Identification of the GDP-*N*-acetyl-D-perosamine producing enzymes from *Escherichia coli* O157:H7

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Abstract **GDP-*N*-acetyl-D-perosamine is a precursor of the LPS-O-antigen biosynthesis in *Escherichia coli* O157:H7. Like other GDP-6-deoxyhexoses, GDP-*N*-acetyl-D-perosamine is supposed to be synthesized via GDP-4-keto-6-deoxy-D-mannose, followed by a transamination- and an acetylation-reaction catalyzed by PerA and PerB. In this study, we have overproduced and purified PerA and PerB from *E. coli* O157:H7 in *E. coli* BL21. The recombinant proteins were partly characterized and the final product of the reaction catalyzed by PerB was shown to be GDP-*N*-acetyl-D-perosamine by chromatography, mass spectrometry, and ^{1}H-NMR. The functional expression of PerB provides another enzymatically defined pathway for the synthesis of GDP-deoxyhexoses, which is needed to further study the corresponding glycosyltransferases in vitro.**
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1. Introduction

D-Perosamine (D-Rha*p*4N), a 6-deoxyhexose, as well as its *N*-acyl-derivatives are important components of lipopolysaccharides in, amongst others, *Vibrio cholerae* O1, *Brucella* spp., *Citrobacter freundii* F90, *Salmonella enterica* O30, and *Escherichia coli* O157 [1–3]. The O-antigen of *E. coli* O157:H7, a participating causative agent of enterohemorrhagic diarrhea, consists of a four sugar repeated subunit containing *N*-acetyl-D-perosamine, L-fucose, D-glucose, and *N*-acetyl-D-galactose [→ 2-α-D-PerNAc-(1 → 3)-α-L-Fuc-(1 → 4)-β-D-Glc-(1 → 3)-α-D-GalNAc-(1→)], which is attached to the lipid A core unit [4]. The introduction of *N*-acetyl-D-perosamine into the oligosaccharide chain is dependent on its prior activation as GDP-hexose and subsequent modification. The synthesis of GDP-*N*-acetyl-D-perosamine starts from GDP-D-mannose, which is derived from mannose-6-phosphate in two steps catalyzed by the mannose-6-phosphate mutase (ManB)

and the guanosine diphosphomannose pyrophosphorylase (ManC). The two further enzymatic steps characteristic for the formation of GDP-D-perosamine follow that of other 6-deoxyhexoses starting with a NAD(P)$^+$ dependent NDP-hexose 4,6-dehydratase reaction that forms NDP-6-deoxy-D-4-hexulose. This intermediate can be further modified by epimerization-, reduction- or transamination-reactions [5]. In case of GDP-D-perosamine it could be shown that GDP-D-mannose is converted by a GDP-mannose-4,6-dehydratase into GDP-4-keto-6-deoxy-D-mannose [6], and then transaminated by a pyridoxal-phosphate dependent GDP-D-perosamine synthetase [7]. The final step towards GDP-*N*-acetyl-D-perosamine requires an acetyltransfer to the C4 amino group (Fig. 1). Genes for the synthesis of nucleotide-activated sugars are generally organized together within the gene cluster for the particular bacterial polysaccharide [8]. The DNA-sequence of the gene cluster for O-antigen biosynthesis in *E. coli* O157:H7 has been described and analyzed by the Reeves group [9]. It contains 12 genes encoding for the nucleotide-sugar biosynthesis, for glycosyltransferases, a flippase, and an O-antigen polymerase (Fig. 2). The *wbdR* (*perB*) gene in this cluster is supposed to encode for the GDP-D-perosamine *N*-acetyltransferase based on sequence homology to other acetyltranferases [9]. Surprisingly, the *wbdR (perB)* gene is exclusively found in the O-antigen cluster of *E. coli* O157. No equivalent gene of *perB* could be found within the gene cluster of *S. enterica* O30 or *C. freundii* F90 [10], although all three strains show the identical structure of their O-antigen [11,12] and also the same organization of their O-antigen gene cluster, except the additional *perB* gene in the *E. coli* cluster (Fig. 2).

The aim of this study was to prove the responsibility of PerB for the formation of GDP-*N*-acetyl-D-perosamine in *E. coli* O157:H7. Here, we describe the identification of the genes *per* (*perA*) and *wbdR* (*perB*) from the O-antigen gene cluster of *E. coli* O157:H7 after expression in *E. coli* BL21(DE3) pLysS. By using overexpressed and affinity purified proteins, we showed that Per (PerA) is a glutamate and pyridoxal-5-phosphate dependent aminotransferase producing GDP-α-D-perosamine and that PerB is a *N*-acetyltransferase transferring an acetyl-residue from acetyl-CoA onto GDP-α-D-perosamine. Our report describes the first functional expression and in vitro assay of an *N*-acetyltransferase acting on a GDP-activated hexose as well as on a D-Rha*p*N4 sugar residue. While we were preparing this manuscript another group published a report on the characterization of the GDP-D-perosamine synthetase (PerA) form *E. coli* O157:H7, which presented similar observations on the heterologous PerA protein [13].

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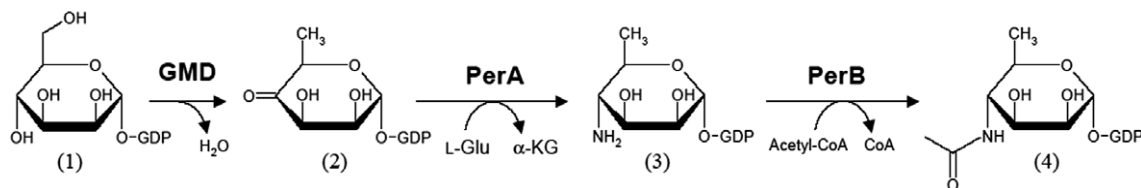*E-mail address:* imbca@imb.uni-stuttgart.de (C. Albermann).

Fig. 1. Pathway for the biosynthesis of GDP-*N*-acetyl-α-D-perosamine (4) from GDP-α-D-mannose (1) via GDP-4-keto-6-deoxy-D-mannose (2) and GDP-D-perosamine (3). GMD = GDP-D-mannose 4,6-dehydratase; PerA = GDP-perosamine synthetase; PerB = GDP-perosamine *N*-acetyltrans-ferase.
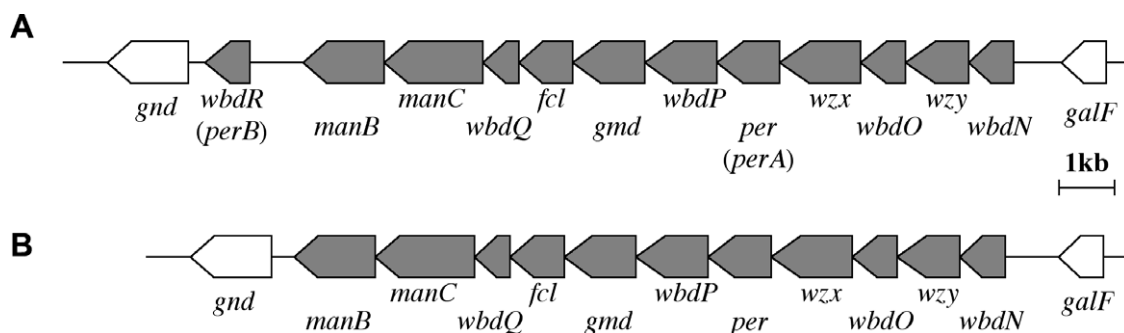

Fig. 2. Organization of O-antigen gene cluster of *E. coli* O157:H7 (A) and *S. enterica* O30 and *C. freundii* F90 (B) (18), including putative genes of the nucleotide activated hexose pathways (*manB*, *manC*, *gmd*, *fcl*, *per*, *wbdR*, *wbdQ*), three putative genes for glycosyltransferases (*wbdN*, *wbdO*, *wbdP*), O-antigen polymerase (*wzy*), and a flippase (*wzx*).

2. Materials and methods

2.1. Bacterial strains, growth conditions, and media

The bacterial strains used in this study were *Escherichia coli* DH5α and *E. coli* BL21 (DE3) pLysS. Strains of *E. coli* were grown at 37 °C in LB medium. Antibiotics were used at the following final concentrations: ampicillin 100 μg/ml, chloramphenicol 25 μg/ml.

2.2. Cloning of perA and perB

The DNA sequences of *per* (*perA*) and *wbdR* (*perB*) were amplified by polymerase chain reaction (PCR) from chromosomal DNA of *E. coli* O157:H7 by using the following oligonucleotides: *perA* (perA-up 5′-CAGTTAAATATAAGCATATGAAAATGAAAT-ATATACC, perA-down 5′-CGCCCCACTCGTAAAATCCATCTG-GATCCAACGC); *wbdR* (perB-up 5′-CTTTGGAGATGGGCATAT-GAATTTGTATGGTATTTTTGGT, perB-down 5′-CATTAGTCC-CATTTGGATCCTGTTTTCGCATTCCCATTA). The PCR-products were further treated with NdeI and BamHI and were ligated into expression vectors pET11a and pET16b (Novagen), which were hydrolyzed with NdeI and BamHI, respectively [14]. Recombinant plasmids (pET11-perA, pET16-perA, pET11-perB, pET16-perB) were obtained and transformed into competent cells of *E. coli* DH5α. For overexpression of the proteins the recombinant plasmids were retransformed into competent *E. coli* BL21 (DE3) pLysS cells. The respective cloning of the *gmd* gene from *E. coli* K12 in plasmid pCAW21.1 had been described previously [15].

2.3. Overproduction of GDP-D-mannose 4,6-dehydratase (Gmd), GDP-4-keto-6-deoxy-D-mannose-4-aminotransferase (PerA), and GDP-perosamine N-acetyltransferase (PerB)

For the overproduction of Gmd, PerA, and PerB, *E. coli* BL21 (DE3) pLysS cells with the recombinant plasmids were grown in LB-media to an optical density of 0.6 at 540 nm. The cells were induced with isopropyl-β-thiogalactoside (24 mg/l) for 90 min. Subsequently, the cells were harvested by centrifugation, washed twice in ice-cold 50 mM Tris/HCl-buffer, pH 7.5, and suspended in extraction buffer (50 mM Tris/HCl-buffer, pH 7.5, 150 mM NaCl, 10 mM MgCl$_2$, 5 mM β-mercaptoethanol, 5 mM ethylendiaminetetraacetic acid). After the disruption by sonication the crude extract was clarified by centrifugation at 30 000 × *g* for 30 min (S30).

2.4. Purification of His-tagged fusion proteins

The His-tag PerA and the His-tag PerB fused proteins were purified as soluble protein by affinity chromatography, using Ni-NTA-agarose (Qiagen, Germany). The crude extract (containing His-tag PerA or His-tag PerB) was loaded on a Ni-NTA-agarose-column (15 mm × 60 mm) which was equilibrated with equilibration buffer (50 mM Tris/HCl-buffer, pH 7.5, 150 mM NaCl, 10 mM MgCl$_2$, 5 mM β-mercaptoethanol). The loaded column was washed with washing buffer (50 mM Tris/HCl-buffer, pH 7.5, 150 mM NaCl, 10 mM MgCl$_2$, 5 mM β-mercaptoethanol, 50 mM imidazol). The His-tag PerA protein was eluted with 50 mM Tris/HCl-buffer, pH 7.5 with 10 mM MgCl$_2$, 300 mM NaCl, 5 mM β-mercaptoethanol, 200 mM imidazol. The His-tag PerB protein was eluted with 50 mM Tris/HCl-buffer, pH 7.5 with 10 mM MgCl$_2$, 50 mM NaCl, 5 mM β-mercaptoethanol, 250 mM imidazol. After elution, the protein-containing fractions were analyzed by SDS–PAGE [16] to check the purity and the molecular mass. Subsequently the imidazol was removed by PD-10 column (GE-Healthcare, Germany). This column was equilibrated with 50 mM Tris/HCl-buffer, pH 7.5 containing 10 mM MgCl$_2$, 5 mM β-mercaptoethanol, 100 mM NaCl, and 10% glycerol.

2.5. Characterization of recombinant proteins

Protein size determination was performed by size exclusion chromatography on a FPLC system with a Superdex 200 HR 10/30 gel filtration column (GE-Healthcare, Germany). Mobile phase: 50 mM Tris/HCl-buffer, pH 7.5 containing 100 mM NaCl; flow rate 0.5 ml/min. For the calibration curve the following proteins were applied: ribonuclease 13.7 kDa, chymotrypsinogen A 25 kDa, ovalbumin 43 kDa, BSA 67 kDa, alcohol dehydrogenase 150 kDa, β-amylase 200 kDa, apoferritin 443 kDa, thyroglobin 669 kDa.

2.6. HPLC, HPLC-MS, and TLC

Acetyl-CoA, CoA, guanosine monophosphate, guanosine diphosphate, GDP-α-D-mannose, GDP-4-keto-6-deoxy-D-mannose, GDP-α-D-perosamine, and GDP-α-*N*-acetyl-D-perosamine were separated by high performance liquid chromatography (HPLC) and detected by UV-photometry (Dionex, Germany) at 254 nm. As mobile phase a phosphate-buffer (30 mM potassium-phosphate, pH 6.0; 5 mM tetrabutylammoniumhydrogen sulfate, 2% acetonitrile) and acetonitrile was used. As stationary phase a RP C$_{18}$ Lichrospher 100,

250 × 4 mm (Trentech, Germany) was used. HPLC-MS analysis was carried out on a HPLC coupled to a UV-detector and a mass spectrometer (LCMS-2010EV/dual ion source DUIS2010, Shimadzu, Germany). Samples were injected onto a RP C_{18} Lichrospher 100 (250 × 4 mm) column and eluted by a gradient of solvent A (50 mM ammonium formate, 0.0384% octylamine, 1% methanol) and solvent B (methanol) as mobile phase. The gradient ran from 0% B to 50% B over 40 min with a flow rate of 1 ml/min. The TLC-analysis was carried out as described previously [7].

2.7. Enzyme assays

The measurement of the GDP-D-mannose 4,6-dehydratase and GDP-perosamine synthetase activity as well as the kinetic measurement of the GDP-perosamine synthetase were described previously [7]. Activity of the GDP-perosamine N-acetyltransferase was determined by the quantitative HPLC-analysis of GDP-4-N-acetyl-D-perosamine, CoA, GDP-D-perosamine, and acetyl-CoA. The standard assay contained 50 mM Tris/HCl buffer, pH 8, 10 mM $MgCl_2$, 3 mM GDP-α-D-perosamine, 3 mM acetyl-CoA and different amounts of PerB in a volume of 100 µl. Protein concentrations were determined according to Bradford [17]. The reactions were performed at 37 °C and measured at different times between 0 and 30 min. The reactions were stopped by adding 100 µl ice-cold methanol to the reaction mixture. For the HPLC analysis the probes were centrifuged and filtered to remove the precipitated protein. For the kinetic measurement 0.38 nM His-tag PerB was used in the standard assay containing 0.1–2 mM GDP-D-perosamine or 0.2–6 mM acetyl-CoA, respectively.

2.8. Stability of the recombinant enzymes

The thermostability of PerA and PerB was investigated using the enzyme pool after gel filtration by PD10-columns. 20 µl of the protein solution were incubated at different temperatures (30–80 °C) for 1–20 min. Subsequently, the resulting solutions were used in standard enzyme assays (see above).

2.9. Synthesis of GDP-α-N-acetyl-D-perosamine

The preparative enzyme assay contains 4 mg GDP-D-perosamine, 50 mM Tris/HCl-buffer, pH 7.5, 10 mM $MgCl_2$, purified His-tag PerB with an acetyltransferase-activity of 0.8 µkat in a final volume of 500 µl. This mixture was incubated for 60 min at 37 °C. The proteins were removed by boiling for 1 min and subsequent centrifugation at 10 000 × g for 30 min. The purification of GDP-α-N-acetyl-D-perosamine from the side product CoA was achieved by several HPLC runs. The chromatography was performed with a reverse phase C_{18} Lichrospher 100 (250 × 4 mm) column and eluted by a gradient of solvent A (50 mM ammonium formiate, 0.0384% octylamine, 10% methanol, pH 6) and solvent B (methanol) as mobile phase. The gradient ran from 10% B to 50% B over 40 min with a flow rate of 1 ml/min. The GDP-α-N-acetyl-D-perosamine containing fractions were pooled and extracted against 1 vol. of ethylether. The water phase was separated and lyophilized (yield 2.5 mg). The enzymatically synthesized GDP-α-N-acetyl-D-perosamine was analyzed by nuclear magnetic resonance (NMR)-spectroscopy: ^{1}H-NMR, ^{1}H,^{1}H-COSY (500 MHz, D_2O) δ (ppm) = 1.2 (d, H − $6''$, $^3J_{5''6''}$ = 6.2 Hz); 1.95 (s, acetyl, CH_3); 3.30 (dd H − $2''$, $^3J_{2''3''}$ = 11 Hz, $^3J_{1''2''}$ = 8.1 Hz); 3.35 (dd, H − $3''$; $^3J_{3''4''}$ = 3.7 Hz, $^3J_{2''3''}$ = 11 Hz); 3.51 (m, H − $4'$, $^3J_{3''4''}$ = 3.7); 3.71 (m, H-$5''$); 4.25 (m, 2H, H-$5a'$ H-$5b'$); 4.52 (m, H-$4'$); 4.59 (dd, H-$3'$); 4.7 (dd, H-$2'$, $^3J_{1'2'}$ = 6.1 Hz, $^3J_{2'3'}$ = 3.6 Hz); 4.95 (dd, H − $1''$, $^3J_{1''2''}$ = 8.1 Hz); 5.9 (d, H − $1'$, $3J_{1'2'}$ = 6.1 Hz); 8.1 (s, H-1).

3. Results and discussion

3.1. Cloning and expression of the putative genes for GDP-N-acetyl-D-perosamine synthesis

The two open reading frames perA and perB are predicted to encode a GDP-perosamine synthetase and a GDP-perosamine-N-acetyltransferase [9,10]. The PerA-protein has 54% amino acid identity to the known and characterized GDP-perosamine synthetase (RfbE) from Vibrio cholerae O1 [7]. PerA possesses a pyridoxal-5-phosphate binding domain, which is supposed to be a group VI (secondary metabolic) aminotransferase and is in the cluster of orthologous proteins COG0399 [18]. The perB gene encodes for a protein (221 aa) which shows the typical primary structure of a left-handed parallel β helix [19] and is in the cluster of orthologous proteins COG0110 [18]. Proteins of this group have been shown to acetylate different compounds using acetyl-CoA as donor substrate [20–22].

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WbbJ Rs      ------------------MTLIGVYGASGFGREVMPLVREQMRAAGQP-YEVVFVDDGA  40
WbbJ Pae     MPLYCRYYPWQIPPTHGDDMDLYGIVGAGGFGREVIPLANKNLRMVSQGNFRLVFIDDGD  60
WbdR EcO157  -------------------MNLYGIFGAGSYGRETIPILNQQIKQECGSDYALVFVDDVL  41
LpxA Af      ---------------------------MTVQIHPLAIVDSSVQIGEGCTIGPFAVIG  30

WbbJ Rs      DGGAGNGHRVLTYPQFLAEPVADKRLCFAIAASQVREKLAARAVSDGIACLDVRAANTVV  100
WbbJ Pae     VAKNVNGYDVLTTEKFLAQKAGERFFNIAIGNSRIREKVCNILLDGGARPFSISASNAVV  120
WbdR EcO157  AGKKVNGFEVLSTNCFLKAPYLKKYFNVAIANDKIRQRVSESILLHGVEPITIKHPNSVV  101
LpxA Af      AGVEIGDHCRIGANTVIEGPCRLGAHNQIFQFASVGTAPQDLGYAGEPTTLEIGSHN-TI  89

WbbJ Rs      LDAVEIGTGAVLCPFVTLTSNVRIGKHFHANIYAYVAHDCVIGDYVTFAPGVKCNGNVVI  160
WbbJ Pae     LDGNELAEGSILCPFSMVTSNTRIGKFFHANIYSYVAHDCEIGDFVTFAPSVKCNGNVRI  180
WbdR EcO157  YDHTMIGSGAIISPFVTISTNTHIGRFFHANIYSYVAHDCQIGDYVTFAPGAKCNGYVVI  161
LpxA Af      REFVTINRGTVKG-----GGTTRIGHHNLLMAYCHVAHDCSIGDQVVMANAATLAGHVSV  144

WbbJ Rs      EDHAYVG-TGAVLKQGKPGAPLVIGKGAVVG------MGAVVTRDVPAGTTVVGNPARPL  213
WbbJ Pae     ESHAYIG-TGAVIKQGTPEHPIVIGEGAVVG------MGAVVTKSVPAGAVVVGNPAKPL  233
WbdR EcO157  EDNAYIG-SGAVIKQGVPNRPLIIGAGAIIG------MGAVVTKSVPAGITVCGNPAREM  214
LpxA Af      EDHAILGGLSAVHQYARVGAHAILGGGTMAPLDIPPFMMAAGNHASLHGINVRGLARRGI  204

WbbJ Rs      VK------------------------------------------------------  215
WbbJ Pae     VRKEVAG-------------------------------------------------  240
WbdR EcO157  KRSPTSI-------------------------------------------------  221
LpxA Af      PRETILQIKRAYRLLFRSGLRLEDAMDEVSQRGLNAPEVAYLLDFIRNSRRGITRP  260
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Fig. 3. ClustalW alignment of the WbdR protein from E. coli O157:H7 (WbdR EcO157), WbbJ from R. solanacearum UW551 (WbbJ Rs), WbbJ from P. aeruginosa (WbbJ Rs), and LpxA from Acidithiobacillus ferrooxidans (LpxA Af). Positions with more than 50% amino acid identity of the four sequences are labeled.

PerB shows highest sequence similarity to an UDP-*N*-acetyl-bacillosamine 4-acetyltransferase from *Ralstonia solanacearum* UW551 (70%), to a putative acetyltransferase from *Pseudomonas aeruginosa* (72%) and it shares 50% similarity with the UDP-*N*-acetylglucosamine-3-*O*-acyltransferase LpxA from *Acidithiobacillus ferrooxidans* (Fig. 3). PerB also shows homology (45%) to the characterized UDP-*N*-acetylglucosamine-4-*N*-acetyltransferase PglD from *Campylobacter jejuni* [22]. Both genes, *perA* and *perB*, were amplified by PCR and were cloned into the expression vector pET11a and pET16b, respectively. Recombinant plasmids (pET11-perA, pET16-perA, pET11-perB, and pET16-perB) were transformed into *E. coli* BL21 (DE3) pLysS, where their expression under induced conditions resulted in the overexpression of large amounts of the recombinant proteins. The overproduction of PerA and PerB was confirmed by SDS–PAGE analysis (data not shown), as well as the corresponding N-terminal His-tag versions (Fig. 4, lanes 1 and 4).

3.2. Purification and properties of the His-tag fusion proteins PerA and PerB

The N-terminal His-tag versions of PerA and PerB proteins were further purified to homogeneity by Ni-NTA-affinity chromatography. The elution of the recombinant proteins from the affinity column was accomplished by increasing the imidazole concentration. The use of buffer containing 200 mM imidazole and 300 mM NaCl was efficient to elute the His-tag PerA protein from the column. The His-tag PerB protein was not eluted under the same conditions even when 250 mM imdazole and 300 mM NaCl were used. The elution was achieved by reducing the NaCl concentration to 50 mM in the presence of 250 mM imdazole. This implicates a strong hydrophobic interaction of PerB with the Ni-NTA-agarose material. The imidazole was removed from the protein fractions by gel filtration. The protein fractions each contained only one band in SDS–PAGE analysis, whose apparent molecular masses agreed well with the calculated masses of the proteins, His-tag PerA

44.33 kDa, His-tag-PerB 26.26 kDa (Fig. 4). Gel permeation analysis was carried out to determine the molecular mass of the purified His-tag fusion proteins under native conditions. By comparison with known molecular weight standards the apparent mass of His-tag PerA is 458 kDa and of His-tag PerB 68 kDa. For this it follows, that the native form of His-tag PerA and PerB is a decamer and a trimer, respectively. The finding that His-tag PerA forms a decamer structure differs to the structure of His-tag RfbE from *V. cholerae* which forms a tetramer. The trimer structure of His-tag PerB agrees with the structure of other proteins belonging to the hexapeptide acyltransferase superfamily [19].

3.3. perA encodes the GDP-perosamine synthetase

The in vitro activity of PerA was assayed by the conversion of GDP-4-keto-6-deoxy-D-mannose into GDP-D-perosamine, which was followed by the use of HPLC and mass spectrometry analysis. The substrate of the GDP-perosamine synthetase GDP-4-keto-6-deoxy-D-mannose was prepared by the enzymatic conversion of GDP-D-mannose to GDP-4-keto-6-deoxy-D-mannose by recombinant GDP-mannose 4,6-dehydratase. The enzymatic conversion of GDP-D-mannose to GDP-4-keto-6-deoxy-D-mannose is shown in Fig. 5A and B and the product was identified by electrospray mass spectrometry (Fig. 6A). By the use of the amino-donor L-glutamate and in presence of pyridoxal-phosphate as co-enzyme and recombinant PerA only, GDP-4-keto-6-deoxy-D-mannose is completely (>98%) converted into a new product (Fig. 5B and C). The product peak was identified as GDP-D-perosamine by its mass ($m/z = 587$ [M−H]$^-$) (Fig. 6B) and by co-chromatography with a standard obtained from previous work [7] (Fig. 5E). In the negative control using cell-free extract of *E. coli* BL21 (DE3) pLysS with either plasmid vector, pET11a or pET16b, no activity of GDP-perosamine synthetase could be detected under the same conditions. The reaction catalyzed by the His-tag PerA protein has its pH-optimum at pH 7.5. The kinetic properties of the His-tag PerA protein for the sub-
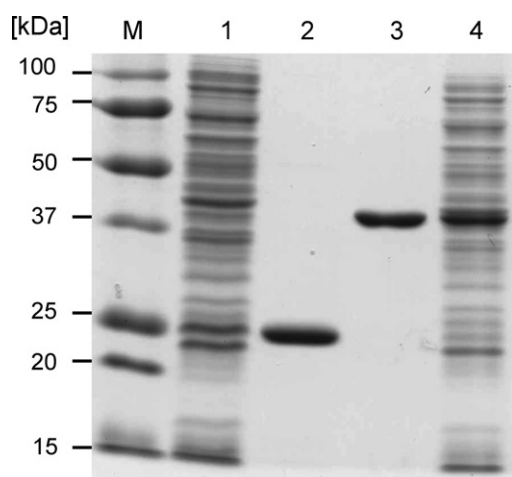

Fig. 4. SDS–PAGE analysis of crude extracts from induced cells and of the affinity purified proteins. The following protein fractions are shown in the lanes: (1) S30 of *E. coli* BL21(DE3) pLysS pET16-perA, 2 h after induction; (2) His-tag PerA after Ni-NTA-purification and gel filtration; (3) His-tag PerB after Ni-NTA-purification and gel filtration; (4) S30 of *E. coli* BL21(DE3) pLysS pET16-perA, 2 h after induction; (M) molecular weight marker.
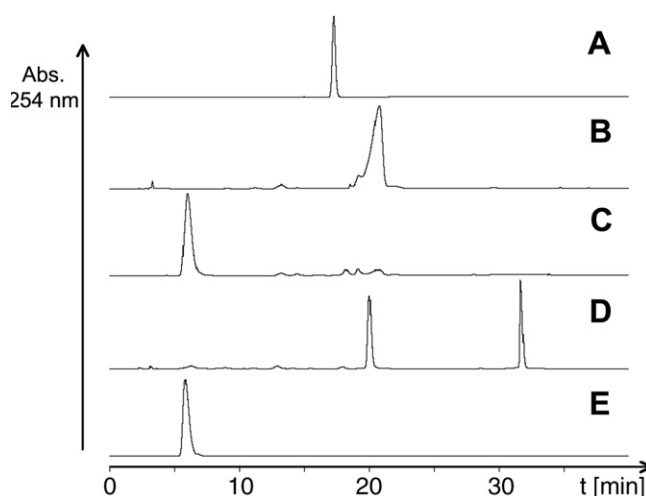

Fig. 5. HPLC analysis of the enzymatic reactions catalyzed by GMD, PerA and PerB. (A) GDP-α-D-mannose standard; (B) GMD catalyzed reaction to GDP-4-keto-6-deoxy-D-mannose (21 min); (C) PerA catalyzed reaction to GDP-α-D-perosamine (6 min) (D) PerB catalyzed reaction to GDP-*N*-acetyl-α-D-perosamine (20 min) and coenzyme A (32 min); (E) GDP-α-D-perosamine standard.
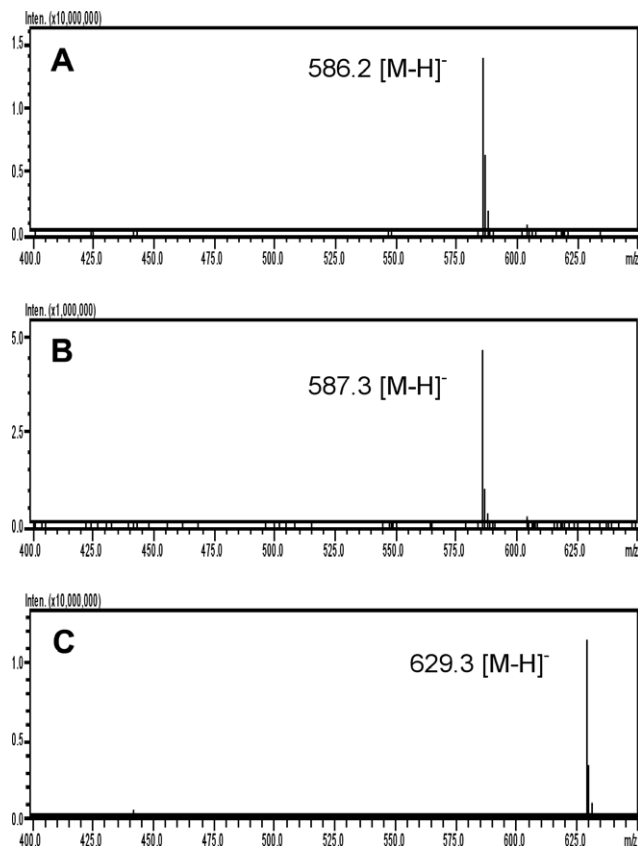
Fig. 6. Mass spectrometry analysis after HPLC separation (see Fig. 4). The mass spectra derived after electrospray ionisation of the product peaks (negative mode): (A) GDP-4-keto-6-deoxy-D-mannose; (B) GDP-α-D-perosamine; (C) GDP-N-acetyl-α-D-perosamine.

strate GDP-4-keto-6-deoxy-D-mannose and the cosubstrate L-glutamate were determined using a photometric assay. The K_M and V_{max} values of GDP-4-keto-6-deoxy-D-mannose were 0.07 mM and 220 nkat/mg protein, respectively. The K_M and V_{max} values of L-glutamate were 2.3 mM and 172 nkat/mg protein, respectively. The kinetic data correspond to the K_M-values of the Wang-group [13]. These values are also approximately in the same range as respective values of RfbE from *V. cholerae* O1 (GDP-4-keto-6-deoxy-D-mannose: K_M 0.06 mM, V_{max} 42 nkat/mg) [7].

3.4. perB encodes the GDP-perosamine N-acetyltransferase

To assay the putative acetyltransferase activity of PerB, we used cell-free extract of *E. coli* BL21 (DE3) pLysS pET11-PerB or pET16-PerB or the purified His-tag PerB. As substrate, the GDP-D-perosamine standard or the GDP-D-perosamine containing reaction mixture derived from the PerA enzyme assay were tested. As acetyl-donor acetyl-coenzyme A was used. The HPLC results showed that the peaks of GDP-D-perosamine and acetyl-CoA disappeared during the reaction. On the other hand, two new peaks appeared (Fig. 5C and D) (acetyl-CoA: retention time 34 min, not shown). By co-chromatography with a standard, the peak at 32 min (Fig. 5D) could be identified as CoA. The peak at 20 min retention time represents GDP-N-acetyl-D-perosamine, which has the detected mass of 629 $[M-H]^-$ (Fig. 6C). The reaction catalyzed by the His-tag PerB protein has its pH-optimum at pH 8.5. The

analysis of His-tag PerB by Michaelis–Menden kinetic for the substrate GDP-D-perosamine showed a K_M of 0.32 mM and V_{max} of 28 mkat/mg protein. The K_M and V_{max} values for the cosubstrate acetyl-CoA were 1.8 mM and 21 mkat/mg protein, respectively, indicating that PerB catalyzed the acetylation of perosamine with a high efficiency. No acetylation of GDP-D-perosamine could be detected in control experiments under the same conditions using cell-free extract of *E. coli* BL21 (DE3) pLysS pET11a or pET16b. To prove the acetylation of the C4 amino group of the sugar residue, a thin layer chromatography (TLC) analysis with ninhydrin staining of the monosaccharide was carried out. The monosaccharides were obtained by acid hydrolysis of the corresponding GDP-sugars. After TLC separation and staining with ninhydrine the monosaccharide derived from GDP-D-perosamine shows a typical "Ruhemann's purple" colored spot (R_f = 0.19) indicating a primary amino group of the compound. The monosaccharide derived from N-acetyl-D-perosamine showed a pale yellow spot (R_f = 0.52). This indicates that the sugar-residue derived from the PerB reaction contains no primary amino-group.

3.5. Thermal stability of the recombinant His-tagged versions of PerA and PerB

The thermal stability of the recombinant proteins was investigated using His-tag protein preparations after the gel filtration run. His-tag PerA holds 55% residual activity at standard assay conditions after 20 min incubation at 50 °C. After 10 min incubation at 60 °C His-tag PerA was completely inactivated. PerB showed a quite high thermal stability. After 20 min incubation at 65 °C His-tag PerB holds 76% and after 20 min at 75 °C 10% residual activity. A complete inactivation was achieved after 10 min incubation at 80 °C. The storage stability at 4 °C under the same buffer conditions after 30 days yielded in a residual activity of 68% in case of His-tag PerA and of 95% in case of His-tag PerB. The results indicate that these enzymes, in particular PerB, are quite robust.

3.6. Synthesis and isolation of GDP-N-acetyl-D-perosamine

To identify the product of the PerB reaction by proton NMR spectroscopy, we synthesized GDP-N-acetyl-D-perosamine on a milligram scale. Pure GDP-D-perosamine and acetyl-CoA were converted into GDP-N-acetyl-D-perosamine using purified His-tag PerB. After removal of the protein and separation of the side product by HPLC, the final product was analyzed by ^{1}H-NMR and ^{1}H,^{1}H-COSY experiments. In comparison to the ^{1}H-NMR of GDP-D-perosamine [7], the spectrum of GDP-N-acetyl-D-perosamine shows an additional peak at 1.95 ppm representing the protons of the N-acetyl residue. Furthermore, the chemical shift of the H4″ (3.51 ppm) shows a more deshielded proton than the H4″ proton (3.01 ppm) in GDP-D-perosamine, which confirms the acetylation of the C4 amino group.

The intention of this work was to identify the function of the genes *perA* and *perB* from *E. coli* O157:H7 to confer their ability for the synthesis of GDP-N-acetyl-D-perosamine from the central intermediate GDP-4-keto-6-deoxy-D-mannose. Our results clearly demonstrate the function of PerA and PerB to catalyze the synthesis of GDP-N-acetyl-D-perosamine in vitro. Due to the demonstrated functions we suggest to rename *per* as *perA* and *wbdR* as *perB*.

To produce novel glycoconjugates or oligosaccharides by the use of glycosyltransferases, it is necessary to synthesize nucleotide-activated sugars in a large scale. Other GDP-activated sugars, like GDP-D-mannose, GDP-D-perosamine, GDP-L-fucose, GDP-D-rhamnose, GDP-L-colitose, and GDP-6-deoxy-D-talose, are already available fully on the basis of enzymatic reactions with the respective recombinant enzymes [23–27]. The identification of PerA and PerB allows the study of corresponding glycosyltransferases and thus we think it gives the opportunity to develop an O157 antigen-based, structurally defined glycoconjugate vaccine.

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