Nitrogen-fixing bacterium *Burkholderia brasiliensis* produces a novel yersiniose A–containing O-polysaccharide

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*Burkholderia brasiliensis*, a Gram-negative diazotrophic endophytic bacterium, was first isolated from roots, stems, and leaves of rice plant in Brazil. The polysaccharide moiety was released by ammonolysis from the *B. brasiliensis* lipopolysaccharide (LPS), allowing the unambiguous characterization of a 3,6-dideoxy-4-C-(1-hydroxyethyl)-D-xylo-hexose (yersiniose A), an uncommon feature for *Burkholderia* species. Here we report the isolation and characterization of the structure of the bacterial cell wall component containing yersiniose A–containing O-antigen was identified by sugar and methylation analyses and NMR spectroscopy. Our results show that the repeating oligosaccharide motif of LPS O-chain consists of a branched tetrasaccharide with the following structure: \( \alpha-D-Rhap-(1\rightarrow 3)-[\alpha-YerA-(1\rightarrow 2)]-\alpha-D-Rhap-(1\rightarrow 3)-\alpha-D-Rhap-(1\rightarrow) \).

**Key words:** *Burkholderia/lipopolysaccharide/nitrogen-fixing bacterium/yersiniose*

**Introduction**

The genus *Burkholderia* comprises species that are found in soil and rhizosphere (Viallard et al., 1998), as human pathogens (Vandamme et al., 1997), and as endophytic microorganisms, including phytopathogenic (Urakami et al., 1994) and nitrogen-fixing bacteria (Gillis et al., 1995). The diazotrophic *Burkholderia* species have been found in natural endophytic association with grasses, *B. vietnamiensis* (Gillis et al., 1995), *B. tropicalis* (PPE8) (Baldani et al., 1997a), and *B. brasiliensis* (M130) (Baldani et al., 1997b). More recently, however, the identification of two species, *B. caribensis* and *B. fugorum*, able to fix nitrogen using the same strategy for establishing the symbiosis with legumes (Moulin et al., 2001; Vandamme et al., 2002) shows that the ability to establish a symbiotic interaction is probably widespread.

The best studied nitrogen-fixing bacteria–plant interaction is the *Rhizobium*/legume symbiosis. The extensive literature on the complex molecular mechanism of this interaction suggests an important role for glycopolymers, such as bacterial lipopolysaccharide (LPS) (Cullimore and Dénarie, 2003; Lerouge and Vanderleyden, 2004). The LPS is required for effective symbiosis (Gao et al., 2001), playing an important role in recognition (Noel and Duelli, 2000); for root hair infection (Dazzo et al., 1991), nodule invasion, and bacterial adaptation to the nodule microenvironment (Frayssie et al., 2003; Lerouge and Vanderleyden, 2001); and for avoiding host defense responses (Lerouge and Vanderleyden, 2001). Mutations of genes coding for rhizobial LPS has revealed the biological importance of the O-antigen structure on the symbiont–plant interaction (Brink et al., 1990; Gao et al., 2001; Jabbouri et al., 1996; Noel and Duelli, 2000; Noel et al., 1986, 2000). Mutants that lack the O-chain polysaccharide of their LPS are symbiotically defective (Frayssie et al., 2003; Lerouge and Vanderleyden, 2001). In addition, it has been shown that structural changes in LPS occur during symbiotic process and that most of these changes appear to take place in the O-chain polysaccharide (Noel et al., 1996, 2004). The LPS recognition may be mediated by host-encoded receptor, as demonstrated for the interaction of *R. leguminosarum* bv. trifoli LPS by trifolli A (Noel et al., 2004). Interestingly, quinovosamine (2-amino-2,6-dideoxyhexose) has been identified as a potent saccharide hapten inhibitor of trifolii A–Rhizobium polysaccharide association, suggesting that the interaction between the bacterial LPS and plant receptor is structure-specific (Dazzo and Wopereis, 2000; Hrabak et al., 1981). Moreover, LPS/trifolii A recognition was shown to be a host species–specific event (Dazzo et al., 1991).

Despite the growing understanding of the role of bacterial polysaccharides in the establishment of symbiosis, the involvement of glycopolymers in the endophytic interaction of nitrogen-fixing bacteria has not been determined. To understand the basis of this alternative association, characterization of the structure of the bacterial cell wall components is required. Here we report the isolation and characterization of a novel O-antigen containing yersiniose A (YerA) synthesized by *B. brasiliensis*.

**Results**

**Isolation and composition of LPS of *B. brasiliensis***

LPS was isolated by hot aqueous phenol treatment from a cell wall preparation of *B. brasiliensis* and purified by ultracentrifugation, yielding 1.28 mg per g of cells (wet weight). Gas chromatography (GC) and GC mass spectrometry (MS) of trimethylsilyl (TMS)-methylglycoside derivatives from purified LPS showed the presence of rhamnose (Rha) and an unidentified C4-branched 3,6-dideoxyhexose as major components and traces of...
heptose and glucose. GC-MS analysis of the TMS (-)-2-butyl glycosides derived from intact LPS showed that Rha residues were D-configuration.

For isolation of the C4-branched 3,6-dideoxyhexose, the B. brasiliensis LPS was submitted to hydrolysis with 1% acetic acid, sufficient to release significant amounts of this compound, which was purified by gel filtration chromatography on a Bio-Gel P-6 column (Figure 1, peak II), and identified as YerA by electron impact (EI)- and chemical ionization (CI)-MS and nuclear magnetic resonance (NMR) spectroscopic analyses. The EI-MS of the alditol acetate derivative of this C4 branched monosaccharide showed prominent fragment ions at m/z 95, 109, 113, 143, 155, and 215, identical with those found for alditol acetates derivatives of Yer present in the LPS isolated from species of Legionella and Yersinia (Gorshkova et al., 1984; Sonesson and Jantzen, 1992). CI-MS of the TMS-methylglycosides showed the presence of two pseudo-molecular ions at m/z 351 (M+H+), and m/z 368 (M+NH4+), the expected masses for TMS-methylglycosides of Yer.

The 1H-NMR spectrum (Figure 2A) of the purified Yer (Figure 1, peak II) showed the presence of several isomers (Figure 2A, inset), however, on reduction with sodium borohydride a single spin system was observed (Figure 2B), indicating that a single C4-branched 3,6 dideoxy-hexose was present in the intact LPS. As shown in Figure 2A, the most intense spin system showed an anomeric signal at 4.539 ppm with \( J_{\text{H1,H2}} = 8 \) Hz, indicating a diaxial relationship between H1 and H2. In the correlation spectroscopy (COSY) and total correlation spectroscopy (TOCSY) spectra (result not shown) the H2 signal correlated with a deoxy group characterized by H3 protons located at 1.675 ppm and 2.067 ppm, H3ax and H3eq, respectively. The methylene portion was supported by the coupling constant \( J_{3a,3e} = 13.21 \), \( J_{3a,3c} = 4.7 \), and \( J_{3a,3c} = 13.21 \) and by the correlation of the H3 protons with the methylene \(^{13}\)C resonance at 35.34 ppm in the heteronuclear multiple quantum coherence spectrum (data not shown). No other correlation involving the H3ax or H3eq was observed. The long relaxation time for the signal at 75.7 ppm in the 1D \(^{13}\)C spectrum supports the presence of a quaternary C4.

Two other spin systems were observed in the COSY spectrum. The resonance at 1.167 ppm correlates with a resonance at 4.002 ppm, corresponding to the methyl group (CH3-6) and the H5 of a 6-deoxyhexose. The second system comprises a methyl group at 1.202 ppm (CH3-8), which correlates with the resonance at 3.759 ppm (H7). These results suggest a 3,6-dideoxy-β-hexose with a hydroxyl-ethyl appendage on C4, which was confirmed by the rotating frame nuclear Overhauser enhancement spectroscopy (ROESY) spectrum, which shows a strong nuclear Overhauser enhancement (NOE) from H1 to the H5 at 4.002 ppm and from H1 to the H3ax consistent with a β-D-xylo configuration with the 6-methyl equatorial. The methyl group (H8) presents a strong NOE to the H3eq and a less intense NOE with the H3ax, indicating that this spin system comprises the side chain attached at the C-4. In addition H7 makes a strong NOE with H6 confirming the β-D-xylo configuration. The configuration of the hydroxyethyl chain attached to the C4 was determined by the H3ax and H3eq chemical shifts as being L-glycerol (Zubkov et al., 1992).

GC and GC-MS analyses of the material eluted of the void volume of P-6 column chromatography (Figure 1, peak I) was mainly composed of Rha and Yer in molar ratio 6:1. These observations suggest that the O-polysaccharide isolated by the conventional methodology using mild acid conditions is partially degraded. Together, sugar analysis, MS, and NMR data indicated the presence of Rha and YerA in the O-polysaccharide from LPS isolated from B. brasiliensis. The fatty acid composition of the LPS showed the presence of myristic, 2-hydroxymyristic, and 3-hydroxypalmitic acids.

Alternatively, the B. brasiliensis LPS was hydrolyzed with ammonium hydroxide, yielding only one carbohydrate-containing fraction on the P-6 column (void volume). The compositional analysis of this fraction by GC and GC-MS of the trimethylsilylated methylglycosides, in conjunction with analysis of alditol acetate derivatives, showed that the presence of Rha and YerA in a molar ratio of 3:1.

**Determination of the sugar linkages of intact LPS**

To determine the linkages between sugars, a methylation analysis of intact LPS was carried out by analysis the O-acetylated partially O-methylated methylglycosides, which were characterized by GC and CG-MS. The derivatives were observed (Figure 3A), arising from terminal YerA, 2-O-, 3-O-, and 2,3-di-O-substituted Rhap. These data suggest that the repeating unit of B. brasiliensis LPS is a branched tetrasaccharide structure. To determine the arrangements of substituents at the 2,3-di-O-Rhap, intact LPS was subjected to different trifluoroacetic acid (TFA) hydrolysis conditions prior to the methylation analysis, resulting in a time-dependent in Yer release. Two carbohydrate-containing fractions were observed on Bio Gel P-4 chromatography when the LPS was treated with 40 mM TFA at 100°C for 1 h. Sugar analysis of the fraction eluted in the void volume showed the presence of Rha, whereas in the included fraction it contained Rha and Yer residues.

Methylation analysis of Rha-containing fraction (P-4 column, void volume) gave rise to 3-O- and 2-O-substituted-Rhap residues in a molar ratio of 2:1.
These methylation data combined with those from intact LPS (Figure 3A), unequivocally, show that the 3→)Rha is further substituted by Yer on O-2 position, because the release of Yer from the LPS resulted in the conversion of 2,3-di-O-substituted Rha to 3-O-substituted Rha.

Characterization of glycosyl sequence of the O-polysaccharide repeating unit

As mild acid hydrolysis with 1% acetic acid used to selectively cleave KDO ketosidic linkage also releases Yer residues, the structure of the repeating unit was determined by NMR analysis of the polysaccharide portion obtained by ammonolysis of the intact LPS. As expected, ammonolysis yielded a single carbohydrate-containing fraction on Bio-Gel P-6 column chromatography. GC-MS of TMS-methylglycoside derivatives showed Rha and Yer as the major components, together with trace of glucose and heptose. The 1D 1H NMR spectrum of this fraction (Figure 4) contained four low field signals, between 4.80 and 5.30 ppm, due to four anomeric protons; resonances between 4.23 and 3.40 ppm arising from ring protons; a triplet and a double doublet at 1.96 and 1.82 ppm, respectively; and high-field...
resonances between 1.34 and 1.11 ppm from methyl groups. The 2D COSY, TOCSY, and heteronuclear single quantum coherence (HSQC) spectra (Table I) showed the presence of three α-Rha residues. The downfield anomeric resonance Rha(A) at 5.248 and 101.32 ppm was assigned 2,3-disubstituted α-Rha, consistent with downfield shifts of the C2 and C3 resonances at 77.89 and 78.25 ppm. Anomeric resonances of Rha(B) at 4.965 and 102.42 ppm were assigned 3-substituted α-Rha, supported by its lowfield C3 resonance at 78.31 ppm. The 2-substituted α-Rha(C) with anomeric resonances at 5.215 and 101.32 ppm was characterized by a C2 resonance at 78.42 ppm. Finally, the α-YerAp anomeric H1 resonated at 5.038, with C1 at 99.89 ppm.

These data are consistent with those from the methylation analysis (Figure 3), suggesting a repeating unit containing 2,3-O-, 3-O-, and 2-O-α-Rhap residues, and a terminal α-YerAp unit. The α-anomeric configuration of Rhap residues was supported by chemical shifts of H1, H3, and H5, at lower field (Jansson et al., 1989) and by the absence of H1, H3, and H5 intraresidue correlations in the 2D ROESY experiment (Figure 5). The α-configuration of YerAp was defined by the coupling constant $J_{1,2}$ and by the H1 and C1 chemical shifts. The configuration of the hydroxyethyl chain attached to the C-4 was determined as being L-glycero (YerA) (Zubkov et al., 1992), as seen for the free sugar isolated from the LPS hydrolyzed with 1% acetic acid (Figure 2A).

The 2D ROESY experiment (Figure 5) revealed the presence of NOE contacts between the anomeric proton resonances between 1.34 and 1.11 ppm from methyl groups. The 2D COSY, TOCSY, and heteronuclear single quantum coherence (HSQC) spectra (Table I) showed the presence of three α-Rha residues. The downfield anomeric resonance Rha(A) at 5.248 and 101.32 ppm was assigned 2,3-disubstituted α-Rha, consistent with downfield shifts of the C2 and C3 resonances at 77.89 and 78.25 ppm. Anomeric resonances of Rha(B) at 4.965 and 102.42 ppm were assigned 3-substituted α-Rha, supported by its lowfield C3 resonance at 78.31 ppm. The 2-substituted α-Rha(C) with anomeric resonances at 5.215 and 101.32 ppm was characterized by a C2 resonance at 78.42 ppm. Finally, the α-YerAp anomeric H1 resonated at 5.038, with C1 at 99.89 ppm.

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The 2D ROESY experiment (Figure 5) revealed the presence of NOE contacts between the anomeric proton

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**Table I.** $^1$H and $^{13}$C NMR assignments for polysaccharide portion

<table>
<thead>
<tr>
<th>Chemical shift</th>
<th>Residue</th>
<th>α-Rha(A)</th>
<th>α-YerA</th>
<th>α-Rha(B)</th>
<th>α-Rha(C)</th>
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<tbody>
<tr>
<td>H-1</td>
<td></td>
<td>5.248</td>
<td>5.031</td>
<td>4.965</td>
<td>5.215</td>
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<tr>
<td>C-1</td>
<td></td>
<td>101.32</td>
<td>99.89</td>
<td>102.42</td>
<td>101.32</td>
</tr>
<tr>
<td>H-2</td>
<td></td>
<td>4.127</td>
<td>3.961</td>
<td>4.185</td>
<td>4.083</td>
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<td>C-2</td>
<td></td>
<td>77.89</td>
<td>72.48</td>
<td>70.28</td>
<td>78.42</td>
</tr>
<tr>
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<td></td>
<td>3.907</td>
<td>1.825/1.957</td>
<td>3.843</td>
<td>3.948</td>
</tr>
<tr>
<td>C-3</td>
<td></td>
<td>78.25</td>
<td>30.07</td>
<td>78.31</td>
<td>70.34</td>
</tr>
<tr>
<td>H-4</td>
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<td>3.708</td>
<td>3.566</td>
<td>3.890</td>
<td>3.516</td>
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<td>C-4</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-5</td>
<td></td>
<td>4.135</td>
<td>4.226</td>
<td>3.890</td>
<td>3.879</td>
</tr>
<tr>
<td>C-5</td>
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<td></td>
<td>67.79</td>
<td></td>
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</tr>
<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-8</td>
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<td>1.189</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-8</td>
<td></td>
<td></td>
<td>15.61</td>
<td></td>
<td></td>
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</table>

Protons were assigned from TOCSY and COSY spectra and the $^{13}$C was assigned through proton-detected heteronuclear correlation. *

Tentative assignment.
of α-YerAp and α-Rhap(A) H2 at 4.127 ppm, confirming that the location of the α-YerAp side chain is on O-2 position of α-Rhap(A). The interresidue correlation between the anomeric proton of α-Rhap(A) and the H3 of α-Rhap(B) at 3.843 ppm is consistent with the substitution of the O-3 position of α-Rhap(B) by α-Rhap(A). Furthermore, the presence of NOE contacts between the anomeric proton of α-Rhap(B) and the α-Rhap(C) H2 at 4.083 ppm show that the position O-2 of α-Rhap(C) is substituted by α-Rhap(B) (Figure 5). Finally, an interresidue NOE between α-Rhap(C) H1 and α-Rhap(A) H3 at 3.907 ppm defines the Rhap(C) α1→3Rhap(A) sequence.

On the basis of these results, Scheme I presents the structure proposed for the repeating unit of specific polysaccharide from the LPS of B. brasiliensis.

### Discussion

The study of nitrogen-fixing bacteria is of great agronomical and ecological importance. Several recent works have advanced our understanding of the biological impact of glycomolecules in the chemical dialogue involved in the symbiotic interaction (Krusell et al., 2002; Limpens et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003). Even though little is known about the involvement of glycomolecules in endophytic association, knowledge of their structures is an important step in unraveling the molecular mechanisms involved in this process. In this work we isolated and characterized a new O-polysaccharide of the LPS synthesized by B. brasiliensis strain M130. Our studies of this antigenic polymer revealed a branched polymer with a tetrasaccharide repeating motif. The repeat unit is a branched tetrasaccharide with the sequence \(-2\alpha-d-Rhap-(1\rightarrow3)-(\alpha-YerAp-(1\rightarrow3)])\alpha-d-Rhap-(1\rightarrow3)-\alpha-d-Rhap-(1\rightarrow3).

The approach used in the structural analysis allowed the characterization of Yer as a component of the B. brasiliensis LPS, a novel feature for Burkholderia polysaccharides and more frequently encountered in the O-polysaccharides of LPS produced by species of Yersinia (Gorshkova et al., 1976, 1983, 1989) and Legionella (Sonesson and Jantzen, 1992). Furthermore, the presence of 3,6-dideoxy-4-C-branched sugars in the cell wall components has already been described for B. caryophylli, as caryophyllose (3,6,10-trideoxy-4-C-[d-glycero-1-hydroxyethyl]-D-erythro-D-gulose) (Adinolfi et al., 1996) and for Mycobacterium gastri as tridecose (3,6-dideoxy-4-C-[1,3-di-O-methylpropyl]-α-hexopyranose) (Gilleron et al., 1994).

Mild acid hydrolysis has long been the method of choice for isolation of O-polysaccharides from LPS. An interesting finding of our work is that the hydrolysis of the B. brasiliensis LPS with 1% acetic acid releases Yer residues, yielding an O-polysaccharide with nonstoichiometric substitution. The hydrolysis of Yer under dilute acid treatment may explain the results found by Gorshkova et al. (1989) that YerA was present in nonstoichiometric amounts in the O-antigen isolated from Yersinia frederiksenii LPS. Cleavage of other susceptible glycosidic linkages under identical acid conditions should be investigated. Using ammonolysis we were able to isolate the B. brasiliensis polysaccharide while maintaining the fidelity of the repeating structure, a general feature that applies to most bacterial polysaccharides (Whitfield, 1995).

The exact role of LPS molecules in endophytic interaction is still obscure, but our data may allow parallels to be drawn with the involvement of LPS in symbiont–plant association. The presence of YerA residues in the B. brasiliensis LPS might confer a higher hydrophobicity for the polysaccharide, which may be important for interaction between the bacterial and plant cell surface. In agreement with this hypothesis, we have shown that the polysaccharide interacts with the reverse-phase LC-18 SPE column. Furthermore the results obtained by Jabbari et al. (1996) showed that mutants lacking smooth LPS, provoking a loss of hydrophobicity of the bacterial membrane, infect Vigna nodules in the usual way but do not produce efficient bacteroids. In this context, Kannenberg and Carlson (2001) observed structural modifications in the LPS of R. leguminosarum during bacteroid development. The importance of hydrophobic character given to the O-antigen by the presence of
deoxy-sugars can also be exemplified by the findings that \textit{R. etli} mutant CE16, expressing a LPS lacking the O-antigenic 2-amino-2,6-dideoxyglucose (quinovosamine) forms nonfixing pseudonodules on \textit{Phaseolus vulgaris} (Noel and Duelli, 2000). In addition, the presence of these unusual deoxy-sugars in the LPS polymers, especially on terminal position, confers immunological specificity of the O-antigen, contributing to the wide variety of antigenic types between species and even strains in Gram-negative bacteria (Liu and Thorson, 1994).

Another characteristic of the O-polysaccharide described in this article is the presence of Rha residues, frequently encountered in O-polysaccharide of LPS of several \textit{Burkholderia} species, including \textit{B. cepacia}, \textit{B. gladioli}, \textit{B. vietnamiensis}, and \textit{B. plantarii} (Céranolta and Montrozier, 1997; Galbraith and Wilkinson, 1997; Gaur et al, 1998; Zähringer et al, 1997). The Rha is the main or the only constituent of the O-specific polysaccharide in \textit{Azospirillum brasilienis} (Fedonenko et al, 2002), \textit{Pseudomonas syringae} (Ovod et al, 1999; Zdrovenko et al, 2003), and \textit{Xanthomonas campestris} pathogens (Senchenkova et al, 1999) and is thus common for phytopathogenic bacteria. This observation may be an indication that this sugar plays an important role in the recognition and interaction between bacteria and plants. Impairment of Rha biosynthesis by knocking out the dTDP-L-Rha synthase of \textit{Azorhizobium cauliformans} disabled symbiosis with \textit{Sesbania rostrata} (Gao et al, 2001).

Current data from \textit{Rhizobium}–legume symbiosis support some of the general proposed functions for LPS and underscore the importance of LPS structural versatility and adaptability. In this work, appropriate methodology applied to the isolation of the O-antigen from \textit{B. brasilienis} LPS allowed a novel YerA-containing polysaccharide to be characterized. This structural knowledge can contribute to further investigation of the participation of glycomolecules in the plant–endophyte association and highlights the uniqueness of polysaccharides expressed by this bacterium.

Materials and methods

Bacterial strain and growth conditions

\textit{B. brasilienis} strain M130, isolated originally from the roots of rice plants growing in Brazil, was obtained from the Culture Collection of Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA, Rio de Janeiro, Brasil) and maintained on agar plate containing manniitol-salts-yeast extract medium (Mattos et al, 2001). The bacterium was grown in a synthetic medium (Mattos et al, 2001), under constant aeration, at 28°C to late exponential phase (72 h).

Preparation of LPS

After removal of capsular polysaccharide (Stephan et al, 1995) the cells were mechanically disrupted by ultrasonic treatment and the cell wall fraction recovered by centrifugation (8000 \times g, 4°C, 15 min). The LPS was extracted from the cell wall preparation by hot aqueous phenol, recovered from the aqueous phase after exhaustive dialysis and lyophilization. The dry material containing LPS was suspended in water to 3% (w/v) and purified by ultracentrifugation (105,000 \times g, 4°C, 16 h) (Westphal and Jann, 1965). The intact LPS was characterized by carbohydrate and fatty acid compositon, methylation analysis, and NMR spectroscopy.

Treatment of LPS by acid conditions

For mild acid hydrolysis, LPS (50 mg) was treated with 1% acetic acid at 100°C for 1 h. After cooling, the sample was centrifuged (3000 \times g, 4°C, 30 min), and the precipitated lipid A was removed. The water-soluble product was lyophilized, dissolved in water, and chromatographed on a Bio Gel P-6 (fine) column (90 cm \times 1.7 cm) equilibrated with 0.05 M pyridine acetae buffer (pH 4.6). Two carbohydrate-containing fractions were detected by phenol/sulfuric acid assay (Dubois et al, 1956). The O-polysaccharide partially hydrolyzed was recovered from the void volume and a monosaccharide fraction in the included volume. An aliquote of the monosaccharide fraction was reduced with sodium borohydrde at room temperature for 2 h. The excess borohydride was destroyed by addition of BioRad (Heruces, CA) AG50X8 H+ (200–400 mesh) and the boric acid removed by repeated evaporation with methanol. The native and reduced monosaccharides were lyophilized for further NMR spectroscopic analysis.

For selective release of monosaccharide from intact LPS, various conditions of hydrolysis were assayed. The LPS (3 mg) was submitted to 40 mM TFA, at 100°C for 1–8 h. The products were evaporated, dissolved in water, and subjected to chromatography on BioGel P-4 column. The carbohydrate-containing fractions, detected by phenol/sulfuric acid assay (Dubois et al, 1956), eluted in the void and included volumes, were analyzed by GC and GC-MS. For methylation analysis, the product of LPS was treated with 40 mM TFA at 100°C for 1 h and recovered in void volume.

Preparation of polysaccharide moiety from LPS by amonolysis

The LPS (50 mg) was hydrolyzed in 10 M ammonium hydroxide (4 ml) at 150°C for 18 h (Barr and Lester, 1984). After cooling, the product was concentrated by evaporation under N2, lyophilized, and dissolved in water; the insoluble components were removed by centrifugation (3000 \times g, 4°C, 30 min). The supernatant was evaporated and dissolved in water, and the polysaccharide recovered by lyophilization after gel filtration chromatography on Bio Gel P-6 eluted with water or on a reverse-phase LC-18 SPE column (Supelco, Bellefonte, PA) eluted with a solution containing 40% isopropanol and 5% acetic acid.

Carbohydrate analysis

Monosaccharides from intact LPS, polysaccharide moiety, or O-polysaccharide chain were analyzed as their TMS-methyl glycosides after methanolysis with 50 mM HCl in methanol at 80°C for 18 h (Sweeley et al, 1963). The monosaccharides were also analyzed as alditol acetate derivatives, after acid hydrolysis with 40 mM TFA at 100°C for 6 h, reduction with sodium borohydrde, and acetylation with...
derivatives was 120 to 240 reagent gas used in the CI. The temperature program for and an ionization current of 0.2 mA. Ammonia was the EI was performed using an ionization potential of 70 eV quadruple mass spectrometer (Shimadzu, Kyoto, Japan). DB-1 capillary column, interfaced with a GC-MS-QP5050 as the carrier gas. GC-MS analysis were performed on a DB-1 fused silica (30 m × 0.5 M HCl in methanol at 80°C for 18 h) and acetylated at room temperature for 18 h (Sweeley et al., 1963). Peaks were identified by their retention time compared to authentic standards and by GC-MS.

Lipid analysis
For fatty acid analysis, the LPS (500 mg) was methanolyzed (0.5 M HCl in methanol at 80°C for 18 h), and fatty acid methyl esters (FAMEs) were extracted into heptane and analyzed by GC after O-trimethylsilylation with bis-(trimethylsilyl)trifluoracetamide/pyridine (1:1, v/v) at room temperature for 1 h (Sweeley et al., 1963). Peaks were identified by their retention time compared to authentic standards and by GC-MS.

Methylation analysis
The methylation analysis of intact and TFA-treated LPS was carried out according to Parente et al. (1985), using dimethyl sulfoxide/lithium methylsulfinyl carbamion and methyl iodide. The permethylated materials were methanolyzed (50 mM HCl in methanol at 80°C for 18 h) and acetylated at room temperature for 18 h. The resulting O-acetylated partially O-methylated methylglycosides were identified by GC and GC-MS.

GC and GC-MS
GC was performed on a Varian Star 3400 gas chromatograph equipped with a capillary column (25 m × 0.2 mm) DB-1 fused silica (30 m × 0.25 mm) with hydrogen (10 psi) as the carrier gas. GC-MS analysis were performed on a Shimadzu GC 17 A gas chromatograph, equipped with a DB-1 capillary column, interfaced with a GC-MS-QP5050 quadruple mass spectrometer (Shimadzu, Kyoto, Japan). EI was performed using an ionization potential of 70 eV and an ionization current of 0.2 mA. Ammonia was the reagent gas used in the CI. The temperature program for the analysis of TMS-methyl glycoside and alditol acetate derivatives was 120 to 240°C at 2°C min⁻¹ and for FAME was 110 to 280°C at 3°C min⁻¹. For the analysis of TMS-(-)-2-butylglycosides the temperature program was 135 to 200°C at 1°C min⁻¹.

NMR spectroscopy
NMR spectra were obtained on a Bruker DRX 600 with a 5-mm triple resonance probe at 25°C, or on a Varian Inova 500 spectrometer equipped with a 5 mm inverse-detection heteronuclear probe, at 30°C. The samples were deuterium exchange by repeated lyophilization from deuterated water (M&G Chemicals, Stockport, U.K.; 99.9% deuterium, 500 ml) and dissolved in 0.5 ml D₂O. Proton NMR spectra were assigned from COSY and TOCSY (Griesinger et al., 1988) experiments. Information on the sequence and linkage of the sugar residues were obtained from ROESY (Bax and Davis, 1985). TOCSY and ROESY experiments were collected with 32 transients of 2k data points and 512 × 2 increments in F1, with mixing times of 80 and 150 ms, respectively. Carbon chemical shifts were assigned from the HSQC (Wider and Wuthrich, 1993) spectra recorded with carbon decoupling. Proton chemical shifts were referenced to internal acetate anion at 1.908 ppm and 13C chemical shifts to external methanol at 50 ppm.

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Abbreviations
COSY, correlation spectroscopy; CI, chemical ionization; EI, electron impact; FAME, fatty acid methyl ester; GC, gas chromatography; HSQC, heteronuclear single quantum coherence; LPS, lipopolysaccharide; MS, mass spectrometry; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser enhancement; ROESY, rotating frame nuclear Overhauser enhancement spectroscopy; TFA, trifluoroacetic acid; TMS, trimethylsilyl; TOCSY, total correlation spectrometry.

References


