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Biosynthesis Pathway of ADP-l-glycero-β-d-manno-Heptose in Escherichia coli

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Received 21 August 2001/Accepted 17 October 2001

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-n-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-n-β-d-heptose. Our results demonstrate that the synthesis of ADP-n-β-d-heptose in Escherichia coli requires three proteins, GmhA (sedoheptulose 7-phosphate isomerase), HldE (bifunctional n-β-d-heptose 7-phosphate kinase/n-β-d-heptose 1-phosphate adenylyltransferase), and GmhB (n,β-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 heptoses 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-2ulosonic 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 phenotype (18, 36).

The complete biosynthesis pathway of the L,D-heptose pre-
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 Eide 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,D-heptose from d,D-sedoheptulose 7-phosphate in the gram-positive bacterium Aneurinibacillus thermoacidophilus DSM 10155 was recently characterized (20). We demonstrated that two independent enzymes catalyze the originally proposed mutase step. Aneurinibacillus thermoacidophilus DH5α::Tn10 (Life Technologies) by homologous recombination. Primers CGBD1 (5'-attB1-GCGGAAATCTTCACTTCAACGAAAGGATTGGCCAGGA GCGTACCC-3') and CGC2 (5'-attB2-GCGGATATTAGGGAGTCG-3') were used for the amplification of the putative bifunctional kinase/adenylyltransferase gene hidd (formerly rfeE). Primers CCGC1 (5'-attB1-GCGGAGAATCTTCACTTCAACGAAAGGATTGGCGGAGGGA ACGTACCC-3') and CCGC2 (5'-attB2-GCGGATATTAGGGAGTCG-3') were used for the amplification of the putative phosphatase gene gmbH (formerly yaeD), and 1b1 and a2b2 are the minimal 25-bp sequences required for efficient homologous recombination. The products were cloned into 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 hidd and gmbH, respectively, and were used for overexpression of the corresponding His-tagged protein products in E. coli BL21-SI. The gmbH gene was also amplified with primers 575 (5'-TCCCGGCGGATTGCCCAAGTAGGTGGCGAAGA-3') and 576 (5'-GATGGCGACATTTCCTGGGGTTTTGGCGTCC-3') to construct a DNA fragment containing an Smal site (underlined) and a HindIII site (underlined). The amplification PCR fragment was digested with Smal and cloned into the plasmid 2372, which was also cleaved with Smal. The resulting plasmid, pFM4, carried the gmbH protein terminally fused to the GST moiety. The A. thermoacidophilus DSM 10155 gmbH gene was amplified by using the primers ATCF1 (5'-CGAGGAATCTTCAACGAAAGGATTGGCGGAGGGA ACGTACCC-3') and ATCF2 (5'-GCGGAGAATCTTCACTTCAACGAAAGGATTGGCGGAGGGA ACGTACCC-3') in one step by using the HiTrap chelating column. The purification of proteins were essentially identical to those described (20). Sugar phosphates were investigated by using pulsed electrochemi- cal detection, and UV detection was induced by ultracentrifugation, the bifunctional kinase/pyrophosphorylase Hidd (synonymous to l,D-heptose formation) was purified in two steps by using HiTrap Chelating and MonoQ columns, while the phosphatase GmbH was purified in one step by using the HiTrap chelating column. The proteins were concentrated by ultrafiltration and stored at 4°C or, after stabi-
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 MgCl₂. 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,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 d,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 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-d,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-d,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. 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. thermoaerophilus GmhA.
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,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,D-heptose 1-phosphate to ADP-D,D-heptose can be expected. However, ADP-D,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-anomeric 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,D-heptose 1,7-bisphosphate intermediate into D,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-β,D-heptose is nucleotide activation. Synthetic D,D-heptose 1-phosphate was incubated with the RfaE protein and ATP, resulting in a peak comigrating with synthetic ADP-D,D-heptose (14.2 min, Fig. 3A). However, no activation was detected when the same reaction was performed with either D-α,D-heptose 1-phosphate (Fig. 3B) or D,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 shown).
A deletion of the E. coli K-12 gnhB gene is associated with an altered LPS core phenotype. Based on the biochemical data obtained from the in vitro synthesis of nucleotide-activated heptose, it was predicted that a mutation in the E. coli gnhB 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 gnhB gene. In one of these strains, BD1, the deletion was marked with a Km\(^{-}\) gene cassette, while in the other strain, BD2, the Km\(^{-}\) gene was excised from the chromosome resulting in an unmarked gnhB deletion. The deletion of gnhB 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::Tn10 mutant KCS2926 (Fig. 4, lane 2) (34). The results suggest that the deletion of gnhB 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 gnhB genes from A. thermoaerophilus DSM 10155 and E. coli K-12, respectively.

A. thermoaerophilus was incubated with GmhA from E. coli and the HisB protein. One of the homologs of the GmhB protein is the thermoaerophilus.
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-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 MCS8 and Z2491, two separate genes encode each of the domains of the bifunctional RfE 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-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 δ-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 δ-D-heptose 7-phosphate, resulting in δ-α-D-heptose 1,7-bisphosphate in the A. thermoaerophilus pathway and δ-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 δ-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-δ,D-heptose and ADP-L,D-heptose.

The elucidation of the pathway of nucleotide-activated δ,D-heptose and L,D-heptose requires a reassessment of the 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 (δ-α,D-heptose 1,7-bisphosphate phosphatase), which are common to both pathways (Fig. 5). The new nomenclature hld (for δ-α,D-heptose synthesis) is proposed for the genes hldaA (δ-α,D-heptose 7-phosphate kinase) and hldC (δ-α,D-heptose 1-phosphate guanylyltransferase) of the gram-positive bacterium A. thermoaerophilus DSM 10155 and their homologs (Fig. 5, left). The nomenclature hdl (for l-β,D-heptose synthesis) is proposed for the genes hldE (bifunctional δ-β,D-heptose 7-phosphate kinase/δ-β,D-heptose 1-phosphate adenyllyltransferase, formerly rfaE) and hldD (ADP-β,D-heptose epimerase, formerly waaD or rfaD). In addition, in cases where the bifunctional δ-β,D-heptose 7-phosphate kinase/δ-β,D-heptose 1-phosphate adenyllyltransferase is encoded by separate genes, such as in N. meningitidis and Ralstonia eutropha, they could be named hldaA (δ-β,D-heptose 7-phosphate kinase) and hldC (δ-β,D-heptose 1-phosphate adenyllyltransferase, 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.

ACKNOWLEDGMENTS

We thank Sonja Zayni and Andrea Scheberl for excellent technical assistance and Tracey Hunt for a critical reading of the manuscript. We also thank Peter Reeves for useful discussions regarding the heptose pathway gene nomenclature.

The construction of mutants BD1 and BD2 was part of an undergraduate research project conducted by Beth Dunn. This work was supported by the Australian Science Fund (projects P12906-MOB and P14209-MOB to P.M.) and by the Natural Science and Engineering Research Council of Canada (to M.A.V.).

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