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Characterization of a lipopolysaccharide O antigen containing two different trisaccharide repeating units from *Burkholderia cepacia* serotype E (O2) ☆

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Abstract

The O antigen of the lipopolysaccharide of *Burkholderia cepacia* serotype E (O2) was shown by a combination of methylation analysis, partial hydrolysis, NMR, and mass spectrometric methods to be a high molecular weight polysaccharide composed of two different trisaccharide repeating units in the ratio 2:1. The major trisaccharide component is composed of two α-D-mannopyranosyl and one β-D-galactopyranosyl residues with the structure,

$$[\rightarrow 2)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow 2)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Gal}p\text{-}(1\rightarrow]_n$$

The minor trisaccharide component is a D-mannan composed of two α- and one β-D-mannopyranosyl residues with the structure,

$$[\rightarrow 2)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow 2)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Man}p\text{-}(1\rightarrow]_n$$

Keywords: O Antigen, lipopolysaccharide; *Burkholderia cepacia*, Methylation analysis; 2D NMR (COSY, NOESY) experiments; GLC–MS of alditol acetates; FABMS

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1. Introduction

Burkholderia (Pseudomonas) cepacia is an important opportunistic pathogen which causes pulmonary infections in children and young adults with cystic fibrosis (CF). In some patients colonization with *B. cepacia* leads to rapid clinical deterioration and death, despite treatment with multiple antimicrobial drug regimens to which the bacteria are susceptible. It is difficult to understand the epidemiology of *B. cepacia* colonization in CF patients because of the marked phenotypic variability exhibited by clinical strains; however, available data [1] suggest that the CF patient infected with *B. cepacia* is the major source of infection for other CF patients. Several different typing schemes [2–5] have been proposed in order to understand the epidemiology of *B. cepacia* and to provide rational approaches to the monitoring and prevention of the spread of infections. The O, H typing system of Heidt et al. [2] was found to be both sensitive and specific [6]; however, McKevitt et al. [7] found many *B. cepacia* strains either agglutinated in multiple antisera or were non-typable. This finding led to the development of a serotyping scheme, based upon agglutination reactions with homologous and heterologous antisera, in which the *B. cepacia* strains were separated into five serotypes A–E [7]. When each of the five diluted antisera was absorbed with its homologous isolated lipopolysaccharide (LPS), the residual antisera no longer agglutinated homologous strains, demonstrating that, in this system, serotype specificity resides in the LPS component of the bacterial cell [7].

McKevitt et al. [7] also attempted to serotype type strains from the Heidt classification scheme. It is reported that *B. cepacia* serotype O1 agglutinated with serotype C antiserum, serotypes O2 and O5 both agglutinated in serotype E antiserum, and serotypes O4 and O6 both agglutinated in serotype A antiserum. The structural analyses of the O antigens of serotypes A, C, D, and E were undertaken to determine the molecular basis for their specificities and serological cross reactions. Strains of serotype B produce a rough LPS.

We now report the structure of the O antigen of *B. cepacia* serotype E, which is identical to that of serotype O2 of the Heidt typing scheme.

2. Results and discussion

Lipopolysaccharide (7.0 g) was obtained by extraction of *B. cepacia* serotype E cells (836 g, wet weight) by a modified phenol–water procedure [8], followed by ultracentrifugation of the dialysed, concentrated, aqueous phase. SDS–PAGE analysis [7] of the LPS showed a banding pattern typical of S-type LPS. Partial acid hydrolysis of the LPS (1 g), with 2% acetic acid at 100°C and gel-filtration of the products on Sephadex G-50 gave an O polysaccharide which eluted in the void volume (K_{av} 0.22, 450 mg) and a core oligosaccharide (K_{av} 0.56, 18.2 mg). The O polysaccharide had $[\alpha]_D$ + 47°. Anal. Found: C, 39.2; H, 5.54; N, 0.58. Complete acid hydrolysis of the O polysaccharide and GLC–MS of the derived alditol acetates gave D-Man and D-Gal (3 : 1), the absolute configurations of the residues being established by GLC of their trimethylsilylated (*R*)-(+)-methylbenzylamine derivatives. Methylation analysis of the polysaccharide

Table 1
Methylation data for *B. cepacia* serotype E O antigen and derived oligosaccharides

Methylated sugars [b] as alditol acetates	T_{GM} [c]	Molar ratios [a]		
		I	II	III
1,2,3,5,6-Gal	0.77		0.2	0.6
2,3,4,6-Man	1.00		1.0	1.0
3,4,6-Man	1.19	2.7	1.1	
2,3,6-Gal	1.23	1.0		
2,4,6-Man	1.25	0.5		

[a] I, native polysaccharide; II, trisaccharide; III, disaccharide.
[b] 2,3,4,6-Man represents 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-mannitol etc.
[c] Retention times relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

(Table 1) showed it to contain O-2 substituted D-Man*p*, O-4 substituted D-Gal*p* and O-3 substituted D-Man*p* residues in the molar ratios 2.7 : 1.0 : 0.5.

The low-field region of the ^{1}H NMR spectrum of the OPS (Fig. 1a) contained three major signals at 5.17, 5.06, and 4.52 (d, $J_{1,2}$ 8.3 Hz) ppm and three minor signals at 5.34, 5.17, and 4.80 ppm. The ^{1}H NMR spectrum of the O polysaccharide was assigned via a COSY experiment, the six signals in the anomeric region of the ^{1}H NMR spectrum being labelled **a** → **f** in order of decreasing chemical shift. On the basis of its coupling constants and chemical shift (Table 2), residue **f** (4.52 ppm, $J_{1,2}$ 8.3 Hz) was identified as having the β-D-*galacto* configuration. Residues **a** → **e** were identified from the observed small magnitude (< 4 Hz) of the vicinal coupling constants $J_{1,2}$ and $J_{2,3}$ (Table 2), which are indicative of pyranose ring systems having *manno* configurations [9]. The anomeric region of the ^{13}C NMR spectrum contained three major signals at 103.0, 101.0, and 100.7 ppm (relative intensities 1.0 : 1.0 : 1.2) and two minor signals at 101.4 and 98.9 ppm, which were assigned via a ^{13}C–^{1}H chemical shift correlation experiment and by comparison with published data [10]. The resonances for C-2**a,b,c,d**, C-4**f**, and C-3**e** were significantly deshielded (77.9–79.3, 77.9 and 80.7 ppm, respectively, Table 3), indicating these residues to be substituted at the corresponding positions. The anomeric configurations of the glycosidic linkages were determined by measurement of the J_{C1-H1} coupling constants: α-D-Man*p* (residues **a,b,c,d**) 173.5–174.0 Hz; β-D-Man*p* (residue **e**) 158.0 Hz; β-D-Gal*p* (residue **f**) 164.0 Hz.

The sequences of the sugar residues giving rise to the major and minor groups of resonances were established from a NOESY experiment. The occurrence of transglycosidic NOE demonstrated connectivities between sugar residues within each series of resonances (Table 4), which established the sequences,

b → **d** → **f** *or* → 2)-α-D-Man*p*-(1 → 2)-α-D-Man*p*-(1 → 4)-β-D-Gal*p*-(1 → ,

and

c → **a** → **e** *or* → 2)-α-D-Man*p*-(1 → 2)-α-D-Man*p*-(1 → 3)-β-D-Man*p*-(1 →

The occurrence of intraresidue NOE between H-1, and H-2 of residues **a,b,c,d** confirmed their α-D configurations, whereas NOE between H-1 and H-3 and between H-1 and H-5 of residues **e** and **f** confirmed their β-D configurations (Table 4).

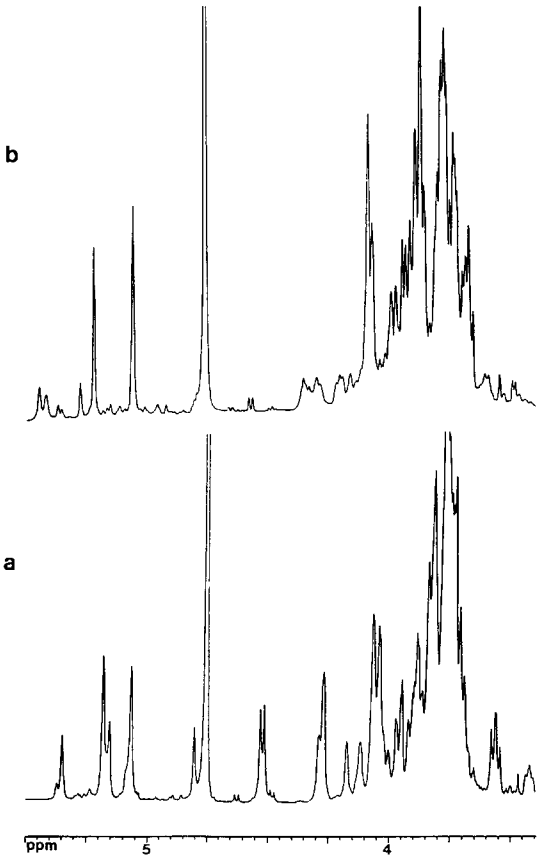

Fig. 1. (a) ^{1}H NMR spectrum of the *O* polysaccharide of *B. cepacia* serotype E. (b) ^{1}H NMR spectrum of the reduced trisaccharide **1** from the O polysaccharide of *B. cepacia* serotype E.

Treatment of the OPS with M TFA for 4 h at 95°C gave a trisaccharide **1** and a disaccharide **2**, after fractionation of the products by gel-permeation chromatography on Bio-Gel P2. FAB analysis, in the positive-ion mode, of the reduced **1** and **2** gave $[M + Li]^+$ ions at m/z 513 and 351, respectively. Methylation and subsequent analysis by GLC–EIMS of reduced **1** gave fragment ions inter alia at m/z 171, 187, 203, 391, and 439 (Fig. 2). Methylation analysis of reduced **1** and **2** gave the results shown in Table 1. The ^{1}H NMR spectrum of **1** had two singlets in the low-field region at 5.06 and 5.10 ppm, together with two signals at 5.45 and 4.63 ($J_{1,2}$ 7.5 Hz) for the α-D and β-D anomers of the D-Gal*p* residue, respectively, which disappeared on reduction of **1** with $NaBH_4$ (Fig. 1b). These results are consistent with the following structures for **1** and **2**,

α-D-Man*p*-(1 → 2)-α-D-Man*p*-(1 → 4)-D-Gal **1**

α-D-Man*p*-(1 → 4)-D-Gal **2**

Table 2
^{1}H NMR chemical shift data for the O polysaccharide of *B. cepacia* serotype E [a]

→ 2)-α-D-Man*p*-(1 → 2)-α-D-Man*p*-(1 → 4)-β-D-Gal*p*-(1 →
b **d** **f**
→ 2)-α-D-Man*p*-(1 → 2)-α-D-Man*p*-(1 → 3)-β-D-Man*p*-(1 →
c **a** **e**

Sugar residue	H-1 ($J_{1,2}$)	H-2 ($J_{2,3}$)	H-3 ($J_{3,4}$)	H-4 ($J_{4,5}$)	H-5	H-6 ($J_{5,6}$) ($J_{6,6'}$)	H-6′ ($J_{5,6'}$)
a	5.34 (< 1.0)	4.12 (3.8)	4.01 (8.3)	3.68	3.97	3.81	3.81
b	5.17 (< 1.0)	4.26 (3.8)	3.88 (8.3)	3.71	3.99 [b]	3.81	3.81
c	5.15 (< 1.0)	4.30 (3.8)	3.87 (8.3)	3.71	3.97	3.81	3.81
d	5.06 (< 1.0)	4.06 (3.8)	3.96 (8.3)	3.76	3.97 [b]	3.81	3.81
e	4.80 (< 1.0)	4.17 (3.8)	3.73 (8.3)	3.80	3.42	3.75	3.92
f	4.52 (8.3)	3.56 (10.4)	3.74 (4.2)	4.04 (< 1.0)	4.07	3.76	3.84

[a] Measured at 27°C from internal acetone (2.225 ppm).
[b] These assignments may be reversed.

One remaining question is whether the two components of the serotype E OPS are present as blocks of the major trisaccharide unit interspersed with blocks of the minor trisaccharide unit, or whether the minor component unit occurs every third repeating unit. The NOESY experiment allowed a distinction to be made between these two alternatives. Within the limits of detection of this experiment, no cross peaks were

Table 3
^{13}C NMR chemical shift data for the O polysaccharide of *B. cepacia* serotype E [a]

→ 2)-α-D-Man*p*-(1 → 2)-α-D-Man*p*-(1 → 4)-β-D-Gal*p*-(1 →
b **d** **f**
→ 2)-α-D-Man*p*-(1 → 2)-α-D-Man*p*-(1 → 3)-β-D-Man*p*-(1 →
c **a** **e**

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6
a	101.4	79.3	70.7	67.4	73.9	61.2
b	101.0	78.0	70.4	67.4	76.7 [b]	61.2
c	100.7	77.9	70.4	67.4	73.9	61.2
d	100.7	79.3	70.7	67.4	73.9 [b]	61.2
e	98.9	71.0	80.7	66.7	73.9	61.6
f	103.0	71.0	73.9	77.9	75.8	61.2

[a] Measured at 27°C from internal acetone (31.07 ppm).
[b] These assignments may be reversed.

Table 4
Proton NOE data for the O polysaccharide of *B. cepacia* serotype E [a]

Observed proton			
Anomeric proton	Intraresidue NOE	Transglycosidic NOE	Partial sequence
1a	**2a**	**3e**	**a → e**
1b	**2b**	**2d,1f**	**b → d**
1c	**2c**	**2a,1e**	**c → a**
1d	**2d**	**4f**	**d → f**
1e	**3e,5e**	**2c**	**e → c**
1f	**3f,5f**	**2b**	**f → b**

[a] Measured from the 2D NOESY spectrum of the native polysaccharide at 27°C.

observed between any H-1 of the major series of resonances and H-2 of the α-D-Man*p* or H-3 of the β-D-Man*p* of the minor series of resonances. Thus, blocks of the major and minor repeating units are expressed either within a single polymeric chain or as separate polymers in the ratio 2 : 1.

Hydrolysis of the O polysaccharide from *B. cepacia* serotype O2 gave D-Man and D-Gal in the ratio of 2.89 : 1.0. Methylation analysis gave results consistent with the presence of O-2, O-3, and O-4 substituted hexopyranosides (results not shown). The ^{1}H and ^{13}C NMR spectra of the O polysaccharide from serotype O2 were found to be identical to those of the O antigen from serotype E (within ± 0.2 ppm); ^{1}H NMR: 5.18, 5.05, 4.49 ($J_{1,2}$ 7.5 Hz) ppm (integration 1.0) and 5.31, 5.14, and 4.77 ppm (integration 0.5); ^{13}C NMR: 103.2, 101.2, 100.9 (major), and 101.5, and 99.1 ppm (minor). Thus, the O polysaccharide from *B. cepacia* serotype O2 is composed of identical trisaccharide repeating units to that of the O antigen of serotype E. Attempts to fractionate the O polysaccharide from the serotype O2 into two separate O chains by gel chromatography on either Sephacryl S-300 or octyl-Sepharose were unsuccessful. Furthermore, the ratio

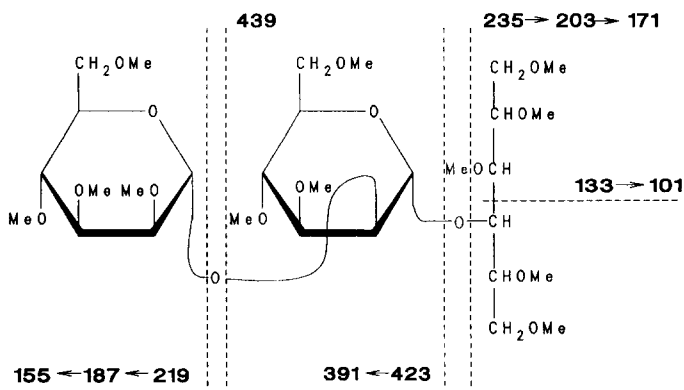

Fig. 2. Fragment ions observed on analysis by GLC–EIMS of reduced and methylated trisaccharide **1**.

of major to minor components remained consistent even when grown under markedly different conditions.

The production of two O polysaccharides has been observed in other bacterial species. *Klebsiella pneumoniae* serotype O1 [11] expresses two structurally distinct D-galactan O antigens. In some strains of *Salmonella* spp., the recognized O antigen is coexpressed with a structurally distinct polysaccharide termed T1 antigen [12]. Simultaneous expression of two O polysaccharides has also been detected in strains of *Pseudomonas aeruginosa* [13], *Pseudomonas pseudomallei* [14,15], *Salmonella boecker* [16] and a variety of *Escherichia coli* strains appear to synthesize both a neutral and an acidic O antigen [17].

With the completion of the structural analyses of the OPS of serotypes A [18], C, D [19], and E it is now possible to explain most of the observed serological cross reactions. Serotypes C, D and E correspond to serotypes O1 [20], O3 [21], and O2 (Table 4), respectively, of the Heidt classification scheme. However, the structure of the OPS of serotype O5 [21] is $[\rightarrow 4)$-α-L-Rha$p$-$(1 \rightarrow 3)$-β-D-Man$p$NAc-$(1 \rightarrow]_n$ and thus would not be expected to agglutinate with serotype E antiserum.

3. Experimental

Production of lipopolysaccharide and O polysaccharide.—*B. cepacia* serotype E (K63-2, NRCC 4705) culture was supplied by Dr D. E. Woods, Department of Microbiology and Infectious Diseases, University of Calgary Health Sciences Centre, Calgary, Alberta, Canada. These cells were grown in fermenters (28 and 75 L, Microferm, New Brunswick Scientific) using a medium of 3.7% brain heart infusion (Difco) at 37°C, 200 rpm, and aeration at 25 L/min for 18 h. Cells were killed by the addition of phenol to 0.75% final concentration and harvested using a Sharples centrifuge. LPS was isolated from the saline-washed cells (yield 836 g, wet weight) by the aq phenol extraction procedure [8]. The O polysaccharide and core oligosaccharide were obtained by treatment of the LPS with 2% v/v acetic acid (2 h, 100°C) and by fractionation of the water-soluble products by Sephadex G-50 gel-filtration chromatography as previously described [22].

B. cepacia serotype O2 (CIP 8236) culture was supplied by Dr H. Monteil, Institut de Bacteriologie de la Faculté de Medecine, Université Louis Pasteur, 6700 Strasbourg. The bacteria were grown for 24 h at 30°C in a 20 L fermenter containing nutrient broth No. 2 (Oxoid Ltd., Basingstoke, UK), 300 rpm and aeration at 20 L/min. The cells were separated from the culture medium by continuous refrigerated centrifugation (Sharples, France) and then disintegrated using a continuous-flow Dyno-Mill KDL (W.A. Bachofen AG, Basel, Switzerland). Lipopolysaccharide (13.5%) was extracted from the cells (97 g wet weight) using hot, aq phenol as previously described [23]. Mild acid hydrolyses of the LPS were done in the presence of 1% sodium dodecyl sulfate [24] or in 1% acetic acid at 100°C for 2 h, and the water-soluble products were fractionated by gel-permeation chromatography on Sephadex G-50.

Nuclear magnetic resonance spectroscopy.—All measurements were made on solutions in D_2O, at 27°C using Bruker AMX 500 or AM 200 spectrometers (serotype E)

and Bruker WH 400 or JEOL JNM-GX270 (serotype O2). Proton spectra were obtained using a spectral width of 2.2 KHz and a 90° pulse. Chemical shifts are expressed relative to internal acetone (2.225 ppm).

Broad-band decoupled ^{13}C spectra were obtained at 125 MHz with a spectral width of 27 KHz and a 90° pulse employing WALTZ decoupling [25]. Chemical shifts are expressed relative to internal acetone (31.07 ppm).

Two-dimensional homonuclear chemical shift correlated (COSY) and nuclear Overhauser enhancement (NOESY) experiments were carried out as previously described [26]. A mixing time of 400 ms was employed for the NOESY experiment. The heteronuclear ^{13}C–^{1}H chemical shift correlations were measured in the ^{1}H-detected mode via multiple quantum coherence ($^{1}H\{^{13}C\}$HMQC) with a Bruker 5-mm inverse broad-band probe, using reverse electronics as previously described [27].

Analytical methods.—For analysis of constituent sugars, samples (1 mg) of polysaccharide (serotype E) were hydrolysed with 4 M TFA for 1 h at 125°C, and the excess acid was removed by evaporation under a stream of nitrogen or, samples of polysaccharide (serotype O2) were hydrolysed with 2 M HCl for 2 h at 105°C and neutralized with Dowex 50 (H^+) and Dowex 1 (HCO_3^-) resins. The identities of the glycoses were determined by GLC–MS of their derived alditol acetates [28].

The configurations of the glycoses were established by GLC–MS of their trimethylsilylated (*R*)-(+)-methylbenzylamine derivatives [29].

Optical rotations were measured at 20°C using a Perkin–Elmer model 243 polarimeter.

Analytical GLC–MS was performed with a Hewlett–Packard model 5958B gas chromatograph, fitted with an OV-17 fused silica capillary column (Quadrex Corp.), in the electron-impact mode, with an ionization potential of 70 eV and employing the following temperature program: 180°C for 2 min, then 2°C/min to 240°C.

FABMS analyses were carried out using a Jeol AX505-H mass spectrometer in the positive-ion mode, with an accelerating voltage of 2 kV, and a mass resolution of 1500. A Xe atom beam of 6 kV was used to sputter and ionize the sample.

Methylation analyses.—Methylation of samples (2 mg) was carried out by the method of Hakomori using dimsyl potassium in dimethyl sulfoxide [30], or the method of Ciucanu and Kerek [31], and the products subsequently purified by partition between dichloromethane and water. The methylated products were hydrolysed (as above) and analysed by GLC–MS as acetylated alditol derivatives.

Partial hydrolysis.—*B. cepacia* serotype E O polysaccharide (111 mg) in M TFA was heated for 4 h at 95°C. The excess acid was removed by evaporation, under reduced pressure, and the remaining traces were neutralized with NH_4OH. The lyophilized product was dissolved in water and fractionated on a Bio-Gel P2 column, and the glycose-positive fractions were pooled and rechromatographed to give two pure oligosaccharides, **1** (K_{av} 0.69, 8.4 mg) and **2** (K_{av} 0.86, 6.8 mg).

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