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Note

# Revised structure of the repeating unit of the O-specific polysaccharide from *Azospirillum lipoferum* strain SpBr17 ☆

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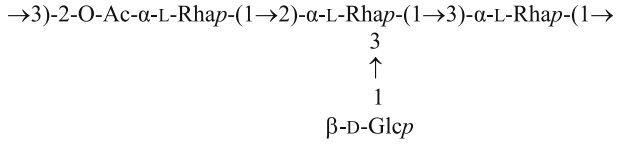
ABSTRACT

A neutral O-polysaccharide liberated by mild acid hydrolysis of a lipopolysaccharide isolated from *Azospirillum lipoferum* SpBr17 was investigated using 1D and 2D $^{1}H$ and $^{13}C$ NMR spectroscopy, including HSQC, HMBC, and NOESY as well as SDS–PAGE electrophoresis along with sugar and methylation analyses. The structure of the O-specific polysaccharide repeating unit was established as follows:

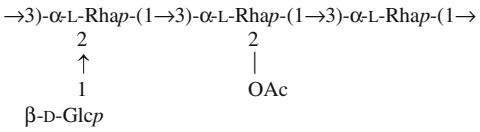



The presented structure is a revised version of the formula that was published earlier in the Abstracts of the 9th International Congress on Nitrogen Fixation in Cancun (Mexico, 1992).



Azospirilla are Gram-negative diazotrophs living in close association with roots of grasses, cereals, and other monocotyledonous plants. They are known as plant growth-promoting bacteria (PGPB) as they secrete into the soil many active substances that have a positive influence on plant development and growth.[1] Currently, seven species belonging to the *Azospirillum* genus have been described: *Azospirillum lipoferum*, *A. brasilense*, *A. amazonense*, *A. halopraeferens*, *A. irakense*, *A. largimobile*, and *A. doebereinerae*.[1–3] Carbohydrate polymers such as EPS, CPS, and LPS play an important role in the interactions between plant and bacteria.[1] Studies concerning the structural characteristics of *Azospirillum* lipopolysaccharides, the main constituents of the outer leaflet of the outer membrane of Gram-negative bacteria, have been performed only for six strains: *A. lipoferum* SpBr17,[4] Sp59b,[5] and SR65,[6] *A. brasilense* Sp245[7] and S17,[8] as well as *A. irakense* KBC1.[9]

The structure of the O-specific polysaccharide from *A. lipoferum* SpBr17 was presented at and published in the Abstracts of the International Congress on Nitrogen Fixation in Cancun (Mexico, 1992).[4] The polysaccharide chain had been deduced to be a rhamnan containing exclusively α-(1→3) glycosidic bonds. This linear polymer was partly O-acetylated and every third L-Rha residue was decorated with β-D-Glc. This structure, as depicted below, had been determined based only on methylation analysis, 1D $^{13}C$ NMR, and data available in the literature.




These results have been cited repeatedly by researchers working on *Azospirillum* surface polysaccharides.[5–8,10] Recently, we have published the structure of *A. lipoferum* SpBr17 lipid A.[11] During preparation of the lipid A moiety, a large amount of the O-specific polysaccharide was obtained. Therefore, we decided to reinvestigate its structure using 2D NMR techniques, to verify if the formula proposed years before was correct. The studies started with routine chemical analysis. Using GLC–MS analysis of alditol acetates, we confirmed the presence of glucose and rhamnose. The estimated quantitative proportion between both sugars was 1:3, respectively. The liberated glucose had a D absolute configuration and rhamnose had an L absolute configuration. A methylation analysis revealed the occurrence of terminal glucose and 3-substituted as well as double (2 and 3)-substituted rhamnose in the ratios of 1:2:1. All these data confirmed our old results.

☆ The results have been presented at the 3rd Baltic Meeting on Microbial Carbohydrates, Sigtuna, Sweden, 2008.

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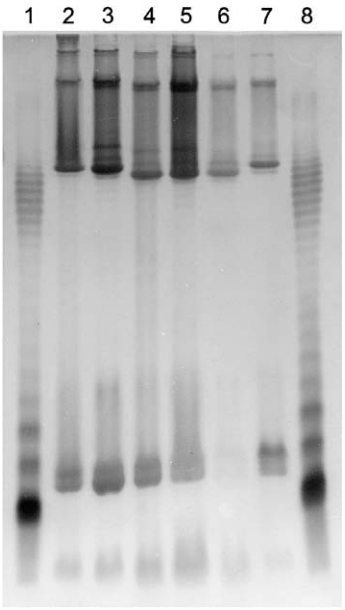


**Figure 1.** SDS–PAGE (13%, w/v) analysis of lipopolysaccharides from *Azospirillum lipoferum* strains. Lanes: 2 and 3—SpBr17 LPS (1 μg and 2 μg, respectively); 3—Gl7 (1 μg); 4—D22 (1 μg); Gl8 (1 μg); D74 (1 μg). Lanes 1 and 8—S-LPS from *Salmonella typhimurium* 902 (1 μg). These *Azospirillum* strains were chemically characterized by Choma et al.[25]

The degraded polysaccharide was subjected to a complete analysis by means of nuclear magnetic resonance. The $^{13}C$ NMR and $^{1}H$ NMR (Fig. 2) spectra of the dgPS were assigned using 2D NMR data from $^{1}H$, $^{1}H$ COSY, TOCSY, and $^{1}H$, $^{13}C$ HSQC experiments. All the chemical shifts are listed out in Table 1. Sugar anomeric configurations were determined by measuring $J_{C1,H1}$ coupling constants (Table 1) and were verified by the presence of corresponding cross-peaks on the NOESY spectrum. A relatively low $J_{C1,H1}$ value (161.5 Hz) indicated a β configuration for glucose (**D**), whereas the three rhamnoses (**A**, **B**, **C**; with $J_{C1,H1}$ > 170 Hz) were α-linked within the O-specific polysaccharide. In the case of glucose, the **D**H1/**D**H3 and **D**H1/**D**H5 intramolecular NOE signals were observed, whereas rhamnose protons (**A**H1, **B**H1, and **C**H1) strongly interacted through space with **A**H2, **B**H2, and **C**H2, respectively.

The monosaccharide sequence in the repeating unit was established by an analysis of correlations between anomeric protons and glycosidic linkage carbons on the HMBC spectrum (Fig. 3). Corresponding proton–proton correlations (**A**H1/**B**H2, **B**H1/**C**H3, **C**H1/**A**H3, and **D**H1/**B**H3) were observed on the NOESY spectrum (data not shown).

The downfield shift (to the anomeric region) of the **A**H2 proton as well as detection of characteristic signals from $CH_3$—21.9 ppm and $CH_3$—2.216 ppm indicated the presence of an O-acetyl group attached to the C-2 position of the **A** rhamnose. Signals marked with asterisks (Figs. 2 and 3) represent the anomeric proton of the A residue deprived of O-acetyl. Comparing the integrals of both variations of **A**H1 peaks, it can be concluded that about 90% of the repeating units are O-acetylated.

Taking together all the results presented above, we propose a revised structure of the O-specific PS repeating unit (Fig. 4).

There are some differences between the revised and the previously published structures: (i) the rhamnan is not a linear homopolymer composed exclusively of (1→3)-linked α-L-rhamnopyranose, but a (1→2) glycosidically bounded rhamnose followed by two rhamnoses connected via (1→3) linkages, (ii) the position of the O-acetyl group is revised, (iii) the glucose residue is attached to rhamnose by a (1→3) glycosidic bond instead of the previously established (1→2) bond.[4]

Lipopolysaccharide preparations from *A. lipoferum* SpBr17 and four other strains from this genus were separated on SDS–PAGE showing two main subfractions (Fig. 1). The fast migrating one presumably represented an LPS composed of lipid A and a core oligosaccharide. The high molecular weight subfraction contained a complete LPS with an O-specific polysaccharide containing approximately 20 repeating units, as estimated by comparing the electrophoretic profiles of the azospirilla LPS with a standard (*Salmonella*) LPS.

The revised structure of the repeating unit appears to be almost identical to that recently published by Fedonenko et al..[6] The slight difference concerns the acetylation pattern, as the O-antigen from *A. lipoferum* SR65[6] was free of O-acetyl groups. Another related structure has also been shown by Fedonenko et al. at the 3rd Baltic Meeting on Microbial Carbohydrates in Sigtuna (Sweden). The repeating unit (O-acetylated in 60%) was identified in an *A. lipoferum* Sp59b surface polysaccharide.[12] So the same or a very similar structure is synthesized by different strains of *Azospirillum* and can

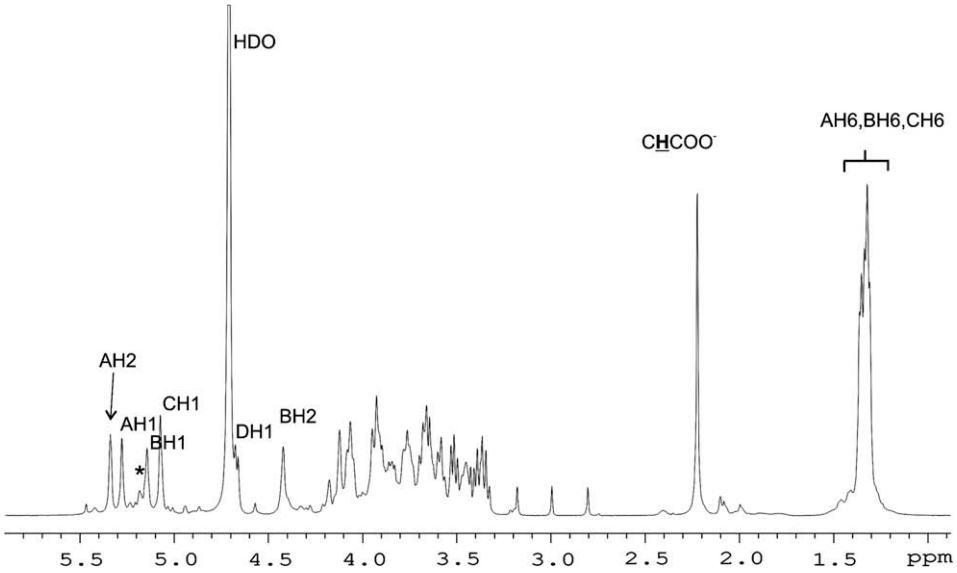


**Figure 2.** 500 Hz $^{1}H$ NMR spectrum of the O-polysaccharide from *A. lipoferum* SpBr17. **A**, **B**, **C**, **D** residue labels; see Table 1. *—the **A**1 proton from the **A** residue without an O-acetyl group.

**Table 1**
$^{1}H$ and $^{13}C$ NMR data for the O-specific polysaccharide derived from the *A. lipoferum* SpBr17 lipopolysaccharide

| Residue | Structure | Nucleus | δ (ppm) $J_{H,H}$ (Hz) $J_{C1,H1}$ (Hz) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 5 | 6 | 6′ | $CH_3CO$– |
| **A** | →3)-2-*O*-Ac-α-L-Rha*p*-(1→ | H | 5.279<br>s | 5.347<br>br s | 4.074<br>~10 | 3.645<br>~10 | 3.848<br>~6 | 1.331 | | 2.216 |
| | | C | 100.0<br>176.6 | 73.2 | 76.8 | 73.0 | 70.6 | 17.9 | | 21.9 |
| **B** | →2,3)-α-L-Rha*p*-(1→ | H | 5.148<br>s | 4.434<br>br s | 4.060<br>~10 | 3.685<br>~10 | 3.926<br>~6 | 1.352 | | |
| | | C | 102.2<br>174.8 | 78.4 | 81.2 | 72.5 | 70.2 | 18.0 | | |
| **C** | →3)-α-L-Rha*p*-(1→ | H | 5.076<br>s | 4.125<br>br s | 3.779<br>~10 | 3.591<br>~10 | 3.751<br>~6 | 1.308 | | |
| | | C | 103.4<br>171.0 | 71.3 | 78.7 | 72.8 | 70.9 | 17.9 | | |
| **D** | β-D-Glc*p*-(1→ | H | 4.676<br>~10 | 3.394<br>~10 | 3.527<br>~10 | 3.348<br>~10 | 3.460<br>~10 | 3.660 | 3.940 | |
| | | C | 105.3<br>161.5 | 74.9 | 76.9 | 71.2 | 77.0 | 62.3 | 62.3 | |

s, singlet; br s broad singlet.

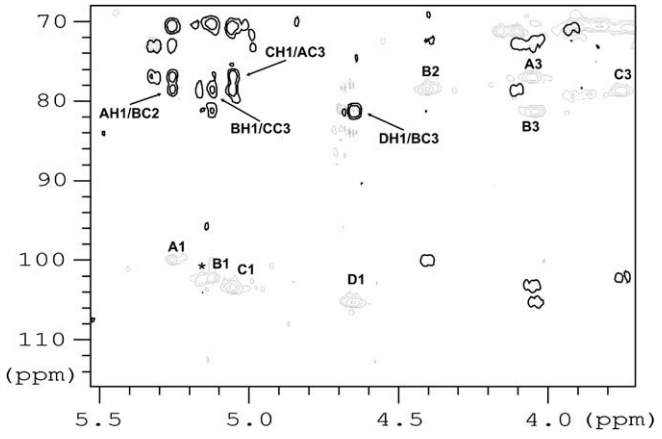


**Figure 3.** Part of the HMBC (thick lines) and the HSQC (thin lines) spectra of the O-polysaccharide from *A. lipoferum* SpBr17. Correlations via one bond are marked with capital letters accompanied by Arabic numerals. Intramolecular correlations are marked with residue names as well as the interacting atoms. The asterisk marks a cross-peak of **A**1 from the **A** residue deprived of an acetyl group.

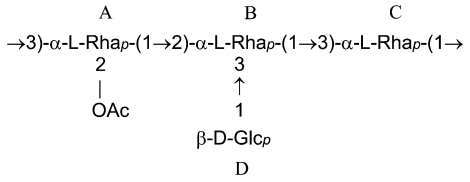


**Figure 4.** A revised structure of the repeating unit of an O-polysaccharide from *A. lipoferum* SpBr17.

be part of a lipopolysaccharide or an extracellular polysaccharide. A similar phenomenon has been observed and described for *Escherichia coli* and other enterobacteria.[13,14]

## 1. Experimental

### 1.1. Bacteria cultivation, LPS and O-antigen isolation

*A. lipoferum* strain SpBr17 (ATCC 29709) was obtained from D. Kulińska's collection, Warsaw Agricultural University (Warsaw, Poland). This strain had originally been isolated by Döbereiner and co-workers.[15] Bacteria were grown to the early stationary phase of growth at 28 °C in a sodium lactate-containing medium, as previously described by Choma et al.[16] Cells were harvested by centrifugation, washed twice with saline and once with distilled water. To remove free lipids and membrane phospholipids, the cell pellet was subjected to the Bligh-Dyer delipidation procedure.[17] After incubation for 2 h at room temperature with intensive stirring, the mixture was centrifuged at 10,000*g* for 20 min. The delipidated bacterial mass was washed twice with a freshly prepared single-phase Bligh-Dyer mixture.

The LPS was recovered from the delipidated cells by the classic hot phenol–water method.[18] The water layer containing the LPS was dialyzed against tap and distilled water. The crude LPS was purified by repeated ultracentrifugation at 105,000*g* for 4 h. A solution of the LPS (5 mg $mL^{-1}$) in aqueous 1% acetic acid was kept at 100 °C for 2 h. The lipid precipitate was removed by centrifugation, and the polysaccharides were separated by gel permeation chromatography on a Sephadex G50 fine column (80 cm × 1.6 cm) with 1% acetic acid as an eluent.

### 1.2. Sugar analysis

The polysaccharide was hydrolyzed with 2 M TFA (100 °C, 4 h). The sugars were converted into alditol acetates.[19] The absolute configurations of the sugars were determined using (−)-2-butanol for glycoside preparation.[20] Methylation was done according to the method of Hakomori[21] and the methylated polysaccharides were purified on a Sep-Pak $C_{18}$ cartridge.[22] The resulting material was hydrolyzed in 2 M TFA (100 °C, 4 h) and reduced with $NaBD_4$. The partially methylated alditols were converted into acetate derivatives.

### 1.3. General methods

GLC–MS was carried out on a Hewlett-Packard gas chromatograph (model HP5890A) equipped with a capillary column (HP-5MS, 30 m × 0.25 mm) and connected to a mass selective detector (MSD model HP 5971). Helium was the carrier gas and the temperature program was initially 150 °C for 5 min, then raised to 310 °C at a ramp rate of 3 °C $min^{-1}$, final time of 20 min. Polyacrylamide gel electrophoresis (PAGE) was performed with sodium dodecyl

sulfate,[23] and the slab was silver-stained after oxidation with periodic acid according to Tsai and Frasch.[24]

### 1.4. NMR spectroscopy

$^{1}H$ and $^{13}C$ NMR experiments were performed in $D_2O$ solutions with acetone as an internal standard ($\delta_H$ 2.225 ppm, $\delta_C$ 31.45 ppm). 1D and 2D (DQF-COSY, TOCSY, and NOESY) $^{1}H$ NMR and $^{1}H/^{13}C$ gHSQC (gradient enhanced-HSQC) and gHMBC experiments were carried out on a Varian Unity plus 500 instrument at 50 °C using standard Varian software. The qHMBC spectrum was recorded with a 55 ms delay for the evolution of long-range couplings. $J_{C1,H1}$ coupling constant values were determined directly from the proton-coupled 1D $^{13}C$ NMR spectrum.

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