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The structure of the O-specific polysaccharide from the lipopolysaccharide of *Burkholderia anthina*

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ABSTRACT

The pathogenic mechanisms of Gram-negative infection in cystic fibrosis are only just beginning to be explored at molecular level. Several virulence factors have been defined, one of the most important is the lipopolysaccharide molecule. In order to fully understand the mechanisms of bacterial infection and host recognition a full structure/activity study of lipopolysaccharide is needed. In the present paper, we define the complete structure of the O-specific polysaccharide from the lipopolysaccharide from *Burkholderia anthina*, an uncommon pathogen of cystic fibrosis patients.

1. Introduction

The genus *Burkholderia* comprises more than 30 species, animals and plant pathogens as well as environmentally bacteria, which inhabit diverse ecological niches and have been isolated from soil, water, plants, insects, industrial settings, hospital environments and from infected humans.[1] Several *Burkholderia* strains have attracted considerable interest from the biotechnological and agricultural industry for bioremediation of recalcitrant xenobiotics, plant growth promotion and biocontrol purposes. *Burkholderia* species have emerged as problematic opportunistic pathogens in cystic fibrosis (CF) patients and immunocompromised individuals.[1,2] These species comprise a heterogeneous group of strains, genetically distinct, each known as a genomovar. The *B. cepacia* complex (BCC) consists of nine described species which share a high degree of 16S rDNA (98–100%) sequence similarity and only moderate levels of DNA–DNA hybridisation: *B. cepacia, B. multivorans, B. cenocepacia, B. stabilis, B. vietnamiensis, B. dolosa, B. ambifaria, B. anthina* and *B. pyrrocinia*.[3,4] Species belonging to the BCC are problematic CF pathogens because of their high resistance to antibiotics, making respiratory infection difficult to treat and impossible to eradicate. Infection by these bacteria is associated with higher mortality in CF patients and poor outcomes following lung transplantation as compared to those with pre-transplant *Pseudomonas aeruginosa* infection. Many species are highly epidemic and spread easily within CF centres from one patient to another leading to considerable anxiety and segregation policies.[3,4]

The distribution of BCC species among isolates from CF patients is clearly disproportionate.[3] In the most comprehensive study published so far, *B. cenocepacia* and *B. multivorans* account for the vast majority of BCC infections in CF patients from the USA (45.6% and 38.7% of patients, respectively) whereas a minority of patients harbour *B. vietnamiensis, B. dolosa* and *B. cepacia* (5.9%, 3.8% and 3.1%, respectively). Remarkably, carriage of *B. stabilis, B. ambifaria, B. anthina* or *B. pyrrocinia* was low (1% of patients) and this is difficult to explain in terms of clinical significance especially because they are encountered more often in natural habitats than in clinical settings. For example, no strong causal association has so far been established between the presence of such species in pulmonary secretions and the health decline in CF patients.[3]

In our opinion, a pre-requisite for a more complete understanding is the chemical study of the virulence factors that are pivotal in the infection, microbial recognition and inflammation. The pathogenic mechanisms leading to poor clinical outcomes are only just beginning to be explored at molecular level. Certainly, bacterial lipopolysaccharides (LPSs) are key players in lung infection by Gram-negative bacteria and so we have started a study of the chemical structure of all LPSs related to the BCC complex in order to get a complete picture of such important virulence factors.[2,5]

From a structural aspect, the *Burkholderia* LPSs show the same general chemical architecture seen in all the Gram-negative bacteria, that is, it does neither depend on the bacterial activity (pathogenic, symbiotic, commensal), nor on its ecological niches: (human, animal, soil, plant, water). From a biosynthetical and

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chemical point of view, three different components are usually identified in the general structure of LPS: a polysaccharide known as O-specific polysaccharide (OPS) which is covalently linked to an oligosaccharide part (core), in turn, linked to a glycolipid portion (lipid A). The lipid A is part of the outer leaflet of the external membrane whereas the sugar moiety is oriented outwards. LPSs are also called smooth LPS (S-LPS) if they possess the OPS or rough LPS (R-LPS or lipooligosaccharide, LOS) if the OPS is lacking.

Within this frame, we report in this work the OPS structure of *B. anthina* strain LMG20983 (CF patient from UK).

2. Results

2.1. Isolation and characterisation of the OPS

Cells of *B. anthina* were extracted using the hot phenol–water[6] procedure and the LPS was detected in the water phase and further purified by digestion with nucleases, protease and by gel permeation chromatography. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of the purified LPS indicated the classical ladder-like distribution of the LPS molecules.[7] The lipopolysaccharide was hydrolysed using typical mild acidic conditions, and the O-polysaccharide fraction (OPS) was collected by centrifugation as supernatant and purified by gel permeation chromatography on a Sephacryl S-100 column. Chemical analyses, carried out by GC–MS analysis of the acetylated *O*-methyl glycoside and of the alditol acetate derivatives, yielded two main monosaccharides, rhamnose (Rha) and galactose (Gal) (ratio 2:1) assignable to the repeating unit of the OPS and, in minor amount, heptose and 3-deoxy-D-*manno*-oct-2-ulopyranosonic acid (Kdo) evidently belonging to the core region.

The absolute configuration of the two residues was deduced from GC–MS analysis of the peracetylated (+)-2-octanol glycosides and resulted to be D for Gal and L for Rha.[8]

Methylation analysis[9] was carried out to establish the site of linkages of the monosaccharide residues and showed the presence of the derivatives of 3-substituted rhamnopyranose, 2-substituted rhamnopyranose and 2-substituted galactopyranose, all three present in nearly a stoichiometric ratio.

The OPS was examined by NMR and the ^{1}H NMR spectrum (Fig. 1) appeared rather straightforward. The assignment of the ^{1}H and ^{13}C resonances for the OPS was performed by the analysis of the 2D NMR spectra. In particular, the proton resonances were assigned from DQF-COSY, TOCSY and NOESY spectra, and, on the basis of these data, ^{13}C resonances were assigned by ^{1}H,^{13}C-HSQC and ^{1}H,^{13}C-HMBC spectra. Three different spin residues were visible, chemical shift values are shown in Table 1.

Spin system **A** was identified as a 2-substituted-α-rhamnose residue since, in TOCSY spectrum (Fig. 2), scalar correlations of the ring protons with methyl signals in the shielded region at 1.18 ppm were visible. The *manno* configuration of **A** spin system was established by $^{3}J_{H1,H2}$ and $^{3}J_{H2,H3}$ values (below 3 Hz) whereas the α-configuration was assigned by the *intra*-residual NOE contact of H-1 with H-2 and chemical shift of its H-5 and C-5.[10] The downfield displacement of C-2 signal in the HSQC spectrum (Fig. 3) evidently indicated glycosylation at this position.

Residue **B** was identified as 2-substituted-α-galactose as indicated by the clear TOCSY scalar correlations between the anomeric signal and the other ring protons until H-4 resonance. The H-5 proton signal was identified through the diagnostic NOE connectivity with both H-3 and H-4, whereas the correlation of H-5 with the H-6s oxy-methylene signals completed the attribution. Even in this case, H-5/C-5 resonances were also indicative of α-configuration whereas C-2 signal downfield shift testified glycosylation at this position.

Spin system **C** was identified as 3-substituted-α-rhamnose. It showed, as **A** residue, TOCSY scalar correlations to the methyl signal at 1.18 ppm. Its α-*manno* configuration was established by $^{3}J_{H,H}$ ring proton coupling constants and H-5 and C-5 chemical shifts. In this case C-3 signal was found shifted downfield and indicated glycosylation at C-3 of **C** residue.

The sequence of the residues **A–C** within the repeating unit was deduced analysing the dipolar couplings in the NOESY spectrum (Fig. 2) where the following NOE contacts were found: H-1 of **A** with H-2 of **B**, H-1 of **B** with H-3 of **C**, H-1 of **C** with H-2 of **A**. In the same way, in the HMBC spectrum (Fig. 2) it was possible to detect scalar correlation between H-1/C-1 of **A** with H-2/C-2 of **B**, H-1/C-1 of **B** with H-3/C-3 of **C**, H-1/C-1 of **C** with H-2C-2 of **A**.

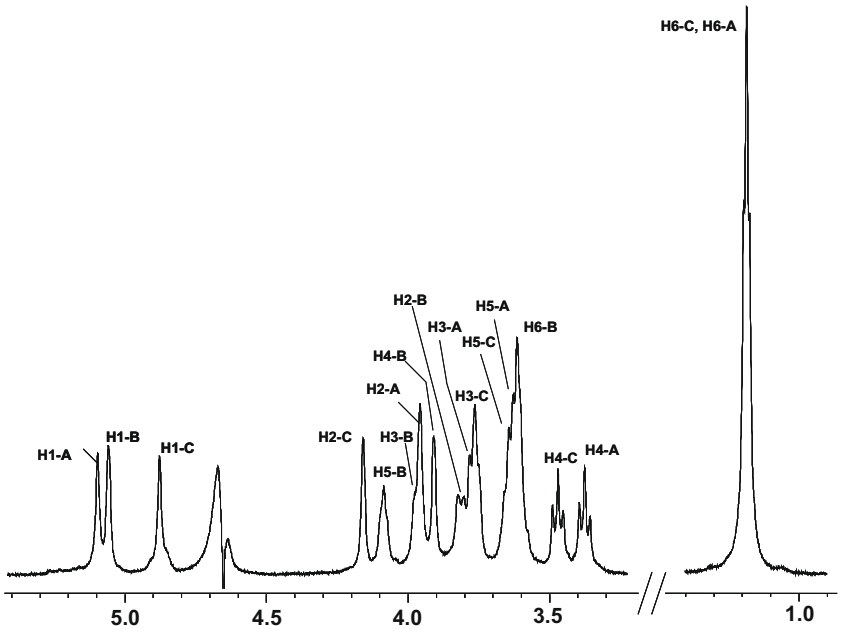

Figure 1. The ^{1}H NMR of the OPS from *B. anthina*. Capital letters refer to the spin systems described in Table 1.

Table 1
^{1}H and ^{13}C chemical shifts (ppm) of the OPS from *B. anthina*

Unit	Chemical shift δ ($^{1}H/^{13}C$)					
	1	**2**	**3**	**4**	**5**	**6**
A	5.09	3.95	3.76	3.37	3.63	1.18
2-α-L-Rha	100.7	78.3	70.1	72.0	69.4	17.1
B	5.05	3.81	3.97	3.90	4.08	3.61
2-α-D-Gal	94.6	74.3	69.3	69.6	71.0	60.9
C	4.87	4.15	3.75	3.47	3.64	1.18
3-α-L-Rha	102.4	66.6	74.6	70.4	69.3	17.1

Chemical shifts are relative to acetone and (^{1}H, 2.225 ppm; ^{13}C, 31.45 ppm). Spectra are recorded in D_2O at 300 K.

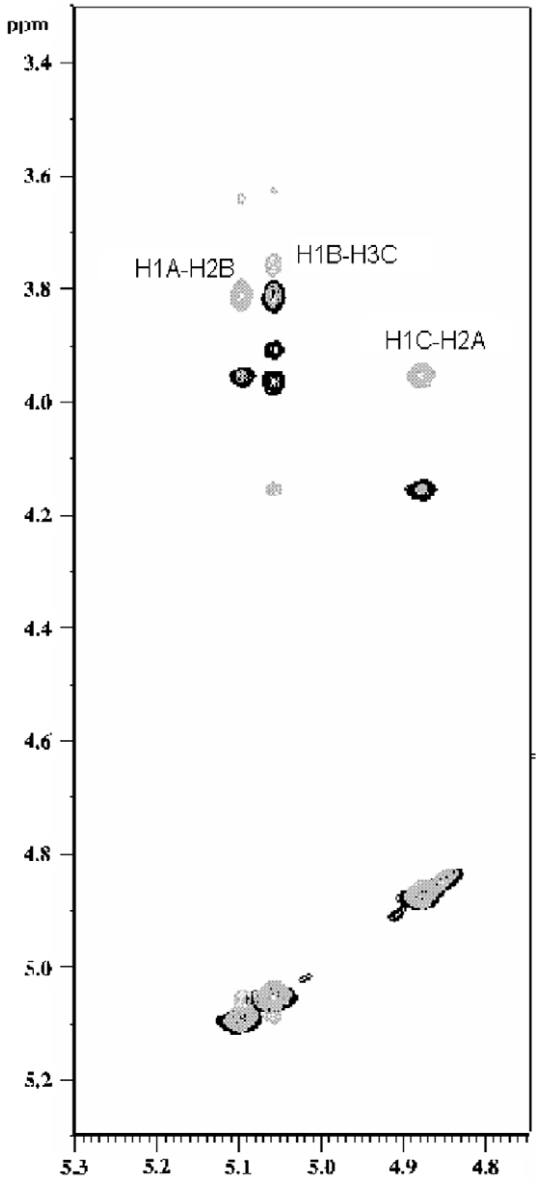

Figure 2. TOCSY (black tone) and NOESY (grey tone) spectra of the OPS from *B. anthina*. Capital letters and numbers refer to the spin systems described in Table 1. The interresidual dipolar correlations are shown.

In summary, chemical and methylation analyses, together with NMR spectroscopy allowed to establish the structure of OPS of the LPS from *B. anthina* as reported below:

C A B

$$[\rightarrow 3)\alpha\text{-L-Rha-}(1\rightarrow 2)\alpha\text{-L-Rha-}(1\rightarrow 2)\text{-}\alpha\text{-D-Gal-}(1\rightarrow]_n$$

This is the first report on the chemical nature of the endotoxin produced by *B. anthina.*

3. Experimental procedures

3.1. Bacterial growth and isolation of the LPS and LOS fraction

Strain LMG 20983 *B. anthina* was obtained from the Belgium Coordinate Collection for Microbes and derived from a patient suffering from CF. Cells were routinely grown on Tryptic Soy-agar plates, at 28 °C. For growth in liquid medium, 5–10 colonies from a fresh plate were inoculated in 500 mL of Tryptic Soy medium, and grown up to saturation overnight at 28 °C under constant shaking. This saturated culture was diluted into 2 L of the same fresh medium up to 0.02 OD_{600}. Cells were recovered by centrifugation at 3000 *g*, for 15 min, at 4 °C, washed with water, ethanol, acetone and ethylic ether, two times for each solvent, and then lyophilised.

Dried cells were extracted according to conventional protocols for LPS extraction.[6] Briefly, cells (2.560 g) were extracted according to the hot phenol/water procedure that yielded two fractions. All extracts were dialysed and screened for LPS content by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE 12%) stained with silver nitrate, as described.[7] LPS was detected only in the water extract. This fraction was digested with nucleases and proteinase furnishing 144 mg of LPS (yield 5.6% of the cells).

3.2. Isolation of the OPS from the LPS

An aliquot (20 mg) of the LPS from the water extract was hydrolysed in order to obtain the O-polysaccharide chain. The sample was treated with 0.1 M sodium acetate buffer (pH 4.4) at 100 °C for 3 h, and centrifuged (3000*g*, 4 °C, 1 h). The supernatant (14 mg, 70% of LPS) was purified by gel-permeation chromatography on a Sephacryl S100-HR column (Pharmacia, 90 cm × 1.5 cm) using 0.05 M ammonium bicarbonate as eluent. Elution was monitored with a Waters differential refractometer.

3.3. General and analytical methods

Monosaccharide analyses, methylation analyses and absolute configuration determination were carried out on the oligosaccharide and polysaccharide fractions by GLC and GC–MS methods according to conventional procedures.[11–13]

3.4. NMR spectroscopy

For structural assignments, spectra on the native OPS fraction were recorded on a solution of 7 mg in 0.6 mL D_2O at 300 K. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX-600 spectrometer equipped with a cryogenic probe and calibrated with internal acetone [δ_H 2.225, δ_C 31.45]. Nuclear Overhauser enhancement spectroscopy (NOESY) was measured using data sets ($t_1 \times t_2$) of 4096 × 1024 points, and 16 scans were acquired. A mixing time of 200 ms was used. Double quantum-filtered phase-sensitive COSY experiments were performed with 0.258 s acquisition time, using data sets of 4096 × 1024 points, and 64 scans were acquired. Total correlation spectroscopy experiments (TOCSY) were performed with a spinlock time of 100 ms, using data sets ($t_1 \times t_2$) of 4096 × 1024 points, and 16 scans were acquired. In all homonuclear experiments the data matrix was zero-filled in the F1 dimension to give a matrix of 4096 × 2048 points and was resolution enhanced in both dimensions by a shifted sine-bell function before

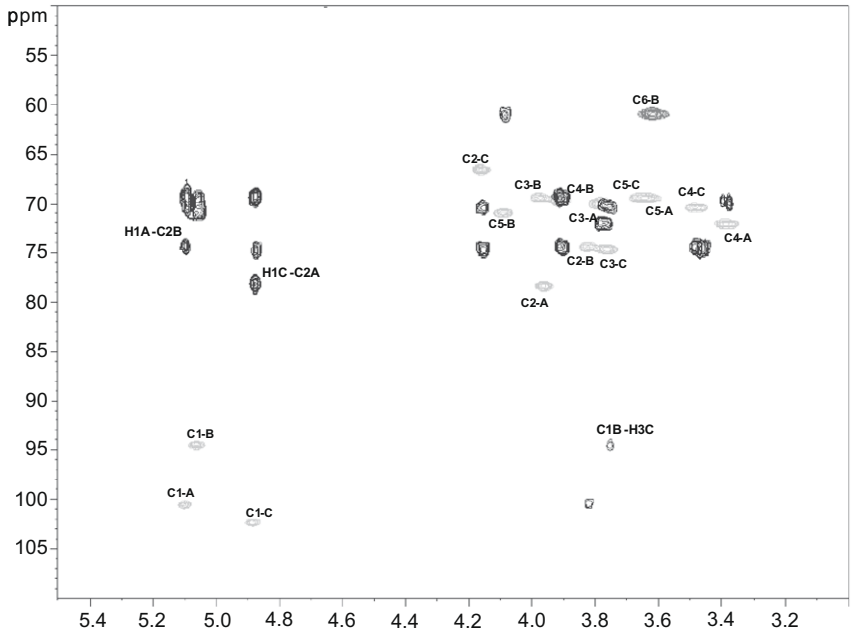

Figure 3. Section of $^{1}H,^{13}C$-HSQC (grey) and HMBC (black) spectra of the OPS from *B. anthina*. Capital letters and numbers refer to the spin systems described in Table 1. The more important direct and long-range scalar correlations are shown.

Fourier transformation. Coupling constants were determined on a first order basis from 2D phase-sensitive double quantum filtered correlation spectroscopy (DQF-COSY).[14,15] Heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC) experiments were measured in the ^{1}H-detected mode via single quantum coherence with proton decoupling in the ^{13}C domain, using data sets of 2048 × 512 points, and 64 scans were acquired for each t_1 value. Experiments were carried out in the phase-sensitive mode according to the method of States et al.[16] A 60 ms delay was used for the evolution of long-range connectivity in the HMBC experiment.

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