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Structure of the O-specific polysaccharide from *Burkholderia pickettii* strain NCTC 11149

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Abstract

A polymeric fraction (the putative O antigen) has been isolated from the lipopolysaccharide of the type strain of *Burkholderia pickettii*. The components of the polymer and their molar proportions were: L-rhamnose (3), 2-acetamido-2-deoxy-D-glucose (1), and *O*-acetyl (1). By means of NMR studies and chemical degradations, the basic repeating-unit of the polymer was identified as a linear tetrasaccharide of the structure shown. The *O*-acetyl group is probably located at position 2 of the 3-substituted α-L-Rha*p*. Similar polymers constitute O antigens in the related species *Burkholderia solanacearum*.

→ 3)-β-D-Glc*p*NAc-(1 → 2)-α-L-Rha*p*-(1 → 2)-β-L-Rha*p*-(1 → 3)-α-L-Rha*p*-(1 →

Keywords: *Burkholderia pickettii*; Lipopolysaccharide; O antigen

1. Introduction

Species which constitute the genus *Burkholderia* were recently removed from the genus *Pseudomonas* [1], and comprise organisms actually or potentially pathogenic to plants or humans. Examples include *Burkholderia pseudomallei* (the causal agent of melioidosis in man [2]), *Burkholderia cepacia* (a cause of onion rot and a frequent coloniser of the lungs of cystic fibrosis patients [3]), and *Burkholderia solanacearum* (the cause of wilt in tobacco and other plants [4]). A further member of the genus, closely related to *B. solanacearum* [5], is *Burkholderia pickettii*, some strains of which

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are opportunistic pathogens in man. Chemotaxonomic characters which distinguish most *Burkholderia* spp. include the presence of two forms each of phosphatidylethanolamine and an ornithine amide lipid [1,6], though the latter lipid is absent from *B. solanacearum* and *B. pickettii*, giving rise to differences in the total cellular fatty acids [1,6–9]. Lipopolysaccharides (LPSs) of *Burkholderia* spp. also display unusual features, such as the presence of a 2-octulosonic acid (Ko) as well as 3-deoxy-D-*manno*-2-octulosonic acid (Kdo) [10], and of distinct polymeric fractions in some serovars of *B. cepacia* [11] and *B. pseudomallei* [12]. Structures have been established for O antigens from several species, including *B. solanacearum* [13–19], but not *B. pickettii*. Here we report the structure of the antigen from the type strain of the latter organism.

2. Results and discussion

LPS was obtained from defatted cell walls by extraction with hot, aqueous phenol (yield, 13-15%). The major monosaccharide components of the LPS were identified as rhamnose, glucose, heptose, and 2-amino-2-deoxyglucose. During mild acid hydrolysis of the LPS, the suspension turned dark-brown, possibly due to the release and decomposition of 4-amino-4-deoxyarabinose, as observed in similar studies of LPS from *B. cepacia* [20]. The yield of polymeric material isolated from the hydrolysate by gel permeation chromatography was in the range 29-34%. Products from different batches of cells gave the same ^{1}H NMR spectrum.

The major monosaccharide components of the polymeric material were L-rhamnose and 2-amino-2-deoxy-D-glucose, but glucose and an unidentified compound (**X**) were present as significant minor components. Compound **X** had a mobility slightly greater than rhamnose on PC, which made separation difficult, and reacted similarly with aniline hydrogenoxalate. It also appeared that compound **X** was not separated from other components in the hydrolysate by high-performance anion-exchange chromatography (HPAEC) nor by GLC of the alditol acetates. When the mixture of monosaccharides was examined by ^{1}H NMR spectroscopy, minor, unattributed anomeric signals were found at δ 4.76 and 5.21. The $J_{1,2}$ values were 1-2 Hz, indicating that compound **X** was another 6-deoxyhexose with equatorial H-2. The peak areas for the anomeric signals for glucose and compound **X** were about equal, and less than 20% of those for 2-amino-2-deoxyglucose (the less abundant major component). It was concluded that glucose and compound **X** were derived from the core oligosaccharide of the LPS or from a separate, minor polymer, and that the proportions were too low to interfere with structural studies of the major polymer.

The ^{1}H NMR spectrum of the polymeric material showed the presence of both *N*- and *O*-acetyl groups (δ 2.03 and 2.18, respectively; ratio 1:1), and the IR spectrum contained an ester C=O band at 1730 cm^{-1}. Unresolved major signals (each 1 H) were found in the anomeric region of the NMR spectrum at δ 5.27, 5.07, 4.97, and 4.75, together with a doublet ($J_{1,2}$ 8.3 Hz, 1 H) at δ 4.72. Methyl signals at δ 1.31 (3 H) and 1.22 (6 H) pointed to the presence of three rhamnose residues. After *O*-deacetylation, four major anomeric signals (each 1 H) were present at δ 5.32 (unresolved), 5.00 (unresolved), 4.83 (unresolved), and 4.75 ($J_{1,2}$ 8.5 Hz), and methyl signals at δ 2.07

(s), 1.31 ($J_{5,6}$ ~5 Hz), 1.22 ($J_{5,6}$ 6.6 Hz), and 1.21 ($J_{5,6}$ ~7 Hz). The ^{13}C NMR spectrum of the *O*-deacetylated polymer contained three signals in the anomeric region at δ 102.75 (2 C, $^1J_{CH}$ apparently ~174 Hz, but vide infra), 100.57 ($^1J_{CH}$ ~180 Hz), and 98.22 ($^1J_{CH}$ 159 Hz). Other features of interest in the spectrum were *N*-acetyl signals at δ 175.10 and 23.07, the signal for C-2 of Glc*p*NAc at δ 56.47, and one for an unsubstituted hydroxymethyl carbon (C-6 of Glc*p*NAc) at δ 61.45. Taken together, these data indicate (a) that the intact polymer is based on a tetrasaccharide repeating-unit of Rha*p* (3) and Glc*p*NAc (1) residues, with regiospecific mono-*O*-acetylation, (b) that the Glc*p*NAc residues have the β configuration, and (c) that at least some of the Rha*p* residues have the α configuration. The confusion caused by superposition of the two signals with δ_C 102.75 was removed by a C-H correlation spectrum (HMQC), which showed that one of them corresponded to the β-Glc*p*NAc residue (δ_H 4.75) and the other to an α-Rha*p* residue (δ_H 5.00). One of the remaining two Rha*p* residues had the α configuration (δ_H 5.32, δ_C 100.57) and the other the β configuration (δ_H 4.83, δ_C 98.22).

Methylation analysis of the polymer confirmed that all residues were pyranosides and showed that the GlcNAc and one Rha were 3-substituted, and that the other two Rha were 2-substituted. Consistent with this, only the GlcNAc and one Rha survived oxidation of the *O*-deacetylated polymer with periodate. Methylation analysis of the oligomeric product (SD1) from a Smith degradation showed that the surviving Rha was unsubstituted and the GlcNAc still 3-substituted and, hence, that the original polymer contained the disaccharide unit **1**. The partial structure **2** for SD1 was supported by the ^1H NMR spectrum, which included anomeric signals, each 1 H, at δ 4.98 (unresolved) and 4.70 ($J_{1,2}$ 8.5 Hz), together with two methyl signals (*N*-acetyl and Rha H-6).

→3)-L-Rha*p*-(1→3)-β-D-Glc*p*NAc-(1→

1

L-Rha*p*-(1→3)-β-D-Glc*p*NAc-(1→2)-Gro

2

α-L-Rha*p*-(1→3)-β-D-Glc*p*NAc-(1→2)-α-L-Rha*p*

3

Other structural features of the *O*-deacetylated polymer were established by further analysis of the NMR spectra. Almost all proton resonances (Table 1) were readily identified with the aid of COSY, relayed COSY, HMQC, and NOE difference spectra. The β-GlcNAc residue (*d*) was obvious from the value of $J_{1,2}$ and assignments were facilitated by the intra-residue NOE contacts (with H-2, H-3, and H-5), and by the C-H correlations (for H-2, H-3, and H-6a/6b, in particular). The chemical shift data for residue *d* are almost identical to those for the GlcNAc residue in the structural element **3** of the *Shigella flexneri* type Y antigen [21].

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

Atom	Rha (*a*)	Rha (*b*)	Rha (*c*)	GlcNAc (*d*)
1 H	5.32	5.00	4.83	4.75
C	100.57	102.75	98.22	102.75
2 H	4.16	3.82	4.12	3.84
C	79.94	68.94 [b]	76.99	56.47
3 H	3.97	3.96	3.74 [c]	3.65
C	70.41	73.32	74.17 [c]	82.27
4 H	3.84	3.32	~ 3.43 [c]	3.51
C	- [d]	- [d]	- [c,d]	69.08 [b]
5 H	4.28	4.22	3.43	3.45
C	68.53	69.38	- [d]	76.56
6 H	1.21	1.22	1.31	3.76; 3.89
C	16.16	17.54	17.51	61.45

[a] Residues (*a*-*d*) are identified in structure **4**. Spectra were obtained at 45 °C and 600 MHz (^{1}H) or 150 MHz (^{13}C) for the polymer in ^2H_2O. Values for chemical shift are given relative to internal acetone (δ_H set at 2.22 and δ_C at 31.00). Signals for the *N*-acetyl group were at 175.10 and 23.07 ppm.
[b] Assignments may be interchanged.
[c] Tentative assignment.
[d] Value unassigned, but one of the following: 73.27, 73.07, 72.86, or 72.48 ppm.

The β-Rha residue (*c*) was also clear from the value (159 Hz) of $^1J_{CH}$ for H-1/C-1, the intra-residue NOE contacts (with H-2, H-3, and H-5), and the high-field location of the signal for H-5 [22]. A conclusive assignment for H-4*c* was more problematic. In the COSY and relayed COSY spectra, there were cross-peaks with H-6*c* at δ 3.43 (H-5*c*), 3.74 (stronger in the relayed COSY spectrum, H-4*c* and/or H-3*c*?), and 4.12 (H-2*c*, weak in both). A signal at δ 3.74 had been assigned to H-3*c* from the relayed COSY spectrum (weak cross-peak with H-1*c*) and from the intra-residue NOE effect. Thus, it appeared that the signal for H-4*c* was coincident either with H-3*c* or H-5*c*. The provisional assignment (Table 1) is based on (a) the impression from the 1D spectrum that the region δ 3.40-3.50 contained signals for 3 H, compared with 2 H in the region δ 3.70-3.80, (b) the expectation that H-4*c* would have δ ~ 3.4 [23], and (c) the presence in the HMQC spectrum of correlations for only 21 of the expected 22 methylene and methine protons in the repeating unit, with only one correlation for a proton with δ 3.74 (H-3*c*). The values tabulated for residue *c* are in fact in good agreement with those reported [24] for a similar β-Rha*p* residue in a D-rhamnan from *B. cepacia*, allowing for a systematic displacement of ~ 0.15 ppm for all protons.

For the two α-Rha residues (*a* and *b*), the assignments of the separate sequences H-1 to H-3, and H-4 to H-6, were straightforward, but linking them was complicated by the close juxtaposition of the two H-3 signals. The selection given (Table 1) appeared the more likely, but interchange of the H-4 to H-6 sets could not be precluded, and would give a better fit for H-4*a* [21]. In any event, the δ values for one H-4 and both H-5 signals were rather high compared with some literature data for α-Rha*p* residues in similar environments.

Most of the ^{13}C signals (Table 1) could be assigned from the HMQC spectrum which

provided shift correlations with the assigned protons. The outstanding problems were a consequence of (a) ambiguity about the signals for H-3*c* and H-4*c*, (b) the proximity of the signals for C-2*b* and C-4*d*, and (c) the presence in the HMQC spectrum of correlations for only 3 of the 4 carbon atoms with chemical shifts in the range δ 72.48 to 73.27. Despite these problems, C-2*a*, C-2*c*, and C-3*d* are clearly identified as sites of glycosylation from the large downfield shifts of these resonances compared with those for the free monosaccharides. Also, inter-residue NOE effects on irradiation of anomeric protons unambiguously revealed the following disaccharide elements: $a \rightarrow c$, $b \rightarrow d$, and $d \rightarrow a$. Irradiation of H-1*c* produced weaker NOE effects (of similar intensity) on H-2*b* and H-3*b*. Residue *b* must be the 3-substituted α-Rha, and the NOE effect at H-2 is the expected one of substitution at the adjacent position by the β-L-Rha*p* residue *c* [25]. The relatively small glycosylation effect for C-3*b* is also consistent with the $c \rightarrow b$ sequence [26], although a magnitude larger than the 2-3 ppm found was expected. In summary, the repeating unit of the *O*-deacetylated polymer can be assigned structure **4**.

d *a* *c* *b*

→ 3)-β-D-Glc*p*NAc-(1→2)-α-L-Rha*p*-(1→2)-β-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→

4

To locate the *O*-acetyl substituent, the NMR spectra for the native and the *O*-deacetylated polymer were compared. GlcNAc was readily eliminated as the *O*-acetylated residue, as the chemical shifts for H and C at positions 4 and 6 were almost identical in both sets of spectra. The Rha residue *a* could also be ruled out for similar reasons, and the provisional assignments made for H-3*c* and H-4*c* (unaffected by acetylation) tended to preclude this Rha residue also. The NMR signal for the ring proton at the acetoxylated position was identified from an HMQC spectrum as that in the anomeric region with δ 5.07, which correlated with a signal at $\delta \sim 68$ in the ^{13}C NMR spectrum. The lack of resolution of the former signal, and the apparent resistance of the *O*-acetyl group to migration during mild acid hydrolysis of the polymer, suggested that the proton was H-2 of the 3-substituted Rha residue *b*. In support of this assignment, the COSY spectrum appeared to contain a cross-peak between the signals with δ 5.07 and δ 4.97 (H-1*b*). Also the signal for C-1*b* in the native polymer was about 1.5 ppm upfield compared with that in the *O*-deacetylated product, consistent with substitution at O-2*b*. This inference was confirmed by periodate oxidation of the native polymer, followed by methylation analysis. Only the 3-substituted Rha residue survived oxidation, showing that neither 2-substituted Rha residue was protected by *O*-acetylation. Although full Smith degradation, including a final reduction ($NaBH_4$), gave a product (SD1) lacking the *O*-acetyl group (which was presumably lost during reduction or acid hydrolysis), the location of the substituent seems to be firmly established.

The tetrasaccharide repeating-unit **4** is similar to those of several other bacterial O antigens. Linear polymers of the same composition, but different in some structural details, are found in *Shigella flexneri* [21], *Serratia marcescens* O18 [27] and O22 [28], and strains of *B. solanacearum* [13,14,17]. Branched polymers based on these or similar backbones are also found in some of these species and others, including some serogroups

of *Salmonella arizonae*, *Escherichia coli*, and *Acinetobacter baumannii* [29]. It is not known whether this common type of architecture reflects biosynthetic economy or confers a biological advantage on the organisms.

3. Experimental

Growth of bacteria, and isolation and fractionation of the LPS.—Strain NCTC 11149 of *B. pickettii* was grown in Nutrient Broth No. 2 (Oxoid, 20 L) for 24 h at 37 °C with aeration at 20 L min^{-1} and stirring at 250 or 300 rpm. The yields of wet cells from three batches were in the range 63-65 g. The cells were disintegrated, and the cell walls were purified (yields, 2.1-4.1 g), defatted, and used for the isolation of LPS (yields, 233-589 mg) as in related studies [27–29]. Samples of LPS were subjected to mild hydrolysis (aq 1% AcOH, 100 °C, 1.5 h), during which the suspension turned dark-brown, and the polymeric fraction was obtained by chromatography of the water-soluble products on Sephadex G-50 in pyridine-AcOH buffer (pH 5.4), monitoring the eluate for total carbohydrate (phenol-H_2SO_4 method).

NMR spectroscopy.—Spectra (1D and 2D) for products (the native and the *O*-deacetylated polymer, the Smith-degradation product SD1) in D_2O were recorded with a Jeol JNM-GX270, a Bruker WH-400, or a Varian DXR600S spectrometer. In general, the NMR data reported were obtained at 45 °C with acetone (δ_H 2.22, δ_C 31.00) as the internal reference; the ^1H NMR data for SD1 were recorded at 70 °C. Standard pulse sequences were used to obtain COSY, relayed COSY, HMQC, and NOE difference spectra. Gated decoupling was used to obtain J_{CH} values for anomeric signals.

Determination of monosaccharide composition.—Conditions of hydrolysis used were: (a) 2 M HCl, 105 °C, 2 h (for neutral sugars); (b) 6.1 M HCl, 105 °C, 4 h (for amino sugars); (c) 2 M trifluoroacetic acid, 98 °C, 16 h (for both classes [30]). Products were analysed by PC, HPAEC (Dionex), high-voltage paper electrophoresis (pH 5.3), auto-analysis for amino compounds (Locarte), and GLC of the alditol acetates. Absolute configurations were assigned by acid-catalysed glycosidation with enantiopure butan-2-ol [31] or octan-2-ol [32], followed by acetylation and GLC of the glycoside acetates.

Chemical degradations.—*O*-Deacetylation was carried out by treatment of the native polysaccharide with 0.1 M NaOH at room temperature for 16 h, followed by passage of the hydrolysate through a column of Dowex 50 (H^+) resin. Methylation analyses were carried out by standard procedures, and monitored by GLC and GLC-MS of the methylated alditol acetates [27–29]. Methods used for periodate oxidation and Smith degradation of the polymer, including reduction ($NaBH_4$) after the selective acid hydrolysis, were also those used in similar studies [27–29].

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