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Chemical analysis of *Azospirillum* lipopolysaccharides

A. Choma[1], R. Russa[1], H. Mayer[2], and Z. Lorkiewicz[1]

[1] Institute of Microbiology, M. Curie-Skłodowska University, 20-033 Lublin, Akademicka 19, Poland
[2] Max-Planck-Institut für Immunbiologie, Stübeweg 51, D-7800 Freiburg, Federal Republic of Germany

Abstract. Lipopolysaccharides (LPS) were extracted by hot phenol-water from five strains each of *Azospirillum lipoferum* and *Azospirillum brasilense*. Rhamnose, glucose, glucosamine and 3-deoxy-D-mannooctulosonic acid were comon sugar constituents of all LPS preparations. 2-O-Me-fucose, 3-O-Me-fucose, 3-O-Me-rhamnose and 2-O-Me-galactose were found in LPSs of some *A. brasilense* strains. Fatty acid spectra from all LPSs studied were almost identical with predominance of 3-hydroxymyristic and 3-hydroxypalmitic acids. 3-Hydroxypalmitic acid was the only amide-linked fatty acid. Lipopolysaccharides isolated from *A. brasilense* showed higher heterogeneity in sugar composition than those from *A. lipoferum*.

Key words: *Azospirillum lipoferum* – *Azospirillum brasilense* – Lipopolysaccharides – O-Methyl sugars – Fatty acids

Azospirillum strains are nitrogen fixing bacteria found to be closely associated with roots of several important *Gramineae*. The genus comprises two species, *A. lipoferum* and *A. brasilense* (Tarrand et al. 1978). The latter has been subdivided into two groups according to the denitrification ability (Neyra et al. 1977). *A. lipoferum* is predominantly associated with maize roots, while *A. brasilense*, especially non-denitryfying strains (nir$^-$), are associated with rice and wheat (Baldani and Döbereiner 1980).

The bacterial surface is believed to play a role in the establishment of the bacterium-plant association. Outer membrane components of *Azospirillum* have not yet been examined in detail. So far only one report on the chemical composition of the lipopolysaccharide from an *Azospirillum* strain is available (Choma et al. 1984). We analyzed the sugar and fatty acid composition of LPSs from several different strains belonging to both *Azospirillum* species. These strains have been also recently investigated with respect to the presence of plasmids and bacteriocins (Skorupska et al. 1985).

Materials and methods

Bacterial strains and growth conditions. All bacterial strains were obtained from D. Kulińska (Agriculture University, Warsaw). Eight of them were isolated in Poland by D. Kulińska (Kulińska 1983) and two reference strains SpBr17 (ATCC 29709) and Sp7 (ATCC 29145) were originally isolated by J. Döbereiner (Tarrand et al. 1978). Among the ten *Azospirillum* strains studied five were identified as *A. lipoferum* and five as *A. brasilense*. Bacteria were grown to early stationary phase of growth as described previously (Choma et al. 1984).

LPS isolation and purification. Acetone-dried cells were extracted with 45% aqueous phenol. The lipopolysaccharides (LPSs) were purified by repeated ultracentrifugations at 105,000 × *g* (Westphal and Jann 1965). The purification procedure of the crude LPS from strain Sp7 was modified by supplementary digestion with ribonuclease (Koch-Light, Heverhill, England) and pronase (Serva, Heidelberg, FRG). The material was fractionated on Sephadex G-200 column (100 × 1.5 cm) using pyridine-acetate buffer pH 4.5 (water:acetic acid:pyridine – 1,000:10:4; v/v/v). The purity of LPS preparations was tested by UV spectroscopy and colorimetric measurements of the protein content (Lowry et al. 1951).

Sugar analysis. For identification and quantitative determination of neutral sugars, the LPS samples were hydrolyzed in 0.5 M H_2SO_4 at 100° C for 4 h. Sugars converted to alditol acetates (Sawardeker et al. 1965) were analyzed by glc on ECNSS-M column (3% ECNSS-M on Gas Chrom Q, 100–120 mesh, 0.2 × 200 cm) at 190° C, as well as on Sp1000 capillary column (25 m; 0.2 mm i.d.) using hydrogen as carrier gas. Gas chromatography-mass spectrometry (glc-ms) analyses were carried out on a Finnigan model MAT 1020B combined mass spectrometer/gas chromatograph equipped with a fused silica capillary column CP Sil5 (25 m; 0.2 mm i.d.; 0.12 μm film thickness). Electron impact mass spectrometry was used with an electron energy of 70 eV. For glc-ms analysis sugar mixtures of LPS hydrolysates were dissolved in ^2H_2O and reduced with NaB^2H_4 (Merck, Darmstadt, FRG). Identification was based on the relative retention times and the mass spectra. For quantitative calculations, xylose was used as internal standard. Demethylation was performed as described previously (Choma et al. 1984).

Amino sugars were released by 10 h hydrolysis with 4 M HCl at 100° C. Uronic acids were looked for in hydrolysates obtained as for liberation of neutral sugars. 3-Deoxy-D-mannooctulosonic acid was liberated by hydrolysis with 1% acetic acid at 100° C for 2 h. For high-voltage electrophoresis (Kickhöfen and Warth 1968) a pyridine:acetic acid:water

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Offprint requests to: A. Choma

Abbreviations. glc, gas liquid chromatography; ms, mass spectrometry; LPS, lipopolysaccharide; dOclA, 3-deoxy-D-mannooctulosonic acid; 3-OH-16:0, 3-hydroxypalmitic acid; nir$^-$, nitrite reductase negative; nir$^+$, nitrite reductase positive.

Table 1. Relative sugar composition of lipopolysaccharides isolated from ten strains of *Azospirillum* (mole per mole of 3-hydroxypalmitic acid).

Strain	*A. lipoferum*					*A. brasilense*				
Substance	SpBr17[a]	Gl7	Gl8	D22	D74	Gl4 nir$^-$	Sp7 nir$^+$	D49 nir$^+$	D50 nir$^+$	D80 nir$^+$
Total sugars[b]	84.2	50.0	29.4	57.1	45.7	40.8	31.4	38.4	44.8	51.5
2-O-Methyl-fucose							3.3			
3-O-Methyl-fucose						0.35	0.2			
3-O-Methyl-rhamnose						0.29				
Rhamnose	32.2	15.6	12.3	19.9	14.5	6.6	6.5	9.3	17.1	24.8
Fucose		1.3	0.3	tr	1.6	10.2	13.3	tr	7.3	27.2
Xylose	0.4		tr			1.3	1.8			
2-O-Methyl-galactose						2.8	0.2			
Mannose	0.3	tr	1.7			3.0	0.8	26.5	0.4	
Galactose	0.3						4.3			
Glucose	11.4	9.4	7.3	11.8	8.3	17.6	3.0	18.3	9.5	15.7
D-Glycero-D-mannoheptose	0.7	0.6	0.7		1.0	0.3	0.5	tr	1.3	
L-Glycero-D-mannoheptose	0.4	0.5	1.3		0.8	0.5	0.7	tr	0.8	
dOclA	0.34	0.63	0.43	0.55	0.40	0.60	1.60	0.89	0.82	0.60
GlcN	2.1	2.2	4.1	4.9	2.9	2.9	4.3	8.0	1.1	2.7
Phosphorus	1.3	1.8	0.3	1.4	1.6	5.9	7.1	4.8	7.6	6.9

nir$^+$ nitrite reductase positive *nir*$^-$ nitrite reductase negative

[a] Data from Choma et al. (1984)

[b] % of dry LPS weight

(10:4:86; v/v/v; pH 5.3) or pyridine: formic acid:acetic acid:water (1:1.5:10:90; v/v/v/v; pH 2.8) was used.

Glucosamine, 3-deoxy-D-mannooctulosonic acid and phosphorus contents were determined as described previously (Choma et al. 1984).

Fatty acid analysis. Fatty acids were released from LPS preparations by two-step hydrolysis (4 M HCl, 100° C, 4 h followed by 1 M KOH, 100° C, 1 h). Ester-linked fatty acids were liberated from LPS by alkaline methanolysis (0.5 M $NaOCH_3$ in CH_3OH, 20° C, 20 h). Amide-linked fatty acids were released from de-O-acylated preparations by successive hydrolysis with HCl and KOH as above (Brade and Galanos 1982). Fatty acids, extracted with $CHCl_3$ and methylated with diazomethane were analyzed by capillary glc on OV1 column (25 m, 0.2 mm i.d.). Additionally, glc-ms analyses were performed using the same equipment as for sugar analysis. Heptadecenoic acid was added as an internal standard.

Results

The yields of lipopolysaccharides (LPSs) from *Azospirillum* strains, except strain Sp7, were approximately 1% of bacterial dry weight. The amount of proteins in LPS preparations varied from 0% to about 5%. The contamination of LPSs with nucleic acids did not exceed 1%. Table 1 shows the molar ratios of the sugar components of the LPSs isolates. Neutral sugar contents were calculated from glc of alditol acetates. The recalculation to the molar ratios was based on the amount of 3-hydroxypalmitic acid in each LPS preparation. All *A. lipoferum* LPSs contained significant amounts of rhamnose and glucose. The approximate molar ratio of these sugars was 3:2 with the exception of strain SpBr17 (3:1). In some preparations small amounts of xylose, mannose and galactose (Table 1) were detected. As an exception, the LPS from strain D22 contained neither D-glycero-D-manno- nor L-glycero-D-mannoheptose.

The LPSs from *A. brasilense*, like those from *A. lipoferum* contained also rhamnose and glucose. Except for D49 LPS other LPS preparations from *A. brasilense* contained also fucose as the major constituent. LPS from strain D49 contained high amount of mannose, whereas in the LPS of strain Sp7 galactose and small amount of other neutral sugars were present.

An unidentified compound (X) with retention time of 0.145 relative to glucitol hexaacetate (ECNSS-M column) was detected in LPS from strain Sp7. For identification, sugars, obtained by hydrolysis of LPS from strain Sp7, were reduced with NaB^2H_4 and analyzed as alditol acetates using capillary glc-ms. Figure 1 A shows the mass spetrum of compound X. The fragments m/e 118, 275, 215, 173 indicate a 2-O-methyl-6-deoxyhexose. Co-chromatography with authentic 2-O-methyl-fucose proved its nature as 2-O-methyl-6-deoxygalactose (2-O-methyl-fucose) (Weckesser et al. 1975b). By glc-ms analysis small amounts of 3-O-methyl-6-deoxyhexose (Fig. 1 B) and 2-O-methyl-hexose (Fig. 1 C) (see also Fig. 2) were additionally found. The same 2-O-methyl-hexose was detected in LPS from strain Gl4 (Table 1). Demethylation showed that the sugar detected was 2-O-methyl-galactose. Moreover, this LPS preparation contained traces of two 3-O-methyl-6-deoxyhexoses. One of them had the same retention properties as 3-O-methyl-6-deoxyhexose present in strain Sp7 LPS. Chromatographic data pointed to 3-O-methyl-fucose and 3-O-methyl-rhamnose.

The presence of charged sugar components was investigated by high-voltage electrophoresis (Choma et al. 1984). GlcN and dOclA were identified in all preparations of LPS of *A. lipoferum* LPS. The ratios of GlcN/3-OH-16:0 ranged from 2 to 5, while those of dOclA/3-OH-16:0 never exceeded 1.

The LPSs from all *A. brasilense* strains contained GlcN but at differing molar ratios with respect to 3-OH-16:0. Similarly to the findings with *A. lipoferum*, LPSs from

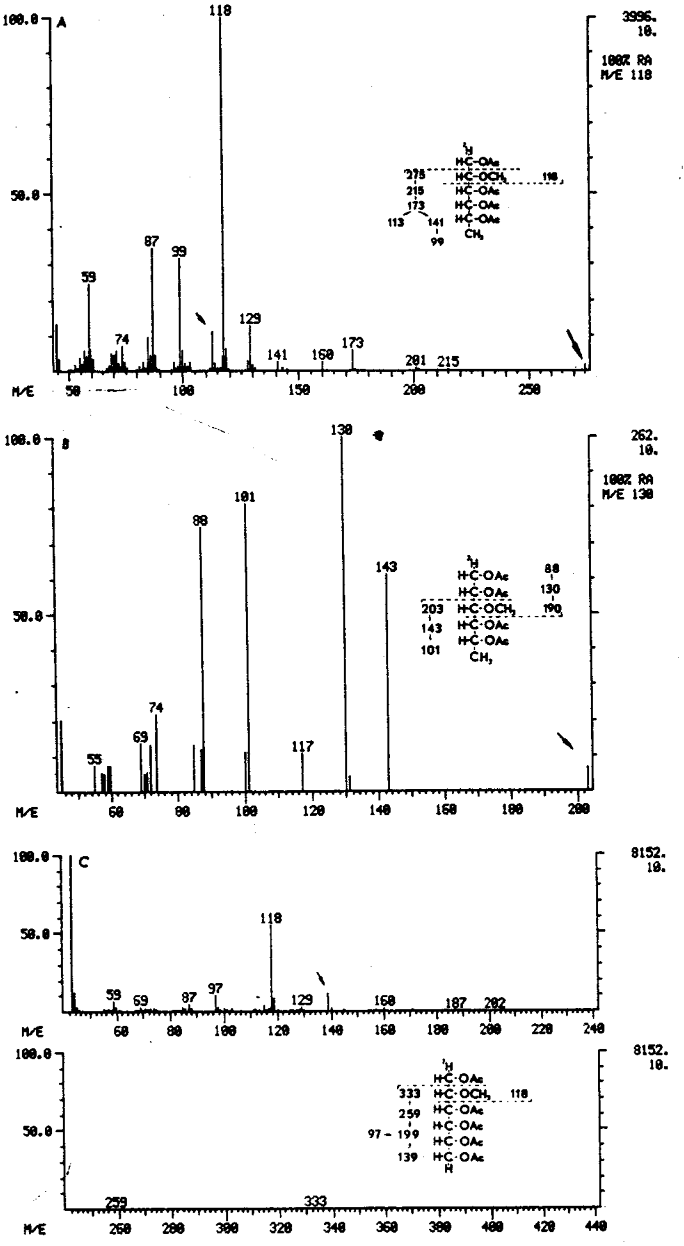

Fig. 1 A – C. Mass-spectra of alditol acetates of O-methyl sugars present in *Azospirillum* LPS; **A** 2-O-methyl-6-deoxyhexitol (2-O-methyl-fuctiol) **B** 3-O-methyl-6-deoxyhexitol (3-O-methyl-fucitol) **C** 2-O-methyl-hexitol (2-O-methyl-galactitol) Mass spectrometry of alditol acetates was carried out as described in the text. Arrows indicate less intensive ions significant for determination of methoxy group localization

A. brasilense strains, except that from D50, had more than one mole of GlcN per mole of 3-OH-16:0. The molar ratios show that *A. brasilense* LPSs contained higher amount of dOclA than those of *A. lipoferum*. Besides GlcN and dOclA, other charged sugars were not detected.

3-Hydroxypalmitic and 3-hydroxymyristic acids were found to be the major fatty acids in all LPSs (Table 2). All LPS preparations contained also myristic, palmitic and octadecenoic acids. Stearic acid occured in three preparations. Two unsaturated fatty acids, i. e. Δ^2-tetradecenoic and Δ^2-hexadecenoic were found in hydrolysates of all LPSs. They, probably, represented artifacts formed during hydrolysis of hydroxy fatty acids. The identity of the fatty acids were confirmed by ms.

Only 3-hydroxypalmitic acid and its artifact was found to be amidelinked. The others, liberated by alkaline methanolysis, were esterlinked.

A. brasilense LPSs contained about four times more phosphorus than *A. lipoferum* LPSs.

Discussion

The symbiotic associations between host plants and bacteria are the result of a specific interaction. So far most attention has been paid to *Rhizobium*-legumes interactions. According to the lectin cross-bridging model (Hrabak et al. 1981) host plant lectins located on the root surface recognize carbohydrate receptors which might be found in LPSs or extracellular polysaccharide of *Rhizobium* cell surface.

Azospirillum strains are adsorbed to root hairs of grasses grown in the absence of nitrogen (Umali-Garcia et al. 1981). The mechanism of the association is not yet determined. Surface components of *Azospirillum* will certainly be involved in the adherence of this bacterium to plant roots.

We haven't detected extracellular polysaccharides in *Azospirillum* cultures by precipitation with acetone/ethanol. Therefore, we studied LPSs of *Azospirillum* in detail.

LPSs were readily extracted by phenol-water from cells of nine out of the ten *Azospirillum* strains studied. The isolation of LPS from strain Sp7, however, required proteolytic enzyme treatment, probably due to a strong association of the lipopolysaccharide with proteins. It should be noted, however, that pronase pretreatment had already been used in isolation of LPS from *Spirillum serpens* (Chester and Murray 1975) and *Campylobacterium jejuni* cells (Naess and Hofstad 1982).

Two LPS preparations (of strains Gl4, Sp7) contained O-methyl sugars. The presence of O-methyl sugars could be critical to a more lipophilic state of LPS. So far 2-O-methylhexoses and O-methyl-6-deoxyhexoses were detected mainly in *Rhodopseudomonas* (Weckesser et al. 1970, 1973, 1975 b), *Klebsiella* (Björndal et al. 1970) and *Rhizobium* species (Carlson et al. 1978; Planque et al. 1979; Zevenhuizen et al. 1980; Hrabak et al. 1981). *Azospirillum lipoferum* LPSs are easily distinguished from those of *A. brasilense: A. lipoferum* preparations consist mainly of glucose and rhamnose, whereas a more complex sugar composition is found in LPSs of *A. brasilense*. Rhamnose, fucose, galactose, mannose and glucose are probably constituents of O-chains of *A. brasilense* LPSs. These sugars are distributed in *A. brasilense* LPSs in different proportions. We can assume that *A. brasilense* strains possess O-specific chains which are characteristic of and specific for a few strains only.

Most of the *Azospirillum* strains studied contained D-glycero-D-mannoheptose. This compound has also been found in certain mutants of *Salmonella* (Lehmann et al. 1973; Branes and Kay 1983), *E. coli* (Coleman and Leive 1979), in wild type *Proteus* (Bagdian et al. 1966) *Serratia* (Bridgen and Wilkinson 1983), *Yersinia* (Samuelson et al. 1974) and *Rhodopseudomonas gelatinosa* (Weckesser et al. 1975 a). Only 3-hydroxypalmitic acid is bound to lipid A via amide bonds. This linkage connects 3-hydroxypalmitic acid with glucosamine, which is the only amino sugar detected in *Azospirillum* LPSs. It is supposed that some part of GlcN

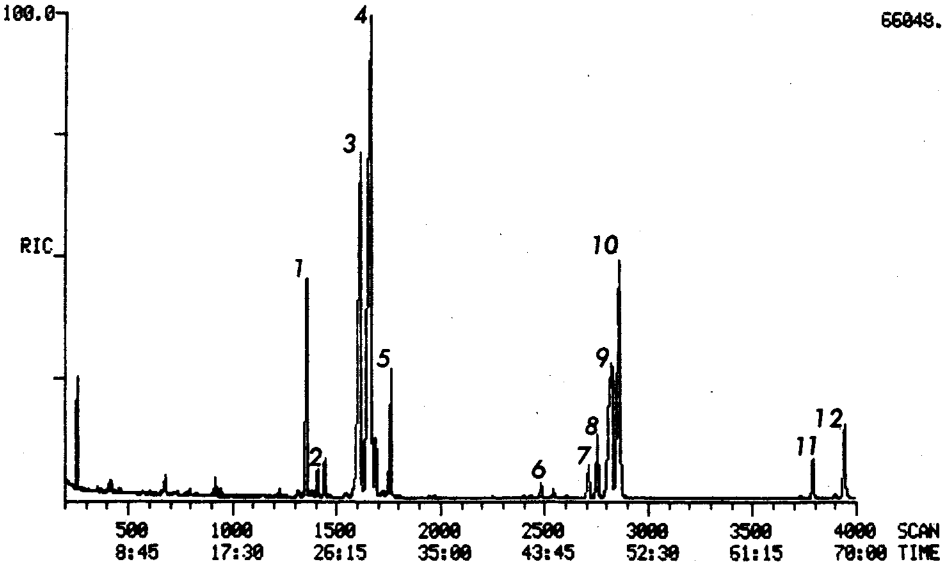

Fig. 2
Gas chromatographic analysis of alditol acetates derived from LPS of *A. brasilense* Sp7 (CP Sil5 capillary column, 25 m, 0.2 mm i.d.; film thickness, 0.12 µm; carrier gas, helium; temperature program, 100° C – 300° C at 2.5° C/min). *1* 2-O-methyl-fucose, *2* 3-O-methyl-fucose, *3* rhamnose, *4* fucose, *5* xylose, *6* 2-O-methyl-galactose, *7* unidentified compound, *8* mannose, *9* glucose, *10* galactose, *11* D-glycero-D-mannoheptose and *12* L-glycero-D-mannoheptose

Table 2. Relative fatty acid composition (3-OH-16:0 = 1) of lipopolysaccharides of *Azospirillum* strains

Strain	*A. lipoferum*					*A. brasilense*				
Fatty acid	SpBr17[a]	Gl7	Gl8	D22	D74	Gl4 nir$^-$	Sp7 nir$^+$	D49 nir$^+$	D50 nir$^+$	D80 nir$^+$
Total fatty acids[b]	7.3	7.3	4.2	6.4	8.6	5.2	1.8	2.7	4.6	3.5
3-Hydroxymyristic	1.19	1.26	1.07	1.06	1.28	1.07	0.81	1.17	1.35	1.32
3-Hydroxypalmitic	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Myristic	0.07	0.03	0.08	0.02	0.03	0.04	0.03	0.06	tr	tr
Palmitic	0.42	0.34	0.22	0.30	0.19	0.34	0.46	0.51	0.32	0.22
Stearic	0.07				tr				tr	
Δ^2-Tetradecenoic	0.13	0.16	0.05	0.52	0.13	0.08	0.07	0.15	0.11	0.17
Δ^2-Hexadecenoic	0.16	0.09	0.11	0.08	0.12	0.10	0.13	0.09	0.12	0.13
Δ^2-Octadecenoic	0.11	0.16	0.31	0.28	0.16	0.32	0.45	0.50	0.32	0.02

nir$^+$ nitrite reductase positive *nir*$^-$ nitrite reductase negative

[a] Data from Choma et al. (1984)

[b] % of dry LPS weight

might be located outside the lipid A backbone, since molar contents of GlcN were usually higher than those of the amide-bound fatty acids (3-OH-16:0). Almost identical spectra of fatty acids for different preparations suggested that the lipid moieties of LPSs were almost identical. Lipopolysaccharides were hydrolyzed with 1% acetic acid to separate lipid A from polysaccharide. Unfortunately, lipid A was not precipitated during this treatment. The modified procedure used for preparation of free lipid A of *Acinetobacter* (Brade and Galanos 1982) appeared also unsuitable. This is probably due to too mild hydrolysis of LPS or increased solubility of the separated lipid A.

Low level of detectable dOclA found in other bacteria (Brade 1985) might also be due to difficultues in separation of lipid A from the polysaccharide chain (Choma et al. 1984; Muhieiskaia et al. 1985).

Serologically *A. lipoferum* represents a homogenous group, but *A. brasilense* strains can be divided at least into three subgroups (De-Polli et al. 1980). The serological division, based on heatstable antigens, corresponds with the ability of bacteria to form associations with different host-plants. The chemical homogeneity of LPSs of *A. lipoferum* and heterogeneity of *A. brasilense* LPSs are in good agree ment with the serological data. The small number o *A. brasilense* strains makes it impossible to divide then into subgroups. Our results show that *Azospirillum* strain belonging to different host-plant groups can differ in thei lipopolysaccharide composition. This fact may have som significant impact on the *Azospirillum*-plant interaction.

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