Structural studies of the O-specific polysaccharide from Plesiomonas shigelloides strain CNCTC 113/92

Jolanta Czaja1, Wojciech Jachymek2, Tomasz Niedziela1, Czeslaw Lugowski1, Eva Aldova3 and Lennart Kenne2

1L. Hirschfeld Institute of Immunology and Experimental Therapy, Wroclaw, Poland; 2Swedish University of Agricultural Sciences, Uppsala, Sweden; 3National Institute of Public Health, Prague, Czech Republic

The structure of the O-specific side chain of the lipopolysaccharide (LPS) of Plesiomonas shigelloides, strain CNCTC 113/92 has been investigated by NMR spectroscopy, matrix-assisted laser desorption/ionization time of flight mass spectrometry and sugar and methylation analysis. It was concluded that the polysaccharide is composed of a hexasaccharide repeating unit with the following structure:

\[ \rightarrow 4)-\beta-\text{d}-\text{Hepp}(1\rightarrow 3)-6\text{d}-\beta-\text{d}-\text{Hepp}(1\rightarrow 4)-\alpha-l-\text{Rhap}(1\rightarrow 3)-\beta-\text{d}-\text{GlcPNac}(1\rightarrow \]

\[ \overset{3}{\alpha-l-\text{Rhap}} \quad \overset{2}{\beta-\text{d}-\text{Galf}} \]

in which d-\beta-\text{d}-\text{Hepp} is d-glycero-\beta-\text{d}-\text{manno}-heptopyranose and 6d-\beta-\text{d}-\text{Hep} is 6-deoxy-\beta-\text{d}-\text{manno}-heptopyranose. This structure represents a novel hexasaccharide repeating unit of bacterial O-antigen that is characteristic and unique to the Plesiomonas shigelloides strain. Using the high-resolution magic angle spinning technique, \(^{1}H\)-NMR spectra were also obtained for the O-polysaccharide components of isolated LPS and in their original form directly on the surface of bacterial cells.

Keywords: high-resolution magic angle spinning (HR-MAS); lipopolysaccharide; matrix-assisted laser desorption/ionization time of flight (MALDI-TOF); O-antigen; Plesiomonas shigelloides.

Plesiomonas shigelloides (previously Aeromonas shigelloides) is a ubiquitous, facultatively anaerobic, flagellated, Gram-negative, rod-shaped bacterium which has been isolated from a variety of sources such as freshwater, surface water and many wild and domestic animals, and is particularly common in tropical and subtropical habitats [1]. DNA–DNA hybridization tests [1] showed that all P. shigelloides strains are closely related to each other thus constituting a separate well defined genus within the family Vibrionaceae. P. shigelloides shares biochemical and antigenic properties with Enterobacteriaceae and Vibrionaceae; however, genetically it shows only 8% and 7% similarity, respectively [1].

Infections with P. shigelloides have been strongly associated with drinking untreated water [2,3], eating uncooked shellfish or with travel to developing countries [4,5]. Recent studies have suggested that P. shigelloides is an opportunistic pathogen in immunocompromised hosts [6] especially neonates [6–10]. It has been associated with diarrhoeal illness [11] and other diseases in normal hosts as well. P. shigelloides has been isolated from a variety of clinical specimens including cerebrospinal fluid, wounds and the respiratory tract. Reported cases of meningitis and bacteraemia [10] caused by P. shigelloides have been of special interest because of their seriousness. P. shigelloides causes both localised infections originating from infected wounds and gastrointestinal infections, which can disseminate to other parts of the body [12].

The serotyping scheme of P. shigelloides proposed by Shimada and Sakazaki [13], and Aldova et al. [14–17] includes 102 O-serotypes, some O-antigens showing cross-reactivity with antisera directed against lipopolysaccharides (LPS) of Shigella sonnei, Shigella dysenteriae strains 1, 7 and 8, Shigella boydi strains 2, 9 and 13, and Shigella flexneri strain 6 [13,18]. Two P. shigelloides strains were found to share the structure of O-antigens with those of S. flexneri and S. dysenteriae [19].

Although the antigenic schemes of P. shigelloides have been extensively studied with serological methods as mentioned above, the unique structures of O-specific polysaccharides are not known (except that of strains 22074 and 12254) [19].
As the O-polysaccharide defines serogroup specificity, we now report on structural studies of the O-specific antigen isolated from *P. shigelloides* strain CNCTC 113/92 LPS. We also compare the NMR spectra of the isolated O-specific polysaccharide with those of bacteria of the same strain, the latter obtained with high-resolution magic angle spinning (HR-MAS) NMR [20], confirming the presence of the carbohydrate structure studied on the bacteria.

**EXPERIMENTAL PROCEDURES**

**Bacteria**

*P. shigelloides* strain CNCTC 113/92, classified as serovar O54:H2 [13–16], was obtained from the collection of the National Institute of Public Health, Prague, Czech Republic. The bacteria were grown as described previously [21]. After growth for 48 h, they were harvested by centrifugation, washed three times with saline and freeze-dried. For HR-MAS NMR analysis, they were also grown on agar, harvested, washed three times with D2O and placed directly in the rotor without any further treatment. For the HR-MAS heteronuclear single quantum coherence (HSQC) NMR experiment, bacteria were grown as described above but with D-[1-13C]glucose as the carbon source and the volume was scaled down to 4 mL.

**LPS and O-specific polysaccharide**

LPS was extracted from bacterial cells by the hot phenol/water method [22] and purified as reported previously [21]. The yield of LPS was 2% of the dry bacterial mass. The O-specific polysaccharide yield, 15% of LPS, was isolated and purified as described [21].

**Analytical procedures**

LPS was analysed by SDS/PAGE by the method of Laemmli [23] with previously described modifications [24]. LPS bands were visualised by the silver staining method [25]. Sugars were analysed as their alditol acetates by GC-MS [21,26]. The absolute configurations of the sugars were determined as described by Gerwig et al. [27,28] using (±)-2-butanol for the formation of 2-butyl glycosides. The trimethylsilylated butyl glycosides were then identified by comparison with authentic samples [produced from the respective sugar and (±)-2-butanol] on GC-MS. The 6d-D-Hept authentic sample was kindly provided by Dr. K. Matsumoto, Tokyo University, Japan.

**Table 1.** 1H-NMR and 13C-NMR chemical shifts (p.p.m.) of the *P. shigelloides* strain CNCTC 113/92 O-specific polysaccharide. OAc, 2.17/174.6; NAc, 2.05/175.2.

<table>
<thead>
<tr>
<th>Residue</th>
<th>Chemical shift</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1/C1</td>
<td>4.54 3.89 3.58 3.33 3.45 3.77 3.98</td>
</tr>
<tr>
<td>H2/C2</td>
<td>4.74 3.96 3.74 3.96 3.54 3.95</td>
</tr>
<tr>
<td>H3/C3</td>
<td>4.83 3.79 3.83 3.62 4.00 1.24</td>
</tr>
<tr>
<td>H4/C4</td>
<td>4.91 3.94 4.18 3.56 4.38 1.32</td>
</tr>
<tr>
<td>H5/C5</td>
<td>5.02 5.59 4.06 3.51 3.50 1.75 2.17 3.79</td>
</tr>
<tr>
<td>H6, H6’/C6</td>
<td>5.30 4.09 4.05 3.98 3.83 3.67 3.70</td>
</tr>
<tr>
<td>H7, H7’/C7</td>
<td>-</td>
</tr>
</tbody>
</table>

**Fig. 1.** Analysis of *P. shigelloides* strain CNCTC 113/92 LPS. (A) SDS/PAGE: a 15% polyacrylamide gel with a 5% stacking gel was used. LPS suspension in 50 mm Tris/HCl/10 mm EDTA buffer, pH 6.8, containing 4% SDS, 10% glycerol and 0.005% bromophenol blue was boiled and then applied (1 µg) to the gel. LPS bands were visualised by silver-staining [25]. (B) MALDI-TOF mass spectrum of the O-specific polysaccharide of the *P. shigelloides* strain CNCTC 113/92. The spectrum was obtained in the negative mode and with 2.5-dihydroxybenzoic acid as matrix.
provided by A. Zamojski and Z. Pakulski (Institute of Organic Chemistry, PAN, Warszawa, Poland). Methylation was performed by the method of Hakomori [29], and methylated sugars were analysed by GC-MS as previously described [21]. GC-MS was carried out with a Hewlett-Packard 5971A system using an HP-1 fused-silica capillary column (0.2 mm × 12 m) and a temperature program 150 → 270 °C at 8 °C/min.

NMR spectroscopy

NMR spectra of bacterial cells and LPS suspensions in 2H2O were obtained using the HR-MAS technique on a Bruker DRX 600 spectrometer. The bacteria were placed in the rotor in ~30 μL corresponding to 0.5–1 mg bacterial cells after freeze-drying. All HR-MAS NMR experiments were carried out at 5 kHz spin rate at 35 °C (the measured temperature of the pressurised air used for sample spinning) with the Bruker 4 mm HR-MAS probe and a ZrO2 rotor. One-dimensional 1H-NMR spectra of bacteria and LPS were acquired using a Carr-Purcell-Meibom-Gill pulse sequence [90-τ-180-τ]-acquisition] as a T2-filter to remove broad signals of lipids and solid-like bacterial cells [20]. The total delay time, counted as n(2τ), was 10 ms. NMR spectra of the isolated polysaccharides were obtained for 2H2O solutions at 35 °C on Bruker DRX 400 and DRX 600 spectrometers. All spectra were obtained using acetone (δH 2.225, δC 31.05) as internal reference. The polysaccharides were repeatedly exchanged with 2H2O with intermediate lyophilisation before analysis. The data were acquired and processed using standard Bruker software.

The signals were assigned by one-dimensional and two-dimensional experiments [COSY, clean-TOCSY, NOESY, heteronuclear multiple bond correlation (HMBC), heteronuclear multiple or single quantum coherence (HMQC and HSQC) and HSQC-distortionless enhancement by polarization transfer (DEPT) with and without carbon decoupling]. In the clean-TOCSY experiments the mixing times used were 30, 60 and 100 ms. The delay time in the HMBC and the mixing time in the NOESY experiments were 60 and 200 ms, respectively.

RESULTS AND DISCUSSION

The LPS of _P. shigelloides_, isolated by conventional methods, showed on SDS/PAGE analysis a smooth character (Fig. 1A). The different bands observed on the silver stained gel correspond to LPS fractions consisting of different numbers of oligosaccharide repeating units.

The polysaccharide was liberated by mild acidic hydrolysis and isolated by gel filtration on Bio-Gel P-10. Monosaccharide analysis of the polysaccharide together with determination of the absolute configuration gave D-galactose, L-rhamnose, D-GlcNAc, 6-deoxy-D-manno-heptose and D-glycero-D-manno-heptose in the proportions 1.0 : 1.2 : 0.4 : 0.6 : 0.6. Methylation

Fig. 2. 600-MHz 1H-NMR spectra of the O-specific polysaccharide of _P. shigelloides_ strain CNCTC 113/92. (A) Purified polysaccharide; (B) isolated LPS suspension; (C) intact bacterial suspension in 2H2O. Spectra in (B) and (C) were recorded using the HR-MAS NMR technique. The capital letters refer to carbohydrate residues as shown in the structure and tables and the numerals refer to protons in the respective residue.
Fig. 3. 600-MHz HSQC-DEPT spectrum of the isolated O-specific polysaccharide of *P. shigelloides* strain CNCTC 113/92. The cross-peaks are labelled as in Fig. 2.

Fig. 4. 600-MHz and NOESY HMBC spectra of the O-specific polysaccharide of *P. shigelloides* strain CNCTC 113/92. (A) Part of the NOESY spectrum; (B) and (C) Fragments of the 600-MHz HMBC NMR spectrum. The cross-peaks are labelled as in Fig. 2.
analysis of the polysaccharide showed the presence of 3-substituted GlcNAc, 3,4-disubstituted d-glycerol-β-d-manno-Hepp, 4-substituted L-Rhap, 3-substituted 6-deoxy-d-manno-Hepp and terminal d-Gal in the proportions 0.7 : 1.0 : 1.6 : 1.0 : 0.7. The substitution positions and the ring forms were supported by NMR data (see below). The matrix-assisted laser-desorption/ionization time-of-flight (MALDI-TOF) mass spectrum of the polysaccharide (Fig. 1B) showed a series of ions up to \( m/z \) 15 000 separated by \( \approx 1067 \) Da. The six sugars, one Gal, two Rha, one GlcNAc, one Hep and one dHep, give together a mass of only 1025 Da and the difference from the observed 1067 Da could be explained by an additional O-acetyl group as a component in the repeating unit. This result is consistent with the SDS/PAGE analysis, as both techniques show patterns typical of a polysaccharide, formed from repeating units, with different chain lengths.

**Table 2.** Selected intraresidue and inter-residue NOE connectivities from the anomeric protons of the isolated O-specific polysaccharide of *P. shigelloides* strain CNCTC 113/92.

<table>
<thead>
<tr>
<th>Residue</th>
<th>H1 (δH p.p.m.)</th>
<th>Connectivities to Inter-residue atom/residue</th>
</tr>
</thead>
<tbody>
<tr>
<td>A ( \rightarrow ) 3-β-ν-GlcNAc-(1→</td>
<td>4.54</td>
<td>3.96 H4 of B ( \rightarrow ) 3,4-β-glycerol-β-d-manno-Hepp-(1→</td>
</tr>
<tr>
<td>B ( \rightarrow ) 3,4-β-glycerol-β-d-manno-Hepp-(1→</td>
<td>4.74</td>
<td>3.96 H2</td>
</tr>
<tr>
<td>C ( \rightarrow ) 4-α-t-Rhap-(1→</td>
<td>4.83</td>
<td>3.58 H3</td>
</tr>
<tr>
<td>D ( \rightarrow ) 4-α-t-Rhap-(1→</td>
<td>4.91</td>
<td>3.74 H3</td>
</tr>
<tr>
<td>E ( \rightarrow ) 3-2-O-Ac-6d-β-n-Hepp-(1→</td>
<td>5.02</td>
<td>3.62 H3</td>
</tr>
<tr>
<td>F ( β-ν-Gal(1→</td>
<td>5.30</td>
<td>3.56</td>
</tr>
</tbody>
</table>

**NMR analysis of the native polysaccharide**

The \(^1\)H-NMR (Fig. 2A) and HSQC-DEPT-NMR (Fig. 3) spectra of the isolated O-specific polysaccharide contained signals for six anomeric protons and carbons confirming a hexasaccharide repeating unit. (The sugar residues are indicated by capital letters as shown in the structure below and these letters refer to the corresponding sugars throughout the text, tables and figures.)

As all the \(^1\)H-NMR spectra were complex and contained unresolved signals, the major signals and spin systems were assigned by COSY, TOCSY with different mixing times, and HSQC experiments (Table 1). By comparing the chemical shifts with previously published NMR data for the respective monosaccharides [30–33] and considering the \(^3\)J\(_{H,C}\)-values, estimated from the cross-peaks in the two-dimensional spectra,

**Table 3.** Selected intraresidue and inter-residue \(^3\)J\(_{H,C}\)-connectivities from the anomeric atoms of the isolated O-specific polysaccharide of *P. shigelloides* strain CNCTC 113/92.

<table>
<thead>
<tr>
<th>Residue</th>
<th>H1/C1 (δH/δC p.p.m.)</th>
<th>Connectivities to Intrareidue atom/residue</th>
</tr>
</thead>
<tbody>
<tr>
<td>A ( \rightarrow ) 3-β-ν-GlcNAc-(1→</td>
<td>4.54/100.7</td>
<td>3.96 H4 of B ( \rightarrow ) 3,4-β-glycerol-β-d-manno-Hepp-(1→</td>
</tr>
<tr>
<td>B ( \rightarrow ) 3,4-β-glycerol-β-d-manno-Hepp-(1→</td>
<td>4.74/97.8</td>
<td>78.3 C2</td>
</tr>
<tr>
<td>C ( \rightarrow ) 4-α-t-Rhap-(1→</td>
<td>4.83/101.9</td>
<td>81.5 C3</td>
</tr>
<tr>
<td>D ( \rightarrow ) 4-α-t-Rhap-(1→</td>
<td>4.91/96.7</td>
<td>75.4 C5</td>
</tr>
<tr>
<td>E ( \rightarrow ) 3-2-O-Ac-6d-β-n-Hepp-(1→</td>
<td>5.02/100.3</td>
<td>81.3 C2</td>
</tr>
<tr>
<td>F ( β-ν-Gal(1→</td>
<td>5.30/109.3</td>
<td>78.9 C4, H2</td>
</tr>
</tbody>
</table>
Fig. 5. (A) Anomeric region of the HMOC HR-MAS NMR spectrum recorded with 1\(^{13}\)C biosynthetically enriched bacteria and (B) part of the \(^1\)H-NMR spectrum of the isolated O-specific polysaccharide.

for the coupling between ring protons, the sugars could be identified and their anomeric configuration determined. Starting with the signal for the anomeric proton, H-1, the COSY spectrum identified the H-2 signal and the TOCSY spectra the H-3 to H-6/7 signals. By these procedures all six spin systems containing an anomeric proton signal were determined. From the assigned \(^1\)H signals and the one-bond C-H connectivities, the carbon signals were assigned in the gradient-enhanced HSQC-DEPT spectrum (Fig. 3) in which the CH\(_2\) moieties of the sugars were readily identified as negative cross-peaks. Then the substitution positions of the respective monosaccharides were determined from the relative high chemical shifts of the signals of the substituted carbons when compared with values for chemical shifts of the unsubstituted residues. Residue A with the H-1/C-1 signals at \(\delta\) 4.54/100.7 p.p.m., \(J_{\text{H-1,H-2}} = 7.8\) Hz was assigned as a 3-substituted \(\beta\)-d-GlcPNAc residue based on the chemical shift of the C-2 signal (\(\delta\) 56.7), the high chemical shift of the C-3 signal (\(\delta\) 81.5) and the large vicinal couplings between all ring protons. Residue B with the H-1/C-1 signals at \(\delta\) 4.74/97.8 p.p.m., \(J_{\text{H-1,H-2}} < 2\) Hz was recognized as a 3,4-disubstituted \(\alpha\)-d-glycero-\(\beta\)-d-manno-Hepp residue from the small vicinal couplings between H-1, H-2 and H-3 and the chemical shifts of C-3 (\(\delta\) 75.4), C-4 (\(\delta\) 71.7) and C-5 (\(\delta\) 78.5) signals, similar to those of a 3,4-disubstituted \(\beta\)-d-Manp residue [30]. Residue C with the H-1/C-1 signals at \(\delta\) 4.83/101.9 p.p.m., \(J_{\text{H-1,H-2}} < 2\) Hz was recognized as a 4-substituted \(\alpha\)-L-Rhap residue from the signal for an exocyclic CH\(_3\) group (\(\delta\) \(\text{H} 1.24\) and \(\delta\) \(\text{C} 17.6\)) and the small vicinal couplings between H-1, H-2 and H-3 in the sugar ring and the high chemical shift of the C-4 signal (\(\delta\) 81.3). Residue D with the H-1/C-1 signals at \(\delta\) 4.91/96.7 p.p.m., \(J_{\text{H-1,H-2}} < 2\) Hz was also recognized as a 4-substituted \(\alpha\)-L-Rhap residue for the same reasons. Residue E with the H-1/C-1 signals at \(\delta\) 5.02/100.3 p.p.m., \(J_{\text{H-1,H-2}} < 2\) Hz was assigned as a 3-substituted 2-O-acetyl-6-deoxy-\(\beta\)-d-manno-Hepp residue from the small coupling between H-1, H-2 and H-3, the characteristic large downfield shift of the H-2 signal (\(\delta\) 5.59) indicating an ester substitution in this position, the relatively high chemical shift of the C-3 signal (\(\delta\) 78.3) and finally signals for the exocyclic CH\(_2\) group (\(\delta\) 1.75, 2.17/34.4) [31]. The O-acetyl position was further supported by an HMBC connectivity between the carbonyl carbon of the O-acetyl group and H-2 of residue E. Residue F with the H-1/C-1 signals at \(\delta\) 5.30/109.3 p.p.m., \(J_{\text{H-1,H-2}} < 2\) Hz was assigned as a terminal \(\beta\)-d-Galp residue from characteristic high chemical shifts of the C-1, C-2 and C-4 signals (\(\delta\) 109.3, 83.3 and 82.9, respectively) [32] and the similarities of the chemical shifts of H-2 (\(\delta\) 4.09), H-3 (\(\delta\) 4.05) and H-4 (\(\delta\) 3.98) to published values.

The \(J_{\text{C\text{\text{1}},\text{H\text{\text{1}}}}}\) values obtained from an HMOC experiment confirmed the \(\alpha\)-pyranosyl configuration for both the \(\alpha\)-Rha residues (\(J_{\text{C\text{\text{1}},\text{H\text{\text{1}}}}} \approx 172\) Hz for both), the \(\beta\)-pyranosyl configuration for \(\text{d-glycero-d-manno-Hepp}\) (163 Hz) and 6-deoxy-\(\text{d-manno-Hepp}\) (164 Hz) and the \(\beta\)-furanosyl configuration for the terminal Gal (178 Hz). The \(J_{\text{C\text{\text{1}},\text{H\text{\text{1}}}}}\) value for \(\text{d-GlcPNAc}\) (169 Hz) is higher than expected, but the other NMR data support the \(\beta\)-configuration. Thus the results are in agreement with data from the monosaccharide and methylation analyses. As the isolated O-specific polysaccharide contained a relatively high percentage (\(\approx 10\%\)) of components from the core, all the NMR spectra also contained signals from the core oligosaccharide.

Each disaccharide element in the repeating unit was identified by NOESY (Fig. 4A, Table 2) and HMBC (Fig. 4B, C, Table 3) experiments showing inter-residue connectivities between the adjacent sugar residues. Inter-residue NOEs were found between H-1 of B and H-3 of E, H-1 of E and H-4 of C, H-1 of C and H-3 of A, H-1 of A and H-4 of B, H-1 of F and H-4 of D, H-1 of D and H-3 of B (Table 2 and Fig. 4A). The HMBC spectra (Fig. 4B) showed cross-peaks for the three-bond coupling between the anomeric proton and the carbon at the linkage position and between the anomeric carbon and the proton at the linkage position (Table 3), which confirmed the disaccharide elements found by the NOESY experiment. Thus the results suggest the following structure for the repeating unit of the \(P.\) \(\text{shigelloides}\) strain CNCTC 113/92 O-polysaccharide:  

\[
\begin{align*}
&\text{B} & & \text{E} & & \text{C} & & \text{A} \\
&(\rightarrow4)-\text{d-}\beta\text{d-}\text{Hepp} & (\rightarrow1-3)-\text{d6-}\beta\text{d-}\text{Hepp} & (\rightarrow1-4)-\alpha\text{L-}\text{Rhap} & (\rightarrow1-3)-\beta\text{d-}\text{GlcPNAc} & (\rightarrow1-\text{OAc}) \\
3 & & 2 & & 1 \\
\uparrow & & \uparrow & & \downarrow \\
1 & & & & 0 \\
\text{D} & & \alpha\text{L-}\text{Rhap} & & \downarrow \\
4 & & 1 \\
\text{E} & & \beta\text{d-}\text{Galp} & & \end{align*}
\]
HR-MAS NMR analysis of the LPS and bacteria
To compare the chemical structure of the isolated O-specific polysaccharide with that found in isolated LPS and as it is found in bacteria, suspensions of LPS and bacteria were subjected to HR-MAS NMR analysis. With this technique it is possible to obtain (1H and H,C-COSY) NMR spectra of the more flexible O-specific polysaccharides even if they are bound to lipids or cell walls [20]. The 1H-NMR (Fig. 2A) spectrum of the isolated O-specific polysaccharide of P. shigelloides strain CNCTC 113/92 contained signals for six anomeric protons. The corresponding signals were also observed in the 1H-NMR spectra recorded for the suspensions of LPS (Fig. 2B) and bacteria (Fig. 2C). If the bacteria are grown on \( ^{\text{d}}\text{[1,13C]}\text{glucose, }^{13}\text{C} \) will be incorporated into each sugar as C-1 and also in other positions on heptoses, higher sugars and acetyl groups, thus simplifying the NMR spectra and increasing sensitivity. Five of the C/H signals from the anomeric atoms could also be observed in the HMOC spectrum obtained by the HR-MAS NMR experiment, confirming the existence of this isolated structure in bacteria (Fig. 5).

ACKNOWLEDGEMENTS
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21. Petersson, C., Niedziela, T., Jachymek, W., Kenne, L., Zarzecki, P. & Lugowski, C. (1997) Structural studies of O-specific polysaccharide of Hafnia alvei strain PCM 1206 lipopolysaccharide containing \( ^{\text{d}}\text{[1,13C]}\text{glucose, }^{13}\text{C} \) will be incorporated into each sugar as C-1 and also in other positions on heptoses, higher sugars and acetyl groups, thus simplifying the NMR spectra and increasing sensitivity. Five of the C/H signals from the anomeric atoms could also be observed in the HMOC spectrum obtained by the HR-MAS NMR experiment, confirming the existence of this isolated structure in bacteria (Fig. 5).

