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Note

Structure of the acidic microcapsular glycan from the reference strain (C.D.C. 6320-58) for *Serratia marcescens* serotype O14:K12

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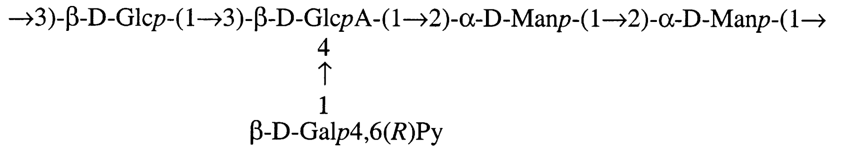
Abstract

The acidic polysaccharide from *Serratia marcescens* serogroup O14:K12 was analyzed by means of chemical studies and NMR spectroscopy and its repeating unit structure found to be



$$\begin{array}{rcl} \rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 3)\text{-} & \beta\text{-D-Glc}p\text{A} & \text{-}(1\rightarrow 2)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow 2)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow \\ & 4 & \\ & \uparrow & \\ & 1 & \\ & \beta\text{-D-Gal}p4,6(R)\text{Py} & \end{array}$$

O-Acetyl groups are proposed to be present in non-stoichiometric amounts on O-6 on one of the hexose residues in the main chain. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Serratia marcescens; Acidic surface glycan; O-Antigen

Of the 29 reference strains for the known heat-stable surface antigens of *Serratia marcescens*, 16 produce both neutral and acidic glycans, 1 produces only acidic glycan, 2 lack both O-antigen and acidic glycan, and the remaining 10 produce only neutral glycans [1]. The neutral polymers are the true O-antigens [side chains of the lipopolysaccharides (LPSs)], while the acidic glycans appear to be microcapsular, K-type antigens. The latter are serodominant, and define 16 of the 29 recognized serogroups in a hybrid scheme with which cross reactions are frequently observed in agglutination tests [2]. The serodifferentiation and the cross-reactions between the O12 and O14 reference strains [3,4] can be explained by the presence in each of a different acidic glycan, but the same neutral glycan. The neutral polymer [5,6] has a disaccharide repeating-unit, **1**. The O14 (O14:K14) acidic glycan is a partially acetylated glucomannan [5] with a branched repeating-unit, **2** (the *O*-acetyl group was not located), while the O12 (O14:K12) acidic polymer was diagnosed in a preliminary study [7] as a galactoglucomannan.

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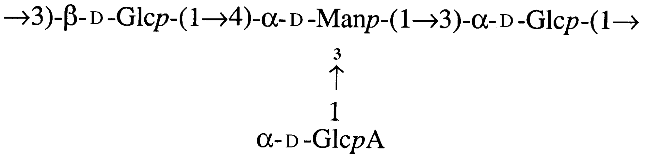
$\rightarrow 4)\text{-}\alpha\text{-D-Gal}p\text{NAc-}(1\rightarrow 2)\text{-}\beta\text{-D-Rib}f\text{-}(1\rightarrow$

1



$$\begin{array}{ccc} \rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-} & \alpha\text{-D-Man}p & \text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow \\ & 3 & \\ & \uparrow & \\ & 1 & \\ & \alpha\text{-D-Glc}p\text{A} & \end{array}$$

2

Phenol–water extraction of a 20 L culture of *S. marcescens* gave 2.5 g of the acidic glycan in an admixture with LPS. The acidic glycan was obtained by precipitation with cetyltrimethylammonium bromide. After dissolution in 3 M NaCl and precipitation with ethanol a more than 90% pure acidic glycan in a yield of 10% was obtained. Attempts to purify the polysaccharides by dilute acetic acid treatment (to cleave off the lipid A residue thereby obtaining the O-chains separable from the acidic glycan by GPC or ion-exchange chromatography) followed by gel filtration were partly successful, but resulted in significant removal of pyruvate as evident from the ^{1}H NMR spectra of the acidic polymer before and after the split.

The neutral monosaccharide components found in a hydrolysate were Man, Glc and Gal in the ratios 47:28:30, as analyzed by GLC of the alditol acetates. Enzymatic assays confirmed the ratios and showed that each hexose was the D-isomer. The presence of a hexuronic acid ($\sim$ 10%) was indicated by a colorimetric assay, and GlcA was identified by paper chromatography (PC) and high-voltage electrophoresis (HVE). The absolute configuration of D-GlcA was determined using GLC of the (+)-2-octyl glycoside acetates [8]. Only a minor amount of hexosamine was detected. The presence of *O*-acetyl signals was indicated in the ^{1}H NMR spectrum (Fig. 1) by a signal at δ 2.20 (1.5 H). A signal for a pyruvate group was observed in the spectrum at δ 1.50 (3 H [9]) and pyruvic acid in hydrolysates was detected by both enzymatic assay and HVE.

Five major anomeric signals were detected (each $\sim$ 1 H) for the acidic glycan in the ^{1}H NMR spectrum, at δ 5.43, 5.21 (both not resolved), δ 4.98, 4.65 and 4.58 (all d, $J_{1,2}$ 7.3–7.4 Hz). Smaller broad signals were at-

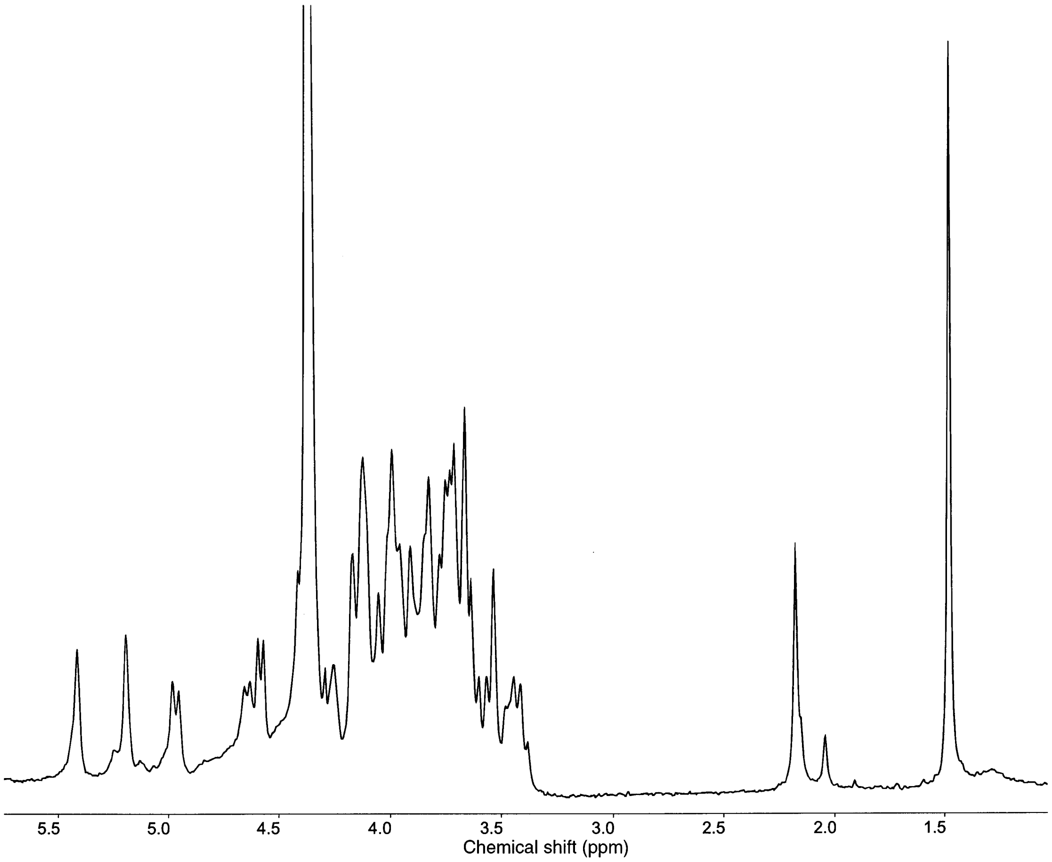


Fig. 1. 600-MHz ^{1}H NMR spectrum of *S. marcescens* serotype O14:K12 microcapsular glycan.

Table 1
^{1}H and ^{13}C NMR chemical shifts for the *S. marcescens* O14:K12, acidic glycan. Additional signals for pyruvate are at δ_H 1.50, δ_C 26.0 (CH_3), δ_C 101.7 (C) and δ_C 176.8 (COOH) [a]

Sugar residue	Chemical shift (δ)					
	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6/C-6
→2)-α-D-Man*p*-(1→	5.43	4.14	4.02	3.76	3.94	
A	(n.r.)					
	101.0	79.2	71.2	67.8	71.8	61.7
→2)-α-D-Man*p*-(1→	5.21	4.26	3.91	3.74	3.84	
B	(n.r.)					
	100.4	78.4	70.6	67.8	74.0	61.7
→3)-β-D-Glc*p*-(1→	4.98	3.42	3.68	3.58	3.48	3.77 3.93
C	(7.4)					
	102.2	73.2	83.0	71.3	76.6	61.4
→3)-β-D-Glc*p*A-(1→	4.65	3.72	4.13	4.13	4.01	
D 4	(7.3)					
↑	102.0	74.2	79.9	77.4	77.4	176.0
β-D-Gal*p*4,6(*R*)Py-(1→	4.58	3.66	3.70	4.18	3.55	3.99 4.09
E	(7.4)					
	102.4	71.8	72.6	71.8	67.3	65.7

[a] $^{3}J_{H,H}$ values are shown in parentheses. n.r., not resolved.

tributed to the neutral glycan. The partial substitution of the *O*-acetyl group did not significantly affect the spectrum as no additional signal from the acetoxylated site could be found and as no splitting of anomeric signals was observed. This suggested that the *O*-acetyl group was located on a hexose at position 6. Further support for this came from a minor carbon signal at δ 64.2 assigned to an acetylated hydroxymethyl group. The signals also disappeared on treatment with alkali showing that they came from acetate esters. Anomeric signals in the ^{13}C NMR spectrum of the intact polymer were found at δ 102.4 ($^{1}J_{C,H}$ 162 Hz), 102.2 ($^{1}J_{C,H}$ 165 Hz), 102.0 ($^{1}J_{C,H}$ 167 Hz), 101.0 ($^{1}J_{C,H}$ 177 Hz) and 100.4 ($^{1}J_{C,H}$ 175 Hz). An additional signal at δ 101.7, was assigned to the pyruvate acetal carbon, as indicated from its absence in the HSQC spectrum. Three signals for unsubstituted hydroxymethyl carbons with δ values in the range 61.7–61.4 were also observed. The signals for the *O*-acetyl and pyruvate methyl groups were found at δ 20.9 and 26.0, respectively, thereby also defining the configuration of the pyruvate acetal carbon as *R* [10].

On methylation analysis of the glycan, with carboxyl reduction after the methylation using triethylborodeuteride, three peaks were detected by GLC of the partially methylated alditol acetates. In the first peak the derivative from a 2-substituted hexopyranose residue co-eluted with that from a 3-substituted hexopyranose residue. The configuration of the sugars was evident from the NMR spectra (see below) and was *manno* and *gluco*, respectively. The second peak in the methylation analysis corresponded to a 4,6-disubstituted galactose residue, attributed to a terminal sugar residue carrying pyruvic acid. NMR evidence (given below) supported this. The last peak in the methylation analysis, a 2,6-di-*O*-methylhexose, corresponded to a 3,4-disubstituted glucuronic acid residue, i.e., a branch point residue and contained the expected deuterium atoms. From these results it was indicated that five monosaccharides were present in the repeating unit, two mannose residues and one residue each of glucose, galactose and glucuronic acid.

The greater part of the ^{1}H and ^{13}C NMR spectra were assigned using COSY, TOCSY, and HSQC spectra and the residues are coded as **A**–**E** (Table 1). The two residues **A** (δ 5.43) and **B** (δ 5.21) had *manno* configuration and were α-linked judging by their high δ value, and their $J_{H\text{-}1,H\text{-}2}$ and $J_{C\text{-}1,H\text{-}1}$ values. They were assigned as 2-substituted from significant glycosylation shifts of the corresponding carbon signals (δ 79.2 and 78.4). The three remaining sugars had $J_{1,2}$ values and $J_{C\text{-}1,H\text{-}1}$ values correspondent to β configuration. The signals from residue **E** (δ 4.58), had chemical shifts close to those of methyl β-galactoside-4,6-pyruvate [10] and hence it was assigned to a terminal galactose residue. Thus, the two remaining sugars, **C** and **D** must be glucose and glucuronic acid. They had the *gluco* configuration as followed from the size of the cross peaks in the COSY spectrum which were large (Fig. 2). Residue **C** could be assigned to the glucose residue as it had signals corresponding to seven protons in the spin system, and consequently **D** was the glucuronic acid residue. The linkage positions were established from large displacements of signals from C-3 (δ 83.0) for the glucose residue, and from C-3 (δ 79.9) and C-4 (δ 77.4) for the glucuronic acid residue. Assignment of glucuronic acid signals was difficult because of overlap in the ^{1}H NMR region δ 4.01–4.13. The signals for H-2 and H-3 were assigned via the COSY and the relayed COSY spectrum, respectively. However, overlap at δ ~4.13, made the assignment of signals from C-3 and C-4 ambiguous. From the HSQC–TOCSY spectrum, on the δ 79.9 trace, only three ^{1}H NMR chemical shifts for ring proton signals were observed, at δ 4.13, 4.01 and 3.72, indicating an overlap of signals. The HSQC spectrum contained five ^{13}C NMR signals in the region 77–80 ppm, two of which could be assigned to the mannose linkage carbons in residues **A** and **B** and one to C-5 in **D**. A HMBC correlation between δ_C 176.0 and δ_H 4.01 aided the assignment of signals from C-6 and H-5. Then, two signals remained at δ 4.13/77.4 and 4.13/79.9, in agreement with the HSQC–TOCSY spectrum. Consequently, two ^{1}H NMR signals from the GlcA residue appear at δ 4.13. The overlap made the assignment of C-3 and C-4 difficult. To resolve the ambiguity for C-3 and C-4 signals a HSQC spectrum at pH 1 was recorded, whereby the signal for C-5 appeared at δ 74.4 (instead of δ 77.4) and the signal for C-4 at δ 75.4, establishing that the C-4 signal is at δ 77.4 at neutral pH.

The sequence of residues was determined from the following inter-residual long-range ^{13}C,^{1}H-correlations, between anomeric protons and linkage carbons observed in an HMBC spectrum: **A**–**C** (δ_H 5.43 and δ_C 83.0), **B**–**A** (δ_H 5.21 and δ_C 79.2) **C**–**D** (δ_H 4.98 and δ_C 79.9), **D**–**B** (δ_H 4.65 and δ_C 78.4), and **E**–**D** (δ_H 4.58 and δ_C 77.4). Further corroboration for the establishment of the **A**–**C** ele-

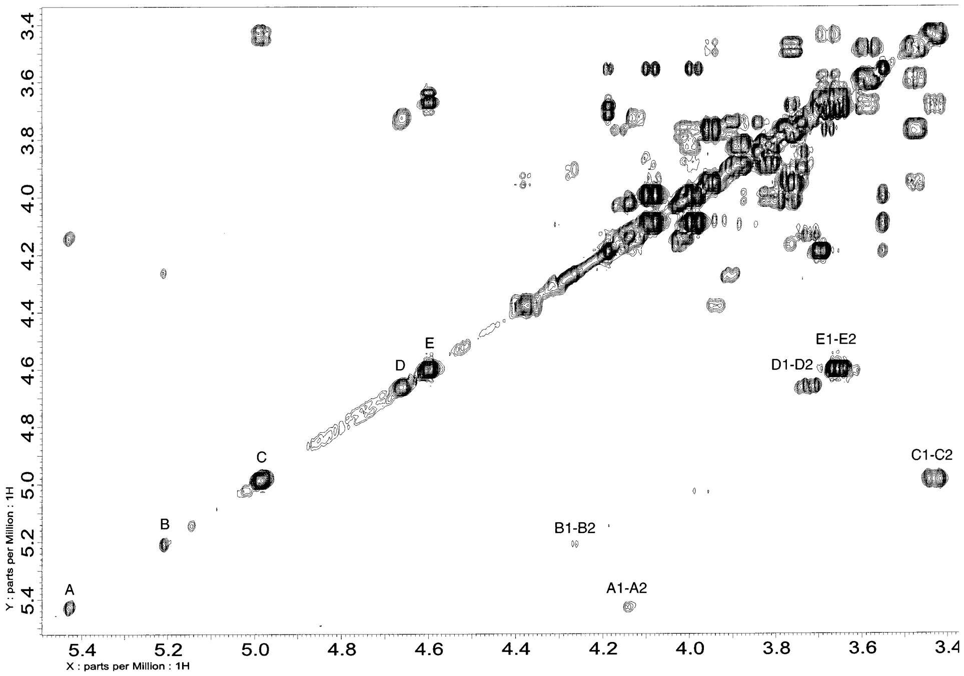
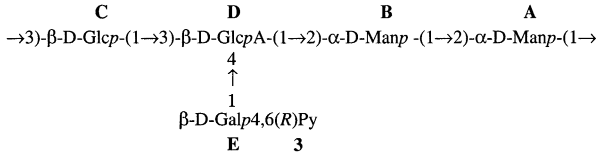


Fig. 2. The 3.4–5.5 ppm region of the 600 MHz COSY spectrum of the *S. marcescens* serotype O14:K12 microcapsular glycan.

ment was obtained from a correlation between δ_C 101.0 and δ_H 3.68 (C-1 in **A** and H-3 in **C**). This defines all five glycosidic linkages and the structure can be postulated as **3**.



$$\begin{array}{llll}
\mathbf{C} & \mathbf{D} & \mathbf{B} & \mathbf{A}\\
\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow & 3)\text{-}\beta\text{-D-Glc}p\text{A-}(1\rightarrow & 2)\text{-}\alpha\text{-D-Man}p\text{ -}(1\rightarrow & 2)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow\\
 & 4 & & \\
 & \uparrow & & \\
 & 1 & & \\
 & \beta\text{-D-Gal}p4,6(R)\text{Py} & & \\
 & \mathbf{E} & &
\end{array}$$

3

Strong inter-residual NOE correlations between anomeric protons and protons on linkage carbons were observed inter alia for the elements **A**–**C** (δ 5.43/3.68) and **B**–**A** (δ 5.21/4.14). The latter correlation is ambiguous as the signals for H-3 and H-4 in **D** both appear at δ 4.13, i.e., close, but is in agreement with HMBC data. A weak correlation between H-1 in **C** and H-3 in **D** (δ 4.98/4.13) was also observed. Two correlations were observed for the **D**–**B** linkage, namely from H-1 to H-2 (δ 4.65/4.26) and from H-1 to H-1 (δ 4.65/5.21). This is typical for the kind of stereochemistry of this linkage [11,12]. A correlation between the anomeric proton in **B** and the equatorial H-1 proton next to the linkage in **A**, corroborating the element **B**–**A**, was also observed (δ5.21/5.43). All these data are consistent with structure **3**. In addition, it is proposed that the polysaccharide contains *O*-acetyl groups in non-stoichiometric amounts, at position 6 of a glucose or mannose residue.

The glycan is unusual among *Serratia* capsule polysaccharides in that it has two sources of acidity, both a uronic acid and a pyruvate group. The dual acidity in a glycan from *S. marcescens* has been found only once earlier, for the O18 serotype [13]. Pyruvate has also been found only once previously in *S. marcescens*, namely in O4 [14].

1. Experimental

Growth of bacteria, and isolation of the acidic glycan.—*S. marcescens* strain C.D.C. 6320-58 was grown in a 20-L batch cultures as described [6]. The polysaccharide material (2.5 g) was extracted from defatted cell walls by the aq phenol method [15]. The crude extract was dissolved in deionized water and the acidic polysaccharide was precipitated with an aq 5% solution of cetyltrimethylammonium bromide. The precipitate was collected by centrifugation (3000 rpm), dissolved in 3 M NaCl, the polysaccharide precipitated in EtOH, and the material collected by centrifugation. After dissolution in water, dialysis and lyophilization, the acidic polysaccharide (~10%), containing small amounts of the neutral glycan, was obtained.

General methods.—Hydrolysis of native and methylated O14:K12 was accomplished with 2 M CF_3CO_2H at 120 °C for 2 h. The sugars in the hydrolysates were converted into alditol acetates and partially methylated alditol acetates. Methylation was carried out with methyl iodide in Me_2SO in the presence of sodium methylsulfinylmethanide for 12 h [16]. Carboxyl reduction of methylated polysaccharide was performed by treatment with 1 M lithium triethylborodeuteride in THF at 0 °C for 1 h. GLC separations of alditol acetates were carried out with a fused-silica capillary column (25 m × 0.25 mm) of DB-5 on an HP5970 instrument, using a temperature program, 160 °C (1 min) → 250 °C at 3 °C/min [17,18]. GLC–MS (EI) was performed on a HP5890 GC-Nermag R10-10H quadrupole MS. Partially methylated alditol acetates were separated on a DB-5 capillary column (25 m × 0.25 mm), using a temperature program, 130 °C (1 min) → 250 °C at 3 °C/min. PC and HVE were carried out on Whatman No. 1 paper, with solvent *A*, 5:5:3:1 EtOAc–pyridine–water–AcOH, or *B*, 13:5:4 EtOAc–pyridine–water, for PC, and buffer *C*, 5:2:43 pyridine–AcOH–water (pH 5.3), or *D*, 1:10:89 pyridine–AcOH–water (adjusted to pH 2.7 with formic acid) [19] for HVE. Detection reagents used were ninhydrin, alkaline $AgNO_3$ and aniline hydrogenoxalate.

Glucuronic acid was identified by PC (solvent *A*) and HVE (buffers *C* and *D*), by colorimetric assay [20] and by GLC of the (+)-2-octyl glycoside [8]. Pyruvic acid was assayed after treatment of the polymer with 1 M HCl at 100 °C for 1 h by HVE (buffer *C*) and enzymatic assay [14].

NMR spectroscopy.—1D NMR spectra (^1H and ^{13}C) were obtained using JEOL EX 270 and Varian Unity 600 spectrometers for samples dissolved in 99.96% D_2O containing 20 mM phosphate buffer at pD 7.4 (pH 7.0). ^1H NMR spectra were recorded at 70 °C with sodium 3-trimethylsilylpropanoate-d_4 as an internal reference (δ 0.00). ^{13}C NMR spectra were recorded at 50 °C with acetone as an internal reference (δ 31.00). The relayed COSY spectra were run with delay times of 30 and 60 ms. The NOESY and TOCSY experiments were run with mixing times of 100 and 200 ms, and 30, 60 and 90 ms, respectively. The HMBC spectrum was obtained with a delay time of 60 ms. The delay times in the HSQC–TOCSY experiment were 50 and 100 ms.

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