of cap5F) in pPAP1.2EX (EcoRI) were ligated into pJC69 carrying cap5G gene in pL150. The constructs pPAP1.2EX were used to transform \textit{E. coli} DH10B, and the plasmid pKOR2 was isolated. The plasmids pPAP27 and pPAP28 were confirmed by DNA sequencing.

\section*{Protein Overexpression and Purification}

Expressions were carried out at 37 °C using terrific broth (32), supplemented with 50 μg/ml kanamycin for WbjB and WbjC, with 25 μg/ml kanamycin for Cap5E, Cap5F, or Cap5G, or with 250 μg/ml ampicillin for WbjC. The cultures were grown to an \(A_{600nm} \) of 0.6, and expression was induced with isopropyl-1-thio-β-D-galactopyranoside at a final concentration of 1 mM. WbjB, WbjD, Cap5E, and Cap5F expression were carried out for 4 h at 37 °C, WbjC was expressed for 4 h at 37 °C, and Cap5G was expressed for 4 h at 30 °C. The cells were disrupted on ice by ultrasonication. Cell debris and membrane fractions were removed by ultracentrifugation at 300,000 \( \times \) g. Purifications using nickel chelating columns were per-
**Table II**

| Primer      | Primer sequence                  |
|-------------|----------------------------------|
| wbjB1F (+)  | 5'-GGACTCGGAGATATGGAAGAAGTCACTCGG3' |
| wbjB2R (-)  |                                  |
| wbjC1R (+)  | 5'-GGAGGTCTCCATAGCAGTGTGGTGG3'    |
| wbjC2R (+)  |                                  |
| wbjD1F (+)  | 5'-GAGAAGCTTTTGCACTGGCTGG3'       |
| wbjD2R (-)  |                                  |
| cap5E-R (+) | 5'-GATGCGCTGATCCATGGGGTGG3'       |
| cap5E-F (+) | 5'-GGTACCCGCTATCCATGGGGTGG3'      |
| KKI (+)     |                                  |
| KK2 (-)     | 5'-CAAAGATCCATCTCCATGGGGTGG3'     |
| 5E- (+)     | 5'-GGAGGTCTCCATAGCAGTGTGGTGG3'    |
| 5E-R (-)    | 5'-GATGCGCTGATCCATGGGGTGG3'       |
| cap5F-R (+) | 5'-GATGCGCTGATCCATGGGGTGG3'       |
| cap5F-F (+) | 5'-GATGCGCTGATCCATGGGGTGG3'       |

* Forward and reverse primers are represented by plus and minus signs, respectively.

* The underlined sequences represent the restriction sites used to clone the PCR products.

formed as recommended by the manufacturers. Purified WbjC and WbjD could be obtained at 200 μM imidazole, whereas WbjB, Cap5E, Cap5F, and Cap5G were eluted from the column at 300 mM imidazole. Dithiothreitol was added to a final concentration of 1 mM, and the proteins were stored at −20°C after the addition of 40% glycerol. The purity of the enzymes was checked by SDS-PAGE analysis on a 10% gel, and N-terminal protein sequencing was performed on Cap5E, Cap5F, and Cap5G to verify the purified proteins.

**Capillary Electrophoresis (CE) Analysis of UDP-L-FucNAc Biosynthesis**—CE analysis was performed with a P/ACE MDQ Glycoprotein system with UV detection (Beckman Coulter, Fullerton, CA). The samples were separated at 22 kV in 25 mM borate buffer (pH 9.0) at 37°C. Typically, the reactions contained 0.5 mM UDP-L-FucNAc, 0.1 mM NADP+, and an excess of hydride donor (NADH or NADPH). For CE analysis, the UDP-D-GlcNAc concentration was 0.1 or 0.5 mM. The separation was achieved using a 90-cm bare fused silica capillary. The outlet of the capillary was tapered to a 10-μm tip with a separation voltage of 30 kV typically applied at the injection end of the capillary. The cultures were grown overnight, and LPS was analyzed by NMR. The protein was removed by ultrafiltration with Centriplus cartridges. The purification of the reaction product was performed as described above for the keto-intermediate. The fractions corresponding to UDP-2-acetamido-2,6-dideoxy-t-talose were pooled, acidified to pH 4.5 with Dowex 50, lyophilized completely, and analyzed by NMR.

5.5 μM (3.5 μg) of UDP-n-GlcNAc were converted quantitatively to the putative UDP-2-acetamido-2,6-dideoxy-t-talose using 200 μg each of WbjB and WbjC and equal molar amounts of NADPH. The reaction was performed overnight in 20 mM Tris buffer (pH 8.0) at 37°C. The enzyme from the reaction mix was removed by ultrafiltration with Centrisil cartridges. The purification of the reaction product was performed as described above for the keto-intermediate. The fractions corresponding to UDP-2-acetamido-2,6-dideoxy-t-talose were pooled, acidified with Dowex 50, lyophilized completely, and analyzed by NMR.

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**Results and Discussion**

**Synthesis and Preparation of Intermediate Products**—5.5 μmol (3.5 μg) of UDP-n-GlcNAc were converted quantitatively to a mixture of keto-sugars using 100 μg of WbjB. The reaction was carried out in 20 mM triethylammonium bicarbonate buffer (pH 8.0) for 4.5 h at 37°C. The protein was removed by ultrafiltration with Centrisil cartridges (Millipore, Bedford, MA). For analysis of the keto-mixture, the sample was acidiﬁed to pH 4.5 with Dowex 50 (Bio-Rad) to get rid of CO2, and concentrated to 100 μl by lyophilization. This concentrated compound was analyzed by NMR. The keto-sugar that migrated faster than NADP+, as judged by CE analysis, was puriﬁed over an Econo-Pac High Q anion exchange column with a linear triethylammonium bicarbonate gradient (0–500 mM). Fractions containing the keto-sugar were pooled, acidified with Dowex 50, lyophilized completely, and analyzed by NMR.

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UDP-L-FucNAc Biosynthesis

**Table III.** Enzymes involved in UDP-L-FucNAc biosynthesis

| Bacterial strain          | Enzymes encoded by three contiguous genes in the different bacteria (accession numbers) |
|---------------------------|---------------------------------------------------------------------------------------|
| *P. aeruginosa* PA103     | WbjB (AAD45265) WbjC (AAD45266) WbjD (AAD45267)                                      |
| *P. aeruginosa* IATS O17  | WbjB (AAD72955) WbjC (AAD72788) ORF 9 (AAM27576) ORF 10 (AAM27578) ORF 11 (AAM27577) |
| *P. aeruginosa* IATS O04  | ORF 8 (AAM27575) ORF 20 (AAM27601) ORF 21 (AAM27602) Cap5E (AAC46089) Cap5F (AAC46089) |
| *P. aeruginosa* IATS O11  | Cap5G (AAC46090) Cap5S (AAB49436) Cap8G (AAB49436)                                 |
| *S. aureus* Reynolds/Newman | Cap5E (AAC46088) Cap5F (AAC46089) Cap5G (AAC46090)                                 |
| *S. aureus* Becker        | Cap5E (AAC46088) Cap5F (AAC46089) Cap5G (AAC46090)                                 |
| *S. pneumoniae* TIG4      | Cps4M (J) (AAK74531) Cps4N (K) (AAK74532)                                             |
| *S. pneumoniae* WCH34     | Fnl1/ORF13 (AAK20679) Fnl2/ORF14 (AAK20680)                                          |
| *B. fragilis* 638R        | WcgJ (AAD56743) WcgK (AAD56744) WcgL (AAD56744)                                      |
| *P. maltocida* PM70       | WbjB (AAK03092) WbjC (AAK03093)                                                     |
| *L. interrogans* Copenhageni | ORFC10 (AAK19896) ORFC32 (AAK19897)                                                 |
| *L. interrogans* Pomona   | ORFP10 (AAL49148) ORFP11 (AAL49148)                                                |

*Proteins appearing in the same column of this table are highly homologous to each other.*

**RESULTS**

**Sequence Analyses**—A common feature of bacterial gene clusters associated with the biosynthesis of L-FucNAc is the presence of three consecutive genes with no assigned function (Table III). These genes are also part of the genomes of *Pasteurella multocida* PM70 and *Leptospira interrogans* serovars Copenhageni and Pomona. Whether these two bacteria contain L-FucNAc in their surface polysaccharide is not known. The proteins encoded by *wjbB*/*cap5E* and *wjbC/cap5F* or their homologs belong to the SDR protein family (40). Both WjbB/Cap5E and WjbC/Cap5F have the nucleotide-binding signature GXGXGGXG near their N terminus (referred to as the Rossmann fold) essential for binding of the cofactor NAD+ or NADP+ (41). Another typical feature of the SDR protein family is the so-called catalytic triad, SMK in the case of WjbB/Cap5E and SYK in WjbC/Cap5F. WjbB/Cap5E is moderately homologous to WbpM, which is putatively involved in UDP-N-acetyl-d-fucosamine/quinovosamine biosynthesis and shows reaction on UDP-α-GlcNAc (42). Thus, we postulated WjbB/Cap5E to be the enzyme catalyzing the first step involving UDP-α-GlcNAc. Because proteins of this family are highly homologous, it is very difficult to predict their exact function. The third putative L-FucNAc biosynthesis protein is WjbD/Cap5G, which shows homology to UDP-GlcNAc 2-epimerases involved in the biosynthesis of UDP-α-ManNAc (39). To determine whether the gene products of *wjbB/cap5E*, *wjbC/cap5F*, and *wjbD/cap5G* are actually involved in the biosynthesis of nucleotide-activated L-FucNAc and to determine the order in which they would act in the pathway, two approaches have been used: overexpression of the corresponding proteins to carry out in vitro enzyme assays and knocking out the genes, evaluating the phenotype of the mutants, and performing complementation studies.

**Expression and Purification of the UDP-L-FucNAc Biosynthesis Enzymes**—WjbB, WjbC, WjbD, Cap5E, and Cap5F were expressed at high levels at 37 °C. High level expression resulting in soluble Cap5G could only be achieved at 30 °C. After sonication and ultracentrifugation, the majority of the proteins proved to be in the supernatant, enabling high yield affinity purification on chelating columns. After addition of 40% glycerol, the enzymes were stable at −20 °C for several weeks. The apparent molecular masses (M_r) of the proteins, determined by SDS-PAGE, were in good agreement with the calculated molecular masses, i.e. WjbB, 40.7 kDa; WjbC, 43.6 kDa; and WjbD, 43.9 kDa (Fig. 1A). Purified recombinant Cap5E, Cap5F, and Cap5G are shown in Fig. 1B. The predicted molecular masses
of these recombinant proteins are 39.9, 43.7, and 45.1 kDa, respectively. N-terminal protein sequencing of Cap5E (MFDD-KILL), Cap5F (LTLNIVIT), and Cap5G (MEKLKLMTIV) correlated with the predicted initial eight to ten amino acids for these proteins. The purified proteins were used for functional characterization of the biosynthesis pathway of nucleotide-activated L-FucNAc.

WbjB/Cap5E Is a Trifunctional Enzyme Catalyzing the First Three Enzymatic Reactions—Sequence analysis suggested that WbjB was the first enzyme in the pathway with UDP-α-GlcNAc as substrate. Indeed, in the WbjB enzyme-substrate reaction, the peak corresponding to UDP-α-GlcNAc disappeared almost completely, and two new peaks appeared on CE analysis (Fig. 2A). NADP⁺ was an essential cofactor in WbjB catalysis. Addition of NAD⁺ or the absence of any added cofactor resulted in less than 5% substrate conversion. Despite performing time course experiments to determine which of the two peaks appeared first, it was not possible to make any assignment. Both products were formed simultaneously in a 1:3 ratio (Fig. 2B, intermediates 1 and 2). The achieving of an equilibrium would be characteristic of an epimerase reaction. Two other related sugar nucleotides, UDP-N-acetyl-D-galactosamine and UDP-α-glucose, also were tested as substrates with WbjB and cofactor, but no reaction could be detected. To further verify that WbjB is the enzyme catalyzing the first reaction step, purified WbjC or WbjD were also incubated with UDP-α-GlcNAc; again no substrate conversion was discerned.

CE-MS was used to characterize further the two reaction products formed by WbjB. Although the reaction appeared to be close to quantitative, only a minor part of UDP-α-GlcNAc was converted to a product with a molecular mass of 588 (negative mode; Fig. 3A). Peaks at m/z 563, 545, and 547 are due to the UDP moiety. The peak at m/z 742 results from NADP⁺ and the peaks at m/z 588, 590, and 606, respectively.

**TABLE IV**

| Peaks arising from UDP-α-GlcNAc | Assignment |
|---------------------------------|------------|
| 608                             | UDP-α-GlcNAc parent peak |
| 405                             | UDP moietoy |
| 204                             | n-GlcNAc moietoy |
| 186                             | n-GlcNAc minus H₂O |
| 168                             | n-GlcNAc minus 2H₂O |
| 144                             | Deacetylated n-GlcNAc minus H₂O |
| 138                             | n-GlcNAc minus 2H₂O and formaldehyde |

| Peak arising from UDP-2-acetamido-4-keto-2,6-dideoxy sugar in the ketal form | Assignment |
|---------------------------------------------------------------------------|------------|
| 608                          | Parent peak |
| 405                          | UDP moietoy |
| 204                          | Sugar moietoy |
| 186                          | Sugar minus H₂O |
| 168                          | Sugar minus 2H₂O |
| 144                          | Deacetylated sugar minus H₂O |

| Peak arising from nonhydrated UDP-2-acetamido-4-keto-2,6-dideoxy sugar | Assignment |
|------------------------------------------------------------------------|------------|
| 590                       | Parent peak |
| 405                       | UDP moietoy |
| 186                       | Sugar moietoy |
| 168                       | Sugar minus H₂O |

| Peak arising from UDP-2-acetamido-2,6-dideoxy sugars | Assignment |
|-----------------------------------------------------|------------|
| 592                     | Parent peak |
| 405                     | UDP moietoy |
| 188                     | Sugar moietoy |
| 170                     | Sugar minus H₂O |

| Peaks arising during time course experiment | Assignment |
|--------------------------------------------|------------|
| 204                                        | n-GlcNAc and hydrated 2-acetamido-4-keto-2,6-dideoxy sugar moietoy |
| 186                                        | Either sugar minus H₂O |
| 168                                        | Either sugar minus 2H₂O |
| 144                                        | Either sugar deacetylated minus H₂O |
| 138                                        | n-GlcNAc minus 2H₂O and formaldehyde |
| 126                                        | Either sugar deacetylated minus 2H₂O |
formed in the WbjB catalyzed reaction with the same molecular mass as UDP-D-GlcNAc was not UDP-D-GlcNAc (Fig. 4, A and B). The fragment ion at \( m/z \) 138 characteristic of N-acetylhexose sugars having a hydroxyl group at C-6 disappeared almost completely. A time course experiment was performed to investigate this reaction further. CE-MS/MS/MS was carried out at \( m/z \) 608 (first generation ions) through \( m/z \) 204 (second generation ions) to analyze the hexose moiety of the nucleotide-activated sugar. Indeed, a significant decrease of the fragmentation ion at \( m/z \) 138 was observed (Fig. 5). The presence of a 6-deoxy-sugar with a molecular mass equivalent to that of its parent 6-hydroxy-sugar provided the evidence for the addition of water somewhere on the hexose moiety. CE-MS/MS analysis of the peak at \( m/z \) 590 (positive mode) demonstrated that the second reaction product is UDP-2-acetamido-4-keto-2,6-dideoxy sugar in the nonhydrated form (Fig. 4 C). Both CE and CE-MS analyses also were performed using the S. aureus homolog Cap5E, with results identical to those obtained for WbjB (data not shown).

To obtain unequivocal evidence of the identity of these intermediates, NMR experiments have been performed. The putative keto-intermediate that migrated faster than NADP\(^+\) was purified by anion exchange chromatography. It was lyophilized to dryness and dissolved in D\(_2\)O. Although it was fairly unstable, we were able to assign the structure of this intermediate sugar nucleotide; the \(^1\)H spectrum is presented in Figs. 6A and 7A. One-dimensional selective experiments on the sugar resonances yielded accurate coupling constants. A value of \( J_{1,2} \) of 3.6 Hz indicated that H-1 was equatorial, having the \( \alpha \)-configuration. A large \( J_{2,3} \) value of 10.8 Hz indicated that H-2 and H-3 were trans to each other. The strong NOE between H-5 and H-3 indicated that H-3 and H-5 were both axial (Fig. 7D). From the HSQC spectrum, the protonated carbons were assigned.

From the HMBC spectrum, the quaternary carbon at C-4 and the NAc-C=O resonances were assigned. Because this sample was dissolved in pure D\(_2\)O, the NH resonance was not observed. The chemical shifts of hydrogens and carbons, as well as the \(^1\)H-\(^1\)H coupling constants, are given in Table V. The \(^1\)H, \(^13\)C, and \(^31\)P chemical shifts for the UDP moiety are similar to the one reported before (34). The sugar nucleotide finally arrived at is UDP-2-acetamido-2,6-dideoxy-\( \alpha \)-D-xylo-4-hexulose.

The second intermediate detected by CE analysis could not be purified in the same manner as the first because of instability. To analyze this sugar, the WbjB reaction mix was concentrated on the lyophilizer after removing the enzyme by ultrafiltration. D\(_2\)O was added to the concentrate, and the keto-mixture was analyzed by NMR (Figs. 6B and 8A). Although no intact UDP-sugar could be detected because of degradation, the structure of the putative keto-sugar could be resolved by analyzing the degradation products. From a series of one-dimensional TOCSY experiments on all of the anomeric peaks identified by a HSQC spectra, a resonance at 5.24 ppm could be identified as belonging to an expected keto-sugar. A small \( J_{2,3} \) value of 3.5 Hz indicated that H-2 and H-3 are gauche to each other. The strong NOE between H-1 and H-5 indicated that H-1 and H-5 were both axial (Fig. 8C). The lack of NOE between H-3 and H-5 and between H-3 and H-1 indicated that H-3 is equatorial (Fig. 8, C and D). From the HSQC
spectrum the protonated carbons were assigned. From the HMBC spectrum, the quaternary carbon at C-4 and the NAc-C=O resonances were assigned. The NMR data are given in Table V. The presence of this degradation product in this mixture is consistent with the second intermediate being UDP-2-acetamido-2,6-dideoxy-β-D-arabino-4-hexulose. The expected UDP-2-acetamido-2,6-dideoxy-β-D-lyxo-4-hexulose could not be detected, either because of instability or because of an equilibrium highly in favor of the two compounds described above.

The reduction reaction was further investigated by CE-MS analysis, which in the negative mode showed a decrease of m/z 606 (hydrated 2-acetamido-4-keto-2,6-dideoxy-sugar), the disappearance of m/z 588 (2-acetamido-4-keto-2,6-dideoxy-sugar), and the appearance of a new ion at m/z 590 (Fig. 3C). This is consistent with a reduction reaction. Additional evidence was obtained by CE-MS/MS at m/z 592 in the positive mode, resulting in the expected fragmentation pattern (Fig. 4D). Again, CE as well as CE-MS experiments using the *Staphylococcus* protein Cap5F gave identical results (data not shown). Finally, the reduction product was purified by anion exchange chromatography and analyzed by NMR spectroscopy (Figs. 6C and 10A). From one-dimensional TOCSY experiments, the small coupling constants for protons within the ring indicated a talose configuration. The observed coupling constants for \(J_{1,2} = 1.5 \text{ Hz}, J_{2,3} = 3.2 \text{ Hz}, J_{3,4} = 3.6 \text{ Hz},\) and \(J_{4,5} = 1.0 \text{ Hz}\) were similar to their respective \(J\) values of 1.5, 3.0, 3.0, and 1.0 Hz observed for synthetic talose oligosaccharides (43). The strong NOE between H-1 and H-3, between H-1 and H-5, and between H-3 and H-5 indicated that H-1, H-3, and H-5 were axial (Fig. 10, C and D). From the HSQC spectrum, the protonated carbons were assigned. From the HMBC spectrum, the NAc-C=O resonances were assigned. The NMR data are

**Table V**

| Compound                | \(^1H\) and \(^{13}C\) chemical shifts, \(\delta\) (ppm), and proton coupling constants, \(J_{H,H}\) |
|------------------------|-----------------------------------------------------------------------------------------------|
| xylol-Sugar            | \(\delta_H^{1H} 5.45, \delta_C^{13C} 41.11, 3.82, 4.13, 1.25, 2.07 \) \(J_{H,H} 22.2, 175.2\) |
| arabino-Sugar          | \(\delta_H^{1H} 5.24, \delta_C^{13C} 41.15, 3.7, 3.96, 1.23, 7.43, 2.05 \) \(J_{H,H} 22.9, 175.8\) |
| 6-Deoxy-TalNAc         | \(\delta_H^{1H} 5.25, \delta_C^{13C} 4.4, 3.95, 3.72, 3.77, 1.29, 7.34, 2.06 \) \(J_{H,H} 23.5, 175.1\) |
given in Table V. Taken together, the reduction product generated by WbjC is UDP-2-acetamido-2,6-dideoxy-β-L-talose.

WbjD/Cap5G Is a Putative C-2-Epimerase Catalyzing the Formation of UDP-L-FucNAc—As described above, WbjD shows moderate homologies to UDP-α-GlcNAc 2-epimerases. Indeed, this enzyme appears to catalyze an equilibrium reaction, as would be expected of an epimerase. The peak corresponding to the 2-acetamido-2,6-dideoxy sugar synthesized in the first three reaction steps decreased significantly, and a new peak migrating slightly faster appeared (Fig. 2, D and F). Approximately 45% of the reduction product was converted to this new peak. Again, this final product could be observed either when all enzymes and cofactors were added simultaneously at the beginning of the reaction or when the reaction steps were carried out consecutively (Fig. 9D).

CE-MS analysis was carried out to investigate this new product. As expected from an epimerization reaction, no changes could be observed compared with the mass spectra obtained from the WbjC-catalyzed reaction (Fig. 3D). The fragmentation pattern in CE-MS/MS analysis at m/z 592 (positive mode) looked exactly the same as before the epimerization step (Fig. 4D; m/z 592 spectra only shown once). This is consistent with the formation of the 2-epimer of UDP-TalNAc, i.e. UDP-L-FucNAc. The Staphylococcus homolog Cap5G yielded the same results, except for inactivity of the enzyme at 37 °C. The Cap5G reaction had to be performed at 30 °C (data not shown).

Three Enzymes Catalyze a Five-step Reaction Cascade Converting UDP-α-GlcNAc to a Product Consistent with UDP-L-FucNAc—We propose the following reaction scheme for the biosynthesis pathway of nucleotide-activated L-FucNAc (Fig. 11). WbjB/Cap5E catalyzes 4,6-dehydration, 5-epimerization, and 3-epimerization of the precursor UDP-α-GlcNAc, resulting in as many as three intermediates, namely UDP-2-acetamido-2,6-dideoxy-α-D-xylo-4-hexulose, UDP-2-acetamido-2,6-dideoxy-β-L-arabinono-4-hexulose, and UDP-2-acetamido-2,6-dideoxy-β-L-lyxo-4-hexulose, which are present in the ketal form. The next step is reduction at C-4 to yield UDP-2-acetamido-2,6-dideoxy-β-L-talose, catalyzed by WbjC/Cap5F. Finally, the UDP-2-acetamido-2,6-dideoxy-β-L-talose 2-epimerase WbjD/Cap5G partially converted UDP-2-acetamido-2,6-dideoxy-β-L-talose to a product that is consistent with UDP-L-FucNAc in a C-2-epimerization reaction.

Inactivation of wbjB Abrogates B-band O Antigen Production in P. aeruginosa PA103—A nonpolar gene knockout of wbjB has been constructed by insertional mutation with a gentamycin resistance cassette and allelic replacement. Strain BK103B with wbjB::aacC1 on its chromosome no longer produces B-band O antigen. In the silver-stained LPS-SDS-PAGE gel, only the banding pattern of A-band O antigen is visible (Fig. 12A). The presence of A-band O antigen was confirmed by Western immunoblotting analysis using the mAb N1F10 (Fig. 12D). Moreover, LPS of the mutant does not react with polyclonal or mAbs to O11 B-band (Fig. 12B and C). It has to be noted that mAb MF55-1 reacts with low molecular weight O11 B-band. Production of the serospecific O antigen in the mutant strain BK103B could be restored by adding P. aeruginosa wbjB or the S. aureus gene cap5E on an E. coli/P. aeruginosa shuttle vector (Fig. 12, A–C).

Knockout of cap5E or cap5F and cap5G Results in Nonencapsulated S. aureus Mutants—S. aureus strain NCTC 8325...
carries a complete cap5 locus but fails to produce CP5. Genomic analysis of the cap5E gene from this strain (sequence of 8325 available at www.genome.ou.edu/staph.html) revealed that it carried a single amino acid mutation (Met134 to Arg134) within Cap5E. Wann and colleagues (44) demonstrated that cap5E provided in trans restored CP5 expression to NCTC 8325-4. We insertionally inactivated the cap5E gene by deleting 126 bp of the gene and replacing it with an ermB cassette. Allelic replacement of the native gene with this disrupted copy resulted in a CP-negative phenotype. Complementation of this mutation was observed when a plasmid containing the cap5 promoter region and genes cap5A through cap5F was introduced into mutant F4. No complementation was observed with the same plasmid containing only the cap5 promoter region and replacing the gene and replacing it with an ermB cassette. Allelic replacement of the native gene with this disrupted copy resulted in a CP-negative phenotype. Complementation of this mutation was observed when a plasmid containing the cap5 promoter region and genes cap5A through cap5F was introduced into mutant F4. No complementation was observed with the same plasmid containing only the cap5 promoter region and replacing the gene.

**DISCUSSION**

This report describes for the first time the biosynthesis steps from the precursor UDP-N-acetyl-D-glucosamine to a nucleotide precursor sugar that is consistent with UDP-l-FucNAc; five distinct enzymatic steps, catalyzed by three enzymes, are sufficient. The deduced amino acid sequences of the gene products from *P. aeruginosa* and *S. aureus* are highly homologous. WbjB and Cap5E show 67% identity and 81% similarity over 332 amino acids. Similarly, WbjC and Cap5F share 40% identity and 57% similarity over 370 amino acids. The last enzymes in the pathway, WbjD and Cap5G, were also highly homologous, with 57% identity and 71% similarity over 377 amino acids.

Two putative pathways for the biosynthesis of UDP-l-FucNAc have been proposed in the literature, both involving UDP-N-acetyl-D-ManNAc as the precursor (15, 20). Hypothesizing putative pathways based solely on *in silico* analysis is prone to error, because occasionally the primary protein sequences of enzymes are highly homologous even though they catalyze completely different conversions. This is especially true for SDR proteins, which include dehydratases, dehydrogenases, epimerases, isomerases, and reductases. Members of this family play crucial roles in the biosynthesis of nucleotide-activated sugars throughout prokaryotes and eukaryotes, in steroid biosynthesis, and in alcohol metabolism (40). Typically, they have the nucleotide-binding motif GXGGXXG or GXGGXXG near the N-terminus that is essential for coenzyme binding (41). Moreover, they all share a motif YXXK, the active site. This motif can be extended to the so-called catalytic triad SYK. Recently, SMK has been described as a slight modification of this triad in WbpM from *P. aeruginosa*, an enzyme that is putatively involved in UDP-N-acetyl-D-fucosamine/quinosamine biosynthesis from UDP-N-GlcNAc (42). FlaA1 from *Helicobacter pylori*, catalyzing the same reaction as WbpM, has an SYK catalytic triad (45). Interestingly, of the two SDR proteins involved in UDP-l-FucNAc biosynthesis, one has SMK (WbjB/Cap5E) and the other has SYK (WbjC/Cap5F). WbjB/Cap5E, WbjC/Cap5F, and WbpM/FlaA1 are highly homologous and yet catalyze distinct reaction steps in nucleotide-activated sugar biosynthesis. Thus, it is not surprising that UDP-l-FucNAc biosynthesis follows a pathway different from those proposed previously by Jiang et al. (15) and Lee and Lee, respectively (20). Limitations of the proposals have been taken into account by these research groups. The most efficient way to analyze biochemical pathways is through enzymatic assays. For the third enzyme involved in UDP-l-FucNAc biosynthesis, it was easier to propose a putative function based solely on its significant homologies to UDP-N-GlcNAc 2-epimerases, which do not belong to the diverse SDR protein family.

Our results showed that WbjB/Cap5E has at least two functions with both 4,6-dehydratase and 5-epimerase activities. Most likely, it also catalyzes epimerization at C-3. Bifunctionality within the SDR protein family also was reported for Gmd from *Aneurinibacillus thermoacrophilus*, which has been described as 4,6-dehydratase/4-reductase converting GDP-D-mannose to GDP-3-rhamnose (46), and for hFX, a 3,5-epime-
UDP-L-FucNAc Biosynthesis

UDP-\(L\)-FucNAc is involved in the biosynthesis of GDP-\(L\)-fucose (47). The first reaction step catalyzed by WbjB/Cap5E is 4,6-dehydration, which appears to be quantitative, because CE analysis revealed no UDP-\(L\)-GlcNAc left on conversion. The second reaction step of WbjB/Cap5E is an equilibrium reaction, as would be expected for an epimerase reaction.

Using CE, coupled CE-MS, and NMR analyses, we were able to show that the first two intermediates, UDP-2-acetamido-2,6-dideoxy-\(L\)-xylopyranose, and 3-epimerase activities. The lack of the exo-moiety after 4,6-dehydration led to the conclusion that WbjB/Cap5E most likely has 4,6-dehydratase, 4,6-dehydratase, and 3,5-epimerase activity. Time course experiments with CE-MS/MS clearly demonstrated that the peak at \(m/z\) 606 is not UDP-\(L\)-GlcNAc. Loss of a fragment ion of \(m/z\) 138 indicated the removal of the hydroxyl group at C-6, because a 30-fold shift (formation of formaldehyde) is characteristic of hexoses. Thus, water has been added to the hexose moiety after 4,6-dehydration. There are two possibilities for this scenario: formation of a geminal diol at the ketogroup at C-4 or ring opening to result in a nucleotide-activated sugar in the open form. Because the final assignments could not be accomplished using an MS approach, we decided to purify the two putative keto-intermediates for NMR analysis, despite their expected instability (46, 48, 49). The purification of the intermediate compound, which migrated faster than UDP-\(L\)-GlcNAc, involved 4,6-dehydration, which was achieved by anion exchange chromatography. The compound proved to be unstable, partially decomposing to the monosaccharide and UDP, but its structure could be elucidated using nanoprobe NMR. One-dimensional TOCSY and one-dimensional TOCSY-NOESY allowed assignment of the hydrogens on C-1, C-2, C-3, C-5, and C-6, as well as on the acetyl group. Lack of a hydrogen at C-4 and lack of a second \(^{13}C\) signal in the keto-ranged indicated the presence of a ketal at C-4, thus excluding the possibility of an open sugar. The quaternary carbon could be assigned by HMBC. The other carbons were assigned with HSQC. Thus, the structure of this intermediate compound is UDP-2-acetamido-2,6-dideoxy-\(\alpha\)-\(D\)-xylo-4-hexulose, resulting from 4,6-dehydration of UDP-\(L\)-GlcNAc. Anion exchange chromatography did not yield a second compound. Therefore, we concentrated a WbjB reaction mix and, after addition of \(D_2O\), analyzed this mixture by NMR spectroscopy. Using similar selective techniques as with the other intermediate compound, we were able to assign a second structure, UDP-2-acetamido-2,6-dideoxy-\(L\)-arabinohexose, which is formed by 5-epimerization of UDP-2-acetamido-2,6-dideoxy-\(\alpha\)-\(D\)-xylo-4-hexulose. These data suggested that WbjB/Cap5E most likely has 4,6-dehydratase, 5-epimerase, and 3-epimerase activities. The lack of the expected final product of this reaction cascade, namely UDP-2-acetamido-2,6-dideoxy-\(\beta\)-\(L\)-\(\alpha\)-\(L\)-arabinohexose, may be due to the highly unstable lyxoox fetus. The high instability of the lyxoox fetus may be due to the highly unstable lyxoox fetus product or an equilibrium favoring the first two intermediates, UDP-2-acetamido-2,6-dideoxy-\(\alpha\)-\(D\)-xylo-4-hexulose and UDP-2-acetamido-2,6-dideoxy-\(\beta\)-\(L\)-arabinohexose. An alternative possibility would be a bifunctional enzyme WbjC/Cap5F with both 3-epimerase and 4-reductase activities. However, because we are not able to purify either UDP-2-acetamido-2,6-dideoxy-\(\beta\)-\(L\)-arabinohexose or UDP-2-acetamido-2,6-dideoxy-\(\beta\)-\(L\)-\(\alpha\)-\(L\)-arabinohexose, we are not able to investigate this scenario further.

The next reaction step in the biosynthesis of UDP-\(L\)-FucNAc involves the conversion of the intermediate sugars into a new compound that co-migrated with the precursor UDP-\(d\)-GlcNAc in CE analysis. NADH as well as NADPH could be used as hydride donor. CE-MS analysis showed a new peak at \(m/z\) 590, which would be expected after reduction of the keto-intermediate. CE-MS/MS experiments provided further evidence that the reduction was on the hexose moiety of the nucleotide-activated sugars. To complete identification of this compound, we purified the reduction product using anion exchange chromatography. The sugar was concentrated and, after addition of \(D_2O\), analyzed by NMR. Unlike the unstable keto-intermediates, this sugar turned out to be highly stable. One-dimensional TOCSY, one-dimensional NOESY, and other techniques demonstrated that the WbjC/Cap5F reduction product is UDP-2-acetamido-2,6-dideoxy-\(\beta\)-\(L\)-talose, sometimes referred to as UDP-L-pneumosamine or UDP-1-N-acetylpneumosamine (PneNAc) (50). Interestingly, the CP of S. pneumoniae type 5 is composed not only of \(\alpha\)-glucose and \(\alpha\)-glucuronic acid but also of 2-acetamido-2,6-dideoxy-\(\alpha\)-\(D\)-xylo-4-hexulose, 1-PneNAc, and 1-FucNAc (50). Thus, three of the intermediates and final products of the pathway described in the present study are part of the type 5 capsular polysaccharide. Unfortunately, no nucleotide sequence data of S. pneumoniae type 5 biosynthesis locus are yet available. Based on the biochemical characterization of the Wbj and Cap proteins, one can predict that homologs of WbjB/Cap5E, WbjC/Cap5F, and WbjD/Cap5G will be present in the corresponding S. pneumoniae type 5 gene cluster.

The last step of the UDP-\(L\)-FucNAc biosynthesis pathway would require 2-epimerization of UDP-2-acetamido-2,6-dideoxy-\(\beta\)-\(L\)-talose. WbjD/Cap5G is moderately homologous to the S. aureus UDP-\(d\)-GlcNAc 2-epimerase (Cap5P). As expected, WbjD/Cap5G catalyzed UDP-2-acetamido-2,6-dideoxy-\(\beta\)-\(L\)-talose to produce a new sugar peak, as detected by CE analysis. Further experiments have been performed using CE-MS. The fragmentation pattern did not change on this reaction from the one obtained for UDP-2-acetamido-2,6-dideoxy-\(\beta\)-\(L\)-talose, which is consistent with epimerization. As mentioned above, UDP-2-acetamido-2,6-dideoxy-\(\beta\)-\(L\)-talose proved to be a very stable sugar, unlike the final reaction product. When purified UDP-2-acetamido-2,6-dideoxy-\(\beta\)-\(L\)-talose was incubated with WbjD, UDP was produced by decomposition of the final product UDP-L-FucNAc to the monosaccharide and the nucleotide as observed by CE. Based on this experience and the following reasons, we were not able to pursue NMR analysis of UDP-L-FucNAc, the putative product of the WbjD reaction. First, the unstable nature of this sugar nucleotide posed difficulty in its preparation. Second, the epimerization activity of WbjD yielded a mixture of UDP-TalNAc and UDP-Fuc2NAc. Our CE (Fig. 2F) and CE-MS/MS results (Fig. 3D) support these findings. The proportion of UDP-Fuc2NAc was small in the mixture, thus making it difficult to purify and resolve the two compounds. Another piece of evidence to support our interpretation that WbjD and its homolog Cap5G in S. aureus are C2-epimerases is the fact that 1-FucNAc is a common sugar residue shared by the O-polysaccharide of P. aeruginosa O11 and the type 5 capsular polysaccharide of S. aureus. Therefore, once we were able to prove that UDP-TalNAc is formed by the activity of WbjC/Cap5F, we clearly correlated the activities of the first two enzymes to the modification of C-3, C-4, C-5, and C-6 of the substrate, UDP-GlcNAc. The only logical reaction to follow is C2-epimerization to produce UDP-Fuc2NAc, and our data obtained from CE and CE-MS/MS support this interpretation.

It was intriguing to observe that UDP-L-FucNAc acted as an inhibitor of WbjB/Cap5E. When all of the enzymes were added simultaneously, more NADPH was left in the reaction mixture than when the enzymes were added sequentially, allowing each
to go to completion. CE-MS clearly demonstrated that some UDP-α-GlcNAc was still left after the simultaneous reaction. No UDP-α-GlcNAc could be detected when the reactions were done consecutively. Feedback inhibition is a common way to regulate nucleotide-activated sugar biosynthesis, as in GDP-\(\beta\)-FucNAc, in which the final product, GDP-\(\beta\)-FucNAc, acts as an inhibitor of Gmd (51).

The cofactor dependence of WbjB/Cap5E proved to be crucial. The absence of any added cofactor or the addition of NAD\(^{+}\) instead of NADPH resulted in almost no conversion at all. The conversion observed (<5%) was most likely due to NADPH bound to the enzyme even during protein purification. In contrast, WbjC/Cap5F used both NADH and NADPH as hydride donors in the reduction reaction. Because of the lack of commercially available substrate, kinetic parameters have not been investigated. 4-Keto-sugars have frequently been described in the literature as unstable (46, 48, 49). Our report confirms these studies, because the keto-intermediates in- stead of NADP\(^+\) have commercially available substrate, kinetic parameters have not been investigated. 4-Keto-sugars have frequently been described in the literature as unstable (46, 48, 49).

The construction of a knockout mutant of the first gene (wbjb) involved in the biosynthesis of UDP-\(\beta\)-FucNAc clearly demonstrated that the corresponding enzyme WbjB is unmis takably involved in the biosynthesis of B-band O antigen in \(P.\) aeruginosa serotype O11. Inactivation of wbjb resulted in complete loss of B-band O antigen, as detected by silver staining of an SDS-PAGE resolved LPS and by Western immunoblotting analysis with polyclonal or monoclonal antibodies to serotype O11 (Fig. 12, A–C). The banding pattern of LPS isolated from the mutant BK103B was due to A-band O antigen, as was proven by the use of mAb N1F10 specific for A-band O antigen (Fig. 12, A and D). The \(P.\) aeruginosa gene wbjb and its \(S.\) aureus homolog cap5E were able to restore production of B-band O antigen, respectively, when provided on an \(E.\) coli\(p\). aeruginosa shuttle vector pUCP27. The three \(cap5\) genes cap5E, cap5F, and cap5G are all required for capsule synthesis in \(S.\) aureus. In this study, the insertional inactivation of cap5F and cap5G confirmed the essential role of these genes in CP5 production. A previous study had identified that a naturally occurring mutation in strain 8325-4 could be mapped to cap5E (39). However, it is apparent only from analysis of the recently published genetic data of the WbjC/Cap5F-catalyzed reduction reaction. The pathway is multifunctional, a coupled assay using both monosaccharides and UDP. Because the first enzyme acting in the pathway is multifunctional, a coupled assay using both monosaccharides and UDP. Because the first enzyme acting in the pathway is multifunctional, a coupled assay using both monosaccharides and UDP. Because the first enzyme acting in the pathway is multifunctional, a coupled assay using both monosaccharides and UDP. Because the first enzyme acting in the pathway is multifunctional, a coupled assay using both monosaccharides and UDP. Because the first enzyme acting in the pathway is multifunctional, a coupled assay using both monosaccharides and UDP.
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