

Fatty Acids Present in the Lipopolysaccharide of *Rhizobium trifolii*

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Approximately 70% of the fatty acids recovered after acid or alkaline hydrolysis of the lipopolysaccharide of *Rhizobium trifolii* were hydroxy fatty acids identified as hydroxymyristic and hydroxypalmitic acids. Palmitic acid was the only saturated fatty acid found in the lipopolysaccharide of *R. trifolii*. Octadecenoic and a small amount of hexadecenoic acids were also identified. The results of BF₃ methanolysis and hydroxylaminolysis suggest that hydroxypalmitic acid is *N*-acyl bound.

The genus *Rhizobium* is composed of strictly aerobic, gram-negative rods divided into six species on the basis of the host plant specificity. Extracellular polysaccharides of *Rhizobium* have been extensively studied (2, 5, 10), whereas only a few papers have dealt with the immunochemistry of the somatic antigens in nodule bacteria. It was found that the lipopolysaccharide (LPS) of *Rhizobium trifolii* contained 2-keto-3-deoxyoctonate, glucuronic acid, glucose, mannose, galactose, fucose, rhamnose, heptose, glucosamine, and a firmly bound lipid (7, 11, 13). The phospholipids identified in *Rhizobium japonicum* are phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine, and cardiolipin (6). Myristic, palmitic, and octadecenoic acids were found to be the major fatty acids, whereas a C₁₇ cyclopropane fatty acid has been identified from a biotin-inhibited culture of *R. japonicum* (3). Since the only report of fatty acids in the nodule bacteria has been confined to *R. japonicum*, it was important to identify the fatty acids in *R. trifolii*. The present study was undertaken to analyze the composition of fatty acids in LPS of *R. trifolii*.

MATERIALS AND METHODS

Cultivation and isolation of LPS. The smooth, mucoid strain *R. trifolii* 24SM and its rough mutant designated 24AR were used.

Bacteria were grown in medium 4 containing the following components (in grams per liter of distilled water): K₂HPO₄, 3.6; KH₂PO₄, 0.4; MgSO₄, 0.05; NaCl, 0.5; ferric ammonium citrate, 0.005; Casamino Acids, 3.0; glucose, 10.0; and a final pH of 7.6. Bacteria were cultivated with moderate shaking in 25 ml of the medium for 18 h at 28 C and then transferred to 800 ml of the medium. After 18 h of

incubation, with shaking, the culture was transferred to 8 liters of the medium. Microorganisms were then incubated with aeration up to the stationary phase (3 days), harvested by centrifugation, washed twice in physiological saline, and frozen. Portions of frozen batches of each culture were extracted with 45% aqueous phenol at 68 C for 15 min, cooled, and centrifuged according to the technique of Lüderitz et al. (14). The aqueous phase was dialyzed against distilled water and freeze dried. The extract of strain 24AR was suspended in distilled water and sedimented at 80,000 × *g* for 7 h. The pellets were suspended in water and centrifuged at 105,000 × *g* for 3 h. Instead of being centrifuged, LPS of strain 24SM was fractionated on a Sepharose 2B column (2 by 40 cm) and eluted with 0.01 M NH₄HCO₃ (16). Fractions containing carbohydrates were determined by the Dische procedure with indole and H₂SO₄. Afterwards the collected fractions were examined in the precipitation test and freeze dried. LPS (3 mg) was next extracted twice with 5-ml portions of diethyl ether followed by a repeated extraction with acetone and then chloroform to remove neutral lipids.

Acid hydrolysis of LPS. LPS (5 mg) was suspended in 2 ml of 1 N HCl and heated at 100 C for 5 h. The reaction mixture was extracted three times with 0.5 ml of petroleum ether, and the extracts combined and dried over anhydrous sodium sulfate were evaporated under reduced pressure.

Hydrolysis of LPS with NaOH. LPS (3 mg) was suspended in 0.5 ml of 4 N NaOH and heated in sealed vials at 100 C for 5 h. The hydrolysates then were acidified with 1 ml of 4 N HCl and the fatty acids were extracted with petroleum ether.

Hydroxylaminolysis. LPS (3 mg) was suspended in 1 ml of a filtered mixture consisting of 4% NH₂OH·HCl in ethanol and 8% NaOH in ethanol (1:1, vol/vol). The resulting suspension was heated at 63 C for 4 min, chilled, and centrifuged. The supernatant fluid containing the fraction of the ester-bound fatty acids as hydroxamates was acidified to pH 1.0 and heated at 100 C for 1 h (17). The free fatty acids

were extracted with petroleum ether. The sediment containing *N*-acylated fatty acids was hydrolyzed in 0.5 ml of 4 N NaOH at 100 C for 5 h, acidified, and extracted with petroleum ether.

Preparation of the fatty acid methyl esters. Fatty acids isolated from 4 mg of LPS were suspended in 0.3 ml of BF₃-methanol (14%, wt/vol), heated at 100 C for 2 min, and cooled, and 0.1 ml of hexane and 5 ml of a saturated NaCl solution were added to the reaction mixture, which was vigorously shaken. The hexane upper phase was separated and concentrated under reduced pressure, and the residue was dissolved in 30 μ liters of hexane. The fatty acid methyl esters were also obtained by direct treatment of LPS (10 mg) with 14% BF₃ in methanol (2 ml) at 75 C in a screw-cap tube for 1 h. The reaction mixture was extracted three times with 0.2-ml portions of diethyl ether after the addition of 3 ml of water. The combined extracts were washed with water, concentrated under reduced pressure, and dissolved in hexane (15).

Hydrogenation of the unsaturated fatty acids. The dried fatty acids (0.2 mg) were dissolved in 2 ml of absolute ethanol and supplemented with 10% Pd on charcoal (3 mg) as catalyst. The reaction mixture was hydrogenated with stirring for 3 h at 22 C. After centrifugation, the solvent was evaporated and the residue was esterified with BF₃-methanol (14).

Oxidation. The dried fatty acids (0.2 mg) dissolved in acetone (1 ml) were mixed with 2% KMnO₄ (1 ml) and stirred for 2 h at 20 or 35 C. Then, 2 ml of ethanol and 0.5 ml of 1 N NaOH were added and the resulting mixture was stirred for 45 min at 20 C. After centrifugation, the supernatant was acidified and the fatty acids as well as oxidation products were extracted with chloroform.

Trimethylsilylation. Fatty acid methyl esters (0.2 mg) were dissolved in 0.1 ml of the reagent TMSi-P-Serva, a mixture of trimethylchlorosilane, hexamethyldisilazane, and pyridine (3:1:9, vol/vol/vol), and left at 22 C for 30 min (15). This solution was heated at 100 C for 1 min and cooled, and 0.3 ml of hexane was added. The resulting trimethylsilylated (TMS) mixture was washed three times with water, and the separated hexane phase was concentrated at room temperature under a stream of nitrogen.

Effect of desaturation of hydroxy acids. Samples (0.2 mg) of hydroxy acid methyl esters were dissolved in 0.2 ml of pyridine, cooled to 0 C, and supplemented with 0.05 ml of POCl₃ (4). The reaction mixture was left at 22 C for 10 h and then chilled, and 0.5 ml of water was cautiously added followed by three extractions with petroleum ether. The ether extracts were washed successively with 2 N HCl and water and concentrated under a stream of nitrogen. The reaction product was subsequently hydrogenated.

In some experiments, P₂O₅ was used to desaturate fatty acids (12). Fatty acids (2 mg) were mixed with 25 mg of P₂O₅ and 2 ml of benzene. The mixture was heated at 65 C for 1 h and cooled, and 1 ml of water was added cautiously. The aqueous phase was separated and washed with benzene. The benzene extracts were pooled, the solvent was removed, and the reac-

tion products were hydrogenated and esterified as described.

Gas chromatography. Gas-liquid chromatography was performed on a GCHF18.3-4 instrument fitted with flame ionization detectors. Separations were performed at 198 C on stainless-steel columns (200 by 0.4 cm) containing 10% EGSS-X on Gas Chrom P (100/20 mesh; Serva) or 3% JXR (300 by 0.4 cm) on Gas Chrom Q (100/20 mesh; Applied Science Laboratories Inc.). Nitrogen (40 ml/min) served as carrier gas. The conditions described give sharp, well-resolved, and relatively narrow peaks for the moving components, thus improving the quantitative accuracy of measurement. The quantitative evaluation of the gas chromatographic analyses was determined from the peak area (peak height times width at half height). The relative percentage of each peak was calculated from the ratio of its area to the sum of the areas of all the peaks.

RESULTS

The results of gas-liquid chromatography of the fatty acids of *R. trifolii* LPS are shown in Table 1. The major fatty acids of LPS isolated from strain 24SM were characterized by relative retention times (r_p) of 2.77 (peak 3) and 4.8 (peak 4), amounting to 40 to 47 and 25%, respectively, of the total fatty acids. Three of the four minor peaks were identified as the methyl esters of palmitic acid (peak 1; $r_p = 1.0$; 3 to 6.6%), hexadecenoic acid ($r_p = 1.2$; 1.3 to 2.8%), and octadecenoic acid (peak 2; $r_p = 2.04$; 7.7 to 17.7%). The fatty acid with the relative retention time 9.65 (5%) was uncharacterized. In the analysis on the nonpolar column (JXR),

TABLE 1. Composition of fatty acids of the LPS of *Rhizobium trifolii*

No. of major peaks	Retention times relative to palmitic acid	Content ^a			
		24SM		24AR	
		NaOH	HCl	NaOH	HCl
1	0.62			1.0	
	1.00	3.1	6.6	2.3	8.1
	1.2	2.8	1.3	0.6	
	1.6	1.5	2.2		
2	2.04	7.7	17.7	3.0	7.2
	3	2.77	47.3	40.0	63.2
4	3.6	2.2		1.4	
	3.9		2.1		
	4.8	25.3	25.0	27.0	25.0
	6.2	1.6			10.0
	8.3	1.0			
	9.65	5.1	5.0		

^a Gas-liquid chromatography (EGSS-X) of methyl esters of the fatty acids released after hydrolysis of LPS with 1 N HCl, 4 N NaOH. Results are expressed as percentages of the total fatty acids.

the palmitic, hexadecenoic, and octadecenoic acids were identical with the corresponding standard methyl esters. The identity of the hexadecenoic and octadecenoic acids was also confirmed in the experiment with the use of hydrogenation.

The recovery of octadecenoic acid from LPS was greatest by direct transesterification with BF_3 , whereas acid and alkaline hydrolysis yielded a significantly reduced recovery.

In gas-liquid chromatograms of LPS treated with BF_3 , fatty acid with a retention time of 4.8 was not detected (Table 2). Hydroxylaminolysis of LPS indicated that the fatty acid with a retention time of 4.8 was an *N*-acyl-bound fatty acid. Similar results were obtained in the experiments on the composition of the fatty acids liberated after saponification of the lipid A from strain 24SM (0.5 N NaOH in methanol, 30 min at 20 C). The soluble fraction obtained after hydroxylaminolysis, i.e., containing substantially *O*-acyl fatty acids, was composed of five components with r_p values of 1.0, 1.77, 2.04, 2.77, and 4.8. The hydroxylaminolysis caused an increase of the content of the first four components. The major components of the *N*-acyl-bound fraction were the fatty acids with $r_p = 4.8$ and 2.77. If the ratio of the mixture $\text{NH}_2\text{OH}\cdot\text{HCl}\text{-NaOH}$ used in hydroxylaminolysis was changed from 1:1 to 4:5, the percentage of the fatty acid 3 in the sediment after hydroxylaminolysis was reduced from 45 to 9% in contrast to the constituent of the peak 4, whose area increased from 40 to 68% of total fatty acids.

A mild oxidation of the total fatty acids resulted in the appearance of lauric acid ($r_p = 0.33$; 7.5%) with a corresponding increase in the percentage composition of myristic and palmitic acids. Additionally, octadecenoic acid and fatty acids with a retention time of 2.77 and 4.8 were reduced by the mild oxidation. A more drastic oxidation (35 C) of the total fatty acids resulted in the identification of lauric acid as the major oxidation product along with a new fatty acid with a retention time of 0.42.

Components 3 and 4 on the nonpolar column showed significantly short retention times ($r_p = 0.82$ and 1.8, respectively).

The fatty acids with retention times of 2.77 and 4.8 on the EGSS-X column were shifted to new positions with retention times of 0.72 and 1.3, respectively, after trimethylsilylation. The TMS-fatty acid with a retention time of 0.72 was almost identical to authentic TMS- α -hydroxy myristic acid. However, authentic α -

TABLE 2. Methyl esters of fatty acids obtained by transesterification or hydroxylaminolysis of *R. trifolii* LPS

Retention time	Content ^a					
	24SM			24AR		
	BF_3	<i>O</i> -Ac	<i>N</i> -Ac	BF_3	<i>O</i> -Ac	<i>N</i> -Ac
0.57	0.8			1.0	1.0	0.6
0.62					2.7	1.3
0.75	1.0					
0.91	0.6		1.7			
1.00	11.0	10.2	4.4	16.0	9.6	4.8
1.32	1.3	0.5		2.8	2.5	1.3
1.6	1.0		4.4	1.2	9.0	
1.77		3.1	2.6			2.6
2.04	29.0	22.3	1.3	22.7	6.2	
2.4					5.6	
2.77	45.3	54.0	45.5	48.0	36.6	53.9
3.3					7.9	2.1
3.6	5.1			2.4		
3.9				2.0	9.3	
4.8		10.0	40.0		1.5	30.0
5.4					5.2	
6.8					1.2	2.4

^a Results are expressed as a percentage of the total fatty acids in each fraction. Abbreviations: *O*-Ac, *O*-acyl fatty acid; *N*-Ac, *N*-acyl fatty acid.

hydroxy myristic acid had a characteristic retention time of 2.0 in contrast to the retention time of 2.77 for the unknown fatty acid.

The fatty acid samples containing TMS-hydroxy fatty acid methyl esters were methanolized in BF_3 -methanol at 100 C for 2 min. Fatty acids with retention times of 0.72 and 1.3 disappeared, and there appeared fatty acids having retention times of 2.77 and 4.8. These results indicate that fatty acids with retention times of 0.72 and 1.3 are TMS-hydroxy fatty acids methyl esters. *N*-acyl-bound fatty acids obtained by the treatment of LPS with $\text{NH}_2\text{OH}\cdot\text{HCl}\text{-NaOH}$ (4:5) were desaturated with POCl_3 , and pyridine to determine the number of carbons. After subsequent hydrogenation, a significant increase of the palmitic acid was observed. This indicated that the component with $r_p = 4.8$ was hydroxypalmitic acid.

A decline of the content of the hydroxy acid with $r_p = 2.77$ was observed after the desaturation-hydrogenation with P_2O_5 . At the same time there appeared three new peaks corresponding to lauric acid ($r_p = 0.33$; 8.6%), myristic acid ($r_p = 0.57$; 22.7%), and an unidentified compound with $r_p = 2.4$.

The results of trimethylsilylation and desatu-

ration-dehydrogenation indicated that the fatty acid of $r_p = 2.77$ was hydroxymyristic acid. The relative retention of β -hydroxymyristic acid of LPS from *Salmonella typhimurium* 902 was also equal to 2.77.

DISCUSSION

In LPS from *Salmonella*, *Escherichia*, *Klebsiella*, *Serratia*, *Proteus*, and *Citrobacter*, the major fatty acids—lauric, myristic, and palmitic—are ester linked, whereas 3-hydroxymyristic acid is an amide-bound component of lipid A (15). In *Pseudomonas*, apart from the 2-hydroxydodecanoic acid, all the hydroxy acids are β -hydroxy acids, and 3-hydroxydodecanoic acid predominated in most of the tested species. Odd-numbered hydroxy acids were minor components in *P. diminuta* and *P. pavonacea* and major components (C_{13} acids) in *P. rubescens* (18). The absence of 3-hydroxymyristic acid, a characteristic component of the LPS of other gram-negative bacteria, was particularly striking in *P. aeruginosa* and *P. synchyanea* (8, 18). The bound lipid of *Veillonella* contained the odd-numbered acids 3-hydroxytridecanoic and tridecanoic acid as the major constituents of ester-bound acids, whereas 3-hydroxypentadecanoic acid was the *N*-acyl-bound derivative (9).

We found that the composition of the fatty acids of *R. trifolii* LPS was different from those of *Salmonella*, *Escherichia*, and *Aerobacter* as well as *Pseudomonas*. The most characteristic feature of *Rhizobium* LPS was the high concentration of hydroxy acids (70% of the total fatty acids). In *Azotobacter agilis* and *P. aeruginosa* the content of hydroxy acids was also high (8, 12). However, there were differences between *Rhizobium* and *Azotobacter* in hydroxy acid components. In *Azotobacter agilis*, 3-hydroxydodecanoic, 3-hydroxytridecanoic, and 2-hydroxydodecanoic acids were identified, whereas in *R. trifolii* hydroxymyristic and hydroxypalmitic acids predominated. The fatty acid composition for *R. trifolii* 24SM measured from fatty acid methyl esters prepared from HCl and NaOH hydrolysates versus that given for the direct BF₃ methanolysis strongly suggest that hydroxypalmitic acid is the *N*-acyl-bound acid. The same conclusion was reached on the basis of hydroxylaminolysis experiments with LPS. Various species of microorganisms, belonging to the same genus, often show significant differences in respect of the fatty acid composition. In *Neisseria catarrhalis*, β -hydroxylauric acid prevailed, in contrast to β -hydroxymyristic acid,

which was the major fatty acid component of the lipid A of *Neisseria perflava* (1). Important differences were also found between *R. trifolii* and *R. japonicum* in regard of the fatty acid components. *R. japonicum* did not contain, whereas *R. trifolii* showed, a high concentration of hydroxymyristic and hydroxypalmitic acids. However, it must be emphasized that in the case of *R. trifolii*, lipids were isolated from LPS, whereas results of *R. japonicum* were obtained with lipids of whole cells. The palmitic acid was the only saturated fatty acid identified in the LPS of *R. trifolii*.

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