Biosynthesis of dTDP-3-acetamido-3,6-dideoxy-α-D-galactose in Aneurinibacillus thermaoerophilus L420-91T

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The glycans of LPS of Gram-negative bacteria resemble O-antigens of LPS of Gram-negative organisms (3). Usually both macromolecules consist of a glycan chain containing identical repeating units, a core saccharide, and a linkage region, which is bound either to the S-layer protein or, as in LPS, to lipid A. Whereas the biosynthesis of LPS is well characterized (4), the exact mechanisms involved in biosynthesis of glycans of bacterial S-layer glycoproteins are still unknown. There is, however, preliminary evidence that both systems follow comparable pathways. For formation of the glycan chains in the cytoplasm in both systems nucleotide-activated sugars serve as precursors in the glycosylation reaction (5). It is proposed that the pre-assembled glycan chains are transported across the cytoplasmic membrane in a lipid-bound state, either linked to undecaprenol (LPS) (4) or dolichol (glycoproteins) (6). Although experimentally not yet determined, final transfer of the glycan chains to the nascent S-layer polypeptide is assumed to take place on the outer side of the cytoplasmic membrane, comparable with archaeal S-layers (7).

The repeating unit of the glycan chain of Aneurinibacillus thermaoerophilus L420-91T (8, 9), a member of the Bacillus/Clostridium group, consists of the sugars D-rhamnose and 3-acetamido-3,6-dideoxy-D-galactose (3-N-acetylufucosamine (D-Fuc3NAc)). The backbone of each repeating unit is built by four D-rhamnose units, and two of them are substituted by α-1,2-linked D-Fuc3NAc units (10, 11). Both constituting sugars are well known components of the LPS of Gram-negative bacteria (12–14). Derivatives of D-Fuc3NAc have also been found in the LPS of clinical strains of Vibrio cholerae (15) and Acinetobacter baumannii (16), where the acetyl group is replaced by modified amino acids and acyl residues, respectively.

Recently, we were able to characterize the biosynthesis of the GDP-D-rhamnose, one of the sugar components of the glycan chain of A. thermaoerophilus L420-91T (17). The other constituent, D-Fuc3NAc, was first described in cell-free extracts of Xanthomonas campestris by Volk and Ashwell (18). They proposed that D-glucose will eventually be converted into dTDP-D-Fuc3NAc and description of a novel type of isomerase capable of synthesizing dTDP-6-deoxy-d-xylohex-3-ulose from dTDP-6-deoxy-d-xylohex-4-ulose.

Glycosylated and non-glycosylated S-layer1 proteins represent the outermost cell envelope components of organisms of the domains Bacteria and Archaea (1, 2). Structurally, S-layer glycoproteins from Gram-positive bacteria resemble O-antigens of LPS of Gram-negative organisms (3). Usually both macromolecules consist of a glycan chain containing identical repeating units, a core saccharide, and a linkage region, which is bound either to the S-layer protein or, as in LPS, to lipid A. Whereas the biosynthesis of LPS is well characterized (4), the exact mechanisms involved in biosynthesis of glycans of bacterial S-layer glycoproteins are still unknown. There is, however, preliminary evidence that both systems follow comparable pathways. For formation of the glycan chains in the cytoplasm in both systems nucleotide-activated sugars serve as precursors in the glycosylation reaction (5). It is proposed that the pre-assembled glycan chains are transported across the cytoplasmic membrane in a lipid-bound state, either linked to undecaprenol (LPS) (4) or dolichol (glycoproteins) (6). Although experimentally not yet determined, final transfer of the glycan chains to the nascent S-layer polypeptide is assumed to take place on the outer side of the cytoplasmic membrane, comparable with archaeal S-layers (7).

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ably involved in dTDP-\(\text{D-Fuc}\)3NAc biosynthesis were first identified in the dTDP-\(\text{rhamnose}\) pathway for review, see Ref. 20). These are glucose-1-phosphate thymidylyltransferase (RmlA) and dTDP-glucose-4,6-dehydratase (RmlB), which catalyze the reaction of glucose 1-phosphate and thymidine triphosphate to dTDP-glucose and in the second step to dTDP-\(\text{D-xylohex-4-ulose}\). This latter compound serves as substrate for a series of reactions, including the formation of dTDP-\(\text{D-Fuc}\)3NAc (21). Activities of RmlA and RmlB in crude extracts of \(A.\ \text{thermoaerophilus}\) L420-91T indicated a possible involvement of these two enzymes in the biosynthesis of dTDP-\(\text{D-Fuc}\)3NAc (22). Recently, the gene cluster responsible for biosynthesis of X. \text{campestris} has been identified (14). Downstream of the dTDP-\(\text{rhamnose}\) genes the authors described a cluster of 15 genes to be involved in the biosynthesis of the LPS glycan chain of X. \text{campestris}. Two genes of the cluster, namely \text{wx}C\(\text{M}\) and \text{wx}K\(\text{C}\), were assumed to code for enzymes that synthesize dTDP-\(\text{D-Fuc}\)3NAc. \text{wx}C\(\text{M}\) was proposed to be bifunctional and to catalyze both the isomerization and the transacylation reaction. The second ORF, \text{wx}K\(\text{C}\), was assumed to be responsible for the transamination reaction. So far none of the described genes has been functionally expressed nor have the gene products been biochemically characterized.

This report is the first functional characterization of the biosynthesis pathway of dTDP-\(\text{D-Fuc}\)3NAc in the S-layer glycoprotein glycan of the Gram-positive thermophile \(A.\ \text{thermoaerophilus}\) L420-91T. We could show that five enzymes, including a novel type of isomerase, are involved in the biosynthesis of this nucleotide-activated precursor of \(\text{D-Fuc}\)3NAc.

**EXPERIMENTAL PROCEDURES**

**Materials**—dTDP-\(\text{D-glucose}\), \(\text{t-glutamate}\), acetyl-CoA, SH-CoA, 5,5'-dithio-bis(2-nitrobenzoic acid) (Ellman's reagent) were from Sigma. GSTrap, HiTrap chelating, Mono Q HR 5/5, and Sephadex G-10 columns were purchased from Amersham Biosciences. All primers used in these studies were synthesized by Invitrogen. Glutathione (reduced form) and imidazole were purchased from ICN Chemicals (Eschwege, Germany).

**Bacterial Strains and Culture Conditions**—\(A.\ \text{thermoaerophilus}\) L420-91T was grown in SVIII media at 55°C. All enzymes were determined with 5 nmol of FdtC and 5 nmol of PLP, and 50 nmol of \text{t-glutamate} and was performed with and without addition of FdtC.

**Kinetic Measurements**—To test the function of the transacylase 5 nmol of FdtC was incubated with 50 nmol of acetyl-CoA and 50 nmol of dTDP-\(\text{D-Fuc}\)3NAc. Assays were performed at 37°C and were analyzed by reverse phase (RP-HPLC) with 0.5 mM NaH2PO4 solution buffer, pH 6.0, as the mobile phase at a flow rate of 0.8 ml/min (29). The eluate was monitored with a UV detector at 254 nm. Approximately 50 nmol of dTDP-\(\text{D-xylohex-4-ulose}\) were synthesized as described previously (30). To synthesize dTDP-\(\text{D-Fuc}\)3NAc, 20 nmol of dTDP-\(\text{D-xylohex-4-ulose}\), 1 µmol of PLP, 50 nmol of \text{t-glutamate}, 200 nmol of FdtA, and 200 nmol of FdtB were incubated in 5 ml of 50 mM \(\text{K}_{2}\text{HPO}_4\) buffer, pH 7.4, and 5 mM MgCl2 at 37°C for 1 h. The volume was reduced by vacuum evaporation to approximately 1 ml, and the material was purified by RP-HPLC with a 50 mM ammonium acetate buffer, pH 6.0, as the mobile phase. To remove the HPLC buffer the solution containing dTDP-\(\text{D-Fuc}\)3NAc was frozen and lyophilized. An overall yield of 80% of dTDP-\(\text{D-Fuc}\)3NAc could be achieved.

**Kinetic Measurements**—To determine the kinetic constants of FdtC the reaction between the free sulffhydryl group of SH-CoA, a reaction product during the transacylation, and Ellman’s reagent (5,5'-dithio-bis(2-nitrobenzoic acid)) (31) was measured continuously as the increase in absorbance at 412 nm on a Hitachi U-2010 (Maidenhead, UK) spectrophotometer. The resulting enzymatic activities were calculated with a molar absorption coefficient of 14,150 M\(^{-1}\) cm\(^{-1}\) according to the instructions of the manufacturer. A standard assay was performed in a 0.1 ml reaction mixture, containing 100 mM \(\text{K}_{2}\text{HPO}_4\) buffer, pH 7.4, containing 0.5 mM FdtC, 0.5 mM 5,5'-dithio-bis(2-nitrobenzoic acid), 25–200 µM dTDP-\(\text{D-Fuc}\)3NAc, 600 µM acetyl-CoA, and 3 µM FdtC. To determine kinetic constants for acetyl-CoA the same assay was used, but the acetyl-CoA concentration was varied from 25 to 200 µM, and the dTDP-\(\text{D-Fuc}\)3NAc concentration was kept constant at 600 µM. To obtain the apparent Michaelis constant directly, the gFdtC construct was overexpressed and purified. GFR was determined directly by protein determination using the program Sigma Plot (SPSS Science, Chicago, IL).
Identification of the Genes Involved in the Biosynthesis of dTDP-\(\text{d-Fucp3NAc}\) 

Previously we reported cloning and characterization of the two genes that are responsible for the formation of GDP-\(\text{d-Fucp3NAc}\) from GDP-\(\text{d-mannose}\) in \(A.\ thermoaerophilus\) L420-91\(^T\) \((17)\). Assuming that the genes for the biosynthesis of the nucleotide-activated precursors of the glycan chain are clustered, we used chromosome walking to locate the genomic region for the biosynthesis of dTDP-\(\text{d-Fucp3NAc}\) \((24)\). Approximately 9 kilobases downstream of the \(rmd\) gene, which encodes an enzyme involved in the formation of GDP-\(\text{d-rhamnose}\) \((17)\), two overlapping PCR fragments (2 and 3.5 kilobases) were obtained. Subsequent sequencing of both fragments revealed a genomic region containing seven ORFs (Fig. 1; GenBank\textsuperscript{TM} accession numberAY205257). Using BLAST searches, the first ORF of that region, consisting of 139 amino acids, showed homologies to a putative bifunctional enzyme of \(X.\ campestris\), namely \(\text{Xcm}^\circ\). Its C terminus is predicted to code for an isomerase and the N terminus for a transacetylase \((14)\). The putative isomerase presumably responsible for the conversion of dTDP-6-deoxy-\(\text{d-xylohex-4-uloside}\) into nucleotide-linked hex-3-ulose isomer showed in addition to homologies to \(X.\ campestris\) genes \((AF204145)\), homologies to genes from \(L.\ anguillarum\) \((AP005373)\), \(L.\ borgpetersenii\) \((AF316500)\), \(S.\ fradiae\) \((U08223)\), \(B.\ fragilis\) \((AF125164)\) and \(E.\ coli\) O91 \((AY035396)\). With the exception of \(X.\ campestris\), the presence of \(\text{d-Fucp3N}\) (either acetylated or acetylated) has not been reported in the LPS or lipooligosaccharides of the Gram-negative representatives. \(S.\ fradiae\), however, is a Gram-positive organism without LPS in the cell envelope. Interestingly, for \(E.\ coli\) O91 as well as for \(S.\ fradiae\), the gluco epimer of \(\text{d-Fucp3N}\), 3-acetamido-3,6-dideoxy-\(\text{d-glucose}\) (\(\text{d-Quip3N}\)), was found to be part of either the repeating units of LPS O-antigens or of the antibiotic tylosin \((32)\). The second ORF (192 amino acids) was predicted to code for an acyl-acetyltetraetherase. A BLAST search revealed high homologies to several acyl- and acetyltransferases that are involved in the metabolism of nucleotide-activated sugar precursors. The third ORF (363 amino acids) showed high similarities to genes of the \(\text{DegT/Dnrt/EryC1/StrS}\) family of aminotransferases. Highest homologies were achieved with sequences from organisms that possess genes similar to the putative isomerase \(\text{fdtA}\) (Table I). The presence of all three enzymes in these organisms would thus indicate the possible occurrence of modified \(\text{d-Fucp3N}\) residues in the cell envelope or metabolite glycan structures. Downstream of the transaminase two unidentified ORFs showed weak similarities to glycoyl transferases and a putative integral membrane protein. The role of these two genes was not the subject of this study. Sequence alignment of the last two ORFs of the gene cluster showed high similarity to \(\text{glucose-1-phosphate thymidyltransferase (RmlA)}\) and dTDP-\(\text{d-glucose dehydratase (RmlB)}\). These two enzymes are known to be involved in the well-characterized biosynthesis of nucleotide-activated \(\text{rhamnose of LPS (20, 33)}\) as well as of the S-layer glycoprotein of Gram-positive organisms \((22)\). They synthesize the precursors for the biosynthesis of \(\text{e.g. d-TDP-fucose (34)}\) and dTDP-6-deoxy-\(\text{L-talose (35)}\). None of the encoded proteins possesses membrane-spanning domains or signal peptides for secretion, implying that the biosynthesis of this nucleotide-activated sugar occurs in the cytoplasm.

For the newly identified genes we suggest the following names: \(\text{fdtA}\) for the putative isomerase, \(\text{fdtB}\) for the transaminase, and \(\text{fdtC}\) for the transacetylase. In this designation \(f\) codes for fucosamine, \(d\) for the \(D\) configuration and \(t\) (= three) indicates the substituted C-3 atom of the sugar ring. Based on the results from our work in \(A.\ thermoaerophilus\) L420-91\(^T\) \((22)\) and also from previous studies on \(X.\ campestris\) \((14, 18, 36)\), we propose a general biosynthesis pathway for \(\text{d-Fucp3N}\) as depicted in Fig. 2.

Overexpression and Purification of \(\text{fdtA}\), \(\text{fdtB}\), and \(\text{fdtC}\)—Before overexpression and purification of the enzymes the sequences of the corresponding plasmids containing the open reading frames of interest were verified by nucleotide sequencing. To confirm the function proposed for the three enzymes involved in the biosynthesis of dTDP-\(\text{d-Fucp3N}\) they were heterologously overexpressed in \(E.\ coli\) and purified by affinity chromatography and anion exchange chromatography. To overcome problems with protein stability, \(\text{fdtA}\) and \(\text{fdtC}\) were expressed with a GST tag and purified using GSTrap columns. In addition, \(\text{fdtA}\) was also expressed as N-terminal (His)_\(n\)-tagged fusion protein as was \(\text{fdtB}\). Both recombinant proteins could be highly purified on a HiTrap chelating column (Ni\(^2+\)). To improve the purity of the enzymes an anion exchange chromatography step on a Mono Q column was applied subsequently. Expression and purification was monitored by SDS-PAGE and showed that the apparent molecular mass of all proteins was in agreement with the values deduced from the nucleotide sequences (Fig. 3). To increase the stability, the enzymes were concentrated by ultrafiltration before storage at 4 °C or −20 °C and supplemented with 50% glycerol. The concentrated enzyme preparations were stable at 4 °C for several months with only a marginal decrease of their enzyme activity (data not shown).

\(\text{fdtA}\) Is Responsible for the Isomerization of dTDP-6-deoxy-\(\text{d-xylohex-4-uloside}\) into dTDP-6-deoxy-\(\text{d-xylohex-3-uloside}\)—This conversion is supposed to be the essential step in the formation of dTDP-\(\text{d-Fucp3N}\) in vivo. However, it was not possible to isolate this intermediate product by HPLC methods, including reverse phase and ion exchange chromatography. RP-HPLC analysis of dTDP-6-deoxy-\(\text{d-xylohex-4-uloside}\) showed an elution...
profile identical to that described in a recent work (35). The isomerase product dTDP-6-deoxy-D-xylohex-3-ulose revealed an identical elution profile, and thus, distinction from the 4-keto educt was impossible (Fig. 4B). The activity of FdtA was monitored by a simultaneous incubation with dTDP-6-deoxy-D-xylohex-4-ulose with FdtB and the required co-substrates, as described under "Experimental Procedures." The resulting product, dTDP-D-Fucp3N, was detectable by HPLC analysis (Fig. 4C). The activities of FdtA as GST- and His-tagged proteins were also examined, and both fusion proteins showed comparable activities.

The direct conversion of dTDP-6-deoxy-D-xylohex-4-ulose into the 3-keto product by the action of FdtA was studied by NMR spectroscopy. Before the interpretation of the NMR data, a full spectral assignment of the starting material was performed. It matched favorably with the literature data (37) and

### TABLE I
Homology of putative proteins involved in biosynthesis of derivatives of dTDP-D-Fucp3N or dTDP-D-Quip3N

| Organism     | Identity/similarity (accession No.) | FdtA (AAO06351) | FdtB (AAO06353) | FdtC (AAO06352) |
|--------------|-------------------------------------|-----------------|-----------------|-----------------|
| X. campestris| 37/59 (AAK53472)                    | 41/56 (AAK53470)| 61/77 (AAK53472)|                 |
| C. jejuni    | 55/73 (AAK12959)                    | 66/77 (AAK12958)| 62/78 (AAK12958)|                 |
| T. elongatus | 50/66 (BAC08895)                    | 53/74 (BAC08894)| 59/79 (BAC08894)|                 |
| Li. anguillarum | 57/74 (AA81630)                    | 62/80 (AA881632)| 59/74 (AA881631)|                 |
| Le. interogens | 50/67 (AAK19911)                 | 53/69 (AAD28182)| 59/74 (AAD28182)|                 |
| Le. borgpetersenii | 48/66 (AAD12964)         | 53/70 (AAD12965)| 59/74 (AAD12965)|                 |
| S. fradiae   | 35/52 (U08223)                      | 48/63 (Q54142)  | 59/74 (AAD6740) | 29/47 (AAD6747) |
| B. fragilis  | 39/60 (AAD6739)                    | 53/72 (AAD6740) | 59/74 (AAD6740) |                 |
| E. coli      | 50/67 (AAK60451)                    | 53/72 (AAK60453)| 59/74 (AAD6740) |                 |

**X.**, Xanthomonas; **C.**, Campylobacter; **T.**, Thermosynechococcus; **Li.**, Listonella; **Le.**, Leptospira; **S.**, Streptomyces; **B.**, Bacteroides; **E.**, Escherichia.

Fig. 2. Pathway for the synthesis of dTDP-D-Fucp3N from D-glucose 1-phosphate and dTTP. I, D-glucose 1-phosphate; II, dTDP-D-glucose; III, dTDP-6-deoxy-D-xylohex-4-ulose; IV, dTDP-6-deoxy-D-xylohex-3-ulose; V, dTDP-D-Fucp3N. RmlA, glucose-1-phosphate thymidyltransferase; RmlB, dTDP-D-glucose-4,6-dehydratase; FdtA, dTDP-6-deoxy-D-xylohex-4-ulose isomerase; FdtB, dTDP-6-deoxy-D-xylohex-3-uloseaminase; FdtC, dTDP-D-Fucp3N acetylase.

Fig. 3. Coomassie Blue-stained SDS-PAGE analysis showing the purified enzymes. Each protein was incubated at 100 °C for 10 min in 0.1% SDS and 1% 2-mercaptoethanol and applied to a 12% SDS-polyacrylamide gel. A, FdtA; B, FdtB; C, FdtC. About 1 μg of protein is loaded in each lane.

Fig. 4. RP-HPLC profiles of the enzymatic synthesis of dTDP-D-Fucp3N. A, no enzyme added to dTTP-D-glucose; B, RmlB added to dTDP-D-glucose to form dTDP-6-deoxy-D-xylohex-4-ulose; dTDP-6-deoxy-D-xylohex-3-ulose showed an identical elution pattern; C, dTDP-D-Fucp3N as the reaction product of FdtA and FdtB; D, addition of FdtC to dTDP-D-Fucp3N to synthesize dTDP-D-Fucp3NAc.
indicated a ~4.5:1 ratio of hydrate to keto form of dTDP-6-deoxy-D-xylohex-3-ulose. H,H-COSY spectroscopy revealed a clear connectivity from H-1 to H-2 and H-3 for both forms, thus eliminating the occurrence of 3-keto precursors within the detection limits. Analysis of the incubation product of FdtA with dTDP-6-deoxy-D-xylohex-4-ulose at pH 7.4 indicated the formation of dTDP-6-deoxy-D-xylohex-3-ulose. As shown in Fig. 5 conversion into the 3-keto form occurred rapidly; however, a full assignment could not be achieved due to the small signal intensity and severe signal overlap of the remaining substrate. Comparison of the data with a non-enzymatic control reaction and assignment of signals separated from the bulk region allowed a partial assignment of the spectra. Whereas in the non-catalyzed reaction no significant conversion was seen after 2 h, the NMR spectrum of the enzymatic reaction performed at 37 °C already indicated after 5 min the formation of a new component (Fig. 5). The anomic proton of this reaction product was observed at lower field (5.86 ppm) with a $^3$J$_{H_1,P}$ coupling constant of 7.0 Hz and a $^3$J$_{H_1,H_2}$ coupling constant of 4.5 Hz. The signal of H-2 at 4.93 ppm gave a coupling to the anomic phosphate but no further spin-spin coupling to position 3, which in conjunction with the observed downfield shift would be consistent with the presence of a neighboring keto group at C-3. Moreover, an additional 6-deoxy signal was observed at 1.31 ppm ($^3$J$_{H_6,H_5}$ 6.5 Hz), which was shown by COSY and heteronuclear multiple quantum coherence experiments to be correlated to H-5/C-5 signals at 4.46 ppm/66.6 ppm, respectively, indicating a change of configuration at the neighboring C-4 carbon (relative to dTDP-glucose). These data are also distinctly different from those published for dTDP-6-deoxy-D-ribohex-3-ulose at pH 7.4 (36 h). The combined evidence would, thus, be in accordance with previous studies on transaminases (39).

Among several amino group donors tested, including L-glutamine, L-alanine, and L-aspartate, only L-glutamate resulted in a simultaneous incubation of dTDP-6-deoxy-D-xylohex-4-ulose with FdtA and FdtB. FdtB requires PLP and L-glutamate as co-factors. Divalent metal ions are not required, which is in accordance with previous studies on transaminases (39).

To obtain better insight into the isomerization reaction and the specific activity of FdtB the 4-keto substrate was incubated without the addition of FdtA. Incubation of the reaction mixture at pH 7.4 in the absence of co-factors/co-substrates was monitored by NMR spectroscopy. The spectra recorded in a time course of 24 h did not show differences to a non-enzymatic control reaction (compare with Fig. 5A). Incubation of 4-keto substrate with FdtB and the co-factors yielded over a time span of 36 h ~30% conversion to dTDP-D-Fucp3N.

To test the substrate specificity of the transaminase further, the isomerase reaction was conducted for 7 h before the addition of FdtB, which would also allow the onset of nonspecific isomerization reactions. Work-up of the reaction mixture and NMR analysis revealed a single product, dTDP-D-Fucp3N, similar to the experiments where both enzymes had been added to the substrate simultaneously.

The structure of the product generated by the action of the enzymes FdtA/FdtB could be fully assigned on the basis of the NMR spectroscopic data (Table II, substance V). Thus, for the 3-amino-3-deoxy-derivative dTDP-D-Fucp3N, the signal at 53.4 ppm was due to a nitrogen-bearing carbon atom being correlated to a H-3 signal, displaying a large trans-diaxial coupling constant $^5$J$_{H_3,H_5}$ (11.1 Hz) and a small value for the coupling constant to H-4 ($^2$J$_{H_3,H_4}$ 3.2 Hz). This is consistent with a 3-amino-3,6-dideoxy-D-xylo configuration. In addition, the $^{13}$C NMR signals of C-4 and C-5 experienced an up-field shift to 68.9 and 68.3 ppm, respectively.

FdtC Catalyzes the Transfer of an Acetyl Group to dTDP-D-Fucp3N to Form dTDP-D-Fucp3NAc—The transacetylation reaction represents the last step of the formation of nucleotide-
activated d-Fucp3NAc. The commonly used acetyl donor of these reactions acetyl-CoA was also used in this experiment. Isolation of the final product was achieved by RP-HPLC (Fig. 1) and this process involved two well characterized enzymes from the biosynthetic pathway of either nucleotide-activated L-rhamnose (40, 41), dTDP-d-fucose (34), dTDP-6-deoxy-L-talose (35), or dTDP-4-acetamido-4,6-dideoxy-L-glucose (42), namely RmlA and RmlB. These two enzymes catalyze the formation of dTDP-d-glucose and dTDP-6-deoxy-d-xylulose-4-ulose, respectively. The latter compound represents the precursor for the sugar biosynthetic pathway of d-Fucp3NAc.

Because of the fact that only couple assays were possible for the isomerase and transaminase reactions, no kinetic constants were determined. However, reaction kinetics were analyzed for the transacetylase reaction. For the determination of the kinetic constants of FdtC with acetyl-CoA and dTDP-d-Fucp3N Ac Ellman’s reagent was used. Both substrates exhibited comparable affinity to FdtC. The Michaelis-Menten constants \( K_{m} \) were 66.7 \((\pm 15.9)\) and 61.0 \((\pm 4.4)\) \(\mu M\) for dTDP-d-Fucp3N Ac and acetyl-CoA, respectively. The turnover numbers, \( k_{cat} \), were 2.3 \((\pm 0.2)\) and 3.1 \((\pm 0.1)\) s\(^{-1}\).

**General Properties of the Enzymes**—All purified enzymes showed maximum activities between 50 and 55 °C, although some activity could also be detected at 30 °C. FdtB was also shown catalyze the reverse reaction using \( \alpha \)-ketoglutarate and dTDP-d-Fucp3N Ac as the substrate instead of L-glutamate and dTDP-6-deoxy-d-xylulose-4-ulose. By adding FdtA to the reaction mixture the equilibrium shifted toward dTDP-6-deoxy-d-xylulose-4-ulose. The reverse reaction was monitored by HPLC (data not shown). However, a reverse reaction catalyzed by FdtC could not be detected.

**DISCUSSION**

d-Fucp3NAc is a sugar component of S-layer glycoproteins (10) and LPS of several Gram-negative organisms. It was first described in *X. campestris* in 1963 (18). Based on this work Shibae (19) postulated that the formation of d-Fucp3NAc involves two well characterized enzymes from the biosynthetic pathway of either nucleotide-activated \( \alpha \)-rhamnose (40, 41), dTDP-d-fucose (34), dTDP-6-deoxy-L-talose (35), or dTDP-4-acetamido-4,6-dideoxy-L-glucose (42), namely RmlA and RmlB. These two enzymes catalyze the formation of dTDP-d-glucose and dTDP-6-deoxy-d-xylulose-4-ulose, respectively. The latter compound represents the precursor for the sugar biosynthetic pathway of d-Fucp3NAc.

In a previous report the gene cluster and the enzymes involved in the biosynthesis of GDP-\( \alpha \)-rhamnose in *A. thermoaerophilus* L420-91\(^{\circ}\) have been identified and characterized biochemically (17). Chromosome walking revealed the presence of five open reading frames downstream of the GDP-\( \alpha \)-rhamnose operon, presumably responsible for the biosynthesis of dTDP-d-Fucp3NAc. Two of the ORFs showed high similarities to *rmlA* and *rmlB*, and two others were homologous to the protein families of transaminases and transacetylases. The fifth gene in the cluster showed homology with a putative enzyme, which is assumed to catalyze potentially an isomerase reaction (14). To characterize the enzymes involved in the biosynthetic pathway they were expressed in *E. coli* and purified.

FdtA catalyzes the isomerization step in the biosynthetic pathway of dTDP-d-Fucp3NAc. In this reaction the 4-keto intermediate is isomerized to the 3-keto product, with d-xylulose configuration. Several attempts to isolate the reaction product...
of the isomerase reaction dTDP-6-deoxy-d-xylohex-3-ulose by HPLC were unsuccessful. Thus, to confirm the function of this enzyme, on-line NMR measurements were applied. These experiments showed that FdtA catalyzes the rapid formation of dTDP-6-deoxy-d-xylohex-3-ulose in small amounts. This finding points toward the importance of a tightly controlled product formation in organisms such as X. campestris, which besides dTDP-d-Fucp3NAC also synthesizes dTDP-L-rhamnose from the same source of precursor. Here we provide the first functional description of a specific isomerase that catalyzes the transition of dTDP-6-deoxy-d-xylohex-4-ulose into dTDP-6-deoxy-d-xylohex-3-ulose.

FdtA shares no homologies with known isomerase families, and no common motifs could be detected. Because the enzyme lacks metal ions and oxido reduction co-factor binding motifs, the isomerization reaction catalyzed by FdtA may act by a novel mechanism. We identified gene homologs of fdtA in several Gram-negative organisms, although their specific functions are not known yet. Because of the importance of the isomerase reaction step, the presence of FdtA may imply that dTDP-6-deoxy-d-ribohex-3-ulose could be achieved chemically via enolization without the addition of an isomerase (37). For an efficient turnover of the substrate, however, the presence of FdtA is indispensable. Our experiments indicated that incubation of dTDP-6-deoxy-d-xylohex-4-ulose with FdtB and the corresponding co-substrates at pH 7.4 leads over time only to dTDP-d-Fucp3NAC.

FdtB turned out to be a member of the Deg/Dnr/J/EryC1/Str5 family of aminotransferases that contains among others 3-amino-5-hydrobenzoic acid synthase, an enzyme required for the biosynthesis of ansamycin antibiotics. Crystal structure analysis of this enzyme revealed the presence of a PLP binding site, which is conserved among the aspartate aminotransferase structural family and is also present in FdtB. This specific site is characterized by a serine residue that is followed by three to five residues before the active site lysine (S_X_3_K) (45). In the BLAST search the amino acid sequence around the PLP binding site revealed a highly conserved region, obviously a common feature of aminotransferases, which use nucleotide-activated 6-deoxy sugars as substrate. Besides the DNA sequences described in Table I, similar transferases may also be involved in the biosynthesis of several antibiotics that contain C-3-amidated 6-deoxy sugars, such as oleandomycin (46), daunomycin (47), erythromycin (48), and tylosin (32). The formation of C-3 amidated 6-deoxy sugars also requires the presence of an isomerase-like enzyme, which until today was found only in the...
biosynthetic pathway of tylosin of S. fradiae (49).

The nucleotide-activated form of D-Fuc3N may also play an important role in the formation of acetylated sugar precursors as described in this study but also in other acylated carbohydrates. In several Gram-negative organisms the corresponding LPS was found to contain β-Fuc3N acylated with e.g. (R,R)-3-hydroxy-3-methyl-5-oxoproline such as in V. cholerae 05 (15) or 1-2-acetoxypropionamido (50) and 1-3-hydroxybutyryl groups (16) in A. baumannii. The biosynthesis of these modified β-Fuc3N units may, therefore, have similar if not identical pathways. However, because of the lack of genetic data and functional characterization of these pathways this assumption remains to be further substantiated.

FTdC catalyzes the transfer of an acetyl group to dTDP-D-Fucp3NAc Biosynthesis

Therefore, exploration of their potential biotechnological application than similar enzymes from mesophilic organisms (22).

A. baumannii remains to be further substantiated.

Functional characterization of these pathways this assumption obtained may be caused by temperatures below the optimum temperature of that enzyme. These data are in agreement with these data are in agreement with the left-

C-terminal domain is responsible for the CoA-dependent acetylation of glycine and catalyzes the transfer of the acetyl group (52).

Therefore, x-ray crystal structure analysis of FdtC catalyzes the transfer of an acetyl group to dTDP-D-Fucp3NAc Biosynthesis

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