Biosynthesis Pathway of ADP-L-*glycero*-β-D-*manno*-Heptose in *Escherichia coli*

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The steps involved in the biosynthesis of the ADP-L-*glycero*-β-D-*manno*-heptose (ADP-L-β-D-heptose) precursor of the inner core lipopolysaccharide (LPS) have not been completely elucidated. In this work, we have purified the enzymes involved in catalyzing the intermediate steps leading to the synthesis of ADP-D-β-D-heptose and have biochemically characterized the reaction products by high-performance anion-exchange chromatography. We have also constructed a deletion in a novel gene, *gmhB* (formerly *yaeD*), which results in the formation of an altered LPS core. This mutation confirms that the GmhB protein is required for the formation of ADP-D-β-D-heptose. Our results demonstrate that the synthesis of ADP-D-β-D-heptose in *Escherichia coli* requires three proteins, GmhA (sedoheptulose 7-phosphate isomerase), HldE (bifunctional D-β-D-heptose 7-phosphate kinase/D-β-D-heptose 1-phosphate adenylyltransferase), and GmhB (D,D-heptose 1,7-bisphosphate phosphatase), as well as ATP and the ketose phosphate precursor sedoheptulose 7-phosphate. A previously characterized epimerase, formerly named WaaD (RfaD) and now renamed HldD, completes the pathway to form the ADP-L-β-D-heptose precursor utilized in the assembly of inner core LPS.

Lipopolysaccharide (LPS) is a major component of the outer membrane of gram-negative bacteria (28). It has a tripartite structural organization consisting of lipid A, a conserved core oligosaccharide region, and an O-specific polysaccharide chain or O antigen. In the majority of gram-negative bacteria, the core oligosaccharide can be subdivided into an outer core, generally composed of hexoses and hexosamines, and an inner core made of 3-deoxy-D-*manno*-oct-2-ulosonic acid and L,D-heptose units. LPS plays an important role in maintaining the structural integrity of the bacterial outer membrane by interacting with outer membrane proteins and divalent cations (15), thereby providing a barrier against the entry of toxic hydrophobic compounds into the bacterial cell (27). *Escherichia coli* mutants defective in the biosynthesis of 3-deoxy-D-*manno*-oct-2-ulosonic acid are nonviable, whereas those impaired in L,D-heptose synthesis survive in vitro, although they display a pleiotropic phenotype referred to as "deep rough" (17). This phenotype is characterized by an extreme sensitivity to very low concentrations of novobiocin, detergents, and bile salts (32). Deep rough mutants also have defects in F plasmid conjugation and generalized transduction by the bacteriophage P1 (6, 16). *Haemophilus influenzae* heptose-deficient mutants were found to be serum sensitive and displayed a reduced virulence in vivo (18, 36).

The complete biosynthesis pathway of the L,D-heptose precursor has not been elucidated. Eidels and Osborn (11) proposed a four-step pathway for the synthesis of NDP-L,D-heptose, which is still widely accepted in the literature (see reference 13 for a review). It includes (i) conversion of D-sedoheptulose 7-phosphate to D,D-heptose 7-phosphate by a phosphoheptose isomerase; (ii) formation of D,D-heptose 1-phosphate by a phosphoheptose mutase; (iii) activation of the D,D-heptose 1-phosphate intermediate to NDP-D,D-heptose by an NDP-heptose synthetase; and (iv) epimerization of the NDP-heptose to form the final product, NDP-L,D-heptose. Subsequent studies involving the isolation of ADP-D,D-heptose and ADP-L,D-heptose from *Shigella sonnei* and *Salmonella enterica* serovar Typhimurium indicated that ADP is the activating nucleotide (20–22). In the absence of purified ADP-heptose, Kadrmas and Raetz (19) used ADP-mannose as a substrate for the *E. coli* heptosyltransferase I (WaaC). More recently, it has been clearly demonstrated that heptosyltransferases I and II (WaaF) from *E. coli* accept ADP-L-β-D-heptose and ADP-D-β-D-heptose as substrates, although the efficiency of the transfer reactions with the D-β-D isomer is markedly reduced (14, 35).

In gram-negative bacteria, functional studies have only been performed for the isomerization reaction and the epimerization step (3, 9, 26), while the conversion of D,D-heptose 7-phosphate to D,D-heptose 1-phosphate and a functional proof of the activating step have not been demonstrated. The D-sedoheptulose 7-phosphate isomerase activity was described in *S. enterica* serovar Typhimurium (12), and the corresponding gene, *gmhA*, has been cloned both from *E. coli* and from *H. influenzae* (3, 4). The amino acid sequence of the GmhA polypeptide is highly conserved in different gram-negative bacteria (33). The epimerization step is catalyzed by the WaaD (formerly RfaD) protein (5), which has also been crystallized (8). We

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have recently shown that the *E. coli rfaE* gene product consists of two distinct domains that may be involved in the biosynthesis of D,D-heptose 1-phosphate, as well as the activating step (34). It was demonstrated that one of the RfaE domains shares structural features with members of the ribokinase family, while the other domain has conserved features present in nucleotidyltransferases (34). The demonstration of a protein domain corresponding to a putative sugar kinase suggested that the original pathway for NDP-heptose biosynthesis as proposed by Eidels and Osborn may not be accurate and, at the same time, predicted the existence of an additional phosphatase step (33).

The complete biosynthesis pathway of GDP-D-α-D-heptose from D-sedoheptulose 7-phosphate in the gram-positive bacterium *Aneurinibacillus thermoaerophilus* DSM 10155 was recently characterized (20). We demonstrated that two independent enzymes catalyze the originally proposed mutase step. A D,D-heptose 7-phosphate kinase adds a phosphate group at the C-1 position, and subsequently a D,D-heptose 1,7-bisphosphate phosphatase removes the phosphate group at the C-7 position. The GDP-activated D,D-isomer serves as a precursor for the incorporation of the heptose into the glycan moiety of a surface layer (S-layer) glycoprotein produced by *A. thermoaerophilus* (20). Amino acid sequence analysis of completely sequenced genomes revealed that the *A. thermoaerophilus* phosphatase is highly conserved among different gram-negative bacteria (20), in agreement with a previous suggestion that a phosphatase reaction is also required for the synthesis of ADP-D,D-heptose and ADP-L,D-heptose in these microorganisms (34). In the present study, we report the reconstruction in vitro with purified enzyme components of the complete biosynthesis pathway for ADP-D-β-D-heptose in *E. coli*. We also provide genetic evidence demonstrating that the function of a novel phosphatase gene in *E. coli* K-12, now designated *gmhB* (formerly *yaeD*), is required for the synthesis of ADP-D-β-D-heptose. Furthermore, we propose a new gene nomenclature to account for the differences and similarities between the components of the pathways leading to the formation of ADP-L-β-D-heptose and GDP-D-α-D-heptose in gram-negative and gram-positive bacteria, respectively.

MATERIALS AND METHODS

Chemicals. ATP, GTP, D-sedoheptulose 7-phosphate and dithiothreitol were obtained from Sigma (Vienna, Austria). HiTrap Chelating, HiPrep Desalting and MonoQ HR5/5 columns were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). The Gateway system was obtained from Life Technologies (Vienna, Austria). D-α-D-Heptose 1-phosphate, D-β-D-heptose 1-phosphate, ADP-D-α-D-heptose, and ADP-D-β-D-heptose were synthesized as described elsewhere (35).

Bacterial strains and growth conditions. *E. coli* MG1655 (K-12 F$^-$ λ$^-$) was purchased from the American Type Culture Collection (Manassas, Va.). *E. coli* DH5α [K-12 F$^-$ φ80d *lacZ*ΔM15 *endA1 recA1 hsdR17* (r$_K^-$ m$_K^-$) *supE44 thi-1 gyrA96 relA1* Δ(*lacZYA-argF*) *U169*] and *E. coli* TG1 [*supE thi-1* Δ(*lac-proAB*) Δ(*mcrB-hsdSM*)5 (r$_K^-$ m$_K^-$) (F′ *traD36 proAB lacI^{q}Z*ΔM15)] (Stratagene, La Jolla, Calif.) were used for plasmid propagation. For enzyme overexpression *E. coli* BL21-SI [F$^-$ *ompB hsdS$_B$*(r$_B^-$ m$_B^-$) *gal dcm endA1 proUp*::T7 RNAP::*malQ-lacZ* Tet^s] (Life Technologies) was used. *E. coli* KCS2926 (*rfaE*::Tn*10*) has been described elsewhere (34). Bacteria were cultured in Luria broth, supplemented when necessary with ampicillin, kanamycin, or tetracycline at final concentrations of 100, 50, and 20 μg/ml, respectively.

Analytical techniques. Nucleotide-activated sugars and monosaccharide phosphates were analyzed by high-performance anion-exchange chromatography (HPAEC) on a CarboPac PA-1 column (Dionex, Sunnyvale, Calif.) as previously

described (20). Sugar phosphates were investigated by using pulsed electrochemical detection, and UV detection at 254 nm was used for nucleotide-activated sugars. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed for proteins according to the original method of Laemmli (23) with minor modifications. Gels were stained with Coomassie brilliant blue R250. Protein concentrations were determined by the method of Bradford (2). LPS was isolated as described by Marolda et al. (24), and analyzed by Tricine-SDS-PAGE (30). Commercially cast 16% polyacrylamide gels were purchased from Novex (San Diego, Calif.), and LPS bands were visualized by silver staining (24).

Plasmid constructions. DNA recombinant procedures were performed according to standard methods described by Sambrook et al. (29). PCR was carried out by using a PCR Sprint thermocycler (Hybaid, Ashford, United Kingdom). Oligonucleotide primers for the amplification of DNA fragments containing the *E. coli* heptose biosynthesis genes were designed with *attB1* or *attB2* sites for the insertion into the Gateway donor vector pDONR201 (Life Technologies) by homologous recombination. Primers CGBD1 (5′-*attB1*-GCGAGAATCTCTAT TTCCAAGGAATGAAAGTAACGCTGCCAGAG-3′) and CGBD2 (5′-*attB2*-ATCTGTGAACCGCTTTCC-3′) were used for the amplification of the putative bifunctional kinase/adenylyltransferase gene *hldE* (formerly *rfaE*). Primers CGC1 (5′-*attB1*-GCGAGAATCTCTACTTCCAAGGAAAGGTGGCGAAGA GCGTACC-3′) and CGC2 (5′-*attB2*-GCGCATTATAGGGAGTCG-3′) were used for the amplification of the putative phosphatase gene *gmhB* (formerly *yaeD*). *attB1* and *attB2* are the minimal 25-bp sequences required for efficient homologous recombination. PCR products were cloned into pDONR201, and the resulting plasmids, pCHE1 and pCHB1, were used to transfer the gene sequences into pDEST17 (His fusion vector) via homologous recombination. These experiments gave rise to pCHLDE3 and pCGMHB3, encoding *hldE* and *gmhB*, respectively, which were used for overexpression of the corresponding His-tagged protein products in *E. coli* BL21-SI. The *gmhB* gene was also amplified with primer 575 (5′-TCC<u>CCCGGG</u>AGTGGCGAAGAGCGTACCCGCA-3′) that contains an *Sma*I site (underlined) and primer 558 (5′-GATCCGA ATTCTGCCGGTTTTTGCTGCTT-3′). The amplified DNA fragment was digested with *Sma*I and cloned into the glutathione *S*-transferase (GST)-fusion vector pGEX-2T (Amersham Pharmacia Biotech), which was also cleaved with *Sma*I. The resulting plasmid, pFM4, expressed the GmhB protein N-terminally fused to the GST moiety. The *A. thermoaerophilus* DSM 10155 *gmhB* gene was amplified by using the primers ATC1F (5′-CG<u>GAATTC</u>ATAGGAAGGCGCA AAAGG-3′) and ATC2R (5′-CGC<u>GGATCC</u>TTACTTTAGCTTTGCAACAC C-3′), where the underlined sequences correspond to the *Eco*RI and *Bam*HI restriction sites, respectively. The digested PCR fragment was ligated into *Eco*RI/*Bam*HI-cut pEXT20 (10) to yield the plasmid pCAT1.

Construction of *E. coli* strains carrying a deletion of the *gmhB* gene. The one-step replacement method described by Datsenko and Wanner (7) was used to construct a *gmhB* deletion in *E. coli* K-12 W3110. In this procedure, recombination requires the λ phage Red recombinase, which is synthesized by an inducible promoter on a curable thermosensitive plasmid, and PCR primers that provide the homology to the targeted gene. A PCR fragment was generated with pKD4 (7) as a template and the primers 342 (5′-GAGCGTACCCGCAATT TTTCTTGACCGTGATGGCACCATTAAT<u>GGTGTAGGCTGGAGCTGCT TCG</u>-3′) and 343 (5′-CAGGCTATTTAACACCCAATCCGCCGCGTTTT CTGCTTCAG<u>CATATGAATATCCTCCTTAG</u>-3′). The underlined sequences correspond to 21 bases that are specific for pKD4, while the remaining sequence corresponds to the ends of *gmhB*. The amplified DNA fragment contained a kanamycin resistance (Km^r) gene flanked by FLP (protein encoded by the *FLP* gene of the 2μm plasmid of *Saccharomyces cerevisiae*) recognition target sites and 40 bases at each end that are specific for the ends of the *gmhB* gene. The PCR product was transformed into *E. coli* W3110 containing pKD46, which encodes the Red recombination system of the λ phage (7). One of the Km^r mutants, BD1, was isolated, and the replacement of *gmhB* for the Km^r gene was verified by PCR. A second derivative was obtained by inducing the excision of the antibiotic resistance gene by introducing a plasmid expressing the FLP recombinase (7). This experiment resulted in strain BD2, which carries a chromosomal unmarked deletion of *gmhB*.

Enzyme purification. The growth conditions of *E. coli* BL21-SI cells carrying expression plasmids and the purification of proteins were essentially identical to those recently described for the purification of GmhA and GmhB (previously named GmhC) from *A. thermoaerophilus* DSM 10155 (20). After cell lysis and removal of the membranes by ultracentrifugation, the bifunctional kinase/pyrophosphorylase HldE (synonymous to L,D-heptose formation) was purified in two steps by using HiTrap Chelating and MonoQ columns, while the phosphatase GmhB was purified in one step by using the HiTrap chelating column. The proteins were concentrated by ultrafiltration and stored at 4°C or, after stabili-

zation with 50% glycerol, at −20°C. The purity of the enzymes was verified by SDS-PAGE analysis.

Enzyme assays. All assays were performed in 0.5-ml PCR tubes (Axygen Scientific, Union City, Calif.). Approximately 10 nmol of D-sedoheptulose 7-phosphate or D,D-heptose 1-phosphate was used for enzyme assays; 50 nmol of D-glucose 1,6-bisphosphate was used in a negative control to test the specificity of D,D-heptose 1,7-bisphosphate phosphatase GmhB. Equimolar amounts of ATP with respect to D-sedoheptulose 7-phosphate and D,D-heptose 1-phosphate were used in the kinase reaction, as well as in the pyrophosphorylase reaction. The assay buffer contained 20 mM Tris-HCl (pH 8.0) and 10 mM MgCl$_2$. Enzymes (500 ng of each of the purified proteins) were added, and after incubation at 37°C for different reaction times (5 to 120 min), the samples were analyzed by HPAEC as described previously (20). The total sample volume was 10 μl for sugar phosphate analysis and 100 μl for investigation of nucleotide-activated sugars. Detailed descriptions of the reactions are given in the figure legends.

RESULTS AND DISCUSSION

Overexpression and purification of ADP-heptose biosynthesis enzymes from E. coli K-12. In the gram-positive bacterium A. thermoaerophilus DSM 10155, the four genes encoding the enzymes involved in the biosynthesis of GDP-D-α-D-heptose are part of a single operon (20). In contrast, the proposed four biosynthesis genes for catalysis of the five reaction steps to produce nucleotide-activated L,D-heptose in E. coli K-12 are located at four different loci. The D-sedoheptulose 7-phosphate isomerase encoded by gmhA is a monocistronic gene near the proAB locus (3). The rfaE gene encoding the putative bifunctional D,D-heptose 7-phosphate kinase/D,D-heptose 1-phosphate adenylyltransferase (20), is part of an operon together with genes coding for enzymes involved in nitrogen assimilation (34). The putative D,D-heptose 1,7-bisphosphate phosphatase gene, previously referred to as gmhC in A. thermoaerophilus DSM 10155 (20) and yaeD in E. coli K-12 (1), has been identified by sequence homology comparisons and is located in E. coli next to the rrnH rRNA operon. The predicted gene products from A. thermoaerophilus and E. coli share 40% amino acid sequence identity and they are homologous to the histidinol-phosphate phosphatase family (data not shown). Finally, the ADP-L,D-heptose epimerase gene, waaD (formerly rfaD) is part of the waa gene cluster encoding enzymes for the biosynthesis of the LPS core oligosaccharide.

To elucidate the intermediate steps in the biosynthesis of ADP-L,D-heptose in E. coli, the enzymes putatively catalyzing steps 2 to 4 of the reaction cascade were overexpressed as histidine-tagged fusion proteins and purified as indicated in Materials and Methods. The molecular masses of the purified, denatured proteins, determined by SDS-PAGE analysis, were in agreement with the calculated molecular masses (54.6 and 24.9 kDa for RfaE and GmhB, respectively; Fig. 1). The purified proteins were utilized for functional characterization of the biosynthetic pathway of nucleotide-activated D,D-heptose in E. coli.

In vitro synthesis of ADP-D-β-D-heptose. The biosynthetic steps leading to ADP-D-β-D-heptose were analyzed by HPAEC as described in an earlier study (20). Reaction mixtures were incubated at 37°C for different times and showed only marginal differences in the product yield. For practical reasons with regard to the HPAEC analysis, the duration of all reactions was standardized to 45 min. Due to the lack of commercially available D,D-heptose 7-phosphate, this sugar was prepared in situ from D-sedoheptulose 7-phosphate by using purified A.
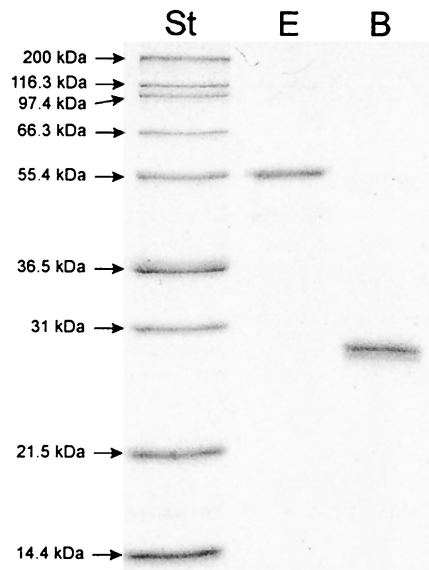

FIG. 1. SDS-PAGE analysis of the purified enzymes HldE and GmhB. Lanes: lane St, molecular mass standards, myosin (200 kDa), β-galactosidase (116.3 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66.3 kDa), glutamic dehydrogenase (55.4 kDa), lactate dehydrogenase (36.5 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa); lane E, histidine-tagged D-β-D-heptose 7-phosphate kinase/D-β-D-heptose 1-phosphate adenylyltransferase HldE; lane B, histidine-tagged D,D-heptose 1,7-bisphosphate phosphatase GmhB.

thermoaerophilus GmhA isomerase (20). Approximately 20% of D-sedoheptulose 7-phosphate was converted to D,D-heptose 7-phosphate in an equilibrium reaction (Fig. 2A). To analyze the second step, D-sedoheptulose 7-phosphate was incubated with A. thermoaerophilus GmhA, the putative bifunctional kinase-pyrophosphorylase RfaE from E. coli K-12, and ATP. Figure 2B shows that the peaks representing D-sedoheptulose 7-phosphate and D,D-heptose 7-phosphate decreased, while a new peak appeared at a retention time of 30.7 min. As a control for enzyme specificity, D-sedoheptulose 7-phosphate was incubated with only the RfaE protein and ATP, resulting in no change of the peak pattern (data not shown). Peaks corresponding to sugar bisphosphates are very small because the signal height corresponding to the free hydroxyl groups that are electrochemically detected by HPAEC decreases with a decreasing number of free OH groups. Synthetic D-β-D-heptose 1-phosphate eluted before synthetic D-α-D-heptose 1-phosphate, and the same was true for the corresponding D,D-heptose 1,7-bisphosphate anomers (20). Thus, the 30.7-min retention time observed for the heptose 1,7-bisphosphate product obtained with the A. thermoaerophilus GmhA and the E. coli RfaE was in agreement with D-β-D-heptose 1,7-bisphosphate. Since the retention time of the D-α-D-heptose 1,7-bisphosphate synthesized with GmhA and the kinase enzyme from A. thermoaerophilus was 31.2 min (20), we concluded that the anomeric configuration of the final product, D-β-D-heptose 1,7-bisphosphate or D-α-D-heptose 1,7-bisphosphate, is specified by the kinase reaction step. This conclusion is also in agreement with the lack of amino acid sequence homologies between the kinase domain of RfaE and the A. thermoaerophi-
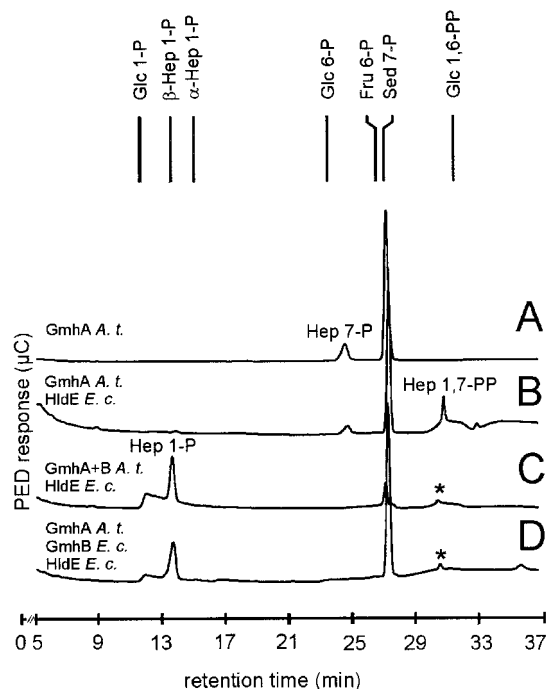
FIG. 2. HPAEC analysis of the enzymatic synthesis of D-β-D-heptose 1-phosphate by pulsed electrochemical detection. (A) D-Sedoheptulose 7-phosphate, converted with the isomerase GmhA from *A. thermoaerophilus* DSM 10155. (B) D-Sedoheptulose 7-phosphate, converted with GmhA from *A. thermoaerophilus* DSM 10155, the kinase/pyrophosphorylase HldE from *E. coli* K-12 and ATP. (C) D-Sedoheptulose 7-phosphate, converted with GmhA and the phosphatase GmhB from *A. thermoaerophilus* DSM 10155, HldE from *E. coli* K-12 and ATP. (D) D-Sedoheptulose 7-phosphate, converted with GmhA from *A. thermoaerophilus* DSM 10155, HldE and GmhB from *E. coli* K-12 and ATP. The peaks labeled with an asterisk are byproducts not representing D-β-D-heptose 1,7-bisphosphate (for more details, see reference 20).

FIG. 3. HPAEC analysis of the activation of D,D-heptose using UV detection. (A) D-β-D-Heptose 1-phosphate, converted with the kinase/pyrophosphorylase HldE from *E. coli* K-12 and ATP. (B) D-α-D-Heptose 1-phosphate, converted with HldE and ATP. (C) D-β-D-Heptose 1-phosphate, converted with HldE from *E. coli* K-12 and GTP. (D) D-Sedoheptulose 7-phosphate, converted with the isomerase GmhA from *A. thermoaerophilus* DSM 10155, HldE from *E. coli* K-12, and ATP. (E) D-Sedoheptulose 7-phosphate, converted with GmhA and the phosphatase GmhB from *A. thermoaerophilus* DSM 10155, HldE from *E. coli* K-12, and ATP. (F) D-Sedoheptulose 7-phosphate, converted with GmhA from *A. thermoaerophilus* DSM 10155, HldE and GmhB from *E. coli* K-12, and ATP.

lus kinase enzyme (data not shown), suggesting that these two enzymes belong to different protein families.

The third step of the reaction was proposed to be the removal of the phosphate group at the C-7 position by a phosphatase. This was first investigated by using the isomerase GmhA and the phosphatase GmhB from *A. thermoaerophilus* in combination with the bifunctional *E. coli* RfaE enzyme and ATP. The peaks corresponding to D-sedoheptulose 7-phosphate, D,D-heptose 7-phosphate and D,D-heptose 1,7-bisphosphate either decreased or disappeared completely, and a new peak was detected, displaying the same retention time as synthetic D-β-D-heptose 1-phosphate (13.6 min, Fig. 2C). Since ATP was present in equimolar amounts with respect to D-sedoheptulose 7-phosphate, minor conversion of D-β-D-heptose 1-phosphate to ADP-D-β-D-heptose can be expected. However, ADP-D-β-D-heptose shows no signal with pulsed amperometric detection used for detection of sugar phosphate. Another reaction, where D-sedoheptulose 7-phosphate was incubated with the isomerase GmhA from *A. thermoaerophilus* and the *E. coli* RfaE and GmhB proteins in the presence of ATP, resulted in the same change in the peak pattern as in the reaction with the phosphatase from *A. thermoaerophilus* (Fig. 2D). Again, the D,D-heptose 1-phosphate was in the β-ano-
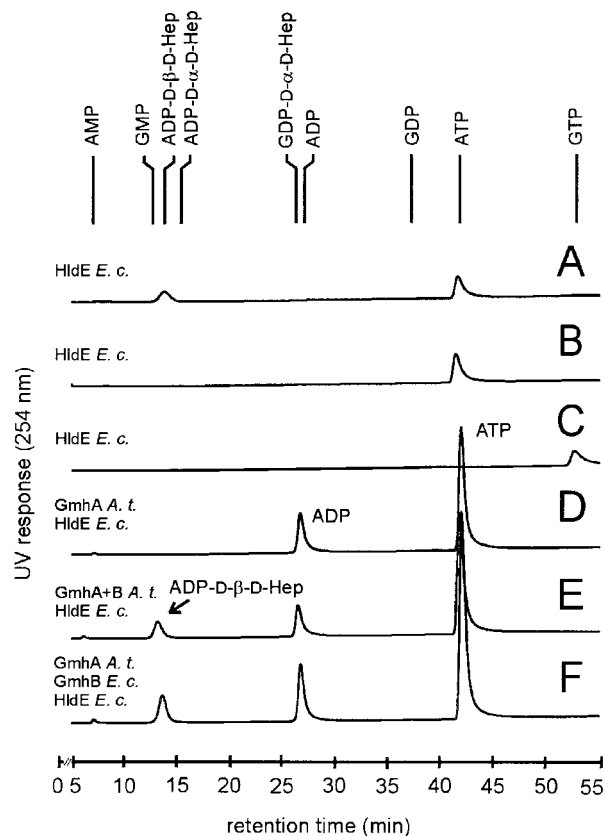
meric configuration. A negative control was performed by using α-D-glucose 1,6-bisphosphate for the phosphatase reaction, showing that this sugar bisphosphate was not a substrate for the phosphatase from *E. coli* K-12 (data not shown). We concluded from these experiments that the phosphatases from *A. thermoaerophilus* and *E. coli* K-12 converted the D-β-D-heptose 1,7-bisphosphate intermediate into D-β-D-heptose 1-phosphate, indicating that these enzymes are insensitive to the anomeric configuration of the D,D-heptose 1,7-bisphosphate.

The next reaction step in the biosynthetic pathway leading to ADP-L-β-D-heptose is nucleotide activation. Synthetic D-β-D-heptose 1-phosphate was incubated with the RfaE protein and ATP, resulting in a peak comigrating with synthetic ADP-D-β-D-heptose (14.2 min, Fig. 3A). In contrast, no activation was detected when the same reaction was performed with either D-α-D-heptose 1-phosphate (Fig. 3B) or D-β-D-heptose 1-phosphate and GTP (Fig. 3C). Furthermore, other combinations of D,D-heptose 1-phosphates and standard nucleoside triphosphates different from ATP did not result in activation (data not
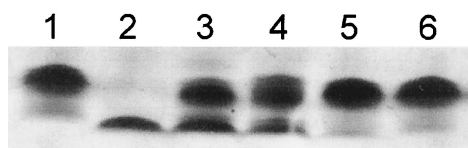
FIG. 4. LPS analysis of *E. coli* K-12 W3110 and the *gmhB* deletion mutant. LPS was extracted and processed from the following *E. coli* K-12 strains: lane 1, W3110 (wild-type); lane 2, KCS2926 (*rfaE*:: Tn*10*); lane 3, BD1 (Δ*gmhB*:: Km); lane 4, BD2 (Δ*gmhB*); lane 5, BD2(pCAT1); and lane 6, BD2(pFM4). Plasmids pCAT1 and pFM4 encode *gmhB* genes from *A. thermoaerophilus* DSM 10155 and *E. coli* K-12, respectively.

shown). To determine whether the phosphatase activity is essential for nucleotide activation, D-sedoheptulose 7-phosphate was incubated with GmhA from *A. thermoaerophilus* and the *E. coli* RfaE in the presence of ATP. The nucleotide-HPAEC results did not show a new peak in the range of ADP-activated sugars (Fig. 3D). The ADP peak in the chromatogram results from the phosphate transfer, catalyzed by the kinase. ADP-D-β-D-heptose was also synthesized directly from D-sedoheptulose 7-phosphate involving the three enzymes analogous to the reactions described in Fig. 2C and 2D. ADP-D-β-D-heptose could be detected by using the GmhB phosphatase from either *A. thermoaerophilus* DSM 10155 (Fig. 3E) or *E. coli* K-12 (Fig. 3F).

A deletion of the *E. coli* K-12 *gmhB* gene is associated with an altered LPS core phenotype. Based on the biochemical data obtained from the in vitro synthesis of ADP-D-β-D-heptose, it was predicted that a mutation in the *E. coli gmhB* gene would express a heptoseless LPS core. To verify this hypothesis, we constructed derivatives from the wild-type *E. coli* K-12 strain W3110 carrying a deleted *gmhB* gene. In one of these strains, BD1, the deletion was marked with a Km^r gene cassette, while in the other strain, BD2, the Km^r gene was excised from the chromosome resulting in an unmarked *gmhB* deletion. The deletion of *gmhB* in both mutants was confirmed by PCR analysis and Southern blot hybridization (data not shown). LPS extracted from strains W3110, BD1, and BD2 was examined by Tricine-SDS-PAGE and silver staining. LPS from strains BD1 and BD2 (Fig. 4, lanes 3 and 4) displayed a fast-migrating band that is absent from the W3110 LPS sample (Fig. 4, lane 1). The fast-migrating band coincided in molecular mass with that of the heptoseless LPS formed by the *hldE*::Tn*10* mutant KCS2926 (Fig. 4, lane 2) (34). The results suggest that the deletion of *gmhB* caused a partial defect in the synthesis of the LPS core, resulting in the formation of heptoseless and heptose-rich forms. This phenotype could be corrected by the introduction of plasmids pCAT1 and pFM4 (Fig. 4, lanes 5 and 6, respectively), which encode cloned *gmhB* genes from *A. thermoaerophilus* and *E. coli* K-12, respectively. The genetic complementation experiment is in agreement with the biochemical function proposed for GmhB. However, the lack of a complete heptoseless phenotype suggests the existence of another function in *E. coli* K-12 that can at least partially compensate for the synthesis of a complete LPS core oligosaccharide. One of the homologs of the GmhB protein is the bifunctional protein HisB that carries a histidinol-phosphate phosphatase activity. Construction of a *gmhB* deletion in *E. coli*
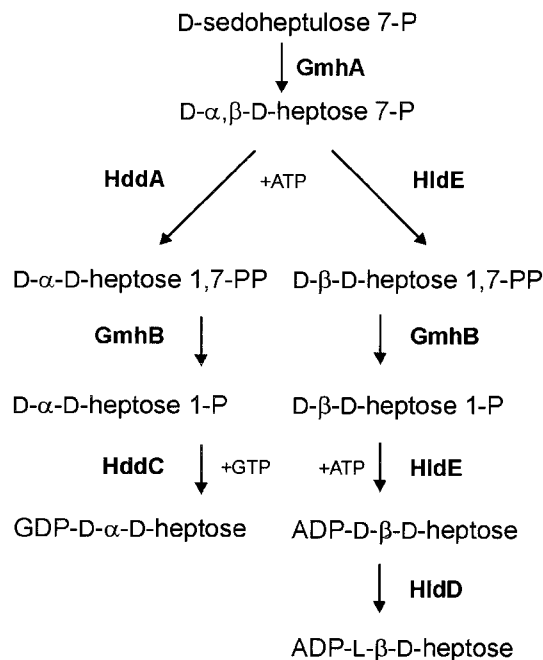

FIG. 5. Comparison of the biosynthetic pathways for GDP-D-α-D-heptose in the gram-positive bacterium *A. thermoaerophilus* DSM 10155 (left) and ADP-L-β-D-heptose in *E. coli* K-12 and other gram-negative bacteria (right). GmhA, sedoheptulose 7-phosphate isomerase; GmhB, D-α,β-D-heptose 1,7-bisphosphate phosphatase; HddA, D-α-D-heptose 7-phosphate kinase; HddC, D-α-D-heptose 1-phosphate guanylyltransferase; HldA, D-β-D-heptose 7-phosphate kinase; HldE, bifunctional D-β-D-heptose 7-phosphate kinase/D-β-D-heptose 1-phosphate adenylyltransferase; HldD, ADP-D-β-D-heptose epimerase.

SØ874, which carries a deletion of the *his* operon (25), showed the same LPS phenotype as in strains BD1 and BD2. Therefore, we postulate that an additional phosphatase activity distinct from HisB, may exist in *E. coli* K-12, which could partially compensate for the *gmhB* deletion.

Concluding remarks and proposed new nomenclature for genes involved in nucleotide-activated heptose synthesis. To our knowledge, this is the first report of the elucidation of the complete pathway for ADP-D-β-D-heptose biosynthesis in *E. coli* (Fig. 5), which involves kinase and phosphatase intermediary steps, as it was shown for the synthesis of GDP-D-α-D-heptose in *A. thermoaerophilus* (20). The phosphatase step, mediated by the *gmhB* gene product, is clearly essential for the nucleotide activation reaction, since treatment of D,D-heptose 7-phosphate with only the bifunctional enzyme and ATP did not yield ADP-activated sugar products. When the phosphatase was present in the system, the product resulting from the nucleotide transfer reaction comigrated with synthetic ADP-D-β-D-heptose. Despite efforts by several investigators in the past, the presence of the *gmhB* phosphatase gene in *E. coli* could not be detected by mutagenesis followed by the screening for novobiocin-sensitive mutants, which usually denotes the presence of a heptoseless LPS core. This may be explained in part by our finding that the deletion of *gmhB* did not confer a complete heptoseless LPS core phenotype, suggesting the presence of another yet-unidentified phosphatase activity that may partially compensate for the synthesis of a complete core. Cur-

rent efforts are dedicated to the isolation and characterization of this gene and its product.

The pathways described in this work and also in a recent study (20) differ greatly from the majority of the classical pathways leading to the formation of nucleotide-activated sugars, which usually involve a mutase step catalyzing the intramolecular transfer of a phosphate group from the distal carbon to the C-1 position (11, 13). This phosphate subsequently reacts with NTP, resulting in the synthesis of an NDP-sugar precursor. In the majority of gram-negative bacteria examined to date, the genes encoding the enzymes for the synthesis of ADP-L-β-D-heptose are scattered throughout the genome. However, in *Campylobacter jejuni* NCTC 11168 and *Helicobacter pylori* strains 26695 and J99, these genes are found in discrete clusters. In the *Neisseria meningitidis* strains MC58 and Z2491, two separate genes encode each of the domains of the bifunctional RfaE enzyme (data not shown), and the *gmhB* phosphatase gene homolog has recently been identified, showing a distinct heptoseless phenotype, which suggests that there are no complementing phosphatase activities present in this organism (31). The presence of a cluster of genes in the gram-positive bacterium *A. thermoaerophilus* DSM 10155 encoding all of the enzymes for the biosynthesis of GDP-D-α-D-heptose greatly facilitated the assignment of the enzymes involved in this novel kinase-phosphatase cascade (20). The results of the present study in *E. coli* and our previous findings with *A. thermoaerophilus* (20) highlight not only similarities but also very important differences between the D,D-heptose and the L,D-heptose synthesis pathways (Fig. 5): (i) the kinase enzyme in each pathway has specificity for the anomeric form of D,D-heptose 7-phosphate, resulting in D-α-D-heptose 1,7-bisphosphate in the *A. thermoaerophilus* pathway and D-β-D-heptose 1,7-bisphosphate in *E. coli*; (ii) the phosphatase activity in both pathways is independent of the anomeric configuration of the bisphosphate sugar; (iii) the nucleotidylyltransferases in both pathways belong to two completely different protein families; and (iv) no epimerization step is required prior to the transfer of D-α-D-heptose onto the S-layer protein in *A. thermoaerophilus*. The β-anomeric configuration of the ADP-heptose is also in perfect agreement with the results of Zamyatina et al. (35) and Gronow et al. (14), indicating that heptosyltransferases from *E. coli* only process the β-anomers of ADP-D,D-heptose and ADP-L,D-heptose.

The elucidation of the pathway of nucleotide-activated D,D-heptose and L,D-heptose requires a reassessment of the gene nomenclature to develop a consistent nomenclature scheme, which takes into account the similarities and the differences among the two heptose pathways, and at the same time follows the general principles for bacterial polysaccharide gene nomenclature as outlined in the Bacterial Polysaccharides Genes Database (http://www.microbio.usyd.edu.au/BPGD/default.htm). We propose that the previous nomenclature of *gmh* (for glycero-mannose-heptose synthesis) should be maintained to design the genes *gmhA* (sedoheptulose 7-phosphate isomerase) and *gmhB* (D-α,β-D-heptose 1,7-bisphosphate phosphatase), which are common to both pathways (Fig. 5). The new nomenclature *hdd* (for D-α-D-heptose synthesis) is proposed for the genes *hddA* (D-α-D-heptose 7-phosphate kinase) and *hddC* (D-α-D-heptose 1-phosphate guanylyltransferase) of the gram-positive bacterium *A. thermoaerophilus* DSM 10155 and their

homologs (Fig. 5, left). The nomenclature *hld* (for L-β-D-heptose synthesis) is proposed for the genes *hldE* (bifunctional D-β-D-heptose 7-phosphate kinase/D-β-D-heptose 1-phosphate adenylyltransferase, formerly *rfaE*) and *hldD* (ADP-D-β-D-heptose epimerase, formerly *waaD* or *rfaD*). In addition, in cases where the bifunctional D-β-D-heptose 7-phosphate kinase/D-β-D-heptose 1-phosphate adenylyltransferase is encoded by separate genes, such as in *N. meningitidis* and *Ralstonia eutropha*, they could be named *hldA* (D-β-D-heptose 7-phosphate kinase) and *hldC* (D-β-D-heptose 1-phosphate adenylyltransferase, formerly *aut*). This new nomenclature will permit a rational grouping of the homologs of all of these genes into gene and protein families and will facilitate comparative studies on the evolution of these genes, as well as future structure-function characterizations of the enzymes. This is especially important taking into account that the elucidation of the complete pathway for ADP-L,D-heptose biosynthesis and the characterization of each of these enzymes pave the way for the development of novel enzyme inhibitors with potential antimicrobial activity for the control of infections by gram-negative bacteria.

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