Expression Cloning and Characterization of the C28 Acyltransferase of Lipid A Biosynthesis in Rhizobium leguminosarum*

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An unusual feature of lipid A from plant endosymbionts of the Rhizobiaceae family is the presence of a 27-hydroxoyctosanolic acid (C28) moiety. An enzyme that incorporates this acyl chain is present in extracts of Rhizobium leguminosarum, Rhizobium etli, and Sinorhizobium meliloti but not Escherichia coli. The enzyme transfers 27-hydroxoyctosanolate from a specialized acyl carrier protein (AcpXL) to the precursor Kdo2 ((3-deoxy-D-manno-octulosonic acid)-lipid IVa). We now report the identification of five hybrid cosmid that direct the overexpression of this activity by screening ~4000 lysates of individual colonies of an R. leguminosarum 3841 genomic DNA library in the host strain S. meliloti 1021. In these heterologous constructs, both the C28 acyltransferase and C28-AcpXL are overproduced. Sequencing of a 9-kb insert from cosmids pSSB-1, which is also present in the other cosmid, shows that the phosphatase activity, as well as proteins involved in the production of the C28 acyltransferase, which is C28-AcpXL-dependent and utilizes (3-deoxy-D-manno-octulosonic acid)-lipid IVa, as the acceptor. The activity is close to each other but not contiguous. Nine other open reading frames around lpxXL were also sequenced. Four of these encode orthologs of fatty acid and/or polyketide biosynthetic enzymes. AcpXL purified from S. meliloti expressing pSSB-1 is fully acylated, mainly with 27-hydroxoyctosanolate. Expression of lpxXL in E. coli behind a T7 promoter results in overproduction in vitro of the expected R. leguminosarum acyltransferase, which is C28-AcpXL-dependent and utilizes (3-deoxy-D-manno-octulosonic acid)-lipid IVa as the acceptor. These findings confirm that lpxXL is the structural gene for the C28 acyltransferase. LpxXL is distantly related to the lauroyltransferase (LpxL) of R. etli for the C28 acyltransferase. LpxXL is distantly related to the lauroyltransferase of the Rhizobiaceae family is the presence of a 27-hydroxoyctosanolate chain (5) in acyloxyacyl linkage at position 2' (2, 3), modified with β-hydroxybutyrate; and 4) replacement in some species (2, 3) of the reducing end glucosamine unit with a 2-deoxy-2-aminoacolonic acid residue (4) (Fig. 1).

Whether or not the remarkable lipid A species of R. leguminosarum and R. etli play a role in root cell infection and nodulation is unknown. It has been proposed that lipid A of Gram-negative plant pathogens (which is similar to that of Escherichia coli) may activate the innate immune system of plants just as E. coli lipid A activates innate immunity in animals (2, 3). The R. leguminosarum and R. etli lipid A species lack the structural features (i.e., the phosphate groups and the proper secondary acyl chains) needed for stimulation of innate immunity in animals (Fig. 1) (2, 3, 6). Structural modification of lipid A might allow the endosymbionts to avoid activating the plant immune system, thereby promoting bacteroid survival.

To evaluate the function of lipid A during symbiosis, a structure-activity study would be informative. The construction of mutant strains with defined alterations in lipid A structure requires a detailed understanding of the enzymology and genetics of lipid A biosynthesis. In previous studies (7), we have shown that the first seven enzymes of the lipid A pathway (1, 8), which generate the key intermediate Kdo2-lipid IVa (Fig. 2) in E. coli, are also present in R. etli and R. leguminosarum. However, additional enzymes are present in R. etli and R. leguminosarum (9, 10) to catalyze alternative processing of Kdo2-lipid IVa. Unique enzymes of R. etli and R. leguminosarum reported so far include the following: 1) phosphatases to remove the 4'-1-phosphate moieties of Kdo2-lipid IVa (9, 11, 12); 2) a deacetylase to remove the ester-linked fatty acid at the 3-position (13, 13); 3) a unique long chain acyltransferase that uses the special acyl carrier protein AcpXL to incorporate the 27-hydroxoyctosanolic acid residue (Fig. 2) (10); and 4) a system for oxidizing the proximal glucosamine to aminglucosamine (3). Enzymes for incorporating the 4'-galacturonic acid and β-hydroxybutyrate residues remain to be identified.

We now describe the expression cloning of a 9-kb DNA fragment of R. leguminosarum that encodes the C28 acyltransferase, as well as proteins involved in the production of the C28 donor, ACP-XL. By assaying lysates (10) of individual members of a R. leguminosarum 3841 genomic cosmide library (14) harbored in Sinorhizobium meliloti 1021 (15), five clones overexpressing C28-acyltransferase activity were identified. The acyltransferase, which is detected using Kdo2-[4'32P]lipid IVa as

The abbreviations used are: Kdo2, (3-deoxy-D-manno-octulosonic acid)-lipid IVa, FPLC, fast protein liquid chromatography; HPLC, high pressure liquid chromatography; Mes, 4-morphlineethanesulfonic acid.

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the acceptor (12, 16) (Fig. 2), is membrane-bound, whereas the donor substrate C28-AcpXL is soluble (10). Sequencing of the relevant DNA insert, which is the same in all five isolates, shows clustering of the genes encoding AcpXL and the long chain fatty acyltransferase (LpxXL) together with four other proteins related to enzymes involved in the fatty acid elongation and/or polyketide biosynthesis. Mass spectrometry of the AcpXL, purified from one of the above clones, shows that it is fully acylated with a long chain fatty acid. The \textit{R. leguminosarum} \textit{lpxXL} gene can be overexpressed in \textit{E. coli} behind the T7 promoter, and the product is catalytically active. The availability of acpXL, lpxXL, and the other genes required to acylate AcpXL should facilitate the re-engineering of lipid A structures present in strains of \textit{E. coli} and \textit{Rhizobium}.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Materials—\textit{[^32P]}ATP was obtained from PerkinElmer Life Sciences. Silica Gel 60 (0.25-mm) thin layer plates were purchased from EM Separation Technologies. Triton X-100 and bicinechonic acid were purchased from either Sigma or Mallinckrodt. Restriction enzymes were purchased from Difco. All other chemicals were reagent grade and were obtained from either Sigma or Mallinckrodt.**

**Preparation of Cell-free Extracts and Membranes—**Mid-logarithmic phase cells were harvested by centrifugation at 8,000 \texttimes \text{g} for 15 min. All procedures were carried out at 0–4 °C. The cell pellets were resuspended in 50 mM Hepes, pH 7.5, to give a final protein concentration of 5–10 mg/ml. To make crude cell extracts, the cells were broken by two passages through a French pressure cell at 18,000 pounds/square inch, and the debris was removed by centrifugation at 8,000 \texttimes \text{g} for 15 min. Membranes were prepared from the crude cell extracts by centrifugation at 149,000 \texttimes \text{g} for 60 min. The membrane pellet was suspended in 50 mM Hepes, pH 7.5, at a protein concentration of ~5–10 mg/ml, and the washed membranes were collected by another centrifugation at 149,000 \texttimes \text{g} for 60 min. The washed membranes were again suspended in 50 mM Hepes, pH 7.5, at a protein concentration of ~5–10 mg/ml. The soluble fraction from the initial centrifugation of the crude cell extract at 149,000 \texttimes \text{g} for 60 min was centrifuged a second time to remove residual membranes. Samples of washed membranes and membrane-free cytosol were stored in aliquots at ~80 °C. Protein concentrations were determined using bicinchoninic acid were from Pierce. Yeast extract and tryptone were purchased from EM Separation Technologies. Triton X-100 and Biovar phaseoli were from New England Biolabs, PCR reagents from Stratagene, and enzymes were purchased from either Roche Molecular Biochemicals or Invitrogen. All other reagents and enzymes were purchased from either Roche Molecular Biochemicals or Invitrogen.

**Bacterial Strains and Growth Conditions—**\textit{E. coli} biovar phaseoli CE3, \textit{R. leguminosarum} 8401, and \textit{S. meliloti} 1021 (15) were described in previous studies (9, 11, 17). All \textit{Rhizobium} and \textit{Sinorhizobium} strains were grown at 30 °C in TY medium, which contain 5 g/liter tryptone, 3 g/liter yeast extract, 10 mM CaCl$_2$, and 20 \mu g/ml nalidixic acid. For the growth of strains CE3 and 1021, streptomycin (200 \mu g/ml) was also added to the medium. The clones and subclones in \textit{S. meliloti} 1021 were grown under additional selection with tetracycline (12.5 \mu g/ml). The \textit{Rhizobium} cells were grown to an 
\textit{A$_{600}$} of 0.8–1.2 before they were harvested.

Plasmid pET23b and \textit{E. coli} strain BLR(DE3)pLysS were purchased from Novagen. BLR(DE3)pLysS/pET23b and BLR(DE3)pLysS/pSSB-101 were grown from a single colony in 1 liter of LB medium (18) (10 g/liter tryptone, 5 g/liter yeast extract, and 10 g/liter NaCl containing 100 \mu g/ml ampicillin) at 37 °C until \textit{A$_{600}$} reached ~0.6. The cultures were then induced with 100 \mu g/ml isopropyl-1-thio-\beta-D-galactopyranoside and incubated with shaking at 225 rpm for an additional 3 h at 37 °C.

**Preparation of Cell-free Extracts and Membranes—**Mid-logarithmic phase cells were harvested by centrifugation at 8,000 \times \text{g} for 15 min. All procedures were carried out at 0–4 °C. The cell pellets were resuspended in 50 mM Hepes, pH 7.5, to give a final protein concentration of 5–10 mg/ml. To make crude cell extracts, the cells were broken by two passages through a French pressure cell at 18,000 pounds/square inch, and the debris was removed by centrifugation at 8,000 \times \text{g} for 15 min. Membranes were prepared from the crude cell extracts by centrifugation at 149,000 \times \text{g} for 60 min. The membrane pellet was suspended in 50 mM Hepes, pH 7.5, at a protein concentration of ~5–10 mg/ml, and the washed membranes were collected by another centrifugation at 149,000 \times \text{g} for 60 min. The washed membranes were again suspended in 50 mM Hepes, pH 7.5, at a protein concentration of ~5–10 mg/ml. The soluble fraction from the initial centrifugation of the crude cell extract at 149,000 \times \text{g} for 60 min was centrifuged a second time to remove residual membranes. Samples of washed membranes and membrane-free cytosol were stored in aliquots at ~80 °C. Protein concentrations were determined using bicinchoninic acid were from Pierce. Yeast extract and tryptone were purchased from EM Separation Technologies.
were determined by the bicinchoninic acid method (19) using bovine serum albumin as the standard.

**Preparation of Radiolabeled Substrates**—Kdo₂-[⁴⁻³²P]-lipid IV₆, Kdo₂-[¹⁻³²P]-lipid IV₆, and [⁴⁻³²P]-lipid IV₆ were prepared using the *E. coli* 4'-kinase and the appropriate Kdo transferase, as described previously (12, 20). Aqueous dispersions of these lipids were stored at −20 °C, and prior to use they were subjected to sonic irradiation in a bath sonicator for 90 s.

**Assay of the C₂₈ Acyltransferase**—The reaction was carried out in 10 µl under optimized conditions at 30 °C for 10 min (32, 35) in 50 mM Hepes, pH 8.2, 0.2% Triton X-100, and 10 µM Kdo₂-[⁴⁻³²P]-lipid IV₆ (20,000–50,000 cpm/µmol). Various protein fractions were added as indicated. Following the incubation, 4-µl samples were withdrawn and spotted directly onto thin layer chromatography plates that were developed in the solvent chloroform/pyridine/88% formic acid/water (30:30:1:1, v/v). The position of the reaction product was visualized by autoradiography and the radioactivity of the spots was quantified using a PhosphorImager, equipped with ImageQuant software.

Screening of *R. leguminosarum* Genomic Library for C₂₈ Acyltransferase Overproduction—The cosmids (pLAFR-1) (21) library of *R. leguminosarum* 3841 genomic DNA (∼20–25 kb) (14) in *E. coli* 803 host (22) was generously provided by Dr. J. Downie of the John Innes Institute, Norwich, UK. It was not possible to assay colony lysates of the *E. coli* host directly, because *R. leguminosarum* promoters are not recognized in *E. coli*. Accordingly, the entire library was first transferred into *S. meliloti* 1021 by tri-parental mating (23). *E. coli* strain 803 harboring the cosmids library served as the cosmid donor, whereas *E. coli* MT616 helper strain 803 harboring the cosmids library served as the cosmid donor, whereas *E. coli* MT616 helper strain served as the cosmid donor, whereas *E. coli* MT616 (24) (the helper strain) provided the transfer functions.

*S. meliloti* 1021 (the recipient) was grown on TY agar with nalidixic acid (20 µg/ml) and streptomycin (200 µg/ml) selection for 24 h. *E. coli* strain 803 harboring the cosmid library was grown on LB agar containing tetracycline (12.5 µg/ml) for 12 h, and the *E. coli* MT616 helper strain was grown on LB agar with chloramphenicol (30 µg/ml) for 12 h. The bacteria were then scraped off their respective plates and resuspended in TY medium (0.5 ml per plate) with no antibiotics. The *S. meliloti* 1021 (the recipient), the *E. coli* strain 803 harboring the cosmid library (donor), and the *E. coli* MT616 helper strain were mixed in a test tube in the approximate ratio of 3:1:1, v/v. A portion (0.5 ml per plate) of the mixture was then spread in a circular area (≈5 cm diameter) on TY agar plate without antibiotic selection. Mating was allowed to take place for 24–36 h at 30 °C. The mixture was scraped off the plate surface in TY medium containing 20% glycerol and then stored at −80 °C. The successful transfer of the cosmid library into *S. meliloti* 1021 was verified by analysis of the cosmids isolated from several randomly chosen transformed recipient colonies grown on a TY agar plate containing nalidixic acid (20 µg/ml), streptomycin (200 µg/ml), and tetracycline (12.5 µg/ml).

To examine a large number of *S. meliloti* 1021 cells harboring the individual members of the *R. leguminosarum* library in the pLAFR-1 cosmid, the glycerol stock of the mating mixture was thawed and appropriately diluted to obtain 50–100 colonies per TY agar plate, supplemented with nalidixic acid (20 µg/ml), streptomycin (200 µg/ml), and tetracycline (12.5 µg/ml). The plates were incubated for 72 h at 30 °C to obtain individual colonies. For large scale screening, several thousand such colonies were re-grown one at a time for 24 h with constant shaking at 30 °C in separate wells of 96-well microtiter plates in 150 µl of TY medium, containing nalidixic acid (20 µg/ml), streptomycin (200 µg/ml), and tetracycline (12.5 µg/ml). A 50-µl portion of the culture from each well was transferred to another microtiter plate and stored at −80 °C. The remaining cells were collected in each of the wells of the original microtiter plate by centrifugation at 3,660 × g for 15 min at 4 °C. The supernatants were decanted, and the cells were resuspended in 50 µl of 50 mM Hepes, pH 7.5. In order to generate concentrated lysates, the cells were broken by incubating with lysozyme (1 mg/ml) and EDTA (10 mM) for an hour at 4 °C (both added from concentrated stocks). Portions of the lysates (5 µl from each well) from four microtiter plates were combined to give 96 pools of four lysates in a fresh microtiter plate (20 µl per well final volume).

The pooled cell lysates were assayed for their ability to acylate Kdo₂-[⁴⁻³²P]-lipid IV₆ in the following manner. A fresh 96-well microtiter plate was prepared in which each well contained 2 µl of 250 mM Mes buffer, pH 6.5, 0.5% Triton X-100, 10 mM EDTA, 1.0 µM Kdo₂-[⁴⁻³²P]-lipid IV₆ and 8 µl of pooled cell lysate, which was added last to give final volume of 10 µl. These plates were incubated at 30 °C for 2 h, and a portion of each reaction mixture (5 µl) was then spotted onto a Silica Gel 60 thin layer chromatography plate (Fig. 3A, lanes 1–8). A negative control reaction without enzyme (Fig. 3A, left lane) and positive controls containing either an *R. leguminosarum* 3841 or an *S. meliloti* crude extract (prepared by passage of cells through a French pressure cell) as the enzyme source were also spotted on each plate. The *R. leguminosarum* 3841 lysate provided standards for the various metabolites generated from Kdo₂-[¹⁻³²P]-lipid IV₆ in extracts of *R. leguminosarum* (Fig. 3, lane R.L.). The plates were developed in the solvent chloroform/pyridine/88% formic acid/water (30:70:16:10, v/v) and exposed to an imaging screen overnight. An Amersham Biosciences Storm PhosphorImager, equipped with ImageQuant software, was used to detect metabolism of the Kdo₂-[¹⁻³²P]-lipid IV₆ substrate to either more hydrophilic or more hydrophobic products. *S. meliloti* 1021/pS581 was representative of several hybrid cosmids that directed expression of
The 6-kb pSSB-1 DNA insert was linearized using the Qiaex II gel extraction kit. All other techniques involving phosphatase, and T4 DNA ligase were used according to the manufacturer. Restriction endonucleases, shrimp alkaline phosphatase, and T4 DNA ligase were used according to the manufacturer. Restriction endonucleases, shrimp alkaline phosphatase, and T4 DNA ligase were used according to the manufacturer.

Construction of plasmids pSSB-4 and pSSB-101 from LpxXL, the R. leguminosarum Lipid A C28 Acyltransferase

Plasmids or cosmids were introduced into E. coli HB101 by tri-parental mating, as outlined above for the library screening. Different strains of E. coli served as plasmid donors, and HB101, verified by restriction digestion with EcoRI (6.0 and 4.0 kb), was then transformed into competent cells of E. coli BLRI (DE3)/pLyS8 (Novagen).

For hydrolysis at pH 4.5, 2 µl of each reaction mixture was combined with 18 µl of chloroform/methanol (2:1 v/v), and 2 µl of 1.25 M NaOH. The mixture was incubated at room temperature for 30 min, after which time 2 µl of 1.25 M HCl was added. After mixing, a 5-µl portion was applied to a thin layer plate.

For acid hydrolysis, 2 µl of each reaction mixture was combined with 18 µl of 0.1 M HCl in a 0.65-mL Microfuge tube, which was sealed with a clip. The tube was floated in a boiling water bath for 30 min. Next, 1.6 µl of 1.25 M NaOH and 2 µl of 10% SDS were added. A 5-µl sample was then applied to the thin layer plate.

For hydrolysis at pH 4.5, 2 µl of each reaction mixture was combined with 10 µl of 50 mM sodium acetate, pH 4.5, 2 µl of 10% SDS, and 3 µl of H$_2$O. The tube was heated in a boiling water bath as described above. Following the incubation, a 5-µl portion was applied directly to the thin layer plate. This plate, containing all the samples described above, was then developed in the solvent chloroform/pyridine/88% formic acid/water (30:70:16:10, v/v) and exposed to an imaging screen overnight.

Partial Purification of C28-AcpXL from Wild-type R. leguminosarum 8401—A membrane-free cytosol from R. leguminosarum 8401 (a 2-liter mid-log phase culture grown to an A$_{600}$ of 0.8) was used as the source of C28-AcpXL. Partial purification of C28-AcpXL was achieved using the first three steps of the procedure described by Brozek et al. (10): ultrafiltration through Centri-prep 100 and Centri-prep 50 membranes, heat treatment at 65 °C for 15 min, and DEAE-Sepharose column chromatography. Purification of C28-AcpXL from S. meliloti 1021/pSSB-1—The purification of C28-AcpXL from the overproducing strain S. meliloti 1021/pSSB-1 was carried out at 4 °C. A cytosolic fraction was prepared from S. meliloti 1021/pSSB-1 cells (2-l culture grown to an A$_{600}$ of 0.8). The cells were broken by two passages through a French pressure cell at ~5 mg/ml protein in 25 mM Hepes, pH 7.5, followed by centrifugation at

![Diagram of plasmid construction](https://via.placeholder.com/150)
149,000 \times g for 60 min. The membrane pellet was removed, and the ultracentrifugation was repeated with the supernatant to remove residual membrane fragments. The final cytosolic fraction, consisting of 135 mg of protein in 40 ml, was applied to a 15-ml DEAE-Sepharose column (Amersham Biosciences) that was equilibrated in 25 mM Hepes, pH 7.5. The flow-through was collected and re-circulated once through the column. Unbound proteins were then washed out with 25 mM Hepes, pH 7.5, containing 0.1 M NaCl, until \( A_{280} \) returned to baseline. Elution was carried out with a 400-ml linear gradient from 0.1 to 0.5 M NaCl in 25 mM Hepes, pH 7.5. The fractions (4-ml each) were analyzed by native PAGE and also assayed for their ability to stimulate long chain acyltransferase activity together with washed membranes of \( R. \) leguminosarum 3841 as the source of long chain acyltransferase. The active fractions were pooled (16 ml total), de-salted, and concentrated (25-fold) by centrifugation (3,660 \times g) in a Millipore Ultrafree-15 centrifugal filter device (Biomax-30K NMWL membrane) at 4 °C. The concentrated material was diluted to 4 ml (4.0 mg of protein/ml) with 25 mM Hepes, pH 7.5, and used for the next purification step.

The second anion-exchange chromatography was carried out with FPLC (Amersham Biosciences). The concentrated active fraction from the DEAE-Sepharose column was loaded onto an 8-ml Source-Q15 FPLC column (protein capacity 25 mg/ml and maximum pressure 750 pounds/square inch) (Amersham Biosciences), pre-equilibrated with 25 mM Hepes, pH 7.5. A flow rate of 3 ml/min was maintained during the chromatography. The column was washed for 10 min with 25 mM Hepes, pH 7.5, to remove unbound proteins, followed by a wash with 0.1...
m NaCl in 25 mM Hepes, pH 7.5, for 40 min. The elution of proteins was carried out with a linear gradient from 0.1 to 0.3 m NaCl in 25 mM Hepes, pH 7.5, for 160 min. Elution with 0.3 m NaCl was continued for another 40 min, followed by 0.5 m NaCl in 25 mM Hepes, pH 7.5, for 40 min. Four-mL fractions were collected. Fractions containing most of the AcpXL activity (total volume of 12 ml) were pooled and concentrated (−20 fold) by centrifugation (3,660 × g) in a Millipore Ultrafree-4 centrifugal filter device (BioMax-30K NMWL membrane) at 4 °C to a final volume of 0.6 ml (protein concentration of ∼1.0 mg/ml).

Size-exclusion chromatography was used as the final purification step. The FPLC system (Amersham Biosciences) with a Superdex-200 HR10/30 gel filtration column (Amersham Biosciences) was used for this purpose. The concentrated active fraction (0.5 ml) from the Source-Q15 column was loaded onto the Superdex column, which was pre-equilibrated with 25 mM Hepes, pH 7.5. The column was developed with the same buffer for 48 min. A constant flow rate of 0.5 ml/min was maintained, and 0.6-ml fractions were collected. The column fractions were analyzed as above. Active fractions were stored at −80 °C.

PAGE—Denaturing gel electrophoresis was carried out according to Laemmli but without SDS. Samples were not denatured or reduced prior to loading. Electrophoresis was performed at a constant current of 25 mA, using a Tris glycine buffer without SDS. Gels were stained with Coomassie Blue. Polyacrylamide gel electrophoresis in the presence of 2.5 M urea was used to analyze samples of ACP and AcpXL, as described previously (28).

N-terminal Sequencing and Mass Spectrometry of Purified AcpXL—A sample (∼10 µg in 300 µl) of purified AcpXL in 25 mM Hepes buffer, pH 7.5, was concentrated to 2-fold, and its buffer was exchanged with de-ionized water by using Microcon-30 device at 20,000 × g and 4 °C. The concentrated material was then diluted to the original volume with de-ionized water; the cycle was repeated three times. The final volume was 150 µl, and it contained 682 pmol of AcpXL, as estimated from the amino acid analysis of the sample.

N-terminal amino acid sequencing and mass spectrometry of purified AcpXL were carried out at the Harvard Microchemistry Facility by Dr. William S. Lane. The mass analysis was performed following a final fractionation on a 10-cm microcapillary system, packed with Perose 10R2 reverse-phase resin, that was interfaced with a Finnigan TSQ7000 triple quadrupole mass spectrometer, set up for electrospray ionization mass analysis (10, 29). Individual fractions from the microcapillary HPLC separation were analyzed. The uncharged intact molecular mass of the AcpXL was obtained by deconvolution of the spectrum.

RESULTS

Screening of a R. leguminosarum Genomic DNA Library for Kdo2-Lipid IVα-modifying Enzymes—Attempts to clone the R. leguminosarum long chain acyltransferase (Fig. 2) by PCR amplification of conserved sequences present in previously characterized lipid A late acyltransferase genes, such as lpxL or lpxM (30–32), or efforts to detect R. leguminosarum DNA restriction fragments by low stringency hybridization using E. coli lpxL as the probe, were unsuccessful. An expression cloning strategy was therefore developed. A cosmids library of R. leguminosarum 3841 genomic DNA (∼20–25-kb inserts) in pLAFR-1, harbored in E. coli 803, was transferred into S. meliloti 1021 by tri-parental mating. This was necessary because the E. coli transcription machinery does not efficiently recognize R. leguminosarum promoters. The screening procedure was designed to find clones directing the overexpression of the 4’-phosphatase, the 1-phosphatase, or the C28 late acyltransferase of R. leguminosarum (Fig. 3). Among extracts derived from ∼4000 individual clones, we were in fact able to identify five that greatly overexpressed apparent C28 acyltransferase activity, using Kdo2-[4’-32P]lipid IVα as the acceptor. A typical result is shown in Fig. 3, in which extract c of pool 8 represents the desired hit. C28 acyltransferase was overexpressed more than 100-fold above the S. meliloti vector background and was easily detectable, even under the suboptimal assay conditions of the screening assay (Fig. 3). A key factor that contributed to the robust overexpression of C28 acyltransferase activity is the close linkage of the C28 acyltransferase and the acpXL structural genes, as determined by DNA sequencing of the insert (see below).

No clones overexpressing the 4’-phosphatase or the 1-phosphatase were detected among the ∼4000 cosmid that were screened. These phosphatases may be toxic to S. meliloti, since its lipid A is phosphorylated, like that of E. coli (33).

The cosmid from the five clones directing the apparent overexpression the C28 acyltransferase were isolated. Based on their insert sizes (∼22-kb by EcoRI digestion) and restriction patterns (not shown), they appeared to be very similar. One of the clones was designated S. meliloti 1021/pSSB-1 and was further characterized. To confirm the screening results, crude extracts of late log phase cultures of S. meliloti 1021, S. meliloti 1021/pLAFR-1, S. meliloti 1021/pSSB-1, R. etli CE3, and R. leguminosarum 3841, prepared with a French press, were assayed under conditions optimized for C28 acyltransferase in wild-type cells. Comparable C28 acyltransferase activity was seen in all three parental strains (Fig. 5). The lipid A phosphatases were detected only in CE3 and 3841 extracts (Fig. 5). The vector pLAFR-1 had no effect on acyltransferase activity in S. meliloti 1021 extracts (Fig. 5, lanes 5 and 6 or lanes 11 and 12), but the hybrid cosmid pSSB-1-directed —100-fold overexpression of acyltransferase activity (Fig. 5, lanes 4 and 10), even though no exogenous C28-AcpXL (10) was provided. Nearly complete conversion of 10 µM Kdo2-[4’-32P]lipid IVα to the putative product Kdo2-[4’-32P]C28-lipid IVα was seen after only 10 min (Fig. 5, lane 10) at an extract concentration of 1.2 mg/ml.

Characterization of the S. meliloti 1021/pSSB-1 Reaction Product—The rapidly migrating product generated by extracts of S. meliloti 1021/pSSB-1 (Fig. 5, lanes 4 and 10) was subjected to hydrolysis under mild alkaline (0.1 m NaOH), strong acid (0.1 m HCl at 100 °C), or mild acid (pH 4.5 at 100 °C) conditions. As shown in Fig. 6A, both the substrate and the product collapsed to the same slowly migrating species following mild alkali hydrolysis, demonstrating that the putative C28 chain incorporated by extracts of S. meliloti 1021/pSSB-1 is attached to Kdo2-[4’-32P]lipid IVα via an ester linkage. Furthermore, the identical migration (Fig. 6A) following mild base treatment excludes the possibility of one dephosphorylation in extracts of S. meliloti 1021/pSSB-1. As indicated in Figs. 3 and 5, 1-dephospho- Kdo2-[4’-32P]lipid IVα migrates only slightly faster than Kdo2-[4’-32P]C28-lipid IVα. Hydrolysis of the product in 0.1 m HCl (34, 35) or 12.5 mM sodium acetate at 100 °C (36) (Fig. 6B) confirmed that the hydrophobic modification in the product generated by extracts of S. meliloti 1021/pSSB-1 is attached to the lipid IVα moiety and not to the Kdo or the 1-phosphate groups, which are both removed by hydrolysis in 0.1 m HCl. Furthermore, the C28 acyltransferase of S. meliloti 1021/pSSB-1 does not utilize [4’-32P]lipid IVα as a substrate (data not shown), consistent with the substrate specificity of other lipid A late acyltransferases, such as LpxL and LpxP (30, 37, 38). Taken together, the product analysis (Fig. 6) strongly suggests that S. meliloti 1021/pSSB-1 overexpresses only the C28 acyltransferase and not some other Kdo2-[4’-32P]lipid IVα-modifying enzyme.

Overexpression of Both AcpXL and LpxXL in S. meliloti 1021/pSSB-1—The long chain acyltransferase (LpxXL) of R. etli and R. leguminosarum is membrane-bound, but its donor substrate, C28-AcpXL, is cytoplasmic (10). Hence, the massive overexpression of the long chain acyltransferase activity seen in extracts of S. meliloti 1021/pSSB-1 (Fig. 5) in the absence of added C28-AcpXL suggested that pSSB-1 might encode both AcpXL and LpxXL or, alternatively, that the insert
of pSSB-1 up-regulates *S. meliloti* LpxXL, *S. meliloti* AcpXL, or both.

In order to address these possibilities, washed membranes (which are almost devoid of cytosolic proteins) and twice ultracentrifuged cytosol (which is largely depleted of membrane fragments) were assayed from *S. meliloti* 1021/pSSB-1 and *S. meliloti* 1021/pLAFR-1 (the vector control). The cytosol and membrane fractions were tested for their ability to support C28 acyltransferase activity by themselves or in various combinations (Fig. 7). As shown in lanes 1–5, cytosol or membranes by themselves displayed very little activity, with the *S. meliloti* 1021/pLAFR-1 fractions being virtually the same as the no enzyme control (lane 1). Importantly, the cytosol of *S. meliloti* 1021/pSSB-1 very efficiently stimulated LpxXL activity when incubated together with *S. meliloti* 1021/pLAFR-1 membranes, compared with *S. meliloti* 1021 pLAFR-1 cytosol (Fig. 7, lanes 8 and 9 versus lanes 12 and 13). Furthermore, the membranes of *S. meliloti* 1021/pSSB-1 were much more active than those of *S. meliloti* 1021/pLAFR-1, irrespective of the source of cytosol (Fig. 7, lanes 6, 7, 10, and 11 versus lanes 8, 9, 12, and 13). These findings strongly suggest that *S. meliloti* 1021/pSSB-1 overexpresses both a membrane-associated enzyme and a cytosolic factor needed for C28 acylation.

To confirm that LpxXL is overexpressed in *S. meliloti* 1021/pSSB-1, washed membranes from selected strains were assayed in the presence of excess added AcpXL, which was purified from *R. leguminosarum* 8401 through the DEAE-Sephacrose step, as described previously (10). About 5–7-fold higher LpxXL-specific activity (Table I) was detected in washed membranes of *S. meliloti* 1021/pSSB-1 versus matched membranes from various wild-type or vector control cells.

Fractionation of membranes from *S. meliloti* 1021/pSSB-1 by isopycnic sucrose density gradient centrifugation (39) revealed that the C28 acyltransferase is localized exclusively in the inner membrane, as in wild-type *R. leguminosarum* 3841 and *R. etli* CE3 (data not shown), consistent with the involvement of an acyl-ACP donor.

### Table I

| Strain                      | Specific activity (nmol/min/mg) |
|-----------------------------|---------------------------------|
| *R. etli* CE3               | 0.52                            |
| *R. leguminosarum* 3841     | 0.63                            |
| *S. meliloti* 1021          | 0.25                            |
| *S. meliloti* 1021/pSSB-1   | 1.69                            |
| *S. meliloti* 1021/pLAFR-1  | 0.23                            |

*Purification to Near-homogeneity of AcpXL Overexpressed in S. meliloti 1021/pSSB-1—PAGE of the cytosolic proteins from *S. meliloti* 1021, *R. leguminosarum* 3841, *R. etli* CE3, *S. meliloti* 1021/pLAFR-1, and *S. meliloti* 1021/pSSB-1 (Fig. 8A) revealed massive overexpression of a protein in *S. meliloti* 1021/pSSB-1 (lane 6) that migrated with a standard preparation of C28-AcpXL (as indicated by the lower arrow). The putative AcpXL band was barely visible in the other strains (Fig. 8A, lanes 1–4). In *S. meliloti* 1021/pLAFR-1 an unknown band migrating just above AcpXL is seen, but careful inspection of the gel shows that this unknown is also present in the cytosol of *S. meliloti* 1021/pSSB-1 and may therefore be expressed from the vector itself.

The massive overexpression of C28-AcpXL in *S. meliloti* 1021/pSSB-1 (Fig. 8A) facilitated the development of a simple purification procedure. Cytosol from *S. meliloti* 1021/pSSB-1 was loaded onto a DEAE-Sephacrose column, and the proteins were eluted with a linear salt gradient. AcpXL emerged in a very sharp peak at 250 mM NaCl. The active fractions were pooled, desalted, and loaded onto a Source Q-15 FPLC column. AcpXL bound strongly and eluted at 200 mM NaCl. The pooled active fraction was passed through Sephacryl-200 column, and...
AcpXL was eluted as a discrete peak. The native gel electrophoresis analysis of the pooled fractions after each step is shown in Fig. 8B and clearly demonstrates that the AcpXL preparation is nearly homogeneous.

The identity and purity of the final AcpXL preparation was confirmed by N-terminal sequencing. The predominant sequence was TATFDKVADIA, with a secondary sequence (MTATFDKVAD-I) attributed to a minor component in which the N-terminal methionine is retained, consistent with the amino acid sequence analysis.

The purified AcpXL from S. meliloti 1021/pSSB-1 is MRVTATFDKVA-DIA (15).

AcpXL Overexpressed in S. meliloti 1021/pSSB-1 Is Fully Modified with a C28 or C30 Hydroxyacyl Chain—The purified AcpXL was resolved into two major components by microcapillary reverse phase HPLC (Fig. 8C, peaks B and D), representing over 95% of the total, and two minor species (Fig. 8C, peaks A and D). Each species was analyzed by electrospray ionization mass spectrometry. The spectrum of component B is shown in Fig. 9. When the observed spectrum (Fig. 9A) is deconvoluted (Fig. 9B), the predominant molecular weight (10909.0) matches the calculated molecular weight \( M_r \) of 10909.4 for an AcpXL species that lacks its N-terminal methionine but is derivatized with a 4'-phosphopantetheine and a 27-hydroxyoctacosanoic acid (C28) residue. A minor species (Fig. 9) is 131 atomic mass units larger, corresponding to the same modified protein in which the N-terminal methionine is retained, consistent with the amino acid sequence analysis.

The predominant molecular weight \( M_r \) of 10937.0 in the deconvoluted spectrum of peak C in Fig. 8C (data not shown) matches the calculated molecular weight \( M_r \) of 10937.4 for an AcpXL species, lacking its N-terminal methionine but containing a 4'-phosphopantetheine and a 29-hydroxytriacontanoic acid (C30) moiety. The mass for the minor component A seen in Fig. 8C was estimated to be 10484.0 (data not shown), consistent with an AcpXL species, lacking the N-terminal methionine and containing an unmodified 4'-phosphopantetheine moiety. No reliable mass spectrum could be obtained for peak D (Fig. 8C).

Experiments, similar to those described above, had been carried out previously (10) with AcpXL purified from wild-type R. leguminosarum. The latter consists of at least seven major fractions with holo-AcpXL as the major component (~40% of the total) and AcpXL acylated with 27-hydroxyoctacosanoic acid (C28) as the second most abundant species (~35%) (10). In contrast, our recombinant AcpXL consists mainly (>95%) of two acylated species (C28 and C30). It appears that all the holo-AcpXL molecules synthesized in S. meliloti 1021/pSSB-1 are rapidly acylated with (ω-1)-hydroxylated C28 or C30 acyl chains.
An in vivo system for producing a fully acylated acyl carrier protein in relatively high yields is unprecedented. A plausible explanation is that the proteins responsible for loading and extending nascent acyl chains on AcpXL are co-expressed in S. meliloti 1021/pSSB-1. Indeed, a number of open reading frames that resemble enzymes involved in fatty acid or polyketide biosynthesis are located near lpxXL on the pSSB-1 insert (see below).

Subcloning of the lpxXL Gene from the ~22-kb Insert of pSSB-1—Four overlapping fragments of the ~22-kb R. leguminosarum genomic DNA insert present in pSSB-1 were subcloned using either EcoRI or HindIII digestion (Fig. 10) into the pRK404A shuttle vector (Fig. 4). The resulting plasmids (pSSB-2, pSSB-3, pSSB-4, and pSSB-5) (Fig. 10) were then individually transferred from E. coli to S. meliloti 1021 by tri-parental mating. Compared with wild-type S. meliloti 1021 extracts, only the crude cell lysates of S. meliloti 1021 harboring either pSSB-2 or pSSB-4 exhibited high levels of acyltransferase activity. Further analysis of these subclones was carried out with membrane-free cytosol preparations and washed membranes. These assays (data not shown) showed that S. meliloti 1021/pSSB-2 overexpresses AcpXL in its cytosol but does not overexpress the membrane-bound acyltransferase, whereas S. meliloti 1021/pSSB-4 overexpresses only the long chain acyltransferase in its membranes (consistent with Fig. 10).

Gene Sequencing in the Vicinity of lpxXL and acpXL—DNA sequencing of the inserts in pSSB-2 and pSSB-4, as well as the downstream region around orf6 in pSSB-1 (Fig. 10), revealed 11 putative open reading frames (Fig. 10). Tables II and III list some relevant homologues of the predicted polypeptides encoded by the 11 open reading frames (Fig. 10). The open reading frames orf114 and orf240 (Fig. 10) (Table III) had been described previously (10, 40). Comparison of the deduced amino acid sequence of orf114 with the translated genes from the data base did not reveal a statistically significant relationship to any other known protein. The orf240 gene product belongs to CRP/FNR family of transcriptional

**Fig. 9. Mass spectrometry of the major acyl-AcpXL component isolated from S. meliloti 1021/pSSB-1.** A shows the mass spectrum of the major AcpXL component resolved by microcapillary HPLC (peak B in Fig. 8C). B is the deconvoluted data from A, indicating the molecular weights of the two related acyl-AcpXL species present in peak B (Fig. 8C), differing by the presence or absence of the N-terminal methionine residue.
The specific activity of the recombinant C28 acyltransferase in E. coli BLR(DE3)/pLysS/pSSB-101 membranes is 42 nmol/min/mg, which seems low given the high apparent level of expression (Fig. 12A). Extracts of E. coli BLR(DE3)/pLysS cells, expressing pLpxXL behind the T7 promoter on plasmid pSSB-101, catalyze robust C28-AcpXL-dependent acylation of Kdo$_2$-32P-lipid IV$_A$ (Fig. 12B, lanes 4 and 5 versus lanes 6 and 7). The C28 acyltransferase activity in membranes of E. coli BLR(DE3)/pLysS/pSSB-101 has a pH optimum and a Triton X-100 requirement similar to that observed with the native C28 acyltransferase of R. leguminosarum (not shown). The recombinant enzyme is localized in the inner membrane, and the acylation reaction (Fig. 12B) is linearly dependent upon time and protein concentration (not shown). The recombinant acyltransferase requires the presence of two Kdo moieties in its acceptor substrate, as no activity is seen with lipid IV$_A$ or Kdo-lipid IV$_A$ (not shown). E. coli ACPs bearing 12–16-carbon acyl chains do not function as donors.

**TABLE II**

Selected R. leguminosarum LpxXL orthologues and related proteins in the NCBI non-redundant database

| Organism          | Homology$^a$ (Gaps) | E values |
|-------------------|---------------------|----------|
| A. tumefaciens$^b$| 227/254/309         | 10$^{-129}$ |
| S. meliloti        | 227/263/309         | 10$^{-136}$ |
| B. melitensis$^b$ | 178/215/303         | 3$\times$10$^{-85}$ |
| M. loti$^b$       | 169/211/297         | 8$\times$10$^{-95}$ |
| R. prowazekii      | 80/154/304 (15)     | 2$\times$10$^{-31}$ |
| C. trachomatis     | 84/119/309 (29)     | 9$\times$10$^{-9}$ |
| E. coli LpxL(MshB) | 70/109/265 (15)     | 2$\times$10$^{-6}$ |
| E. coli LpxL(HtrB) | 69/118/301 (31)     | $<10^{-2}$ |

$^a$ Homology is given as number of identities/number of positives/number of residues (including gaps) in the related segment when compared with R. leguminosarum LpxXL, a hypothetical protein of 311 amino acid residues. The number of gaps generally increases with increasing E values.

$^b$ These organisms contain an AcpXL orthologue and a cluster of open reading frames that are transcribed in the same direction. Although BLASTp searches with the predicted product of orf6 did not yield any obvious homologies to polypeptides of known function. Likewise, firm predictions of the biochemical functions of the proteins encoded by orf7 and orf8 were not possible.

**Heterologous Expression of LpxXL and Characterization of the Enzyme**—Unequivocal demonstration that pLpxXL encodes the long chain acyltransferase of R. leguminosarum is provided by heterologous expression in E. coli (Fig. 12A), extracts of which do not normally transfer the C28 chain of C28-AcpXL to Kdo$_2$-32P-lipid IV$_A$ (Fig. 12B, lanes 2 and 3). However, washed membranes of E. coli BLR(DE3)/pLysS cells, expressing pLpxXL behind the T7 promoter on plasmid pSSB-101, catalyze robust C28-AcpXL-dependent acylation of Kdo$_2$-32P-lipid IV$_A$ (Fig. 12B, lanes 4 and 5 versus lanes 6 and 7).
B. melitensis indicate that necessitating the expression cloning strategy shown in Fig. 3. lpxL interaction with primers designed from the containing a Kdo disaccharide (30, 31, 37, 38). However, at-
LpxP of E. coli resembles the late lipid A acyltransferases LpxL, LpxM, and 
ria (45, 46, 48) that contain AcpXL (Table II). Spectrometry of the purified recombinant acyl-AcpXL (Figs. 8 
crocapillary HPLC followed by electrospray ionization mass 
mcomplete for purification to near-homogeneity was devised (Fig. 8). Mi-
LpxXL acyltransferase formally re-
Orf114 (114) Hypothetical protein of unknown function, R. 
leguminosarum (114) S11952 100/100/114 10−85 
Orf240 (240) Probable Fnr-type transcriptional regulator, R. 
leguminosarum (240) P24290 100/100/240 10−126 
AcpXL/Orf10 (109) Specialized acyl carrier protein AcpXL, R. 
leguminosarum (92) AAC32203 100/100/92 10−38 
Orf1 (124) (3R)-Hydroxymyristoyl-ACP dehydratase, FabZ, E. coli 
(151) P21774 33/45/116 10−7 
Orf2 (401) 3-Oxoacyl-ACP synthase II (KAS II), FabF, E. coli 
(413) P39435 22/39/263 10−5 
Orf3 (428) 3-Oxoacyl-ACP synthase II (KAS II), FabF, E. coli 
(413) P39435 33/49/417 10−51 
Orf4 (346) Probable alcohol dehydrogenase, Aquifex aeolicus 
(343) AAC07327 39/57/276 10−43 
Putative NADPH:quinone oxidoreductase, Arabidopsis 
thaliana (324) BAB08996 36/62/250 10−30 
Orf5/LpxXL (311) Lipid A biosynthesis: Kdo2-laurolipid IVa 
acyltransferase, MabB (LpxM), E. coli (323) P24205 70/109/265 10−6 

DISCUSSION

As shown in Fig. 2, R. leguminosarum membranes contain a Kdo-dependent C28 acyltransferase that utilizes the conserved lipid A precursor Kdo2-lipid IVa as its acceptor (9, 10). The enzyme does not function with 12–16 carbon acyl-ACPs derived from E. coli but, instead, requires its own dedicated acyl carrier protein, termed AcpXL (10). By using an expression cloning strategy, we have now identified an R. leguminosarum genomic DNA fragment containing a cluster of open reading frames involved in the incorporation of 27-hydroxyoctacosanoate into lipid A. This fragment includes the structural genes for the C28 acyltransferase LpxXL, the acyl carrier protein variant AcpXL, and several enzymes that may be involved in the biosynthesis of the (ω-1)-hydroxylated C28 acyl chain (5) that is attached to AcpXL (10) (Table III). Similar clusters are present in the genomes of M. loti (47), S. meliloti (15), and other proteobacteria (45, 46, 48) that contain AcpXL (Table II).

The R. leguminosarum LpxXL acyltransferase formally resembles the late lipid A acyltransferases LpxL, LpxM, and LpxP of E. coli in its strong preference for acceptor substrates containing a Kdo disaccharide (30, 31, 37, 38). However, attempts to clone the C28 acyltransferase using PCR amplification with primers designed from the LpxL gene, or detection of R. leguminosarum genomic DNA fragments by Southern hybridization with lpxL derived probes, were all unsuccessful, necessitating the expression cloning strategy shown in Fig. 3. Direct protein sequence comparisons (Tables II and Fig. 11) indicate that lpxL and lpxXL encode very distant orthologues, which presumably share a common catalytic mechanism despite their differences with respect to acyl chain length and Acp subtype specificity (10, 30). The T7 construct (Fig. 12) that directs overexpression of R. leguminosarum C28 acyltrans-
fiberase activity in E. coli confirms that lpxXL is indeed the structural gene for the acyltransferase. This overproducing strain should also greatly facilitate the purification and character-
ization of the C28 acyltransferase.

Prior to the present work, the donor substrate for the C28 acyltransferase was not readily accessible, since the only source of C28-AcpXL was the cytosol of wild-type Rhizobium 
cells (10). However, large amounts of acyl-AcpXL were found to accumulate in S. meliloti 1021/pSSB-1, and a simple method for purification to near-homogeneity was devised (Fig. 8). Mi-
9) revealed negligible amounts (<0.5%) of free AcpXL. About 60% of the protein is acylated 27-hydroxyoctasanoate (C28) and 40% with 29-hydroxytriacanotate (C30).

The mass spectrometry of the recombinant acyl-AcpXL (Fig.
9) furthermore strongly suggests that the (ω-1)-hydroxy group is already in place prior to C28 transfer to Kdo2-lipid IVa (as proposed in Fig. 2). The stereochemistry and origin of the (ω-1)-hydroxyl group remains to be established. It is conceivable that this hydroxyl group is left intact during the initial steps in the biosynthesis of the long acyl chain on AcpXL. There may be a distinct set of enzymes that carry out the conversion of C2 to C4 on AcpXL, some of which might be encoded in the AcpXL/LpxXL gene cluster (Fig. 10). Alternatively, β-hydroxybutyrate may be transferred directly from β-hydroxybutyryl-
coenzyme A to free AcpXL. In either case, the dehydratases of the fatty acid biosynthesis cycle (41–43, 51) are apparently unable to act on the 4-carbon β-hydroxybutyryl chain intermediate attached to AcpXL.

As shown in Fig. 1, the (ω-1)-OH moiety of the C28 acyl chain present on mature R. leguminosarum or R. etli lipid A is itself further acylated with a β-hydroxybutyrate substituent (2, 3). However, there is no such moiety on the (ω-1)-OH moiety of the acyl-AcpXL isolated from wild-type R. leguminosarum cytosol or from S. meliloti 1021/pSSB-1. The origin of the pendant β-hydroxybutyrate group in R. leguminosarum lipid A (Fig. 1) is unknown. One interesting scenario is that the same β-hydroxybutyryl-AcpXL species that primes C28 biosynthesis also serves as the donor for the β-hydroxybutyrate cap.

Long acyl chains bearing a hydroxyl group at the ω-1-position are present in the lipid A molecules of all members of the Rhizobaceae family (5) so far examined. Similar oxygenated acyl chains are also found on lipid A species from a number of other Gram-negative facultative intracellular organisms, such as B. melitensis and Legionella pneumophila (52, 53). The biological significance of such oxygenated long acyl chains remains elusive. Given that the number, distribution, and length of the secondary acyl chains on a lipid A molecule often influence the extent of immune stimulation (or inhibition) (6, 54), it may be that the C28 secondary acyl chain of Rhizobium lipid A likewise modulates the plant innate immune response, perhaps thereby facilitating symbiosis.

Relatively low endotoxic activity is observed in animal sys-
tems with lipopolysaccharides from L. pneumophila and Bru-
cella abortus (55, 56). The long acyl chains of these lipopolysac-
Charides may interfere with toll receptor binding (57–59), or they may prevent digestion by host lipases (60). We have observed that the secondary 27-hydroxyoctacosanoate chain of \textit{R. leguminosarum} or \textit{R. etli} lipid A is not readily cleaved \textit{in vitro} by the mammalian acyloxyacyl hydrolase (60) (data not shown).

Cloning of the C28 acyltransferase and the other putative genes related to the biosynthesis of the \((\alpha-1)/H9275\)-hydroxylated long chain fatty acids on AcpXL paves the way for genetic studies of the biological significance of this unusual acyl chain. Characterization of the properties of \textit{Rhizobium} mutants lacking one or more of these genes will be of special interest with respect to the efficiency of root infection, nodulation, and nitrogen fixation. An evaluation of the immunostimulatory and biophysical properties of C28-containing lipid A species may further help to explain the occurrence of this unique structure in \textit{Rhizobium} lipid A. In preliminary experiments purified components B and D possess very low stimulatory activity in mouse cell cytokine production assays.\(^2\)

The existence of an unusual lipid A species in plant endosymbionts like \textit{R. leguminosarum} or \textit{R. etli} lipid A is not readily cleaved \textit{in vitro} by the mammalian acyloxyacyl hydrolase (60) (data not shown).

Cloning of the C28 acyltransferase and the other putative genes related to the biosynthesis of the \((\alpha-1)/H9252\)-hydroxylated long chain fatty acids on AcpXL paves the way for genetic studies of the biological significance of this unusual acyl chain. Characterization of the properties of \textit{Rhizobium} mutants lacking one or more of these genes will be of special interest with respect to the efficiency of root infection, nodulation, and nitrogen fixation. An evaluation of the immunostimulatory and biophysical properties of C28-containing lipid A species may further help to explain the occurrence of this unique structure in \textit{Rhizobium} lipid A. In preliminary experiments purified components B and D possess very low stimulatory activity in mouse cell cytokine production assays.\(^2\)

\(^2\) N. Que, S. Basu, C. Raetz, and S. Vogel, unpublished data.
they be distinguished from lipid A species introduced by plant pathogens or plant endosymbionts.

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