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Note

Structure of the O16 antigen of *Stenotrophomonas maltophilia*

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Abstract

A polysaccharide containing D-ribose, *N*-acetyl-D-glucosamine, and *N*-acetyl-D-mannosamine was isolated from the phenol-soluble lipopolysaccharide extracted from defatted cell walls of the reference strain (560) for serogroup O16 of *Stenotrophomonas maltophilia*. The results of methylation analysis, chemical degradations, and NMR spectroscopy showed that the polysaccharide is based on a branched trisaccharide repeating-unit of the structure shown below. Although ribose was absent from about half of the units in the isolated polymer, the regularity and spacing of the ladder observed on SDS-PAGE of the parent lipopolysaccharide indicate that this was an artefact of the mild acid hydrolysis used to release the polymer. On the other hand, the effects of mild alkaline hydrolysis on the polymer indicated partial O-acetylation.

α-D-Rib*f*
1
↓
4
→3)-β-D-Man*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→

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

Stenotrophomonas maltophilia has risen to prominence as an opportunist pathogen and an agent of nosocomial infections, difficult to combat because of the multidrug resistance of many strains.[1,2] Amongst the methods available for epidemiological monitoring is serotyping of the lipopolysaccharide (LPS)-based, heat-stable O antigens.[3,4] Structures have been reported for the O-specific polymers from 12 of the 31 defined serotypes (Refs. 5–7 and references cited therein), including the prevalent serotypes (O3, O10 and O19). Most of the polymers have branched repeating-units, commonly with lateral pentosyl substituents. The O16 antigen, found in about 4% of 900 human and environmental isolates examined,[4] provides a further example of this type of structure.

When defatted cell walls (6.85 g) of *S. maltophilia* strain 560 were extracted with hot aqueous phenol, similar amounts of LPS were recovered from both the aqueous and the phenolic layer obtained on cooling. The LPS from the aqueous phase (362 mg; yield, 5.3%)

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was of the R-type. The monosaccharide components identified were Glc, Man, GlcN and GalN (known components of the core and lipid A regions[8,9]), and no polymeric material was released on mild acid hydrolysis. The LPS used for structural studies was that isolated from the phenolic phase (356 mg; yield, 5.2%). When examined by SDS-PAGE, the LPS gave a regular ladder pattern typical of S-type LPS containing a variable number of repeating units in the polymeric side-chain. The spacing of the rungs was comparable with that for the O6 antigen analysed simultaneously; the O6 antigen is based on a trisaccharide repeating-unit of D-Xyl, D-GlcNAc and L-Rha.[10] When the O16 LPS was subjected to mild acid hydrolysis, followed by GPC of the water-soluble products (yield, 69%) on Sephadex G-50, a polymeric fraction was obtained (yield from LPS, 40%). Heterogeneity of the material was apparent, both from its monosaccharide composition and the ^{1}H NMR spectrum. In addition to the major components, Rib, GlcN and ManN (each as the D isomer), smaller proportions of core sugars (Glc, Man, GalN) and Rha were detected. In the ^{1}H NMR spectrum, the anomeric region was complex and there was evidence for *O*-acetyl groups (δ 2.15 and 2.16) as well as major *N*-acetyl signals (δ 2.02–2.06), relative intensities $\sim$1:3.5. Support for the presence of ester function(s) was provided by the IR spectrum (band at 1726 cm^{-1}) and the effects of mild alkaline treatment of the polymer (loss of the ^{1}H NMR signals at δ 2.15 and 2.16, simplification of the anomeric region).

Apart from residual minor signals, the anomeric region of the ^{1}H NMR spectrum of the O-deacetylated polymer (Fig. 1) included

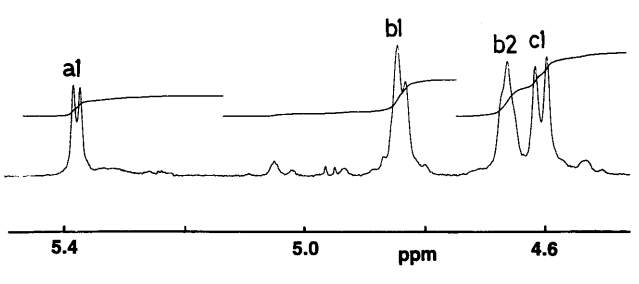

Fig. 1. Anomeric region of the ^{1}H NMR spectrum of the O-deacetylated O16 polymer. The spectrum was recorded at 400 MHz and 70 °C with acetone (δ_H 2.22) as the internal reference.

signals for protons at δ 5.38 ($J_{1,2}$ 4.5 Hz, $\sim$0.5 H, **a1**), 4.83 (two overlapping, unresolved signals, total 1 H, **b1**) and 4.60 ($J_{1,2}$ 7.6 Hz, 1 H, **c1**). The broad, unresolved signal (Fig. 1) at δ 4.66 (1 H) was shown (COSY) to correspond to H-2 of residue **b**, while the occurrence of each hexosamine as its *N*-acetyl derivative was indicated by the signals at δ 2.02 (3 H), 2.05 and 2.07 (total 3 H). Residue **b** was identified as Man*p*NAc and residue **c** as β-Glc*p*NAc by means of COSY and ^{13}C–^{1}H correlation spectra leading to C-2 in each case (δ 50.64 and 55.99, respectively, consistent with carbon attached to nitrogen[11]). The appearance of some signals of residue **b** (including those for H-1 and H-2) gave the impression that the Man*p*NAc residues occurred in different chemical environments, and this was confirmed by the COSY spectrum, which showed two cross-peaks between H-2 and H-3 (δ 4.26 and 4.02). Subsequent work (vide infra) established that only the latter cross-peak was present in the spectrum of the polymer from which Rib had been removed. Thus, the major source of heterogeneity in the O-deacetylated O16 antigen could be attributed to incomplete ribosylation. As SDS-PAGE of the parent LPS had indicated a regular structure based on a trisaccharide repeating-unit, the results for the isolated polymer probably reflect partial loss of Rib (by cleavage of labile furanosidic linkages) during acid hydrolysis of the LPS, rather than partial incorporation of the sugar, or the existence of a pentasaccharide repeating-unit in which only alternate Man*p*NAc residues are substituted by the pentose. The value of 4.5 Hz for $J_{1,2}$ of residue **a** is consistent with its identification as α-Rib*f*,[12] and this was corroborated by ^{13}C NMR data (vide infra).

Confirmation of a branched structure with partial ribosylation of the main chain was provided by methylation analysis, monitored by GLC and GLC–MS of the methylated alditol acetates. Products derived from unsubstituted Rib*f*, 4-substituted Glc*p*NAc, 3-substituted Man*p*NAc and a disubstituted Hex*p*NAc residue were identified by GLC comparison with standards and/or the fragmentation pattern obtained on MS. No reference sample for the disubstituted Hex*p*NAc

Table 1
^{1}H NMR data [a] for the polymeric product from Smith degradation of the O-deacetylated O16 antigen

Atom	Residue	
	→3)-β-D-Man*p*NAc-(1→ **b′**	→4)-β-D-Glc*p*NAc-(1→ **c′**
H-1	4.80	4.57 [b]
H-2	4.62	3.72
H-3	4.00	~3.68
H-4	3.58	~3.69
H-5	3.43	3.49
H-6	3.77, 3.88	3.70, 3.84

[a] Values for chemical shifts relative to internal acetone (δ 2.22) at 70 °C and 600 MHz. Additional signals from *N*-acetyl groups at δ 2.00 and 2.01.
[b] Signal seen as a doublet ($J_{1,2}$ ~7 Hz) at 270 MHz, but as a multiplet (virtual coupling) at 600 MHz.

Table 2
NMR data [a] for the O-deacetylated O16 antigen

Atom		Residue		
		α-D-Rib*f*-(1→ **a**	→3,4)-β-D-0Man*p*NAc-(1→ **b**	→4)-β-D-Glc*p*NAc-(1→ **c**
1	H	5.38	~4.83	4.60
	C	104.63	~100.3	98.73
2	H	4.14	~4.66	3.74
	C	72.15	~50.6	55.9
3	H	4.04	4.26	3.71
	C	70.63	nd	73.27
4	H	4.11	(3.74)	3.70
	C	85.63	nd	79.90
5	H	~3.65, ~3.72	~3.46	3.52
	C	nd	~77.1	75.35
6	H		~3.80, ~3.91	~3.73, ~3.87
	C		~61.5	61.15

[a] Values for chemical shifts relative to internal acetone (δ_H 2.22, δ_C 31.07) at 70 °C and 400 MHz; nd, not determined; value in brackets is tentative. Additional signals from *N*-acetyl groups at δ_H 2.02, 2.05 and 2.07 (total 6 H), δ_C 175.19, 175.08, 23.23 and 22.92.

(expected to correspond to Man*p*NAc at the branch point) was available, but the absence from the mass spectrum of primary fragments with *m*/*z* 275 and 189 appeared to rule out the 3,6-disubstituted compound. Thus, the Man*p*NAc residue at the branch point was inferred to be 3,4-disubstituted (the derivative from which produces no distinctive fragments).

Characterisation of the disaccharide repeating-unit of the polymer backbone as structural element **1** was achieved by Smith degradation of the O-deacetylated material. A homogeneous polymer containing only 4-substituted Glc*p*NAc (**c′**) and 3-substituted Man*p*Ac (**b′**) was produced. Signals in the ^{1}H NMR spectrum were assigned (Table 1) with the aid of 2D correlations (COSY and relayed COSY). The values of $^1J_{CH}$ for the anomeric signals at δ 4.80 and 4.57 were 165 and 166 Hz, respectively, consistent with the β configuration for both residues.

→3)-β-D-Man*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→
b′ **c′**

1

a
α-D-Rib*f*
1
↓
4
→3)-β-D-Man*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→
b **c**

2

Attempts to confirm ribosylation at position 4 of Man*p*NAc, and **2** as the repeating unit of the O-deacetylated O16 polymer, by detailed interpretation of its NMR spectra (^{1}H and ^{13}C), were hindered by the heterogeneity of the material, the consequential multiplicity of peaks (particularly in the ^{13}C spectrum), signal overlap and the absence of some cross-peaks from 2D spectra. Data are summarised in Table 2. As expected, the proton assignments for residue **c** are essentially the same as for the corresponding residue **c′** in the Smith degradation product, and the downfield location of the signal for C-4 confirms 4-substitution of the Glc*p*NAc. In the case of residue **a**, the complete proton spin system was readily followed in the COSY spectrum and the corresponding carbon signals were assigned by ^{13}C–^{1}H correlation (Fig. 2), except for C-5, for which no unique cross-peak was apparent. The NMR data are consistent with the identification of residue **a** as unsubstituted α-D-Rib*f*.[12–14] As seen (Fig. 2), the ^{13}C signals for

residue **a** are of relatively low intensity and, unsurprisingly, no cross-peaks were detected for H-3 or H-4 of the ribosylated Man*p*NAc residue **b**. However, correlations for positions 3 (δ_H 4.02/δ_C 77.88) and 4 (δ_H 3.61/δ_C 65.90) of the monosubstituted residue (equivalent to **b′**) are diagnostic for 3-substitution.

A further attempt to confirm ribosylation of the Man*p*NAc residue by application of the reaction sequence N-deacetylation–deamination–reduction ($NaBH_4$) to the O16 polymer was unsuccessful. Only free 2,5-anhydromannitol and glucitol, derived directly from non-ribosylated units of Glc*p*NAc and Man*p*NAc, respectively, were detected by HPAEC and by GLC of the peracetates. Nevertheless, it is clear that the O16 antigen is based on the branched trisaccharide unit of structure **2**, probably with non-stoichiometric O-acetylation (less than one acetyl group per repeating unit).

The O16 antigen of *S. maltophilia* resembles others previously characterised in its branched structure with a lateral pentosyl substituent, although linear structures have been found for the O7,[6] O20[15] and O25[7] polymers. As with the O1[9] and O21[7] polymers containing α-D-Ara, the substituent in the O16 polymer is the unusual α-furanosyl form, whereas in polymers containing xylose (D or L) it is the β-pyranosyl form. Partial O-acetylation has only been reported for the O1 and O16 polymers. Recent studies[16,17] have explored the phenotypic and genomic diversity within strains of *S. maltophilia*, and may lead to taxonomic changes or the redesignation of reference strains in due course.

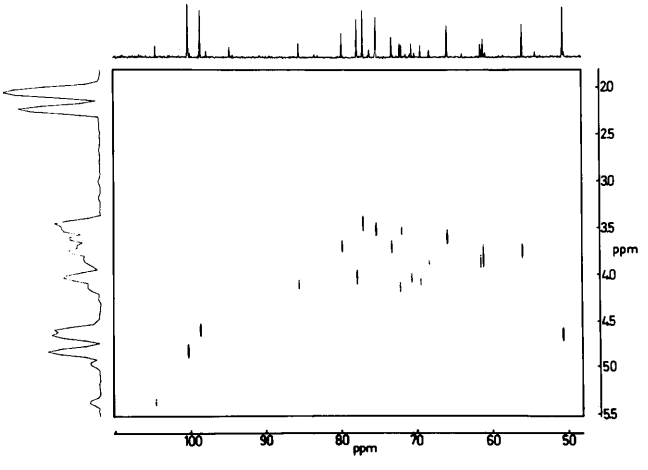

Fig. 2. HETCOR spectrum of the O-deacetylated O16 polymer. Corresponding parts of the ^1H and ^{13}C spectra are displayed along the vertical and horizontal axes, respectively. Signals for the *N*-acetyl groups are omitted.

1. Experimental

Growth of bacteria, and isolation and fractionation of the LPS.—Strain 560 of *S. maltophilia* (serotype O16)[3] was grown in Nutrient Broth No. 2 (Oxoid, 20 L) for 16 h at 37 °C with stirring at 300 rpm and aeration at 20 L min^{-1}. The cells (wet weight 167 g) were harvested (Sharples) and disintegrated mechanically (Dyno Mill KDL). Cell walls were isolated, purified, freeze-dried (yield, 7.3 g) and extracted with 2:1 $CHCl_3$–MeOH at rt for 2 h. The defatted cell walls (6.85 g) were treated with hot, aq phenol as in related studies[5–10,15] and LPS was recovered from both phases after exhaustive dialysis and clarification by centrifugation. Samples of LPS were subjected to mild acid hydrolysis (1% AcOH, 100 °C, 2 h) and polymeric products were isolated by GPC of the water-soluble material on Sephadex G-50.

Monosaccharide analysis.—Conditions used to release monosaccharides were treatment with 2 M HCl at 105 °C for 2 h (neutral sugars), 6.1 M HCl at 105 °C for 4 h (amino sugars), or 2 M CF_3CO_2H at 98 °C for 16 h (both classes[18]). When necessary, amino sugars were separated from neutral sugars by adsorption onto Dowex 50 (H^+) resin and elution with 2 M HCl. Free monosaccharides were identified by HPAEC on CarboPac PA100 (Dionex) eluted with 16 mM NaOH (neutral sugars) or 10 mM NaOH (amino sugars), and by capillary GLC of the alditol acetates on a column (25 m) of BP10. Configurations of sugars were determined by GLC (BP1 and BP10) of the (−)-but-2-yl glycoside acetates, after separation by HPLC on HPX-87H (Bio-Rad) by elution with 5 mM H_2SO_4 at 40 °C, followed by N-acetylation[19] of the amino sugars.

Structural methods.—Treatment with 0.1 M NaOH at rt was used for O-deacetylation of the O16 polymer. Methylation analyses, monitored by GLC and MS of the methylated alditol acetates (deuterated at C-1), were car-

ried out by standard procedures.[20–22] Smith degradation of the polymer (25 mg) involved treatment with 50 mM $NaIO_4$ (10 mL) at 4 °C for 5 days, addition of ethylene glycol, reduction ($NaBH_4$), dialysis, hydrolysis with 1 M CF_3CO_2H at rt for 16 h, and recovery of the polymeric product (15 mg) by GPC on Sephadex G-15. N-Deacetylation–deamination[20,23] of the reduced ($NaBH_4$) O16 polymer was also followed by GPC of the products on Sephadex G-15. SDS-PAGE of whole LPS was carried out by the method of Laemmli[24] using a discontinuous Tris–glycine buffer system in a Protean II cell (Bio-Rad), and silver staining of the bands.[25] NMR spectra (1D, COSY, relayed COSY and HETCOR) for samples in D_2O were recorded at 70 °C with JEOL JNM-GX270, Bruker WH-400 or Varian DXR600S instruments and acetone (δ_H 2.22, δ_C 31.07) as internal reference. The IR spectrum for the O16 polymer in KCl was recorded with a Perkin–Elmer 993 spectrometer.

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