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Structure of the O3 antigen of *Stenotrophomonas* (*Xanthomonas* or *Pseudomonas*) *maltophilia*

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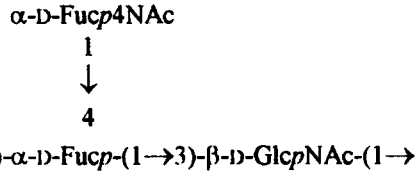
Abstract

The O antigen isolated from the lipopolysaccharide of a strain of *Stenotrophomonas* (*Xanthomonas* or *Pseudomonas*) *maltophilia* serogroup O3 was found to contain 4-acetamido-4,6-dideoxy-D-galactose, D-fucose, and *N*-acetyl-D-glucosamine. By means of chemical degradations and NMR spectroscopy the repeating unit of the O-specific polymer was determined to be a branched trisaccharide repeating-unit of the structure shown.



Keywords: *Stenotrophomonas maltophilia*; Lipopolysaccharide; O Antigen

1. Introduction

As a result of extensive taxonomic studies, the organism originally named as *Pseudomonas maltophilia* was transferred to the genus *Xanthomonas* as *Xanthomonas maltophilia* [1]. This decision did not gain universal approval [2], and the correct position of the species remained unclear because of similarities to both genera. A subsequent review of the properties of the genus *Xanthomonas* and the species

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Xanthomonas maltophilia [3] led to the proposal of a new generic name, *Stenotrophomonas*, to include a single species, *S. maltophilia*. *S. maltophilia* is an increasingly important opportunist pathogen, being frequently isolated from a variety of clinical sources [4–6], and notably resistant to a wide range of antibiotics [7–9]. A scheme for typing clinical isolates by their heat-stable (lipopolysaccharide) O antigens [10] was developed to aid epidemiological monitoring of the organism. Structures of the repeating units in the O antigen polymers have so far been reported for six strains [11–16]. The repeating units are all branched and contain between three and five sugars, including relatively uncommon ones such as D-arabinose, D-fucose, 6-deoxy-L-talose, L-xylose, 3-acetamido-3,6-dideoxy-D-galactose, and 4-acetamido-4,6-dideoxy-D-mannose. We now report the structure of the O3 antigen, the most common example in the typing study, accounting for 21% of the isolates [10].

2. Results and discussion

The lipopolysaccharide (LPS) of *S. maltophilia* strain 56 (serogroup O3) was extracted as a water-soluble product (yield, 10%) by aqueous phenol treatment of the defatted cell walls. Various acid treatments of the LPS at 100 °C were used in attempts to release lipid A. No visible reaction was apparent when 1% AcOH was used, while the suspension turned black with 0.25 M trifluoroacetic acid. However, a similar treatment of the LPS with 10% AcOH gave a white precipitate, which was removed by centrifugation. The water-soluble material produced (yield, 54%) was fractionated on Sephadex G-50 to give a polymeric fraction (overall yield, ca. 40%).

Preliminary analyses identified two of the major monosaccharide components of the polymer as D-fucose (Fuc) and 2-acetamido-2-deoxy-D-glucose (GlcNAc); trace amounts of rhamnose and glucose were also detected. Blackening of acid hydrolysates of LPS and of the polymeric fraction indicated the presence and resulting destruction of an unstable 4-amino sugar. In an attempt to protect this component, the polymer was treated with 10 M HCl at 80 °C for 30 min [16]. High-voltage paper electrophoresis (HVE) of the hydrolysate at pH 5.3 revealed an additional basic component with m_{GlcN} ca. 1.1, which reduced $AgNO_3$, gave a yellow spot with ninhydrin, a pink spot with the Ehrlich reagent, and a fluorescent yellow–brown spot with heat alone. These reactions are characteristic of 4-amino sugars [17]. GLC-MS of the aminodeoxyalditol acetate (primary fragment ions with m/z 288 and 158, inter alia) indicated that the sugar was a 4-amino-4,6-dideoxyhexose. The *galacto* configuration was apparent from the ^{1}H NMR spectrum of the *N*-acetyl derivative (Fuc4NAc), obtained by *N*-acetylation of the amino sugars in the hydrolysate, and separation from the Fuc and GlcNAc by means of HPLC on HPX-87P. Assignments for the signals were made by inspection and selective decoupling. The ^{1}H NMR data obtained (Table 1) were in good overall agreement with those reported by Knirel et al. [18], except for the chemical shift of H-2 in α-Fuc*p*4NAc (3.72 ppm in Table 1; 3.90 ppm in ref. [18]). The ^{13}C NMR signals (Table 2) could also be matched with those reported [18]. The D configuration was assigned to the Fuc4NAc from its positive optical rotation [18].

NMR analysis of the polymer established the presence of a regular trisaccharide

Table 1
^{1}H NMR data [a] (δ in ppm, J in Hz) for 4-acetamido-4,6-dideoxygalactose (α:β ca. 1:2.7)

Residue	H-1	H-2	H-3	H-4	H-5	H-6
α-Fuc p4NAc	5.28	3.72	4.07	4.30	4.40	1.10
	$J_{1,2}$ 4.1	$J_{2,3}$ 10.6	$J_{3,4}$ 4.5	$J_{4,5}$ n.d. [b]	$J_{5,6}$ ~ 6.6	
β-Fuc p4NAc	4.63	3.39	3.86	4.27	3.96	1.14
	$J_{1,2}$ 8.0	$J_{2,3}$ 10.3	$J_{3,4}$ 4.6	$J_{4,5}$ 1.7	$J_{5,6}$ 6.4	

[a] Values for chemical shifts at 270 MHz and 70 °C relative to an external reference of sodium 4,4-dimethyl-4-silapentane-1-sulfonate. The N-acetyl signals had δ_H 2.10.
[b] n.d., Value not determined.

Table 2
^{13}C NMR data [a] (δ in ppm) for 4-acetamido-4,6-dideoxygalactose

Residue	C-1	C-2	C-3	C-4	C-5	C-6
α-Fuc p4NAc	93.03	69.31 [b]	69.13 [b]	54.89	65.82	16.49
β-Fuc p4NAc	97.22	72.83 [c]	72.66 [c]	54.50	70.70	16.49

[a] Values for chemical shifts at 100 MHz and 22 °C relative to an internal reference of 1,4-dioxane (δ_C 67.40). The N-acetyl signals had δ_C 176.33 and 22.67.
[b,c] Pairs of signals for which assignments are tentative, and hence may be interchanged.

repeating-unit. The ^{1}H NMR spectrum contained three anomeric signals (each 1 H) at δ 5.34 (unresolved), 5.03 ($J_{1,2}$ ~ 3.6 Hz), and 4.72 ($J_{1,2}$ ~ 8.4 Hz), as well as a signal at δ 4.69 (m, 1 H), and methyl signals at δ 2.10, 2.03 (both singlets, N-acetyl), 1.29 (d, $J_{5,6}$ 6.4 Hz), and 1.17 (d, $J_{5,6}$ 6.0 Hz). These results indicate that two of the residues are α-linked and one is β-linked. This was confirmed by the ^{13}C NMR spectrum (Fig. 1) which contained 22 major, discrete signals, including signals for anomeric carbons of pyranosyl residues at δ 103.71 ($^1J_{CH}$ 161 Hz), 100.58 ($^1J_{CH}$ 171 Hz), and 100.08 ($^1J_{CH}$ 171 Hz), two N-acetyl groups (δ 176.15, 174.81, 23.25, and 22.80), an unsubstituted hydroxymethyl carbon (δ 61.53), two carbons attached to nitrogen (δ 55.09 and 54.94), and two other methyl groups (δ 16.72 and 16.63).

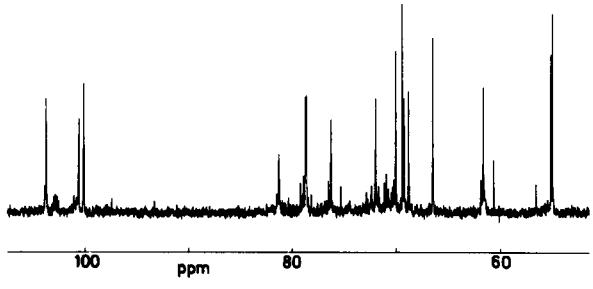

Fig. 1. ^{13}C NMR spectrum of the O3 polymer. The spectrum for the sample in D_2O was recorded at 100 MHz and 70 °C with acetone (δ_C 31.07) as the internal reference. In addition to the signals shown, the spectrum contained acetyl signals (δ 176.15, 174.81, 23.25, and 22.80) and two methyl signals (δ 16.72 and 16.63).

Table 3
NMR data [a] for the native O3 polymer

Atom		Residue		
		→ 3,4)-α-Fuc-(1 →	α-Fuc4NAc-(1 →	→ 3)-β-GlcNAc-(1 →
		a	**b**	**c**
1	H	5.34	5.03	4.72
	C	100.58	100.08	103.71
2	H	3.91	3.66	3.86
	C	69.15	69.94	55.09
3	H	4.02	3.99	3.70
	C	78.64	68.69	81.21
4	H	3.92	4.25	~ 3.70
	C	78.56	54.94	71.86
5	H	4.01	4.69	3.44
	C	69.30	66.39	76.18
6	H	1.29	1.17	~ 3.91, ~ 3.77
	C	16.63	16.72	61.53

[a] Values for chemical shifts at 70 °C (^{1}H 400 MHz) relative to an internal reference of acetone (δ_H 2.22; δ_C 31.07). The *N*-acetyl signals had δ_H 2.10 and 2.03, δ_C 176.15, 174.81, 23.25, and 22.80.

Methylation analysis of the polymer gave only two products, derived from 3,4-di-substituted Fuc*p* and 3-substituted Glc*p*NAc residues; the expected derivative from Fuc4NAc was absent presumably because of the acid-lability of the residue. These results suggest a branched trisaccharide repeating-unit with Fuc4NAc as a lateral substituent. This was confirmed by Smith degradation of the polymer resulting in the destruction of only the Fuc4NAc during oxidation. Methylation analysis of the polymeric degradation product (SD) revealed that the Fuc and GlcNAc residues were now both 3-substituted, thus proving that the Fuc4NAc substituent had been attached directly to position 4 of the Fuc branch point in the original polymer. The ^{1}H NMR spectrum of SD contained signals for two anomeric protons, δ 5.27 (unresolved) and 4.74 ($J_{1,2} \sim 8$ Hz), from which it could be concluded that the Fuc4NAc residues had the α configuration in the native polymer. Anomeric configurations for the Fuc and GlcNAc residues were determined by further interpretation of the NMR data for the native polymer and SD (Tables 3 and 4, respectively).

For this purpose, the monosaccharide residues in the O3 polymer were labelled **a**–**c**,

Table 4
NMR data [a] for the Smith-degradation product (SD)

Residue	H-1	H-2	H-3	H-4	H-5	H-6
→ 3)-α-Fuc-(1 →	5.27	3.86	3.77	3.86	3.97	1.20
→ 3)-β-GlcNAc-(1 →	4.74	3.84	3.70	3.70	3.46	3.87, 3.75

[a] Values for chemical shifts at 270 MHz and 70 °C relative to an internal reference of acetone (δ_H 2.22). The *N*-acetyl signal had δ_H 2.04.

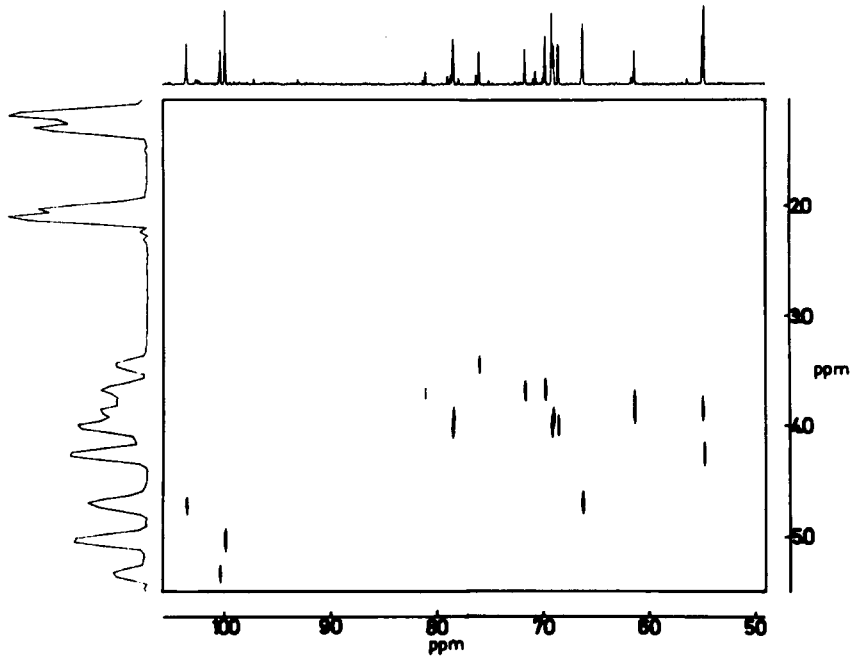

Fig. 2. HETCOR spectrum of the O3 polymer. Signals corresponding to methyl groups are omitted.

in order of decreasing chemical shift for the anomeric signals (Table 3). H-2 of the β-linked residue **c** (δ 3.86) was located from a COSY spectrum, and the corresponding C-2 (δ 55.09) from a HETCOR spectrum (Fig. 2), thus identifying the residue as GlcNAc and allowing the assignment of the α configuration to Fuc as well as Fuc4NAc. Residue **b** was identified as the Fuc4NAc, using similar correlations leading to C-4 through COSY, relayed COSY, and HETCOR spectra. The glycosylation effect on the chemical shift of C-3 of GlcNAc (ca. 6.4 ppm downfield compared with the corresponding carbon in the free monosaccharide [19]) is consistent with substitution by an α-D-Fuc*p* [20]. The glycosylation effects at C-3 (~ 8.3 ppm) and C-4 (~ 5.8 ppm) of Fuc confirm these as positions of substitution at the branch-point residue. Thus the repeating unit of the O3 polymer could be assigned the structure **1**.

α-D-Fuc*p*4NAc
1
↓
4
→3)-α-D-Fuc*p*-(1→3)-β-D-Glc*p*NAc-(1→
1

The repeating unit of the O3 antigen in *S. maltophilia* is comparable with others from this species [11–16] due to its branched nature and the presence of unusual monosaccharides. D-Fuc4NAc has been previously found as a component in the O-specific chain of *Escherichia coli* O10 [21] and that of *Pseudomonas fluorescens* biovar A strain IMV 1152 [18,22], and also the enterobacterial common antigen [23–25]. LPS containing D-Fuc is also relatively uncommon, being present in the O antigen from *Burkholderia* (*Pseudomonas*) *cepacia* serotype E [26], notable in that it has a disaccharide repeating-unit identical to that of the degradation product (SD) from the O3 antigen of *S. maltophilia*.

3. Experimental

Growth of bacteria, and isolation and fractionation of the LPS.—*S. maltophilia* strain 56 [10] was grown in Nutrient Broth No. 2 (Oxoid, 20 L) for 16 h at 37 °C with aeration at 20 L min^{-1} and stirring at 300 rpm. Mechanical disintegration of the cells (wet weight, 168 g), repeated washing and enzymic treatment of the particulate fraction [11], and lyophilisation of the product produced the cell walls (dry weight, 13.0 g). LPS (yield, 1.33 g) was isolated by treatment of the defatted cell walls with hot aqueous phenol as in related studies [11–15]. Hydrolysis of the LPS with aq 10% AcOH at 100 °C for 1.5 h released insoluble lipid A, whereupon the water-soluble products were fractionated on Sephadex G-50 to yield the polymeric side chain.

General methods.—Solvent systems used for PC were: *A*, EtOAc–pyridine–water (13:5:4); *B*, 1-butanol–EtOH–water–concd NH_3 (49:10:40:1, upper phase); *C*, EtOAc–pyridine–AcOH–water (5:5:1:3). HVE was performed using pyridine–AcOH–water (5:2:43, pH 5.3) as the buffer. Acetylated alditols and but-2-yl glycosides were separated by GLC using fused-silica capillary columns (25 m) of BP1 and BP10, respectively, in a Carlo Erba Mega 5160 chromatograph. GLC-MS was carried out on a BP10 column situated in a Finnigan 1020B instrument. Mixtures of free monosaccharides were resolved by high-pH anion-exchange chromatography (HPEAC) using a CarboPac PA100 column eluted with 10 mM NaOH, and by HPLC on HPX-87P (Bio-Rad) eluted with water at 85 °C, followed by ^1H NMR analysis.

NMR spectra for samples in D_2O were obtained by using Jeol JNM-GX270, Bruker WH-400, and Varian DXR600 spectrometers. ^1H NMR spectra of monosaccharides were obtained at 70 °C and 270 MHz with sodium 4,4-dimethyl-4-silapentane-1-sulfonate as an external reference. ^1H NMR data for product SD were obtained under the same conditions, but with acetone (δ_H 2.22) as an internal reference.

Additional 2D spectra (COSY, relayed COSY, and TOCSY) for this product were recorded at 600 MHz. The ^{13}C spectrum for Fuc4NAc was recorded at 22 °C and 100.6 MHz, and referenced to an internal standard of 1,4-dioxane (δ_C 67.40). One-dimensional (^1H and ^{13}C, including the proton-decoupled variation) and two-dimensional (COSY, relayed COSY, and HETCOR) NMR spectra for the native O3 polymer were obtained at 70 °C using a Bruker WH-400 spectrometer with acetone (δ_H 2.22, δ_C 31.07) as an internal reference.

Identification of monosaccharide composition.—Neutral sugars were released by treatment with 2 M HCl at 105 °C for 2 h, amino sugars with 6.1 M HCl at 105 °C for 6

h, both types with 2 M trifluoroacetic acid at 98 °C for 16 h [27], and Fuc4NAc with 10 M HCl at 80 °C for 30 min. Fuc was detected by PC (solvents *A* and *B*), HPEAC, and HPLC, and by GLC and GLC-MS of the alditol acetate. The same range of methods, supplemented by HVE and by PC (solvent *C*) and HPLC of the *N*-acetyl derivative, was used to establish the identity of GlcN. These assignments were confirmed by the ^{1}H NMR spectra, and the D configuration for both sugars was established by GLC of the but-2-yl glycoside acetates [28], by the CD curve of the alditol acetate [29], and by polarimetry. The presence of a component undergoing reactions typical of a 4-amino sugar was initially detected by HVE. The *N*-acetylated derivative [30] was isolated by HPLC and identified as Fuc4NAc from NMR analysis, and the D configuration determined by polarimetry (dextrorotation).

Structural methods.—Methylation analyses, monitored by GLC and GLC-MS of the methylated alditol acetates, were performed according to standard procedures [31–33]. Smith degradation of the polysaccharide (31 mg) involved treatment with 50 mM $NaIO_4$ (8 mL) at 4 °C for 6 days. The resulting polymeric product (16 mg) was recovered by chromatography on Sephadex G-15 and purification by HPLC on TSKgel G-Oligo-PW (Bio-Rad) eluted with water at 0.7 mL min^{-1}.

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