The lipopolysaccharide of *Rhizobium leguminosarum* differs from that of other Gram-negative organisms. *R. leguminosarum* lipid A lacks phosphate groups, but it contains a galacturonic acid residue at the 4'-position and an aminoglucosamine moiety in place of the usual glucosamine 1-phosphate unit. *R. leguminosarum* lipid A is esterified with a peculiar long chain fatty acid, 27-hydroxyoctacosanoate, not found in enteric Gram-negative bacteria, and the inner core of *R. leguminosarum* mannose and galactose in place of heptose. Despite these differences, the biosynthesis of *R. leguminosarum* lipid A is initiated by the same seven enzyme pathway as in *Escherichia coli* (Raetz, C. R. H. (1993) J. Bacteriol. 175, 5745–5753) to form the phosphorylated precursor, (Kdo)₂-lipid IV₇A, which is then processed differently. We now describe several novel *Rhizobium*-specific enzymes that recognize and modify (Kdo)₂-lipid IV₇A, which is then processed differently. We now describe several novel *Rhizobium*-specific enzymes that recognize and modify (Kdo)₂-lipid IV₇A. The 1- and 4'-phosphatases were detected using (Kdo)₂-[³²P]-lipid IV₇A and (Kdo)₂-[⁴⁺³²P]-lipid IV₇A, respectively, as shown by release of ³²P. In the presence of GDP-mannose and/or UDP-galactose, membranes of *R. leguminosarum* first transferred mannose and then galactose to (Kdo)₂-[⁴⁺³²P]-lipid IV₇A. In addition, at least two hydrophobic metabolites were generated from (Kdo)₂-[⁴⁺³²P]-lipid IV₇A in a manner that was dependent upon both membranes and a cytosolic factor from *R. leguminosarum*. These compounds are attributed to novel acylations of (Kdo)₂-[⁴⁺³²P]-lipid IV₇A. *E. coli* membranes and cytosol did not catalyze any of the unique reactions detected in *R. leguminosarum* extracts. Our findings establish the conservation and versatility of (Kdo)₂-lipid IV₇A as a lipid A precursor in bacteria.

Lipopolysaccharides (LPSs), or endotoxins, comprise the outer leaflet of the outer membranes of Gram-negative bacteria (1–7). LPS consists of three covalently linked domains. These are lipid A, the hydrophobic portion of the molecule that anchors LPS in the membrane, a core oligosaccharide consisting of inner and outer regions, and a repeating 0-antigen. Biosynthesis of the lipid A portion is essential for cell viability (2, 3, 8, 9). In addition, lipid A is responsible for the toxic effects observed when LPS is introduced into the mammalian bloodstream (3, 5, 10–12). The endotoxin activity of lipid A results from the overproduction of cytokines by the immune system in response to lipid A (3, 5, 10–12). These biological effects of *Escherichia coli* lipid A (Fig. 1) require the presence of several key structural features: both phosphate groups, the glucosamine disaccharide, and all the fatty acyl chains, especially the aclyoyxacyl residues (3, 5, 10, 12).

Although lipid A varies slightly in structure among different animal pathogens (1, 13), the above hallmark structural features are generally conserved. However, the lipid A from the nitrogen-fixing bacterium *Rhizobium leguminosarum* differs strikingly from that of *E. coli* (14). It lacks the phosphate present in the 4'-phosphate and an aminoglucosamine moieties in place of the proximal glucosamine 1-phosphate (Fig. 1) (14). Preliminary structural studies suggest that it does not possess any acylxacyl residues (14), but instead, contains an unusual very long fatty acid, 27-hydroxyoctacosanoic acid (15). *R. leguminosarum* lipid A therefore lacks all the key structural features thought to be necessary for immunostimulation and toxicity in mammals (1, 3, 5, 10). As yet, the immunostimulatory properties of *R. leguminosarum* lipid A have not been explored.

In addition to its distinct lipid A moiety, *R. leguminosarum* also possesses a different core structure (Fig. 2) (16). While the Kdo region is likely to be identical to that of *E. coli* LPS (17), the inner core of *R. leguminosarum* contains mannose instead of heptose, as well as galactose and galacturonic acid residues (Fig. 2) (16).

Despite these marked differences in its LPS structure, *R. leguminosarum* extracts nevertheless contain all seven enzymes previously identified in *E. coli* for the biosynthesis of (Kdo)₂-lipid IV₇A, an important precursor of LPS (Fig. 3) (17). The fact that *R. leguminosarum* and *E. coli* make the same intermediate en route to LPS suggests that *R. leguminosarum* contains other enzymes, not present in *E. coli*, for the further processing of (Kdo)₂-lipid IV₇A to its own unique lipid A. We have previously reported a 4'-phosphatase activity in extracts of *R. leguminosarum* that acts on (Kdo)₂-lipid IV₇A (18). We now present evidence for a 1-phosphatase, a mannosyl transferase, a galactosyl transferase, and a long chain acyl transferase. All these transformations of (Kdo)₂-lipid IV₇A occur in extracts of *R. leguminosarum*, but not of *E. coli*. The availability of these new enzymes will facilitate the preparation of novel endotoxin analogs for studies of lipid A activation of animal cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—[³²P]ATP and [³²P]P were products of DuPont NEN. Sugar nucleotides, nucleotide triphosphates, dithiothreitol, PEPES, MES, and Kdo were obtained from Sigma. Other items were purchased from the following companies: Pierce Chemical Co. (Triton X-100 and bicinchoninic acid); Difco Laboratories (yeast extract and tryptone); EM Science (Silica Gel-60 thin layer plates, 0.25 mm); and Amicon (Centri centrifugation devices). Chloroform, pyridine, and acetic acid were Mallinkrodt reagent grade.

**Bacterial Strains and Growth Conditions**—*R. leguminosarum* biovar
viciae 8401 was obtained from J. A. Downie (John Innes Institute, Norwich, United Kingdom). R. leguminosarum biovar phaseoli CE3 was a gift of D. Noel (Marquette University, Milwaukee, WI). This strain was recently reclassified as Rhizobium etli (20). Rhizobium meliloti 1021 was obtained from Sharon Long (Stanford University). E. coli K12 strain R477 has been used for previously studies of lipid A biosynthesis in our laboratory (21). All rhizobia were grown on TY medium (17), containing 5 g of tryptone and 3 g of yeast extract per liter, and 10 mM CaCl2. Rhizobia were selected with 20 µg/ml nalidixic acid and 200 µg/ml streptomycin, when appropriate. All strains were grown at 30 °C.

Preparation of Cell-free Extracts—Bacterial cultures were harvested in late logarithmic phase (A550nm = 0.6–1.0) by centrifugation at 8,000 × g for 15 min, and the cell pellet was resuspended in 50 mM HEPEs, pH 7.5, to give a final protein concentration of 5–15 mg/ml. The cells were broken by passage through a French pressure cell at 18,000 p.s.i. Unbroken cells and debris were removed by another centrifugation at 8,000 × g for 15 min. Extracts were prepared and handled at 0–4 °C. Protein concentrations were determined with biocinchonic acid (22), using bovine serum albumin for the standard curve.

Subcellular fractions were prepared by centrifugation of the crude extract in 25 mM HEPEs, pH 7.5, at 150,000 × g for 60 min. The supernatant was removed by aspiration. The supernatant was centrifuged a second time, and the second small pellet of residual membranes was discarded. The first membrane pellet was resuspended in the original volume of buffer. The resuspended membranes were centrifuged a second time, and the buffer wash was discarded. The final washed membranes were resuspended in ~20% of the original volume of crude extract from which they were made. The final preparations are referred to as the “cytosol” and the “washed membranes” in subsequent experiments.

Preparation of Radioactive Substrates—(Kdo)2-[4-32P]-lipid IVα and (Kdo)2-[1-32P]-lipid IVα were prepared as described (18), and stored at −20 °C. Prior to use, the aqueous lipid dispersions were subjected to sonic irradiation in a bath sonicator for 2 min. (Kdo)2-[4-32P]-lipid IVα was made at high specific radioactivity (105 cpm/nmol) (23), and was diluted with non-radioactive (Kdo)2-lipid IVα, prepared as described (24), to an activity of 20,000–50,000 cpm/nmol in the assay mixture. (Kdo)2-[1-32P]-lipid IVα was prepared at a lower specific radioactivity (18) and was usually diluted further.

Assays of (Kdo)2-lipid IVα Metabolism—Reaction mixtures contained 50 mM MES, pH 6.5, 0.5% Triton X-100, 10 mM dithiothreitol, 10 mM NaCl, and crude cell-free extracts or subcellular fractions, as indicated, in a 10-µl final volume. The reactions were incubated for 60 min at 30 °C, and 5-µl samples were withdrawn and applied to thin-layer chromatography plates. After drying the spots with a cool air stream, the plates were developed in CHCl3:pyridine, 88% formic acid, water (30:70:16:1, v/v). Exposure to imaging screens at room temperature or to x-ray film at −80 °C was carried out overnight. Extent of conversion of substrate to product(s) was measured using a Molecular Dynamics PhosphorImager operated with ImageQuant software.

Size Fractionation of the Cytosol—A membrane-free cytosol was prepared as described above, except that during growth the cells were supplemented with 0.2% glucose and harvested at A550nm of approximately 2.5. This was done to increase the number of cells, but it had no effect on the biochemical activities present in the cytosol. Approximately 10 ml of cytosol (12.1 mg/ml protein) was applied to a total of eight Centricon C50 units, which were then centrifuged at 10,000 × g for 60 min. The units were centrifuged for about 4 h at 10,000 × g at 4 °C, according to manufacturer’s directions, until 7.5 ml (containing 2.0 mg/ml protein) had emerged. Theoretical, this fraction of the cytosol should contain proteins less than 100,000 daltons in size, and is referred to as “C50 filtrate.” About 7.0 ml of this material was applied to four Centricon C50 units, which were then centrifuged at 8,000 × g for 15 min. The “C50 retentate” (1.0 ml, 6.9 mg) which should contain proteins with native molecular masses between 50 and 100 kDa, was removed and stored in aliquots at −80 °C. The retentate is concentrated about 7 times on the basis of volume, as compared to the initial cytosol. Small molecules should not have become concentrated in the retentate, but should be at the same concentration as in the initial cytosol. The C50 filtrate (3.8 ml, 4.1 mg) presumably contains proteins less than 50 kDa in size, as well as other small molecules.

RESULTS

Rationale for Probing the Metabolism of (Kdo)2-lipid IVα in Extracts of R. leguminosarum—We have previously identified a key late intermediate in E. coli LPS biosynthesis, designated (Kdo)2-lipid IVα (Fig. 3) (24). In E. coli, (Kdo)2-lipid IVα is an acceptor for both the lauryl and the myristoyl moieties that are present in aciloxaeyl linkage (Fig. 1), as well as for the heptose residues of the inner core (Fig. 2) (25–27). Since (Kdo)2-lipid IVα is also generated in extracts of Rhizobium (17), it is a plausible substrate for several Rhizobium-specific lipid A modifications (Figs. 1–3). One of these Rhizobium-specific reactions, a 4′-phosphatase (18), has already been identified. Here, we investigate whether or not Rhizobium-specific reactions might exist that recognize (Kdo)2-lipid IVα as a substrate (Fig. 3).

Conversion of (Kdo)2-lipid IVα to Several Novel Products with or without GDP-Mannose and UDP-Galactose—In the absence of added sugar nucleotides, (Kdo)2-[4-32P]-lipid IVα is rapidly converted to several products when incubated with crude R. leguminosarum extracts. A major product is 32P α, which is produced by the 4′-phosphatase (Fig. 4, lane 2) (18). The lipid product generated by the 4′-phosphatase from (Kdo)2-[4-32P]-lipid IVα is unlabeled, and therefore it is not seen in Fig. 4. However, under the conditions employed, several more hydrophobic bands are also observed (Fig. 4, lane 2). As shown below, we believe that the band labeled “a” and “a”’ is a mixture of (Kdo)2-[4-32P]-lipid IVα derivatives that either are further acylated (metabolite a) or are dephosphorylated at the 1-position (metabolite a’). These can be resolved from each other by
modifying the conditions of thin layer chromatography. Small amounts of an additional, very hydrophobic compound designated a* can also be seen (Fig. 4, lane 2).

To look for core sugar extensions beyond Kdo, (Kdo)\(_2\)-lipid IV\(_A\) was incubated with crude extracts of \(R.\ leguminosarum\) 8401 and the potential sugar donors, GDP-mannose and/or UDP-galactose (Fig. 4, lanes 3–5). In the presence of GDP-mannose, both the (Kdo)\(_2\)-lipid IV\(_A\) and its more hydrophobic derivative(s) shifted to more slowly-migrating positions (Fig. 4, lane 3, metabolites b and b'), consistent with the addition of a mannose residue. In the presence of UDP-galactose, no additional metabolites of (Kdo)\(_2\)-lipid IV\(_A\), beyond those formed by the crude extract alone, were observed (Fig. 4, lane 4 compared to lane 2). When both sugar nucleotide donors were present, however, an additional, even more slowly migrating derivative of (Kdo)\(_2\)-lipid IV\(_A\) was generated (Fig. 4, lane 5, metabolite c).

We postulate that metabolite b represents the addition of mannose to (Kdo)\(_2\)-lipid IV\(_A\), and c represents the further addition of galactose to mannosyl-(Kdo)\(_2\)-lipid IV\(_A\). These results support the proposed core structure of \(R.\ leguminosarum\) LFS (Fig. 2), according to which galactose in the presence of a bifunctional Kdo transferase in extracts of \(R.\ leguminosarum\), as in \(E.\ coli\) (17). Heptose (\(t\)-glycerol-\(t\)-manno-heptose) is absent in the proposed structure of the \(R.\ leguminosarum\) core, and it is replaced with mannose and galactose (16). In addition, the \(R.\ leguminosarum\) inner core is rich in galacturonic acid (16), which is absent in \(E.\ coli\). Additional sub-stoichiometric modifications of the inner core of \(E.\ coli\) with phosphate, ethanolamine phosphates, or additional sugars (3, 5) are not indicated.

An additional advantage of using (Kdo)\(_2\)-lipid IV\(_A\) as the substrate is that the lipid product generated by the 4'-phosphatase reaction can now be visualized. Thus, the prominent, more rapidly migrating derivative of (Kdo)\(_2\)-lipid IV\(_A\) observed in lane 2 of Fig. 5 (metabolite p) mainly reflects the action of the 4'-phosphatase (18). As noted above, metabolite p cannot be detected in the experiment of Fig. 4, since p is not radioactive after (Kdo)\(_2\)-lipid IV\(_A\) has been dephosphorylated at position 4'. Metabolites a and p are distinct monodephosphorylated isomers of (Kdo)\(_2\)-lipid IV\(_A\) that happen to migrate with about the same Kdo domain, since it also dephosphorylated earlier precursors in the lipid A pathway, such as lipid IV\(_A\) and lipid X (1–3) (data not shown).

In the presence of GDP-mannose, or GDP-mannose plus UDP-galactose (Fig. 5, lanes 4 and 5), several more slowly migrating metabolites are formed from (Kdo)\(_2\)-lipid IV\(_A\) (designated q, b, and c), similar to what is seen with (Kdo)\(_2\)-lipid IV\(_A\) (Fig. 4). When only UDP-galactose is added as the co-substrate with (Kdo)\(_2\)-lipid IV\(_A\), no metabolites beyond those seen in lane 2 are formed (data not shown). These results support the conclusion, also arrived at with (Kdo)\(_2\)-lipid IV\(_A\), that first mannose and then galactose is transferred to (Kdo)\(_2\)-lipid IV\(_A\), consistent with the proposed core domain of \(R.\ leguminosarum\). The mannosylated derivations were designated a* and a**.
two phosphates are present (28, 29). In *R. meliloti* (Kdo)\(_2\)-[4-\(^{32}\)P]-lipid IV\(_A\) as the probe, crude extract of *R. leguminosarum* 8401 was present at 1.5 mg/ml. GDP-mannose and UDP-galactose were added at 1.0 mM, as indicated.

The mannosyl and galactosyl transferase activities, like the phosphatases, appeared in the washed membranes (Fig. 6, lanes 2–5), but not in the cytosol (Fig. 6, lanes 6–9). When washed membranes and cytosol were recombined (Fig. 6, lanes 10–13), however, significantly larger amounts of several more hydrophobic derivatives of (Kdo)\(_2\)-[4-\(^{32}\)P]-lipid IV\(_A\) were generated than when (Kdo)\(_2\)-[4-\(^{32}\)P]-lipid IV\(_A\) was incubated with membranes alone. This result is seen most clearly in Fig. 6, lanes 14–19, in the absence of added sugar nucleotides. When both membranes and cytosol were used (Fig. 6, lanes 18 and 19), there was a substantial increase in the amounts of at least two more hydrophobic metabolites (a’/a’ and a”) of (Kdo)\(_2\)-[4-\(^{32}\)P]-lipid IV\(_A\), as compared to assays in which membranes alone or cytosol alone were used (Fig. 6, lanes 14–17). The most hydrophobic of these metabolites (a”) was not detected at all in the incubations containing membranes or cytosol alone (Fig. 6, lanes 1–9). Its formation was also stimulated severalfold by the presence of 1 mM ATP (Fig. 6, lane 19).

**Size Fractionation of the Cytosolic Factor Required for the Generation of the Hydrophobic Derivatives**—We wanted to investigate further the formation of the more hydrophobic metabolites of (Kdo)\(_2\)-[4-\(^{32}\)P]-lipid IV\(_A\) involving the interaction of the membranes and the cytosol of *R. leguminosarum* (Fig. 6). The whole cytosol was first fractionated by centrifugation through a 100-kDa sizing membrane, as described under “Experimental Procedures.” The filtrate was then centrifuged through a 50-kDa membrane, concentrating the soluble macromolecules that range in size from approximately 50 to 100 kDa by about 7-fold relative to the unfraccionated cytosol.

Next, (Kdo)\(_2\)-[4-\(^{32}\)P]-lipid IV\(_A\) was incubated with washed membranes and various size-fractionated pools of the cytosol (Fig. 7). In this reconstituted system, in which more extensive conversion of substrate to hydrophobic products was possible, one could discern two closely migrating substances just above (Kdo)\(_2\)-[4-\(^{32}\)P]-lipid IV\(_A\) (designated a and a’), and one sub-
stantially higher migrating product (a'). In the earlier chromatograms (for instance in Figs. 4 and 6), these two closely migrating compounds (a and a*) had not been resolved from one another. Separation was enhanced in the experiment of Fig. 7 by using chromatography solvent that was 2–3 days old and by developing the chromatogram all the way to the top of the plate. Thus, we found that what appeared initially as a single band in Figs. 4 and 6 was actually a combination of two substances (a and a* in Fig. 7), which were produced in varying amounts depending on the exact assay conditions.

Membranes alone (Fig. 7, lane 2) produced only product a' from (Kdo)_2-[4'-32P]-lipid IV_A. Whole cytosol alone was inactive, as were the two size-fractionated pools of the cytosol (Fig. 7, lanes 3–5). However, when combined, the washed membranes and the cytosol produced all three hydrophobic metabolites (a, a*, and a') in the presence of both washed membranes and cytosol. The active component of the cytosol ran through a 100-kDa molecular filtration device. However, it was retained and concentrated by a 50-kDa filtration membrane (Fig. 7, lanes 6 and 7 versus lane 8).

We suggest that metabolite a' in Fig. 7 is a derivative of (Kdo)_2-[4'-32P]-lipid IV_A that is dephosphorylated at position 1. Metabolite a contains a distinct modification that makes it more hydrophobic than (Kdo)_2-[4'-32P]-lipid IV_A. Formation of

FIG. 7. Both membranes and a cytosolic component are required for conversion of (Kdo)_2-[4'-32P]-lipid IV_A to certain more hydrophobic metabolites. Assay conditions were as described under "Experimental Procedures" with washed membranes of 8401 at 0.5 mg/ml and various cytosolic fractions, as indicated. Lane 1, no added membranes or cytosol; lane 2, membranes only; lane 3, 1.2 mg/ml whole cytosol only; lane 4, 1.0 mg/ml C100 filtrate of the cytosol only; lane 5, 1.0 mg/ml C50 retentate of the cytosol only; lane 6, membranes plus 1.2 mg/ml whole cytosol; lane 7, membranes plus 1.0 mg/ml C100 filtrate of the cytosol; lane 8, membranes plus 1.0 mg/ml C50 retentate of the cytosol. More rapidly migrating metabolites of (Kdo)_2-[4'-32P]-lipid IV_A are designated a, a', and a'. The C50 filtrate was inactive either with or without washed membranes.

FIG. 8. Transformations of (Kdo)_2-[4'-32P]-lipid IV_A observed in extracts of 8401 but not of E. coli. Assay conditions were as described under "Experimental Procedures," using 10 mM (Kdo)_2-[4'-32P]-lipid IV_A as the probe. Crude extracts of 8401 or E. coli R747 were present at 2.0 mg/ml, and ATP, GDP-mannose, and UDP-galactose were added at 1.0 mM, as indicated. These reactions were incubated for 30 min.
metabolite a reflects a novel acylation of (Kdo)₂-[4'-32P]-lipid IVₐ with a long fatty acid. Mild alkaline hydrolysis of metabolite a with 0.2 M NaOH results in the formation of a decaclylation product that is the same as the one that is obtained by alkaline hydrolysis of (Kdo)₂-[4'-32P]-lipid IVₐ. This observation is consistent with the presence of an additional ester-linked acyl chain in metabolite a. Furthermore, metabolite a migrates significantly faster than monolauroyl-(Kdo)₂-

**DISCUSSION**

An overview of the proposed reactions that account for the processing of (Kdo)₂-lipid IVₐ observed in extracts of *R. leguminosarum* is shown in Figs. 3 and 9. In addition to the 4'-phosphatase (18), the present work provides evidence for the following *Rhizobium*-specific enzymes: 1) a mannosyl and a galactosyl transferase that act sequentially on (Kdo)₂-lipid IVₐ (Figs. 4, 5, and 6). The mannosyl transferase activity is unique, macromolecular long chain acyl donor. Following this reasoning, metabolite a in Fig. 7 might be (Kdo)₂-[4'-32P]-lipid IVₐ that is then dephosphorylated at the 1-position and acylated with a long chain fatty acid.

**Transformations of (Kdo)₂-[4'-32P]-lipid IVₐ Observed in Extracts of *R. leguminosarum* Do Not Occur in *E. coli* Extracts—To determine if the reactions observed in *R. leguminosarum* extracts are also present in *E. coli*, (Kdo)₂-[4'-32P]-lipid IVₐ was incubated with extracts of *E. coli* using the same assay conditions developed above for *R. leguminosarum* (Fig. 8). A typical pattern of metabolites, both with and without ATP, GDP-mannose, and UDP-galactose, was observed with *R. leguminosarum* (Fig. 8, lanes 3 and 4), and all of the (Kdo)₂-[4'-32P]-lipid IVₐ was consumed in the complete system (Fig. 8, lane 3). In contrast, no derivatives of (Kdo)₂-[4'-32P]-lipid IVₐ were observed when matched *E. coli* extracts were substituted (lane 2). These results establish that the metabolites described here represent novel enzymatic transformations of (Kdo)₂-[4'-32P]-lipid IVₐ that could not have been observed previously using the *E. coli* system (2, 3).

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3 Brozek, K. A., Carlson, R. W., and Raetz, C. R. H. (1996) *J. Biol. Chem.* 271, 32126–32136.
lipid IV\textsubscript{A} is a key, generally conserved intermediate of LPS assembly in diverse microbial systems.

Several additional enzymes besides the ones that we have identified (Figs. 3 and 9) must exist in \textit{R. leguminosarum} to generate its unique lipid A. For instance, there must be an enzyme that transfers galacturonic acid to the 4′-position after dephosphorylation. So far, attempts to demonstrate galacturonic acid transfer from UDP-galacturonic acid to 4′-dephosphorylated (Kdo)\textsubscript{2}-lipid IV\textsubscript{A} (metabolite \textbf{p} of Fig. 9) have not been successful. There must also be enzymatic mechanisms for the oxidation of the 1-position of the lipid A disaccharide (Fig. 1) to generate the aminogluconate moiety. Attempts to demonstrate oxidation following 1-dephosphorylation of (Kdo)\textsubscript{2}-lipid IV\textsubscript{A} (i.e. of metabolite \textbf{a′} of Fig. 9) have not yielded positive results. An enzymatic precedent for the oxidation of the anomic carbon at the reducing end of an oligosaccharide has been reported in the case of cellobiose dehydrogenase (30).

To study the relationship between the function of \textit{Rhizobium} LPS and its special structural features, the isolation of mutants in the new enzymes that we have discovered will be required. Such mutants might have a lipopolysaccharide structure more closely resembling that of \textit{E. coli}. The reasons for the special set of enzymes that we have found in \textit{Rhizobium} might become clear by characterizing such mutants. Special structural modifications of lipid A might be required for root hair infection, nodule formation, or maintenance of symbiosis (31). Perhaps, some plants can mount an “immune” response to bacteria containing phosphorylated lipid A residues.

In parallel with purification and mutant isolation, we intend to clone the genes encoding the enzymes that we have discovered and to express them in \textit{E. coli}. In this way it may be possible to modify the structure of lipid A in living cells of \textit{E. coli}. It will be very interesting to determine whether or not \textit{E. coli} can grow with lipid A having the unusual structural features associated with \textit{R. leguminosarum}. The approach of modifying lipid A structure in living cells may provide new insights into the biological functions of lipid A and the assembly of outer membranes.

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