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Structures of the O4 and O18 antigens of *Stenotrophomonas maltophilia*: a case of enantiomeric repeating units

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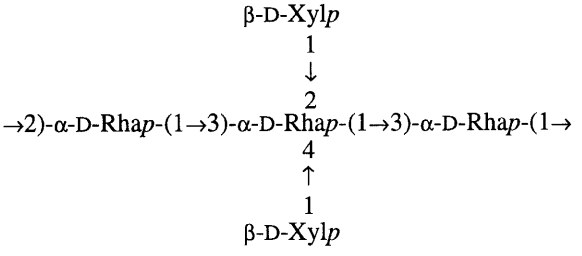
Abstract

The O-specific side-chain polymers of lipopolysaccharides from the reference strains for *Stenotrophomonas maltophilia* serogroups O4 and O18 are both xylosylated rhamnans. In the O4 polymer, both sugar components are the D isomers, whereas the O18 polymer contains only the L isomers. By means of NMR spectroscopy, methylation analysis and Smith degradation, the repeating unit of the O4 polymer was identified as a doubly-branched pentasaccharide of the structure shown below. The O18 polymer is based on the enantiomeric pentasaccharide, but the xylosyl substituent at the 4-position is apparently absent from some units. The polymers closely resemble the O antigens found in *Xanthomonas campestris* pathovars.



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Keywords: *Stenotrophomonas maltophilia*; Lipopolysaccharide; O antigens; *Xanthomonas*

1. Introduction

The genus *Stenotrophomonas* was created[1] to accommodate the organism originally known as *Pseudomonas maltophilia* and subsequently as *Xanthomonas maltophilia*.[2] Two additional species have been recently added to the genus.[3,4] In addition to continuing interest[5,6] in its genomic and phenotypic diversity, the species has potential for bioremediation and the biological control of plant pathogens,[6–8] and has emerged as a multidrug-resistant opportunistic pathogen responsible for nosocomial infections[9] and a particular threat to the cystic fibrosis population.[10,11]

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The phenotypic similarities between *S. maltophilia* and *Xanthomonas* spp. include unusual compositional and structural features of the respective lipopolysaccharides (LPS). Thus, as reviewed,[12] lipid A contains a distinctive range of branched-chain fatty acids, GalA is released during mild acid hydrolysis of the LPS, and the core oligosaccharide lacks heptose but includes phosphorylated D-Man. It is becoming apparent that the similarities extend to the O-specific antigens, which often contain Xyl and/or Rha (either enantiomer) in both *Xanthomonas*[13–17] and *S. maltophilia*.[18–24] During the course of a systematic study of the O antigens of *S. maltophilia* (Ref. 25 and references cited therein), we have characterised the O4 and O18 polymers as xylosylated rhamnans based on enantiomeric repeating-units.

2. Results and discussion

Isolation and composition of O-specific polymers.—LPS of the O4 and O18 reference strains of *S. maltophilia* were recovered from the aqueous phase (yields: O4, 29%; O18, 19%) following extraction of the defatted cell walls with hot, aqueous phenol. Mild acid hydrolysis of the LPS released water-soluble material (yields: O4, 62%; O18, 53%) from which the polymeric fractions were isolated by GPC on Sephadex G-50 (yields from LPS: O4, 50%; O18, 34%). The monosaccharide components of the O4 polymer were D-Rha and D-Xyl (molar ratio, 3.0:2.0), whereas those of the O18 polymer were L-Rha and L-Xyl (molar ratio, 3.0:1.6). Consistent with this difference, the specific rotations ($[\alpha]_D$) of the polymers were similar in value but opposite in sign: O4, $+40.1°$; O18, $-34.1°$.

Structure of the O4 polymer.—The presence of a regular structure based on a pentasaccharide repeating-unit was evident from the NMR spectra. The ^{1}H spectrum contained four major signals for anomeric protons, at δ 5.26, 5.16, 4.85 (each 1 H, unresolved) and 4.44 (2 H, $J_{1,2}$ 7.3 Hz) and three methyl doublets (Rha H-6) at δ 1.37 ($J_{5,6}$ 6.2 Hz), 1.30 ($J_{5,6}$ 6.2 Hz) and 1.28 ($J_{5,6}$ 6.2 Hz). Similarly, the ^{13}C spectrum (Fig. 1) contained 28 discrete signals, including signals for five anomeric carbons, at $\delta \sim 104.3$, 104.19, 103.26, 101.08 and 100.88, and three methyl carbons (Rha C-6) at δ 17.71,

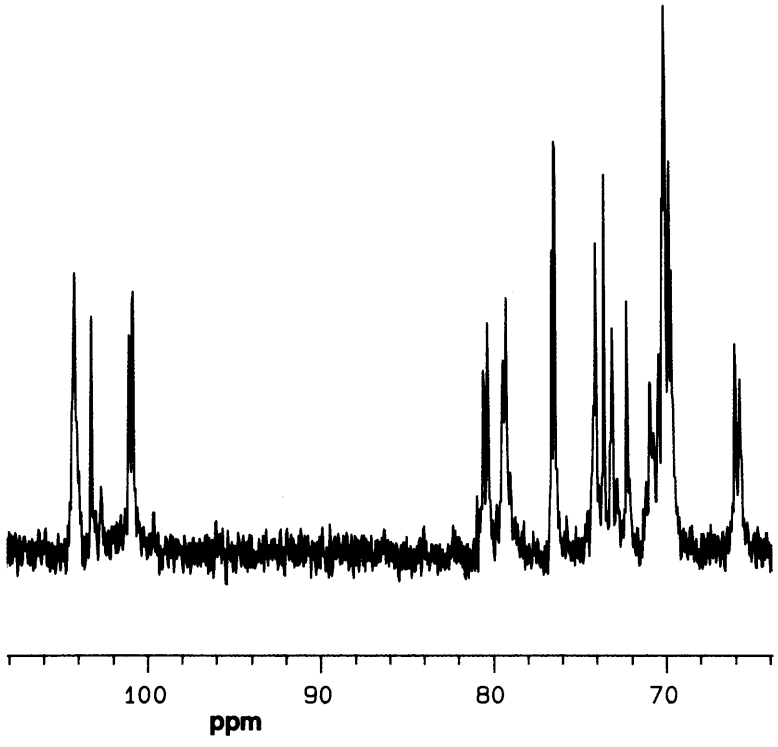


Fig. 1. ^{13}C NMR spectrum of the O4 polymer. The spectrum for the sample in D_2O was recorded at 150 MHz and 70 °C with acetone (δ_C 31.07) as internal reference. In addition to the signals shown, the spectrum contained those for three methyl carbons (δ 17.71, 17.56 and 17.42).

Table 1
NMR data [a] for the O4 polymer

Atom		Residue				
		→2)-α-Rha-(1→ **a**	→2,3,4)-α-Rha-(1→ **b**	→3)-α-Rha-(1→ **c**	β-Xyl-(1→ **d**	β-Xyl-(1→ **e**
1	H	5.26	5.16	4.85	4.44	4.44
	C	101.08	100.88	103.26	104.19	~104.3
2	H	4.02	4.16	4.15	3.43	3.32
	C	80.37	79.29	70.98	74.14	73.67
3	H	3.87	4.06	3.84	3.44	3.44
	C	70.43	~74.3	~79.5	76.67 [b]	76.50 [b]
4	H	3.50	3.72	3.57	3.62	3.62
	C	73.20	80.59	72.32	70.22 [b]	70.14 [b]
5	H	3.87	4.00	3.78	3.28, 3.98	3.28, 3.98
	C	~70.0	69.68	69.86	66.06 [b]	65.73 [b]
6	H	1.30	1.37	1.28		
	C	17.42 [b]	17.56 [b]	17.71 [e]		

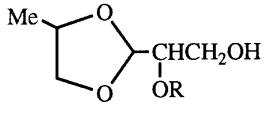
[a] Values for chemical shifts relative to internal acetone (δ_H 2.22, δ_C 31.07) in spectra recorded at 70 °C and 600 MHz (^{1}H).
[b] Sets of signals for which assignments may be interchanged.

17.56 and 17.42. The data are consistent with the presence in the repeating unit of two β-D-Xyl*p* residues and three D-Rha*p* residues, at least two having the α configuration.

Methylation analysis of the polymer, monitored by GLC and MS of the methylated alditol acetates, gave the derivatives from unsubstituted Xyl*p*, 2-substituted Rha*p*, 3-substituted Rha*p* and 2,3,4-trisubstituted Rha*p* residues. That both Xyl residues were lateral substituents of a linear rhamnan was shown by Smith degradation of the O4 polymer, to give two oligomeric products (SD1 and SD2) largely resolved by GPC on Sephadex G-15. The major product (SD2) was assigned the partial structure **1**, based on its ^{1}H NMR spectrum (two anomeric protons giving unresolved signals at δ 5.05 and 4.96), the results of methylation analysis, and MS (EI and FAB) of the permethylated compound.[24] The minor product (SD1) was shown by EI- and FABMS[24] to be the dirhamnosyl derivative of a 1,3-dioxolane (**2**), formed by transacetalation during the hydrolytic step of the degradation.[17,22,24]

D-Rha*p*-(1→3)-D-Rha*p*-(1→2)-Gro

1



2 R = D-Rha*p*-(1→3)-D-Rha*p*-(1→

The remaining structural features were established by detailed interpretation of the NMR spectra for the polymer, assisted by 2D correlations (COSY, relayed COSY, NOESY and HMQC). For this purpose, the residues were coded **a**–**e** (Table 1), in order of decreasing chemical shift for the anomeric protons. Spin systems for the three Rha residues were readily traced in the COSY spectrum (Fig. 2) and each could be assigned the α configuration from the chemical shift for H-5[26] and the intra-residue contact between H-1 and H-2 in the NOESY spectrum. Positions of substitution were clear from the downfield shifts of ^{13}C signals (Fig. 3) compared with those for the free monosaccharide.[27] ^{1}H and ^{13}C NMR signals for the two Xyl residues (**d** and **e**) were identical or very similar, and are entirely consistent with the literature for terminal β-Xyl*p*.[28] The linear trisaccharide repeating-unit of structure **3** was defined by the following inter-residue contacts in the NOESY spectrum: **a** H-1 with **b** H-3; **b** H-1 with **c** H-3; **c** H-1 with **a** H-2 (and H-1). The contacts of H-1 of **d** and **e** with H-2 and H-4 of residue **b** confirmed the location of the xylosyl substituents and structure **4** for the repeating unit of the O4 polymer.

→2)-α-D-Rha*p*-(1→3)-α-D-Rha*p*-(1→3)-α-D-Rha*p*-(1→

3

β-D-Xyl*p*
1
↓
2
→2)-α-D-Rha*p*-(1→3)-α-D-Rha*p*-(1→3)-α-D-Rha*p*-(1→
4
↑
1
β-D-Xyl*p*

4

Structure of the O18 polymer.—Although the O18 polymer contained Rha and Xyl in about the same proportions (~ 3:2) as the O4 antigen, both sugars in the former case had the L configuration (in contrast to D for O4), and the ^{1}H NMR spectrum of the polymer provided evidence of heterogeneity which was not removed by further GPC on Bio-Gel P100. The anomeric region was complex and contained at least six significant signals of varied intensity. Four of them, with δ 5.26 (~ 0.4 H, **a**′), 5.16 (~ 1 H, **b**′), 4.85 (~ 0.5 H, **c**′) and 4.44 (d, $J_{1,2}$ ~ 7 Hz, ~ 1.4 H, **d**′/**e**′), matched those for the anomeric protons in the O4 polymer; additional unresolved signals (also α-Rha H-1) were present at δ 5.07 (~ 0.2 H) and 4.97 (~ 0.5 H). The Rha:Xyl ratio calculated from the summed, integrated anomeric signals was 3.0:1.6, in agreement with the value obtained by direct analysis. Despite the complexity of the spectrum, it was possible by 2D NMR (COSY, relayed COSY, TOCSY) to track the spin systems for each of the major anomeric signals **a**′ to **d**′/**e**′ and thereby confirm the presence in the O18 product of residues enantiomeric with those in the O4 polymer. Also, the NOESY spectrum confirmed that the backbone of the O18 polymer was an L-rhamnan based on a trisaccharide repeating-unit with the sequence of residues →**a**′→**b**′→**c**′→ glycosidically linked as in the O4 polymer (structure **3**). This was confirmed by a Smith degradation, which gave products analogous to **1** and **2** from the O4 polymer.

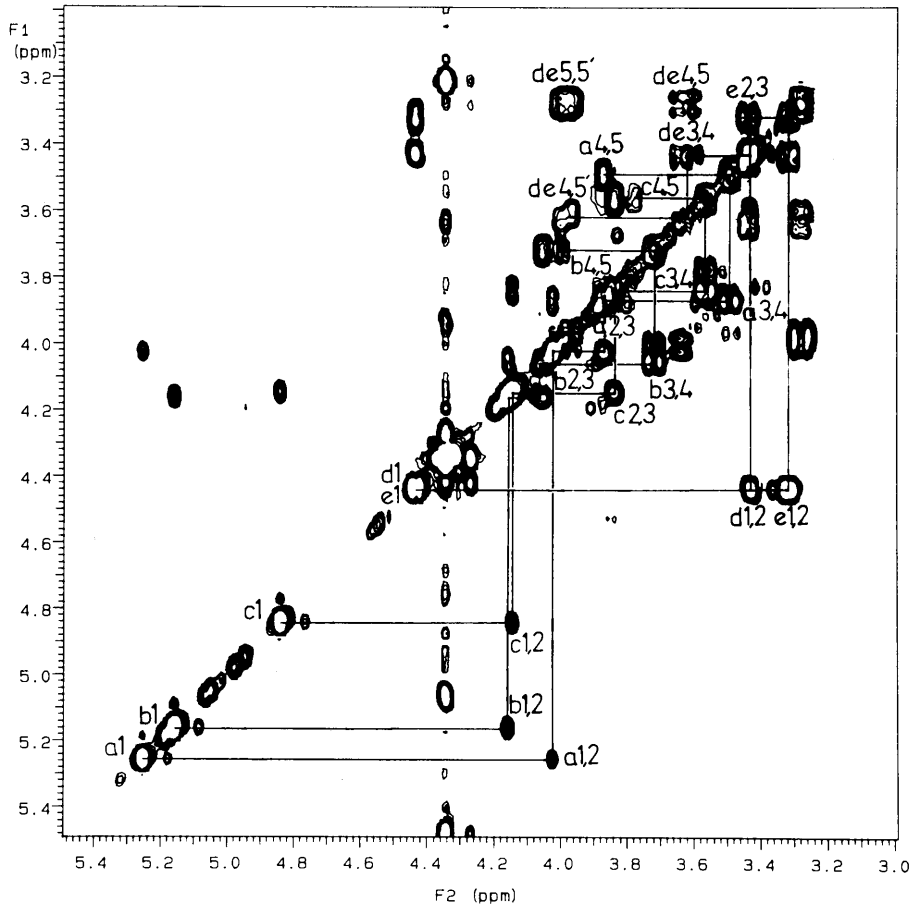


Fig. 2. COSY spectrum of the O4 polymer. Signals derived from the methyl groups are omitted.

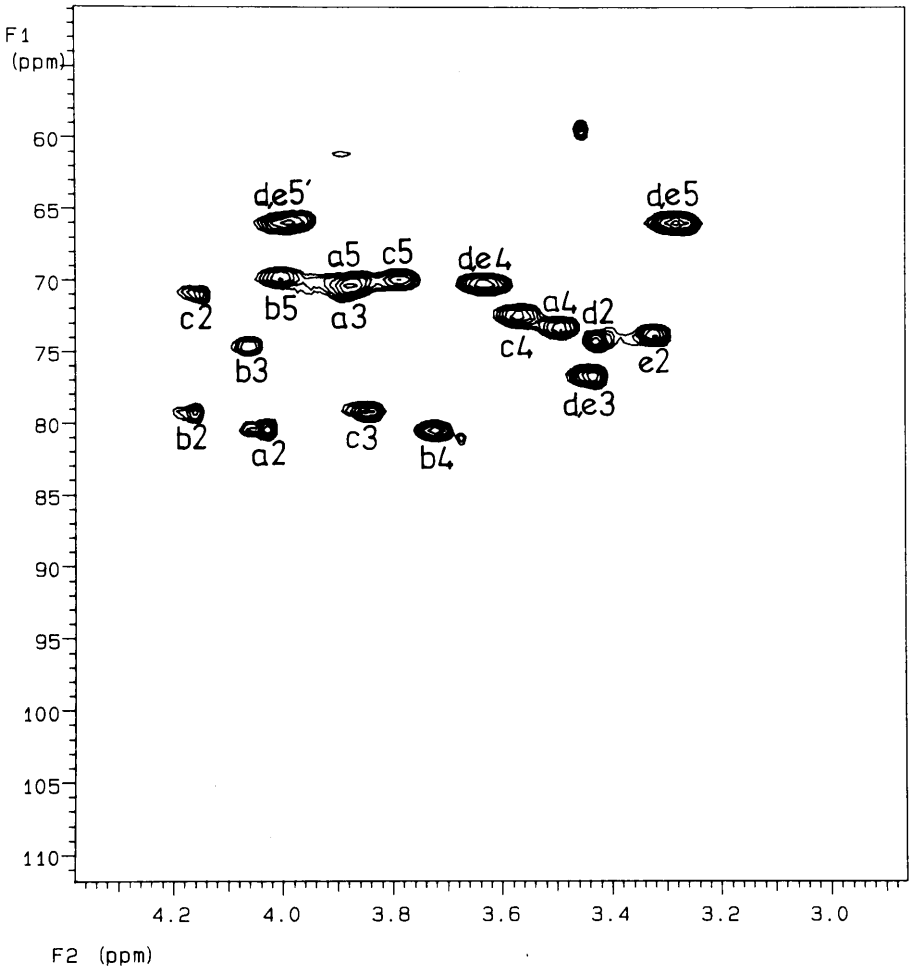


Fig. 3. HMQC spectrum of the O4 polymer. Correlations for anomeric and methyl signals are omitted.

Methylation analysis gave five significant products, the four described for the O4 polymer plus that derived from 2,3-disubstituted Rha*p*. Thus, possible explanations for the heterogeneity of the O18 polymer include (a) absence of the β-L-xylosyl substituent from the 4-position of the Rha residue at the branch point in some of the repeating units; (b) additional xylosylation of either of the monosubstituted Rha residues (**a**′, **c**′); (c) contamination by another Rha-rich polymer. Explanation (a), but not (b), is consistent with the higher Rha:Xyl ratio for the O18 polymer compared with the O4 product, though the unequal sizes of the anomeric signals designated **a**′, **b**′ and **c**′ require a more complex assignment. Whatever the precise details of the structural basis for the heterogeneity, it remains clear that the repeating unit **5** forms a major part of the O18 polymer.

Comparison with other O antigens.—This study brings the number of serogroups of *S. maltophilia* to 15 for which the structure of the O antigen has been published. Of the type strains for the 16 remaining defined serogroups, one (O15) produces an R-type LPS and should be excluded from the scheme, while O5 produces the same polymer as O2, and in both cases 4-*O*-methylmannose is a minor component not previously[23] detected (Galbraith, L.; Winn, A. M.; Wilkinson, S. G. unpublished results). The present study again highlights the occurrence of both enantiomers of Rha and Xyl in O antigens from strains of *S. maltophilia*. Linear D-rhamnans based on the repeating unit **3** constitute the LPS-associated common antigen of *Pseudomonas aeruginosa*[29–31] and are found as O antigens in some pathovars of *Pseudomonas syringae*[32,33] and a clinical isolate of *Burkholderia cepacia*[34] as well as *S. maltophilia* O7.[24] The same trisac

charide also forms the backbone or a structural element of the O antigens of diverse Gram-negative bacteria.[35,36] In particular, structure **4** for the O4 antigen of *S. maltophilia* is identical with that for an O antigen of *Alcaligenes faecalis*.[37] Similar L-rhamnans carrying one or two xylosyl substituents (D or L) have been described recently for O antigens from pathovars of *Xanthomonas campestris*,[15–17] and the monoxyosylated repeating unit which is a minor component in *X. campestris* pv. *begoniae* GSPB 525 may be present in the O18 antigen of *S. maltophilia*. Xylose-substituted polymers rich in Rha also occur in some strains of another phytopathogen, *Burkholderia solanacearum*.[35] As LPS is implicated in the virulence and host-specificity of phytopathogens,[38] it is intriguing that closely-related xylorhamnans occur as O antigens in *S. maltophilia* as well as xanthomonads, when lack of phytopathogenicity of the former organisms is one of the differences which led to creation of the genus *Stenotrophomonas*.[1]

3. Experimental

Growth of bacteria, isolation and fractionation of the LPS.—*S. maltophilia* strains 363-4 (serotype O4) and 788-3 (serotype O18) were grown in Nutrient Broth no. 2 (Oxoid, 20 L) at 37 °C for 16 h, with stirring at 300 rpm and aeration at 20 L/min. The cells (wet weights, 158 and 109 g, respectively) were disintegrated mechanically, and the purified cell walls (dry weights, 8.0 and 7.0 g, respectively) were treated with 2:1 $CHCl_3$–MeOH at rt for 2 h. LPS (yields, 2.30 and 1.30 g, respectively) was extracted from the defatted cell walls by using hot, aq phenol as in previous studies[20–25] and was recovered from the aqueous phase. Treatment of the LPS with aq 1% HOAc at 100 °C for 1.5 h (O4) or 2.5 h (O18) released the insoluble lipid A, and fractionation of the water-soluble products by GPC on Sephadex G-50 gave the polymeric O antigens.

General methods.—Mixtures of monosaccharides were separated by HPAEC on a CarboPac PA100 column (Dionex) eluted with 16 mM NaOH. Alditol acetates, methylated alditol acetates and (−)-but-2-yl glycoside acetates were resolved by GLC using a fused-silica capillary column (BP1 or BP10; 25 m) in a Carlo Erba Mega 5160 instrument. GLC–MS was carried out with a BP10 column fitted to a Finnigan 1020B instrument. MS (EI and FAB) was also carried out at the EPSRC Mass Spectrometry Service Centre (University of Wales, Swansea) with *m*-nitrobenzyl alcohol as the matrix (FAB).

NMR spectra for samples in D_2O were obtained at 70 °C and referenced to internal acetone (δ_H 2.22, δ_C 31.07). ^1H NMR data for the Smith degradation products were recorded with a JEOL JNM-GX270 spectrometer. Other 1D spectra (^1H and ^{13}C) and all 2D spectra (COSY, relayed COSY, TOCSY, NOESY and HMQC) were obtained at the University of Edinburgh with a Varian DXR600S spectrometer. An AA-10 automatic polarimeter (Optical Activity Ltd.) was used to measure optical rotations for aqueous solutions at 24 °C.

Analysis of monosaccharide composition.—Sugars were released by treatment of polymers with 2 M CF_3CO_2H at 98 °C for 16 h.[39] Monosaccharides were identified by HPAEC of the free sugars, and by GLC of the alditol acetates. The configuration was established by GLC of the acetylated (−)-but-2-yl glycosides.[40]

Structural methods.—Methylation analyses, monitored by GLC and GLC–MS of the methylated alditol acetates, were carried out by standard procedures.[41–43] Smith degradation of polymer samples (50 mg) involved treatment with 50 mM $NaIO_4$ (20 mL) at 4 °C for 5 days. Following the addition of ethane–1,2-diol, reduction ($NaBH_4$) and dialysis, the product was hydrolysed with 1 M CF_3CO_2H at rt and again reduced ($NaBH_4$). Fractionation by GPC on Sephadex G-15 gave the oligomeric products SD1 and SD2.

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