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Note

The O-chain structure from the LPS of the bacterium *Naxibacter alkalitolerans* YIM 31775^T

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Abstract—The O-chain polysaccharide of the lipopolysaccharide from the bacterium *Naxibacter alkalitolerans* strain YIM 31775^T was characterized. The structure was studied by means of chemical analysis and 2D NMR spectroscopy and shown to be built up by the following tetrasaccharide repeating unit:

→3)-α-D-Fuc*p*NAc-(1→2)-β-D-Qui*p*3NHBu-(1→2)-α-D-Rha*p*-(1→4)-β-D-Gal*p*-(1→

where HBu is hydroxy-butanoyl.

Keywords: *Naxibacter alkalitolerans*; Lipopolysaccharide; O-Antigen, NMR spectroscopy; 3-Hydroxy-butanoyl

In the course of a screening programme for new antibiotics, a Gram-negative strain, YIM 31775^T, which contained genes encoding both type I and type II polyketide biosynthesis pathways, was isolated from a soil sample collected from Lijiang, Yunnan Province, China.[1] 16S rRNA gene sequence analysis showed that the isolate belonged to the class '*Betaproteobacteria*'. The morphological, physiological and chemical characteristics, and phylogenetic analyses, showed that the strain should be classified as representing a novel member of the family '*Oxalobacteraceae*', order '*Burkholderiales*', class '*Betaproteobacteria*' and the name *Naxibacter alkalitolerans* was proposed.[1]

One of the major chemical hallmarks of Gram-negative bacteria are lipopolysaccharides (LPSs),[2,3] the main constituents of the outer leaflet of the outer cell membrane of Gram-negative bacteria, thus, directly in contact with the external environment.[2] This paper describes the structural elucidation of the O-polysaccharide fraction from the LPS of *N. alkalitolerans* YIM 31775^T.

The cells of *N. alkalitolerans* YIM 31775^T were extracted using the hot phenol–water procedure[4] 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 electrophoretic analysis of the purified LPS indicated the presence of a lipopolysaccharide bearing an O-antigen and showed the banding pattern formed by the repeating units. The lipopolysaccharide was hydrolyzed using mild acidic conditions, and the O-polysaccharide fraction was collected as the supernatant after centrifugation and purified by gel permeation chromatography on a Sephacryl S-100 column. Chemical analyses, carried out by GC–MS of the acetylated *O*-methyl and *O*-oct-2-yl glycoside derivatives, yielded four different monosaccharides, 2-amino-2,6-dideoxy-galactose (fucosamine, FucN), 3-amino-3,6-dideoxy-glucose (quinovosamine, Qui3N), 6-deoxy-mannose (rhamnose, Rha) and galactose (Gal), all in D configuration. In addition, at a higher retention time of the GLC

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of acetylated *O*-methyl glycoside analysis, it was possible to detect a tiny amount of Qui3N that was acylated by its original acyl moiety, a hydroxy-butanoyl group not cleaved by methanolysis (see below). Methylation analysis showed the presence of 2-substituted Qui3N, 2-substituted Rha, 4-substituted Gal and 3-substituted FucN. The 3-hydroxy-butanoyl absolute configuration was determined to be *S* by GLC of trifluoroacetylated *R*-2-octyl esters.[5]

The ^{1}H NMR spectrum (Fig. 1) of the polysaccharide appeared rather simple and the product was eventually identified by 2D NMR analysis. In the anomeric region four signals were identifiable as belonging to four different spin systems, **A**–**D,** ranging from 5.57 and 4.32 ppm whereas in the high field region a diastereotopic methylene signal and several methyl signals were present. Chemical shifts of each residue were assigned utilizing DQF-COSY, TOCSY, ROESY and HSQC experiments (Table 1). Anomeric configurations were assigned on the basis of the chemical shifts and of $^{1}J_{C\text{-}1,H\text{-}1}$ values derived from a coupled ^{1}H,^{13}C-HSQC.

Spin systems **A** was identified as FucN. The H-1/C-1 chemical shifts were diagnostic of α-anomeric orientation while H-2 proton correlated in the HSQC spectrum to a nitrogen bearing carbon signal. Its ring configuration was inferred on the basis of coupling constant values of ring protons, in particular, $^{3}J_{3,4}$ (3 Hz). Moreover, H-5 of **A** spin system was only detectable by NOESY experiment since in the TOCSY spectrum the low $^{3}J_{4,5}$ impaired any magnetisation transfer over H-4. Residue **B** appeared to have a small $^{3}J_{1,2}$ coupling constant value (about 1.4 Hz) deduced from the DQF-COSY spectrum that, together with the diagnostic H-5/C-5 chemical shift values,[6] suggested the α-*manno* configuration. In the TOCSY spectrum, starting from H-2 proton signal, all correlations within the ring protons

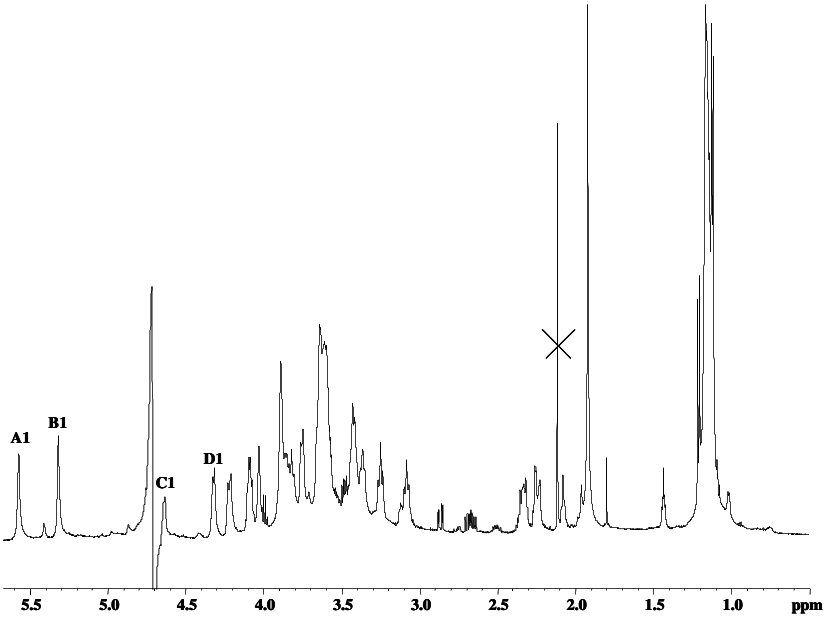

Figure 1. ^{1}H NMR spectrum of the purified O-polysaccharide from *N. alkalitolerans*. Letters refer to the anomeric proton signals as in Table 1.

Table 1. ^{1}H and ^{13}C chemical shifts (ppm) of the O-chain from *N. alkalitolerans*

Residue	**1**	**2**/NH	**3**/NH	**4**	**5**	**6**
3-α-FucN	5.57	4.21/8.36	3.63	3.89	3.85	1.14
A	96.3	48.9	79.2	72.5	67.7	17.5
2-α-Rha	5.31	4.03	3.74	3.25	3.59	1.16
B	101.4	81.3	70.5	73.2	70.7	18.0
2-β-Qui3N	4.63	3.37	3.81/8.16	3.08	3.42	1.17
C	106.0	77.6	56.9	74.2	74.2	18.1
4-β-Gal	4.32	3.43	3.65	3.88	3.63	3.61
D	106.6	72.0	74.0	77.4	76.6	61.5
Acetyl	—	1.98				
	176.0	24.0				
3-Hydroxy-butanoyl	—	2.24, 2.33	4.08	1.12		
	172.6	46.5	66.1	23.5		

Values are referred to the internal standard acetone measured in the conditions described in Section 1.

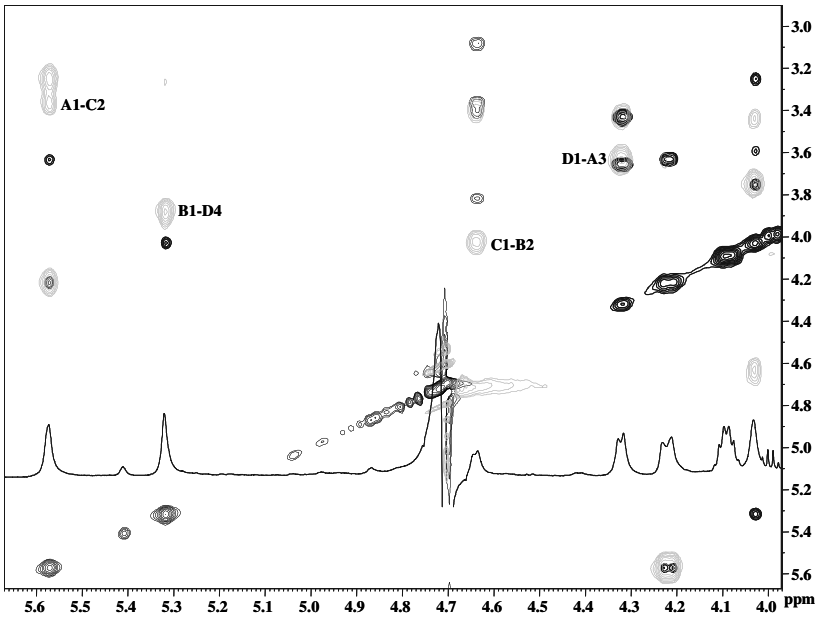

Figure 2. Section of the anomeric region of the ROESY (grey) and TOCSY (black) spectra of the O-chain from *N. alkalitolerans*. ^{1}H NMR spectrum is overlapped. Annotations only refer to diagnostic interresidual contacts.

and up to the methyl proton signal were visible. This led to the identification of **B** residue as α-Rha.

Residue **C** was established to be β-*gluco* configured since all its ring proton signals showed large ring coupling constant values (around 10 Hz) and furthermore H-3 signal was correlated in the HSQC spectrum to a nitrogen bearing carbon. These data allowed us to identify it as β-Qui3N.

Residue **D** was identified as β-Gal. Actually, its ring identification was similar to the one inferred for residue **A**, whereas the anomeric coupling constant value (8.0 Hz) clearly indicated β-orientation. The 3-hydroxy butirroyl moiety was identified by COSY spectrum where the correlations were apparent from the methyl signal to an oxymethine proton and from this latter to the diastereotopic methylene signals around 2 ppm.

The ROESY spectrum (Fig. 2) suggested the sequence of the tetrasaccharide repeating unit. Actually, H-1 **A** gave NOE effect with H-2 **C** while H-1 **C** gave NOE with H-2 **B**. On the other hand, the H-1 signal of residue **B** gave a dipolar coupling with the H-4 of **D** residue, whereas the H-1 of **D** residue gave NOE contact with the H-3 of residue **A**. All of these data suggested the sequence [→**A**→**C**→**B**→**D**→] that, together with methylation data and glycosylation shifts obtained by HSQC spectrum (Table 1), indicated the following repeating unit structure:

→3)-α-D-Fuc*N*p-(1→2)-β-D-Qui3N*p*-(1→2)α-D-Rha*p*-(1→4)-β-D-Gal*p*-(1→

A **C** **B** **D**

The location of the two acyl groups, acetyl and 3-hydroxy butirroyl, remained to be established. With this aim, a complete set of 2D NMR experiments was carried out in H_2O/D_2O 9:1. In this way, it was possible to observe and to assign the amide proton signals located on the two 2-deoxy-2-acylamido residues, FucN and Qui3N, and from these, by watergate-NOESY spectrum (Fig. 3), to detect the dipolar coupling of the amide proton signals with the proton signals of the acyl groups. In particular, the amide proton signal belonging to Qui3N residue gave NOE effect with the methylene proton signals of the butirroyl moiety and, on the other hand, the amide proton of the FucN residue gave NOE effect with the methyl acetyl group. These data indicated the presence of the acetyl group on FucN and butirroyl group on Qui3N.

In conclusion we have established the structure of the O-polysaccharide chain of the lipopolysaccharide from the Gram-negative bacterium *N. alkalitolerans* strain YIM 31775^{T} as the following:

→3)-α-D-Fuc*p*NAc-(1→2)-β-D-Qui*p*3NHBu-(1→2)α-D-Rha*p*-(1→4)-β-D-Gal*p*-(1→

A **C** **B** **D**

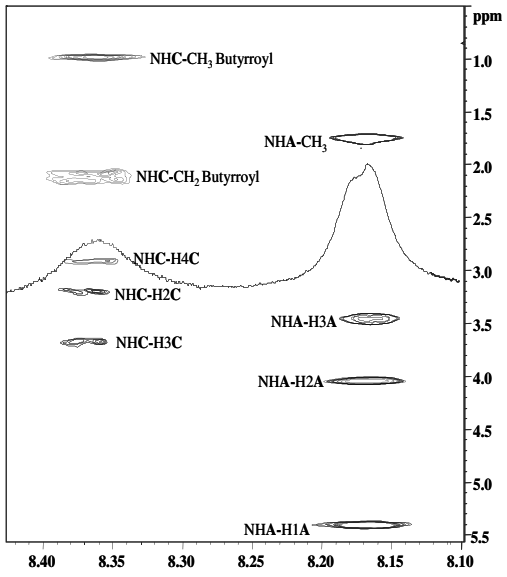

Figure 3. Section of the amide proton region of the 2D ROESY spectrum of the O-chain from *N. alkalitolerans*. ^{1}H NMR spectrum is overlapped, spectra were registered at 285 K. Annotations refer to diagnostic interresidual contacts.

1. Experimental

1.1. Growth of bacteria, isolation of LPS and OPS

The bacterium strain YIM 31775^{T} was cultured in a YIM 38 broth (malt extracts 10 g, yeast extracts 4 g, glucose 4 g, vitamin mixture, water 1000 mL, pH 7.2) for 36 h on a shaker.[1] The cells were collected by centrifugation, washed with saline and lyophilized (3 g). LPS were extracted from freeze-dried bacterial cell walls using the phenol–water method.[4] The phase partitioning was repeated twice and the water phase, containing LPS was lyophilized (100 mg of crude product). To further purify the LPS, RNAse (Roche), was added and the solution incubated at 37 °C for 2 h. The solution was vortexed and centrifuged at 12,000*g* for 15 min to yield 80 mg of LPS. In order to obtain the O-polysaccharide chain, an aliquot of LPS was hydrolyzed with aq 1% AcOH for 2 h at 100 °C and centrifuged (11,000 rpm, 4 °C, 1 h). The supernatant thus obtained (OPS fraction, 45 mg, 90% of LPS) was purified by gel permeation chromatography on a Sephacryll S100-HR column (90 cm × 1.5 cm) using 0.05 M ammonium bicarbonate as the eluent and monitored with a Waters differential refractometer.

1.2. NMR spectroscopy

1D and 2D ^{1}H NMR spectra were recorded on a solution of 3 mg in 0.6 mL of D_2O, at 298 K using a Bruker DRX-600 equipped with a cryogenic probe. The spectra were calibrated with internal acetone δ_H 2.225, δ_C 31.45. Rotating frame Overhauser enhancement spectroscopy (ROESY) was measured using the data sets ($t_1 \times t_2$) of 4096×256 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 the data sets of 4096×512 points, and 64 scans were acquired. Total correlation spectroscopy experiments (TOCSY) were performed with a spinlock time of 120 ms, using the data sets ($t_1 \times t_2$) of 4096×256 points, and 16 scans were acquired. In all homonuclear experiments, the data matrix was zero-filled in the *F*1 dimension to give a matrix of 4096×2048 points and was resolution enhanced in both dimensions by a shifted sine-bell function before Fourier transformation. Heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC) experiments were measured in the ^{1}H-detected mode with proton decoupling in the ^{13}C domain, using the data sets of 2048×256 points, and 100 scans were acquired for each t_1 value. The experiments were carried out in the phase-sensitive mode according to the method of States et al.[7] A 60 ms delay was used for the evolution of a long-range connectivity in the HMBC experiment. In all heteronuclear experiments the data matrix was extended to 2048×1024 points using the forward linear prediction extrapolation.[8]

In the experiments performed in H_2O, the spectra were recorded on a solution of 2 mg in 0.5 mL of H_2O/D_2O 9:1, at 285 K, the water signal was removed using watergate W5 pulse sequence with double echo as described.[9] Wg-nuclear Overhauser enhancement spectroscopy (NOESY) spectra were measured using the data sets ($t_1 \times t_2$) of 4096×256 points and with a mixing time of 200 ms, wg-total correlation spectroscopy experiments (TOCSY) were performed with a spinlock time of 100 ms, using the data sets ($t_1 \times t_2$) of 4096×256 points.

1.3. Gas chromatography

GC was performed on a Hewlett-Packard 5890 instrument, SPB-5 capillary column (0.25 mm × 30 m, Supelco), for compositional and methylation analyses the temperature program was 150 °C for 5 min, then 5 °C min^{-1} to 300 °C, for absolute configuration analysis was 150 °C for 8 min, then 2 °C min^{-1} to 200 °C for 0 min, then 6 °C min^{-1} to 260 °C for 5 min.[10]

1.3.1. Compositional and methylation analysis. The monosaccharides were identified as acetylated *O*-methyl glycosides derivatives: briefly, samples were methanolyzed with 2 M HCl/MeOH at 85 °C 20 h, dried under reduced pressure and then acetylated with acetic anhydride in pyridine at 80 °C for 30 m. After work-up, the sample was analyzed by GLC–MS. The absolute config-

uration of monosaccharides was determined by the GLC of acetylated glycosides of (+)-2-butanol according to the published method.[10] The absolute configuration of 3-hydroxy-butanoyl was determined by the GLC analysis of the trifluoroacetylated 2-octyl ester derivative and compared with authentic sample from *Pseudomonas fluorescens*. Qui3N and FucN were compared with authentic samples derived from *Pseudomonas cichorii*.[11] Methylation analysis was carried out with methyl iodide in dimethyl sulfoxide in the presence of sodium hydroxide.[12] The hydrolysis of the methylated O-polysaccharide was carried out with 2 M TFA (120 °C, 1 h) and the partially methylated monosaccharides, reduced with $NaBD_4$, were converted in alditol acetates with acetic anhydride in pyridine at 80 °C for 30 min and analyzed by GLC–MS.

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