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LIPOPOLYSACCHARIDE FROM THE O14 TYPE STRAIN OF *Serratia marcescens*: STRUCTURAL STUDIES OF A POLYMERIC FRACTION

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ABSTRACT

The lipopolysaccharide from *Serratia marcescens* C.D.C. 4444-60, the reference strain for O-serogroup 14, has been analysed. All of the phosphorus of the lipopolysaccharide appeared to be present in the lipid A region as orthophosphoric mono- and di-ester residues. Mild hydrolysis of the lipopolysaccharide with acid released orthophosphate, a 3-deoxy-2-octulosonic acid, and an unidentified related compound. The major components of the core oligosaccharide were probably D-glucose and L-*glycero*-D-*manno*-heptose; lesser components included the 3-deoxy-2-octulosonic acid, D-*glycero*-D-*manno*-heptose, and 2-amino-2-deoxyglucose. Products of high molecular weight included a polysaccharide having the disaccharide repeating-unit →4)-α-D-Gal*p*NAc-(1→2)-β-D-Rib*f*-(1→. The same polysaccharide was isolated from the lipopolysaccharide of a second O14 strain of *S. marcescens*, but was absent from a third. All three lipopolysaccharides contained polymeric material rich in glucose and mannose.

INTRODUCTION

In recent years, *Serratia marcescens* has emerged as an opportunist pathogen of some importance, which has stimulated the refinement of serological-typing schemes for use in epidemiological investigations. As for other Gram-negative organisms, typing of the heat-stable O-antigen (of which some 20 serogroups have been identified) seems to be preferred for the primary classification of an isolate[1,2]. Nevertheless, the existence of serological cross-reactions and the prevalence of some serogroups (notably O14) frequently demand the additional application of other typing criteria in clinical studies.

At present, information on the surface carbohydrates of *S. marcescens* is both sparse and confusing[3,4], although the usual association of O-antigenicity with lipopolysaccharides is assumed to apply to this species as to other Gram-negative bacteria. No systematic study of the chemistry of the O-antigens has yet been reported, although structures have been obtained for the putative O-specific polysaccharides from three

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strains[5–7]. We now describe structural features of the lipopolysaccharide from the O14 type strain of *S. marcescens*, and make comparisons with the lipopolysaccharides from two other O14 strains.

RESULTS AND DISCUSSION

Lipopolysaccharide was extracted from whole cells and from isolated cell-walls of the reference strain of *S. marcescens* O14:H12 (C.D.C. 4444-60), but only the product from cell walls was used in structural studies. The latter product contained 2-amino-2-deoxyglucose (glucosamine) (2.2%), 2-amino-2-deoxygalactose (galactosamine) (5.2%), major amounts of glucose, ribose, and L-*glycero*-D-*manno*-heptose (or its enantiomer), and smaller amounts of rhamnose, mannose, and D-*glycero*-D-*manno*-heptose (or its enantiomer). Its ^{31}P-n.m.r. spectrum contained three signals, with δ +3.02, −1.39, and −2.34 at apparent pH 11.1 and relative peak-areas in the ratios of ~1:3:2. The δ values, together with the upfield shift of ~4 p.p.m. for the first signal only, on acidification to apparent pH 4.6, indicated that the first signal corresponded to a phosphomonoester residue and the others to phosphodiesters. No signals attributable to pyrophosphate residues were detected. The lipopolysaccharide extracted from whole cells differed from the cell-wall product in its enhanced contents of glucosamine (5.7%) and galactosamine (8.6%), and in the virtual absence of mannose (material containing mannose remained in the supernatant fractions during purification of the lipopolysaccharide by ultracentrifugation).

As noted for the lipopolysaccharide from *S. marcescens* N.C.T.C. 1377 (ref. 7), mild hydrolysis of the cell-wall lipopolysaccharide (1% acetic acid, 100°, 2.25 h) gave a dark-brown suspension, from which crude lipid A and polysaccharide fractions were recovered (respective yields, 17 and 60%). Chromatography of the water-soluble products on Sephadex G-50 and G-10 provided polymeric material (F1), an oligosaccharide fraction (F2), and monomeric degradation-products (F3), representing 60, 27, and 13%, respectively, of the recovered material. The only significant components of fraction F3 were P_i, a 3-deoxy-2-octulosonic acid (KDO), and another compound (*X*) reactive towards periodate–thiobarbituric acid. The KDO was indistinguishable from reference 3-deoxy-D-*manno*-2-octulosonic acid as judged by paper chromatography (solvents *B* and *C*), paper electrophoresis (pH 5.3 and 2.7), and reactivity (colour yield and λ_{max}) in three colorimetric assays.

Compound *X* had a mobility of ~0.8 relative to KDO in paper chromatography (solvents *B* and *C*) and paper electrophoresis (pH 5.3), did not react with ninhydrin, and did not contain phosphorus. The latter results eliminated the phosphorylethanolamine derivative of KDO[8] as a candidate for Compound *X*. Other results indicated that *X* was not a simple glycosyl derivative of KDO: no neutral or amino sugar, nor KDO, was detected after appropriate acid hydrolysis. The spectrum of the products given by *X* in the periodate–thiobarbituric acid reaction was the same as that given by free KDO, and prior treatment with acid only decreased the colour yield. Thus, treatment with 0.1M HCl at 100° for 30 min caused a 29% loss in colour yield, while

TABLE I

COMPOSITION OF THE CORE-OLIGOSACCHARIDE FRACTION F2 FROM THE LIPOPOLYSACCHARIDE OF STRAIN C.D.C. 4444-60

Component	*Content (%)*		
Phosphorus	0.3		
Total carbohydrate[a]	66		
Total heptose[b]	35		
L-*glycero*-D-*manno*-Heptose[c]	32.4		
D-*glycero*-D-*manno*-Heptose[c]	8.7		
D-Glucose	22.2[b]	20.2[c]	20.0[d]
Ribose[c]	2.8		
Rhamnose[c]	1.1		
Glucosamine	5.8		
Galactosamine	3.2		
KDO	0.5[e]	8.0[f]	

[a]Determined by the phenol–H_2SO_4 method and expressed as glucose. [b]Determined by the cysteine–H_2SO_4 method. [c]Determined by g.l.c. of the alditol acetate. [d]Determined by using D-glucose oxidase. [e]Determined by the periodate–thiobarbituric acid method. [f]Determined by the diphenylamine method.

the loss under more drastic conditions (M HCl, 100°, 0.5–4 h)[9] closely paralleled that for free KDO (75% after 4 h). The apparent KDO content of *X* was the same by both the periodate–thiobarbituric acid and the diphenylamine assays, indicating the absence of substitution at positions 4 and 5 in the presumed KDO residue. In the diphenylamine reaction for *X*, there was a bathochromic shift of λ_{max} from 425 nm (KDO) to 438 nm, which could not be attributed to any known neutral-sugar component of the lipopolysaccharide. Whatever the identity of *X*, it does not appear to be a degradation product of KDO, but has been found in mild, acid hydrolysates of all four lipopolysaccharides of *S. marcescens* examined in this laboratory.

Analyses of fraction F2 (Table I) were consistent with its description as a core oligosaccharide. Similar compositions have been reported for the corresponding products from *S. marcescens* strains O8 and Bizio[10] and strain N.C.T.C. 1377 (ref. 7). The virtual absence of phosphorus from fractions F1 (below) and F2 indicated that the signals detected in the ^{31}P-n.m.r. spectrum of the parent lipopolysaccharide arose from residues in the lipid A moiety. The results of further studies of fraction F2 will be reported separately.

Fraction F1, obtained by chromatography of the crude polysaccharide on Sephadex G-50, gave the following analyses: phosphorus, 0.03%; total carbohydrate (as glucose), 69%; galactosamine, 20.1%; glucosamine, 2.2%; D-glucose, 17.2%; ribose, 16%; mannose, 6.0%; L-*glycero*-D-*manno*-heptose, 3.9%; rhamnose, 3.2%; D-*glycero*-D-*manno*-heptose, <1%. The i.r. spectrum contained major amide-bands at 1640 and 1555 cm^{-1}, and a lesser ester-band at 1730 cm^{-1}. The ^{13}C-n.m.r. spectrum supported the inference of *N*- and *O*-acetyl groups, and also indicated that the major constituent of fraction F1 was a polymer having a disaccharide repeating-unit. This

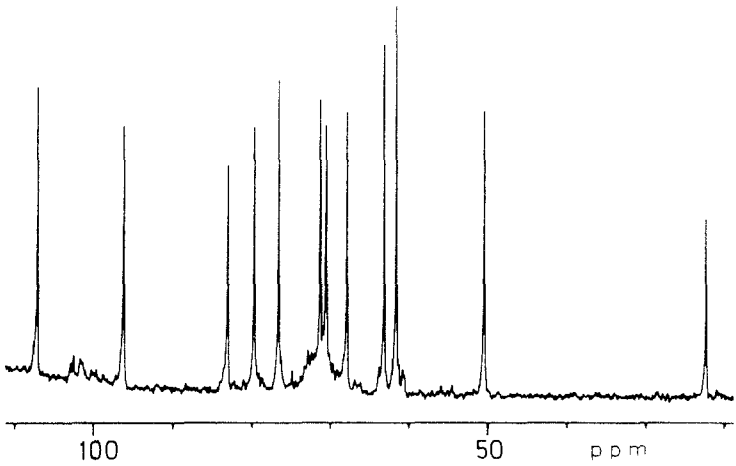

Fig. 1. ^{13}C-N.m.r. spectrum for fraction F1b from the lipopolysaccharide of strain C.D.C. 4444-60. The spectrum for the sample in D_2O was obtained at 100.62 MHz and 50° with complete proton-decoupling. In addition to the signals shown, the spectrum contained a signal for a carbonyl carbon at δ 174.77 with reference to external tetramethylsilane.

polymer (fraction F1b) was separated from the contaminating carbohydrate (fraction F1a) by chromatography on Sephadex G-75: F1a was eluted as a sharp peak soon after the void volume of the column, whereas F1b gave a later, broad peak. Both fractions had qualitatively similar monosaccharide compositions, but F1a was enriched in glucose and mannose, and F1b contained D-galactosamine (34.0%), D-ribose (20.4%), D-glucose (7.3%), L-*glycero*-D-*manno*-heptose (5.1%), and small proportions of the remaining monosaccharides found in whole F1. It is likely that the minor components of F1b are at least partly explained by the presence of a terminal core-oligosaccharide. The ribose content of F1b was almost certainly underestimated, as acid hydrolysates contained a basic product with M_{GalN} 0.62 at pH 5.3, which reacted with ninhydrin, alkaline silver nitrate, and aniline hydrogenoxalate. A compound having the same properties was also detected when a mixture of ribose and galactosamine was examined by electrophoresis (the amount apparently increased when the mixture was treated with acid under the conditions used to hydrolyse F1b).

Fraction F1b had $[\alpha]_D$ +112° (*c* 1.8, water), and gave n.m.r. spectra consistent with a polymer based on a disaccharide repeating-unit of ribose and *N*-acetylgalactosamine residues. The ^{1}H-n.m.r. spectrum included one-proton anomeric signals at δ 5.23 and 5.11 (each with a low value of $J_{1,2}$) and a methyl singlet at δ 2.02 (*N*-acetyl). The ^{13}C-n.m.r. spectrum (Fig. 1) contained signals for the *N*-acetyl group at δ 174.77 and 22.27, together with anomeric signals at δ 107.19 ($^1J_{CH}$ 179 Hz) and 96.08 ($^1J_{CH}$ 172 Hz). The former anomeric signal can be assigned to C-1 of a β-ribofuranosyl residue[11,12], and the latter to C-1 of a 2-acetamido-2-deoxy-α-galactopyranosyl residue. The ^{13}C-n.m.r. spectrum also contained signals at δ 63.12 and 61.64 which showed that the primary hydroxyl group in each residue was unsub-

TABLE II

ASSIGNMENT OF SIGNALS[a] IN THE ^{13}C-N.M.R. SPECTRUM OF FRACTION F1b FROM THE LIPOPOLYSACCHARIDE OF STRAIN C.D.C. 4444-60

Carbon atom	*→4-α-GalpNAc-1→*	*→2-β-Ribf-1→*
C-1	96.08	107.19
C-2	50.41	79.65
C-3	67.86	70.52
C-4	76.59	83.01
C-5	71.30	63.12
C-6	61.64	
-NHC(O)CH_3	174.77	
-NHC(O)CH_3	22.27	

[a]Chemical shifts are given in p.p.m. downfield from external tetramethylsilane.

stituted. The actual linkage positions were identified by methylation analysis, monitored by g.l.c. and by g.l.c.–m.s. of the methylated alditol acetates labelled with deuterium at C-1. Only two major products were obtained, the first of which could be distinguished from that from a 3-substituted ribofuranosyl residue by g.l.c. (column II) and gave a mass spectrum (primary fragments at *m/z* 190 and 161) diagnostic for a 1,2,4-tri-*O*-acetyl-3,5-di-*O*-methylpentitol-*1-d*. The second product was identified from its mass spectrum (primary fragments at *m/z* 233, 203, and 159) as 1,4,5-tri-*O*-acetyl-2-deoxy-3,6-di-*O*-methyl-2-*N*-methylacetamidogalactitol-*1-d*. Structure **1** could therefore be assigned to the repeating unit of fraction F1b. Although periodate was consumed (2.6 μmol.mg^{-1}) by F1b, the molar ratio of *N*-acetylgalactosamine:ribose (1.00:0.72) remained unchanged and at least 74% of each sugar was recovered. Oxidation must therefore have been confined to terminal residues and/or the minor sugar components of F1b. Structure **1** was also supported by a full interpretation of the ^{13}C-n.m.r. spectrum for F1b. Assignments for the ribose residue (Table II) were based on those described for 2-*O*-alkyl derivatives of methyl β-D-ribofuranoside[12], and assignments for the *N*-acetylgalactosamine residue were in satisfactory agreement with those made for similar 4-substituted residues in other polysaccharides[13,14].

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

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In order to test the possibility that fraction F1b corresponded to the O14 specific polysaccharide of *S. marcescens*, lipopolysaccharides from two other O14 strains of the organism were also extracted and fractionated. The results obtained for strain C.D.C. 874–57, which is the same serotype (O14:H12) as the reference strain C.D.C. 4444-60, closely resembled those described above. By chromatography on Sephadex G-75, the polymeric water-soluble products could be separated into a fraction rich

in glucose and mannose (equivalent to F1a), and another fraction rich in ribose and galactosamine but also containing small proportions of other sugars (equivalent to F1b). The ^{13}C-n.m.r. spectrum of the second fraction was identical to that shown in Fig. 1 for F1b. By contrast, the lipopolysaccharide from strain C.D.C. 1783-57 (serotype O14:H9) contained neither ribose nor galactosamine, and the polymeric water-soluble products gave only one, broad, tailing peak on Sephadex G-75. However, analysis of early fractions of this peak showed only glucose and mannose as major components, whereas later fractions were rich in glucose, galactose, and glucosamine, but contained only a little mannose.

The above findings show that fraction F1b cannot represent the O14 specific polysaccharide of *S. marcescens*, and are in accord with previous evidence[3,4] that at least some strains of the organism can produce a complex range of cellular and extracellular polysaccharides. Of special interest is the observation[3,4] that acidic glucomannans have been found as extracellular, capsular, and cellular products. It may be recalled that the mannose-containing material in aqueous phenol extracts of whole cells of strain C.D.C. 4444-60 was not deposited by ultracentrifugation. The possibility that the glucomannans present in the cell-wall "lipopolysaccharides" of the three O14 strains of *S. marcescens* used in the present study represent their common (O) antigen is being explored by both chemical and serological methods.

EXPERIMENTAL

Growth of bacteria, and isolation and fractionation of lipopolysaccharides. — Cells of *S. marcescens* strains C.D.C. 4444-60, 874-57, and 1783-57 were grown under conditions described previously[7] and used for the preparation of cell walls. Lipopolysaccharides were extracted by the aqueous phenol method from whole cells (C.D.C. 4444-60 only) and defatted cell-walls (all three strains), and were purified by standard methods[7,15]. After mild, acid hydrolysis (1% acetic acid, 100°, 2.25 h), the water-soluble products from the lipopolysaccharides were fractionated[7] on columns of Sephadex G-75, G-50, and G-10.

Chromatographic and electrophoretic methods. — The following solvent systems were used for p.c.: *A*, ethyl acetate–pyridine–water (13:5:4); *B*, ethyl acetate–pyridine–acetic acid–water (5:5:1:3); and *C*, ethanol–acetic acid–water (80:1:19). The pyridine–acetic acid buffer systems (pH 5.3 or 2.7) used for paper electrophoresis have been described[16], as have the detection reagents used for chromatograms and electrophoretograms[7]. For g.l.c., glass columns, packed with stationary phases of Silar 10c (I), OS-138 (II), or OV-225 (III), and a fused-silica capillary column of SE-54 (IV) were used.

Analysis of neutral monosaccharides. — Samples were hydrolysed at 105° with 2M HCl (2 h) or M HCl (4 h), and the hydrolysates were neutralised with Dowex 1 (HCO_3^-) resin and then deionised. Monosaccharides were identified by p.c. (solvent *A*) and by g.l.c. of the alditol acetates (column I). Confirmation was provided by g.l.c.–m.s., notably for the 1,6-anhydro derivative of L-*glycero*-D-*manno*-heptose

formed during the acid hydrolysis[17]. Quantitative data were obtained by g.l.c. (using inositol hexa-acetate as the internal standard, and a calibration mixture of monosaccharides "hydrolysed" alongside the sample), by using D-glucose oxidase (EC 1.1.3.4), and by the cysteine–H_2SO_4 reaction[18] (for glucose and total heptose). The phenol–H_2SO_4 reaction[19] was used to estimate total carbohydrate with glucose as the standard. D-Ribose was identified chromatographically[20].

Analysis of hexosamines. — Glucosamine and galactosamine, released by hydrolysis of samples with 6.1M HCl at 105° for 4 h, were identified by the following methods: p.c. (solvent *B*), paper electrophoresis (pH 5.3), g.l.c. (column III) of the aminodeoxyhexitol acetates, ninhydrin degradation[21] followed by p.c. (solvent *A*) of the aldopentoses, and deamination[22] followed by reduction and p.c. (solvent *A*) of the 2,5-anhydrohexitols. Autoanalysis provided confirmation and quantitative data; D-galactosamine was identified and estimated by enzymic assay[23].

Analysis of KDO and Compound X. — These products were examined by p.c. (solvents *B* and *C*), paper electrophoresis (pH 5.3 and 2.7), and colorimetric assays with periodate–thiobarbituric acid[9,24], diphenylamine[25], and semicarbazide[26]. In the case of KDO, the reference compound used in the colorimetric assays had been subjected to the same treatments as the isolated product (heating with 1% acetic acid, chromatography on Sephadex G-50, and preparative p.c.), to reproduce the degradative changes[8,9]. In the case of *X*, the response in the periodate–thiobarbituric assay after "hydrolysis" under various conditions[9] was determined.

Other methods. — Phosphorus was determined by a modification of the method of Bartlett[27], and periodate by the method of Avigad[28]. The oxidation of fraction F1b with 50mM sodium periodate was carried out at 4° for 6 days, and the methylation analysis of F1b was done by standard methods[29,30]. G.l.c.–m.s. of (methylated) alditol acetates was carried out with columns I or IV. The optical rotation of fraction F1b was determined with a Bendix polarimeter model 143A. N.m.r. spectra (^1H, ^{13}C, and ^{31}P) were obtained with a Bruker WH-400 spectrometer as described previously[7,31].

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