A Mannosyl Transferase Required for Lipopolysaccharide Inner Core Assembly in Rhizobium leguminosarum

PURIFICATION, SUBSTRATE SPECIFICITY, AND EXPRESSION IN SALMONELLA waaC MUTANTS

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The lipopolysaccharide (LPS) core domain of Gram-negative bacteria plays an important role in outer membrane stability and host interactions. Little is known about the biochemical properties of the glycosyltransferases that assemble the LPS core. We now report the purification and characterization of the Rhizobium leguminosarum mannosyl transferase LpcC, which adds a mannose unit to the inner 3-deoxy-o-manno-octulosonic acid (Kdo) moiety of the LPS precursor, Kdo2-lipid IVα. LpcC containing an N-terminal His6 tag was assayed using GDP-mannose as the donor and Kdo2-[4-32P]lipid IVα as the acceptor and was purified to near homogeneity. Sequencing of the N terminus confirmed that the purified enzyme is the lpcC gene product. Mild acid hydrolysis of the glycolipid generated in vitro by pure LpcC showed that mannosylation occurs on the inner Kdo residue of Kdo2-[4-32P]lipid IVα. A lipid acceptor substrate containing two Kdo moieties is required by LpcC, since no activity is seen with lipid IVα or Kdo-lipid IVα. The purified enzyme can use GDP-mannose or, to a lesser extent, ADP-mannose (both of which have the α-anomeric configuration) for the glycosylation of Kdo2-[4-32P]lipid IVα. Little or no activity is seen with ADP-glucose, UDP-glucose, UDP-GlcNAc, or UDP-galactose. A Salmonella typhimurium waaC mutant, which lacks the enzyme for incorporating the inner 1-glycero-di-manno-heptose moiety of LPS, regains LPS with O-antigen when complemented with the lpcC gene (13, 14). In E. coli and Salmonella, WaaC(RfaC) adds 1-glycero-di-manno-heptose to the inner Kdo unit at the same place where LpcC adds the mannose residue in R. leguminosarum or R. etli (Fig. 1) (18, 19). Both LpcC and WaaC can employ the analogue ADP-3-deoxy-d-manno-octulosonic acid (Kdo) units represent the minimal LPS substructure required for bacterial viability (1, 2). All three domains of LPS are essential for full virulence during infection of animals and plants (1).

There are striking differences in the structures of diverse LPS molecules, such as those of the plant endosymbionts Rhizobium leguminosarum and Rhizobium etli compared with the enteric bacterium Escherichia coli (2, 7–9). R. leguminosarum and R. etli lipid A molecules lack the phosphate groups found in E. coli lipid A, are modified with a galacturonic acid moiety at position 4', and contain an unusual 28-carbon secondary acyl chain at position 2' (Fig. 1) (7–10). The inner core domains of R. leguminosarum and R. etli lack the 1-glycero-di-manno-heptose residues found in E. coli, Salmonella, and most other Gram-negative bacteria and instead contain a mannose residue directly linked to position 5 of the inner Kdo sugar (Fig. 1) (11–14).

Despite these and other differences, the first seven enzymes that catalyze the formation of the key intermediate Kdo2-lipid IVα (Fig. 2) are the same in both organisms (15). These bacteria diverge in their subsequent processing of Kdo2-lipid IVα. For instance, R. leguminosarum membranes contain phosphatases that remove the 1- and 4'-phosphate moieties late in the pathway (16, 17). The mannose residue of the inner core of R. leguminosarum is incorporated by a GDP-mannose-dependent enzyme, which is encoded by the lpcC gene (13, 14). In E. coli and Salmonella, WaaC(RfaC) adds 1-glycero-di-manno-heptose to the inner Kdo unit at the same place where LpcC adds the mannose moiety in R. leguminosarum or R. etli (Fig. 1) (18, 19). There is, however, no sequence homology between WaaC and LpcC (14, 18, 19), despite the formal structural similarity of heptose and mannoheptose (Fig. 1). WaaC is thought to utilize ADP-1-glycero-di-manno-heptose as its physiological sugar nucleotide donor (2, 18, 20), whereas LpcC employs GDP-mannose (14, 19). Both LpcC and WaaC can employ the analogue ADP-mannose (a natural product from corn) (21, 22) as an alternative substrate in vitro (13, 14, 19). However, bacteria do not normally synthesize ADP-mannose.

Significant orthologs of R. leguminosarum lpcC are present in the genomes of several other important proteobacteria, including S. meliloti (see accompanying manuscript (58)) (23, 24).

TOF, matrix-assisted laser desorption-ionization/time of flight; Kdo, 3-deoxy-d-manno-octulosonic acid; NOE, nuclear Overhauser effect; CAPS, 3-cyclohexylamino/propanesulfonic acid.
structures of L-lipid A and of the inner core oligosaccharides. The closely related structures of t-glycero-d-manno-heptose and d-mannose are highlighted for comparison. There is, however, no sequence similarity between the enzyme that attaches the innermost heptose in E. coli (WaaC) and the enzyme that attaches the mannosone residue in R. leguminosarum (LpcC) (14, 19). Only the major molecular species are indicated, which, in the case of R. leguminosarum, is designated component D-1 (2, 8, 9, 12).

Given the importance of LpcC in the biology of R. leguminosarum (14), its presence in certain human pathogens, and the general lack of biochemical information concerning glycosyl transferases involved in LPS core assembly (2, 31), we have now purified R. leguminosarum LpcC to homogeneity and characterized some of its properties. Pure LpcC is highly selective for GDP-mannose, consistent with the reported structure of the mannose/mannosyl transferase has not yet been confirmed but is presumed to be the same as that seen in the core domain of the LPS isolated from cells (11, 12).

EXPERIMENTAL PROCEDURES

Chemicals and Materials—The [γ-32P]ATP was purchased from PerkinElmer Life Sciences. ADP-mannose, GDP-mannose, Kdo, and HEPES were obtained from Sigma. Bicinchoninic assay reagents and Triton X-100 were purchased from Pierce. Yeast extract and peptone-tryptone were from Difco. Silica Gel 60 thin layer chromatography plates were obtained from Merck. DNA primers and T4 DNA ligase were from Invitrogen, and PCR reagents were from Stratagene. Reagent grade pyridine, chloroform, and methanol were purchased from Mallinckrodt.

Bacterial Strains and Growth Conditions—The E. coli deep rough mutant WBB06 (waaC-waaF) was kindly provided by Werner Brabetz (34). Wild-type strain W3110 was obtained from the E. coli Genetic Stock Center at Yale University. Salmonella typhimurium strains SL1347 (waaC630) (18) and SL3770 (wild-type) were obtained from the Salmonella Genetic Stock Center (University of Calgary, Calgary, Canada). E. coli strain BL21 (DE3)/pLysS was purchased from Novagen. All bacteria were grown in LB broth (10 g of NaCl, 10 g of peptone-tryptone, and 5 g of yeast extract per liter) (35). When necessary, the cultures were supplemented with tetracycline (12 µg/ml) or kanamycin (30 µg/ml).

Molecular Biology Techniques—Plasmids were prepared using the Qiagen Mini Prep Kit (Qiagen). Restriction endonucleases (New England Biolabs), shrimp alkaline phosphatase, and T4 ligase were used according to the manufacturer’s instructions (Invitrogen). Competent cells were prepared for transformation using the calcium chloride method (36, 37).
Constitution of Plasmid pMKCA Expressing LpcC with a His6 tag at the N terminus—The plasmid pMKS6A (13) was used to retrieve the lpcC gene by digesting with BamHI and NdeI. The desired fragment was ligated into the pET28b+ vector (Novagen) behind the T7lac promoter that had been digested with the same enzymes, placing a His6 tag at the N terminus. This construct (pMKH) contains a thymoquinone cleavage site immediately downstream of the His tag.

Construction of the High Copy Plasmid pMKCA Expressing lpcC—The plasmid pMKCA was used for complementation of the waaC-deficient S. typhimurium mutant SL1344 (18) of the E. coli waaC waaF deletion mutant WBB06 (34). pMKH was digested with XhoI and BamHI to retrieve the lpcC gene, and the desired fragment was then cloned behind the lac promoter into the vector pBluescript KS (Stratagene), digested with the same enzymes, to generate pMKCA. The sequence was determined as GSHMPDIRDV. The first two amino acid residues represent the residual thrombin cleavage site, and the last five acid residues represent the residual thrombin cleavage site, and the last five amino acid residues represent the residual thrombin cleavage site, and the last five amino acid residues represent the residual thrombin cleavage site, and the last five amino acid residues represent the residual thrombin cleavage site, and the last five amino acid residues represent the residual thrombin cleavage site, and the last five amino acid residues represent the residual thrombin cleavage site, and the last five amino acid residues represent the residual thrombin cleavage site, and the last five amino acid residues represent the residual thrombin cleavage site, and the last five amino acid residues.

Mannosyl Transferase Assay—The manniosyl transferase LpcC was assayed using the LPS precursor Kdo₂-[4-³²P]lipid IV₆, which was isolated and stored as a frozen aqueous dispersion as described previously (38). Prior to each use, the radiolabeled substrate was subjected to ultrasonic irradiation in a water bath for 1 min. The manniosyl transferase assay was assayed as follows. The reaction mixture (10 µl final volume) contained 50 mM HEPES, pH 7.5, 0.1% Triton X-100, 1 mM GDP mannose, and 10 µM Kdo₂-[4-³²P]lipid IV₆ (3000–6000 cpm/nmol). The reaction was started by the addition of an appropriate amount of enzyme (2.5–5 µg/µl of pure enzyme or about 50 µg/µl of membranes of cells overexpressing lpcC behind the T7lac promoter) and incubated for the indicated times at 30°C. The reactions were stopped by spotting 4-µl samples directly onto a silica gel thin layer plate. After drying the spots at room temperature, the plate was developed in the solvent chloroform, pyridine, 88% formic acid, water (30:70:10:16, v/v/v/v). Following removal of the solvent with a hot air stream, the plate was spotted onto a silica TLC plate. The plate was developed in the solvent chloroform, pyridine, 88% formic acid, water (30:70:16:10, v/v/v/v) and analyzed as described previously to determine the site of radiolabeled product formation.

Purification and Properties of R. leguminosarum LpcC—Purification of His-tagged LpcC over a Nickel Column—One ml of His-Bind resin (Novagen) was prepared for nickel affinity chromatography, as for the preparation of Kdo₂-[4-³²P]lipid IV₆, which was achieved by thin layer chromatography, as for the preparation of Kdo₂-[4-³²P]lipid IV₆ (38). Next, 4 µl of the freshly solubilized mutant sonicate was spotted onto each microcentrifuge tube, and the tubes were incubated in a boiling water bath. At various times, 4-µl samples were withdrawn and spotted onto a silica TLC plate. The plate was developed in the solvent chloroform, pyridine, 88% formic acid, water (30:70:16:10, v/v/v/v) and analyzed as described previously to determine the site of radiolabeled product formation.

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Purification of LpcC from E. coli BLR(DE3)/pLysS/pMKHN

| Step                  | Total activity | Total protein | Specific activity | Yield | Purification |
|-----------------------|----------------|---------------|-------------------|-------|--------------|
| Membranes             | 9017           | 39.9          | 226               | 100   | 1.0          |
| Solubilized membranes | 4716           | 11.2          | 421               | 52    | 1.9          |
| Nickel column         | 540            | 0.15          | 3600              | 6     | 18^a         |

^a The purification relative to membranes of wild-type R. leguminosarum is estimated to be about 3600-fold, given a specific activity of about 1 nmol/min/mg in such preparations (13). One unit is defined as 1 nmol/min at 30 °C.

RESULTS

Purification of His6-tagged LpcC—As shown in Table I, a derivative of LpcC bearing a His6 tag followed by a thrombin cleavage site at its N terminus was expressed behind the T7lac promoter on pMKHN in E. coli BLR/DE3/pLysS, resulting in overproduction of a protein of the expected molecular weight (Fig. 3, lane 1) and robust expression of membrane-associated (GDP-mannose-dependent) mannosyl transferase activity (Table I). LpcC activity is not detectable in wild type membranes or vector controls. These findings are consistent with previous studies of native lpcC expressed in E. coli (14).

Triton X-100 was used to solubilize the mannosyl transferase from the particulate fraction with a 52% recovery of activity (Table I). Relatively little of the overexpressed protein was actually solubilized (Fig. 3, lane 2), indicating that much of it is present in the form of small inclusion bodies. The active, solubilized material was applied to a nickel column prepared in 0.1% Triton X-100. Most of the activity was retained and was eluted by 60 mM imidazole together with a protein of the molecular weight expected for LpcC (Fig. 3, lane 3). The specific activity of the purified material was increased about 15-fold relative to the crude membrane fraction (Table I).

A portion of the purified preparation was cleaved with thrombin, displayed on a 12% SDS gel, and then transferred to an polyvinylidene difluoride membrane. N-terminal sequencing yielded the peptide GSHMMPDIRDV... in which the first two residues arise from the residual thrombin cleavage site and the third is a single His residue derived from the construction of the vector. The sequence MPDIRDV represents the true N terminus of LpcC (14).

Sugar Nucleotide Specificity of LpcC—As shown in Fig. 4 (lanes 2 and 3), 5 μg/ml purified LpcC catalyzes the quantitative conversion of Kdo2-[4-32P]lipid IVα to mannosyl-Kdo2-[4-32P]-lipid IVα in the presence of 1 mM GDP-mannose or 1 mM ADP-mannose in 30 min at 30 °C. In contrast, UDP-GlcNac, UDP-galacturonic acid, or ADP-glucose (Fig. 4, lanes 4, 6, and 7, respectively) support little or no glycosylation of Kdo2-[4-32P]-lipid IVα under these conditions. A modest amount of glycosylated product is seen with UDP-glucose or UDP-galactose (Fig. 4, lanes 5 and 8). However, when these assays are conducted in the linear range with respect to the rate of product formation (data not shown), only GDP-mannose and to a lesser extent ADP-mannose function as substrates (also see accompanying manuscript (58)). We estimate that R. leguminosarum LpcC selectivity is at least 2 orders of magnitude greater for GDP-mannose than for UDP-galactose, UDP-glucose, UDP-GlcNac, UDP-galacturonic acid, or ADP-glucose under our assay conditions. These findings are consistent with the reported structures of the R. leguminosarum and R. etli LPS core domains (11, 12).

Lipid Acceptor Specificity of LpcC—As shown in Fig. 5, only Kdo2-[4-32P]lipid IVα functions as an acceptor substrate for the mannosyl residue. Kdo-[4-32P]lipid IVα (prepared with KdoA from Hemophilus influenzae) (44) and [4-32P]lipid IVα were completely inactive (Fig. 5). The failure of Kdo-[4-32P]lipid IVα to function as an acceptor is somewhat surprising, given that LpcC incorporates the mannosyl unit on the inner Kdo moiety of Kdo2-[4-32P]-lipid IVα (see below and Fig. 2). This unusual substrate specificity could be explained by the direct participation of the carbohydrate moiety of the outer Kdo residue of Kdo2-[4-32P]lipid IVα in LpcC catalysis.

Characterization of the Mannosyl Transferase Reaction Product—To determine the site of mannosyl attachment catalyzed by purified LpcC, the mannosyl-Kdo2-[4-32P]lipid IVα product was subjected to hydrolysis at 100 °C in acetate buffer at pH 4.5 in the presence of SDS (19, 45). This procedure randomly cleaves glycosidic linkages of Kdo while leaving all other glycosidic linkages intact. As shown in Fig. 6, the products of mannosyl-Kdo2-[4-32P]lipid IVα hydrolysis were subjected to thin layer chromatography and PhosphorImager analysis at various times of hydrolysis. No Kdo-[4-32P]lipid IVα was detected as an intermediate at any time point. Instead, a compound migrating as would be expected for mannosyl-Kdo-[4-32P]lipid IVα was observed (Fig. 6). These findings demonstrate that little or no mannosyl is added to the outer Kdo residue of
migrating bands (Fig. 7, cells, as judged by the higher intensity of the most rapidly
plasmid restores O-antigen production in a
waaC
structure lacking O-antigen. Expression of
core illustrated in Fig. 1) generates a truncated LPS substruc-
ture containing mannose residues, and therefore possess
the enzymatic machinery to make GDP-mannose when grown on
nutrient broth (2, 31). As shown in Fig. 7 (lane 2), a mutant of
S. typhimurium
waaC
mutant defective in
Kdo2-LPS in place of the inner heptose unit when
S. typhimurium
S. typhimurium
suggest that mannose can be incorporated into the core of
S. typhimurium
lpcC
expression in mutants lacking waaC.

The antibiotic sensitivity data shown in Table II likewise
have been isolated in the presence of SDS (19). The two Kdo glycosidic linkages are about equally susceptible
to cleavage under these conditions, allowing discrimination between
mannose addition to the outer versus the inner Kdo (19). LpcC modifies
the inner Kdo as shown by the absence of unmodified Kdo-[4-32P]lipid
IV, during the time course of the hydrolysis of the mannosyl-Kdo2-
[4-32P]lipid IV.

Kdo2-[4-32P]lipid IV
by LpcC, consistent with previous structural studies of
mannosyl-Kdo containing core oligosaccharide fragments (11, 12) isolated from cells of R. etli.

Partial Restoration of O-Antigen Synthesis and Antibiotic Resistance in a S. typhimurium waaC Mutant Expressing lpcC—Cells of S. typhimurium
LT-2 synthesize O-antigen units containing mannose residues, and therefore possess
the enzymatic machinery to make GDP-mannose when grown on
nutrient broth (2, 31). As shown in Fig. 7 (lane 2), a mutant of
S. typhimurium
waaC
(18) (which encodes
the heptosyl transferase that adds the inner heptose unit of the
core illustrated in Fig. 1) generates a truncated LPS substruc-
ture lacking O-antigen. Expression of
lpcC
on a multicopy plasmid restores O-antigen production in a
waaC
mutant (Fig. 7, lane 4), although somewhat less efficiently than in wild type
cells, as judged by the higher intensity of the most rapidly
migrating bands (Fig. 7, lane 1 versus lane 4). These findings

To evaluate the LpcC donor substrate selectivity in living
cells, the lpcC gene was expressed in an E. coli mutant
(WBB06) (34) containing a deletion spanning both waaC and
waaF. The latter gene encodes the second heptosyl transferase (Fig. 1) (2, 31). The LPS of WBB06 can be isolated directly by
Bligh-Dyer extraction without prior mild acid hydrolysis, and it
can be analyzed without further modification by MALDI-TOF
mass spectrometry (Fig. 8A). It consists largely of Kdo2-lipid A,
as judged by the signal in the negative mode spectrum attributed
to [M – H]
– at m/z 2237.8, consistent with hexa-acylated
lipid A of E. coli
K-12 (M, 2238.7). When
lpcC
is expressed in living cells of WBB06, grown at 25
°C to stimulate GDP-mannose production, about one-third of the Kdo2-lipid A is derivatized with an additional hexose moiety (presumably a man-
nose, as indicated in Fig. 8C), as judged by the signal at m/z
2400.1. If LpcC had utilized endogenous ADP-1-glycero-d-manno-heptose (which is also present in WBB06) (20), the mass
mannotose unit (Fig. 9B, arrows). The observed NOE between H-1’ and H-2” (Fig. 9B) is identical to that seen in GDP-mannose (data not shown). However, this NOE is expected, regardless of whether H-1’ is in the axial position (β-anomer) or in the equatorial position (α-anomer), since H-2’ in mannose is in the equatorial position. Similarly, the coupling constant between H-1’ and H-2” and also the C-1’ proton and carbon chemical shifts (Table II) are not diagnostic for the α-anomeric stereochemistry (46, 47).

In addition to the lack of NOEs between H-1’ and H-3” as well as between H-1” and H-5” (Fig. 9B, arrows), a very reliable distinguishing criterion for determining the mannose anomeric configuration is the one-bond 1H-13C coupling constant at the C-1’-position (48, 49). The mean one-bond 1H-13C couplings are 170 and 160 Hz for the α- and β-anomers, respectively (48, 49). This 10-Hz difference is attributed to the equatorial disposition of the H-1’ of the α-anomer, which has two gauche interactions with the lone pair orbitals of the ring oxygen, whereas the axial H-1” of the β-anomer has one trans and one gauche interaction. The 1H-coupled 13C NMR spectrum of ADP-mannose revealed a C-1’ one-bond 1H-13C coupling constant of 173.4 Hz, confirming the α-anomeric stereochemistry.

The unequivocal demonstration of the α-anomeric stereochemistry of the mannose residue in ADP-mannose is of considerable interest in light of our previous finding that ADP-mannose can serve not only as an excellent surrogate donor substrate for LpcC (13) but also for purified WaaC (13, 19) in place of the physiologically relevant compound, ADP-1-glycero-D-manno-heptose (20). Based on these observations, one would expect that ADP-1-glycero-D-manno-heptose would likewise possess the α-anomeric configuration, but this point is controversial (20), as discussed below.

**DISCUSSION**

The structures of the lipid A and core domains of *R. leguminosarum* LPS differ significantly from those of *E. coli* LPS (Fig. 1). Nevertheless, except for subtle variations in acyl chain preferences, the first seven enzymes that generate the conserved intermediate Kdo2-lipid IVα (Fig. 2) are identical in both organisms (15). Kdo2-lipid IVα is further acylated both in *E. coli* and *R. leguminosarum* (Fig. 1) (50–53), but in *R. leguminosarum*, it is also dephosphorylated at the 1- and 4’-positions (16, 17) and oxidized at the 1 position (9) in the late stages of LPS assembly to generate the unusual phosphate-free lipid A species that are characteristic of this organism (Fig. 1, component D-1). In both systems, Kdo2-lipid IVα is also glycosylated with multiple core sugars (14, 18) prior to MabA-mediated translocation to the periplasmic surface of the inner membrane (54).

The carbohydrate composition and linkages of the *E. coli* and *R. leguminosarum* core domains are quite different distal to the two Kdo residues (Fig. 1). In *E. coli*, *S. typhimurium* and most other Gram-negative bacteria that have been characterized to date, 1-glycero-D-manno-heptose is attached at position 5 of the inner Kdo moiety (1, 2). In *R. leguminosarum* and *R. etli*, a mannose unit replaces this 1-glycero-D-manno-heptose residue (Fig. 1) (11, 12). In previous studies with crude membrane preparations, we demonstrated that GDP-mannose is utilized as the mannose donor for *R. leguminosarum* and *R. etli* core glycosylation of Kdo2-lipid IVα (Fig. 2), and that *R. leguminosarum* LpcC is the structural gene encoding the mannosyl transferase (13, 14).

We have now purified the mannosyl transferase to homogeneity (Table I), using a His6-tagged LpcC construct expressed behind the T7lac promoter in *E. coli*. The properties of the pure enzyme are similar to those observed previously with crude membrane preparations (also see accompanying manuscript (58)). Of particular interest is the high degree of specificity of

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**FIG. 7.** SDS gel electrophoresis of LPS from *Salmonella* *waaC* (rfaC) mutants complemented with *R. leguminosarum* LpcC. LPS preparations were separated on a 12% SDS gel and silver-stained by the method of Hitchcock and Brown (43). Lane 1, SL3770 (waaC); lane 2, SL1377 (waaC); lane 3, SL1377 with p Bluescript KS +; lane 4, SL1377 with pMKCA.

**TABLE II**

Partial complementation of the antibiotic hypersensitivity of a *S. typhimurium* waaC mutant by lpcC

| Strain                  | Novobiocin | Rifamycin | Bacitracin |
|-------------------------|------------|-----------|------------|
| SL3770 (waaC)           | <6         | 7         | <6         |
| SL1377 (waaC630)        | 12         | 13        | <6         |
| SL1377/pBluescript KS + | 12         | 13        | <6         |
| SL1377/pMKCA (lpcC)     | 12         | 13        | <6         |

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of the resulting LPS species would have been another 30 atomic mass units larger (equivalent to the extra CHOH unit in heptose versus mannose) than what was actually observed (Fig. 8D).

**Anomeric Carbon Configuration in GDP-mannose and ADP-mannose**—Most common nucleotide diphosphate derivatives of D-sugars have the α-anomeric configuration (46, 47). This point is well documented for GDP-mannose (33) but has not been evaluated for ADP-mannose, a natural product synthesized in corn (21, 22).

The 1H, 13C, and 31P NMR assignments for ADP-mannose in D2O are summarized in Table III. Fig. 9A shows the two-dimensional 1H-1H COSY analysis with the mannose through-bond cross-peaks and assignments indicated. As shown in Fig. 9B, a 1H-1H NOESY analysis of ADP-mannose provides the first indication of the expected α-anomeric stereochemistry in its mannose unit, as evidenced by the absence of significant cross-peaks between H-1’ and H-3” or H-1’ and H-5” of the...
magnitude less rapidly under the assay conditions employed (Fig. 4). Only the analogue ADP-mannose can substitute for GDP-mannose (Fig. 1), we wished to determine whether or not lpcC could restore the assembly of a complete LPS molecule in a mutant of S. typhimurium lacking a functional waaC gene. As shown in Fig. 7, the O-antigen ladder that is characteristic of wild-type S. typhimurium is largely restored when lpcC is expressed on a low copy plasmid behind a lac promoter in a S. typhimurium waaC mutant. This result most likely reflects the utilization of GDP-mannose by LpcC in living cells of S. typhimurium and the incorporation of a mannose unit in place of the inner l-\textit{glycero}-d-\textit{manno}-heptose residue.

ADP-mannose was originally described as a natural product isolated from corn (21, 22). Given its ability to substitute for GDP-mannose (Fig. 4) in the LpcC-catalyzed glycosylation of Kdo\textsubscript{2}-lipid IV\textsubscript{X}, we assume that its anomeric stereochemistry must be \textit{\alpha}. To validate this proposal, commercial ADP-mannose (Fig. 9 and Table III) and GDP-mannose (not shown) were evaluated by \textsuperscript{1}H-\textsuperscript{13}C NMR spectroscopy of ADP-mannose

| Position | \( \delta \text{H}, J \) ppm (mult), Hz | \( \delta \text{C}, J \) ppm (mult), Hz |
|----------|----------------------------------------|---------------------------------|
| Adenine  |                                        |                                 |
| 2        | 8.486 (s) 155.455 (d)                  |                                 |
| 4        | 151.706 (s) 72.960 (d)                 |                                 |
| 6        | 121.233 (s) 67.770 (dt) 6.0            |                                 |
| 8        | 8.237 (s) 142.333 (d)                  |                                 |
| Ribose   |                                        |                                 |
| 1'       | 6.118 (s) 7.664 (d) 89.353 (d)         |                                 |
| 2'       | -4.73 (m) 76.854 (d) 4.501 (s)         |                                 |
| 3a       | 6.118 (d) 76.960 (d) 4.501 (s)         |                                 |
| 5a       | 4.372 (m) 86.478 (d) 3.893 (dd) 4.19 (m) |                                 |
| 5b       | -4.19 (m) 67.770 (dt) 6.118 (s)        |                                 |
| Mannose  |                                        |                                 |
| 1'       | 5.485 (dd) 7.8 99.027 (dd) 5.9          |                                 |
| 2'       | 4.021 (m) 7.664 (d) 72.824 (s)         |                                 |
| 3'       | 3.893 (dd) 7.664 (d) 72.370 (d)        |                                 |
| 4'       | 3.893 (dd) 7.664 (d) 76.235 (s)        |                                 |
| 5'       | 3.818 (m) 63.318 (d)                   |                                 |
| 6a'      | -3.83 (m) 63.318 (d)                   |                                 |
| 6b'      | 3.718 (dd) 8.235 (s) 142.333 (d)       |                                 |

\( a \) Data obtained from natural abundance one-dimensional \( ^{13} \text{C} \) spectrum at high digitization. 
\( b \) Carbon-phosphorus coupling constant.

\( \frac{4.5}{4.5} \) Purification and Properties of \( \text{R. leguminosarum} \) LpcC for GDP-mannose versus most other sugar nucleotides, which are utilized at least 2 orders of magnitude less rapidly under the assay conditions employed (Fig. 4). Only the analogue ADP-mannose can substitute for GDP-mannose effectively. As will be shown in the accompanying manuscript (58), a closely related LpcC ortholog found in \( \text{S. meliloti} \) (23, 24) can employ a much broader range of sugar nucleotide donors at comparable rates with the interesting consequence that the \( \text{S. meliloti} \) gene can complement a \( \text{R. leguminosarum} \) lpcC mutation but not vice versa (23).
coupling at C-1\(^{1}\). These criteria are especially useful for determining the anomeric stereochemistry of mannose residues, in which the coupling constants between H-1\(^{1}\) and H-2\(^{1}\) as well as the proton and carbon chemical shifts are not always diagnostic (46, 47). As shown in Fig. 9B, strong NOEs were observed between H-1\(^{1}\) and H-2\(^{2}\) but not between H-1\(^{1}\) and H-3\(^{1}\) or between H-1\(^{1}\) and H-5\(^{1}\) (arrows), which would be expected if the anomeric linkage had the \(\beta\)-configuration. The NOE results were identical with GDP-mannose (not shown). Furthermore, the 173.4-Hz one-bond \(^1\)H-\(^{13}\)C coupling for C-1\(^{1}\) of ADP-mannose is diagnostic for the \(\alpha\)-anomer. Accordingly, LpcC appears to utilize the \(\alpha\)-anomer of both sugar nucleotides.
The demonstration that ADP-mannose has the α-annomer configuration raises the question of whether or not the proposed endogenous ADP-L-glycerod-mannheptose (2) possesses the α- or the β-annomer configuration. The few attempts to validate the structure of ADP-L-glycerod-mannheptose isolated from bacteria have not been altogether conclusive (20, 56, 57), since these preparations were not subjected to the 'H-NOESY analysis shown in Fig. 9. Based on chemical shifts and coupling constants, Gronow et al. (20) proposed that the naturally occurring ADP-L-glycerod-mannheptose has the β-annomer configuration, but given the above observations, this issue should be reinvestigated. Purified WaaC can be effectively utilized to develop ADP-mannose (but not GDP-mannose) (19) as a substrate for the glycosylation of Kdo2-lipid IVα, indicating that it too can utilize the β-annomer. However, other studies with synthetic ADP-L-glycerod-mannheptose preparations and crude membrane preparations containing WaaC (47) have suggested that only the β-annomer is active as the substrate for WaaC. Clearly, additional structural characterization of the natural product and enzymatic studies of the substrate for WaaC. Clearly, additional structural characterization of the natural product and enzymatic studies of WaaC are needed to resolve these issues.

One of the most interesting long term biological implications of the elucidation of the biochemistry of LPS core glycosylation is the possibility of creating hybrid strains of humans and animal pathogens, such as F. tularensis (28), with rationally reengineered cores. Such strains might show reduced virulence towards the human host. Consequences of this possibility of generating such strains should be thoroughly investigated. Such strains might show reduced virulence towards the human host. Consequences of this possibility of generating such strains should be thoroughly investigated. Purification and Properties of R. leguminosarum LpcC

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