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Structures of the O21 and O25 antigens of *Stenotrophomonas maltophilia*

Lesley Galbraith, Stephen G. Wilkinson *

Faculty of Science and the Environment, Department of Chemistry, University of Hull, Hull HU6 7RX, UK

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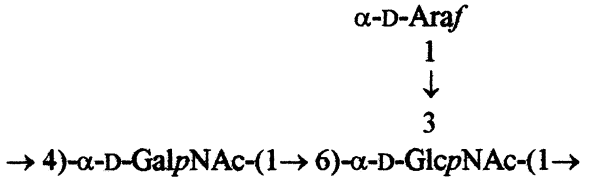
Abstract

The O-specific side-chain polymers from *Stenotrophomonas maltophilia* serogroups O21 and O25 were isolated from the lipopolysaccharides of the reference strains. The O21 polymer contained D-arabinose, 2-amino-2-deoxy-D-glucose and 2-amino-2-deoxy-D-galactose in equal proportions. Methylation analysis and NMR spectroscopy showed that the polysaccharide is based on a branched trisaccharide repeating unit of the structure shown below. The O25 polymer is linear with a disaccharide repeating unit identical to that forming the backbone of the O21 polymer.



Keywords: *Stenotrophomonas maltophilia*; Lipopolysaccharide; O-Specific polymer

1. Introduction

Stenotrophomonas maltophilia is an opportunist pathogen that has an increasing reputation as an agent of nosocomial infection [1,2]. Many strains display multidrug resistance [3,4] and the organism poses a potential threat to the cystic fibrosis population inter alia [5–8]. One method for the epidemiological monitoring of clinical isolates is serotyping of the heat-stable O-antigens [9]. Structural studies of several O-antigens have already been published ([10,11] and references cited therein). As part of an ongoing study of the remaining O-antigenic structures, we have characterised the O-specific side-chain polymer from the lipopolysaccharides (LPSs) of the reference strains for *S. maltophilia* serogroups O21 and O25.

2. Results and discussion

Isolation and composition of O-specific polymers.—LPS was extracted from the defatted cell walls of the reference strains by treatment with hot, aqueous phenol [12], followed by exhaustive dialysis and freeze-drying of the aqueous phase (yields: O21, 13%; O25, 8%). Minor amounts of phenol-soluble LPS were also isolated (yields: O21, 1%; O25, 0.4%).

* Corresponding author. Tel.: +44-1482-465-484; fax: +44-1482-466-410.

E-mail address: s.g.wilkinson@chem.hull.ac.uk (S.G. Wilkinson)

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The polymeric fractions were obtained by mild acid hydrolysis of the LPS, followed by GPC (Sephadex G-50) of the water-soluble products (yields from LPS: O21, 33%; O25, 7%). The relatively high yield (28%) of the oligomeric fraction from the hydrolysate of the O25 LPS showed that the strain produced mainly an R-type product. The main monosaccharide components were identified as D-Ara, D-GalN and D-GlcN (O21), and D-GalN and D-GlcN (O25). Minor components were also identified. The O21 polymer contained Rha, Glc and Xyl, the O25 polymer Rha, Gal, Glc and Man. A likely explanation for the Glc and Man in the O25 polymer is the presence of core material, as further studies of the oligomeric fraction showed that these were the major monosaccharide components.

Structure of the O25 polymer.—The NMR spectra clearly showed that the polymer was based on a disaccharide repeating unit of α-D-Gal*p*NAc and α-D-Glc*p*NAc residues.The ^{1}H spectrum included major signals for anomeric protons (each 1 H) with δ 4.99 ($J_{1,2}$ 3.2 Hz) and δ 4.94 ($J_{1,2}$ 3.4 Hz) and two *N*-acetyl signals with δ 2.08 and 2.06. The ^{13}C NMR spectrum contained 16 major signals, including those for anomeric carbons at δ 99.22 and 98.05, two carbons attached to nitrogen (δ 54.93 and 50.86), two acetyl groups (δ 175.40, 175.15, 22.87 and 22.70), and one unsubstituted hydroxymethyl carbon (δ 61.12). A signal at δ 66.18 was shown (HSQC) to be coupled to two protons (δ ~4.03 and ~3.65) and could therefore be attributed to a substituted hydroxymethyl carbon. For detailed assignment of the signals (Table 1) using 2D spectra (COSY, relayed COSY, TOCSY, NOESY, HMQC and HSQC) the residues were coded **A** and **B,** in order of decreasing chemical shift for the anomeric protons. Residue **A** was readily identified as α-D-Gal*p*NAc from the chemical shift for C-2 [13] and residue **B** as α-D-Glc*p*NAc from the large value (~10 Hz) for $J_{3,4}$ in the proton spectrum. The absence of downfield glycosylation shifts for C-3 and C-4 of **B**, for which δ values were readily established from correlations with the corresponding, well-resolved protons, indicated that the Glc*p*NAc was the 6-substituted residue. Further assignments for residue **A** were complicated by severe overlap of signals at ~4.04 ppm (total of 4 H, including **A** H-3). The attribution of the signal with δ 78.26 to **A** C-4, and that with δ 68.09 to **A** C-3, was based on the expected α and β effects of glycosylation at position 4 (predicted shifts ~78 and 68 ppm, respectively [14]). Glycosylation at position 3 of **A** should result in chemical shifts for C-3 and C-4 of ~74 and 66 ppm, respectively [14]. Thus the NMR evidence points to structure **1** as the repeating unit of the O25 polymer.

Table 1
NMR data [a] for O25 polymer

Atom		Residue	
		→4)-α-Gal*p*NAc-(1→ **A**	→6)-α-Glc*p*NAc-(1→ **B**
1	H	4.99	4.94
	C	98.05	99.22
2	H	4.27	3.93
	C	50.86	54.93
3	H	~4.04	3.86
	C	68.09	71.33
4	H	~4.04	3.62
	C	78.26	70.68
5	H	~4.03	4.28
	C	72.44	72.03
6	H	~3.77, ~3.73	~4.03, ~3.65
	C	61.12	66.18

[a] Values for chemical shifts relative to internal acetone (δ_H 2.22, δ_C 31.07) in spectra recorded at 40 °C. Additional signals due to *N*-acetyl groups at δ_C 175.40, 175.15, 22.87 and 22.70, δ_H 2.08 and 2.06.

A **B**

→4)-α-D-Gal*p*NAc-(1→6)-α-D-Glc*p*NAc-(1→

1

Methylation analysis of the O25 polymer supported this structure. Although the results were complicated by the presence of derivatives of Rha and hexose residues, and the relatively low response for the amino sugar derivatives, GLC–MS data were consistent with the presence in the polymer of 4-substituted and 6-substituted hexosamines, both as pyranose residues.

Structure of the O21 polymer.—Methylation analysis of the polymer, monitored by GLC–

Table 2
NMR data [a] for the O21 polymer

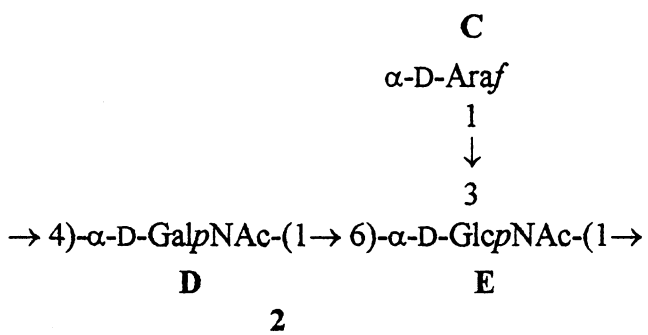
Atom		Residue		
		α-Araf-(1→ C	→ 4)-α-Galp-NAc-(1→ D	→3,6)-α-GlcpNAc-(1→ E
1	H	5.27	5.01	4.93
	C	108.69	97.00	98.40
2	H	4.13	4.28	4.02
	C	81.20	49.81	52.36
3	H	3.94	~4.06	3.94
	C	76.12	66.88	77.62
4	H	3.97	~4.06	3.77
	C	83.79	77.13	69.58
5	H	3.83, 3.73	~4.05	4.34
	C	60.92	71.36	70.78
6	H		~3.73	~4.05, 3.61
	C		59.93	64.79

[a] Values for chemical shifts relative to internal acetone (δ_H 2.22, δ_C 31.07) in spectra recorded at 40 °C. Additional signals due to *N*-acetyl groups at δ_C 174.46, 173.86, and 21.86 (2C), δ_H 2.10 and 2.06.

MS of the methylated alditol acetates (labelled at C-1 with deuterium), showed that it was constructed from a branched trisaccharide repeating unit. The products detected were derived from unsubstituted Araf, 4-substituted Hexp N (or 5-substituted Hexf N), and 3,6-disubstituted Hexp N residues. The retention time of the third product, derived from the branch-point residue, matched that of the product from 3,6-disubstituted GlcN [15]. NMR spectra confirmed that both hexosamines occurred as N-acetylated pyranoses and showed that all three sugars had the α configuration. Thus, the ^{1}H NMR spectrum included signals for three anomeric protons at δ 5.27 (unresolved), 5.01 ($J_{1,2}$ 3.6 Hz) and 4.93 ($J_{1,2}$ 3.0 Hz), and methyl singlets at δ 2.10 and 2.06. The ^{13}C spectrum included three signals for anomeric carbons at δ 108.69, 98.40 and 97.00, and signals from two acetamido groups [δ 174.46, 173.86, 52.36 and 49.81 and 21.86 (2C)]. For more detailed interpretation of the spectra (Table 2), the residues were coded **C**, **D** and **E**, in order of decreasing chemical shift for the anomeric protons.

Residue **C** was readily identified as α-Araf from the five prominent signals in the ^{13}C spectrum and by reference to literature data [16]. Assignments for the corresponding protons were made with the aid of COSY, TOCSY and HMQC spectra. Residue **D** was identified as α-Galp NAc from the chemical shift for C-2 [13] located via the COSY and HMQC spectra. The spin system could not be traced beyond H-3 because of signal congestion at about 4.05 ppm, as found for the corresponding residue **A** in the O25 polymer. The assignments made for C/H at positions 3 and 4 of **D** were based on the arguments used for the O25 polymer, and the evidence from methylation analysis of 4-substitution of the Galp NAc residue in both cases. **D** H-5 and H-6,6′ were correlated from the COSY spectrum and signals for the corresponding carbons identified from HMQC. Similar analyses led to assignments (Table 2) for the 3,6-disubstituted Glcp NAc residue **E**.

The NOESY spectrum showed the following contacts: (a) between **C** H-1 and signals at δ 4.13 (**C** H-2) and ~3.95 (possibly **E** H-3), (b) between **D** H-1 and signals at δ 4.28 (**D** H-2), 4.05 and 3.61 (**E** H-6,6′), (c) between **E** H-1 and signals at δ 4.02 (**E** H-2), 4.06 (presumably **D** H-4) and 3.73 (unassigned). These data point to structure **2** for the repeating unit of the O21 polymer. This was confirmed by selective removal of the Araf substituent by mild acid hydrolysis to give a linear polymer with NMR spectra (^{1}H and ^{13}C) essentially identical to those of the O25 polymer.



2

Neither the O21 nor the O25 serotype of *S. maltophilia* is frequently encountered [9,17]. Although the O antigens are structurally related, no cross-reaction between the serotypes has been reported [9], indicating that the lateral α-D-Araf substituent is part of the dominant epitope defining the O21 serotype. Aldopentoses are relatively unusual as compo-

nents of bacterial O antigens [18], but this feature is present in several antigens from S. *maltophilia*. In addition to the O21 antigen, α-D-Ara*f* occurs in O1 [15], β-D-Xyl*p* in O6 [19], β-L-Xyl*p* in O2 [10] and O10 [20], 3-*O*-Me-β-L-Xyl*p* in O8 [21], and α-D-Rib*f* in O16 (A.M. Winn, S.G. Wilkinson, unpublished results). It is also noteworthy that the O21 antigen of *S. maltophilia* differs from the O antigen of *Ralstonia* (*Pseudomonas*) *solanacearum* strain ICMP 4157 [22] only in the anomeric configuration and the location of the D-Ara*f* substituent.

3. Experimental

Growth of bacteria, isolation and fractionation of LPS.—*S. maltophilia* strains PM-41 (serotype O21) and PM-219 (serotype O25) [9] were grown in Nutrient Broth No. 2 (Oxoid, 20 L) for 21 h at 37 °C, with stirring at 300 rpm and aeration at 20 L min^{-1}. The wet cells (yields 98 and 168 g, respectively) were mechanically disintegrated, the cell walls were isolated (yields 2.8 and 9.3 g), defatted, and the LPS was extracted with hot aq phenol (45%, w/w, 68 °C, 15 min) as in earlier studies [12,23] (yields: 330 and 755 mg). Fractionation of the LPS was achieved by mild acid hydrolysis (aq 1% AcOH, 100 °C, 1.5 h) followed by GPC of the water-soluble products on Sephadex G-50 in pyridine–AcOH buffer (pH 5.4). The eluates were monitored for total carbohydrate by the phenol–H_2SO_4 method [24].

General methods.—NMR spectra (1D and 2D), for the O-specific side-chains and the polymeric product from partial hydrolysis (O21), in D_2O were recorded with a Jeol Lambda LA 400 MHz instrument. Spectra (^1H and ^{13}C) were recorded at 40 °C, with acetone (δ_H 2.22 and δ_C 31.07) as internal reference. Standard pulse sequences were used to record COSY, relayed COSY, HMQC, HSQC, NOESY and TOCSY spectra. GLC was carried out on a Perkin–Elmer Autosystem GLC, and GLC–MS on a Finnigan GCQ mass spectrometer.

Determination of monosaccharide composition.—In general, samples were hydrolysed with 2 M CF_3COOH at 98 °C for 16 h [25]. Products were analysed by PC (13:5:4 EtOAc–pyridine–water, and 5:5:1:3 EtOAc–pyridine–AcOH–water), HPAEC (Dionex DX300, CarboPac PA100 column, 0.16 M or 0.25 M NaOH), and by GLC of the alditol acetates. Absolute configurations were assigned by conversion into but-2-yl glycosides [26] followed by peracetylation and GLC, by enzymic assay of D-GalN [27], and by polarimetry of D-Ara (Optical Activity Ltd., AA-10 automatic polarimeter). Amino sugars in the partially hydrolysed O21 polymer (vide infra) were released by hydrolysis (6.1 M HCl, 105 °C, 4 h), and separated by elution from a Dowex 50 column (H^+ form) with 0.33 M HCl [28]. The eluate was monitored for amino sugars by the methods of Rondle and Morgan [29] and Lewandowski [30].

Chemical degradations.—Partial hydrolysis of the O21 polymer was carried out by treatment with 0.05 M H_2SO_4 (80 °C, 4 h). The hydrolysate was neutralised with $BaCO_3$, filtered then freeze-dried. The products were separated by preparative HPLC (Anachem, TSK gel G-Oligo-PW, water, 0.6 cm^3 min^{-1}). Standard procedures were used for methylation analysis [31–33]. The results were monitored using GLC and GLC–MS.

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References

[1] M. Denton, K.G. Kerr, *Clin. Microbiol. Rev.*, 11 (1998) 57–80.
[2] R. Gopalakrishnan, H.B. Hawley, J.S. Czachor, R.J. Markert, J.M. Bernstein, *Heart Lung*, 28 (1999) 134–141.
[3] S.R. Penzak, B.J. Abate, *Pharmacotherapy*, 17 (1997) 293–301.
[4] W.H. Traub, B. Leonhard, D. Bauer, *Chemotherapy*, 44 (1998) 293–304.

[5] F. Karpati, A.-S. Malmborg, H. Alfredsson, L. Hjelte, B. Strandvik, *Infection*, 22 (1994) 258–263.
[6] J. Wüst, R. Frei, H. Günthard, M. Altwegg, *Scand. J. Infect. Dis.*, 27 (1995) 499–502.
[7] S. Ballestero, I. Vírseda, H. Escobar, L. Suárez, F. Baquero, *Eur. J. Clin. Microbiol. Infect. Dis.*, 14 (1995) 728–729.
[8] M. Denton, *Rev. Med. Microbiol.*, 8 (1997) 15–19.
[9] B. Schable, D.L. Rhoden, R. Hugh, R.E. Weaver, N. Khardori, P.B. Smith, G.P. Bodey, R.L. Anderson, *J. Clin. Microbiol.*, 27 (1989) 1011–1014.
[10] A.M. Winn, S.G. Wilkinson, *Carbohydr. Res.*, 298 (1997) 213–217.
[11] A.M. Winn, S.G. Wilkinson, *FEMS Microbiol. Letts.*, 166 (1998) 57–61.
[12] O. Westphal, K. Jann, *Methods Carbohydr. Chem.*, 5 (1965) 83–89.
[13] P.-E. Jansson, L. Kenne, G. Widmalm, *Carbohydr. Res.*, 188 (1989) 169–191.
[14] A.S. Shashkov, G.M. Lipkind, Y.A. Knirel, N.K. Kochetkov, *Magn. Res. Chem.*, 26 (1988) 735–747.
[15] S.G. Wilkinson, L. Galbraith, W.J. Anderton, *Carbohydr. Res.*, 112 (1983) 241–252.
[16] K. Bock, C. Pedersen, *Adv. Carbohydr. Chem. Biochem.*, 41 (1984) 27–66.
[17] B. Schable, D.L. Rhoden, W.R. Jarvis, J.M. Miller, *Epidemiol. Infect.*, 108 (1992) 337–341.
[18] Y.A. Knirel, N.K. Kochetkov, *Biochemistry* (*Moscow*), 59 (1994) 1325–1383.
[19] A.M. Winn, S.G. Wilkinson, *Carbohydr. Res.*, 272 (1995) 225–230.
[20] A.M. Winn, A.W. Miller, S.G. Wilkinson, *Carbohydr. Res.*, 267 (1995) 127–133.
[21] D.J. Neal, S.G. Wilkinson, *Eur. J. Biochem.*, 128 (1982) 143–149.
[22] N.A. Kocharova, Y.A. Knirel, A.S. Shashkov, N.K. Kochetkov, L.D. Varbanets, *Carbohydr. Res.*, 228 (1992) 315–320.
[23] D. Oxley, S.G. Wilkinson, *Carbohydr. Res.*, 195 (1989) 111–115.
[24] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, *Anal. Chem.*, 28 (1956) 350–356.
[25] C.C. Yu Ip, V. Manam, R. Hepler, J.P. Hennessey, *Anal. Biochem.*, 201 (1992) 343–349.
[26] G. Gerwig, J.P. Kamerling, J.F. Vliegenthart, *Carbohydr. Res.*, 62 (1978) 349–357.
[27] J.M. Sempere, C. Gancedo, C. Asensio, *Anal. Biochem.*, 12 (1965) 509–515.
[28] M.J. Crumpton, *Biochem.J.*, 72 (1959) 479–485.
[29] C.J.M. Rondle, W.T.J. Morgan, *Biochem J.*, 61 (1955) 586–589.
[30] J. Lewandowski, *Anal. Biochem.*, 54 (1973) 325–332.
[31] B. Lindberg, J. Lönngren, *Methods Enzymol.*, 50C (1978) 3–33.
[32] L.R. Phillips, B.A. Fraser, *Carbohydr. Res.*, 90 (1981) 149–152.
[33] A.J. Mort, S. Parker, M.-S. Kuo, *Anal. Biochem.*, 133 (1983) 380–384.