Biosynthesis of a Structurally Novel Lipid A in *Rhizobium leguminosarum*: Identification and Characterization of Six Metabolic Steps Leading from UDP-GlcNAc to 3-Deoxy-D-manno-2-Octulosonic Acid₂-Lipid IV_A

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Lipopolysaccharides (LPSs) are prominent structural components of the outer membranes of gram-negative bacteria. In *Rhizobium* spp. LPS functions as a determinant of the nitrogen-fixing symbiosis with legumes. LPS is anchored to the outer surface of the outer membrane by the lipid A moiety, the principal lipid component of the outer bacterial surface. Several notable structural differences exist between the lipid A of *Escherichia coli* and that of *Rhizobium leguminosarum*, suggesting that diverse biosynthetic pathways may also exist. These differences include the lack of phosphate groups and the presence of a 4'-linked GalA residue in the latter. However, we now show that UDP-GlcNAc plays a key role in the biosynthesis of lipid A in *R. leguminosarum*, as it does in *E. coli*. ³²P-labeled monosaccharide and disaccharide lipid A intermediates from *E. coli* were isolated and tested as substrates in cell extracts of *R. leguminosarum* biovars phaseoli and viciae. Six enzymes that catalyze the early steps of *E. coli* lipid A biosynthesis were also present in extracts of *R. leguminosarum*. Our results show that all the enzymes of the pathway leading to the formation of the intermediate 3-deoxy-D-manno-2-octulosonic acid (Kdo₂)-lipid IV_A are functional in both *R. leguminosarum* biovars. These enzymes include (i) UDP-GlcNAc 3-O-acyltransferase; (ii) UDP-3-O-(R-3-hydroxymyristoyl)-GlcNAc deacetylase; (iii) UDP-3-O-(R-3-hydroxymyristoyl)-GlcN N-acyltransferase; (iv) disaccharide synthase; (v) 4'-kinase; and (vi) Kdo transferase. Our data suggest that the early steps in lipid A biosynthesis are conserved and that the divergence leading to rhizobial lipid A may occur at a later stage in the pathway, presumably after the attachment of the Kdo residues.

Rhizobium spp. are gram-negative, symbiotic bacteria that induce root nodules on leguminous host plants. The infection process is well established (26) and leads to intracellular bacteroids that are able to fix atmospheric nitrogen. Various carbohydrate signals are involved in regulating the development of the symbiosis (16, 24), including Nod factors (15), exopolysaccharide (23, 12), capsular polysaccharide antigens (36), and lipopolysaccharide (LPS) (28, 9). Rhizobial mutations that alter LPS structure also result in impaired infectivity (29, 10).

The outer surface of the outer membrane of gram-negative bacteria consists primarily of the lipid A moiety, which functions as an anchor for LPS. To date, most work on the biosynthesis of lipid A has been confined to *Escherichia coli* and *Salmonella typhimurium* (32). In both of these organisms, lipid A consists of a β-1,6-linked glucosamine disaccharide (Fig. 1), which is phosphorylated at positions 1 and 4' and is acylated with R-3-hydroxymyristate (3-OH-C_{14:0}) at positions 2, 3, 2', and 3'. The two R-3-hydroxymyristoyl groups attached to the nonreducing glucosamine are further esterified with myristate and laurate residues (Fig. 1).

Lipid anchors from several *Rhizobium* and *Bradyrhizobium* strains have been analyzed and found to display various species-dependent structural differences (6). The structure of

Rhizobium leguminosarum lipid A has recently been described (5a), and when it is compared with lipid A of *E. coli* several major differences are also evident (Fig. 1). The sugar moieties are reported as D-galacturonic acid and D-glucosamine for *R. leguminosarum* biovars phaseoli, trifolii, and viciae (6), as D-glucosaminuronic acid for a particular strain of *R. leguminosarum* bv. trifolii (20), as 2,3-diamino-2,3-dideoxy-D-glucose for *Bradyrhizobium japonicum* (11, 25), and as D-glucosamine for *Rhizobium meliloti* (42). For only one strain of *R. leguminosarum* bv. trifolii are these sugars reported to be phosphorylated (37). Whatever its structure, the lipid A sugar backbone is acylated with diverse hydroxy fatty acids (Fig. 1), one of which is a unique 27-hydroxyoctacosanoic acid (27-OH-C_{28:0}) (19). Other 3-hydroxy fatty acids are 3-hydroxymyristic acid (3-OH-C_{14:0}), 3-hydroxypentadecanoic acid (3-OH-C_{15:0}), 3-hydroxypalmitic acid (3-OH-C_{16:0}), and 3-hydroxystearic acid (3-OH-C_{18:0}) (6). The type and quantity of these acyl groups vary among different *Rhizobium* species.

E. coli lipid A biosynthesis (32) begins with UDP-GlcNAc (Fig. 2), which is also utilized for the assembly of peptidoglycan. UDP-GlcNAc is initially 3-O acylated with R-3-hydroxymyristic acid that is transferred from R-3-hydroxymyristoyl acyl carrier protein. Deacetylation then occurs, followed by N acylation with a second R-3-hydroxymyristate moiety to yield UDP-2,3-diacyl-GlcN. A portion of this is hydrolyzed to 2,3-diacyl-GlcN-1-P (lipid X), which condenses with another molecule of UDP-2,3-diacyl-GlcN to give tetraacyldisaccharide-1-phosphate (Fig. 2). In *E. coli* this is phosphorylated at position 4', yielding the tetraacyldisaccharide-1,4'-bis-phosphate (lipid IV_A), the minimal substrate to which Kdo (3-deoxy-D-manno-

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FIG. 1. Schematic structures of the lipid A's, depicted with Kdo residues attached, from *E. coli* (A) and *R. leguminosarum* (B). A significant portion of the 27-OH-C_{28:0} fatty acyl residues may also be esterified at the 27-OH with 3-hydroxybutyric acid (5a).

2-octulosonic acid) residues become attached. Two Kdo residues are then linked to the 6' position of the nonreducing glucosamine by a specific Kdo transferase. Later stages in the pathway include the addition of laurate and myristate residues to generate acyloxyacyl moieties.

An important factor in the elucidation of *E. coli* lipid A biosynthesis was the discovery of mutants that accumulate precursors of lipid A (27, 41). These intermediates can be prepared as radiolabeled probes for demonstrating enzymatic reactions in cell extracts. Initially, [³²P]lipid X and UDP-2,3-diacyl-GlcN, isolated from a temperature-sensitive *E. coli* strain defective in the disaccharide synthase gene (*lpxB*), were used to demonstrate the mechanism by which the β-1,6 linkage of lipid A is generated (34). Given the conservation of the lipid A pathway in *E. coli* and related gram-negative bacteria (43) on the one hand and the significant structural differences between *E. coli* and *R. leguminosarum* lipid A moieties (Fig. 1) on the other, it seemed relevant to investigate the biosynthesis of lipid A in *R. leguminosarum*.

We now report that the initial steps of the lipid A pathway

are conserved in both organisms and show that *R. leguminosarum* is capable of supporting all the key enzymatic reactions needed to synthesize Kdo₂-lipid IV_A from UDP-GlcNAc. These steps included the UDP-GlcNAc 3-O-acyltransferase, the UDP-3-O-(R-3-hydroxymyristoyl)-GlcNAc deacetylase and UDP-3-O-(R-3-hydroxy-myristoyl)-GlcN N-acyltransferase, the lipid A disaccharide synthase, the 4'-kinase, and the Kdo transferase. Our results suggest that lipid A biosynthesis is highly conserved in gram-negative bacteria and that the divergence leading to rhizobial lipid A may occur at a later stage in the pathway, presumably after attachment of the Kdo residues.

MATERIALS AND METHODS

Chemicals and materials. [U-¹⁴C]acetate and [γ-³²P]ATP were obtained from Amersham, and ³²P_i was obtained from New England Nuclear. Silica gel 60 (0.25 mm) thin-layer chromatographic (TLC) plates were purchased from E. Merck, Darmstadt, Germany, and polyethyleneimine-cellulose plates (0.1 mm) were purchased from J. T. Baker Inc., Phillipsburg, N.J. Bio-Sil A silicic acid for column chromatography was obtained from Bio-Rad. Pyridine (Fisher) was redistilled before use. All other chemicals were reagent grade. For autoradiography Kodak AR X-ray film was used with intensifying screens (DuPont Lightning Plus) at -80°C.

Bacterial strains and growth conditions. *R. leguminosarum* biovar viciae 8401 was obtained from J. A. Downie (John Innes Institute, Norwich, England), and *R. leguminosarum* bv. phaseoli CE3 was obtained from D. Noel (Marquette University, Milwaukee, Wis.). Both strains were grown at 30°C either on tryptone-yeast medium containing 5 g of tryptone per liter, 3 g of yeast extract per liter, and 10 mM CaCl₂ or on RMM minimal medium containing 12 mM succinate and 6 mM glutamate (30). When necessary, *Rhizobium* cultures were selected on streptomycin (100 μg/ml). *E. coli* MN7 and R477 are derivative strains of *E. coli* K-12 and were obtained from the *E. coli* Genetic Stock Center, Yale University, New Haven, Conn. Strain MC1061(pSR8), which overexpresses the *E. coli* *lpxB* gene product, has been described previously (14). Unless stated otherwise, the *E. coli* strains were grown at 37°C in Luria-Bertani broth containing NaCl (10 g/liter), yeast extract (5 g/liter), and tryptone (10 g/liter) or in G56 minimal medium (18) supplemented with 0.2% glucose and 0.4% Casamino Acids.

Preparation of cell extracts. Crude cell extracts of *Rhizobium* spp. were prepared by growing cells to late log phase ($A_{550} = 1.0$) at 30°C in tryptone-yeast medium. The cells were harvested by centrifugation (8,000 × *g*) for 10 min at 4°C, washed with cold 50 mM Tris buffer (pH 7.0), and resuspended in the same buffer to give a final protein concentration of 1 to 10 mg/ml. Cells were broken by two passages through a French pressure cell at 18,000 psi, and debris was removed by centrifugation for 15 min at 8,000 × *g*. All procedures were performed at 4°C. Cell extracts of *E. coli* strains were obtained in a similar manner, as described previously (1). Protein concentrations were determined by the bicinchoninic acid method with bovine serum albumin (BSA) as the standard (39).

Preparation of radiolabeled substrates. [α-³²P]UDP-GlcN was prepared enzymatically from GlcN-1-P and [α-³²P]UTP by using bovine liver UDP-glucose pyrophosphorylase and baker's yeast inorganic pyrophosphatase. The product was next N acetylated with acetic anhydride and was purified on a DEAE-Sepharose column (3). After being washed with water, [α-³²P]UDP-GlcNAc was eluted with triethyl ammonium bicarbonate (100 mM). R-3-Hydroxymyristoyl-ACP, [α-³²P]UDP-3-O-(R-3-hydroxymyristoyl)-GlcNAc, and [α-³²P]UDP-

3-*O*-(*R*-3-hydroxymyristoyl)-GlcN were prepared and purified as described previously (22).

[32 P]lipid X (41) was isolated from a temperature-sensitive mutant (MN7) of *E. coli* bearing a lesion in the *lpxB* gene, after in vivo labeling with $^{32}P_i$ as described previously (31). UDP-2,3-diacyl-GlcN was prepared as previously described (8). [32 P]tetraacyldisaccharide-1-P was prepared enzymatically from [32 P]lipid X and UDP-2,3-diacyl-GlcN, by using extracts of an *E. coli* strain that overproduces the disaccharide synthase from vector pSR8 (14). The [32 P]tetraacyldisaccharide-1-P was recovered by Bligh-Dyer fractionation (34) and was further purified on a 2-ml BioSil A column, equilibrated with chloroform. After successive washings with 5 ml of chloroform-methanol (95:5 [vol/vol]) and 5 ml of chloroform-methanol (90:10 [vol/vol]), the tetraacyldisaccharide-1-P was eluted with 5 ml of chloroform-methanol (80:20 [vol/vol]). The purification was monitored by TLC and autoradiography. A small quantity of contaminating lipid X (5 to 10% of the radioactivity) remained after these procedures, but this did not interfere with subsequent 4'-kinase assays and was readily identified on TLC plates by including a lane of purified ^{32}P -labeled lipid X as a standard (see below).

[1- 32 P]lipid IV_A was prepared by enzymatic conversion of [1- 32 P]tetraacyldisaccharide-1-P in the presence of ATP as described previously (35). [4'- 32 P]lipid IV_A was prepared in a similar manner, but using [γ - 32 P]ATP and unlabeled tetraacyldisaccharide-1-P. The products were purified by preparative TLC and were stored at $-80^{\circ}C$ as described previously (35).

Coupled assay for the first three enzymes of lipid A biosynthesis. Assay mixtures contained [α - 32 P]UDP-GlcNAc (2.77×10^5 cpm/nmol, 200 μ M), *R*-3-hydroxymyristoyl-ACP (50 μ M), crude bacterial extract (1 mg/ml), and 40 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 8.0) in a final volume of 20 μ l. After 5 min at $30^{\circ}C$, 5- μ l portions of the reaction mixtures were spotted directly onto silica plates. The spots were thoroughly dried with a cold air stream, and the plates were developed in chloroform-methanol-water-acetic acid (25:15:4:2 [vol/vol]), followed by overnight autoradiography. The products were scraped from the plates and were quantified by liquid scintillation spectrometry.

Assay for UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcNAc deacetylase. The assay mixture for measuring UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcNAc deacetylase activity (3, 22) directly contained [α - 32 P]UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcNAc (9.25×10^6 cpm/nmol, 3 μ M), BSA (1 mg/ml), 40 mM bis-Tris (pH 5.5), and crude bacterial extract (0.8 mg/ml) in 20 μ l. After 5 min at $30^{\circ}C$, 5- μ l portions of the reaction mixtures were hydrolyzed with 1 μ l of 1.25 M NaOH at $30^{\circ}C$ for 10 min. Following neutralization with 1 μ l of 1.25 M acetic acid, protein precipitation with 1 μ l of 5% trichloroacetic acid, and a brief centrifugation, 2- μ l portions were spotted onto flexible, plastic-backed polyethyleneimine-cellulose plates, which were developed in 0.2 M guanidine HCl. Following drying and overnight autoradiography, the product spots were cut out and quantitated by liquid scintillation spectrometry.

UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcN *N*-acyltransferase assays. The specific assay for UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcN *N*-acyltransferase activity was performed as previously described (22). The assay mixture contained [α - 32 P]UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcN (4.44×10^6 cpm/nmol, 1 μ M), *R*-3-hydroxymyristoyl acyl carrier protein (1

FIG. 2. Biosynthetic pathway of lipid A in *E. coli*. Evidence for this scheme has been reviewed previously (33).

TABLE 1. In vivo radiolabeling analysis of *E. coli* and *R. leguminosarum*^a

	³² P labeling (10 ³ cpm/ <i>A</i> ₅₅₀)		¹⁴ C labeling (10 ³ cpm/ <i>A</i> ₅₅₀)	
	<i>E. coli</i>	<i>R. leguminosarum</i> CE3	<i>E. coli</i>	<i>R. leguminosarum</i> CE3
Glycerophospholipid	161.3	133.6 (82.8% ^b)	632.8	1,935.6 (305.9%)
Lipid A	19.0	0.64 (3.37%)	119.2	313.9 (263.3%)
Lipid A/glycerophospholipid	0.118	0.0048 (4.07%)	0.188	0.162 (86.2%)

^a Uptake and incorporation of radiolabel (in counts per minute) in 0.8-ml culture normalized to the optical density (*A*₅₅₀ = 1.0) of the bacterial culture (i.e., 0.8 × 10⁹ cells).

^b Relative value obtained by comparison with the *E. coli* data.

μM), BSA (1 mg/ml), bacterial extract (50 μg/ml), and 50 mM HEPES (pH 8.0) in a final volume of 10 μl. After 5 min at 30°C, 5-μl portions of the reaction mixtures were spotted directly onto silica plates. After drying, the plates were developed in chloroform-methanol-water-acetic acid (25:15:4:2 [vol/vol]). Following overnight autoradiography, the products were scraped from the plates and were quantitated by liquid scintillation spectrometry.

Enzyme assay for the lipid A disaccharide synthase. Lipid A disaccharide synthase was monitored by using a quantitative assay as described previously (31). Assays (total volume, 15 μl) were performed in Eppendorf tubes, and the reaction mixtures contained UDP-2,3-diacyl-GlcN (1 mM), [³²P]lipid X (3.36 × 10⁶ cpm/nmol, 100 μM), 20 mM HEPES (pH 8.0), and bacterial cell extract (1.4 mg/ml). After incubation at 30°C, portions (5 μl) were spotted onto silica TLC plates. After the spots were dried in a stream of cold air, the plates were developed in chloroform-methanol-water-acetic acid (25:15:4:2 [vol/vol]), dried, and analyzed by autoradiography.

Enzyme assay for the lipid A 4'-kinase. Lipid A 4'-kinase activity was assayed as described by Ray and Raetz (35). A typical reaction mixture (20 μl) contained cardiolipin (2 mg/ml), 50 mM HEPES buffer (pH 7.4), Nonidet P-40 (1%), MgCl₂ (10 mM), ATP (10 mM), and the appropriate bacterial protein extract (1 mg/ml). Radiolabeled substrate was included as [1-³²P]tetraacyldisaccharide-1-P (0.18 mM; 36 nCi per reaction mixture) or [γ-³²P]ATP (10 mM; 2 μCi per reaction mixture). Unlabeled tetraacyldisaccharide-1-P (250 μg/ml) was included in the [γ-³²P]ATP reaction mixture. After incubation at 37°C, the reaction was stopped by direct spotting onto the TLC plates. Plates were developed in chloroform-pyridine-88% formic acid-water (70:30:16:5 [vol/vol]) and were analyzed by autoradiography.

Enzyme assay for the lipid A Kdo transferase. The lipid A Kdo transferase assays were performed according to the method of Brozek et al. (7), as modified by Belunis and Raetz (5). Reaction mixtures (20 μl) contained 50 mM Tris buffer (pH 7.5), MgCl₂ (10 mM), Triton X-100 (0.2%), and 100 μM [4'-³²P]lipid IV_A (6.44 × 10⁵ cpm/nmol). CMP-Kdo was generated in situ from CTP (5 mM) and Kdo (2 mM) with 2.6 mU of purified CMP-Kdo synthase, isolated from frozen cells of *E. coli* B (Grain Processing Co., Muscatine, Iowa). Reactions were started by the addition of bacterial protein extracts (0.5 mg/ml), were incubated for 30 min at 30°C, and were terminated by spotting 5 μl of the assay mixture onto silica TLC plates. Plates were then dried and developed in the chloroform-pyridine-88% formic acid-water system described above.

Analysis of lipid A biosynthesis in vivo. The assay for lipid A content in living cells was carried out by labeling 1.0-ml cultures for 30 h at 30°C with ³²P_i (10 μCi/ml) or [¹⁴C]acetate (10 μCi/ml) in tryptone-yeast medium (*R. leguminosarum*) or G56 medium (*E. coli*). Portions of the cultures (0.8 ml) were then extracted with a single-phase Bligh and Dyer solvent

mixture by adding 2.0 ml of methanol and 1.0 ml of chloroform. LPS was associated with the cell debris and was removed by centrifugation for 15 min at 5,000 × g, while the glycerophospholipids remained in the supernatant. The subsequent workup and chromatography to determine the lipid A and glycerophospholipid contents of the cultures were performed as described by Galloway and Raetz (17).

RESULTS

In vivo labeling with [U-¹⁴C]acetate or ³²P_i. Steady-state incorporation of [U-¹⁴C]acetate or ³²P_i into phospholipids and lipid A in 30-h overnight cultures of *E. coli* K-12 and *R. leguminosarum* CE3 cells was measured by the method of Galloway and Raetz (17). Virtually no ³²P_i was incorporated into the rhizobial lipid A (Table 1), a result consistent with the finding that the lipid A from *R. leguminosarum* is nonphosphorylated (5a). In contrast, [U-¹⁴C]acetate incorporation into lipid A in *E. coli* was very similar to that in *R. leguminosarum*. Lipid A-to-glycerophospholipid ratios of 0.180 and 0.162 in *E. coli* and *R. leguminosarum*, respectively, indicate that lipid A constitutes 15 to 20% of total phospholipids in both organisms.

Formation of UDP-2,3-diacyl-GlcN from UDP-GlcNAc and R-3-hydroxymyristoyl-ACP. A coupled assay for observing the formation of acylated metabolites from [α-³²P]UDP-GlcNAc during the early steps of lipid A biosynthesis was previously described by Anderson and Raetz et al. (1-3). [α-³²P] UDP-GlcNAc was prepared and incubated with unlabeled R-3-hydroxymyristoyl-ACP. As noted earlier (3, 14, 22), upon the addition of crude cytosolic protein from *E. coli*, the labeled substrate was rapidly metabolized to mono- and diacylated species, as judged by TLC (Fig. 3). The same products arose with an equivalent protein concentration of an extract from *R. leguminosarum* bv. phaseoli CE3, although somewhat less rapidly (Table 2). The formation of UDP-2,3-diacyl-GlcN requires the proper functioning of the first three enzymes (Fig. 2 and 3) of the lipid A pathway.

To assay directly for [α-³²P]UDP-3-*O*-(R-3-hydroxymyristoyl)-GlcNAc deacetylation in crude extracts, it is convenient to de-O-acylate both the remaining substrate and the product with mild base (22), resulting in the formation of [α-³²P]UDP-GlcNAc (derived from substrate) and [α-³²P]UDP-GlcN (derived from deacetylated reaction product). Separation of [α-³²P]UDP-GlcNAc and [α-³²P]UDP-GlcN was then performed on polyethyleneimine-cellulose plates developed in 0.2 M guanidine HCl. The results (Fig. 4) show that UDP-3-*O*-(R-3-hydroxymyristoyl)-GlcNAc is converted to UDP-3-*O*-(R-3-hydroxymyristoyl)-GlcN by the action of a deacetylase in extracts of both *E. coli* and *R. leguminosarum* CE3. An estimate of the specific activity of the deacetylase showed that the *R. leguminosarum* enzyme was 36% as efficient as the *E. coli* deacetylase (Table 2). A spot due to ³²P-UMP was seen in the *E. coli* assay because of the pyrophosphatase activity of CDP-diacylglycerol hydrolase on [α-³²P]UDP-3-*O*-(R-3-hy-

FIG. 3. Acylation of UDP-GlcNAc in extracts of *E. coli* and *R. leguminosarum*. This coupled assay for the generation of UDP-2,3-diacyl-GlcN demonstrates the activities of the first three steps of lipid A biosynthesis. The assay utilized [α - 32 P]UDP-GlcNAc (2.77×10^5 cpm/nmol; 200 μ M) and *R*-3-hydroxymyristoyl-ACP (50 μ M) in the presence of bacterial cell extracts (1 mg of protein per ml) in a final volume of 20 μ l. Lanes: 1, enzyme-free control replacing the cell extract with an equivalent volume of extraction buffer; 2, crude extract from wild-type *E. coli* R477; 3, crude extract from *R. leguminosarum* CE3. Incubations (5 min at 30°C) were stopped by direct spotting of 5- μ l portions onto a silica gel 60 plate. The plate was developed in chloroform-methanol-water-acetic acid (25:15:4:2 [vol/vol]). Radiolabeled products were visualized by overnight autoradiography at -80°C with an intensifying screen.

droxymyristoyl)-GlcNAc (2). Interestingly, this interfering activity is absent from the rhizobial extract.

Direct evidence that the UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcN is *N* acylated to UDP-2,3-diacyl-GlcN was provided by the results of an assay specific for the *N*-acyltransferase. Purified [α - 32 P]UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcN and *R*-3-hydroxymyristoyl-ACP were incubated with crude extracts of either *R. leguminosarum* or *E. coli* (1 mg/ml). Efficient *N* acylation was observed with both extracts, giving rise to a TLC spot corresponding to [α - 32 P]UDP-2,3-diacyl-GlcN on silica gel 60 plates (Fig. 5). The specific activity of the rhizobial *N*-acyltransferase was estimated to be about one quarter of that of the comparable *E. coli* enzyme (Table 2). Accordingly, the overall efficiency (7.2%) for the coupled assay is in good agreement with the calculated aggregate efficiency (5.2%) for the individual steps (Table 2). We have not yet attempted to optimize the reaction conditions for each of the rhizobial enzymes. For instance, it is likely that the constitutive rhizobial ACP will be a more effective substrate than the *E. coli* ACP employed in our assays.

Formation of [32 P]tetraacyldisaccharide-1-P from UDP-2,3-

TABLE 2. Relative efficiencies of the first three enzymes of lipid A biosynthesis in extracts of *E. coli* and *R. leguminosarum*

CE3	Sp act (pmol/min/mg)	
	<i>E. coli</i> R477	<i>R. leguminosarum</i>
Coupled assay (monoacyl species)	269.2	151.0 (56.1% ^a)
Coupled assay (diacyl species)	310.6	22.5 (7.2%)
Deacetylase	197.0	71.8 (36.4%)
<i>N</i> -Acyltransferase	1,010.0	260.0 (25.7%)

^a Relative value obtained by comparison with the *E. coli* data.

diacyl-GlcN and [32 P]lipid X. [32 P]lipid X was isolated from *E. coli* K-12 mutant MN7, which has a mutation in the gene (*lpxB*) encoding the disaccharide synthase. When grown at nonpermissive temperatures in the presence of $^{32}\text{P}_i$, this strain accumulates [32 P]lipid X (31, 41). Labeled lipid X was isolated by Bligh-Dyer extraction and was used to assay for the presence of the disaccharide synthase.

When [32 P]lipid X was incubated in the presence of unlabeled UDP-2,3-diacyl-GlcN and *E. coli* extract, it was converted into a more hydrophobic product, previously shown to be the tetraacyldisaccharide-1-P (31, 34) (Fig. 2 and 6). No reaction in an enzyme-free control or in the absence of UDP-2,3-diacyl-GlcN was observed. When assayed at an equivalent protein concentration (2 mg/ml), *R. leguminosarum* extracts formed the same product, although the rate of formation was 50 to 60% lower than that with extracts of wild-type *E. coli* (data not shown). In Fig. 6, the *E. coli* control was strain MC1060(pSR8), which overproduces the disaccharide synthase ~200-fold, resulting in nearly complete conversion of [32 P]lipid X to product at the first time point. The products of the *E. coli* and *Rhizobium* reactions comigrated when they were analyzed by two-dimensional TLC, further indicating that they are structurally identical (data not shown).

Phosphorylation of tetraacyldisaccharide-1-P by the 4'-kinase. In *E. coli* the next reaction in the lipid A pathway is the conversion of tetraacyldisaccharide-1-P to lipid IV_A by an ATP-dependent 4'-kinase (35). It was not possible to monitor the disaccharide synthase and the 4'-kinase activities concurrently in a coupled assay. It was therefore necessary to prepare purified [1- 32 P]tetraacyldisaccharide-1-P as the substrate for the 4'-kinase assay. This was accomplished by using UDP-2,3-diacyl-GlcN, [32 P]lipid X, and disaccharide synthase from the overproducing *E. coli* strain MC1060(pSR8), as described in Materials and Methods.

FIG. 4. Deacetylation of UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcNAc in extracts of *E. coli* and *R. leguminosarum*. Reaction mixtures (20 μ l) containing [α - 32 P]UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcNAc (9.25×10^6 cpm/nmol, 3 μ M), BSA (1 mg/ml), 40 mM bis-Tris buffer (pH 5.5) and crude extract protein (0.8 mg/ml) were incubated for 5 min at 30°C. A portion (5 μ l) was then de-O-acylated with NaOH (1 μ l, 1.25 M, 30°C, 10 min). Following neutralization, trichloroacetic acid precipitation, and a brief centrifugation, a portion (2 μ l) of the supernatant was spotted onto polyethyleneimine-cellulose plates and was developed in 0.2 M guanidine HCl. Lanes: 1, enzyme-free control; 2, crude extract from wild-type *E. coli* R477; 3, crude extract from *R. leguminosarum* CE3. Autoradiography was performed as described in the legend to Fig. 3.

To ascertain whether the 4'-kinase activity also occurs in *R. leguminosarum*, the [1 - 32 P]tetraacyldisaccharide-1-P was incubated either with or without MgATP in the presence of bacterial extracts of *E. coli*, *R. leguminosarum* 8401, or *R. leguminosarum* CE3 (Fig. 7). All extracts were used at equivalent protein concentrations (1 mg/ml). A more slowly migrating, less hydrophobic product was observed with the complete incubation system as judged by TLC analysis (Fig. 7), consistent with the attachment of the 4'-phosphate group. For *E. coli* this product was shown previously to be the tetraacyldisaccharide-1,4'-bis-phosphate, i.e., lipid IV_A (35). The products resulting from either the *E. coli* or the rhizobial cell extracts also had identical chromatographic properties when they were analyzed by two-dimensional TLC (data not shown). The lipid

IV_A product was not formed in the enzyme-free controls or in the absence of MgATP (Fig. 7). The formation of lipid IV_A in extracts was time dependent, and the rates of formation were similar for the *E. coli* and the *Rhizobium* strains (Fig. 7).

Mature lipid A from *R. leguminosarum* contains a galacturonic acid residue in place of phosphate at the 4' position (5a). This prompted us to repeat the 4'-kinase assays in the presence of UDP-GalA. However, the inclusion of 500 μ M UDP-GalA in the assay mixtures had no effect on the rate or identity of the products formed under the 4'-kinase assay conditions.

Assays for the 4'-kinase were also performed by using [γ - 32 P]ATP and unlabeled tetraacyldisaccharide-1-P (data not shown). As before, the substrates were incubated with extracts

FIG. 5. Assay of UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcN *N*-acyltransferase activity in extracts of *E. coli* and *R. leguminosarum*. [α - 32 P]UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcN (4.44×10^6 cpm/nmol, 1 μ M) was incubated with *R*-3-hydroxymyristoyl-ACP (1 μ M) in the presence of bacterial cell extracts (50 μ g/ml) in 10 μ l as described in Materials and Methods. Portions (5 μ l) were spotted onto silica plates and were developed in chloroform-methanol-water-acetic acid (25:15:4:2 [vol/vol]). 32 P-labeled spots were visualized by autoradiography as described in the legend to Fig. 3. Lanes: 1, enzyme-free control; 2, crude extract from wild-type *E. coli* R477; 3, crude extract from *R. leguminosarum* CE3.

FIG. 6. Disaccharide synthase activity in extracts of *E. coli* and *R. leguminosarum*. Complete enzyme assay mixtures (15 μ l total volume) consisted of crude bacterial extract (1.4 mg/ml), 32 P-labeled lipid X (3.36×10^6 cpm/nmol), UDP-2,3-diacyl-GlcN (1 mM), carrier lipid X (100 μ M), and 20 mM HEPES (pH 8.0). Portions (5 μ l) were spotted onto silica plates, which were developed with chloroform-methanol-water-acetic acid (25:15:4:2 [vol/vol]). Lanes: 1, [32 P]lipid X standard; 2, *E. coli* MC1061(pSR8) 2-h control without UDP-2,3-diacyl-GlcN; 3 to 5, *E. coli* MC1061(pSR8) with the complete system, spotted 20 min, 60 min, or 2 h after the addition of the extract; 6, *R. leguminosarum* 8401 2-h control lacking UDP-diacyl-2,3-GlcN; 7 to 9, *R. leguminosarum* with the complete system, spotted after 20 min, 60 min, or 2 h, respectively. *E. coli* MC1061(pSR8) is an overproducer of the disaccharide synthase.

of *E. coli* or *R. leguminosarum* bv. phaseoli CE3 or bv. viciae 8401. Negative controls lacked either tetraacyl-disaccharide-1-P or the protein extract, and the previously prepared [1- 32 P]lipid IV_A was used as a standard. All three bacterial extracts gave rise to a new TLC spot due to [4'- 32 P]lipid IV_A, which was absent from the control assay and migrated with [1- 32 P]lipid IV_A.

Conversion of lipid IV_A to Kdo₂-lipid IV_A by Kdo transferase. The next enzymatic step in *E. coli* involves the attachment of two Kdo residues to the 6' position of the nonreducing glucosamine by a CMP-Kdo-dependent Kdo transferase (7, 13). [γ - 32 P]ATP and tetraacyl-disaccharide-1-P were used to prepare the labeled substrate [4'- 32 P]lipid IV_A, as described previously (7). The second substrate, CMP-Kdo, is unstable and must be generated in situ in the Kdo transferase assay. This was achieved from Kdo, CTP, and partially purified CMP-Kdo synthase (7).

Assays for Kdo transferase were started by the addition of crude bacterial extracts of either *E. coli* or *R. leguminosarum* CE3 and were stopped when they were spotted onto silica 60 TLC plates (Fig. 8). The *E. coli* extract converted the substrates to a new product, which ran more slowly on the TLC plates and which was previously shown to be Kdo₂-lipid IV_A (7). An identical spot was formed at a lower rate in the rhizobial assay but was absent in the enzyme-free controls.

DISCUSSION

In gram-negative bacteria UDP-GlcNAc is a precursor of the β -1,6-linked disaccharide backbone of lipid A. The biosynthetic pathway (32, 33) in *E. coli* has been well characterized and proceeds via 3-O-acylation of UDP-GlcNAc, followed by deacetylation, N acylation, disaccharide formation, 4' phos-

phorylation, Kdo addition, and late acylation (Fig. 2). However, little is known about lipid A biosynthesis in gram-negative bacteria with novel lipid A structures.

Nitrogen-fixing symbiotic bacteria of the genus *Rhizobium* are of interest because they have a lipid A which is structurally distinct from that of enteric bacteria. The *R. leguminosarum* and *E. coli* lipid A structures are shown in Fig. 1. The lipid A from *R. leguminosarum* is nonphosphorylated, consistent with the in vivo 32 P-labeling result (Table 1). It contains 2-deoxy-2-aminogluconic acid in place of the reducing glucosamine residue on *E. coli* lipid A. In addition, *R. leguminosarum* lipid A is more diverse in the types of acyl substituents and does not contain acyloxyacyl residues. Galacturonic acid is linked to the 4' position of the nonreducing glucosaminosyl residue, instead of the phosphate on *E. coli* lipid A. The existence of structural differences between *R. leguminosarum* lipid A and lipid A from enteric bacteria poses the question of whether a distinctly different lipid A biosynthetic pathway exists in *Rhizobium* spp.

R. leguminosarum lipid A is quite heterogeneous with regard to the type of ester and amide-linked β -hydroxy fatty acyl groups (Fig. 1). In addition to 3-hydroxymyristate (3-OH-C_{14:0}), these acyl groups can consist of 3-OH-C_{15:0}, 3-OH-C_{16:0}, or 3-OH-C_{18:0} (6). In contrast, the lipid A of *E. coli* contains only 3-OH-C_{14:0} as the fatty acid ester or amide linked to the glucosamine residues. Thus, it is expected that the rhizobial O- and N-acyltransferases will have a broader substrate specificity than do those respective enzymes from *E. coli*, and it is predicted that structural analogs of UDP-2,3-diacyl-GlcN and lipid X with 3-OH-C_{15:0}, 3-OH-C_{16:0}, and 3-OH-C_{18:0} as the fatty acyl residues will also be found in *R. leguminosarum*. With respect to the heterogeneity in *Rhizobium* lipid A acylation, it is interesting to note that the *E. coli* N-acyltransferase, but not the O-acyltransferase, is potentially capable of utilizing R-3-

FIG. 7. Demonstration of 4'-kinase activity in extracts of *E. coli* and *R. leguminosarum*. Complete assay mixtures (20 μ l total volume) contained [32 P] tetraacyldisaccharide-1-P (1.6×10^4 cpm/nmol, 250 μ M), cardiolipin (2 mg/ml), 50 mM HEPES buffer (pH 7.4), Nonidet P-40 (1%), MgCl_2 (10 mM), ATP (10 mM), and crude bacterial extract (1 mg/ml). Reactions were started by the addition of the bacterial extract, incubated at 37°C, and terminated by spotting onto silica gel 60 TLC plates, which were developed with chloroform-pyridine-88% formic acid-water (30:70:16:5 [vol/vol]). Radiolabeled spots were visualized by overnight autoradiography as described in the legend to Fig. 3. Lanes: 1, enzyme-free control; 2, *E. coli* control without MgATP after 2 h of incubation; 3 and 4, complete *E. coli* assays after 20 min and 2 h, respectively; 5, *R. leguminosarum* 8401 control without MgATP after 2 h; 6 and 7, complete *R. leguminosarum* 8401 assays after 20 min and 2 h, respectively; 8, *R. leguminosarum* CE3 control without MgATP after 2 h; 9 and 10, complete *R. leguminosarum* CE3 assays after 20 min and 2 h, respectively.

hydroxy- $\text{C}_{12:0}$ -ACP as a substrate (43). In many other gram-negative bacteria the *N*-acyltransferase also has a broader substrate specificity than does the *O*-acyltransferase (43).

The presence of the unusual, very long chain fatty acid 27-hydroxy- $\text{C}_{28:0}$ in rhizobial lipid A suggests the existence of a unique acyltransferase for the attachment of this component. This fatty acid is exclusively ester linked, and a portion contains a β -hydroxybutyryl group esterified to the 27-hydroxyl group. It is possible that this fatty acyl substituent is added in later biosynthetic steps after the synthesis of Kdo₂-lipid IV_A. It will be necessary to prepare possible substrates, such as 27-OH- $\text{C}_{28:0}$ -ACP, or 27-OH- $\text{C}_{28:0}$ -CoA, to examine the stage at which this fatty acyl group is added to *Rhizobium* lipid A.

The addition of the 4' phosphate group to tetraacyldisaccharide-1-P occurred in the presence of ATP with both *E. coli* and *R. leguminosarum* extracts. The formation of the tetraacyldisaccharide-1,4'-bis-P (lipid IV_A) was unexpected in view of the fact that mature lipid A of *R. leguminosarum* is not phosphorylated and has a GalA residue attached at the 4' position. However, in *E. coli* the 4' phosphate is essential for recognition of lipid IV_A as an acceptable substrate for the Kdo transferase (5, 7). Since the enzymes in the *R. leguminosarum* and *E. coli* lipid A biosynthetic pathways are conserved, it is likely that the rhizobial Kdo transferase requires the 4'-phosphorylated substrate for recognition, even if this phosphate group is removed later in the pathway.

The Kdo transferase of *R. leguminosarum* was found to utilize CMP-Kdo to transfer two Kdo residues to lipid IV_A. This also showed that the rhizobial enzyme is functionally more similar to *E. coli* Kdo transferase than that from *Chla-*

mydia species, which transfers at least three Kdo residues (4). In *E. coli* both Kdo residues are added by a single transferase (13), although the intermediate, Kdo₁-lipid IV_A, is difficult to detect in either the *E. coli* or the *R. leguminosarum* enzyme assays under the conditions employed. It is probable that the addition of the second Kdo residue is more efficient than that of the first, leading to rapid metabolism of Kdo₁-lipid IV_A to Kdo₂-lipid IV_A. This has been shown to be the case in vitro with the *E. coli* Kdo transferase (5).

In addition to peptidoglycan and lipid A synthesis, flavonoid-induced rhizobia also utilize UDP-GlcNAc as a precursor of Nod factors, secreted N-acylated oligosaccharide signals involved in the *Rhizobium*-legume symbiosis (15, 40). These molecules are modified chitin tetra- or pentasaccharides, in which the terminal glucosamine residue is N acylated. Nod factor biosynthesis is determined by the nodulation (*nod*) genes and also proceeds via a de-N-acetylation and presumed re-N-acylation of the terminal *N*-acetylglucosamine residue. The *E. coli* lipid A 3-*O*-acyltransferase (*lpxA*), deacetylase (*lpxC*), and *N*-acyltransferase (*lpxD*) genes have been identified and characterized (33), allowing sequence comparison with the rhizobial *nod* genes. No significant sequence homology was found between *E. coli lpx* genes and rhizobial *nod* genes (data not shown). More specifically, no homology was found between *lpxC* and *nodB*, the latter of which is known to encode the Nod factor de-N-acetylase (21), or between *lpxD* and *nodA*, which has been suggested to encode the Nod factor *N*-acyltransferase (40). This suggests that *Rhizobium* spp. have evolved separate deacetylases and *N*-acyltransferases for the biosynthesis of lipid A and Nod factors.

FIG. 8. Kdo transferase activity in extracts of *E. coli* and *R. leguminosarum*. Kdo transferase assay mixtures contained $[4'\text{-}^{32}\text{P}]\text{lipid IV}_A$ (6.44×10^5 cpm/nmol, 100 μM), 50 mM Tris-HCl buffer (pH 7.5), MgCl_2 (10 mM), and Triton X-100 (0.2%), plus an in situ CMP-Kdo generating system consisting of CTP (5 mM), Kdo (2 mM), and CMP-Kdo synthase (130 mU/ml). Bacterial extracts were added at time zero. Incubations (30°C) were for 30 min, after which time portions (5 μl) were spotted onto silica gel 60 plates. Chromatography and autoradiography are as described in the legend to Fig. 7. Lanes: 1, crude extract from *E. coli* R447 (2 mg/ml); 2, crude extract from *R. leguminosarum* CE3 (2 mg/ml); 3, no enzyme control.

In summary, we have found that the early lipid A biosynthetic steps of *R. leguminosarum* and *E. coli* are highly conserved, even though the mature lipid A's from *R. leguminosarum* and *E. coli* display major structural differences. That the pathway is conserved between such genetically diverse organisms is an indication of its central importance for the viability of gram-negative bacteria. Mutations of genes involved in the early pathway in *E. coli* are all lethal (33), the minimal requirement for viability probably being formation of Re endotoxin (32, 38). The central role of the Kdo addition is accentuated by the fact that in *E. coli* Kdo₂-lipid IV_A can be derivatized with heptose prior to acyloxyacyl addition (38). Conservation of biosynthetic enzymes leading to Kdo₂-lipid IV_A may therefore be a prerequisite for growth of most gram-negative bacteria, and enzymatic steps leading to diverse lipid A moieties probably occur after Kdo attachment. From the structural analysis (5a), we can anticipate that in *R. leguminosarum* these later steps may include 1-phosphatase and 4'-phosphatase, GalA 4' transferase, and enzymes involved in the oxidation of the reducing glucosamine residue to 2-deoxy-2-aminogluconic acid. The latter step may be catalyzed by an enzyme analogous to glucose oxidase or by a two-step mechanism involving the concerted action of a glucosamine dehydrogenase and lactonase. We also expect that *R. leguminosarum* has unique enzymes for the synthesis and transfer of 27-OH-C_{28:0} but probably does not possess the late acyltransferases for the generation of acyloxyacyl substituents on lipid A.

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