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Lipopolysaccharides from *Mesorhizobium huakuii* and *Mesorhizobium ciceri*: chemical and immunological comparative data

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Lipopolysaccharides of two *Mesorhizobium* species of different host specificity were compared: *M. huakuii* and *M. ciceri*. *M. huakuii* sp. was represented by five strains with special consideration of *M. huakuii* IFO 15243^{T}.

SDS/PAGE profiles revealed that all *M. huakuii* LPS preparations contained low molecular mass fractions (LPS-II) of the same molecular size. All of lipopolysaccharides contained high molecular mass fractions (LPS-I). However, the high molecular mass fraction from each strain possessed an individual molecular size distribution pattern. The crossreactivity of blotted lipopolysaccharides with rabbit polyclonal antibodies against *Mesorhizobium huakuii* IFO 15243^{T} whole bacteria indicated the presence of common epitope(s) within the investigated *Mesorhizobium huakuii* strains. Moreover, LPS from *M. huakuii* S52 also reacted with anti *M. ciceri* HAMBI 1750 serum showing that there are epitopes common for different mesorhizobial species.

LPS isolated from *Mesorhizobium huakuii* strain IFO 15243^{T} contained neutral sugars: L-6-deoxytalose, L-rhamnose, D-galactose and D-glucose, aminosugars: D-quinovosamine, D-glucosamine, D-2,3-diamino-2,3-dideoxyglucose and D-galacturonic and D-glucuronic acids. In the LPS preparation, fatty acids typical for *Mesorhizobium* strains were detected. 3-Hydroxydodecanoic, 3-hydroxy-*iso*-tridecanoic, 3-hydroxyeicosanoic, 3-hydroxyheneicosanoic and 3-hydroxydocosenoic acids were the major amide linked fatty acids, while *iso*-heptadecanoic, eicosanoic, docosenoic, as well as 27-hydroxyoctacosanoic and 27-oxooctacosanoic acids were the dominant ester linked fatty residues.

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Abbreviations: Cps, capsular polysaccharides; EPS, exopolysaccharides; Kdo, 3-deoxy-D-*manno*-octulosonic acid; LPS, lipopolysaccharides.

Bacteria from the *Mesorhizobium* species are classified as other rhizobia in the α subgroup of *Proteobacteria* and are Gram-negative soil microorganisms that induce nitrogen fixing nodules on roots of leguminous plants (Jarvis *et al.*, 1997; Malek & Sajnaga 1999; Tighe *et al.*, 2000). Their surface components, especially polysaccharides, such as acidic exopolysaccharides (EPS), lipopolysaccharides (LPS), capsular polysaccharides (CPS), and cyclic β-glucans, play an important role during the free-living stage in soil environment and especially during the development of symbiosis. Changes of environmental growth conditions affect the cell surface polysaccharide constitution (Kannenberg *et al.*, 1998; Kannenberg *et al.*, 2001).

Mesorhizobium huakuii strains induce the formation of indeterminate nodules on host roots of *Astragalus sinicus*. An acidic EPS is required for the successful establishment of this type of nodules. Hisamatsu and coworkers (1997) have proposed that the backbone structure of *M. huakuii*-My6 EPS is very similar to that of EPS-13336 from *Sinorhizobium meliloti.* Also, the cyclic glucans secreted to the medium by *M. huakuii* My6 have a distribution pattern of ring size resembling that of *S. meliloti.*

In comparison with other rhizobial genera, *Mesorhizobium* LPSs have not been detailed. So far, the structure of two O-specific polysaccharides (Russa *et al.* 1995; Choma *et al.*, 2000), cellular fatty acid composition and lipopolysaccharide fatty acid analyses, as well as characteristics of water and phenol soluble fractions of LPS from *Mesorhizobium* have been described (Yokota, *et al.*, 1993; Russa *et al.*, 1995a; Choma *et al.*, 2000a).

Systematic studies of *Mesorhizobium* lipid A have not been completed. However, several authors have indicated that the sugar backbone of mesorhizobial lipid A represents the DAG-type (meaning, it is composed of 2,3-diamino-2,3-dideoxy-D-glucose disaccharide) (Russa *et al.*, 1995a, Urbanik-Sypniewska *et al.*, 2000). This type of lipid A has been found in *Bradyrhizobium* sp. (Lupinus) (Mayer *et al.*, 1989) but not in any other *Rhizobiaceae* LPS preparations.

For a comparative analysis of LPSs we have selected two *Mesorhizobium* species of different host specificity. *M. huakuii* creates nitrogen fixing nodules on *Astragalus sinicus* and *M. ciceri* on *Cicer arietinum.* Special attention was focused on the chemical and immunochemical characteristics of lipopolysaccharide of *Mesorhizobium huakuii* IFO 15243^T (type strain in the Culture Collection of the Institute of Fermentation, Osaka), as compared with other *M. huakuii* and *M. ciceri* lipopolysaccharides using appropriate rabbit antisera. The strains selected for the analysis were isolated in different regions of the world. Four of *M. huakuii* strains are from China, one is from Japan, whereas *M. ciceri* bacteria were isolated from Spanish soil. The diversified geographic origins of the strains give a good opportunity to conduct comparative studies of bacteria (strains) from the same species or belonging to the same genus yet originating from places very distant from one another.

MATERIALS AND METHODS

Bacterial strains and their sources are listed in Table 1. The bacteria were grown at 28°C in liquid mannitol-yeast extract medium 79CA (Vincent, 1970) and were aerated by vigorous shaking. LPSs were extracted from the cells by the hot phenol-water procedure, with the modification of Johnson & Perry (1976).

For sugar analysis, LPS was hydrolysed with 2 M TFA (100°C, 4 h). The sugars were converted into alditol acetates. Acidic sugars were liberated by methanolysis, then carboxyl reduced with $NaBD_4$, hydrolysed with 2 M TFA (100°C, 4 h), repeatedly reduced with $NaBD_4$, finally peracetylated and analysed as alditol acetates (Russa *et al.*, 1995a). For the analysis of amino sugars in the form of peracetylated amino alditols, LPS sample was hydrolysed with 4 M HCl for 8 h at 100°C and

Table 1. Bacterial strains used in this study

Mesorhizobium strain	Other possible designations of the strain	Host plant and geographic origin	Source and references
huakuii IFO 15243^T	CCBAU 2603	*Astragalus sinicus,* China	IFO Nuswantara *et al.*, 1997
huakuii IFO 15244	My-3	*Astragalus sinicus* subsp. *rengei,* Japan	IFO Yokota *et al.*, 1993
huakuii 38		*Astragalus sinicus,* China	CCBAU Chen *et al.*, 1991
huakuii S52		*Astragalus sinicus,* China	CCBAU Chen *et al.*, 1991
huakuii Pl-52		*Astragalus sinicus,* China	CCBAU Chen *et al.*, 1991
ciceri HAMBI 1750 Type strain	UPM-Ca7^T ATCC 51585^T	*Cicer arietinum,* Spain	HAMBI Nour *et al.*, 1994

CCBAU, Culture Collection of Beijing Agricultural University, Beijing, People's Republic of China; HAMBI, Culture Collection of the Department of Microbiology, University of Helsinki, Finland; IFO, Culture Collection of the Institute of Fermentation Osaka, Japan.

then *N*-acetylated prior to the reduction. The absolute configurations of sugars were determined using (-)-2-butanol for glycoside preparation (Gerwig *et al.,* 1978).

Fatty acids were released and converted into methyl esters by methanolysis (2 M HCl in methanol, 85°C, 16 h) of freeze-dried LPS. Preparations of fatty acid methyl esters were trimethylsilylated with SIL-MIX (POCh, Poland) and the reaction mixture was directly injected into a GC-MS apparatus. Ester- and amide-linked fatty acids were distinguished using a method described by Wollenweber and Rietschel (1990) or according to Sonesson *et al.* (1994). Authentic 4-oxo and 27-oxo fatty acids for comparative chromatographic analysis were isolated from appropriate LPS preparations (*M. loti* NZP2213 (Russa *et al.,* 1995a) and *L. pneumophila* serogroup 1 LPS, respectively).

Alditol acetates and fatty acid methyl esters were analysed 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 20 min.

Polyacrylamide gel electrophoresis (PAGE) was performed with sodium dodecyl sulphate (Laemmli, 1970) and the slab was silver stained after oxidation with periodic acid (Tsai & Frasch, 1982).

Two polyclonal rabbit antisera were obtained against whole *M. huakuii* IFO 15243^T cells and *M. ciceri* HAMBI 1750. Rabbits were injected with 0.2 ml of temperature killed bacteria (about 10^9 bacteria per ml). The bacteria were washed twice with and resuspended in phosphate-saline buffer, pH 7.0, prior to boiling. The rabbits were injected weekly, thrice. Three days after the last injection, the rabbits were bled and antisera were prepared according to Carlson and coworkers (1987). Small portions of the ready to use antisera were stored at −20°C.

Lipopolysaccharides separated by SDS/PAGE were transferred electrophoretically from the gel slab to Immobilon P (Millipore). After reaction with the appropriate antiserum the electroblotted material was immunostained with goat anti-rabbit antibodies coupled to alkaline phosphatase (Sigma). The blots were developed with nitroblue tetra-

zolium and 5-bromo-4-chloro-3-indolylphosphate toluidine (Sigma).

RESULTS AND DISCUSSION

The yield of the water soluble lipopolysaccharide from *M. huakuii* IFO 15243^T strain was approximately 0.3% of the bacterial dry mass. A small portion of LPS was found in the phenol phase. Because of insufficient quantity, the material was not analysed further. The neutral, amino and acidic sugar components of *M. huakuii* IFO 15243^T LPS are given in Table 2. The LPS is mainly composed of 6-deoxytalose and rhamnose, both sugars in L-configuration. D-Galactose and D-glucose, as well as L-*glycero*-D-*manno*-heptose, were also found in significant amounts. The absolute configuration of the last mentioned sugar was estimated by chromatographic comparison with the sugars released from *Azospirillum lipoferum* SpBr17 LPS. This microorganism contains both L-*glycero*- and D-*glycero*-D-*manno*-heptose within its LPS (Choma *et al.*, 1987). The amount of 3-deoxy-D-*manno*-octulosonic acid (Kdo), estimated by the GLC method as appropriate alditol acetate, seems to be too low in relation to the amount of aminosugars, especially 2,3-diamino-2,3-dideoxyglucose (DAG) and in comparison with the known rhizobial lipopolysaccharide structures. Possibly it is due to strong hydrolysis conditions used for liberation of these saccharides. The conditions used in our experiment were directed to neutral sugar hydrolysis and were unappropriate for liberation of Kdo.

Table 2. Neutral, acidic and aminosugar composition of LPS from *Mesorhizobium huakuii* IFO 15243^T

Component	Amount μg/mg LPS	Component	Amount μg/mg LPS
Neutral sugars		**Acidic sugars**	
L-6-Deoxytalose	92.6	D-Galactouronic acid	22.7
L-Rhamnose	110.1	D-Glucuronic acid	13.6
D-Mannose	6.8		
D-Glucose	127.2	**Amino sugars**	
D-Galactose	155.1		
L-Glycero-D-mannoheptose	59.0	D-Quinovosamine	12.7
Kdo	2.3	D-Glucosamine	10.6
		2,3-Diamino-2,3-dideoxyglucose	46.2

In our previous studies it was shown that deoxysugars were the only components of the O-specific chain of *M. huakuii* IFO 15243^T LPS (Choma *et al.*, 2000). The molar ratio of L-6-deoxytalose to L-rhamnose in the O-specific polysaccharide was 2:1. The same ratio, estimated for intact LPS, was nearly 1:1. This divergence can be explained when one takes into consideration that L-rhamnose is also a constituent of the core oligosaccharide. The heptose, the hexoses, D-glucuronic acid and aminosugars, with the exception of DAG, are presumably components of the core oligosaccharide as well. Preliminary studies of lipid A from the IFO 15243^T strain isolated by mild acid hydrolysis (1% acetic acid, 1 h, 100°C) showed that DAG is the only aminosugar present in that fraction of LPS. Similar data have been published by Russa *et al.* (1995a) and Urbanik-Sypniewska *et al.* (2000) for LPS from *M. loti* HAMBI 1129 and HAMBI 1148, respectively.

SDS/PAGE analysis of the LPSs from *M. huakuii* IFO 15243^T, IFO 15244, S52, Pl-52 and 38 strains, as well as *M. ciceri* HAMBI 1750, indicated a high heterogeneity of these preparations (Fig. 1). All lipopolysaccharides

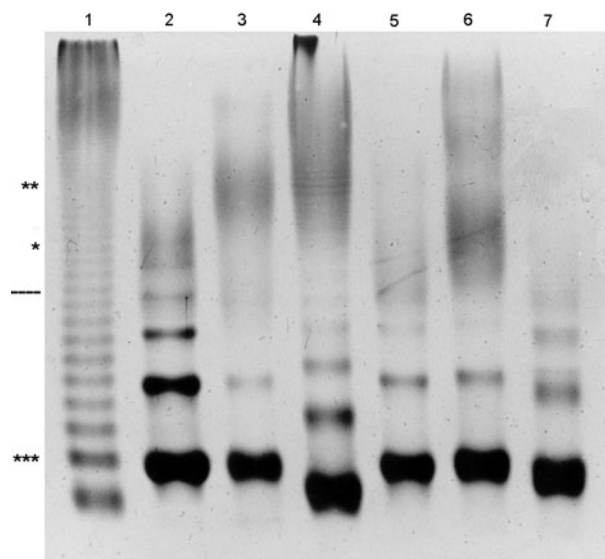

Figure 1. Silver-stained SDS/PAGE profiles of LPS.

Lane 1, *Salmonella montevideo* SH94. (standard); lane 2, *M huakuii* IFO 15243^T; lane 3, *M. huakuii* IFO 15244; lane 4, *M. ciceri* HAMBI 1750; lane 5, *M. huakuii* 38; lane 6, *M. huakuii* S52; lane 7, *M. huakuii* Pl-52. The positions of LPS-I and LPS-II were separated by a dashed line. (–). Asterisk. (*) indicates the position of the most abundant HMW LPS-I fractions of *M. huakuii* IFO 15243^T. Double asterisks. (**) indicate the position of the most abundant HMW LPS-I fractions of *M. huakuii* IFO 15244. Triple asterisks indicate the position of the simplest incomplete LPS (basic core and lipid A).

contained high molecular mass fractions (LPS-I). The distribution of molecular weight of LPS-I was different for each preparation (Fig. 1). For example, the most intense bands of *M. huakuii* IFO 15243^T LPS-I had the same mobility as band No. 15 (counting from the bottom of the slab, indicated in Fig. 1 with an asterisk) from *S. montevideo* SH 94 LPS. The average mass of LPS-I molecules from *M. huakuii* IFO 15244 was higher than that from *M. huakuii* IFO 15243^T LPS and these molecules migrated in a similar manner to the molecules from band No. 20 of *S. montevideo* SH 94 LPS (indicated with two asterisks in Fig. 1). The smallest-sized molecules within LPS-I fractions were found in LPS from *M. huakuii* Pl-52. Intensely stained bands are present in the bottom of the SDS/PAGE electrophoregrams. Low molecular mass fractions in all *M. huakuii* preparations migrated parallel to the band No. 2 of *S. montevideo* LPS (three asterisks). *M. huakuii* LPS preparations, except for IFO 15244 and S52, showed intensely stained second and third low molecular mass bands. The low molecular mass LPSs from *M. ciceri* HAMBI 1750 were found to be even smaller than those from *M. huakuii*, the electrophoregram of this slot is shifted a little down.

It is interesting to note that densitometric analysis of the gel (not shown) showed that the lowest molecular size LPS fractions were also the most abundant and represented more than 30% of LPS in each case.

Figure 2 shows immunoblot of the gel probed with a polyclonal rabbit antiserum against *M. huakuii* IFO 15243^T. LPSs of all

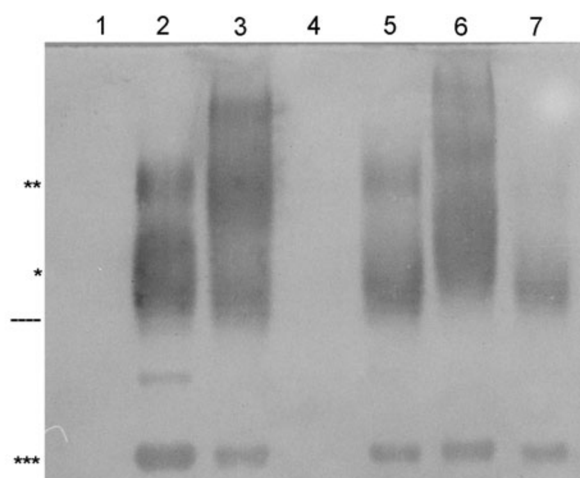

Figure 2. Immunoblot probed with polyclonal rabbit antiserum against *M. huakuii* IFO 15243^T temperature killed whole bacteria.

Lane 1, *Salmonella montevideo* SH94 (standard); lane 2, *M. huakuii* IFO 15243^T; lane 3, *M. huakuii* IFO 15244; lane 4, *M. ciceri* HAMBI 1750; lane 5, *M. huakuii* 38; lane 6, *M. huakuii* S52; lane 7, *M. huakuii* Pl-52. Symbols along the left side of the blot are defined in the legend to Fig. 1.

M. huakuii species could be detected with this antiserum. No other LPS preparation (LPS *M. ciceri* HAMBI 1750 and *S. montevideo* SH 94) was immunostained. Rabbit antisera react strongly with the epitopes from O-polysaccharides and weakly with other LPS fractions. This feature is well known and has been described for *Rhizobium* and *Bradyrhizobium.* The majority of antibodies are directed against the O-chain epitopes and react slightly with LPS composed only of lipid A and the core (Kannenberg *et al.*, 1998). In the case of *Mesorhizobium,* incomplete lipopolysaccharides, containing the core oligosaccharide and lipid A, were stained very weakly while some bands remained unlabeled (Fig. 2). This immunostaining enabled us to visualise the molecular mass distribution of *M. huakuii* high molecular fraction LPSs even better than silver staining. Moreover, because the immunoreaction with the so called "rough" LPS is slight, one can conclude that the LPS material which migrated faster than *S. montevideo* SH 94 LPS band No. 10 (counting from the bottom of the slab, marked with a dashed line) belonged to the set of incomplete *M. huakuii* LPS molecules (LPS-II). If that indeed were the case, then the *Mesorhizobium* LPS bands visible in the bottom part of the slab are representative of fine differences in the core structure. The oligosaccharides could have originated at different stages of the core biosynthesis pathway. The LPS probes of *M. ciceri* HAMBI 1750 and *M. huakuii* S52 reacted with a rabbit antiserum obtained against *M. ciceri* HAMBI 1750 whole bacteria. Also, this antiserum reacted positively exclusively with LPS-I fractions (Fig. 3). It is worth noting that *M. huakuii* Pl-52 as the only one among *M. huakuii* strains crossreacted with antiserum against *M. ciceri* although its reaction with antiIFO 15243T was the same as with other strains.

A polyphasic approach to the determination of the taxonomic position of mesorhizobia evidently indicates a very high similarity between *M. loti* and *M. ciceri* (Wang *et al.*, 1999). Strains of *M. huakuii* were found to be closely related to both of them (Jarvis *et al.*, 1997; Malek & Sajnaga 1999; Wang *et al.*, 1999). Although *M. huakuii* and *M. ciceri* strains are almost identical from the genetic and chemotaxonomic (Choma *et al.*, 2000a; Tighe *et al.*, 2000) point of view, they effectively create nitrogen fixing nodules in differing host plants (*Astragalus sinicus* and *Cicer arietinum,* respectively). The studied strains were isolated in different regions of the world, i.e. *M. huakuii* strains are from China and Japan, whereas *M. ciceri* HAMBI 1750 was isolated from Spanish soil (Chen *et al.*, 1991; Yokota *et al.*, 1993; Nour *et al.*, 1994). Neither the geographic distance separating their places of origin nor the different host plants were a barrier for the development of the same epitopes

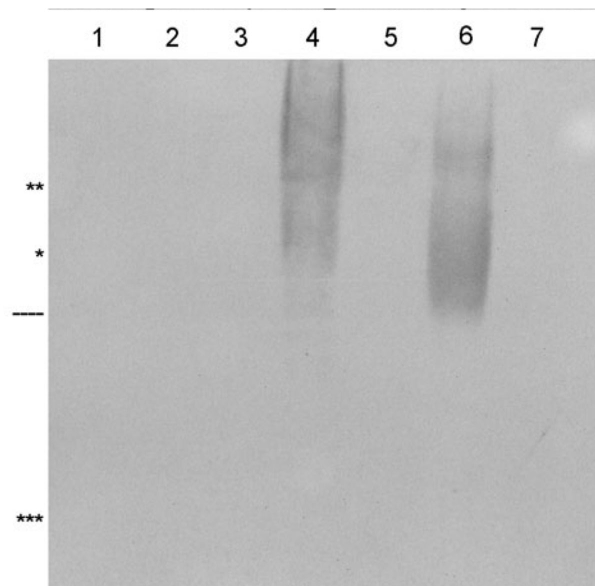

Figure 3. Immunoblot probed with polyclonal rabbit antiserum against *M. ciceri* HAMBI 1750 temperature killed whole bacteria.

Lane 1, *Salmonella montevideo* SH94. (standard); lane 2, *M. huakuii* IFO 15243^{T} ; lane 3, *M. huakuii* IFO 15244; lane 4, *M. ciceri* HAMBI 1750; lane 5, *M. huakuii* 38; lane 6, *M. huakuii* S52; lane 7, *M. huakuii* PL-52. Symbols are defined in the legend to Fig. 1.

during the process of lipopolysaccharide biosynthesis in the HAMBI 1750 and Pl-52 strains. Thus, the results of immune reactions between the described LPS preparations and

M. ciceri HAMBI 1750-specific antibodies may be explained by similarities among the genes determining O-chain synthesis.

The crossreactivity of the antiserum against the IFO 15243T strain with all *M. huakuii* LPS preparations tested seems to be self-evident taking into consideration their close taxonomic positions (Chen *et al.*, 1991). Reactions with the anti IFO 15243T serum indicate a similarity among the structures of the investigated lipopolysaccharides. A preliminary chemical analysis (not presented) pointed to main components shared by the lipopolysaccharides tested (6-deoxytalose, rhamnose, glucose and galactose). A similar chemical structure of LPS fragments renders the search of biochemical pathways leading to the synthesis of whole LPS molecules much more feasible.

The rhizobial O-chain structures published earlier revealed a high degree of diversity. The latest studies on modification of *Rhizobium* LPS during bacteroid development suggest that instead of a specific interaction or a specific site within the O-chain structure, the hydrophobic character of the entire LPS molecule is the crucial factor for normal development of symbiosis (Kannenberg & Carlson, 2001). The bacteria need the complete lipopolysaccharides (i.e. composed of lipid A, core and O-specific chain) to effectively infect the host plants. Therefore, any mutation in the genes responsible for core synthesis leads to the creation of O-chain deprived *nod* mutants (Campbell *et al.*, 2002). Moreover, neither the appropriate structure nor the appropriate length of the O-chain but solely its presence in the LPS in a hydrophobic form is es-

Table 3. Fatty acid composition of LPS from *Mesorhizobium huakuii* IFO 15243^T

Component Amide linked fatty acids	Amount µg/mg LPS	Component Ester linked fatty acids	Amount µg/mg LPS
3-OH-12:0	40.0	12:0	Tr
3-OH-*i*-13:0	69.0	14:0	Tr
3-OH-14:0	1.6	16:0	4.3
3-OH-16:0	11.2	*i*-17:0	8.1
3-OH-18:0	2.9	18:1	0.2
3-OH-19:0	Tr	18:0	0.4
3-OH-20:0	64.4	19:1	0.7
3-OH-*i*-21:0	8.8	20:0	7.8
3-OH-22:1	3.4	20:1	1.9
3-OH-22:0	9.1	21:0	0.8
3-OH-23:1	1.4	22:1	6.8
		22:0	1.8
4-oxo-20:0	5.8	23:1	1.1
4-oxo-i-21:0	1.4	28:1	3.2
4-oxo-22:0	1.2		
		27-OH-28:0	71.9
		29-OH-30:0	1.4
		27-oxo-28:0	5.4

sential in the process of nodulation (Kannenberg *et al.*, 1998; Räsänen *et al.*, 1997). Indeed, in the case of the discussed *M. huakuii* lipopolysaccharides, each preparation possesses an individual pattern of length distribution of the O-chain.

The fatty acid composition of LPS from *M. huakuii* IFO 15243^T is shown in Table 3. All 3-hydroxy fatty acids were found to be amide linked. 3-Hydroxydodecanoic, 3-hydroxy-*iso*-tridecanoic, 3-hydroxyeicosanoic and 3-hydroxydocosenoic acids were the major fatty acids. Additionally, 3-hydroxyheneicosanoic and 3-hydroxydocosenoic acids were present in significant amounts. Similarly to the *M. ciceri* and other *Mesorhizobium* strains (Choma *et al.*, 2000a), *M. huakuii* IFO 15243^T lipopolysaccharide contained amide bound 4-oxo fatty acids (Table 3). However, only 4-oxo-20:0 was present in quantities comparable to the amount of 3-hydroxy fatty acids. Others were present in trace amounts. Moreover, a few nonpolar ester linked fatty acids were identified but only three of them, namely: *i*-17:0, 20:0 and 22:1, occurred in significant amounts. The total amount of nonpolar fatty acids is less than half the quantity of the main ester bound 27-OH-octacosanoic acid. This fatty acid was accompanied by 29-OH-30:0 and 27-oxo-28:0 acids. The presence of ω-1 hydroxy fatty acids with hydrocarbon chains almost twice as long as "the usual" (i.e. 16:0, 18:0) fatty acids is typical for *Rhizobium* and *Bradyrhizobium* with the only exception of *Azorhizobium caulinodans* (Bath *et al.*, 1991). Furthermore, the presence of 27-oxo-28:0 acid is characteristic for *Mesorhizobium* species (Choma *et al.*, 2000a). Assuming that lipid A from *M. huakuii* LPS is composed of 2,3-diamino-2,3-dideoxyglucose exclusively, one might conclude that the mentioned ester linked fatty acids must create acyloxyacyl residues. Such acyloxyacyl moieties have, in fact, been described for *Rhizobium etli* CE3 lipid A (Que *et al.*, 2000; 2000a). Previously, it was believed that bacteria from the *Rhizobiaceae* family can not synthesise acyloxyacyl residues (Bhat *et al.*, 1994). The above mentioned hypothesis should be verified experimentally. Therefore, further studies of the *M. huakuii* lipid A are in progress.

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