* Sinorhizobium meliloti acpXL Mutant Lacks the C28 Hydroxylated Fatty Acid Moiety of Lipid A and Does Not Express a Slow Migrating Form of Lipopolysaccharide*

Larissa A. Sharypova, Karsten Niehaus, Heiko Scheidle, Otto Holst§, and Anke Becker

From the Institute of Genetics, Biology VI, University of Bielefeld, Postfach 100131, Bielefeld D-33501, Germany and the §Division of Structural Biochemistry, Research Center Borstel, Center for Medicine and Biosciences, Borstel D-23845, Germany

Lipid A is the hydrophobic anchor of lipopolysaccharide (LPS) in the outer membrane of Gram-negative bacteria. Lipid A of all Rhizobiaceae is acylated with a long fatty acid chain, 27-hydroxyoctacosanoic acid. Biosynthesis of this long acyl substitution requires a special acyl carrier protein, AcpXL, which serves as a donor of C28 (ω-1)-hydroxylated fatty acid for acylation of rhizobial lipid A (Brozek, K.A., Carlson, R.W., and Raetz, C. R. (1996) J. Biol. Chem. 271, 32126–32136). To determine the biological function of the C28 acylation of lipid A, we constructed an acpXL mutant of Sinorhizobium meliloti strain 1021. Gas-liquid chromatography and mass spectrometry analysis of the fatty acid composition showed that the acpXL mutation indeed blocked C28 acylation of lipid A. SDS-PAGE analysis of acpXL mutant LPS revealed only a fast migrating band, rough LPS, whereas the parental strain 1021 manifested both rough and smooth LPS. Regardless of this, the LPS of parental and mutant strains had a similar sugar composition and exposed the same antigenic epitopes, implying that different electrophoretic profiles might account for different aggregation properties of LPS molecules with and without a long acyl chain. The acpXL mutant of strain 1021 displayed sensitivity to deoxycholate, delayed modulation of Medicago sativa, and a reduced competitive ability. However, nodules elicited by this mutant on roots of M. sativa and Medicago truncatula had a normal morphology and fixed nitrogen. Thus, the C28 fatty acid moiety of lipid A is not crucial, but it is beneficial for establishing an effective symbiosis with host plants. acpXL lies upstream from a cluster of five genes, including msbB (lpxXL), which might be also involved in biosynthesis and transfer of the C28 fatty acid to the lipid A precursor.

Soil bacteria of the family Rhizobiaceae, known as rhizobia, form a nitrogen-fixing symbiosis with leguminous plants. The rhizobia-legume symbiosis is a highly specific and complex developmental process in which both partners undergo differentiation in a concerted way. Bacteria induce the formation of nodules on roots of their host plants and colonize the root tissue as intracellular nitrogen-fixing bacteroids (1). Rhizobial cell surface polysaccharides play an important role during this process (2). LPS1 constitutes an integral part of the outer cell membrane of Gram-negative bacteria. It is therefore intimately involved in the formation of the plant-bacterial interface during symbiosis (3).

Among Rhizobiaceae, a complete LPS structure has only been resolved for Rhizobium etli CE3 and Rhizobium leguminosarum (4–8). Actually, these related bacterial species have identical lipid A core structures and different O antigens (5). The LPS of Sinorhizobium meliloti has been characterized only partially. Its chemical composition has been analyzed many times, but its structure is still unknown (9–13). The LPS of S. meliloti strains 1021 and 102F51 reveals a high content of acidic sugars, namely galacturonic acid, glucuronic acid, and Kdo (3-deoxy-o-manno-octulosonic acid) (11, 13). In addition, the LPS of S. meliloti strain 102F51 contains DHA (3-deoxy-2-heptulosonic acid), considered a component of the O chain (11, 13). However, DHA could be also a component of the outer core.

The LPS of Escherichia coli and other Enterobacteriaceae is also known as endotoxin because of its diverse pathophysiological effects displayed during the infection of animal hosts, such as cytokine production, inflammation, and shock (14, 15). Lipid A is primarily responsible for the endotoxic activity of LPS. Modifications of lipid A may significantly change its toxicity. The lipid A of E. coli and related Enterobacteriaceae is composed of a backbone of two glucosamines replaced by two phosphate groups at positions 1 and 4’, and four residues of R-3-hydroxymyristic acid (C14) in ester and amide linkage at positions 2, 2’, 3, and 3’ (Fig. 1). The hydroxymyristic residues at positions 2’ and 3’ are further replaced by laurate (C12) and myristate (C14) forming acyloxyacyl moieties (14).

Lipid A of rhizobia displays a number of remarkable differences from enteric lipid A. Regardless of significant variations of lipid A structures within the family of Rhizobiaceae there is one common feature that distinguishes their lipid A from that of Enterobacteriaceae: the presence of a long fatty acid chain, 27-hydroxyoctacosanoic acid (27-OH-C28:0) (4). It was detected in various members of this family, including species of Rhizobium, Sinorhizobium, Mesorhizobium, Bradyrhizobium, and Agrobacterium (4, 54, 55).

Fig. 1 shows one of six lipid A components identified in R. etli. All six components share the following structural fea*

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1 The abbreviations used are: LPS, lipopolysaccharide; BSTFA, bis(trimethylsilyl) trifluoroacetamide; DOC, deoxycholate; GC, gas-liquid chromatography; GUS’ and GUS, carriers and noncarriers of the β-glucuronidase gene, respectively; MS, mass spectrometry; R-LPS, rough LPS; S-LPS, smooth LPS.

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1 To whom correspondence should be addressed: Lehrstuhl für Genetik, Fakultät für Biologie, Universität Bielefeld, Postfach 100131, Bielefeld D-33501, Germany. E-mail: Larissa.Sharypova@Genetik.Uni-Bielefeld.DE.
tures: the absence of phosphate groups and the presence of a galacturonic residue at the 4′ position and of a long acyloxyacyl moiety as a secondary acyl substitution of the N-linked hydroxyacyl chain (7, 8). Variation of *R. etli* lipid A species concerns mainly the proximal glucosamine residue and its acyl substitutions (7, 8).

Recently the structure of *Sinorhizobium* sp. NGR234 lipid A was established (56). It appeared to be a remarkable “hybrid” sharing features with enteric lipid A and lipid A of *R. etli*. A similarity to enteric lipid A consisted in the presence of two phosphate substitutions of glucosamine disaccharide. A similarity to lipid A of *R. etli* was observed in the type and location of the acyl substituitions (acyloxyacyl residues) indicated by broad lines.

**Fig. 1. Structures of lipid A from *E. coli*, *R. etli*, and *S. meliloti*.** Lipid A of *E. coli* consists of a glucosamine backbone, two phosphate substitutions, and six acyl substitutions, two of which form acyloxyacyl moieties. Lipid A of the *msbB* mutant of *E. coli* lacks an acyloxyacyl residue containing myristic acid. Lipid A of *R. etli* lacks both phosphate groups but carries instead a single galacturonic residue. The characteristic feature of *R. etli* lipid A is a single amide-linked acyloxyacyl residue containing a long fatty acid chain (27-OH-28:0). Lipid A of *S. meliloti* consists of a glucosamine backbone with two phosphate substitutions and four acyl substitutions. No acyloxyacyl residue was detected (10). Based on the presence of the *acpXL-msbB* (*lpxXL*) gene cluster in the *S. meliloti* genome, we propose that lipid A of this bacterium should carry a long acyl residue in the same position as lipid A of *R. etli* and *Rhizobium* sp. NGR234. Secondary acyl substitutions (acyloxyacyl residues) are indicated by broad lines.
of an acyloxyacyl residue and in the heterogeneity of lipid A species concerning the presence or absence of an acyl substitution at position 3 (56).

The structure of S. meliloti lipid was characterized 14 years ago and probably needs confirmation with modern methods of analysis. Similar to the lipid A of enteric bacteria, lipid A of S. meliloti had a glucosamine backbone with two phosphate substitutions (10) (Fig. 1). No secondary acyl substitutions were found, and a C28 hydroxylated fatty acid chain was identified as a primary ester-linked substitution of glucosamine (10).

Interestingly, S. meliloti and R. leguminosarum lipid A were reported to have the same endotoxic activity on animal hosts as lipid A of enteric bacteria (10, 16). The role of bacterial LPS in symbiotic interactions with plants seems to be profoundly different from its role in pathogenic interactions with animals. There are several lines of evidence that LPS of rhizobia may suppress host defense responses in plant cells and promote symbiotic interaction (2, 17, 18). Studies with rhizobial mutants producing modified lipid A may shed more light on mechanisms of LPS interaction with plant cells. To our knowledge, all LPS mutants of rhizobia studied so far contained modifications either in the core or in the O antigen, and rhizobial mutants affected in lipid A expression have not been analyzed yet.

Recently, the complete genome sequence of S. meliloti 1021 was determined (19). Sequence annotation of the S. meliloti chromosome revealed a number of genes whose products are conserved among Gram-negative bacteria and control early chromosome revealed a number of genes whose products are conserved among Gram-negative bacteria and control early properties of free living and endosymbiotic bacteria. Based on the results of LPS composition analysis as well as results of electrophoretic and immunological comparisons of mutant and wild type LPS, we reconsider here the structure of S. meliloti LPS.

EXPERIMENTAL PROCEDURES

### Bacterial Strains and Growth Conditions

Strains and plasmids used in this work are described in Table I. S. meliloti strains were incubated at 30 °C either on LB or TY or mannitol-glutamate-salts medium (28–30). E. coli strains were incubated at 37 °C on LB medium. Antibiotics were added at the following concentrations (μg/ml): streptomycin (Sm) 600, neomycin (Nm) 120, tetracycline (Tc) 8 for S. meliloti, ampicillin (Ap) 100, kanamycin (Kn) 50, tetracycline 5 for E. coli. The deoxycholate (DOC) sensitivity of S. meliloti strains was tested on LB plates containing 0.1% sodium deoxycholate.

**Construction of S. meliloti acpXL Mutant**—An 188-bp intragenic fragment of the 285-bp coding sequence of acpXL was amplified using Taq polymerase and the primer pair acpXLup (5′-TCGACAAGGTTGCGTTCAC-3′) and acpXLdn (5′-GCGTGACAGCTACATTCGAC-3′). The underlined nucleotides represent XbaI and KpnI restriction sites, respectively, added for convenience of cloning. The amplified DNA product was cloned into the plasmid pBR322. The resulting plasmid pLSacpXL was transferred to S. meliloti recipient strains by S17-1-mediated conjugation. Transconjugants were selected on LB plates containing neomycin and streptomycin. To verify that the genomic acpXL sequence was disrupted by the plasmid, the transconjugants were analyzed by PCR using the primer pair acpXLup K (5′-GGTTACCGCTTCAGGCTTTCCTCAA). The underlined nucleotides represent XbaI and KpnI restriction sites, respectively, added for convenience of cloning. The amplified DNA product was cloned into the plasmid, designated pXK11a, was used for complementation. Conjugational transfer of pXK11a into S. meliloti acpXL mutant L994 was accomplished as described above. Transconjugants were selected on LB plates containing tetracycline and streptomycin.

**Purification of LPS**—Preparative purification of LPS was performed from S. meliloti strains 1021 and L994. S. meliloti strains were grown on TY plates at 30 °C for 2 days. Bacterial cells were harvested from plates and washed twice with 0.9% NaCl. The cell pellet was resuspended in an equal volume of distilled water, and LPS was extracted after a hot phenol-water extraction procedure (31). The material from the water phase was treated with DNase, RNase, and proteinase K, dialyzed against water for 2 days, and freeze dried. Then LPS was dissolved in water to a final concentration of about 40 mg/ml and centrifuged for 20 h at 100,000 × g. The pellet consisting of LPS micelles was dissolved in water and freeze dried.

**Fatty Acid Analysis**—The determination of fatty acids and hydroxy fatty acids was done by gas-liquid chromatography (GC) and mass spectrometry (MS) as described (32) with modifications. Briefly, 200 μg of purified LPS was supplemented with 10 μg of 17:0-Me as internal standard. The samples were dissolved in 1 ml of 4 M HCl and heated for 4 h at 100 °C in a tightly closed Reacti-Vial (Fisher). 1 ml of 5 M NaOH was added, and the mixture was incubated at 100 °C for a further 30 min. After the addition of 3 ml of water, the pH was adjusted to 3 using ~300 μl of HCl. The fatty acids were extracted with 1 ml of chloroform.

### Table I

**Bacterial strains and plasmids used in this work**

| Strain/Plasmid | Relevant characteristics | Source or reference |
|----------------|-------------------------|---------------------|
| S. meliloti    |                         |                     |
| 1021           | Sm<sup>+</sup> derivative of wild type strain SU47 | 22                  |
| 2011           | Sm<sup>+</sup> derivative of wild type strain SU47 | 23                  |
| L943           | 1021 acpXL               | This work           |
| Rm6963         | 2011 lpaA::Tn5           | 24                  |
| L753           | 2011 egIC::gusA          | Footnote 2          |
| E. coli        |                         |                     |
| DH105          | supE44, ΔlacU169 (Δ80 lacZAM15) ΔhisR17 recA1 endA1 gyrA96 thi1-1 relA1 | Invitrogen          |
| S17-1          | MM294, RP4–2-Tc::Mu-Km::Tn7 chromosomally integrated |                     |
| Plasmids       |                         |                     |
| pGEM-T-Easy    | T-overnag vector for cloning PCR products, Ap<sup>+</sup> | Prokera             |
| pK18mob2      | pK18mob derivative with unique KpnI and SmaI sites in polylinker, Km<sup>+</sup> | This work           |
| pLSacpXL      | pK1Smok2 carrying the 188-bp internal fragment of acpXL, Km<sup>+</sup> | This work           |
| pHU231         | pRK290 derivative, broad host range vector, Tc<sup>+</sup> |                     |
| pXK11a        | pPHU231 carrying acpXL under control of its own promoter |                     |
The extraction was repeated three times, and the extracts were pooled and dried in a steam of nitrogen. Methylation of the carbohydrate groups was carried out using diazomethane in ether (0.1 ml), freshly prepared using 1-methyl-3-nitro-1-nitroguanidine (Aldrich), and the MNNG diazomethane generator (Aldrich). The ether was evaporated by a steam of nitrogen, and fatty acid methyl esters carrying free hydroxy groups were replaced by 40 μl of BSTFA (Pierce) in 4 h at 65 °C to produce the particular methylmethylsilylest esters. As a further reference for GC-MS analysis (Sigma, O 004) was subjected to the same derivatization procedure. GC-MS was carried out on a Thermo Trace gas chromatograph equipped with a 30-m SPB-50 (Supelco) and a Polaris electron impact ion trap mass spectrometer (Thermo). The following temperature program was used: 80 °C for 3 min, then 5 °C/min up to 300 °C and holding for 10 min. MS spectra were compared with reference substances and the NIST data base.

Glycosyl Composition Analysis—Analysis of neutral sugars and uronic acids was performed as described previously (33).

Analysis of Crude LPS Preparations and Immunodetection of LPS—LPS from S. meliloti strains was extracted using triethylamine/EDTA or SDS/proteinase K methods (34, 35). LPS samples were fractionated by SDS-PAGE. After electrophoresis in 16.5% acrylamide gels, LPS was visualized by the silver staining method of Tsai and Frisch (36). LPS was electroblotted from SDS-PAGE to an Immobilon-P (polyvinylidene difluoride) membrane (Millipore), pore size 0.45 μm, and detected using peroxidase-conjugated antibodies recognizing this epitope will remain unbound and may be binding of antibodies to epitopes of cell surface polysaccharides. If the adsorption of the antiserum was performed basically as described previously (37).

Generation of Polyclonal Antiserum—S. meliloti strain 2011 (23) was grown in mannitol-glutamate-salts medium overnight, washed twice with phosphate-buffered saline, pH 7.2, and incubated for 5 min at 65 °C. A final titer of 10⁵ cells was used for immunization of rabbits. The antisera was produced following the 2-month standard immunization protocol (www.seqlab.de/customer_services.html) by SEQLAB, Sequenz Laboratories Göttigen GmbH (Germany). The resulting antiserum 2605 showed high reactivity to surface antigens of numerous S. meliloti strains, including 1021.

Adsorption of the Antiserum with Whole Cells of S. meliloti—Coincubation of the antiserum with whole cells of a specific strain results in binding of antibodies to epitopes of cell surface polysaccharides. If the antiserum is incubated with mutant cells lacking a particular epitope, antibodies recognizing this epitope will remain unbound and may be detected by an immunoblot in an assay with material from a wild type strain. Preabsorption of the antiserum was performed basically as described previously (37) with modifications. Because adsorption of antibodies after incubation with strains 2011 and 1021 was incomplete, bacterial cells were boiled for 3 min in phosphate-buffered saline, cooled down, and mixed with the antiserum. Adsorption was performed for 2 h at room temperature with shaking. This resulted in a complete and specific adsorption of antibodies.

Plant Growth Conditions and Assays—Medicago sativa cv. Europe and Medicago truncatula genotype Jemalong seeds were surface sterilized by concentrated sulfuric acid for 10 min and washed several times with large volumes of sterile water. Seeds were germinated on 0.7% water agar plates at room temperature, in the dark. Seedlings were grown on nitrogen-free medium (38). One day after planting, the seedlings were inoculated with overnight cultures of S. meliloti strains that were washed in distilled water. Each plate contained three M. sativa or two M. truncatula seedling was inoculated with 200 μl of cell suspension (10⁶–10⁹ cells). Plants were grown at 20 °C under a 16-h light:8-h dark cycle for 30–40 days.

To confirm that mutant L994 did not revert during the passage through root nodules, we isolated bacteria from nodules and tested them for the expression of the neomycin antibiotic resistance marker of the integrated plasmid pSsgcXL. Nodules elicited by mutant L994 were harvested from roots of both host plants, at least three nodules from each variant of inoculation. Detached nodules were surface-sterilized with 70% ethanol for 1 min, washed with sterile water, and crushed in 0.85% saline buffer. Bacterial suspensions, recovered from nodules, were diluted and streaked on LB plates containing neomycin and streptomycin or only streptomycin.

To distinguish between nodules occupied by the competitor strain L753, carrying β-glucuronidase gene (GUS”), and the tester GUS” strains, roots of alfalfa were washed with phosphate buffer and incubated in the dark for 3–4 h at 37 °C in 50 mM sodium phosphate buffer, pH 7.5, containing 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide and 1% SDS. Nodules occupied by a GUS” strain stained blue; nodules occupied by a GUS” strain remained white or pink.

RESULTS

The acpXL-msbB Gene Cluster May Direct the Biosynthesis of the C28 Fatty Acid Chain and Its Incorporation into the S. meliloti Lipid A—All genes of the acpXL-msbB cluster are oriented in the same direction and probably comprise three transcriptional units: (i) acpXL; (ii) SMc04277, SMc04275, and SMc04270; and (iii) SMc04270 and msbB (Fig. 2). A similar gene cluster was recently identified in R. leguminosarum (39), accession AF510733. acpXL of R. leguminosarum is a specialized acyl carrier protein for a C28 acyl chain and is involved in attachment of this long fatty acid chain to (Kdo)₂-lipid IVₐ, a key lipid A precursor common to different Gram-negative bacteria (21). The LpxXL protein of R. leguminosarum is an acpXL-dependent 28-cytransferase (39). Both S. meliloti MsbB and R. leguminosarum LpxXL are similar to E. coli MsbB (LpxM), an ACP-dependent myristoyltransferase that catalyzes the addition of the secondary myristoyl chain to (Kdo)₂-lipid IVₐ (40). Four other hypothetical proteins encoded by the acpXL-msbB gene cluster show similarity to E. coli enzymes of fatty acid biosynthesis. Smc04277 is similar to 3R-hydroxy-myristoyl-ACP dehydratase (accession P21774), E value 3e-11; Smc04275 and Smc04273 are similar to 3-oxoacyl-ACP synthase II or polyketide synthase (P39435), E values 5e-08 and 6e-65, respectively; Smc04270 is similar to threonine 3-dehydratase (P07913), E value 3e-11, and many other zinc-binding dehydrogenases. The fact that the acpXL-msbB gene cluster of S. meliloti and the acpXL-LpxXL gene cluster of R. leguminosarum show conservation implies that all proteins encoded by the cluster are involved in the same biosynthetic pathway leading to synthesis and incorporation of C28 acyl chain into lipid A. None of the genes comprising the acpXL-msbB gene cluster was ever targeted by a mutation. We decided to focus on the analysis of S. meliloti acpXL gene, expecting that its function should be similar to that of R. leguminosarum acpXL.

acpXL Mutant of S. meliloti Does Not Express Smooth LPS—An acpXL mutant of the S. meliloti strains 1021 was constructed as described under “Experimental Procedures.” LPS from strain 1021 and the acpXL mutant L994 was extracted using the SDS/proteinase K method (35) and analyzed by SDS-PAGE. The cell extract of strain 1021 showed two LPS bands, the upper LPS-1 and the lower LPS-2 band (Fig. 3). Compared with the LPS ladder of Salmonella enterica the molecular mass of LPS-1 and LPS-2 may be estimated as 9 kDa and 4.5 kDa, respectively (data not shown). It is thought that LPS-2 contains rough LPS (R-LPS), whereas LPS-1 contains smooth LPS (S-LPS). Cell extract of mutant L994 only revealed the fast migrating R-LPS (Fig. 3).

The type and yield of LPS from Rhizobium mutants may vary depending on the extraction method (41). Therefore, LPS from the strains of interest was also extracted using the triethylene/EDTA method (34) and hot phenol-water procedure (31). Regardless of the method of LPS isolation, mutant L994, in contrast to its parent 1021, manifested only the fast migrating band of LPS (data not shown).
Protein-free cell extracts were separated in 16.5% acrylamide gel. LPS was visualized by silver staining.

LPS of Mutant L994 Showed the Same Immunological Properties as LPS of Strain 1021—As shown in Fig. 6, both LPS bands from cell extracts of 1021 reacted with the polyclonal antiserum. However, no other immunoreactive material was found. The finding that mutant L994 was nonspecific. LPS of Mutant L994 was missing slow migrating LPS, which, similarly to NGR234, was identified as ester-linked residue (10), also represents the most variable portion of the molecule. The levels of four other fatty acids were comparable in the LPS of both strains (Table II).

LPS of acpXL Mutant L994 and Parental Strain 1021 Have a Similar Sugar Composition—The finding that mutant L994 was missing slow migrating LPS could be interpreted as an inability of the mutant to express O antigen. To reveal sugars comprising S. meliloti O antigen, we compared the glycosyl composition of LPS derived from strains 1021 and L994. Surprisingly, we found that LPS of both strains had the same glycosyl composition, characterized by the presence of two neutral sugars and two uronic acids (Fig. 5 and Table II). The only difference between 1021 and L994 was in the level of glucuronic acid. It was 2-fold higher in LPS of L994 than in LPS of L994. This peak was completely absent (Fig. 4). In addition, we found that the level of 3-OH-14:0 in wild type LPS was 2.6-fold higher than in the mutant LPS (Table II). Interestingly, in Sinorhizobium sp. NGR234, the highest variation of the lipid A structure was connected to presence or absence of this hydroxylated fatty acid (56). Probably, in S. meliloti lipid A, 3-OH-14:0, which, similarly to NGR234, was identified as ester-linked residue (10), also represents the most variable portion of the molecule. The levels of four other fatty acids were comparable in the LPS of both strains (Table II).

To confirm that the observed “rough” phenotype was caused by the acpXL mutation, we performed complementation analysis. Plasmid pXK11a carrying the acpXL gene was constructed as described under “Experimental Procedures.” Upon introduction of pXK11a into L994, transconjugants manifested the same LPS profile as strain 1021 (Fig. 3). The positive result of the complementation analysis evidenced that (i) the 144-bp noncoding upstream region of acpXL was sufficient for normal expression of the gene; (ii) the rough phenotype of mutant L994 was solely caused by the acpXL mutation; and (iii) the mutation within acpXL had no polar effect on the downstream genes.

Lipid A of an acpXL Mutant Lacks the C28 Acyl Chain—To test whether the acpXL mutation affected C28 acylation of lipid A, the LPS extracted from S. meliloti strains 1021 and L994 was subjected to fatty acid analysis using GC-MS. A peak corresponding to a C28 fatty acid was found in the LPS of the wild type strain (Fig. 4). However, in the LPS of the mutant this peak was completely absent (Fig. 4). In addition, we found that the level of 3-OH-14:0 in wild type LPS was 2.6-fold higher than in the mutant LPS (Table II). Interestingly, in Sinorhizobium sp. NGR234, the highest variation of the lipid A structure was connected to presence or absence of this hydroxylated fatty acid (56). Probably, in S. meliloti lipid A, 3-OH-14:0, which, similarly to NGR234, was identified as ester-linked residue (10), also represents the most variable portion of the molecule. The levels of four other fatty acids were comparable in the LPS of both strains (Table II).

TABLE II

| Component                  | LPS of 1021 | LPS of L994 |
|----------------------------|-------------|-------------|
| Fatty acids                |             |             |
| 3-OH-14:0                  | 117         | 117         |
| 3-OH-18:0                  | 59          | 59          |
| 27-OH-28:0                 | Not detectable | Not detectable |
| 16:0                       | 24          | 24          |
| 18:0                       | 7           | 7           |
| 18:1                       | 16          | 16          |
| Carbohydrates              |             |             |
| Glucose                    | 446         | 446         |
| Galactose                  | 55          | 55          |
| Glucuronic acid            | 80          | 80          |
| Galacturonic acid          | 203         | 203         |
| Mannose                    | Traces      | Traces      |

To test whether the antiserum contained antibodies specific to LPS-1 (recognizing O antigen), we adsorbed this antiserum with whole cells of mutant L994. It was expected that cells of the mutant would bind LPS-2-specific antibodies but not LPS-1-specific antibodies. However, when the preadsorbed antiserum was used for immunostaining LPS from S. meliloti 1021, neither LPS-1 nor LPS-2 was detected (Fig. 6B). A failure to reveal antibodies specifically reacting with LPS-1 might be explained by the following possibilities: (i) both forms of LPS had identical antigenic epitopes; (ii) the antiserum did not contain antibodies directed against all structural modules of LPS; and (iii) adsorption of antibodies by bacterial cells was nonspecific.

We cannot exclude the first two possibilities. However, we were able to check the third one, preadsorbing the antiserum by cells of a S. meliloti mutant that had altered antigenic properties. To that aim we used lpeB mutant Rm6963, known to have no or weak reactivity with antibodies against wild type LPS of S. meliloti (24). This mutant is defective in LPS core biosynthesis and produces a truncated core oligosaccharide (24). As shown in Fig. 6C, the antiserum preincubated with
mutant Rm6963 reacted with both LPS bands of 1021 and with the single LPS band of mutant L994. Consequently, the possibility of nonspecific adsorption of antibodies may be excluded. Taken together, the obtained results provide further evidence that mutant L994 expressed the same surface antigens as parental strain 1021 and that only LPS-2 (probably core) contained antigenic epitopes.

**Mutant L994 Displayed Sensitivity to DOC and Was Impaired in Symbiosis with Host Plants—Modifications of LPS structure are often associated with increased sensitivity to DOC. Thus, one of the phenotypes of lpsB mutant Rm6963 was DOC sensitivity (24). Therefore, we decided to test whether our acpXL mutant would also have such a phenotype. S. meliloti strains 1021, L994, and Rm6963, were plated on solid LB medium containing 0.1% DOC. After 2 days of incubation at 28 °C, strain 1021 manifested normal growth, whereas L994 and Rm6963 did not grow at all. Thus, regardless of the different nature of LPS modifications, both mutants had defects in the outer membrane permeability barrier.**

**Symbiotic properties of S. meliloti strains were analyzed in plant assays with aseptically grown M. sativa and M. truncatula plants. Mutant L994, similarly to strain 1021, was able to form nitrogen-fixing nodules on the roots of both host plants. However, in contrast to the parental strain, L994 was impaired in promoting shoot growth. Mutant Rm6963, which was also tested in this experiment, manifested the expected phenotypes: delayed nodulation of M. sativa and formation of nonfixing (ineffective) nodules on M. truncatula (42).**

There remained the possibility that the acpXL mutation caused attenuation of the outer membrane permeability barrier and quantitatively impaired the outer membrane permeability barrier and quantitatively impaired the competitive ability of the strains. Nodulation rate was assayed by counting the numbers of nodules at particular time points after inoculation. As shown in Fig. 7, mutant L994 manifested delayed nodulation compared with parental strain 1021. To study the competitive ability of the strains, we employed S. meliloti strain L753, which can be easily detected inside root nodules because of the expression of an egf1C–gusA fusion.

**RESULTS**

From Acyl “Roots” to Glycosyl “Tops”: What Is the Link between the Missing C28 Acyl Chain and the Missing O Antigen?—The presence of a C28 (ω-1) hydroxylated acyl chain is a taxonomically important feature of Rhizobiaceae and related
bacteria belonging to the α-2 subclass of Proteobacteria (4).

Brozek and coauthors (21) purified the AcpXL protein of *R. leguminosarum* and demonstrated that it facilitated the transfer of the C28 acyl chain to the lipid A precursor. This enzymatic activity was also found in cell extracts of *R. etli* and *S. meliloti* (21). Up until now, AcpXL has only been characterized biochemically but not genetically. In this study, we constructed *S. meliloti* acpXL mutant L994. Fatty acid composition analysis of lipid A from parental strain 1021 and mutant L994 confirmed that AcpXL of *S. meliloti* is involved in C28 acylation of lipid A. Recently, Geiger and Lopez-Lara (43) reported that the *S. meliloti* genome had the capacity to encode six acyl carrier proteins including AcpXL. In the light of this notion, it is important that none of the acyl carrier proteins can replace AcpXL in C28 acylation of lipid A.

Mutant L994 revealed a number of interesting phenotypes, including the lack of slow migrating LPS, LPS-1. However, cells of L994 expressed the same antigenic epitopes as cells of 1021, and LPS extracted from both strains revealed the same glycosyl composition. Because results of LPS immunodetection and its sugar composition analysis did not agree with results of SDS-PAGE demonstrating a clear difference between LPS of 1021 and L994, we decided to revise the conventional view on LPS of *S. meliloti* and looked for a noncontradictory explanation of our observations. Although slow migrating LPS of *S. meliloti* was usually considered as smooth LPS, there is no convincing evidence that it consists of lipid A core plus O antigen. The structure of *S. meliloti* O antigen is unknown, and even with the help of monoclonal antibodies it was impossible to demonstrate that LPS-1 possesses a unique structural domain not present in LPS-2 (44). Following this line of reasoning, we considered the possibility that LPS-1 might be an aggregated form of LPS-2 (presumed rough LPS) stabilized by hydrophobic interactions between C28 acyl chains of lipid A. Consequently, *S. meliloti* mutant L994, missing the C28 acyl moiety of lipid A, would express only a fast migrating form of LPS. Usually, LPS aggregates are resolved under standard conditions of SDS-PAGE or DOC-PAGE. However, it cannot be excluded that structural peculiarities of *S. meliloti* LPS may preclude resolving of the aggregates because aggregation properties of LPS molecules depend greatly on their structure (45). Remarkably, the molecular mass of the slow migrating form of LPS (LPS-1) is approximately two times higher than that of the fast migrating form of LPS (LPS-2), 9 kDa versus 4.5 kDa.

Interestingly, Gudlavalleti and Forsberg (56) demonstrated that S-LPS and R-LPS of *Sinorhizobium* sp. NGR224 differed in their acylation pattern. Both forms of LPS contained lipid A with a long fatty acid chain, but there was a difference in the number of acyl substitutions. Thus, S-LPS was enriched in tri- and tetraacylated lipid A species, whereas R-LPS was enriched in penta- and tetraacylated species and contained no triacylated species at all. It is obvious that a long acyl chain would have stronger influence on physical properties of underacylated LPS molecules than on fully acylated ones. Therefore, if our speculation is true, underacylated LPS molecules containing long acyl substitution would self-aggregate most efficiently. It may be argued that LPS aggregates should have been dispersed under dissociative conditions used for separation of LPS. However, it cannot be excluded that a minor portion of LPS (S-LPS constituted only 4%) was not dissipated completely.

Although we cannot provide experimental evidence to our hypothesis, we would like to mention three groups of facts that also point to an elusive nature of *S. meliloti* O antigen and may support our speculation that slow migrating LPS lacks O antigen and presents an aggregated form of fast migrating LPS.

First, in SDS-PAGE, LPS-1 of *S. meliloti* is often fainter than LPS-2, and sometimes it is nonreproducible (12, 34, 44, 46). Such an irregular mode of expression may be expected if LPS-1 is not a distinct form of LPS but just an aggregate of LPS-2. A defect would result not only in truncation of rough LPS but also in the complete absence of smooth LPS because the O antigen cannot be added to the truncated core. Consequently, the case of the *S. meliloti* lpsB mutant expresses two LPS bands instead of one. This mutant has a defect in LPS core biosynthesis which accounts for faster migration of both bands relative to LPS-1 and LPS-2 of the wild type (47). However, in *R. leguminosarum* and enteric bacteria such a defect would result not only in truncation of rough LPS but also in the complete absence of smooth LPS because the O antigen cannot be added to the truncated core. Consequently, the case of the *S. meliloti* lpsB mutant was considered as exceptional (47). However, in the light of our speculation that the upper LPS band is just an aggregate, there will be no need to seek reasons why alteration of the core did not prevent attachment of an O antigen.

Second, Reubs *et al.* (44), who considered LPS-1 as smooth LPS, i.e. lipid A core plus O antigen, pointed out that the O antigen of *S. meliloti* strains, in contrast to the O antigens of typical Gram-negative bacteria, was nonspecific and nonimmunogenic. Another peculiar feature of *S. meliloti* LPS is an immunodominance of its core (12, 24, 44). In this work, we also found that antisera raised against whole cells of *S. meliloti* contained antibodies reacting with rough LPS (likely core) but contained no antibodies reacting with the O antigen. In *S. meliloti*, K antigens but not O antigens determine the serotypes of individual strains (44). A similar situation is known for the mucosal pathogens *Haemophilus influenzae* and *Neisseria meningitidis* (48–50). These bacteria produce a specific type of LPS, named lipooligosaccharide, which lacks an O antigen but is decorated with nonrepeating oligosaccharide branches (51). *S. meliloti* may also produce lipooligosaccharide because *S. meliloti* mutants deficient in the production of both expolysaccharides and capsular polysaccharide (e.g. *exoB rkpC*) do not have as rough a morphology as rough mutants of *E. coli*.

Third, recent analysis of a collection of *S. meliloti* LPS mutants generated by random Tn5 mutagenesis revealed mutants with diverse LPS profiles. However, none of these mutants showed a rough phenotype in SDS-PAGE. Consequently, not a single gene was assigned to a function in O antigen biosynthesis. To date, *acpXL* mutant L994 is the only rough mutant ever found in *S. meliloti*. The existence of L994 demonstrates that rough *S. meliloti* mutants are viable.

### Table III

| Inoculum, A + B | Experiment 1 | Experiment 2 |
|-----------------|--------------|--------------|
| A (GUS<sup>+</sup>) | B (GUS<sup>+</sup>) | % of GUS<sup>+</sup> nodules | A:B<sup>a</sup> | % of GUS<sup>+</sup> nodules | A:B<sup>a</sup> |
|-----------------|--------------|--------------|-----------------|-----------------|-----------------|
| 1021            | L753         | 1:3          | 20 ± 4.8        | 2.5:1           | 36 ± 5.3        |
| L994            | L753         | 1:3          | 2 ± 1.9         | 2.5:1           | 6 ± 2.9         |

<sup>a</sup> The ratio of strains in the initial inoculum was determined by plating serial dilutions of both strain cultures separately before mixing. Cell titers of all inocula ranged from 10<sup>6</sup> to 10<sup>8</sup> colony-forming units. 80 nodules were analyzed for each variant of inoculation.
To summarize, our results and the data from other authors support the idea that *S. meliloti* does not possess an O antigen. There remains an alternative possibility that the *S. meliloti* O antigen is very unusual, and it is only attached to the lipid A core that is acylated with the C28 fatty acid chain, but not to the lipid A core missing this long acyl substitution.

The C28 Acyl Chain of Lipid A Does Not Play a Specific Role during Symbiosis of *S. meliloti* with Its Host Plants—Since the time of the discovery of a long acyl substitution in LPS of *Rhizobium* (4) it has been intriguing to know, if this C28 acyl substitution plays a role during symbiosis with host plants. To address this question we studied the symbiotic properties of *S. meliloti* aepXL mutant and found that it was able to form nitrogen-fixing nodules on *M. sativa* and *M. truncatula* but showed a delay in nodulation rate and reduced competitive ability.

The importance of *S. meliloti* LPS for the establishment of symbiosis with host plants was first demonstrated using a *lpsB* mutant of 2011, Rm6963 (42). This mutant was unable to form an effective symbiosis with *M. truncatula* and evoked the defense response of plant cells (42). Mutant L994 resembles Rm6963 by its DOC sensitivity, delayed nodulation, and impaired competition on *R. etli* plants. However, the aepXL mutant, in contrast to the *lpsB* mutant, retains wild type cell surface antigens, as was demonstrated by immunoblotting, and also retains the ability to induce nitrogen-fixing nodules on the roots of *M. truncatula*. This comparison of mutants L994 and Rm6963 shows that in relation to symbiosis, attenuation of the C28 acyl substitution have already been detected in a number of human pathogens. A remarkable feature of such lipid A is its reduced toxicity in comparison with wild type lipid A. In particular, msbB mutants of *E. coli* and *S. enteridris* which lack myristoyltransferase activity and produce pentaacylated lipid A instead of hexaacylated molecule (Fig. 1) had a dramatically attenuated ability to stimulate an inflammatory response and did not cause lethality of animal hosts (40, 52, 53). As we found, the C28 acyl substitution of *S. meliloti* lipid A had no specific symbiotic function. However, given a key role of secondary acyl substitutions in eliciting host immune responses, it may be relevant to yet unknown mechanisms of plant-microbe interaction.

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