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Structure of the O20 antigen of *Stenotrophomonas* (*Xanthomonas* or *Pseudomonas*) *maltophilia*

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Abstract

The O-antigen polymer recovered from the reference strain for *Stenotrophomonas* (*Xanthomonas* or *Pseudomonas*) *maltophilia* serogroup O20, by mild acid hydrolysis of the lipopolysaccharide, was found to contain D-rhamnose and D-mannose. By means of chemical degradations and NMR studies, the repeating-unit of the polymer was deduced to be a linear tetrasaccharide with the structure shown.

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

Keywords: *Stenotrophomonas maltophilia*; Lipopolysaccharide; O-Antigen

1. Introduction

The organism once known as *Pseudomonas maltophilia* was assigned to the genus *Xanthomonas* [1] following a detailed taxonomic analysis. However, the correct position of the species remained a matter of dispute [2] because of similarities to organisms in both genera. Re-examination of the problem [3] highlighted fundamental differences and resulted in the creation of a new genus, *Stenotrophomonas*, which contains the single species *Stenotrophomonas maltophilia*.

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The reputation of *S. maltophilia* as an opportunistic pathogen causing nosocomial infections [4,5] has grown, partly because of the organism's resistance to the majority of commonly used antibiotics [6–8]. This situation led to the development of a scheme for typing clinical isolates by their heat-stable O-antigens [9] to aid epidemiological monitoring of the organism. Structural studies of the repeating-units from the antigens in seven strains [10–16] have revealed the presence of regular, branched polymers constructed from a variety of monosaccharides. We now report the structure of the repeating-unit in the O-antigen polymer from the reference strain for serogroup O20.

2. Results and discussion

The lipopolysaccharide (LPS) of *S. maltophilia* strain 143 (serogroup O20) [9] was isolated from the aqueous phase (yield, 12%) after aqueous phenol treatment of the defatted cell walls. The water-soluble material (yield, 61%) released by mild acid hydrolysis of the LPS (aq 1% AcOH, 100 °C, 1.5 h) was fractionated by chromatography on Sephadex G-50. The monosaccharide composition of the resulting polymeric fraction (the putative O20 antigen; yield, 50%) was determined to be D-rhamnose and D-mannose (molar ratio, 3:1).

The presence of a regular structure based on a tetrasaccharide repeating-unit was evident from NMR analysis of the polymer. The ^{1}H NMR spectrum contained four signals (each 1 H) in the region for anomeric protons at δ 5.23, 5.11, 5.08, and 4.74 (all unresolved), and three doublets (Rha H-6) at δ 1.33 (3 H, $J_{5,6}$ 6.0 Hz) and 1.27 (6 H, $J_{5,6}$ 6.2 Hz). The chemical shifts for the anomeric protons suggested the presence of three α-linked and one β-linked pyranoid residues. This was confirmed by the ^{13}C NMR spectrum (Fig. 1), which contained 23 discrete signals (one at δ 17.49 with double intensity), including those for four anomeric carbons at δ 101.55 ($^{1}J_{CH}$ 174 Hz), 101.42 ($^{1}J_{CH}$ 172 Hz), 100.61 ($^{1}J_{CH}$ 170 Hz), and 99.21 ($^{1}J_{CH}$ 159 Hz), one for an unsubsti-

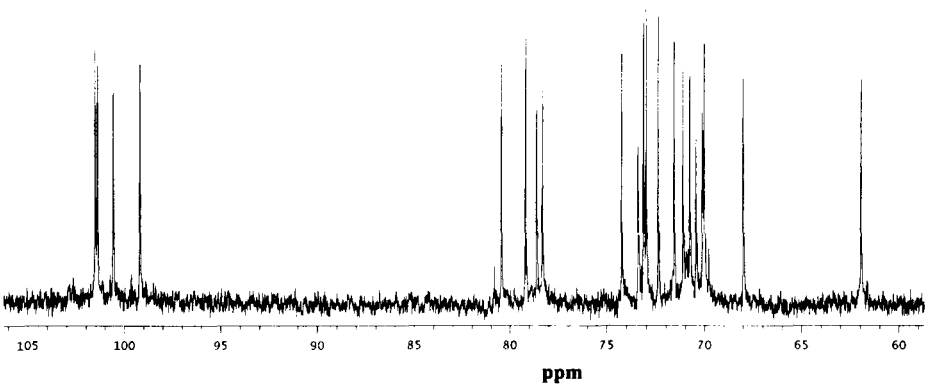


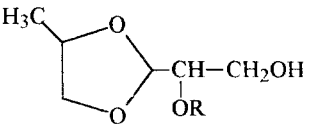
Fig. 1. ^{13}C NMR spectrum of the O20 polymer. The spectrum for the sample in D_2O was recorded at 150 MHz and 70 °C with acetone (δ_C 31.07) as internal reference. In addition to the signals shown, the spectrum contained those for three methyl carbons [δ 17.49 (2 C) and 17.42].

tuted hydroxymethyl carbon (δ 61.95), and those for three methyl groups [Rha C-6, δ 17.49 (2 C) and 17.42].

Three products were obtained by methylation analysis of the polymer (relative peak areas on GLC of the methylated alditol acetates, 1.5:0.7:1.0). These were identified by GLC and MS as the derivatives from 2-substituted Rha*p*, 3-substituted Rha*p*, and 2-substituted Man*p* residues, respectively. Smith degradation of the polymer, followed by reduction of the products ($NaBH_4$), resulted in oxidation of the 2-substituted residues and the isolation of a mixture (SD) of two oligomeric products. The major product was a rhamnosylglycerol, as expected from the classical degradative pathway.

On FABMS of the permethylated compound, pseudomolecular ions with *m/z* 331 (M + Na) and 347 (M + K) were observed, while on EIMS the derivative gave the expected fragment ions [17], including those with *m/z* 189 [aA_1], 157 [aA_2], 125 [aA_3], 103 [bA_1], and 163 [abJ_1]. The minor product from the Smith degradation was apparently the rhamnosyl derivative of a substituted 1,3-dioxolane (**1**, R = Rha*p*), formed by transacetalation during the hydrolytic step of the degradation [18]. The per-*O*-methyl derivative gave the expected pseudomolecular ions with *m/z* 373 (M + Na) and 389 (M + K) on FABMS, and on EIMS produced the [aA] series of ions from terminal Rha*p* and the ions with *m/z* 145 [bA_1], and 205 [abJ_1] from the 1,3-dioxolane unit.

The ^1H NMR spectrum of the mixed oligomeric products (SD) contained only one major signal at δ 4.77 (unresolved) in the region for anomeric protons and a methyl doublet (Rha H-6) at δ 1.32. The chemical shift for the anomeric proton of the surviving rhamnosyl residue (derived from that originally 3-substituted) indicated that it had the β configuration, and hence that all the 2-substituted residues in the parent polymer were α-linked. Also, the formation of **1** during the Smith degradation showed that the repeating-unit of the O20 antigen contained the disaccharide unit of structure **2**.



1

→3)-β-D-Rha*p*-(1→2)-α-D-Rha*p*-(1→

2

The remaining structural details for the repeating-unit were determined by further analysis of the NMR data for the native polymer. In order to assist discussion of the data, the four monosaccharide residues were labelled **a**–**d** (Table 1), in order of decreasing chemical shift for the anomeric protons. Signals for other protons were assigned with the aid of COSY and relayed COSY spectra, and were correlated with the signals for corresponding carbons by means of an HMQC spectrum (Fig. 2). The β-linked residue **d** was confirmed as the 3-substituted Rha*p* by the high-field location (δ 3.43) of the signal for H-5 [19] and the major downfield glycosylation shift for C-3, when compared with the corresponding carbon in free β-Rha*p* [20]. Residue **a** was identified as the 2-substituted α-Man*p* from the assignments for H-4 (δ 3.70) and C-4

Table 1
NMR data [a] for the O20 polymer

Atom		Residue			
		→ 2)-α-Man-(1 →	→ 2)-α-Rha-(1 →	→ 2)-α-Rha-(1 →	→ 3)-β-Rha-(1 →
		a	**b**	**c**	**d**
1	H	5.23	5.11	5.08	4.74
	C	101.55	101.42	100.61	99.21
2	H	4.07	4.08	4.22	4.14
	C	78.63	79.20	78.31	71.56
3	H	4.00	3.89	3.79	3.68
	C	71.10	70.74	70.42	80.46
4	H	3.70	3.46	3.46	3.52
	C	67.97	73.12 [b]	73.39 [b]	72.38
5	H	3.76	3.73	3.73	3.43
	C	74.24	70.01 [c]	70.09 [c]	72.99
6	H	~ 3.76, 3.89	1.27	1.27	1.33
	C	61.95	17.49	17.49	17.42

[a] Values for chemical shifts relative to acetone (δ_H 2.22, δ_C 31.07).
[b,c] Pairs of signals for which the assignments are tentative and may, therefore, be transposed.

(δ 67.97), which could not correspond to Rha*p* [20]. The proton spin system for residue **a** could not be traced beyond H-5 because of the superposition of signals in the COSY spectrum. The remaining assignments were established by the location of C-6 (δ 61.95)

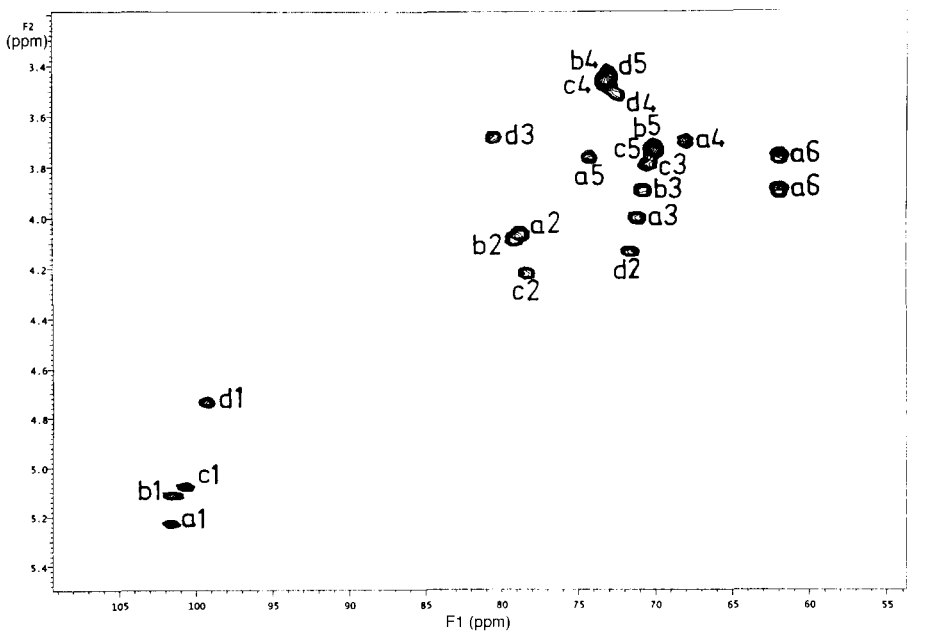


Fig. 2. HMQC spectrum of the O20 polymer. Signals derived from the methyl groups are omitted.

and correlations with H-6a and H-6b (δ 3.76 and 3.89) in the HMQC spectrum. The respective assignments given in Table 1 for C-4 and C-5 in residues **b** and **c**, both 2-substituted α-Rha*p*, are only tentative because of overlap of the relevant cross-peaks in the HMQC spectrum, but the α configuration for both residues is supported by the chemical shifts for H-5 and C-5 [19,20]. For all three 2-substituted residues, the position of substitution was clear from the glycosylation effects at C-2 [20].

The overall sequence of monosaccharide residues in the repeating-unit was determined from a NOESY spectrum. Although detailed interpretation of the NOE effects was complicated by the proximity of signals for H-2 of **a** and **b**, there was a clear inter-residue contact between H-1 of **a** and H-3 of **d**, as well as the expected intra-residue contact with H-2 of **a**. The inference of the disaccharide unit **a** → **d** leads immediately to structure **3** for the repeating-unit in the O20 antigen. NOE responses for H-1 of residues **b** and **c** did not provide conclusive structural information, but H-1 of **d** gave contacts with both H-1 and H-2 of **c** (consistent with substitution of position 2 of D-Rha*p* by β-D-Rha*p* [21]) as well as the intra-residue contacts with H-2, H-3, and H-5. The sequence of residues in the polymer is therefore **a** → **d** → **c** → **b**.

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

3

The repeating-unit of the O20 antigen in *S. maltophilia* differs from others of this species [10–16] in its linear architecture. In contrast to some other O-antigens containing rhamnose [10,12–14], the O20 polymer contains the relatively uncommon D isomer. This isomer is also present in the antigen of strain 555 (O12/O27) [16]. The occurrence of both isomers of a monosaccharide in O-antigens of the same species is rather unusual, but previous examples [22] of D- and L-rhamnose include O-antigens of *Burkholderia* (*Pseudomonas*) *cepacia*, *Pseudomonas syringae*, *Ralstonia* (*Burkholderia* or *Pseudomonas*) *solanacearum*, and *Xanthomonas campestris*.

3. Experimental

Growth of bacteria, and isolation and fractionation of the LPS.—Strain 143 of *S. maltophilia*, the O20 reference strain [9], was grown in Nutrient Broth No. 2 (Oxoid, 20 L) at 37 °C for 24 h with aeration at 20 L min^{-1} and stirring at 300 rpm. The cells (wet weight, 188 g) were disintegrated mechanically, and purified by exhaustive washings and enzymatic treatments [10]. The cell walls (dry weight, 11.2 g) were then treated with 2:1 $CHCl_3$–MeOH at room temperature for 2 h. LPS (yield, 1.34 g) was extracted from the defatted cell walls by the hot, aqueous phenol method as in previous studies [10–15]. Treatment of the LPS with aq 1% AcOH at 100 °C for 1.5 h released insoluble lipid A. Fractionation of the water-soluble products on Sephadex G-50 provided the polymeric material.

General methods.—The solvent system used for PC was 13:5:4 EtOAc–pyridine–water. Mixtures of free monosaccharides were separated by high-pH anion-exchange

chromatography (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) in a Carlo Erba Mega 5160 chromatograph. GLC–MS was carried out on a BP10 column fitted to a Finnigan 1020B instrument. FABMS was carried out at the EPSRC Mass Spectrometry Service Centre (University of Wales, Swansea) with *m*-nitrobenzyl alcohol as the matrix.

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 oligomeric fraction SD were recorded with a JEOL JNM-GX270 spectrometer. The ^1H NMR spectra (1D, COSY, relayed COSY, and NOESY), the ^{13}C spectrum (1D), and HMQC spectra (with and without proton coupling) for the native O20 polymer were obtained at the University of Edinburgh with a Varian DXR600 spectrometer.

Analysis of monosaccharide composition.—Sugars were released by treatment of the polymer with 2 M trifluoroacetic acid at 98 °C for 16 h [23]. Monosaccharides were identified by PC and HPAEC, and by GLC of the alditol acetates. The D configuration for both mannose and rhamnose was established by GLC of the acetylated (−)-but-2-yl glycosides [24].

Structural methods.—Methylation analyses, monitored by GLC and GLC–MS of the methylated alditol acetates, were carried out by standard procedures [25–27]. Smith degradation of the polysaccharide (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 trifluoroacetic acid at room temperature overnight and again reduced ($NaBH_4$). Fractionation on Sephadex G-15 gave the oligomeric product SD (8 mg).

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