Cloning and Overexpression of Glycosyltransferases That Generate the Lipopolysaccharide Core of *Rhizobium leguminosarum*

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The lipopolysaccharide (LPS) core of the Gram-negative bacterium *Rhizobium leguminosarum* is more amenable to enzymatic study than that of *Escherichia coli* because much of it is synthesized from readily available sugar nucleotides. The inner portion of the *R. leguminosarum* core contains mannose, galactose, and three 3-deoxy-D-manno-octulosonate (Kdo) residues, arranged in the order: lipid A-(Kdo)_2-Man-Gal-Kdo-[O antigen]. A mannosyltransferase that uses GDP-mannose and the conserved precursor Kdo_2-[4^32P]lipid IV_A (Kadrmas, J. L., Brozek, K. A., and Raetz, C. R. H. (1996) J. Biol. Chem. 271, 32119–32125) is proposed to represent a key early enzyme in *R. leguminosarum* core assembly. Conditions for demonstrating efficient galactosyl- and distal Kdo-transferase activities are now described using a coupled assay system that starts with GDP-mannose and Kdo_2-[4^32P]lipid IV_A. As predicted, mannose incorporation precedes galactose addition, which in turn precedes distal Kdo transfer. LPS core mutants with Tn5 insertions in the genes encoding the putative galactosyltransferase (lpcA) and the distal Kdo-transferase (lpcB) are shown to be defective in the corresponding *in vitro* glycosylation of Kdo_2-[4^32P]lipid IV_A. We have also discovered the new gene (lpcC) that encodes the mannosyltransferase. The gene is separated by several kilobase pairs from the lpcAB cluster. All three glycosyltransferases are carried on cosmid pJJ1848, which contains at least 20 kilobase pairs of *R. leguminosarum* DNA. Transfer of pJJ1848 into *R. meliloti* 1021 results in heterologous expression of all three enzymes, which are not normally present in strain 1021. Expression of the lpc genes individually behind the T7 promoter results in the production of each *R. leguminosarum* glycosyltransferase in *E. coli* membranes in a catalytically active form, demonstrating that lpcA, lpcB, and lpcC are structural genes.

Lipopolysaccharide (LPS)\(^1\) of Gram-negative bacteria is composed of lipid A (the hydrophobic membrane anchor), the core region (a non-repeating oligosaccharide), and O-antigen (a distal repeating oligosaccharide) (1–4). The O-antigen and much of the core are not required for growth (2, 5–7) under laboratory conditions, but mutants lacking portions of the core, especially the inner core, possess several interesting phenotypes. Inner core mutants often grow more slowly than wild type cells, are hypersensitive to certain antibiotics and display a compromised barrier to hydrophobic compounds (2, 5–7). In addition, the assembly of some outer membrane proteins, such as OmpF and OmpC, is altered in these mutants (8–10). In nitrogen-fixing Gram-negative bacteria, like the Rhizobiaceae, the core region may influence plant host specificity and may function in signaling pathways leading to the formation of root nodules within the host plant (11–13). For instance, in *Rhizobium leguminosarum*, core mutants are able to recognize their plant hosts and form nodules, but these nodules either do not fix nitrogen or do so at greatly reduced rates (14–16).

There is remarkable diversity of LPS core structures in different species of Gram-negative bacteria. The structure of the *Escherichia coli* K-12 core region (Fig. 1) is one of the best characterized (2). Nearly all of the genes required for the biosynthesis of the *E. coli* core have been identified (2, 4, 17). However, because the inner *E. coli* core contains the unusual sugar, L-glycero-d-manno-heptose, the activated nucleotide form of which is not fully characterized, the reactions catalyzed by the enzymes of *E. coli* core biosynthesis have not been studied in depth (2, 18, 19). The core structure of *R. leguminosarum* LPS, as partially displayed in Fig. 1, has been proposed by Carlson and co-workers (20–22). It contains Kdo, mannose, galactose, and galacturonic acid, but lacks heptose. The enzymeology of core assembly in *R. leguminosarum* is more amenable to study than in *E. coli*, given that all the relevant sugar nucleotides are available.

In accordance with Carlson’s structure, we have been able to identify three novel glycosyltransferases unique to extracts of *R. leguminosarum* that incorporate mannose (23), galactose (23), and the outer Kdo (present study) in the expected order (Fig. 2) to the conserved lipid A precursor, Kdo_2-lipid IV_A. We now describe the three structural genes of *R. leguminosarum* that encode these glycosyltransferases. Two of the genes, lpcA and lpcB, adjacent to each other on the chromosome, were reported previously (24, 25), based on mutants with truncated core LPS structures. lpcA was partially sequenced and was proposed to encode the galactosyltransferase because of its homology to other sugar transferases and chemical characterization of LPS isolated from an lpcA::Tn5 insertion mutant (24–26). lpcB was sequenced entirely, and although it showed no homology to any known gene, it was proposed to encode the distal Kdo-transferase based upon the absence of the distal Kdo

\(^{1}\) The abbreviations used are: LPS, lipopolysaccharide; kb, kilobase pair(s); ORF, open reading frame; PCR, polymerase chain reaction; Kdo, 3-deoxy-D-manno-octulosonate.
in the LPS core isolated from an lpcB transposon insertion mutant (24). We now demonstrate by means of our enzyme assays that lpcA does indeed encode the galactosyltransferase and that lpcB encodes the distal Kdo-transferase. In addition, we report a new gene, designated lpcC, encoding the mannosyltransferase, and describe a mutant lacking mannosyltransferase activity. lpcC is located several kb downstream of lpcA and lpcB on the chromosome (Fig. 3). All three Rhizobium genes have been overexpressed using an E. coli T7 promoter-driven system. The recombiant enzymes are catalytically active. The availability of the lpc genes should facilitate the re-engineering of LPS core structures in both E. coli and Rhizobium. The biological significance of core structural diversity in pathogenesis and symbiosis might be revealed using this approach.

**EXPERIMENTAL PROCEDURES**

**Materials and Bacterial Strains**—The following materials and kits were purchased: [γ-32P]ATP (NEN Life Science Products); Heps, GDP-mannose, UDP-galactose, Kdo, and CTP (Sigma); Triton X-100 and Qiaex II gel extraction kit reagents (Stratagene); restriction enzymes (New England Biolabs); [1-14C]mannose, UDP-galactose, Kdo, and CTP (Sigma); Triton X-100 and Qiaex II gel extraction kit reagents (Stratagene); restriction enzymes (New England Biolabs); phosphorimager (Molecular Dynamics); ImageQuant software. 

**FIG. 1.** Partial structures of the E. coli K-12 and the R. leguminosarum core oligosaccharides. The two Kdo residues closest to lipid A, their linkages to each other, and the α-1–5 linkage of the second sugar attached to the inner Kdo are conserved (2, 21, 22). Dashed lines represent partial substituents. Not all details of the proposed structures are shown (2, 21, 22).

**FIG. 2.** Proposed reactions for the biosynthesis of the R. leguminosarum core with the conserved precursor Kdo2-lipid IVα as the acceptor. The presence of the enzymes capable of generating Kdo2-lipid IVα in extracts of R. leguminosarum has been reported (42, 43). The mannosyltransferase, LpcC, is proposed to form the α-1–5 linkage between mannose and the inner Kdo. Next, the galactosyltransferase (LpcA) appears to generate the α-1–6 linkage between galactose and mannose. Finally, the distal Kdo, which is attached to galactose via an α-2–6 linkage, is added by LpcB, a novel kind of Kdo-transferase. In cell extracts, the product of each reaction serves as the substrate for the next. The linkages shown are those reported for the core oligosaccharide isolated from cells of R. leguminosarum etli (21, 22). The actual linkages formed enzymatically in vitro with Kdo2-lipid IVα as the acceptor have not yet been confirmed.

**Preparation of Radiolabeled Substrates**—The 4'-32P]lipid IVα was generated from [γ-32P]ATP and the tetra-acylated disaccharide 1-phosphate precursor, using the E. coli 4'-kinase from membranes of strain BLR(DE3)pLysS/pJK2 (29). The labeled lipid IVα was converted to Kdo2-[4'-32P]lipid IVα using purified E. coli Kdo-transferase (30, 31). The products were purified by preparative thin layer chromatography, and stored at −20 °C as an aqueous dispersion (30, 31). Prior to each use, these substrates were subjected to ultrasonic irradiation in a water bath for 60 s.

**Assay Conditions**—For mannosyltransferase reactions, unless indicated, the standard reaction mixtures (10–40 μl) contained 50 μM Heps, pH 7.5, 0.1% Triton X-100, 10 μM Kdo2-[4'-32P]lipid IVα at 6,000 × g for 15 min. For each liter of late log phase culture (A600 = 1), cell pellets were resuspended in 10 ml of 50 mM Heps, pH 7.5. The cells were broken by passage through a French pressure cell at 18,000 p.s.i., yielding a protein concentration of approximately 10 mg/ml. Cellular debris was removed by centrifugation at 6,000 × g for 15 min. Washed membranes were prepared by a series of two ultracentrifugations at 100,000 × g for 60 min. The membrane pellet was resuspended in a minimal volume (1–2 ml) of 50 mM Heps, pH 7.5. The protein concentrations of the extracts, membranes and cytosol were determined by the bicinchoninic acid assay (28) using bovine serum albumin as the standard.
80,000 cpm/nmol, and 1.0 mM GDP-mannose. The enzyme source, added last to initiate the reaction, was generally 0.3 mg/ml washed *Rhizobium* membranes. Reactions were incubated at 30 °C for 60 min, unless specified. Galactosyltransferase reactions were identical but also included 1.0 mM UDP-galactose in addition to the above components. Distal Kdo-transferase assays contained all the galactosyltransferase reaction components plus 2 mM Kdo, 5 mM CTP, 10 mM MgCl₂, and 1.8 milliunits of partially purified CMP-Kdo synthase per 10 μl. CMP-Kdo is generated in situ because of its short half-life (minutes) (30).

When assaying for the *R. leguminosarum* enzymes expressed in the *E. coli* T7 system, slightly different conditions were used. The mannosyltransferase assay was the same as above except that 0.2 mg/ml washed *E. coli* BLR(DE3)pLysS/pJK6 membranes were used as the enzyme source. To assay the *E. coli* galactosyltransferase construct (pJK7), mannosyl-Kdo₀₋Ⅳ₉ was first generated in a standard 60-min mannosyltransferase reaction utilizing 0.3 mg/ml washed *Rhizobium meliloti* 1021/pIJ1848 (Table I) membranes. Residual *R. meliloti* 1021/pIJ1848 activity was then destroyed by a 20-min incubation at 65 °C. To this reaction mixture, 1.0 mM UDP-galactose and 0.2 mg/ml *E. coli* BLR(DE3)pLysS/pJK7 washed membranes were added. Reactions were then incubated for 30 min at 30 °C. To assay the *E. coli* distal Kdo-transferase construct (pJK5), galactosyl-mannosyl-Kdo 2-IVA was first generated in a standard 60-min galactosyltransferase reaction utilizing 0.3 mg/ml washed *R. meliloti* 1021/pIJ1848 membranes. Residual *R. meliloti* 1021/pIJ1848 activity was then destroyed by a 20-min incubation at 65 °C. To this reaction mixture, 1.0 mM UDP-galactose and 0.2 mg/ml *E. coli* BLR(DE3)pLysS/pJK7 washed membranes were added. Reactions were then incubated for 30 min at 30 °C. Analysis of the Reaction Products by Thin Layer Chromatography—

Reactions were stopped by spotting 5-μl portions of the reaction mixtures onto a silica gel 60 thin layer chromatography plate. After drying in a stream of cold air, plates were developed in the solvent chloroform/pyridine/88% formic acid/water (30:70:16:10, v/v). The amount of product formed was calculated from the percent conversion of radioactive substrate (of known specific radioactivity) to product, quantified using a Molecular Dynamics PhosphorImager.

General Recombinant DNA Techniques—Plasmids were prepared using the Qiagen Spin Prep kit. Restriction endonucleases, shrimp alkaline phosphatase, and T4 DNA ligase were all used according to the manufacturer's instructions. Plasmid DNA was isolated from agarose gels using a Qiaex II gel extraction kit. All other techniques involving manipulation of nucleic acids were from Ausubel et al. (32).

Plasmids were introduced into strains of *Rhizobium* via triparental mating (33). *E. coli* strain 803 (34) or DH5α (35) served as the plasmid donor. *E. coli* strain MT616 (36) provided the transfer vector. The appropriate strain of *Rhizobium* (see below) served as the recipient.

Nucleotide Sequencing of the *lpc*-Region—Sequencing of a portion of the *lpc* gene is homologous to certain LPS core glycosyltransferases has previously been reported (24) (accession no. X94963). Full-length *lpcA* was required to demonstrate the enzymatic activity of LpcA. The 5' terminus of the gene was determined by cycle sequencing using custom-made Cy5-labeled primers to pRU68 (25), Thermo Sequenase, and the ALFExpress automated DNA sequencer.

Nucleotide Sequencing of the *lpc* Region—A restriction map of the 4.4-kb EcoRI fragment containing *dctA* has been constructed (37), and the nucleotide sequence of *dctA* determined (EMBL accession no. Z11529). The region downstream of *dctA* on the 4.4-kb fragment was sequenced on both strands using a combination of sub-cloned fragments and custom primers, and an Applied Biosystems model 373A autosequencer. This sequence can be found under accession no. AF050103.

Construction of RSKnH—To construct strain RSKnH, a kanamycin-resistance cassette was inserted into the HindIII site within the *lpc* open reading frame. The kanamycin cassette was cloned from pUC4KIXX (Amersham Pharmacia Biotech) as a SmalI fragment into pLC20H (38). It was then excised as a HindIII fragment and cloned into HindIII-digested pPN120 to give pRS5. pPN120 is a pLAFR1 derivative carrying the 4.4-kb EcoRI fragment that includes part of *dctB, dctA*, and 2000 base pairs downstream of *dctA* (37). pRS5 was transferred by triparental mating to *R. leguminosarum* strain 3855, and recombination of the kanamycin resistance gene into the genome was forced by introduction of the incompatible plasmid pPH1. Southern hybridizations of EcoRI and KpnI digests of genomic DNA probed with pPN108 (37) were used to confirm that the kanamycin cassette had recombined into the expected location.

Plant Assays—*Pisum sativum* seeds were surface-sterilized by washing with absolute alcohol, followed by soaking for 1 h in 12% sodium hypochlorite, followed by five washes with sterile water. The seeds were allowed to imbibe and then transferred to 550-ml jars containing a sterile moistened mix of fine vermiculite and pumice at a ratio of 3:1. The seeds were inoculated with 1 ml of a suspension of *Rhizobium* cells washed from a fresh GRDM plate (37). The pots were watered with nitrogen-free nutrient solution (37) and grown under controlled environmental conditions in a growth room at 20 °C day/15 °C night on a 12-h day/night cycle. The plant roots were examined for nodules after 3–6 weeks.

Placing *lpcA*, *lpcB*, and *lpcC* under T7 Promoter Control—The cloning of PCR generated *lpcA*, *lpcB*, and *lpcC* DNA into a vector under T7 promoter control is outlined in Fig. 4 (39–41). The forward primers were synthesized with a clamp region, an NdeI restriction site, and a match to the coding strand starting at the translation initiation site.
and Kdo to Kdo-2-[32P]lipid IVₐ in extracts of R. leguminosarum. Three band shifts, corresponding to the addition of mannose, galactose, and Kdo, respectively, to Kdo₂-2-[32P]lipid IVₐ are observed. The incubations were performed under standard conditions with the indicated sugar nucleotides, as described under "Experimental Procedures," and they contained 0.3 mg/ml washed membranes of strain 3841 as the enzyme source. After 60 min at 30 °C, 5-µl portions were spotted onto a silica TLC plate to stop the reactions. Following chromatography, the plate was analyzed with a PhosphorImager.

The reverse primer was synthesized with a clamp region, a BamHI restriction site, and a match to the conserved acceptor,

\begin{align*}
\text{Gal-Mann-Kdo-2-IV}_\text{A} & \quad \text{CMP-Kdo-generating system} \\
\text{GDP-mannose} & \quad + \quad + \quad + \quad + \quad + \quad + \quad + \quad + \\
\text{UDP-galactose} & \quad + \quad + \quad + \quad + \quad + \quad + \quad + \quad + \\
\end{align*}

FIG. 5. Sequential addition of mannose, galactose, and Kdo to Kdo₂-2-[32P]lipid IVₐ in extracts of R. leguminosarum. Three band shifts, corresponding to the addition of mannose, galactose, and Kdo, respectively, to Kdo₂-2-[32P]lipid IVₐ are observed. The incubations were performed under standard conditions with the indicated sugar nucleotides, as described under "Experimental Procedures," and they contained 0.3 mg/ml washed membranes of strain 3841 as the enzyme source. After 60 min at 30 °C, 5-µl portions were spotted onto a silica TLC plate to stop the reactions. Following chromatography, the plate was analyzed with a PhosphorImager.

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\text{UDP-galactose} & \quad + \quad + \quad + \quad + \quad + \quad + \quad + \quad + \\
\end{align*}

RESULTS

Sequential Addition of Mannose, Galactose, and Kdo to the Acceptor Kdo₂-lipid IVₐ in Extracts—Based upon the proposed core structure for R. leguminosarum (Fig. 1), we expected that the order of sugar addition in vitro to the conserved acceptor, Kdo₂-lipid IVₐ (Fig. 2) (42, 45), would be mannose, galactose, and Kdo. Membranes of wild type R. leguminosarum strain 3841 were found to catalyze all three glycosylations (Fig. 5), using the assay conditions previously optimized for the mannose-transferase (44). In these reactions, inclusion of GDP-mannose alone was sufficient to cause a differential shift of the Kdo₂-2-[32P]lipid IVₐ band (lane 3), indicative of mannose addition. Inclusion of UDP-galactose alone or of the CMP-Kdo-generating system alone did not cause any significant reactions to occur (lanes 4 and 5), as judged by the unchanged migration of the Kdo₂-2-[32P]lipid IVₐ. In the reactions shown in lanes 6 and 7, GDP-mannose was present to permit generation of mannol-Kdo₂-2-[32P]lipid IVₐ. In addition, these incubations contained either UDP-galactose (lane 6) or the CMP-Kdo-generating system (lane 7). Inclusion of UDP-galactose together with GDP-mannose caused a second more slowly migrating derivative of Kdo₂-2-[32P]lipid IVₐ to be formed (lane 6), but the CMP-Kdo-generating system by itself had no effect (lane 7). We conclude that galactose is incorporated after mannose, consistent with the proposed core structure, since mannosyl-Kdo₂-2-[32P]lipid IVₐ should be the acceptor for galactose. When GDP-mannose, UDP-galactose, and the CMP-Kdo-generating system were all included together in the same reaction mixture (lane 8), a third more slowly migrating derivative of Kdo₂-2-[32P]lipid IVₐ was produced, presumably reflecting the incorporation of the distal Kdo residue (Figs. 1 and 2). The coupled assay shown in Fig. 5 (lane 8) represents the first direct evidence for the incorporation of the distal Kdo residue in vitro. All of the reactions were dependent upon both the inclusion of the sugar nucleotide donors (lane 2) and the appropriate R. leguminosarum enzyme source (lane 1), in this case membranes of wild type strain 3841.

The Galactosyltransferase Is Encoded by lpcA—Several strains of R. leguminosarum were used in conjunction with the coupled galactosyltransferase activity assay to identify the galactosyltransferase gene (see Fig. 3 and Table I). Wild type VF39 was the parent used in the transposon mutagenesis to produce VF39–86 (16), which lacks galactose in its LPS core. This mutant contains a Tn5 insertion within a gene designated lpcA. VF39–86/pRU68 contains a wild type copy of lpcA in a broad host range vector, and galactose is restored in the LPS core region of VF39–86/pRU68. As shown in Fig. 6 (lanes 2–5), wild type VF39 membranes displayed normal mannosyl- and galactosyltransferase activities that are characteristic of R. leguminosarum. VF39–86 membranes efficiently transferred mannose (lane 7), but they were missing the galactosyltransferase (Fig. 6, lane 9). Wild type copies of lpcA present on the plasmid in VF39–86/pRU68 restored galactosyltransferase activity (Fig. 6, lane 13).

Complete Sequence of lpcA—The complete sequence of lpcA can be found under accession no. X94963. The full-length lpcA gene shows moderate homology to many bacterial glycosyltransferase genes, including some of those involved in the assembly of LPS cores (2, 4, 24). For instance, the lpcA gene displays 30%, 27%, and 26% identity, respectively, to lgtC of Neisseria gonorrhoeae (45), to ipa-12d of Bacillus subtilis (46), and to rfaJ (waaJ) (2, 4) of E. coli. LgtC and RfaJ are known to be galactosyl- and glucosyltransferases.

In previous work (24), the initiation codon was proposed to be an ATG that now appears to be 54 nucleotides downstream of the actual start site. The product expressed from this ATG codon is non-functional (see below). Since there are no other in-frame ATG sites prior to a stop codon further upstream, a GTG start codon was considered as an alternative. Two such GTG sites are present in the DNA sequence. A primer was designed to the most upstream of these GTG codons, resulting in a construct expressing functional galactosyltransferase (see below).

The Distal Kdo-transferase Is Encoded by lpcB—Several different strains of R. leguminosarum (Fig. 3 and Table I) were used in conjunction with band shift assays to confirm the identification of the structural gene encoding the distal Kdo-transferase. Wild type strain 3841 was the parent used in the transposon mutagenesis to produce strain RU301 (25), which contains a Tn5 insertion within the gene designated lpcB. Strain RU301/pRU74 contains a wild type copy of lpcB in a broad host range vector compatible with expression in Rhizobium. Wild type strain 3841 membranes displayed distal Kdo-trans-
ferase activity, as shown in Fig. 7 (lane 2, bottom band). Membranes of strain RU301 transferred mannose and galactose to Kdo2-[γ-32P]lipid IV A but were unable to catalyze the third band shift corresponding to the incorporation of Kdo (Fig. 7, lane 3). The wild type copy of lpcB present on the plasmid in RU301/pRU74 restored the ability to add the distal Kdo (Fig. 7, lane 7).

Cosmid pIJ1848 Contains All Three Core Glycosyltransferases—Rhizobium meliloti may contain LPS with a different core than that found in R. leguminosarum. Whatever the structure, membranes from wild type R. meliloti strain 1021 were tested in the glycosyltransferase assays optimized for R. leguminosarum. As seen in Fig. 8 (lanes 1–4), such membranes possessed very little activity, as judged by their inability to shift the migration of the acceptor, Kdo2-[γ-32P]lipid IV A, in the presence of GDP-mannose, UDP-galactose, and CMP-Kdo.

FIG. 6. The lpcA gene encodes the galactosyltransferase. These reactions were performed under standard conditions and contained the indicated sugar nucleotides. Washed membranes of the indicated strains were added to the reactions at 0.3 mg/ml. The mixtures were incubated at 30 °C for 1 h, and 5-μl portions were spotted onto a silica TLC plate to stop the reactions. The plate was analyzed as in Fig. 5.

FIG. 7. The lpcB gene encodes the distal Kdo-transferase. These reactions were performed under standard conditions and contained the indicated sugar nucleotides or the CMP-Kdo-generating system. Washed membranes of the indicated strains were added to the reactions at 0.3 mg/ml. The mixtures were incubated at 30 °C for 1 h, and 5-μl portions were spotted onto a silica TLC plate to stop the reactions. The plate was analyzed as in Fig. 5.

dicarboxylic acid transport (det). When membranes of R. meliloti 1021/pIJ1848 were assayed for the three core glycosyltransferases of R. leguminosarum, high levels of mannosyltransferase were detected (Fig. 8, lane 6). In addition, the galactosyl- and the distal Kdo-transferases, encoded by lpcA and lpcB respectively, were present (Fig. 8, lanes 7 and 8). These results indicate that the gene encoding the mannosyltransferase may also be located on pIJ1848.

The Mannosyltransferase Gene Maps on the Opposite Side of the dctABD Cluster to lpcA/B—To define the locus of the mannosyltransferase gene more precisely, mannosyltransferase assays were performed on four cosmids expressed in R. meliloti 1021 that partially overlap with pIJ1848. These cosmids (pRU3000, pRU3001, pRU3020, and pRU3022) are described in Table I.

### Table I: Bacterial strains and plasmids

| Strain or Plasmid | Description | Source or Ref. |
|-------------------|-------------|---------------|
| Strain           |             |               |
| 1021             | Wild type R. meliloti | Sharon Long |
| VF39             | Wild type R. leguminosarum bv. viciae | (16) |
| VF39–86          | Strain VF39 lpcA::Tn5 | (16) |
| 3841             | SmK derivative of wild type R. leguminosarum strain 300 bv. viciae | (55) |
| RU301            | Strain 3841 lpcB::Tn5 | (25) |
| 3855             | SmK derivative of wild type R. leguminosarum strain 128C53 bv. viciae | (56) |
| RSKaH            | Strain 3855 lpcC::nptII | This work |
| 803              | E. coli donor strain for triparental mating | (34) |
| DH50             | E. coli donor strain for triparental mating | (35) |
| MT616            | E. coli helper strain for triparental mating; contains pRK600, a ColE1 replicon with RK2 tra genes, SmR | (36) |
| Plasmid          |             |               |
| pKJ1848          | Cosmid from strain 8002; dctABD, lpcABC | (25) |
| pRK415           | Broad host range P-group cloning vector; TcR | (57) |
| pRU68            | pK415 plus 3.8-kb EcoRI/HindIII fragment from pIJ1848 | (25) |
| pRU74            | pK415 plus 2.4-kb EcoRI/PstI fragment from pIJ1848 | (25) |
| pRU3000          | Cosmid from strain 3841 containing dctA, lpcC | (25) |
| pRU3001          | Cosmid from strain 3841 containing dctABD, lpcC | (25) |
| pRU3020          | Cosmid from strain 3841 containing dctABD, lpcABC | (25) |
| pRU3022          | Cosmid from strain 3841 containing lpcAB | (25) |
Croscopic examination of the nodules formed by RSKnH showed with larger pink nodules formed by strain 3855. Electron microscopy of the nodules formed by RSKnH showed the presence of enlarged infection threads with some bacterial release, but no evidence of bacteroid formation (data not shown). Similar observations have been made with other lps mutants of R. leguminosarum (47).

The Mannosyltransferase Is Encoded by lpcC—Selected strains of R. leguminosarum were used in conjunction with mannosyltransferase activity assays to confirm the identity of the functional gene. Wild type 3855 (see Table 1) is the parent of RSKnH, which contains a kanamycin cassette inserted within the lpcC gene in an internal HindIII site. As shown in Fig. 10, wild type 3855 membranes displayed normal mannolysyltransferase activity, whereas membranes of strain RSKnH completely lacked the mannolysyltransferase. This strongly supports the view that the lpcC gene product is the mannolysyltransferase.

T7 Expression Cloning of lpcA, lpcB, and lpcC—Unequivocal demonstration that the lpcC, lpcA, and lpcB genes encode the mannolysyl-, galactosyl-, and distal Kdo-transferases, respectively, is provided by heterologous expression of these genes. E. coli is unable to catalyze these core glycosyltransferase reactions, which are characteristic of extracts of R. leguminosarum (33). Accordingly, E. coli membranes prepared from strains containing the vector pET23a alone did not catalyze efficient GDP-mannose, UDP-galactose, or CMP-Kdo-dependent band shifts of Kdo₂-[4'-32P]lipid IV₂ (or related glycolipids) in our assays (Fig. 11, lanes 2, 6, and 10).

The three lpc genes were cloned individually into an E. coli T7 expression system, as described under “Experimental Procedures.” R. leguminosarum DNA containing each of the lpc open reading frames and a minimal amount of flanking sequence were generated by PCR, and were ligated into the T7 expression vector pET23a. The resulting constructs, pJK5, pJK6, and pJK7, containing lpcA, lpcC, and lpcA respectively, were transformed into the E. coli strain BLR(DE3)pLysS. As shown in Fig. 11, mannolysyltransferase activity was not observed in the absence of an enzyme source (lane 1), in membranes of BLR(DE3)pLysS containing the vector pET23a alone (lane 2), or in the absence of GDP-mannose (lane 3). However, the plasmid pJK6 conferred upon E. coli membranes the ability to catalyze efficient GDP-mannose-dependent transfer of mannose to Kdo₂-[4'-32P]lipid IV₂ (lane 4). In lanes 5–8, mannolysyl-Kdo₂-[4'-32P]lipid IV₂ (prepared enzymatically) was employed as the acceptor substrate for detecting the galactosyltransferase. As anticipated, galactose transfer required an enzyme source (lane 5), and it did not take place in membranes of BLR(DE3)pLysS containing the vector alone (lane 6) or in the absence of UDP-galactose (lane 7). However, the plasmid pJK7 imparted UDP-galactose-dependent galactosyltransferase activity to these E. coli membranes (lane 8) when mannolysyl-Kdo₂-[4'-32P]lipid IV₂ was the acceptor. Finally, in lanes 9–12, galactosyl-mannosyl-Kdo₂-[4'-32P]lipid IV₂ (generated release, but no evidence of bacteroid formation (data not shown). Similar observations have been made with other lps mutants of R. leguminosarum (47).

The Mannosyltransferase Is Encoded by lpcC—Selected strains of R. leguminosarum were used in conjunction with mannosyltransferase activity assays to confirm the identity of the functional gene. Wild type 3855 (see Table 1) is the parent of RSKnH, which contains a kanamycin cassette inserted within the lpcC gene in an internal HindIII site. As shown in Fig. 10, wild type 3855 membranes displayed normal mannolysyltransferase activity, whereas membranes of strain RSKnH completely lacked the mannolysyltransferase. This strongly supports the view that the lpcC gene product is the mannolysyltransferase.

T7 Expression Cloning of lpcA, lpcB, and lpcC—Unequivocal demonstration that the lpcC, lpcA, and lpcB genes encode the mannolysyl-, galactosyl-, and distal Kdo-transferases, respectively, is provided by heterologous expression of these genes. E. coli is unable to catalyze these core glycosyltransferase reactions, which are characteristic of extracts of R. leguminosarum (33). Accordingly, E. coli membranes prepared from strains containing the vector pET23a alone did not catalyze efficient GDP-mannose, UDP-galactose, or CMP-Kdo-dependent band shifts of Kdo₂-[4'-32P]lipid IV₂ (or related glycolipids) in our assays (Fig. 11, lanes 2, 6, and 10).

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**TABLE II**

| Gene   | Organism                   | Product size | Function             | Homologya | Accession no. |
|--------|-----------------------------|--------------|----------------------|-----------|---------------|
| waaK   | *Neisseria meningitidis*    | 354          | N-acetylglucosamine  | 24/43/265 | U58765        |
| dng0bpH| *Bordetella pertussis*      | 390          | Glycosyltransferase  | 27/45/185 | X90711        |
| capM   | *Staphylococcus aureus*     | 380          | Capsule synthesis    | 22/45/183 | U10927        |
| ubaU   | *Salmonella enterica*       | 353          | Mannosyltransferase  | 41/46/77  | X56793        |

**DISCUSSION**

Although the structures of the lipid A and core domains of *R. leguminosarum* LPS differ substantially from those of *E. coli* LPS, the first seven reactions of lipid A biosynthesis, leading to the conserved intermediate Kdo2-lipid IV<sub>A</sub> (Fig. 2), are identical (42, 43). In both systems, Kdo2-lipid IV<sub>A</sub> can be further acylated (2, 48–50), but in *R. leguminosarum*, Kdo2-lipid IV<sub>A</sub> can also be dephosphorylated at the 1- and 4'-positions to generate an unusual lipid A moiety lacking phosphate (23, 43). In both systems, Kdo2-lipid IV<sub>A</sub> can also serve as an acceptor of several distinct core sugars, which are transferred one at a time from sugar nucleotide donors (18, 19, 23, 44). A disadvantage of the *E. coli* core is the presence of 1-glycero-D-mannoheptose (Fig. 1), the activated sugar nucleotide form of which is not fully characterized (2, 18, 19, 44). Consequently, the enzymology of core glycosylation is more amenable to study in extracts of *R. leguminosarum* than of *E. coli*.

The assays described in the present work show that mannose, galactose, and Kdo can be transferred sequentially to Kdo2-lipid IV<sub>A</sub> in extracts of *R. leguminosarum* (Fig. 2). The order of sugar transfer is consistent with the core structure of *R. leguminosarum* LPS proposed by Carlson et al. (Fig. 1) (20–22). Efficient incorporation of these sugars is dependent upon both the inclusion of *R. leguminosarum* membranes and the appropriate sugar nucleotides. In the case of CMP-Kdo, a generating system must be used because of the short half-life of this compound (30, 31, 51, 52).

**FIG. 10.** The *lpc* gene encodes the mannosyltransferase. The reactions contained the standard components and 1 mM GDP-mannose. Washed membranes of the indicated strains were added at 0.3 mg/ml, and the mixtures were incubated at 30 °C for various times. Finally, 5-μl portions were spotted onto a silica TLC plate to stop the reactions. The plate was analyzed as in Fig. 5.

**FIG. 11.** Expression of *lpcA*, *lpcB*, and *lpcC* behind a T7 promoter in *E. coli*. These *E. coli* assays were performed as detailed under "Experimental Procedures." Using *R. meliloti* 1021/pIJ1848 washed membranes, Man-Kdo2-lipid IV<sub>A</sub> was generated as the acceptor substrate for reactions 5–8 and Gal-Man-Kdo2-lipid IV<sub>A</sub> was generated as the acceptor substrate for reactions 9–12. Lanes 1, 5, and 9 contain no acceptor substrate. Lanes 2, 6, and 10 contain 0.2 mg/ml BLR(DE3)pLysS/pET23a membranes. Lanes 3 and 4 contain 0.2 mg/ml BLR(DE3)pLysS/pJK6(*lpcA*) membranes; lanes 7 and 8, 0.2 mg/ml BLR(DE3)pLysS/pJK7(*lpcC*) membranes; and lanes 11 and 12, 0.2 mg/ml BLR(DE3)pLysS/pJK5(*lpcB*) membranes. Each reaction was incubated 30 min at 30 °C before spotting on a TLC plate to stop the reactions. The plate was analyzed as in Fig. 5. The small amount of apparent Kdo transfer seen in lane 10 (vector control) may represent a minor or alternative reaction catalyzed by chromosomal *E. coli* KdA.
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activities in crude extracts of wild type, mutant, or overexpressing strains, quantification of the specific activities of the galactosyl- and the Kdo-transferases is not yet feasible. However, the functional overexpression of these genes using the T7 promoter-driven system (Fig. 11) should facilitate purification of these proteins. With the pure glycosyltransferases, milligram quantities of each intermediate should be accessible, and quantitative assays could then be developed. In addition, the glycosidic linkages in the various products generated from Kdo₂-lipid IV₅₆ in vitro could be verified. These substances could be further used as substrates with which to probe for additional enzymes of R. leguminosarum LPS assembly, such as the putative galacturonyltransferases (Fig. 1) or even the O-antigen ligase. To date, however, attempts to incorporate a galacturonic acid moiety into Kdo₂-lipid IV₅₆ or mannose-Kdo₂-lipid IV₅₆ using UDP-galacturonic acid as the donor, have been unsuccessful.

LpcA and LpcC are members of large families of glycosyltransferases, as judged by Gapped BLAST sequence analysis (53). These proteins are about 350 amino acid residues long, but the homologies are seen only within the last ~270 residues. LpcA shows homology to bacterial and eucaryotic enzymes that function as galactosyl- or glucosyltransferases, consistent with the role of LpcA in R. leguminosarum. LpcC displays homology to more diverse bacterial, archaeal, and eucaryotic sequences. The functions of very few of these LpcC homologues have been studied directly with in vitro enzyme assays. Many proteins with homology to LpcC are not believed to be mannosyltransferases, but are proposed to be GlcNac or galactosyltransferases (Table II). The results of Figs. 5 and 6 show that LpcC is not an efficient galactosyltransferase when Kdo₂-lipid IV₅₆ is the acceptor, although minimal band shifts are occasionally observed in some strains with UDP-galactose as the donor (Fig. 6, lanes 4 and 8). LpcC shows no activity whatsoever with UDP-GlcNac as the sugar donor, despite the fact that the waaK(furA) gene displays very significant homology in a BLAST search (Table II). If we had not demonstrated the biochemical function of LpcC as a mannosyltransferase, one might have concluded (based on genomic sequence analysis) that LpcC is a GlcNac or a galactosyltransferase. The biochemical functions of the many putative glycosyltransferases that have recently been uncovered by genome sequencing need to be studied with targeted mutations and in vitro assays before the assignments of their biochemical functions are viewed as fully established.

An unexpected feature of LpcC is the fact that it displays no sequence similarity to WaaC(WaaC) of E. coli. This is surprising given that in vitro both LpcC and WaaC are thought to transfer mannoside to the same position on the acceptor, Kdo₂-lipid IV₅₆ (19, 44). Although E. coli WaaC cannot use GDP-mannose as the sugar donor, both WaaC and LpcC can employ the analog ADP-mannose as the donor substrate (19, 44). Consequently, it will be very important to validate the structures of the mannosyl-Kdo₂-lipid IV₅₆ products that are generated in vitro by LpcC and WaaC. Assuming that the products are indeed the same, a comparison of the protein x-ray structures of LpcC and WaaC might be very interesting. Unfortunately, there are no structures of any of the members of the LpcA and LpcC families. Very little is known about the structural biology of glycosyltransferases in general, since most of them, like the lpc gene products, are membrane-bound.

In contrast to LpcA and LpcC, the distal Kdo-transferase LpcB has no homologues in any of the current data bases. Other known Kdo-transferases, such as those that add one, two, or three Kdo residues to lipid A precursors (51, 52, 54), do not even display limited similarity. Further studies of the three-dimensional structure and substrate specificity of LpcB may reveal the significance of its unique sequence.

The T7 constructs overexpressing lpcA, lpcB, and lpcC in E. coli should greatly facilitate purification and characterization of these unique glycosyltransferases, which should be useful for the preparation of new LPS structures and endotoxin-like molecules (2, 3). Now that the lpc genes are available and better understood, they will also serve as tools with which to explore the relationship between LPS core structure and function. For instance, it will now be possible to re-engineer the core domains of Gram-negative bacteria using lpc and related genes, and to investigate the effects of structural modifications on pathogenesis and symbiosis.

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