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Structure of the O-specific polysaccharide from *Burkholderia vietnamiensis* strain LMG 6998

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Abstract

The putative O-specific polymer containing D-mannose and L-rhamnose was isolated from the lipopolysaccharide obtained from cells walls of *Burkholderia vietnamiensis* strain LMG 6998. NMR and degradative studies showed that the polymer has a linear trisaccharide repeating-unit of the structure shown. The same polymer carrying an *O*-acetyl group at position 3 of the 4-substituted mannose residue has previously been found as the O antigen in the related species *Burkholderia cepacia* serogroup J.

→3)-α-L-Rha*p*-(1→3)-β-D-Man*p*-(1→4)-α-D-Man*p*-(1→

Keywords: *Burkholderia vietnamiensis*; Lipopolysaccharide; O antigen

1. Introduction

The species *Burkholderia vietnamiensis* was proposed in 1995 to accommodate certain nitrogen-fixing bacterial strains isolated in Vietnam from the rhizosphere of rice [1]. The organisms phenotypically resemble *Burkholderia cepacia*, and some clinical strains of the latter species have been redesignated *B. vietnamiensis*. In addition to nitrogenase activity, *B. vietnamiensis* differs from *B. cepacia* in the range of carbon sources used for growth and in producing ornibactins as the only siderophores [1,2]. *B.*

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cepacia and some other *Burkholderia* spp. are recognised as pathogens of animals or plants, and much effort has been devoted to the identification of their possible virulence factors [3–6]. The lipopolysaccharide (LPS), which is one putative virulence factor for *B. cepacia*, possesses several unusual features, including (a) a low degree of phosphorylation [7], (b) extensive substitution by 4-amino-4-deoxy-L-arabinose [7,8], (c) a low content of 3-deoxy-D-*manno*-2-octulosonic acid (Kdo) [7], (d) the substitution of Kdo by D-*glycero*-D-*talo*-2-octulosonic acid (Ko) [8], and (e) the presence of two distinct polymeric fractions in several reference strains [4]. The clinical importance of *B. cepacia*, the fact that at least some of these unusual structural features of its LPS also apply to other *Burkholderia* spp., and the excision of some strains of *B. cepacia* as *B. vietnamiensis* made it desirable to initiate a study of the LPS from the latter species. Here we report the structure of the putative O antigen from strain LMG 6998 (CCUG 7246), a clinical isolate which was initially identified as *B. cepacia*.

2. Results and discussion

LPS of strain LMG 6998 was extracted by treatment of the defatted cell walls with hot, aqueous phenol, and was mainly isolated from the aqueous phase (yield from whole walls, 28%). The small amount of LPS recovered from the phenolic phase (yield, 2.7%) was not studied further. The putative O-specific polymer was obtained by mild acid hydrolysis (1% acetic acid, 100 °C, 2.5 h) of the LPS, during which the hydrolysate turned dark brown (suggesting the release and destruction of 4-amino-4-deoxyarabinose as for *B. cepacia*), followed by fractionation of the water-soluble products on Sephadex G-50 (yield from LPS, 60%).

Monosaccharide components of the polymer were identified by PC and high-pH anion-exchange chromatography (HPAEC), and by GLC of the alditol acetates, as mannose and rhamnose [molar ratio by HPAEC, 2.3:1]. The rhamnose was isolated by

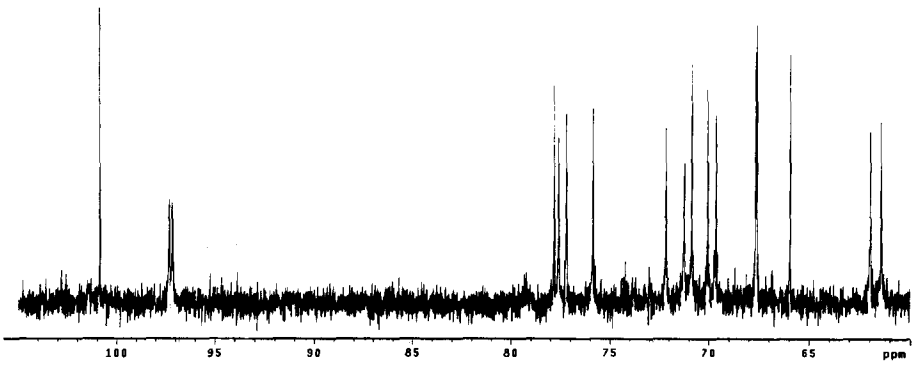


Fig. 1. ^{13}C NMR spectrum of the O-specific polymer. The spectrum for the sample in D_2O was recorded at 150 MHz and 70 °C with acetone (δ_C 31.07) as the internal reference.

preparative HPAEC and its identity confirmed by ^{1}H NMR spectroscopy. Comparison of the values for total mannose (HPAEC) and D-mannose (enzymic assay) showed that only the latter isomer was present. This assignment was confirmed, and the L configuration of rhamnose was determined, by GLC of the diastereoisomeric but-2-yl glycoside acetates.

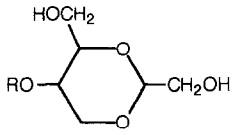
The ^{1}H NMR spectrum of the polymer contained three signals (each 1 H, unresolved) in the anomeric region with δ 5.06, 5.02, and 4.75, and a methyl signal (Rha H-6) with δ 1.29 ($J_{5,6}$ 7.1 Hz). The inference of a trisaccharide repeating-unit containing pyranoid residues [tentatively two α and one β, from the δ values] of Man (2) and Rha (1) was confirmed by the ^{13}C NMR spectrum (Fig. 1), which included signals for three anomeric carbons with δ 100.88 ($^1J_{CH}$ 161 Hz), 97.36 ($^1J_{CH}$ 170 Hz), and 97.21 ($^1J_{CH}$ 170 Hz), two signals for unsubstituted hydroxymethyl carbons (δ 61.95 and 61.44), and one for Rha C-6 (δ 17.58) *inter alia*.

On methylation analysis of the polymer, monitored by GLC and MS of the methylated alditol acetates, the derivatives from 3-substituted Rha*p*, 4-substituted Man*p*, and 3-substituted Man*p* were obtained (relative peak areas, 0.58:1.00:1.11), showing that the polymer was linear. Depolymerisation was achieved by the Smith degradation, which provided a mixture of oligomeric products (SD). The ^{1}H NMR spectrum for SD contained the expected signal for Rha H-6 (δ 1.28, 3 H), but the anomeric region was more complex than expected from a classical degradation of the polymer to a trisaccharide-alditol. There were two unresolved signals, apparently corresponding to H-1 β protons (δ 4.83 and 4.76, ratio 1:2; total 1 H relative to 3 H for Rha H-6), and a broadened, major signal (1 H) for an H-1 α proton (δ 4.98), probably overlapping a minor triplet (δ 5.01). FABMS, and combined GLC-EIMS of permethylated SD confirmed the presence of two products (relative peak areas, ~ 2:1; relative retention times, 0.73:1.00), the major and more volatile one of which corresponded to the expected diglycosylerythritol. On FABMS, this gave pseudomolecular ions with *m/z* 579 $[M + Na]^+$ and 595 $[M + K]^+$, and on EIMS gave the fragment ions [9] with *m/z* 189 $[aA_1]$, 157 $[aA_2]$, 125 $[aA_3]$, 147 $[cA_1]$, 207 $[bcJ_1]$, 351 $[bcA_1]$, 319 $[bcA_2]$, and 411 $[abcJ_1]$ *inter alia*. The disaccharide sequence Rha*p* → Man*p* implied by these results was supported by the presence of fragment ions with *m/z* 189 and 393 $[baA_1]$ in the FAB mass spectrum of permethylated SD, and the results of methylation analysis (which gave the derivatives of unsubstituted Rha*p* and 3-substituted Man*p*). The major product from Smith degradation can therefore be assigned the partial structure **1**. The minor component of SD, the permethylated derivative of which gave pseudomolecular ions with *m/z* 607 $[M + Na]^+$ and 623 $[M + K]^+$ on FABMS, was provisionally identified as the corresponding diglycosyl derivative of a 1,3-dioxane (**2**, R = Rha*p* → Man*p*) or the related 1,3-dioxolane, formed by transacetalation during the hydrolytic step of the degradation [10]. In support of such a structure, EIMS of the permethylated compound gave diagnostic fragment ions with *m/z* 189 $[aA_1]$, 175 $[cA_1]$, 235 $[bcJ_1]$, and 347 $[bcA_2]$ *inter alia*. The probable minor triplet (δ 5.01) in the anomeric region of the ^{1}H NMR spectrum of SD could be attributed to H-2 of the heterocyclic aglycon. The Rha*p* and Man*p* residues in **1** and **2** were assigned the α and β configuration, respectively, as the chemical shift of the anomeric signal for the terminal unit (assigned as δ ~ 4.98)

should be less affected by the identity of the aglycon. Thus, it is possible to propose structure **3** for the repeating unit of the parent polymer.

Rha*p*-(1→3)-Man*p*-(1→2)-Ery-ol

1



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→3)-α-L-Rha*p*-(1→3)-β-D-Man*p*-(1→4)-α-D-Man*p*-(1→

3

The structural conclusions drawn above were reinforced by recourse to additional NMR experiments and assignments. For this purpose, the residues were labelled **a**–**c** in order of decreasing chemical shift for the anomeric protons (Table 1). The chemical shift for Rha H-5 (δ ~ 4), determined from a COSY spectrum, showed that this sugar was α-linked [11] and hence that the β-linked residue (**c**) was mannose. Further correlations by COSY, relayed COSY, and HMQC established that C-3 of **c** had δ 77.83. Comparison of this value with the corresponding chemical shift (δ ~ 74) for free β-Man*p* [12] showed that **c** was the 3-substituted residue. The modest glycosylation shift

Table 1
NMR data [a] for the O-specific polymer

Atom		Residue		
		→ 4)-α-D-Man-(1 → **a**	→ 3)-α-L-Rha-(1 → **b**	→ 3)-β-D-Man-(1 → **c**
1	H	5.06	5.02	4.75
	C	97.36	97.21	100.88
2	H	4.07	4.22	4.26
	C	70.87	67.62	67.66
3	H	4.05	3.94	3.72
	C	70.07	75.88	77.83
4	H	3.95	3.55	3.70
	C	77.60	72.18	65.85
5	H	4.00	3.97	3.48
	C	71.25	69.66	77.22
6	H	3.78, 3.85	1.29	3.76, 3.96
	C	61.44	17.58	61.95

[a] Values for chemical shifts relative to acetone (δ_H 2.22; δ_C 31.07).

for C-3 and the large upfield shift of ~ 4 ppm for C-2 in the same residue pointed to substitution at the 3-position by α-L-Rha*p* (rather than by α-D-Man*p*) consistent with structure **3** [13]. Residue **b** was identified as the L-Rha by its proton spin system, and the α and β effects of α-D-mannosylation at position 3 were comparable with those observed for residue **c**, again in accord with expectation [13]. The much larger glycosylation effect (~ 10 ppm) at C-4 of the α-D-Man*p* residue **a** was also consistent with β-D-mannosylation and the overall sequence → **a** → **b** → **c** → .

The structure **3** established for the repeating unit of the O antigen in *B. vietnamiensis* strain LMG 6998 has been reported previously [14] for a strain of *B. cepacia* serogroup J, although in the latter instance the α-D-Man*p* residue **a** was acetylated at position 3. As some other strains of *B. cepacia* have recently been reclassified as *B. vietnamiensis*, it will be of interest to know whether this applies also to the strain defining serogroup J in the Japanese typing scheme [15].

3. Experimental

Growth of bacteria, and isolation and fractionation of the LPS.—*B. vietnamiensis* strain LMG 6998 was grown in Nutrient Broth No. 2 (Oxoid, 20 L) for 24 h at 34 °C, with aeration at 20 L min^{-1} and stirring at 300 rpm. Cells (wet weight, 87 g) were harvested by continuous centrifugation (Sharples) and disintegrated mechanically (Dyno-Mill KDL). After repeated washing and enzymic purification [16], the cell walls were freeze-dried (yield, 2.55 g) and lipids were extracted with 2:1 $CHCl_3$–MeOH at room temperature. LPS (yield, 723 mg) was isolated by treatment of the defatted cell walls with hot aqueous phenol as in related studies [17]. The O-specific polymer was obtained by mild hydrolysis of the LPS (aq 1% AcOH, 100 °C, 2.5 h), followed by chromatography of the water-soluble products on Sephadex G-50.

General methods.—NMR spectra (^1H and ^{13}C; 1D and 2D) for the O-specific polymer in D_2O were recorded with a Jeol La400 or a Varian DXR600S spectrometer at 70°C with acetone (δ_H 2.22, δ_C 31.07) as the internal reference. Standard pulse sequences were used to obtain COSY, relayed COSY, and HMQC (with and without decoupling) spectra. The ^1H NMR spectrum of the Smith-degradation product (SD) was recorded with a Jeol JNM-GX270 spectrometer. Mixtures of alditol acetates, methylated alditol acetates, permethylated oligosaccharide-alditols, and but-2-yl glycoside acetates were separated by GLC using fused-silica capillary columns (25 m; BP1 or BP10) in a Carlo Erba Mega 5160 chromatograph. GLC-MS was carried out with a Finnigan 1020B instrument. The matrix used for FABMS was *m*-nitrobenzyl alcohol. HPAEC separations of monosaccharides utilised a CarboPac PA100 column in a DX-300 instrument (Dionex). The solvent system used for PC was 13:5:4 EtOAc–pyridine–water.

Determination of monosaccharide composition.—Monosaccharides were released by hydrolysis of the polymer with 2 M CF_3CO_2H at 98 °C for 16 h [18]. The products were identified by PC, HPAEC, GLC of the alditol acetates, ^1H NMR spectroscopy (Rha), and enzymic assay (Man) [19]. For both Man and Rha, absolute configurations were determined by conversion into their but-2-yl glycosides [20] (using racemic butan-2-ol or individual stereoisomers), followed by acetylation and GLC.

Degradative methods.—Methylation analyses, monitored by GLC and GLC-MS of the methylated alditol acetates, were carried out by standard procedures [21–23]. Smith degradation of the O-specific polymer was carried out as in related studies [19].

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