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Occurrence of Lipid A Variants with 27-Hydroxyoctacosanoic Acid in Lipopolysaccharides from Members of the Family *Rhizobiaceae*

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Lipopolysaccharides (LPSs) isolated from several strains of *Rhizobium*, *Bradyrhizobium*, *Agrobacterium*, and *Azorhizobium* were screened for the presence of 27-hydroxyoctacosanoic acid. The LPSs from all strains, with the exception of *Azorhizobium caulinodans*, contained various amounts of this long-chain hydroxy fatty acid in the lipid A fractions. Analysis of the lipid A sugars revealed three types of backbones: those containing glucosamine (as found in *Rhizobium meliloti* and *Rhizobium fredii*), those containing glucosamine and galacturonic acid (as found in *Rhizobium leguminosarum* bv. phaseoli, trifolii, and viciae), and those containing 2,3-diamino-2,3-dideoxyglucose either alone or in combination with glucosamine (as found in *Bradyrhizobium japonicum* and *Bradyrhizobium* sp. [*Lupinus*] strain DSM 30140). The distribution of 27-hydroxyoctacosanoic acid as well as analysis of lipid A backbone sugars revealed the taxonomic relatedness of various strains of the *Rhizobiaceae*.

Bacteria belonging to the family *Rhizobiaceae* are gram negative and are able to form nitrogen-fixing symbiotic relationships with legume plants. There are three distinct genera: the symbiotic nitrogen-fixing *Rhizobium* and *Bradyrhizobium* spp. and the plant pathogenic *Agrobacterium* spp. Quite recently a new genus, so far comprising only the stem-nodulating nitrogen-fixing species *Azorhizobium caulinodans*, was defined (13). Of these genera, the species of *Rhizobium* are taxonomically closely related and show genetic similarities to the genus *Agrobacterium* as evidenced by 16S rRNA homology studies (12). On the other hand, the slowly growing species of *Bradyrhizobium* are rather distantly related to the other two genera as revealed by their low S_{AB} values determined by DNA-rRNA hybridization studies (1). In addition to the nucleotide sequence homology studies, differentiation of various members of the *Rhizobiaceae* has been attempted by several chemotaxonomic approaches such as cellular fatty acid analysis (21, 31), polyacrylamide gel electrophoresis of cellular proteins (19), and composition analysis of extracellular gum (26). However, results of these studies were not sufficient to adequately distinguish between members of the *Rhizobiaceae*. More recently, the backbone sugar composition of lipid A fractions of lipopolysaccharide (LPS) has been used as a taxonomic marker for recognition and relatedness of various nonsulfur bacteria (23). Therefore, in this study, the lipid A fractions from rhizobial LPSs were examined to see whether they represented a marker for determining the relatedness of these bacteria.

The surface polysaccharides, including the LPS, of strains of *Rhizobium* have been hypothesized to be involved in the molecular mechanisms of symbiotic infection (5). In an attempt to elucidate the structure of LPS from rhizobial strains, an unusual very-long-chain hydroxy fatty acid, 27-hydroxyoctacosanoic acid (27-OH-28:0), was discovered to be the major fatty acid constituent of the lipid A region (15). More recently, we have also identified this long-chain hydroxy fatty acid and related long-chain *n*-2 hydroxylated fatty acids in LPSs from almost all of the bacteria belonging to the α-2 subdivision of the class *Proteobacteria* (2). In the present investigation, we have examined LPSs isolated from many species of the *Rhizobiaceae*. The identification of the long-chain hydroxy fatty acid, along with careful studies on the lipid A backbone sugars, clearly revealed the relatedness of different species.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The various bacterial strains of *Rhizobium*, *Bradyrhizobium*, and *Agrobacterium* used in the present study and references for their cultivation conditions are given in Table 1.

Isolation of LPSs. LPSs were obtained from either water phase or phenol phase (11) (in the case of *Bradyrhizobium japonicum* only) after hot phenol-water extraction (30). The LPS isolated was purified either by ultracentrifugation or by treatment with α-amylase and RNase or with proteinase K followed by gel filtration on a Sepharose 4B column (9, 30). Lipid A was isolated from the purified LPS by acid hydrolysis with 1% acetic acid at 100°C for 2 to 5 h.

Identification of 27-OH-28:0. The identification of hydroxy fatty acids was done by transesterification by acid-catalyzed methanolysis (25) and analysis by gas-liquid chromatography (GLC). Briefly, 2.0 ml of 2 M methanolic HCl was added to either the LPS or lipid A samples, and methanolysis was carried out at 85°C for 18 h. After cooling, the solution was evaporated under N_2 and analyzed directly after solvent fractionation or after further derivatization with trifluoroacetic anhydride (3), preparation of phenylcarbamates (17), preparation of trimethylsilyl derivatives (22), or methylation with diazomethane (2).

GLC analysis of fatty acid methyl esters was carried out on a Hewlett Packard 5890-A gas chromatograph equipped with a 30-m DB-1 fused silica column (J & W Scientific) and a flame ionization detector. A temperature program of 80°C for 2 min and then 8°C/min to 290°C and holding for 15 min was used.

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TABLE 1. Bacterial strains, cultivation conditions, and distribution of 27-OH-28:0 among strains of the *Rhizobiaceae*

Strain[a]	Presence of 27-OH-28:0	Reference for cultivation condition
Genus *Rhizobium*		
R. leguminosarum		
bv. viciae LB2, 128C63, 128C53	+	10
bv. phaseoli CE3 CE109	+	10
bv. trifolii ANU843, OH03, 45, TA1	+	11
R. meliloti 10406, 2011	+	12
R. loti IFO 14779T	+	13
R. fredii IFO 14780T	+	13
R. galegae IFO 14965T	+	16
Genus *Bradyrhizobium*		
B. japonicium USDA 123	+	14
Bradyrhizobium sp. (*Lupinus*) strain DSM 30140	+	15
Genus *Agrobacterium*		
A. tumefaciens		
bv. 1 IFO 12667	+	13
bv. 2 IFO 14793	+	13
A. radiobacter		
bv. 1	+	13
bv. 2	+	13
A. rhizogenes		
bv. 1 IFO 14554	+	13
bv. 2 IFO 13257	+	13
A. rubi IFO 13261T	+	13
Genus *Azorhizobium* (*A. caulinodans* IFO 14845)	−	27

[a] T, Type strain.

Sugar composition analysis. Neutral sugars were identified by the alditol acetate method after acid hydrolysis (2 M trifluoroacetic acid at 121°C for 2 h) (33). Uronic acids were identified by mild methanolysis (70°C for 3 h) followed by reduction with sodium borodeuteride, acid hydrolysis (2 M trifluoroacetic acid at 121°C for 2 h), and alditol acetate derivatization. Amino sugars were analyzed by acid hydrolysis (4 M HCl at 100°C for 18 h) with subsequent N acetylation and alditol acetate derivatization (32).

GLC-mass spectrometry (MS) analysis was performed on a Hewlett-Packard 5985 gas chromatography-MS system with an ionizing voltage of 70 eV. Chemical ionization-MS was performed with the ion source temperature at 150°C, with ammonia as the reactant gas.

RESULTS

Occurrence of 27-OH-28:0 in various species of the *Rhizobiaceae*. LPSs or lipid A samples of many strains belonging to *Rhizobium*, *Bradyrhizobium*, and *Agrobacterium* were analyzed for the presence of 27-OH-28:0. The results are summarized in Table 1. Of the several species studied, all samples, with the exception of *Azorhizobium caulinodans*, contained various amounts of the long-chain hydroxy fatty acid. Analysis of samples as fatty acid methyl esters or after further derivatization of the hydroxyl group with diazomethane or trifluoroacetylation gave similar quantitative results. The long-chain hydroxy fatty acid was a major fatty acyl component in *Rhizobium* strains, whereas in *Agrobacterium* and *Bradyrhizobium* strains, it was present in smaller amounts and often along with 29-OH-30:0 or other homologs. Quantitation of 27-OH-28:0 in LPS samples was not possible because of the lack of *n*-hydroxy fatty acid standards required to calculate the response factors. However, some lipid A samples were methanolyzed, and the relative levels of various hydroxy fatty acids were calculated as the percentages of the total peak areas. The results are given in Table 2. All the *Rhizobium* strains contained nearly 35 to 45% 27-OH-28:0. Since some of the LPSs were purified in several different laboratories, the variation in fatty acid distribution among strains of the same biovar may be due to various culture conditions, time of harvest, etc.

In the GLC analysis with a 30-m DB-1 column, the 27-OH-28:0 methyl ester had a relative retention time (T_R) of 1.56 with respect to 20:0, which was used as an internal standard. The GLC-MS data for 27-OH-28:0 are shown in Fig. 1. The chemical ionization-MS is characterized by the presence of *m/z* 455 (M + 1) and *m/z* 472 (M + NH_4^+). The electron impact-MS is characterized by the presence of *m/z* 436 (M − 18, loss of water), *m/z* 404 (M − 50, loss of water and methanol), and *m/z* 410 (M − 44, loss of acetaldehyde), arising from the fragmentation shown in Fig. 1. The location of the hydroxyl group in the long-chain fatty acid was at the *n*-2 position as indicated by *m/z* 59 and *m/z* 117 in the electron impact-MS of the corresponding methoxy and tri-

TABLE 2. Fatty acid composition of lipid A's from *Rhizobium* species

Source of LPS samples	% Fatty acids[a]									
	3-OH-12:0	14:0	3-OH-14:0	3-OH-15:0	3-OH-16:0	16:0	18:1	18:0	3-OH-18:0	27-OH-28:0
R. leguminosarum										
bv. phaseoli CE3	0	0	37	3	12	0	0	0	13	35
bv. phaseoli CE109	0	0	31	3	11	0	0	0	11	44
bv. viciae LB2	0	0	33	5	9	0	0	0	16	38
bv. viciae 128C63	0	0	36	4	8	0	0	0	12	40
bv. viciae 128C53	0	0	38	6	10	0	0	0	11	35
bv. trifolii 45	0	0	33	10	11	0	0	0	11	36
bv. trifolii 0403	0	0	33	10	11	0	0	0	11	36
bv. trifolii ANU843	0	0	33	10	11	0	0	0	11	36
bv. trifolii TA1	0	24	5	4	0	0	0	11	11	56
R. meliloti 2011	0	0	47	2	7	4	4	0	13	24
R. fredii USDA 205	0	0	23	0	0	0	16	0	16	45
B. japonicum 61A123[b]	14	4	25	0	0	9	22	20	0	5

[a] Fatty acid amounts are given as relative percentages of the total peak area.
[b] LPS contains five different *n*-2 hydroxylated fatty acids with 30-OH-31:0 in large amounts.

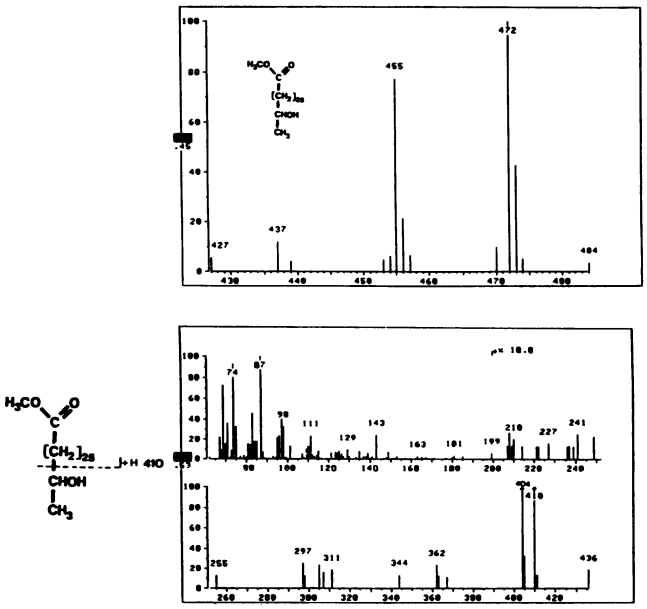

FIG. 1. Chemical ionization (top) and electron-impact (bottom) mass spectra of 27-OH-28:0 methyl ester in LPSs from members of the *Rhizobiaceae*. Ammonia was used as the reactant gas to obtain the chemical ionization spectrum.

methylsilyl fatty acid methyl esters, respectively. These fragments result from the cleavage between *n*-2 and *n*-3 without proton dislocation (22).

Identification of lipid A backbone sugars. Mild acid hydrolysis of LPS samples afforded lipid A and degraded polysaccharides. Sugar analysis of the lipid A fractions from strains belonging to *Rhizobium* and *Bradyrhizobium* revealed the absence of neutral sugars. Amino sugar analysis carried out by acid hydrolysis, N acetylation, reduction, and O acetylation procedures revealed the presence of 2,3-diamino-2,3-dideoxy-D-glucose (DAG) in strains from *B. japonicum* and of DAG and glucosamine in *Bradyrhizobium* sp. (*Lupinus*) strain DSM 30140, whereas lipid A's from *Rhizobium leguminosarum* (bv. phaseoli, trifolii, and viciae), *Rhizobium meliloti*, and *Rhizobium fredii* revealed the presence of glucosamine as the only amino sugar. On a 30-m DB-1 column with a temperature program of 200°C at 3°C/min to 260°C with a 3-min hold at 200°C, the alditol acetates of glucosamine and DAG had T_R values of 1.36 and 1.60 with respect to standard inositol, respectively. The electron impact-MS of DAG was characterized by primary fragments at *m/z* 288, 215, and 144, together with a number of characteristic secondary fragments observed in another study (32). Sugar analysis by trimethylsilylation and trifluoroacetylation of the methyl glycosides revealed the presence of galacturonic acid in lipid A's from *R. leguminosarum* bv. viciae, phaseoli, and trifolii. This was further confirmed by mild methanolysis followed by $NaBD_4$ reduction of the carboxymethylesters. Acid hydrolysis of the resultant material, followed by $NaBH_4$ reduction, O acetylation, and GLC-MS analysis resulted in the alditol acetate of galactitol with two deuterium atoms at C-6. Its electron impact mass spectrum was characterized by *m/z* 147, 219, 291, and 363 arising from cleavages between C-5 and C-4, C-4 and C3, C-3 and C-2, and C-2 and C-1, respectively. The lipid A from these strains contained equimolar proportions of D-galacturonic acid and D-glucosamine. The structural elucidation of the galacturonic acid-containing lipid A is in progress in our laboratories.

TABLE 3. Lipid A types of bacteria belonging to the *Rhizobiaceae*[a]

Type	Species	Presence of:			
		27-OH-28:0	GlcN	GalA	DAG
1	*R. meliloti*	+	+	−	−
	R. fredii	+	+	−	−
2	*R. leguminosarum* bv. viciae	+	+	+	−
	R. leguminosarum bv. phaseoli	+	+	+	−
	R. leguminosarum bv. trifolii	+	+	+	−
3	*B. japonicum*	+	−	−	+
	Bradyrhizobium sp. (*Lupinus*) strain DSM 30140	+	+	−	+

[a] GlcN, Glucosamine; GalA, galacturonic acid.

The results of the lipid A backbone sugar analyses are summarized in Table 3. On the basis of these results, three distinct lipid A types can be defined in the *Rhizobiaceae*. The first group contains only glucosamine as the backbone sugar (as found in *R. meliloti* and *R. fredii*), the second contains D-galacturonic acid and glucosamine (as found in *R. leguminosarum* bv. phaseoli, viciae, and trifolii), and the third contains DAG either alone or in combination with glucosamine (as found in *B. japonicum* and *Bradyrhizobium* sp. [*Lupinus*] strain DSM 30140). We have not yet investigated the backbone sugar composition of lipid A's found in other species, such as *Rhizobium loti*, *Rhizobium galegae*, and the *Agrobacterium* species. *Agrobacterium tumefaciens* has been reported to contain glucosamine in the lipid A fractions (24).

DISCUSSION

The present investigation involving the screening of LPS samples isolated from strains belonging to the *Rhizobiaceae* for the presence of 27-OH-28:0 revealed that all species, with the exception of *Azorhizobium caulinodans*, contain this fatty acyl component in lipid A fractions. The four major groups in the *Rhizobiaceae*, i.e., *Rhizobium*, *Bradyrhizobium*, *Agrobacterium*, and *Azorhizobium*, belong to the α-2 branch of the *Proteobacteria* (12). The taxonomic tree proposed on the basis of 16S and 5S rRNA homology studies has been well supported by the chemical composition analyses of the lipid A moieties (23). Several closely related bacteria have been shown to contain similar lipid A sugar backbones or fatty acid distribution patterns (23). Hence, lipid A is considered a valuable chemotaxonomic marker for ascertaining the relationships of various bacteria. In the case of the *Rhizobiaceae*, Table 2 shows that the relative percentages of hydroxy fatty acids of *R. leguminosarum* strains are quite similar, except for one strain of *R. leguminosarum* bv. trifolii, TA1. The significance of the different fatty acyl pattern for strain TA1 requires further investigation.

The first identification and characterization of 27-OH-28:0 was reported for lipid A from *R. leguminosarum* bv. trifolii (15). The results presented here clearly reveal the distribution of 27-OH-28:0 throughout the *Rhizobiaceae*, with the exception of *Azorhizobium caulinodans*, thereby indicating the close taxonomic relationship of various strains. These chemical studies indicate that *Azorhizobium caulinodans* is

not closely related to other members of the *Rhizobiaceae*. This is supported by DNA-rRNA hybridization studies which show that *Azorhizobium caulinodans* is closely related to *Xanthobacter autotrophicus* and *Xanthobacter flavus* (13). The last two strains lack the very-long-chain hydroxylated fatty acids in their LPSs (2). Also, with a few notable exceptions, we have recently detected 27-OH-28:0 in LPS samples isolated from most of the bacteria belonging to the α-2 cluster of *Proteobacteria* (2). Hence, in addition to the lipid A sugar backbone analysis, screening for 27-OH-28:0 has proven to be a very useful marker for determining taxonomic relationships.

The composition analysis of the lipid A sugar backbones revealed the presence of three distinct lipid A types. The results presented here give only the composition and not the detailed structures. Type 1 consists of *R. meliloti* and *R. fredii* strains which have lipid A's that contain glucosamine as the only glycosyl residue. One report indicates that an *R. meliloti* lipid A may have the classical structure of enterobacterial lipid A, 1,4′-bisphosphorylated β-1,6-linked glucosamine disaccharide (29). Other reports, based on DNA homology studies, show that *R. fredii* is most closely related to *R. meliloti* (14). The type 2 lipid A's contain galacturonic acid and glucosamine, which are common in all the biovars of *R. leguminosarum*. Galacturonic acid has so far not been reported to occur in lipid A samples from any gram-negative bacteria. The type 3 lipid A's contain either only DAG or glucosamine and DAG (mixed lipid A backbone), as has already been reported for *B. japonicum* (11, 24) and *Bradyrhizobium* sp. (*Lupinus*) strain DSM 30140 (24). Recently, we have detected DAG in lipid A's from both nodulating and nonnodulating strains of *B. japonicum* (5a). Other gram-negative bacteria which contain DAG in lipid A, e.g., *Brucella abortus*, *Rhodopseudomonas palustris*, *Rhodopseudomonas viridis*, and *Pseudomonas carboxydovorans*, are closely related to species of *Bradyrhizobium* (23). All these bacteria have been shown to contain 27-OH-28:0 as a major fatty acyl component (2). Hence, the distribution of 27-OH-28:0 is not just restricted to bacteria with DAG or galacturonic acid-containing lipid A's but may also be present in the glucosamine-containing lipid A types. Also, there are lipid A's with DAG but not *n*-2-hydroxylated long-chain fatty acids as found in thiobacilli of the β branch of *Proteobacteria* (e.g., *Thiobacillus ferroxidans*) (4).

Two previous reports describe the sugars present in the lipid A of *R. leguminosarum* bv. trifolii (18, 27). One report (18) describes the lipid A glycosyl residue as glucosaminuronic acid. This sugar residue was found in a purified fraction of the lipid A. Glucosamine was not found in that purified fraction. Since that report, it has been found that the major lipid A fraction contains glucosamine and galacturonic acid as described in this report. The second article states that *R. leguminosarum* bv. trifolii lipid A contains a phosphorylated glucosamine disaccharide (27). Russa et al. (27) also mention the presence of unidentified components. These unidentified components could be the 27-OH-28:0 and galacturonosyl residue described in this report. A discrepancy between our results and those presented in that report is that we find no detectable phosphate in any of the *R. leguminosarum* lipid A's examined. This discrepancy requires further investigation. Further structural work on *R. leguminosarum* lipid A is in progress.

So far there is only one report on the characterization of LPSs from *R. galegae* (20). Various strains have been characterized by comparison of their LPS gel patterns and the whole cell protein patterns. The results have indicated that the *R. galegae* strains form a distinct taxonomic group within the genus *Rhizobium*. Our results show that 27-OH-28:0 is present in the lipid A from *R. galegae*. The types of glycosyl residues which are present in the lipid A backbone remain to be identified.

In addition to lipid A backbone sugars, the core components seem to be rather conserved for the three types. Preliminary analysis of the core fractions revealed that type 1 contains largely glucose and 2-keto-3-deoxyoctulosonic acid and type 3 consists of 4-*O*-methyl mannose, mannose, glucose, and 2-keto-3-deoxyoctulosonic acid (11, 27a). On the other hand, mild acid hydrolysis of type 2 LPS releases two distinct core structures: a tetrasaccharide with mannose, galactose, galacturonic acid, and 2-keto-3-deoxyoctulosonic acid in 1:1:1:1 molar ratio and a trisaccharide with galacturonic acid and 2-keto-3-deoxyoctulosonic acid in 2:1 molar ratio (6–8, 16). Thus, it is possible that the lipid A and the core structures may be identical for LPSs from strains within a type.

The biological significance of lipid A's with 27-OH-28:0 is not known. This unusual fatty acid has the double length of the usual β-hydroxy fatty acids and may stretch through the entire outer membrane. The location of a functional OH group at the penultimate carbon may be helpful in the interaction with components of the inner leaflet of the outer membrane, such as phospholipids and proteins, which in turn may contribute to an increased stability and rigidity of the outer membrane. This is also evident from the fact that the freeze fracture of the outer membrane is not, or only rarely, observed with the *Rhizobiaceae* (28).

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