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Straight and branched (ω-1)-hydroxylated very long chain fatty acids are components of *Bradyrhizobium* lipid A

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Lipopolysaccharides of seven *Bradyrhizobium* strains and three whole-cell fatty acid preparations from bacteria isolated from nodules of *Sarothamnus scoparius* (Common Broom) were studied for the presence of very long chain (ω-1)-hydroxy fatty acids. Several such fatty acids were identified. Among them, straight-chain as well as mono- and dimethyl branched acids with chains in the range from 26 to 34 carbon atoms were found. Pyrrolidides and 4,4-dimethyloxazoline derivatives were used to determine the branching position. Carbons at the (ω-10) and/or (ω-11) positions in alkyl chains were points of attachment of methyl groups. These data complete the structure of bradyrhizobial lipid A with important details. The obtained results can be applied in the chemotaxonomy of *Bradyrhizobium*.

Keywords: *Bradyrhizobium*, symbiosis, LPS, lipid A, fatty acids

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INTRODUCTION

The order *Rhizobiales* comprises Gram-negative bacteria capable of living in symbiosis with leguminous plants (Sawada *et al.,* 2003). This taxon includes, among others, various strains which grow slowly or extra-slowly on typical rhizobial media. These bacteria are classified in the *Bradyrhizobiaceae* family (Young *et al.,* 2001; Sawada *et al.,* 2003). Lipopolysaccharide (LPS) is the major surface component of Gram-negative bacteria. The composition and structure of LPS is crucial for the development of symbiosis between rhizobia and host plants (Kannenberg *et al.,* 1998). Lipopolysaccharide molecules are anchored in the outer membrane by a lipophilic component — lipid A. This part of LPS consists of an aminosugar backbone bearing amide- and ester- or exclusively amide-bound 3-hydroxy fatty acids. Other fatty acid residues are known as secondary substituents creating acyloxyacyl moieties (Zähringer *et al.,* 1999). It has been shown that all rhizobia, with the exception of *Azorhizobium caulinodans,* contain significant amounts of 27-OH28:0 acid in their LPSs (lipids A). This component is present regardless of the type of lipid A backbone (Bhat *et al.,* 1991a; 1991b). Further investigations have revealed the occurrence of other bacteria from the *Rhizobiales* having (ω-1)-hydroxylated fatty acids with hydrocarbon chains twice the usual length (about 30–32 carbon atoms in a chain) as well as their oxo counterparts (Gil-Serrano *et al.,* 1994; Russa *et al.*, 1995; Choma, 1999; Silipo *et al.*, 2004). Very long chain fatty acids (VLCFAs) with a hydroxyl at the penultimate position have been detected in lipid A of members of the α-2 branch of Proteobacteria (Bhat *et al.*, 1991b). Dicarboxylic and oxo VLCFAs have also been found in *Legionella* lipopolysaccharides (Moll *et al.*, 1992; Sonesson *et al.,* 1994a; 1994b; 1994c). Additionally, some of these bacteria synthesized small amounts of 27-OH28:0 fatty acid. It has been postulated that the VLCFAs which stretch through the entire outer membrane play a role in its stabilization and in strong anchoring of the LPS in the membrane. This problem is important in the case of the intracellular lifestyle of the bacteria, where there is a continuous interaction between eukaryotic and bacterial membranes (Lerouge & Vanderleyden, 2001; Becker *et al.*, 2005).

MATERIALS AND METHODS

Bacterial strains and lipopolysaccharide extraction. Bacterial strains and their sources are listed in Table 1. The bacteria were grown at 28 °C in liquid 79CA medium in which mannitol was replaced with glycerol (Vincent, 1970). The culture was aerated by vigorous shaking. Lipopolysaccharides were extracted from the cells by the hot phenol-water procedure with the modification of Johnson and Perry (1976). The water and phenol phases were dialysed extensively against tap and distilled water. Enzymatic degradation of nucleic acids and proteins was carried out using DNase, RNase and proteinase K, respectively, and dialysis was performed again. Lipopolysaccharides were purified by ultracentrifugation (105 000 × *g*, 4 °C, 4 h).

Lipid A fatty acids from *Sarothamnus scoparius* isolates were liberated directly from the delipidated bacterial cells. Extractable lipids were removed from cell pellets by an exhaustive washing with a mixture of chloroform/methanol (2:1, v/v).

Preparation of fatty acid derivatives. Fatty acids were released and converted into methyl esters by methanolysis (2 M HCl in methanol, 85 °C, 16 h) of dried LPS. Preparations of fatty acid methyl esters were trimethylsilylated with silyl reagent (Supelco, cat no. 33038) and the reaction mixture was directly injected into a GLC-MS apparatus. Ester- and amide-linked fatty acids were distinguished using a method described by Wollenweber and Rietschel (1990) or the method described by Sonesson and co-workers (1994). Fatty acids from bacteria isolated from *Sarothamnus scoparius* (Common Broom) roots were prepared on a micro-scale. Briefly, the bacte-

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Abbreviations: DMOX, 4,4-dimethyloxazoline; EI-MS, electron impact-mass spectrometry; FAME, fatty acid methyl ester; GLC-MS, gas-liquid chromatography-mass spectrometry; LPS, lipopolysaccharide; TMS, trimethylsilylate; VLCFA, very long chain fatty acid

ria were cultivated in small volumes (20 ml) of the medium and centrifuged. The bacterial masses were washed several times with chloroform/methanol (2/1; v/v) to remove free lipids. Delipidated cells were solvolized (2 M HCl in methanol, 85 °C, 16 h) to liberate total fatty acids from lipid A. The method for converting fatty acid methyl esters into pyrrolidides had been described by Andersson and Holman (1974). 4,4-Dimethyloxazoline (DMOX) derivatives were prepared in a one-step reaction of LPS with 2-amino-2-methyl-1-propanol (Fay & Richli, 1991) or *via* a reaction of free fatty acids, liberated from LPS by standard acid hydrolysis (4 M HCl, 5 h, 100 °C), with 2-amino-2-methyl-1-propanol (Hamilton & Christi, 2000).

Gas-liquid chromatography-mass spectrometry. Fatty acid derivatives were analyzed routinely with a Hewlett-Packard gas chromatograph (model HP 5890A) equipped with a capillary column (HP-5MS, 30 m × 0.25 mm) and connected to a mass selective detector (MSD model HP 5971). The carrier gas was helium and the temperature program was initially 150 °C for 5 min, then raised to 310 °C at a ramp rate of 5 °C/min, final time 40 min. Isothermic separation of TMS derivatives of fatty acid methyl esters was performed on the same chromatograph and column at a constant temperature of 310 °C.

RESULTS

As highly hydrophobic compounds, lipopolysaccharides of *Bradyrhizobium elkani* USDA 76 and *B. liaoningense* USDA 3622 were extracted to the phenol phase, whereas a majority of LPSs from *B. yuanmingense* CCBAU 10071, *Bradyrhizobium* sp. (Lupinus) USDA 3045 and WM9, and *B. japonicum* USDA 110 and USDA 6 were recovered from the water phase. For example, during the process of extraction of *B. japonicum* USDA 110 cells, LPS was partitioned between the phenol and water phases at a ratio of 1:6 (w/w) (Puvanesarajah *et al.,* 1987; Carrion *et al.*, 1990). The isolated and purified lipopolysaccharides were the source of fatty acids used in further analyses. To simplify the procedure of lipid A fatty acids isolation for chemotaxonomic purpose the fatty acids from *Sarothamnus scoparius* isolates were liberated directly from the delipidated bacterial cells. Fatty acid methyl esters, after conversion of hydroxyls to trimethylsilyloxy ethers, were analyzed using a gas chromatograph connected to a mass spectrometer.

Chromatographic properties and mass spectra of VLCFA. The obtained total ion chromatograms of TMS derivatives of fatty acid methyl ester preparations were very similar to one another. Figure 1 shows a chromatogram of fatty acid methyl esters isolated from *Bradyrhizobium* sp. (Lupinus) USDA 3045 LPS. The peaks for 3-hydroxydodecanoic and 3-hydroxytetradecanoic acids and small amounts of saturated non-polar fatty acids as well as a broad range of very long chain fatty acids which contain hydroxyls at the penultimate position in the aliphatic chain were observed. The latter group of acids yielded two prominent ions in the EI-MS spectra. The ion at m/z 117 originated from splitting the bond between (ω-1) and (ω-2) carbons in the acyl chain. The other one was formed by loss of methyl from the molecular ion ($[M\text{-}15]^+$) and was used to determine the molecular mass or the number of carbon atoms in the individual fatty acids. The results of a compositional analysis of very long fatty acid methyl esters are presented in Table 2. All of the (ω-1)-hydroxylated fatty acids were found to be ester-linked to lipids A. The shortest acyl residue contained 26 carbon atoms in the aliphatic chain; the longest one was 34-OH35:0 acid. Peaks representing 27-OH28:0 or/and 30-OH31:0 acids were most frequently the highest on the chromatograms (see Fig. 1 and Table 2). It is worthy of note that the preparations from *B. elkanii* USDA 76 and *B. liaoningense* USDA 3622 also contained (ω-1)-oxo fatty acids — the oxidized forms of 27-OH28:0 and 29-OH30:0 acids (Table 2). These oxo fatty acids were identified by their characteristic spectra and molecular ions at m/z 452 and 480, respectively.

TMS derivatives of methyl esters of VLCFA from *Bradyrhizobium* migrated along the chromatographic column in pairs (Fig. 1). The first peak in a doublet always represented a fatty acid with an even number of carbon atoms, the second had one carbon atom more. A chromatogram recorded isothermally at 310 °C for trimethylsilyl derivatives of fatty acid methyl esters from *Bradyrhizobium* sp. (Lupinus) USDA 3045 was used to draw logarithmic plots of retention times *versus* number of carbon atoms (not shown). On this plot, points formed two parallel lines representing the correlation for even-numbered fatty acids and for odd-numbered ones, respectively. This chromatographic feature of the fatty acids allowed us to conclude that almost all the VLCFAs belonged to two homologous series. Presumably, bradyrhizobia can synthesize straight and branched-chain fatty acids. This problem was resolved by a fine structure

Table 1. Bacterial strains used in this study

Bradyrhizobium strain	Host plant and geographic origin	Source
B. japonicum USDA 6	*Glycine max,* United States	USDA
B. japonicum USDA 110	*Glycine max,* United States	USDA
B. elkanii USDA 76	*Glycine max,* United States	USDA
B. liaoningense USDA 3622	*Glycine soya, Glycine max,* China	USDA
B. yuanmingense CCBAU 10071	*Lespendeza cuneata,* China	CCBAU
B. sp. (*Lupinus*) USDA 3045	*Lupinus* sp., United States	USDA
B. sp. (*Lupinus*) WM9	*Lupinus* sp., Poland	Dr. W. Malek, Dept. Gen. & Microbiol.
B. sp. (*Sarothamnus*) O5B2	*Sarothamnus scoparius,* Poland	Dept. Gen. & Microbiol.
B. sp. (*Sarothamnus*) O4B2	*Sarothamnus scoparius,* Poland	Dept. Gen. & Microbiol.
B. sp. (Sarothamnus) O3B1	*Sarothamnus scoparius,* Poland	Dept. Gen. & Microbiol.

CCBAU, Culture Collection of Beijing Agricultural University, Beijing, People's Republic of China; USDA, United States Department of Agriculture, Beltsville, Md.; Dept. Gen. & Microbiol., Department of Genetics and Microbiology, Maria Curie-Skłodowska University, Lublin, Poland

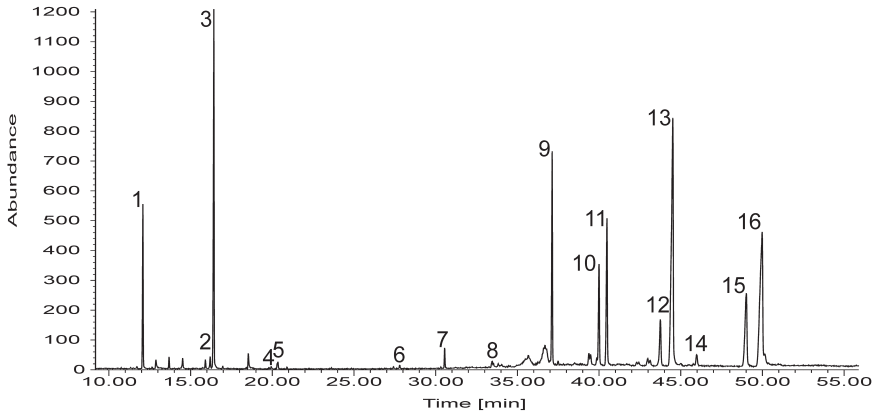

Figure 1. Gas chromatogram of TMS ethers of fatty acid methyl esters isolated from LPS of *Bradyrhizobium* sp. (Lupinus) USDA 3045
Methyl esters of the following fatty acids: **1**. 3-OH12:0; **2**. 16:0; **3**. 3-OH14:0; **4**. 18:1; **5**. 18:0; **6**. 22:0; **7**. 24:0; **8**. 26:0; **9**. 25-OH26:0; **10**. 27-OH28:0; **11**. 28-OH29:0; **12**. 29-OH30:0; **13**. 30-OH31:0; **14**. 31-OH32:0; **15**. 31-OH32:0[a]; **16**. 32-OH33:0. See Materials and Methods for chromatographic conditions. 31-OH32:0[a] -structural isomer of 31-OH32:0.

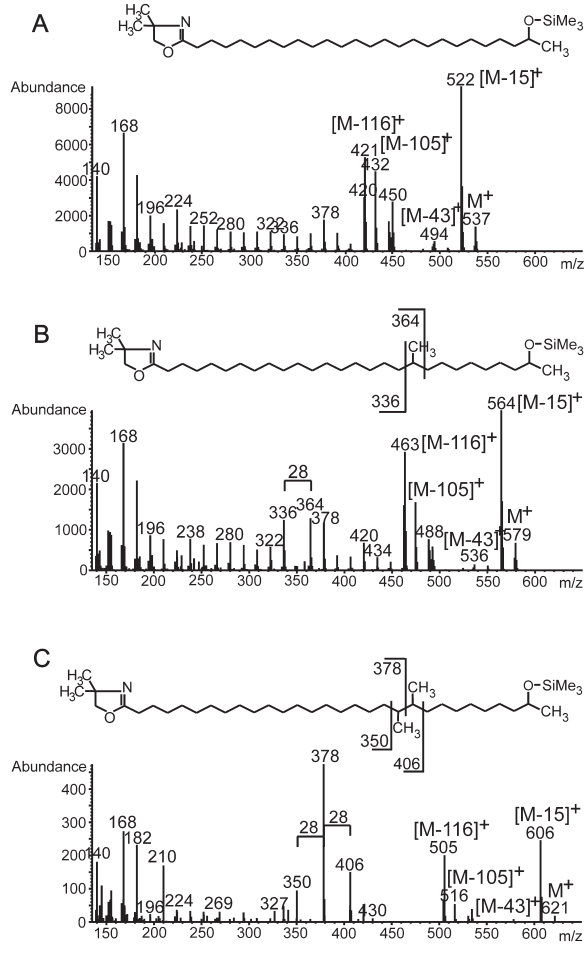

Figure 2. Structures and partial mass spectra of TMS ethers of DMOX derivatives of chosen fatty acids
(A) 25-OH26:0 [25-hydroxyhexacosanoic, 37.13 min], **(B)** 28-OH29:0 [27-hydroxy-19-methyloctacosanoic, 40.49 min] **(C)** 31-OH32:0[a] [29-hydroxy-20,21-dimethyltriacontanoic, 45.97 min]. In square brackets chemical names and retention times are given.

analysis using DMOX and pyrrolidine derivatives of the fatty acids. These methods are widely used for determination of fatty acid structures in mass spectrometric terms. Despite differences in their structure, both types of derivatives had identical molecular mass and the McLafferty ions at m/z 113 as their base peaks. These ions were accompanied by prominent ions at m/z 126. The spectra of the TMS ether pyrrolidides or DMOX derivatives of *Bradyrhizobium* (ω-1)-hydroxylated very long chain fatty acids contained an additional prominent ion at m/z 117 resulting for α-cleavage relative to the trimethylsilyl ether group. The presence of a trimethylsilyloxy group at the (ω-1) position of VLCFA also resulted in the formation of ion fragments at $[M-15]^+$, $[M-87]^+$, $[M-105]^+$, and $[M-116]^+$. The molecular ion was always present but at a low intensity of about 5% (Fig. 2) (Tulloch, 1980; Hamilton & Christi, 2000; Rontani & Aubert, 2008). Mass spectra of the TMS ethers of pyrrolidides exhibited two series of ions the formation of which was attributed to sequential cleavage of the alkyl chain and to an initial trimethylsilyl transfer from the ether group to the ionized ester group. Thus, we additionally observed a prominent ion at m/z 185 (Tulloch, 1980) and ions having the following structures: $H_2C{=}CH{-}(CH_2)_n{-}C[O^+{-}Si(CH_3)_3]N(CH_2{-}CH_2)_2$. This type of rearrangement is impossible in DMOX derivatives. Since the mass spectra of TMS ethers of DMOX derivatives were simple, they were subjected to further analysis. The cleavage of a methyl radical from the DMOX ring giving the ion $[M-15]^+$ and fragmentation leading to a loss of a propyl moiety $[M-43]^+$ only negligibly complicated the high molecular mass region of the spectra, but this problem did not concern us since the most interesting ions had lower masses.

Localization of the methyl branch in VLCFA. On the basis of their similarities, mass spectra of 4,4-dimethyloxazoline derivatives were clustered into several groups. Individual VLCFA clusters are discussed below using specific mass spectra.

Figure 2A shows part of the mass spectrum of the 25-OTMS26:0 DMOX derivative. Apart from the ions mentioned in the previous section, the spectrum contained intensive ions derived from the nitrogen-containing end of the fatty acid derivative, which were 14 units (amu) apart. This pattern was characteristic of all fatty acid derivatives with an even number of carbon atoms (from 26 to 34). The only exception was 31-OTMS32:0 acid migrating at 45.97 min (see Table 2 and text below). Therefore, it can be concluded that fatty acids with long, even-carbon-atom-number chains create a group of straight-chain saturated fatty acids decorated with hydroxyl at the penultimate position.

Figure 2B shows a mass spectrum of 28-OTMS29:0 acid, which is characteristic of fatty acids with a methyl branch at position (ω-10). The branching methyl group was located by the fact that a gap of 28 amu was situat-

Table 2. Composition of very long chain fatty acids (VLCFA) from LPS of bradyrhizobia and from isolates from *Sarothamnus scoparius*.

No.	Fatty acid preliminary names*	type of chain/methyl position**	retention time*** [min.]	*B. japonicum* USDA 110	*B. japonicum* USDA 6	*B. elkanii* USDA 76	*B. liaoningense* USDA 3622	*B. yuanmingense* CCBAU 1071	*B.* sp. (*Lupinus*) USDA3045	*B.* sp. (*Lupinus*) WM9	*B.* sp. (*Sarothamnus*) O5B2	*B.* sp. (*Sarothamnus*) O4B2	*B.* sp. (*Sarothamnus*) O3B1
1.	25-OH26:0	n/–	37.13	10.6	7.8	2.2	4.5	8.8	10.4	13.0	10.1	5.0	17.4
2.	26-OH27:0	br/(ω-10)	37.51	–	–	–	–	tr	tr	–	–	–	–
3.	27-OH28:0	n/–	40.00	10.5	8.2	56.6	46.9	10.6	6.4	12.5	5.5	40.9	8.7
4.	28-OH29:0	br/(ω-10)	40.49	2.8	–	–	–	–	9.7	2.3	0.5	–	4.3
5.	29-OH30:0	n/–	43.76	12.3	20.7	11.8	6.5	16.6	4.2	14.9	14.9	43.5	11.8
6.	30-OH31:0 (two isomers)	br/(ω-10) br/(ω-11)	44.52	16.2	21.9	6.2	17.1	46.1	28.2	32.6	30.8	8.1	33.2
7.	31-OH32:0[a]	Me_2-br/(ω-10), (ω-11)	45.97	0.3	3.8	–	–	–	–	13.3	12.0	–	13.0
8.	31-OH32:0[b]	br/?	47.69	0.1	2.2	–	–	–	–	2.3	0.7	–	–
9.	31-OH32:0[c]	n/–	49.02	22.7	28.6	4.2	3.9	–	10.2	4.5	17.1	2.5	4.5
10.	32-OH33:0	br/(ω-10)	49.98	24.5	6.8	1.4	6.0	18.0	21.7	4.4	5.5	–	7.1
11.	33-OH34:0	?/?	54.32	–	tr	–	–	tr	–	–	1.2	–	–
12.	34-OH35:0	?/?	55.43	–	tr	–	–	tr	–	–	1.6	–	–
13.	27-oxo-28:0	n/–	39.26	–	–	16.3	9.5	–	–	–	–	–	–
14.	29-oxo-30:0	n/–	42.90	–	–	1.3	5.7	–	–	–	–	–	–

Calculations were performed based on chromatograms of TMS ethers of FAME using the internal normalization method. The compositions were given as relative percents. Only very long chain fatty acids were taken into consideration. *Number of carbon atoms in fatty acids was determined based on FAME EI mass spectra. **Type of chain was determined by the analysis of EI-MS spectra of TMS ethers of fatty acid pyrrolidides or DMOX derivatives. ***See Materials and Methods for chromatographic conditions; br, mono-methyl-branched chain fatty acid; Me_2-br, dimethyl-branched chain fatty acid; n, straight chain fatty acid; tr, traces; [a,b,c]structural isomers of 31-OH32:0 (M_m=582 Da).

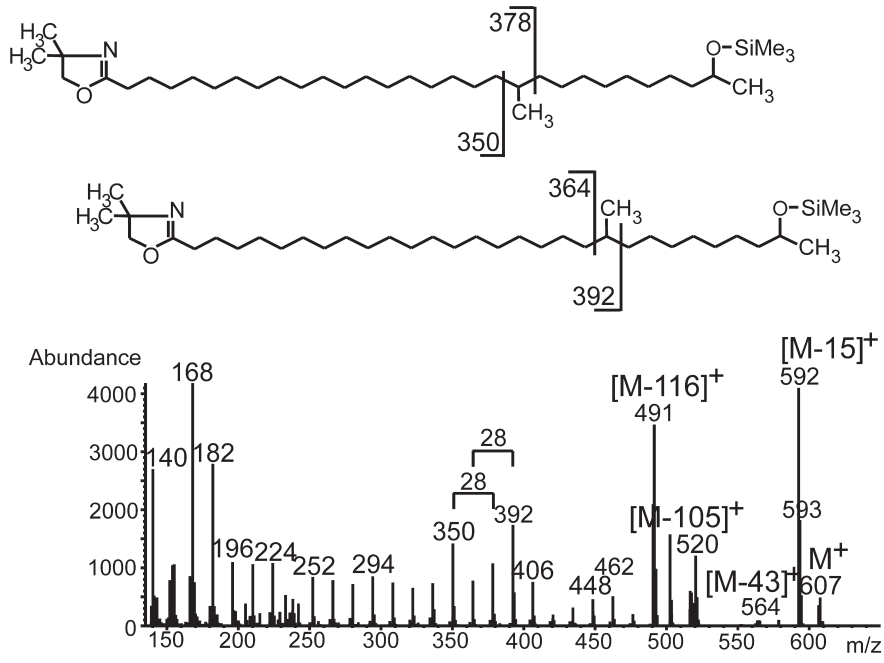

Figure 3. Structures and partial mass spectra of TMS ethers of DMOX derivatives of two unseparated isomers of 30-OH31:0 acid
The two acids were present as peak no. 13 on chromatogram, Fig. 1. Their chemical names are 29-hydroxy-20-methyltriacontanoic and 29-hydroxy-21-methyltriacontanoic acid.

ed between ions at *m/z* 336 and 364. A similar scheme of fragmentation was found for the 26-OH27:0, 28-OH29:0, and 32-OH33:0 acids (see Table 2).

Bradyrhizobium sp. (Lupinus) MW9, *B. japonicum* USDA 6 and USDA 110, as well as two isolates from *Sarothamnus scoparius* produced considerable amounts of double-branched VLCFAs (Table 2). The mass spectrum shown in Figure 2C allowed us to identify two branching methyls at positions (ω-10) and (ω-11) in the alkyl chain.

Figure 3 shows a mass spectrum which can be explained as an overlap of two independent spectra: a spectrum with a 28 amu gap between *m/z* 350 and 378 and a second one with a gap between *m/z* 364 and 392. The difference between the two spectra (and the two compounds) is restricted to the position of the methyl residue. One fatty acid has the methyl at position (ω-10) and the other at position (ω-11). The variable ratios of ion abundances (especially the ions at *m/z* 350, 364, 378, and 392) measured in the different parts of the chromatographic peak (retention time of 44.52 min.) indicated that these two fatty acids were only slightly separated on the column used and under the chosen chromatographic conditions.

Both of the identified oxo VLCFAs had straight and saturated aliphatic chains. The structures of the other fatty acids (retention times of 47.69 min, 54.32 min and 55.43 min) were not established due to their low amounts.

The chemical names of the analyzed VLCFAs are given in the legends to Fig. 2 and Fig. 3.

DISCUSSION

Rhizobial lipids A are generally of low or no toxicity to animal cells and do not stimulate the mammalian immune system to respond by macrophage and lymphocyte proliferation, Shwartzman reaction, and induction of cytokine and TNF synthesis. These features are associated with the completely different chemical structure of lipids A compared to their enterobacterial counterparts (Park *et al.*, 2009). Apart from the differences in the structure and composition of the sugar backbone, the fatty acids directly connected to the polar part of lipid A have a different chain length. These substituents bear a different number and type of secondary or even tertiary acyls. In this paper, special attention was paid to very long chain fatty acids. The first report on the isolation and chemical characterization of 27-OH28:0 from *R. leguminosarum* bv. *trifolii* was published twenty years ago by Hollingsworth and Carlson (1989). Later, Bhat and co-workers (1991a) demonstrated that all species belonging to *Rhizobiaceae*, except for *Azorhizobium caulinodans*, contained this fatty acyl within their LPSs. Since that time, 27-OH28:0 acid has become a valuable taxonomic marker of rhizobia. The results of further studies showed that not only rhizobia but some other microorganisms from the α-2 subgroup of Proteobacteria could synthesize and incorporate (ω-1)-hydroxylated very long chain fatty acids into their lipids A (Bhat *et al.*, 1991b). Special attention was paid to 28-OH29:0 acid from *Rhodopseudomonas palustris* strain le5. Its chromatographic proprieties indicated a branched structure; however, this feature of 28-OH29:0 acid has never been proved by detailed analysis. The group of very long fatty acids was extended to include 29-OH30:0 acid, which was identified in *Rhizobium tropici* LPS (Gil-Serrano *et al.*, 1994), 27-OH28:0 and 27-oxo28:0 in the LPS from *R. leguminosarum* bv. *viciae* (Izmailov *et al.*, 1999), as well as 25-OH26:0, 27-oxo28:0, and 29-OH30:0 in the LPS of *M. huakuii* (Choma, 1999). Based on mass spectrometry and NMR data, the precise linkage of very long chain acyls within the lipid A was determined (Que *et al.*, 2000a; 2000b). Structural analyses of lipids A published to-date support the hypothesis that rhizobia incorporate one VLFA molecule to lipid A as a secondary fatty acid (Que *et al.*, 2000a; Jeyaretnam *et al.*, 2002; Gudlavalleti & Forsberg 2003; Choma & Sowinski, 2004). According to our knowledge, no systematic studies are available on VLCFAs isolated from *Bradyrhizobium* strains. This article fills this gap showing that preparations of fatty acids liberated from *Bradyrhizobium* strains contain significant amounts of various (ω-1)-hydroxylated VLCFAs, always accompanied by 3-hydroxydodecanoic and 3-hydroxytetradecanoic acids, and small amounts of nonpolar saturated acids. It is important to mention here that fast-growing rhizobia incorporate to lipids A different sets of 3-hydroxy fatty acids (Yokota *et al.,* 1993; Tighe *et al.*, 2000). This unique total fatty acid pattern could be used as a chemotaxonomic marker for identifying slow-growing rhizobia isolated from nodules of wild-growing

leguminous plants. Preliminary studies have been done on three recently isolated strains from *Sarothamnus scoparius* nodules. The characteristic fatty acid patterns obtained (Table 2) suggested that these bacteria belonged to the genus *Bradyrhizobium*. Genetic data and biochemical characterization (R. Russa, unpublished) confirmed these results.

The simple and time-effective chromatographic analysis (GLC-MS) once more proved its usefulness in preliminary classification of microorganisms (Tighe *et al.,* 2000). Obviously, the discussed fatty acyl pattern reflects the complicated biochemical machinery characteristic of *Bradyrhizobium*. However, the quantitative and qualitative composition of the cellular fatty acids depends on the conditions under which rhizobia grow (Fraysse *et al.,* 2002). This criterion should always be taken into account during chemotaxonomic investigations.

In this paper, it was proved that bradyrhizobial VLCFAs with an odd number of carbon atoms (though not exclusively — see text) had a branched structure. The branching carbon is at the (ω-10/11) position. Biosynthesis of (ω-1)-hydroxylated VLCFAs with internal methyl branching seems to be a complex process in which the methyl group is transferred from *S*-adenosylmethionine to the double bond of an appropriate olefinic acid. This biosynthetic pathway has been described for tuberculostearic and related acids characteristic of myco- and corynebacteria (Goren, 1972; Couderc *et al.,* 1991). The presence of branching methyl groups in chains of fatty acids causes the membrane to become less ordered and therefore more fluid. Bacteria can adapt to the microchanges in the environment by modifying the proportions between branched and straight chain acyl substituents in lipid A.

Many of the bacteria containing lipid A with VLCFAs lead an intracellular life. Some of them are animal or plant pathogens but others are able to create symbiotic relationships with leguminous plants and can, in the form of bacteroids, convert atmospheric nitrogen into ammonia. The presence of VLCFAs in their lipids A may result in a stronger anchoring of the entire LPS in the outer membrane, which makes the membrane more stable. This feature seems to be advantageous for intracellular living bacteria having continuous intimate contact with the plant cell membranes. In the case of *Bradyrhizobium,* the bacteria have to penetrate the narrow infection thread and transit to bacteroids clustered in groups surrounded by peribacteroidal membranes. In the host tissue, the bacterium/bacteroid cannot afford to lose its own LPS molecules since its stable anchoring is essential for establishing a healthy and effective symbiosis. Note that *S. melilotii* and *R. leguminosarum* mutants deprived of (ω-1)-hydroxylated VLCFAs infected their hosts with a delay. The mutants also changed their growth rates and physiological properties (Sharypova *et al.,* 2003; Vedam *et al.*, 2003; Ferguson *et al.*, 2005; Vanderlinde *et al.*, 2009). It was found that the AcpXL mutant of *R. leguminosarum* bv. *viciae* 3841, unable to incorporate 27-OH28:0 acid into its lipid A in laboratory conditions of growth, was able to partially restore this ability inside the nodule environment (Vedam *et al.*, 2006). It is worth remembering that during the process of nodulation the entire LPS, including lipid A, undergoes modifications which convert it into a more hydrophobic form. Thus the proper structure of LPS is necessary for the formation of determinate nodules. Therefore, it is not surprising that these LPS mutants were arrested at the stage of nodule/tissue penetration (Lerouge & Vanderleyden, 2001; Becker *et al.,* 2005).

Bradyrhizobia are able to synthesize a wide range of VLCFAs and incorporate them into their lipids A. The following facts confirm this: **i)** The proportion between 3-hydroxy fatty acids directly linked to the lipid A backbone and (ω-1)-hydroxylated VLCFAs is approximately 4:2 or even 4:3 for *Bradyrhizobium* in contrast to the ratio of 4:1 for *R. leguminosarum* (Que *et al.*, 2000b) and other rhizobia. **ii)** Moreover, we have data (unpublished) confirming the occurrence of *Bradyrhizobium* species containing lipids A with three or even four secondary VLCFA residues. **iii)** Recently, the complete structure of *B. elkanii* lipid A has been published (Komaniecka *et al.*, 2010), in which VLCFAs, 3-hydroxydodecanoic and 3-hydroxytetradecanoic acids, created two acyloxyacyl residues attached to the distal GlcN3N. The results presented here complete the published bradyrhizobal lipid A structure with important details. Consequently, *Bradyrhizobium* bacteria seem to be very sensitive to the lack of VLCFAs in LPS. Mutants unable to synthesize VLCFAs are under construction. This work is facilitated by the knowledge of the complete genome sequence of *Bradyrhizobium japonicum* USDA 110 including enzymes for VLFA biosynthesis which are encoded by five genes (bll3807-bll3811; http://www.ncbi.nlm.nih.gov or http://blast.genome.jp) and are closely related to corresponding genes in two photosynthetic bradyrhizobia (strains BTAi1 and ORS278) whose genome sequences have also been deposited in the NCBI database.

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