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STUDIES OF LIPOPOLYSACCHARIDES FROM TWO STRAINS (C.D.C. 3607-60 AND IP 421) OF *Serratia marcescens* O13: STRUCTURE OF THE PUTATIVE O13 ANTIGEN

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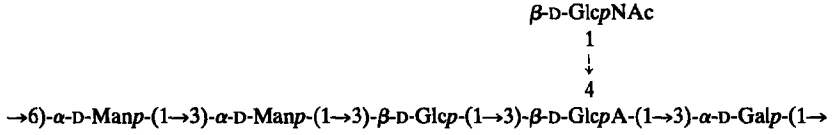
ABSTRACT

Structural studies have been carried out on the putative O-specific polysaccharide of the reference strain (C.D.C. 3607-60) for *Serratia marcescens* O13. Circumstantial evidence that the O13 antigen is a microcapsular, acidic polymer, rather than an integral part of the lipopolysaccharide, has been obtained. Degradative and spectroscopic studies established that the polymer is based on the repeating unit shown, in which the glucuronic acid residue of the linear pentasaccharide carries the lateral 2-acetamido-2-deoxy-β-D-glucopyranosyl substituent in only about half of the units. The same polymer, again with non-stoichiometric substitution, is also produced by strain IP 421 (O13:H7). The latter strain also produces a neutral polymer which appears to constitute the side chain of the lipopolysaccharide. This polymer, which has a disaccharide repeating-unit of 2-substituted β-D-ribofuranosyl and 4-substituted 2-acetamido-2-deoxy-α-D-galactopyranosyl residues, has been isolated previously from the lipopolysaccharides of the reference strains for *S. marcescens* serogroups O12 and O14, and appears to be the antigen known to be shared by these strains.



INTRODUCTION

The increasing importance of *Serratia marcescens* as a cause of nosocomial infections demands reliable and discriminatory methods for the typing of clinical isolates. The prevalence of strains from a few serogroups (notably O14) and the extent of cross-reactions limit the epidemiological value of serotyping by the heat-stable (O) antigens[1]. During the course of a systematic study of the surface polysaccharides of *S. marcescens*[2–7], we have shown that all O14 strains examined (as well as the O6 reference strain) produced an acidic glucomannan thought to be the

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O6/O14 antigen and of (micro)capsular origin[4]. In addition, each O14 strain produced a neutral polymer believed to be the side chain of the lipopolysaccharide, which would normally constitute the O antigen. Three such neutral polymers have been characterised[2,3,6] and each of them has been shown to occur also in strains of other serogroups: O6 (ref. 5), O8 (ref. 7), or O12 (ref. 5). The polymer with a disaccharide repeating-unit of D-ribose and 2-acetamido-2-deoxy-D-galactose, present in the O12 reference strain[5] and the O14 reference strain[3] *inter alia*, is probably the antigen known[8] to be shared by these strains. This antigen is reported[8] to be present also in an O13 strain (IP 421), but not in the O13 reference strain. We have therefore studied the polymers present in "lipopolysaccharides" extracted from both O13 strains in order to confirm the above inference, as well as to characterise the O13 antigen.

RESULTS

Lipopolysaccharide from strain C.D.C. 3607–60. — The yield of lipopolysaccharide from this O13 strain was ~20% of the whole cell wall. The monosaccharide components detected were 2-amino-2-deoxyglucose (9.4%), glucose, galactose, mannose, glucuronic acid, and two heptoses with the chromatographic properties of L-*glycero*-D-*manno*-heptose (major) and D-*glycero*-D-*manno*-heptose (minor): no attempt was made to detect the 3-deoxy-D-*manno*-2-octulosonic acid also expected to be present. On polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate, the lipopolysaccharide gave two major and two minor fast-moving bands, but no "ladder" typical of S-form lipopolysaccharides with variable numbers of repeating units in the side chain.

Like most other lipopolysaccharides from *S. marcescens* studied in these laboratories, the product from strain C.D.C. 3607–60 gave a dark-brown suspension during mild acid hydrolysis. After removal of insoluble material, chromatography on Sephadex G-50 gave an acidic polysaccharide (yield, 30% based on lipopolysaccharide). The polymer was eluted from DEAE-Sepharose CL-6B partly by 0.1M NaCl (26%) but mainly by 0.2M NaCl (74%). As both fractions had the same monosaccharide composition and gave indistinguishable n.m.r. spectra, only the major fraction was used for structural studies.

Quantitative analyses (D-mannose, 37.7; D-glucose, 17.7; D-galactose, 15.4; D-glucuronic acid, 13.0; 2-amino-2-deoxy-D-glucose, 5.0%; heptoses, not detected) suggested that the polymer was based on a pentasaccharide repeating-unit non-stoichiometrically substituted by the hexosamine. Lack of stoichiometry was also indicated by the n.m.r. spectra (Figs. 1 and 2). Thus, the ^{13}C-n.m.r. spectrum (Fig. 1) contained eight signals in the anomeric region at δ 103.50, 103.41, 102.68, 102.57, 101.80, 100.78, 99.09, and 98.19, while the ^{1}H-n.m.r. spectrum (Fig. 2) contained three unresolved signals (δ 5.22, 5.14, and 5.02, each 1 H) and five doublets (each ~0.5 H, $J_{1,2}$ ~8 Hz) in the range δ 4.87 to 4.67. The presence of the 2-amino-2-deoxyglucose as its *N*-acetyl derivative was indicated by signals at δ 22.56 and ~175 (^{13}C) and δ 2.07 (~1.5 H).

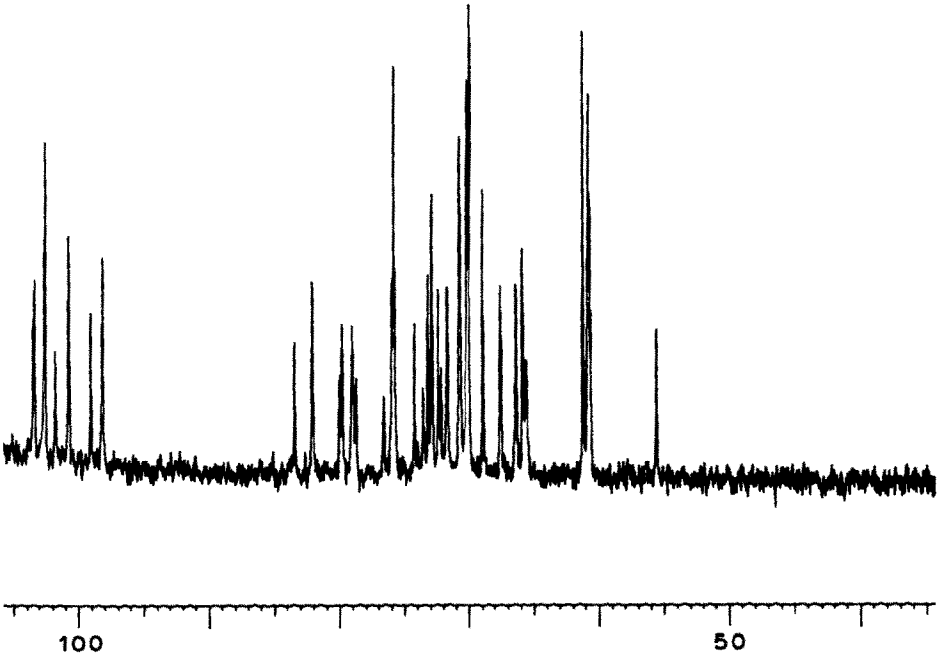


Fig. 1. ^{13}C-N.m.r. spectrum of the acidic polymer from *S. marcescens* C.D.C. 3607-60. The spectrum for the sample in D_2O was obtained at 100.61 MHz and 50° with complete proton-decoupling and external tetramethylsilane as the reference. In addition to the signals shown, the spectrum contained signals for two carbonyl carbons at δ ~175 and a methyl carbon at δ 22.56.

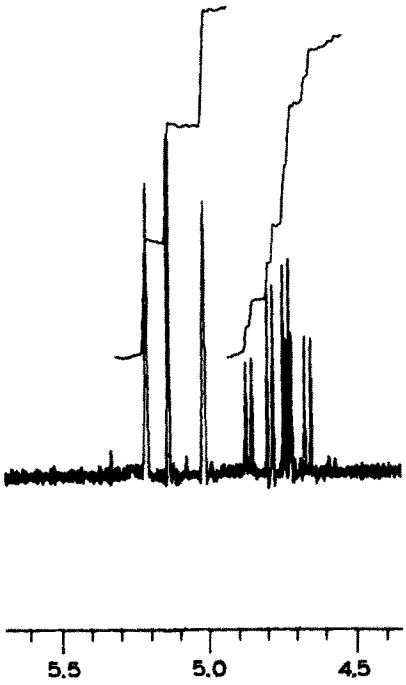


Fig. 2. ^{1}H-N.m.r. spectrum (anomeric region) of the acidic polymer from *S. marcescens* C.D.C. 3607-60. The spectrum for the sample in D_2O was obtained at 400.14 MHz and 80° (internal TSP).

Methylation analysis of the polymer gave the results shown in Table I, column A, and the results obtained when the methylated polymer was reduced prior to hydrolysis are shown in column B. The combined data indicate that the repeating unit of the polymer is a linear pentasaccharide containing one pyranose residue each of L-3-substituted galactose, 3-substituted glucose, 3-substituted mannose, 6-substituted mannose, and 3-substituted glucuronic acid. The data also indicate that about half of the uronic acid residues are also substituted at position 4 by 2-acetamido-2-deoxyglucose. The latter feature, which was responsible for the complexity of the n.m.r. spectra, was confirmed by *N*-deacetylation and deamination of the polymer. The product, which remained polymeric, lacked only 2-amino-2-deoxyglucose of the initial monosaccharide components and, on methylation analysis with reduction of the uronic ester before acid hydrolysis, gave the results shown in Table I, column C. The loss of 2-*O*-methylglucose and the increase in the relative amount of 2,4-di-*O*-methylglucose clearly identify the position of lateral substitution as position 4 of glucuronic acid. The production of a little 2,3,4,6-tetra-*O*-methylgalactose in the methylation analysis was probably a consequence of partial degradation of the polymer during the prolonged base treatment of *N*-deacetylation.

In order to identify the monosaccharide substituted by glucuronic acid, the methylated native polysaccharide was subjected to base-catalysed β-elimination and the product was re-methylated and converted into methylated alditol acetates. The results (Table I, column D), showing that unsubstituted galactopyranose residues are produced by the β-elimination, point to galactose as the monosaccharide adjacent to glucuronic acid. This was confirmed by isolation of the aldobiouronic acid (M_{GlcA} 0.86 at pH 2.7) after partial hydrolysis of the polysaccharide. The ^{1}H-n.m.r. spectrum of the reduced aldobiouronic acid contained a single anomeric

TABLE I

METHYLATION ANALYSIS OF THE ACIDIC O13 POLYSACCHARIDE AND SOME DEGRADATION PRODUCTS[a]

Methylation product[b]	*Relative peak area (g.l.c.)*			
	A	B	C	D
2,3,4,6-Gal			0.26	1.47
2,4,6-Glc	1.00	1.00	1.00	1.00
2,4,6-Man	0.96	1.19	0.92	1.22
2,4,6-Gal	0.72	1.01	0.67	
2,3,4-Man	1.00	1.20	1.02	1.23
2,4-Glc		0.45	0.69	
2-Glc		0.39		
2,3,4,6-GlcNAc	+[c]	+		

[a]Key: *A*, native polymer; *B*, carboxyl-reduced polymer; *C*, *N*-deacetylated, deaminated, and carboxyl-reduced polymer; *D*, product from β-elimination and re-methylation. [b]2,3,4,6-Gal = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol, *etc.* [c]Present but not quantified.

signal at δ 4.55 ($J_{1,2}$ ~8 Hz), showing a β linkage. On the basis of these results, partial structure **1** can be assigned to the (tri)saccharide unit at the branching-point of the polymer.

D-Glc*p*NAc
1
↓
4
→3)-β-D-Glc*p*A-(1→3)-D-Gal*p*-(1→

1

The presence in the main chain of the polymer of only one residue amenable to periodate oxidation (6-substituted mannose) made it possible to determine the monosaccharide sequence by a series of Smith degradations. As expected, half of the mannose and all of the 2-acetamido-2-deoxyglucose were destroyed on treatment of the native polymer with $NaIO_4$. Mild acid hydrolysis of the reduced product gave an oligosaccharide (SD1) with $R_{Stachyose}$ 0.94 (paper chromatography, solvent *A*). In contrast to the parent polymer, the oligosaccharide was homogeneous as judged by its n.m.r. spectra. The ^{1}H-n.m.r. spectrum (Fig. 3) contained four one-proton anomeric signals at δ 5.24 (unresolved), 4.98 (unresolved), 4.80 ($J_{1,2}$ ~8 Hz), and 4.70 ($J_{1,2}$ ~8 Hz), showing that either glucose or galactose was β-linked

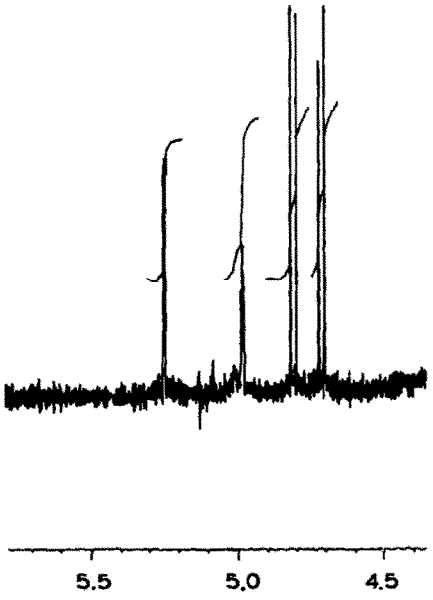


Fig. 3. ^{1}H-N.m.r. spectrum (anomeric region) of the first Smith-degradation product (SD1) of the acidic polymer from *S. marcescens* C.D.C. 3607-60. The spectrum was obtained as for the native polymer (Fig. 2).

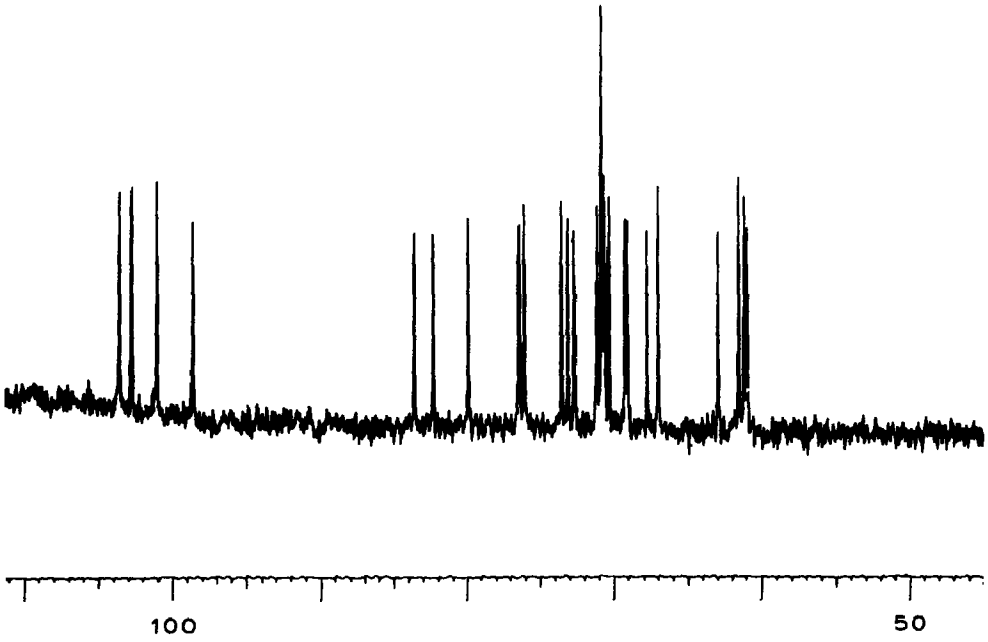


Fig. 4. ^{13}C-N.m.r. spectrum of the first Smith-degradation product (SD1) of the acidic polymer from *S. marcescens* C.D.C. 3607-60. The spectrum was obtained as for the native polymer (Fig. 1). In addition to the signals shown, the spectrum contained a signal for a carbonyl carbon at δ 175.53.

as well as the glucuronic acid. The ^{13}C-n.m.r. spectrum (Fig. 4) contained a total of 26 signals (one being of double intensity), consistent with a composition of three hexoses, a hexuronic acid, and glycerol (derived from 6-substituted mannose).

When product SD1 was subjected to two further Smith-degradations, mannose and glucose were successively destroyed, producing oligosaccharides SD2 and SD3, respectively. The ^{1}H-n.m.r. spectrum of SD2 contained anomeric signals at δ 5.00 (unresolved), 4.77 ($J_{1,2}$ ~8 Hz), and 4.74 ($J_{1,2}$ ~8 Hz), while that of SD3 contained anomeric signals at δ 4.99 (unresolved) and 4.72 ($J_{1,2}$ ~8 Hz). From these results, it could be inferred that the native polysaccharide contained a sequence of partial structure **2**. The loss of the anomeric signals at δ ~5.2 and ~5.1 in the ^{1}H-n.m.r. spectra on going from the native polymer to the degradation product SD2 suggested that both mannose residues were α-linked, and this was confirmed by oxidation of the acetylated polysaccharide with chromium trioxide, during which glucose was the only hexose destroyed. This result also showed that galactose was α-linked (consistent with the ^{1}H-n.m.r. data), while the β configuration could be assigned to the 2-acetamido-2-deoxyglucose on the basis of the ^{1}H-n.m.r. data alone (Table II). Table III gives the assignments of signals in the ^{13}C-n.m.r. spectra for the three Smith-degradation products.

→6)-D-Man*p*-(1→3)-D-Man*p*-(1→3)-β-D-Glc*p*-(1→

2

TABLE II

^{1}H-N.M.R. DATA FOR THE ACIDIC O13 POLYSACCHARIDE AND THE OLIGOSACCHARIDE-ALDITOLS OBTAINED ON THREE CONSECUTIVE SMITH-DEGRADATIONS (SD1, SD2, SD3)

Compound	*^{1}H-N.m.r. data*			
	δ[a]	*$J_{1,2}$ (Hz)*	*Integral*	*Anomeric assignment*
Native polymer	5.22	Unresolved	1.0	→3)-α-Man*p*-(1→
	5.14	Unresolved	1.0	→6)-α-Man*p*-(1→
	5.02	Unresolved	1.0	→3)-α-Gal*p*-(1→
	4.87	8	1.0	→3)-β-Glc*p*-(1→
	4.80	8		
	4.74	8	1.0	→3)-β-Glc*p*A-(1→
	4.73	8		→3,4)-β-Glc*p*A-(1→
	4.67	8	~0.5	β-Glc*p*NAc-(1→
SD1	5.24	Unresolved	1.0	α-Man*p*-(1→
	4.98	Unresolved	1.0	→3)-α-Gal*p*-(1→
	4.80	8	1.0	→3)-β-Glc*p*-(1→
	4.70	8	1.0	→3)-β-Glc*p*A-(1→
SD2	5.00	Unresolved	1.0	→3)-α-Gal*p*-(1→
	4.77	8	1.0	β-Glc*p*-(1→
	4.74	8	1.0	→3)-β-Glc*p*A-(1→
SD3	4.99	Unresolved	1.0	→3)-α-Gal*p*-(1→
	4.72	8	1.0	β-Glc*p*A-(1→

[a]All spectra were recorded at 80° with internal TSP as reference.

TABLE III

^{13}C-N.M.R. DATA[a] FOR THE OLIGOSACCHARIDE-ALDITOLS OBTAINED ON THREE CONSECUTIVE SMITH-DEGRADATIONS OF THE ACIDIC O13 POLYSACCHARIDE

Carbon atom	*Product*	*Chemical shift (p.p.m.)*[a]			
		*α-Man*p	*β-Glc*p	*β-Glc*p*A*	*α-Gal*p
C-1	SD1	100.85	102.60	103.41	98.45
	SD2		102.77	103.55	98.62
	SD3			103.82	98.59
C-3	SD1	70.53	82.13	83.40	79.69
	SD2		76.26	83.68	79.81
	SD3			75.38	79.76

[a]All spectra were recorded at 50° (external tetramethylsilane).

The identification of the structural units **1** and **2**, combined with the anomeric configurations discussed above, allows complete structures to be assigned to the Smith-degradation products SD1 (**3**), SD2 (**4**), and SD3 (**5**), and to the repeating unit (**6**) of the native polymer.

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

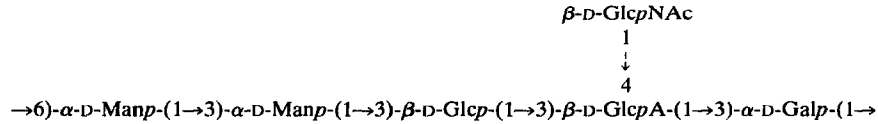
3

β-D-Glc*p*-(1→3)-β-D-Glc*p*A-(1→3)-α-D-Gal*p*-(1→1)-ethylene glycol

4

β-D-Glc*p*A-(1→3)-α-D-Gal*p*-(1→1)-ethylene glycol

5



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Lipopolysaccharide from strain IP 421. — The yield of lipopolysaccharide for this strain was ~12% of the whole cell wall. In addition to the monosaccharide components listed for strain C.D.C. 3607–60, the product contained 2-amino-2-deoxygalactose (8.8%) and ribose. Unlike the lipopolysaccharide from strain C.D.C. 3607–60, the IP 421 product gave a typical S-type "ladder" as well as fast-moving bands on polyacrylamide gel electrophoresis.

After mild acid hydrolysis of the lipopolysaccharide (again accompanied by considerable darkening), a water-soluble polymeric fraction was obtained in 40% yield by chromatography on Sephadex G-50. Further chromatography of this fraction on DEAE-Sepharose provided a neutral polymer and an acidic polymer in approximately equal amounts. The neutral polymer was eluted partly by water but mainly by 0.1M NaCl; the acidic polymer was eluted mainly by 0.2M NaCl, but some was eluted by 0.1M NaCl after the neutral polymer.

(*a*) *Structure of the acidic polymer.* Quantitative analysis of the major fraction gave the following composition: D-mannose, 35.9; D-glucose, 18.1; D-galactose, 17.1; D-glucuronic acid, 12.3; 2-amino-2-deoxy-D-glucose, 5.6%. The minor fraction had the same monosaccharide components, but was not used for quantitative or structural studies. Both the ^{1}H- and ^{13}C-n.m.r. spectra of the polymer were virtually identical to those of the corresponding polymer for strain C.D.C. 3607-60.

Treatment of the polymer with $NaIO_4$ resulted in the destruction of all of the 2-amino-2-deoxyglucose and half of the mannose, but none of the glucose, galactose, or glucuronic acid. Partial acid hydrolysis of the native polymer gave an aldobiouronic acid with the same electrophoretic mobility, composition, and linkage configuration as described for the product isolated from the acidic polymer from strain C.D.C. 3607-60. Thus, the polymers appeared to be identical with repeating-unit **6**, in which about half of the glucuronic acid residues carry the lateral substituent.

(*b*) *Structure of the neutral polymer.* Both fractions of the polymer gave indistinguishable ^{13}C-n.m.r. spectra and had the same monosaccharide composition. The major components were D-ribose (24.9%) and 2-amino-2-deoxy-D-galactose (28.2%, uncorrected), but minor proportions of glucose (4.8%), rhamnose (5.7%), and two heptoses were also present. Evidence that the hexosamine occurred as its *N*-acetyl derivative was provided by bands in the i.r. spectrum at 1640 and 1550 cm^{-1}, signals at δ 174.60 and 22.11 in the ^{13}C-n.m.r. spectrum, and a three-proton singlet at δ 2.04 in the ^1H-n.m.r. spectrum.

That the bulk of the material consisted of a polymer of ribose and 2-acetamido-2-deoxygalactose was apparent from the signals in the anomeric regions of the n.m.r. spectra at δ 106.99 and 95.82 (^{13}C), and at δ 5.25 (unresolved) and 5.12 ($J_{1,2}$ ~3 Hz), equal integrated intensity (^1H). Methylation analysis, using both electron-impact and chemical-ionisation mass spectra to identify the methylated alditol acetates, showed that the polymer consisted of 2-substituted ribofuranosyl and 4-substituted 2-acetamido-2-deoxygalactopyranosyl residues. The anomeric configurations (β and α, respectively) could be assigned on the basis of the n.m.r. data above and a full interpretation of the 13 discrete signals in the ^{13}C-n.m.r. spectrum (Table IV). Thus, the neutral polymer has a disaccharide repeating-unit of structure **7**.

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

7

TABLE IV

^{13}C-N.M.R. DATA[a] FOR THE NEUTRAL POLYMER FROM STRAIN IP 421

Carbon atom	*→4)-α-GalpNAc-(1→*	*→2)-β-Ribf-(1→*
C-1	95.82	106.99
C-2	50.22	79.38
C-3	67.62	70.28
C-4	76.41	82.74
C-5	71.11	62.94
C-6	61.50	
$-NHC(O)CH_3$	174.60	
$-NHC(O)CH_3$	22.11	

[a]The spectrum was recorded at 50° (external tetramethylsilane). Comparable data have been reported elsewhere[3,10].

DISCUSSION

The presence of the acidic polymer with the repeating-unit **6** in both O13 strains studied, and the absence from one of them of the neutral polymer with the repeating-unit **7**, constitutes circumstantial evidence that the acidic polymer is the O13 group antigen. Moreover, the failure to detect an S-type "ladder" on polyacrylamide gel electrophoresis of the lipopolysaccharide from the strain (C.D.C. 3607–60) producing only the acidic polymer indicates that this polymer is not an integral part of the lipopolysaccharide, and that the lipopolysaccharide is of the R-type lacking a side chain. Another indication that the acidic polymer is a separate entity is the failure to detect heptose, a known component of core oligosaccharides in lipopolysaccharides of *S. marcescens*.

The presence of a second (neutral) polymer, of which heptose is a minor component, in strain IP 421 correlates with the different (S-type) pattern obtained on polyacrylamide gel electrophoresis of the lipopolysaccharide. These observations indicate that the neutral polymer constitutes the side chain of the lipopolysaccharide, but is not the O13 group antigen. A similar situation has been described for serogroup O14, where all six strains examined produce an acidic glucomannan[4] (the putative O6/O14 antigen) but also one of three neutral polymers[2,3,6]. One of these polymers, present in the original O14 reference strain (C.D.C. 4444-60), a new reference strain[9] (C.D.C. 1423–74), and a third strain (C.D.C. 874–57) of the same serotype (O14:H12), is the neutral polymer with the disaccharide repeating-unit **7** here reported for strain IP 421. As the polymer also occurs in the O12 reference strain[5], it can reasonably be concluded that it confers the antigenic factor (Co) known[8] to be shared by the O12 and O14 reference strains, and strain IP 421 (O13:H7), but not by the O13 reference strain C.D.C. 3607-60 (O13:H4). The same polymer has also been identified as the O12 antigen of *Pseudomonas aeruginosa*[10], and may be a minor product of the O6 reference strain of *S. marcescens*[5].

EXPERIMENTAL

Growth of bacteria, and isolation and fractionation of the lipopolysaccharides. — *S. marcescens* strains C.D.C. 3607–60 and IP 421 were grown and processed as in related studies[3–7,11]. For strain C.D.C. 3607-60, the yields of wet cells, dry cell walls, and lipopolysaccharide from a 20-L batch culture grown for 16 h at 30° were 233, 7.16, and 1.38 g, respectively. The corresponding values for strain IP 421 were 167, 4.77, and 0.59 g. After mild, acid hydrolysis of the lipopolysaccharides (1% acetic acid, 2.25 h, 100°), the water-soluble products were fractionated by chromatography on Sephadex G-50 and DEAE-Sepharose CL-6B.

General methods. — Chromatography (gel-permeation, ion-exchange, p.c., g.l.c.), electrophoresis on paper or in polyacrylamide gels, and other physicochemical analyses (i.r., n.m.r., m.s.) were carried out essentially as described previously[3–7,11].

Determination of monosaccharide composition. — In general, methods used for the release, identification, and determination of neutral and amino sugars were those used previously[7,11]. Glucuronic acid was identified by p.c. and by paper electrophoresis (pH 2.7) after hydrolysis of polymers with 2M trifluoroacetic acid for 5 h at 105°, followed by rotary evaporation. An aldobiouronic acid produced under these conditions was isolated by passing the hydrolysate down a column of Dowex 1 (AcO^-) resin, eluting the adsorbed acidic products with 2M acetic acid, and separating the disaccharide from free glucuronic acid by chromatography on Sephadex G-15. Total glucuronic acid was determined by the 3-hydroxybiphenyl assay[12]. Monosaccharides were assigned to stereochemical series by g.l.c. of the acetylated oct-2-yl glycosides[13] on a fused-silica capillary column (25 m) of BP1 (S.G.E.). In the cases of hexoses and 2-amino-2-deoxygalactose, the results were confirmed by specific enzymic assays[3,4,6].

Degradative methods. — Periodate oxidation, Smith degradation, and methylation analysis were done as described previously[7]. In some cases, methylated alditol acetates were characterised by their chemical-ionisation mass spectra (with methane as the reagent gas) as well as by the electron-impact spectra. Carboxyl-reduction of acidic polysaccharides was achieved by treating the methylated polymer with $LiBH_4$ in tetrahydrofuran for 4 h at 70°. A β-elimination reaction was carried out on the methylated acidic polymer from strain C.D.C. 3607–60 by an established procedure[14]. The product was re-methylated and converted into methylated alditol acetates as before[7]. Peracetylation of the acidic polymer followed by oxidation with CrO_3 (1 h, 50°) was used to detect β-hexopyranose residues[15]. Recoveries after this treatment were mannose (81%), galactose (82%), and glucose (8%), showing that only the last monosaccharide was β-linked.

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