Heptosyltransferase I, encoded by the rfaC (waaC) gene of Escherichia coli, is thought to add l-glycero-d-manno-heptose to the inner 3-deoxy-d-manno-octulosonic acid (Kdo) residue of the lipopolysaccharide core. Lipopolysaccharide isolated from mutants defective in rfaC lack heptose and all other sugars distal to heptose. The putative donor, ADP-l-glycero-d-manno-heptose, has never been fully characterized and is not readily available. In cell extracts, the analog ADP-mannose can serve as an alternative donor for RfaC-catalyzed glycosylation of the acceptor, Kdo2-lipid IVα. Using a T7 promoter construct that overexpresses RfaC—15,000-fold, the enzyme has been purified to near homogeneity. NH2-terminal sequencing confirms that the purified enzyme is the rfaC gene product. The subunit molecular mass is 36 kDa. Enzymatic activity is dependent upon the presence of Triton X-100 and is maximal at pH 7.5. The apparent Km (determined at near saturating concentrations of the second substrate) is 1.5 mM for ADP-mannose and 4.5 μM for Kdo2-lipid IVα. Chemical hydrolysis of the RfaC reaction product at 100 °C in the presence of sodium acetate and 1% sodium dodecyl sulfate generates fragments consistent with the inner Kdo residue of Kdo2-lipid IVα as the site of mannosylation. The analog, Kdo-lipid IVα, functions as an acceptor, but is mannosylated at less than 1% the rate of Kdo2-lipid IVα. The purified enzyme displays no activity with ADP-glucose, GDP-mannose, UDP-glucose, or UDP-galactose. Mannosylation of Kdo2-lipid IVα catalyzed by RfaC proceeds in high yield and may be useful for the synthesis of lipopolysaccharide analogs. Pure RfaC can also be used together with Kdo2-[4-32P]lipid IVα to assay for the physiological donor (presumably ADP-l-glycero-d-manno-heptose) in a crude, low molecular weight fraction isolated from wild type cells.

Lipopolysaccharide (LPS) is a major component of the outer leaflet of the outer membranes of Gram-negative bacteria (1–4). It is composed of three domains (Fig. 1): 1) a hydrophobic anchor, known as lipid A, that consists of an acylated disaccharide of glucosamine; 2) a non-repeating oligosaccharide, designated the core, that serves as a barrier to many antibiotics; and 3) the O-antigen, that extends outwards from the core and is comprised of a distinct repeating oligosaccharide. All components of LPS are required for the virulence of Gram-negative bacteria (1, 3, 5). The O-antigen and many of the core sugars are not required for viability (1, 3, 6–8), but the lipid A and Kdo residues of the inner core are essential for growth of Escherichia coli and related organisms (9–13).

Most of the genes of core oligosaccharide biosynthesis are contained in the rfa (waa) cluster near minute 82 on the E. coli chromosome (1, 3, 14–16). The functions of these genes have been deduced from genetic studies, in conjunction with partial physical and chemical characterizations of isolated LPS (1, 3). Direct enzymatic studies of E. coli core biosynthesis beyond Kdo have been limited (1) because the structure of the core is not fully established. Consequently, the acceptor substrates of most of the enzymes involved in core glycosylation and the products generated by these enzymes are not fully characterized (1, 17–19). In vitro assays dependent upon time and protein have not generally been developed (1, 17, 20), and key synthetic donors and acceptors are not available (1).

The inner core of E. coli contains 2–3 Kdo residues, 2–3 heptose residues, and several other substoichiometric substituents (Fig. 1) (1–4, 20). Mutants that lack heptose are viable but display a deep rough phenotype (3). They are sensitive to detergents, hydrophobic antibiotics, and rough-specific bacteriophages (3, 7). The incorporation of the first heptose residue into LPS is thought to be catalyzed by the rfaC (waaC) gene product (1, 3), designated heptosyltransferase I (17, 21).

Previous studies of E. coli and Salmonella heptosyltransferase I suffered from some of the above mentioned limitations (17). Since synthetic ADP-l-glycero-D-manno-heptose was not available, partially purified preparations of ADP-heptose, isolated from cells of Shigella sonnei (22), were utilized. However, the heptosyl acceptor employed, Kdo2-lipid IVα, was well characterized (23). Kdo2-lipid IVα is thought to be capable of acquiring a complete core in vitro (24). Even so, the products generated by this in vitro system could only be isolated in radiochemical amounts insufficient for physical analysis (17).

Recently, we have described a new assay for heptosyltransferase I of E. coli, utilizing commercially available ADP-mannose as an alternative donor in place of ADP-l-glycero-D-manno-heptose (21), as shown in Fig. 2. ADP-heptose is a naturally occurring sugar nucleotide found in corn (25, 26). Here, we report the first characterization of the catalytic properties of heptosyltransferase I, using ADP-mannose as the donor and Kdo2-[4-32P]lipid IVα as the acceptor. We have purified RfaC to near homogeneity using this optimized assay system, and we have characterized the product as mannosyl-Kdo2-lipid IVα (proposed structure shown in Fig. 2). We have also devised a new procedure for the isolation of a crude (low molecular weight) sugar nucleotide-containing fraction from various strains of E. coli and Salmonella. Assays utilizing these sugar donors.
**ADP-mannose as a Substrate Analog for Assaying RfaC/WaaC**

### EXPERIMENTAL PROCEDURES

**Materials and Bacterial Strains**—Materials and kits purchased were: [α-32P]ATP (NEB Life Science Products); Heps, Mes, Tris, bovine serum albumin (BSA); Reactive Green 19, ADP-mannose, and all other sugar nucleotides (Sigma); Triton X-100 and bicinechonic assay reagents (Pierce); silica gel 60 thin layer chromatography plates (E. Merck); yeast extract and tryptone (Difco); Wizard Mini-prep kit (Promega); PCR reagents (Stratagene); restriction enzymes (New England Biolabs); shrimp alkaline phosphatase (U. S. Biochemical Corp.); custom primers and T4 DNA ligase (Life Technologies, Inc.); Qiagen II gel extraction kit (Qiagen); polyclayamide gel reagents (National Diagnostics); Centricon centrifugation devices (Amicon); and Immobilon P polyvinylidifluoride membranes (Millipore). All solvents were reagent grade. Radiochemical analysis of thin layer plates was performed with a model 425S Molecular Dynamics PhosphorImager equipped with ImageQuant software.

**EXPERIMENTAL PROCEDURES**

### Experimental Procedures

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The reaction was subjected to 30 cycles of denaturation (1 min, 94 °C), plasmid (designated pJK1) was transformed into a single colony in 1 liter of LB medium (43) containing ampicillin (50 μg/ml). BLR(DE3)pLysS/pET3a and BLR(DE3)pLysS/pJK1 were grown from a reisolated and digested again to verify its structure, and finally transformed vector pET3a, also digested with the same enzymes. The resulting plasmid was first transformed into E. coli SURE cells, verified by restriction mapping, and then transferred into BLR(DE3)pLysS.

FIG. 3. Construction of plasmid pJK1 for overexpression of the rfaC gene product. Details of the amplification of rfaC using the polymerase chain reaction are described under “Experimental Procedures.” PCR primers were designed to amplify the entire rfaC gene and to introduce restriction sites at the ends of the PCR product, as indicated. The primer sequences are as follows: forward, 5′-GGCGGCCGCTTAACGCGGTTTGGAG-3′; reverse, 5′-GGCGGCCGCGATCCTTTATAATGATGTCGCG-3′. The 980-base pair product of the amplification was digested with the restriction enzymes NdeI and BamHI, and ligated into the expression vector pET3a, also digested with the same enzymes. The resulting plasmid was first transformed into E. coli SURE cells, verified by restriction mapping, and then transferred into BLR(DE3)pLysS.

Placing rfaC under T7 Promoter Control—The cloning of PCR-generated rfaC DNA into a vector under T7 promoter control is outlined in Fig. 3 (40–42). The forward primer was synthesized with a GC clamp, an NdeI restriction site, and an rfaC coding strand starting at the translation initiation site (primer sequences are shown in legend for Fig. 3). The reverse primer was synthesized with a GC clamp, a BamHI restriction site, and an rfaC anticoding strand that includes the stop site. The PCR was performed using Pfu polymerase, as specified by the manufacturer. The plasmid pLC10–7 (28, 29) was used as the template. Amplification was carried out in a 25-μl reaction mixture containing 100 ng of template, 20 mM Tris-HCl, pH 8.8, 10 mM KCl, 10 mM (NH₄)₂SO₄, 0.1% Triton X-100, 0.1% BSA, 2 mM MgSO₄, 250 μM of each of the dNTPs, 200 ng of each of primer, and 1.2 units of Pfu polymerase. The reaction was subjected to 30 cycles of denaturation (1 min, 94 °C), annealing (1 min, 55 °C), and extension (1.5 min, 72 °C) in a DNA thermal cycler. The reaction product was analyzed on a 1% agarose gel, digested with NdeI and BamHI, and ligated into the expression vector pET3a that had been similarly digested. The resulting desired hybrid plasmid (designated pJK1) was transformed into E. coli SURE cells, reisolated and digested again to verify its structure, and finally transformed into cells of strain BLR(DE3)pLysS.

Growth Conditions and Cell-free Extract Preparation—BLR(DE3)pLysS/pET3a and BLR(DE3)pLysS/pJK1 were grown from a single colony in 1 liter of LB medium (43) containing ampicillin (50 μg/ml) and chloramphenicol (30 μg/ml) at 37 °C until the A₆₀₀ reached approximately 0.5. The culture was split into two equal portions, and one portion was induced with 100 μM IPTG. Both cultures were incubated with shaking at 225 rpm for an additional 3 h at 37 °C, the A₆₀₀ was recorded, and the cells were harvested by centrifugation for 10 min at 6000 × g at 4 °C. All subsequent steps were performed either on ice or at 4 °C. The cell pellet was resuspended in a minimal volume, typically 10 ml of 50 mM Hepes, pH 7.5, and broken by passage through a 5-mI French pressure cell at 18,000 p.s.i. Unbroken cells and debris were removed by centrifugation for 10 min at 6000 × g. The resulting crude extract supernatant was used to prepare membranes. The crude extract was subjected to ultracentrifugation at 100,000 × g for 60 min. The membrane pellet was resuspended in 1.5 ml of 50 mM Hepes, pH 7.5. The protein content of each fraction was determined by the bicinchoninic acid (BCA) assay (44) using BSA as the standard.

Making Solubilized Membranes—A 1 ml portion containing 8–10 mg/ml protein of the BLR(DE3)pLysS/pJK1 membranes was mixed with an equal volume of 2% Triton X-100 and incubated on ice for 2 h with periodic gentle inversion of the tube. The solubilization mixture was then centrifuged at 100,000 × g for 60 min to remove any unsolubilized proteins. The pellet was resuspended in 750 μl of 50 mM Hepes, pH 7.5, and the protein contents of both the solubilized and unsolubilized fractions were determined by the BCA assay (44).

Reactive Green 19 Column Chromatography of RfaC—One gram of Reactive Green 19 resin suspended in 5 ml of water was equilibrated in a small plastic disposable column with 10 column volumes of equilibration buffer (50 mM Hepes, pH 7.5, 0.1% Triton X-100). A 4-μg sample of solubilized membrane proteins (in 1.25 ml) was diluted 10-fold with 50 mM Hepes pH 7.5, and the material was then applied to the column at a flow rate of 1 ml/min. Fractions of 5.2 ml were collected throughout. Next, the column was washed with 25 ml of equilibration buffer. Elution was carried out in three stages: 1) 25 ml of equilibration buffer plus 0.5 M NaCl; 2) 25 ml of equilibration buffer plus 1.0 M NaCl, and finally, 3) 25 ml of equilibration buffer plus 2.5 M NaCl. The protein content of each fraction was determined using the BCA assay (44). The peak of enzyme activity was determined by assaying each fraction in the linear range under standard conditions for detection of mannose transfer to Kdo₂-[γ-³²P]-Phosphatidylinositol. The protein in certain samples was also visualized by 10% polyacrylamide gel electrophoresis in the presence of SDS, using the Laemmli buffer system (45) in conjunction with Bio-Rad Mini-Protein II electrophoresis equipment.

Preparation of the Purified Protein for NH₂-terminal Sequencing—Approximately 20 μg (400 μl) of Green 19 purified protein was concentrated 40-fold on a Microcon 10 device, according to the manufacturer’s instructions. The concentrated sample was loaded onto a 10% polyacrylamide SDS gel along with a lane containing prestained standards as a control for transfer. Electrophoresis was carried out at 200 V for 50 min in a Laemmli gel buffer system. The gel was then soaked in 10 mM CAPS, pH 11, for 10 min at 4 °C. A polyvinylidene difluoride membrane was prepared while the electrophoresis was in progress for brief soaking in a water bath, and then soaking in 10 mM CAPS, pH 11. A Bio-Rad SD electrophoresis blotter was used according to the manufacturer’s directions at 20 V for 40 min. Protein bands transferred to the membrane were visualized by Coomassie staining, and the band of interest was excised. NH₂-terminal amino acid sequencing of the intact protein was carried out by Dr. John Leszyk of the Worcester Foundation for Experimental Biology, Shrewsbury, MA.

Sodium Acetate Hydrolysates of Mannosamine-Kdo₂-lipid IVα—Two 10-μl reaction mixtures were prepared containing 50 mM Hepes, pH 7.5, 0.1% Triton X-100, 0.4 μM Kdo₂-lipid IVα (1 × 10⁷ cpm/mmol or 40,000 cpm/reaction), and 1 mM ADP-mannose. To only one tube, 0.3 μg/ml purified RfaC was added. Both tubes were incubated for 30 min at 30 °C. Next, 4 μl of 10% SDS and 26 μl of 50 mM sodium acetate pH 4.5 were added (46–48) to both tubes to give a final pH of approximately 5.0, and the tubes were incubated in a boiling water bath. At 0, 1, 2, 5, 10, 20, and 30 min, 5-μl samples were withdrawn and spotted onto a silica TLC plate. The plate was developed and analyzed by Phosphorimager analysis, as described above.

Preparation of Crude (Low Molecular Weight) Sugar Nucleotide Fractions from Living Cells—Five different strains (see below) were studied. Single colonies of each organism were used to inoculate 250-mI LB broth cultures. These cultures were grown at 37 °C until the A₆₀₀ reached approximately 1.0. The cells were centrifuged for 10 min at 6000 × g (4 °C). The cell pellets were each then extracted with 5 ml of 50% ethanol at room temperature and incubated on ice for another 30 min with occasional stirring. The precipitates were removed by 10-min centrifugations at 6000 × g (4 °C). The supernatants were collected and placed in a 100-μl Eppendorf microcentrifuge tube in 4 °C to reduce the half-life of ADP-mannose and to remove the most of the ethanol. Next, 500-μl portions of each of these supernatants were applied to Centricon 3 filtration devices and centrifuged at top speed in an Eppendorf microcentrifuge for 30 min at 4 °C. The flow-throughs, consisting of compounds with molecular weights less than 3000, were collected and used as the source of crude sugar nucleotides for studies of purified RfaC specificity.
Overexpression of the rfaC Gene Using the T7 Promoter—The NdeI restriction site (CATATG) in the multiple cloning cassette of pET3a can be used to insert a piece of foreign DNA with an overlapping transcriptional start site (ATG) (Fig. 3). This positions the inserted gene properly in the context of a strong T7 promoter region and ribosome binding site (41, 42). The inserted DNA in plasmid pJK1 (Fig. 3), generated by PCR as described under “Experimental Procedures,” contains such a NdeI site, and in addition, a BamHI site at the opposite end of the gene to ensure unidirectional cohesive end cloning. Because the T7 promoter drives expression of rfaC in pJK1, a host strain that codes for T7 RNA polymerase, such as BLR(DE3)pLysS, is required to obtain expression.

Extracts were prepared from cells of BLR(DE3)pLysS containing either pJK1 or vector alone (pET3a). Duplicate cultures of each were first grown to an A600 of 0.5, and then for another 3 h either with or without IPTG. The extracts were assayed for RfaC activity using ADP-mannose as the sugar donor. As shown in Table I, the specific activity of the transferase in extracts of BLR(DE3)pLysS/pJK1 grown without IPTG was over 200-fold higher than in extracts of the vector control strain. The presence of IPTG in the growth medium enhanced the expression of the transferase another 50-fold in BLR(DE3)pLysS/pJK1, as judged by assaying the activity (Table I) and analysis by gel electrophoresis (data not shown).

Purification of the Overproduced Transferase—It was observed previously that the rfaC-encoded transferase of wild type E. coli was membrane-associated (21). We therefore used membranes from the overproducing strain BLR(DE3)pLysS/pJK1 for the purification. Only about one quarter of the total activity present in the crude extract of BLR(DE3)pLysS/pJK1 was recovered in the membranes; nevertheless, a 2-fold increase in the specific activity was observed (Table II). Upon solubilization of the membranes with 1% Triton X-100, approximately 40% of the transferase activity was recovered with an additional slight increase in specific activity (Table II). At this stage, as shown by gel electrophoresis (Fig. 4), the transferase comprised a large fraction of the protein present in the solubilized sample, as judged by the presence of an overproduced band at ~36 kDa. It was therefore possible to employ only one chromatography step to obtain a homogeneous protein.

A survey of dye-ligand and ion-exchange resins indicated that Reactive Green 19-agarose had a strong reversible affinity for the transferase (Fig. 5). The transferase eluted at 2.5 mM NaCl, resulting in a 5.5-fold purification (Fig. 5 and Table II). The purity of the final preparation, fractions 38–46, was over 95% as judged by gel electrophoresis (Fig. 4) with an estimated molecular mass of 36,000 Da. The final purification of the transferase relative to the crude cell extracts employed was 14.6-fold, but it was 220,000-fold relative to extracts of wild type cells (Table II). The material purified through the Green 19 step was used as the enzyme source in all subsequent experiments.

Two separate purifications were performed. Preparation 1 (data not shown) was used for the kinetic characterization of the enzyme. Preparation 2 was used to generate Table II and for microsequencing and product analysis by sodium acetate hydrolysis and mass spectrometry.

Amino-terminal Sequence of the Purified Protein—The predominant protein band present in the purified enzyme preparation (Fig. 4) was transferred to a polyvinylidene difluoride membrane, and subjected to amino acid sequencing. The sole NH2-terminal amino acid sequence found was MRV-LIVKTSSMGDVL. This matches exactly the amino acid sequence predicted from the nucleotide sequence of E. coli rfaC (4, 17).

Effect of pH and Detergent on Transferase Activity—Mannosyltransferase activity was measured using purified RfaC in the range of pH 5.5 to 8.9, as shown in Fig. 6A. The pH optimum for this reaction is centered around 7.5. Fig. 6B shows that the detergent Triton X-100 is required for activity. Under standard assay conditions with 10 μM Kdo2-lipid IVα no activity is detected without detergent. Since Kdo2-lipid IVα probably does not form a true solution in water, the detergent likely interacts with the lipid substrate to generate mixed micelles, allowing the enzyme better access to the substrate. At concentrations greater than 0.1% Triton X-100, however, the transferase activity is inhibited, probably because of surface dilution effects (49).

Kinetic Properties of the Purified Enzyme—As seen in Fig. 7, the mannosyltransferase activity of RfaC is linearly dependent upon both time and protein concentration. The reaction is well behaved, and the enzyme can catalyze the quantitative mannosylation of Kdo2-lipid IVα (data not shown).

When the concentration of ADP-mannose was held constant in the assay at 7.5 mM and the concentration of Kdo2-lipid IVα was varied (Fig. 8A), the apparent Km for Kdo2-lipid IVα was calculated to be 4.5 μM. Likewise, when Kdo2-lipid IVα was held constant at 25 μM and the concentration of ADP-mannose was varied, the apparent Km for ADP-mannose was found to be 1.47 mM. The Vmax for the preparation employed in the kinetic analysis (Fig. 8) was ~3000 nmol/min/mg. In other preparations, the Vmax was severalfold higher (data not shown).

Specificity of the Transferase for Its Sugar Nucleotide Donor Substrate—The proposed physiological sugar nucleotide substrate for this reaction, ADP-L-glycerol-1-phospho mannose (22), is not readily available for use in in vitro assays. Because we were able to substitute ADP-mannose in the assay, we also examined the question of whether or not other sugar nucleotides

### RESULTS

#### Overexpression of the rfaC Gene Using the T7 Promoter

The NdeI restriction site (CATATG) in the multiple cloning cassette of pET3a can be used to insert a piece of foreign DNA with an overlapping transcriptional start site (ATG) (Fig. 3). This positions the inserted gene properly in the context of a strong T7 promoter region and ribosome binding site (41, 42). The inserted DNA in plasmid pJK1 (Fig. 3), generated by PCR as described under “Experimental Procedures,” contains such a NdeI site, and in addition, a BamHI site at the opposite end of the gene to ensure unidirectional cohesive end cloning. Because the T7 promoter drives expression of rfaC in pJK1, a host strain that codes for T7 RNA polymerase, such as BLR(DE3)pLysS, is required to obtain expression.

### Specific activities of rfaC in recombinant strains

Specific activities were determined under linear standard assay conditions as described under “Experimental Procedures.” For the strain bearing pET3a, 1 mg/ml crude extract was the enzyme source. For the strain with pJK1, 2 μg/ml crude extract was used.

| Strain of BLR(DE3)pLysS | Specific activity |
|-------------------------|------------------|
|                         | Uninduced        | Induced         |
|                         | nmol/min/mg      | nmol/min/mg     |
| pET3a                   | 0.028            | 0.027           |
| pJK1                    | 6.58             | 331             |

### Purification of RfaC from E. coli BLR(DE3)pLysS/pJK1

| Sample  | Protein | Total protein | Specific activity | Total activity | Purification | Purification Yield |
|---------|---------|---------------|------------------|----------------|--------------|-------------------|
|         | mg/ml   | mg            | nmol/min/mg      | nmol/min       | fold/wild type | fold              |
| Crude pET3A | 20.4     | 235           | 335              | 78.7 × 10^2    | 1            | 100.00            |
| Crude pJK1 | 20.4     | 235           | 335              | 15.2 × 10^3    | 1            | 100.00            |
| Membranes | 15.2     | 30.4          | 691              | 21.0 × 10^3    | 3.14 × 10^3   | 2.06              |
| Solubilized | 3.2     | 10.25         | 877              | 8.99 × 10^3    | 39.9 × 10^3   | 2.62              |
| Membranes | 0.026   | 1.52          | 4875             | 7.39 × 10^3    | 221.6 × 10^3  | 14.55             |
| Green 38–46 | 0.026   | 1.52          | 4875             | 7.39 × 10^3    | 221.6 × 10^3  | 14.55             |
could function as alternate donors. ADP-mannose, GDP-mannose, ADP-glucose, UDP-glucose, and UDP-galactose were all tested at 1 mM under otherwise standard assay conditions. As seen in Fig. 9, transferase activity was only detectable with the ADP-mannose substrate. Even at enzyme levels that were 50 times higher than those used in the standard assay, these other sugar nucleotides could not serve as donors. These results imply that the transferase recognizes the axial OH at the C-2 position of the pyranose ring in the donor sugar. The structure of the nucleotide is also very important for the functioning of *E. coli* RfaC, since GDP-mannose did not substitute for ADP-mannose.

Specificity of *E. coli* RfaC for Lipid Acceptors—As shown in Fig. 10, several lipid acceptors were tested as substrates for purified RfaC. Each was 4'-32P-labeled and present in otherwise standard assay conditions at 10 μM. Kdo2- lipid IVₐ was by far the best substrate of those tested, supporting a specific activity of 2280 nmol/min/mg. Under the conditions of Fig. 10, 50-fold less enzyme was used with Kdo2-lipid IVₐ than with the other acceptors. Nevertheless, measurable glycosylation of Kdo-lipid IVₐ was detected at a rate that was approximately 170 times less (13.5 nmol/min/mg) than with Kdo2-lipid IVₐ. No activity was detected using lipid IVₐ as the substrate.

Sodium Acetate Hydrolysis of Mannosyl-Kdo2-lipid IVₐ Generated by *E. coli* RfaC—Hydrolysis of LPS or its Kdo containing precursors at 100 °C in pH 4.5 sodium acetate buffer containing 1% SDS specifically cleaves all glycosidic linkages involving the anomeric carbon of Kdo (47, 48). The half-life of such linkages under these conditions is 5–10 min. The glycosidic linkages between the glucosamine moieties of lipid A and the phosphomonoester groups at positions 1 and 4' of lipid A are not disturbed. As seen in Fig. 11A, hydrolysis of Kdo2-[4'-32P]lipid IVₐ under similar conditions transiently produces Kdo-[4'-32P]lipid IVₐ. At later times (30 min), [4'-32P]lipid IVₐ becomes the predominant hydrolysis product. However, no Kdo-[4'-32P]lipid IVₐ was detected with mannose as an acceptor substrate, in vivo. Thus, mannose is also incorporated by RfaC. The proposed site of attachment of mannose on the outer Kdo is 0.8 μg of transferase after the Reactive Green 19 step. The band in lane 7 is broadened slightly because of salt in the sample.
fraction from the *Salmonella typhimurium* rfaE mutant SL1102 (Fig. 12, lane 6), which is defective in the biosynthesis of ADP-\(\text{L-}\text{glycero-}\text{D-}\text{manno-}\text{heptose}\) (1, 4), did not support any RfaC-catalyzed modification of Kdo\(_2\)-[4\(^\text{\text{-32P}}\)]lipid IVA. In lanes 2–5, various other crude sugar nucleotide containing fractions were utilized. Lane 2 contains the low molecular weight extract from the wild type *E. coli* strain, D21. The observed band shift with the D21 derived donor indicates that ADP-\(\text{L-}\text{glycero-}\text{D-}\text{manno-}\text{heptose}\) (or something like it) is indeed present in this organism. Lanes 3 and 4 contain the low molecular weight isolates from two rfaC\(_2\) strains, which can supposedly synthesize ADP-\(\text{L-}\text{glycero-}\text{D-}\text{manno-}\text{heptose}\) but cannot transfer it to Kdo\(_2\)-[4\(^\text{\text{-32P}}\)]lipid IVA (1, 4). D21f2 is an *E. coli* mutant, and SA1377 (Fig. 12) is a *S. typhimurium* strain. These organisms appear to accumulate considerable amounts of ADP-\(\text{L-}\text{glycero-}\text{D-}\text{manno-}\text{heptose}\) like material, as evidenced by the massive band shifts (lanes 3 and 4) supported by the low molecular weight fractions isolated from these strains. Lane 5 shows the results obtained with a low molecular weight fraction from SL3600 of *S. typhimurium*, a mutant that is defective in the epimerase (RfaD) that is believed to convert ADP-\(\text{D-}\text{glycero-}\text{D-}\text{manno-}\text{heptose}\) to ADP-\(\text{L-}\text{glycero-}\text{D-}\text{manno-}\text{heptose}\) (54). The low molecular weight material from SL3600 does not support an efficient band shift of Kdo\(_2\)-[4\(^\text{\text{-32P}}\)]lipid IVA when incubated together with pure RfaC, consistent with the absence of heptose in the LPS of SL3600 (1, 4). A subtle, but reproducible, observation is that the crude nucleotides isolated from the *E. coli* rfaC mutant D21f2 generate a product that migrates slightly more rapidly (lane 3) than that formed with the corresponding nucleotides of the *S. typhimurium* rfaC mutant SA1377 (lane 4). This finding suggests that there may be more than one molecular species of heptose donor in living cells. Whatever the explanation, the results of Fig. 12 provide a simple new assay for the isolation and definitive characterization of these elusive sugar donors.

**DISCUSSION**

Recently, we reported that it is possible to assay heptosyltransferase I (RfaC) of *E. coli* in crude cell extracts using ADP-mannose as a Substrate Analog for Assaying RfaC/WaaC (21) as the donor. The lack of available ADP-\(\text{L-}\text{glycero-}\text{D-}\text{manno-}\text{heptose}\) has prevented the characterization of RfaC activity, even though the gene that encodes RfaC has been known for some time (17). ADP-mannose and ADP-\(\text{L-}\text{glycero-}\text{D-}\text{manno-}\text{heptose}\) are very similar in structure (21). Based on the composition.

![Graph A](image1.png)

**Fig. 6.** The effect of pH and Triton X-100 on transferase activity. A, transferase activity was measured under standard conditions in with 0.1 \(\mu g/ml\) purified enzyme at the indicated pH values with 50 mM Mes (pH 5.5, 6.0, 6.5, or 6.8), 50 mM Hepes (pH 7.2 or 7.5), or 50 mM Tris-HCl (pH 8.0, 8.5, or 8.9). B, the requirement for Triton X-100 in this assay was demonstrated under standard assay conditions at pH 7.5.

![Graph B](image2.png)

**Fig. 7.** A quantitative assay for *E. coli* RfaC using ADP-mannose as the donor. Formation of mannosyl-Kdo\(_2\)-[4\(^\text{\text{-32P}}\)]lipid IVA is linear with time and protein concentration. Upon prolonged incubation or in the presence of high enzyme concentrations, the reaction goes nearly to completion. The assays shown here were carried out under standard assay conditions at pH 7.5 using the transferase purified through the Green 19 step.
and structure of the E. coli core (1, 4), the linkage formed by RfaC in vitro is proposed to be α,1–5 to the inner Kdo. The observation that the mannose residue is indeed attached to the inner Kdo (Fig. 11) and that Kdo-[4,9-32P]lipid IVA functions as an alternative, albeit slow, substrate (Fig. 10) supports the proposed structure (Fig. 2). Additional studies will be necessary to confirm the α,1–5 linkage.

By using our ADP-mannose assay to follow activity and by constructing a strain that overproduces transferase activity by 15,000-fold (Table I), we were able to develop a facile purification scheme for RfaC (Table II). Only a 14.5-fold purification of the overexpressed protein was necessary to achieve homogeneity. The pure protein displays a specific activity that is 220,000 times higher than that of wild type crude extracts. NH2-terminal sequencing of the purified protein confirmed that RfaC and the mannosyltransferase activity are indeed identical.

To determine the catalytic properties of RfaC, purified protein was used. The pH optimum for the reaction is 7.5, and a non-ionic detergent, such as Triton X-100, is required for activity (Fig. 6). Bovine serum albumin was used in all assays involving purified protein at <1 μg/ml to prevent inconsistencies due to enzyme adsorption to the sides of the reaction tubes. Inclusion of BSA in these assays typically improved the rate of conversion of substrate to product by 10%. The apparent $K_m$ for Kdo2-lipid IVA was calculated as 4.53 μM. In both cases, the apparent $V_{max}$ is approximately 3 μmol/min/mg using enzyme preparation 1. The lines are drawn using a non-linear least squares fitting to the equation: $V = (V_{max} [S])/([K_m]+ [S])$.

![Fig. 8. Kinetic properties of the purified enzyme. Standard assay conditions were used in these assays, but the substrate concentrations were varied, as indicated. In A, the concentration of ADP-mannose was held constant at 7.5 mM. The apparent $K_m$ for Kdo2-lipid IVA is 4.53 μM. In B, the concentration of Kdo2-lipid IVA was held constant at 25 μM. The apparent $K_m$ for ADP-mannose is 1.47 mM. In both cases, the apparent $V_{max}$ is approximately 3 μmol/min/mg using enzyme preparation 1.](http://www.jbc.org/)

![Fig. 9. Sugar nucleotide specificity of purified RfaC. Reactions were carried out under standard conditions at pH 7.5 using either 10 μM Kdo2-[4,9-32P]lipid IVA, Kdo-[4,9-32P]lipid IVA, or [4,9-32P]lipid IVA. To be certain each substrate was assayed in the linear range, differing amounts of purified RfaC were utilized, as indicated.](http://www.jbc.org/)

![Fig. 10. Lipid acceptor specificity of purified RfaC. Reactions were carried out under standard conditions at pH 7.5 using either 10 μM Kdo2-[4,9-32P]lipid IVA, Kdo-[4,9-32P]lipid IVA, or [4,9-32P]lipid IVA. To be certain each substrate was assayed in the linear range, differing amounts of purified RfaC were utilized, as indicated.](http://www.jbc.org/)

ADP-mannose as a Substrate Analog for Assaying RfaC/WaaC
mannosyltransferase in E. coli constructs able to generate GDP-mannose, but lacking their own rfaC gene. Such mutants should contain mannose in place of the inner heptose normally found in the LPS core. The consequences of this modification on further core extension and outer membrane protein assembly would be of considerable interest. Toward this end, we have recently identified a R. leguminosarum clone that appears to encode the GDP-mannose-dependent transferase and several additional enzymes of R. leguminosarum core assembly (50).

As shown in Fig. 10, lipid IV<sub>A</sub> is not a substrate for the RfaC-catalyzed reaction. Lipid IV<sub>A</sub> lacks the Kdo moiety to which the mannose is attached (Fig. 11). Surprisingly, Kdo-lipid IV<sub>A</sub> is a relatively poor mannose acceptor (Fig. 10) despite the fact that it possesses the Kdo residue to which the mannose is linked (Fig. 11). Kdo-lipid IV<sub>A</sub> does not accumulate and is not available as an acceptor in wild type E. coli cells because it is rapidly converted to Kdo<sub>2</sub>-lipid IV<sub>A</sub> by the bifunctional Kdo transferase (37). The relative inactivity of Kdo-lipid IV<sub>A</sub> as a substrate is not without precedent. The late E. coli acyltransferase, HtrB, similarly catalyzes efficient addition of laurate to Kdo<sub>2</sub>-lipid IV<sub>A</sub> but not to Kdo-lipid IV<sub>A</sub> (51).

The synthesis of Kdo<sub>2</sub>-lipid IV<sub>A</sub> proceeds in a defined, linear sequence of seven enzymatic reactions (1). After this point in the biosynthetic pathway, the late acyltransferases can function, the core can be built up from the proximal heptoses to the more distal sugars, and other substoichiometric modifications can be made (1). It has not been determined in what order these diverse reactions take place in living cells, or even if these reactions are ordered in vivo, since they can occur independently of each other in vitro. It should now be possible to examine the kinetics RfaC using lauroyl-Kdo<sub>2</sub>-lipid IV<sub>A</sub> and Kdo<sub>2</sub>-lipid A (which contains a myristate in addition to the laurate) as acceptors for mannose in place of Kdo<sub>2</sub>-lipid IV<sub>A</sub>. Previous studies with crude ADP-heptose isomers (22) isolated from S. sonnei suggested that Kdo<sub>2</sub>-lipid A might be a much better acceptor than Kdo-lipid IV<sub>A</sub>, but this data could not be quantified due to lack of the purified donor (52).

Under the conditions utilized in our experiments, heptosyltransferase I behaves as a peripheral membrane protein. In some studies, the activity was found to be membrane-associated in wild type cells (21), but in other studies with different buffers the activity partitioned mainly into the cytosol (17). In the overproducer, much of the activity was cytosolic (Table II), but purification of the cytosolic material was not attempted. The data are consistent with the proposal that the enzymes of core assembly function as peripheral membrane proteins at the cytoplasmic face of the inner membrane (4) where they have access to both their cytosolic sugar nucleotide substrates and their lipid acceptors. If a purification of RfaC in the absence of detergent could be devised, x-ray crystallography might be feasible. The further study of the cytosolic RfaC is of considerable importance, as very little structural information is available for glycosyltransferases, most of which are integral membrane proteins.

In previous work, when concentrated crude cytosols and partially purified ADP-heptose preparations (22) were used for the in vitro glycosylation of Kdo<sub>2</sub>-lipid IV<sub>A</sub>, two more hydrophilic products were generated, as judged by thin layer analysis. These likely correspond to the products of heptosyltransferase I (RfaC) and heptosyltransferase II (RfaF) (17). It appears that RfaF cannot efficiently utilize mannosyl-Kdo<sub>2</sub>-lipid IV<sub>A</sub> ADP-mannose, or both as substrates, since only one mannose residue is incorporated into Kdo<sub>2</sub>-lipid IV<sub>A</sub> in crude cell extracts when ADP-mannose is used as the donor (Fig. 9).

At present, RfaC is the last step in the E. coli LPS pathway that can be assayed quantitatively. However, we have now also shown that unfractionated low molecular weight extracts of various bacterial strains do support the modification of Kdo<sub>2</sub>-lipid IV<sub>A</sub> catalyzed by pure RfaC in what appears to be a single
glycosylation (Fig. 12). Wild type E. coli and S. typhimurium cells appear to contain ADP-\(\alpha\)-glycerophosphate-\(\delta\)-manno-heptose (or something like it), as do the rfaC mutants, D21f2 and SA1377 (Fig. 12). Mutants in rfaC cannot transfer the heptose to the lipid A acceptor, and accordingly they appear to accumulate much more of the donor substrate than wild type (Fig. 12, lanes 3 and 4). Mutants in the biosynthetic pathway for the formation of the putative ADP-\(\alpha\)-glycerophosphate-\(\delta\)-manno-heptose, such as SL3600 (\(\text{rfaD}\)) and SL1102 (\(\text{rfaE}\)), do not contain a competent donor pool (Fig. 12, lanes 5 and 6). The actual function of RfaE in the biosynthesis of ADP-\(\alpha\)-glycerophosphate-\(\delta\)-manno-heptose is unknown (1, 4). Mutants in \(\text{rfaD}\) should accumulate ADP-\(\alpha\)-glycerophosphate-\(\delta\)-manno-heptose (54), which apparently is a poor substrate for RfaC (Fig. 12, lane 5), consistent with the heptose-deficient LPS found in such strains (1, 4). The band shift assays shown in Fig. 12 will now finally permit the functional isolation and definitive structural characterization of the heptose donor(s) required for LPS assembly. The availability of these donors should enable to development of quantitative new assays for other putative heptosyltransferases, such as RfaF, RfaQ, and RfaK (1, 4).

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Enzymatic Synthesis of Lipopolysaccharide in *Escherichia coli* : PURIFICATION AND PROPERTIES OF HEPTOSYLTRANSFERASE I

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