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Structural studies of acidic polymers produced by the O23 reference strain of *Serratia marcescens*: presence of amide-linked glutamic acid*

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ABSTRACT

The major fraction of an acidic galactoglucomannan present in lipopolysaccharide extracts from cell walls of the O23 reference strain of *Serratia marcescens* has the tetrasaccharide repeating-unit shown. In a minor fraction, L-glutamic acid was amide-linked to about half of the D-glucuronic acid residues. The possible contributions of the acidic polymers and a neutral polymer produced by the organism to cross-reactions with other serogroups are discussed.

→3)-α-D-Man*p*-(1→3)-α-D-Gal*p*-(1→3)-α-D-Gal*p*-(1→2)-α-D-Glc*p*A-(1→

INTRODUCTION

Like many other strains of *Serratia marcescens*, the reference strain for serogroup O23 produces acidic and neutral glycans, both of which are present in aqueous-phenol extracts of isolated cell walls[1]. We have shown[1] that the repeating unit of the neutral glycan, which is believed to constitute the side chain of the lipopolysaccharide component of the bacterial outer membrane, has the structure **1**. The neutral glycan does not obviously account for the antigenic relationship[2–4] between serogroup O23 and strains of serogroups O12, O13, and O14, in some of which[5–7] the corresponding glycan has the disaccharide repeating-unit **2**. It was therefore of interest to characterise the acidic O23 glycan for comparison with those obtained from the reference strains for serogroups O13 (ref. 7) and O14 (ref.8).

→4)-α-L-Rha*p*-(1→4)-β-D-Gal*p*NAc-(1→
1

→2)-β-D-Rib*f*-(1→4)-α-D-Gal*p*NAc-(1→
2

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RESULTS

Acidic products represented 45% of the polymeric, water-soluble material recovered after mild hydrolysis (aqueous 1% acetic acid, 2.25 h, 100°) of the "lipopolysaccharide" isolated from *S. marcescens* O23 (ref. 1). The major acidic product (fraction I, 67% of the total) was eluted from DEAE-Sepharose CL-6B with 0.3M NaCl, and the minor fraction (II, 33%) with M NaCl (no attempt was made to elute material with an intermediate concentration of NaCl).

Monosaccharide analysis of fraction I showed that galactose and mannose (molar ratio 2.8:1.0) were the major neutral components and that glucuronic acid was also present; traces of glucose and glutamic acid were also detected. Quantitative analysis of the carboxyl-reduced polymer gave the following data: D-galactose (38.7%), D-mannose (18.0%), and D-glucose (14.7%). The increased proportion of mannose in the reduced polymer (galactose:mannose ratio 2.2:1.0) indicated that this sugar was substituted by glucuronic acid in the parent polymer.

The n.m.r. spectra of the acidic polymer showed that it was based on a tetrasaccharide repeating-unit, in which all sugars were present as α-pyranoside residues. Thus, the ^{1}H-n.m.r. spectrum contained four anomeric signals (each 1 H) at δ 5.47 ($J_{1,2}$ 3.2 Hz), 5.23 ($J_{1,2}$ 3.4 Hz), 5.19 ($J_{1,2}$ 3.2 Hz), and 5.05 (unresolved). The ^{13}C-n.m.r. spectrum contained 22 discrete signals (two being of double intensity), including anomeric signals at δ 97.90 ($^1J_{CH}$ 171 Hz), 96.43 ($^1J_{CH}$ ~ 170 Hz), and 95.35 (2 C, $^1J_{CH}$ 171 Hz), a carbonyl signal at δ 175.67, and three signals with δ ~61, corresponding to unsubstituted hydroxymethyl carbon atoms. Methylation analysis of the acidic polymer gave the derivatives from 3-substituted galactopyranosyl and 3-substituted mannopyranosyl residues (molar ratio ~2:1), and similar analysis of the carboxyl-reduced polymer gave an additional product derived from a 2-substituted glucopyranosyl residue.

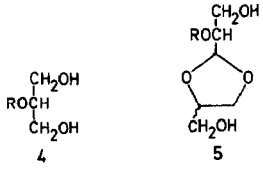
An aldobiouronic acid was isolated by h.p.l.c. after partial acid hydrolysis of the polymer. The disaccharide-alditol obtained by reduction ($NaBH_4$) gave a single anomeric signal [δ 5.20 ($J_{1,2}$ ~4 Hz)] in the ^{1}H-n.m.r. spectrum and released mannitol on total acid hydrolysis. F.a.b-m.s. of the disaccharide-alditol gave the expected pseudomolecular ions with m/z 359 $(M + H)^+$, 376 $(M + NH_4)^+$, 381 $(M + Na)^+$, and 397 $(M + K)^+$. Thus, the aldobiouronic acid could be identified as α-D-Glc*p*A-(1→3)-D-Man, and structure **3** could be proposed for the repeating unit of the acidic polymer in fraction I.

→3)-α-D-Man*p*-(1→3)-α-D-Gal*p*-(1→3)-α-D-Gal*p*-(1→2)-α-D-Glc*p*A-(1→

3

In order to reinforce the structural conclusions, a Smith degradation (including a final treatment of the products with $NaBH_4$) was carried out on the carboxyl-reduced polymer. Although a single peak in the tri- or tetra-saccharide region was obtained by size-exclusion h.p.l.c., ^{1}H-n.m.r. spectroscopy indicated that the product was a mixture

(the anomeric region was more complex than expected for a trihexosylglycerol). After permethylation and reverse phase h.p.l.c., two derivatives were isolated in similar amounts. Methylation analysis and f.a.b.-m.s. [which gave pseudomolecular ions with m/z 747 $(M + H)^+$, 764 $(M + NH_4)^+$, 769 $(M + Na)^+$, and 785 $(M + K)^+$] showed that one product was the derivative of the classical Smith-degradation product of general structure **4** [R = $(Hexp)_3$]. Similar studies of the second methylated compound [which gave pseudomolecular ions with m/z 819 $(M + H)^+$, 836 $(M + NH_4)^+$, 841 $(M + Na)^+$, and 857 $(M + K)^+$, in f.a.b.-m.s.] indicated that it was derived from the 1,3-dioxolane of general structure **5** [R = $(Hexp)_3$]. Such a product would be formed by cyclisation of the ring-opened glucose residue during the hydrolytic step of the Smith degradation, involving transacetalation and participation by the primary hydroxyl group at either C-4 or C-6.



4 5

The monosaccharide components of the minor acidic polymer (fraction II) were the same as those described for fraction I, and identical results were obtained on methylation analysis. However, glutamic acid (which was only a trace component of fraction I) was a significant component of the material in fraction II. A comparison of the anomeric regions in the ^{1}H-n.m.r. spectra for fractions I (Fig. 1) and II (Fig. 2) showed that the signal at lowest field (δ 5.47) for the former had been replaced by two signals (each ~0.5 H, $J_{1,2}$ ~3.5 Hz) at δ 5.52 and 5.48 for fraction II. Also, the spectrum for fraction II contained additional signals at δ 4.50 (~0.5 H, dd, J ~5 and ~9 Hz), 2.50 (~1 H, t, J ~10 Hz), 2.23 (~0.5 H, m), and 2.05 (~0.5 H, m). Selective decoupling showed that these additional signals could be attributed to H_α, the two H_γ, and the prochiral H_β protons of the glutamic acid residue. Consistent with these observations, the ^{13}C-n.m.r. spectrum for fraction II contained the expected signals for C_α (δ 54.63), C_γ (δ 31.63), and C_β (δ 27.31) of the glutamic acid residue, together with four signals for carbonyl carbon atoms at δ 178.81, 177.78, 176.60, and 171.77. Thus, it appeared that fraction II was based on the acidic polymer with the repeating unit **3**, with glutamic acid attached to ~50% of the repeating units. The glutamic acid was identified as the L isomer both by enzymic assay and by h.p.l.c. using a chiral mobile phase[9].

Glutamic acid was not released by mild alkaline treatment of the polymer, and there was no significant absorption at ~1740 cm^{-1} in the i.r. spectrum. Thus, it appeared that the glutamic acid was amide-bound to the polysaccharide via the glucuronic acid residues.

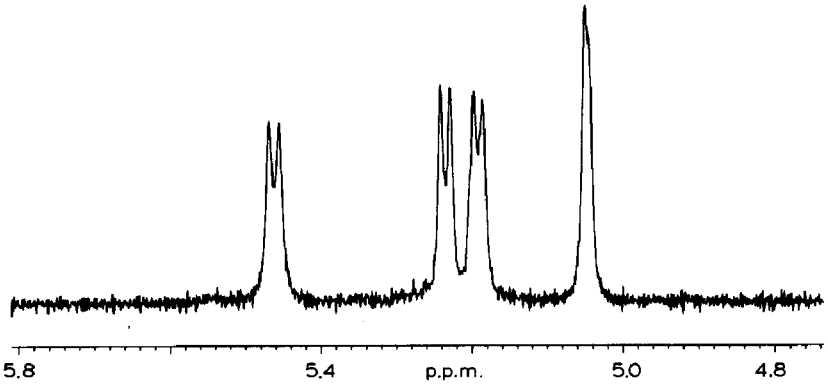


Fig. 1. ^{1}H-N.m.r. spectrum of fraction I of the acidic polymer. The spectrum was recorded at 70° and 270 MHz with sodium 4,4-dimethyl-4-silapentane-1-sulphonate as the external reference.

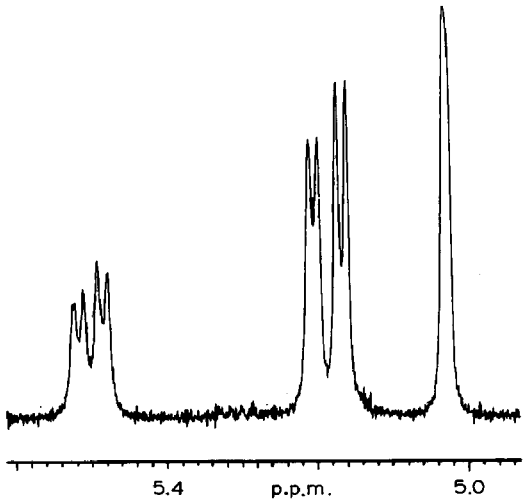
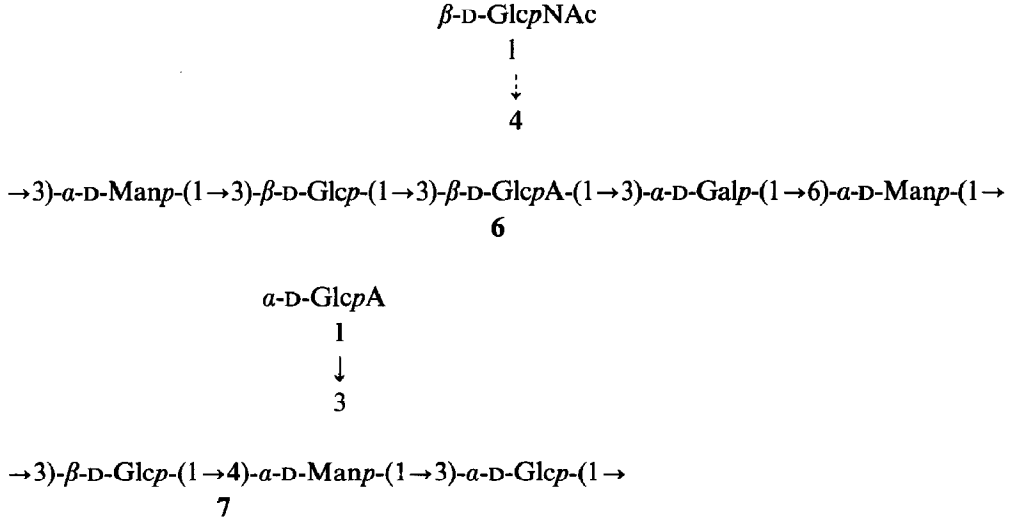


Fig. 2. ^{1}H-N.m.r. spectrum of fraction II of the acidic polymer. The conditions used were the same as for fraction I (Fig. 1).

DISCUSSION

In general, the acidic polysaccharides produced by *S. marcescens* have branched repeating units. Apart from the polymers described here for the O23 reference strain, the only previous exception is the acidic polymer from the O2 reference strain[10], which is a partially acetylated galactorhamnan with a tetrasaccharide repeating-unit. Structure **3**, established for the repeating unit in fraction I of the O23 products, incorporates some residues also present[7] in the O13 polymer with the repeating unit **6**, but there is no obvious reason to believe that the acidic polymers account for the antigenic relationship between the O13 and O23 strains. Also, it should be noted that the O13 reference strain

does not contain the lipopolysaccharide-derived neutral polymer with the disaccharide repeating-unit **2**, which is thought[7] to confer the antigenic factor Co12,13,14 (refs. 3 and 11) associated with serological cross-reactions[2,3]. The O14 acidic polymer[8] with the repeating unit **7** (partially acetylated) is even more dissimilar to the O23 acidic polymer, again indicating that they are not involved in the serological cross-reactions. This inference is supported by the observation[4] that lipopolysaccharide-related cross-reactions with O23 can be detected by immunoblotting for O12 and O14 (which have different acidic polymers but the same neutral polymer **2**).



Reports of amino acids as integral components of bacterial polysaccharides, often in amide linkage to sugar acids, are growing steadily. In some cases, as in the attachment of lysine to D-galacturonic acid[12,13] or D-glucuronic acid[14] in lipopolysaccharides from *Proteus* species, the result is neutralisation of the charge. In other cases where a neutral amino acid (alanine, serine, or threonine) is similarly attached[14–19], the overall charge is unaffected. However, the incorporation of glutamic acid (as in fraction II of the O23 acidic polymer from *S. marcescens*) enhances the acidity of the polymer. The later elution of fraction II (compared with fraction I) from DEAE-Sepharose CL-6B is presumably a consequence of this acidity, and is evidence that it is glutamic acid itself (rather than glutamine) which is incorporated in the polymer. Because the incorporation affected only one anomeric signal in the ^{1}H-n.m.r. spectrum of the polymer (Fig. 2), it was not possible to decide whether fraction II consisted of a family of polymers differing in the extent of substitution by glutamic acid, nor whether substitution was random or regular. Overall, however, fraction II contained about equal proportions of tetrasaccharide units with and without the amino acid.

L-Glutamic acid amide-linked to the 6-position of D-glucuronic acid has recently been reported[20] in the capsular polysaccharide from *Klebsiella* K82. Although both compounds also occur in a structural component of the cell wall of an alkalophilic

Bacillus strain[21], it appears that this material consists of separate polyglutamate and polyglucuronate entities.

EXPERIMENTAL

Growth of bacteria, and isolation and fractionation of lipopolysaccharide. — These operations have been described in a report on the isolation and characterisation of a neutral polymer[1].

Component analyses. — Neutral sugars were released, identified (p.c., g.l.c.), and determined as in related studies[22]. Glucuronic acid was identified by paper electrophoresis[23] at pH 2.7 and, after carboxyl reduction[10], by g.l.c. of the alditol acetate. Absolute configurations of the sugars were determined by g.l.c. of the but-2-yl glycoside acetates[1].

Glutamic acid was released from fraction II by hydrolysis using either 6.1M HCl for 4 h at 105°, or 2M trifluoroacetic acid for 16 h at 105°. The amino acid was identified by p.c. with ethyl acetate–pyridine–water–acetic acid (5:5:3:1), paper electrophoresis with pyridine–acetic acid–water (5:2:43, pH 5.3), by chiral h.p.l.c.[9], and by enzymic assay using glutamate dehydrogenase (EC 1.4.1.3) as described by the supplier (Boehringer Mannheim GmbH). According to the latter assay, fraction II contained 8.2% of L-glutamic acid.

General methods. — N.m.r. spectra (^1H and ^{13}C) of samples in D_2O were recorded with a Bruker WH-400 or JEOL JNM-GX270 spectrometer. The ^1H-n.m.r. data were obtained at 270 MHz and 70° with sodium 4,4-dimethyl-4-silapentane-1-sulphonate as the external reference. The ^{13}C-n.m.r. data were obtained at 100.6 MHz and 27°, with or without gated decoupling, and with tetramethylsilane as the external reference (fraction I), or at 67.8 MHz and 21° with 1,4-dioxane as the internal reference (fraction II). The i.r. spectrum of fraction II dispersed in KCl was recorded with a Perkin–Elmer 783 spectrometer. H.p.l.c. was carried out with Gilson apparatus, g.l.c. with a Carlo Erba Mega 5160 chromatograph, and g.l.c.–m.s. with a Finnigan model 1020B instrument. F.a.b.-m.s. of samples in a thioglycerol matrix was carried out with a ZAB-E mass spectrometer (VG Analytical).

Degradative methods. — Methylation analyses were carried out following standard procedures[1,10]. Glucuronate residues in methylated polymers were reduced by treatment with $LiAlH_4$ in tetrahydrofuran for 4 h at 70°. A Smith degradation of fraction I was carried out[1] and, after reduction ($NaBH_4$) and methylation, the products were separated by h.p.l.c.[24] with methanol–water (3:2) as the eluant. An aldobiouronic acid produced by partial hydrolysis (2M trifluoroacetic acid, 100°, 3 h) of fraction I was isolated by h.p.l.c. using a TSKgel G-Oligo-PW column (Anachem) eluted with 0.2M sodium acetate (1 mL.min^{-1}), followed by treatment of the eluate with Dowex 50 resin (H^+ form). A mild alkaline treatment[8] of fraction II was used to test for the presence of ester-linked glutamic acid. Although the amino acid was not removed by this treatment, the ^1H-n.m.r. spectra of the product (as the ammonium salt) showed improved resolution compared with those of the starting material. In particular, downfield shifts of signals for the glutamic acid residue revealed the doublet of doublets for H_α which had previously been hidden by the HOD signal.

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