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THE STRUCTURE OF THE ACIDIC POLYSACCHARIDE SECRETED BY *Klebsiella aerogenes* TYPE 54 STRAIN A3[*]

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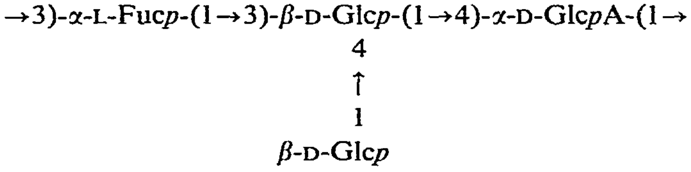
ABSTRACT

The polysaccharide secreted by *Klebsiella aerogenes* type 54 strain A3 was isolated, methylated, the ester carboxyl-reduced, and the product partially hydrolyzed. The resulting, partially *O*-methylated oligosaccharides were reduced and ethylated, and the mixture of products was fractionated by l.c. The l.c. fractions containing per-*O*-alkylated oligosaccharide-alditols were analyzed by e.i.-m.s. Pure per-*O*-alkylated oligosaccharide-alditols were also analyzed by ^{1}H-n.m.r. spectroscopy. The products obtained by base-catalyzed degradation and subsequent ethylation of the per-*O*-methylated polysaccharide were fractionated by l.c. The main product isolated was analyzed by e.i.-m.s., c.i.-m.s., and ^{1}H-n.m.r. spectroscopy. The results of these studies, in conjunction with results of analytical methods commonly used in the elucidation of polysaccharide structures, unambiguously characterized the primary glycosyl structure of the polysaccharide. Base-labile substituents, previously reported to be present in the polysaccharide, were not studied. Structure **1** revises, and complements, previously reported structures.



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

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INTRODUCTION

Several papers have been published[1-6] describing the structure of the acidic polysaccharide secreted by *Klebsiella aerogenes* type 54 strain A3, formerly named *Aerobacter aerogenes* strain A3. The polysaccharide was one of the first bacterial polysaccharides recognized to have a simple repeating-unit[1,2], and it was the first bacterial polysaccharide found to contain formyl groups[5,6]. A structure for the polysaccharide, except for the anomeric configuration of one glycosidic linkage, has been proposed[2]; however, we have found that it is incorrect. Except for alkali-labile substituents, we now report the complete structure of the acidic polysaccharide secreted by *K. aerogenes* type 54 strain A3.

EXPERIMENTAL

The isolated polysaccharide[2] was a gift from Dr. H. E. Conrad of the University of Illinois, Urbana, IL 61801. The polysaccharide was extensively dialyzed against tap water to remove residual salts, and then lyophilized.

Glycosyl composition analysis. — The content of neutral sugars in the polysaccharide was determined by g.l.c.–m.s. after hydrolysis with 2M trifluoroacetic acid (TFA) for 2 h at 120°, reduction of the sugars with $NaBH_4$, and acetylation[7] of the alditols. Glycosyluronic acids were analyzed by the colorimetric method of Blumenkrantz and Asboe-Hansen[8].

Determination of the absolute configurations of the glycosyl residues. — The polysaccharide (1 mg) was hydrolyzed with 2M TFA for 1 h at 120°, and the products were analyzed by the method of Gerwig *et al.*[9].

Methylation of the polysaccharide. — The polysaccharide (50 mg) was dried in a vacuum oven for 8 h at 40°. Dimethyl sulfoxide (15 mL) was added, and the slurry was sonicated overnight. Potassium dimethylsulfinyl anion[10] (5 mL, 3M) was added, and the mixture was stirred for 6 h. The solution was cooled until it solidified, methyl iodide (2 mL) was added, and the mixture was stirred for 4 h at room temperature. The excess of methyl iodide was evaporated, and the residue was dialyzed against tap water for 2 days. The per-*O*-methylated polysaccharide was then lyophilized, taken up in chloroform, the suspension filtered through glass-fiber paper, and the filtrate evaporated to dryness.

Glycosyl-linkage composition analysis. — Per-*O*-methylated polysaccharide (250 μg) was hydrolyzed with 2M TFA for 2 h at 120°, the sugars were reduced ($NaBD_4$), and the alditols acetylated. The identity of the partially *O*-methylated, partially *O*-acetylated alditols was established by their g.l.c. retention-times and by their mass spectra[11]. *O*-Methylated polysaccharide was carboxyl-reduced with lithium aluminum deuteride as described[12], and the product subjected to the same analysis.

Preparation of partially O*-methylated, partially* O*-ethylated oligosaccharide-alditols.* — *O*-Methylated, carboxyl-reduced ($LiAlD_4$) polysaccharide (40 mg) was partially hydrolyzed with 2M TFA (10 mL) for 2.5 h at 80°, the sugars were reduced ($NaBD_4$), and the alditols *O*-ethylated as described[13].

Fractionation of the per-O-alkylated oligosaccharide-alditols by l.c. — The mixture of per-*O*-alkylated oligosaccharide-alditols was fractionated by liquid chromatography (l.c.) in a Whatman, Partisil 5, ODS column, using 11:9 acetonitrile–water as the solvent. The refractive index of the column effluent was monitored, and 0.5-mL fractions were collected.

Base-catalyzed degradation[14,15] *of the O-methylated polysaccharide, and fractionation of the products by l.c.* — *O*-Methylated polysaccharide (50 mg) was dissolved in freshly distilled dimethyl sulfoxide (2 mL). Potassium dimethylsulfinyl anion (2 mL, 2M) was added with cooling, and the mixture was stirred for 24 h at room temperature. The solution was cooled until it solidified, and ethyl iodide (1 mL) was added. The mixture was then agitated for 2 h in an ultrasonic bath before it was chromatographed on a column of LH-20, using 1:1 methanol–chloroform as the solvent. The carbohydrate-containing fractions were identified by using the anthrone colorimetric assay for neutral sugars. The fractions containing purified components were combined, and then evaporated to dryness. The residue was fractionated in a column of Du Pont Zorbax, ODS, using 1:1 acetonitrile–water as the solvent. The refractive index of the column effluent was monitored, and 0.5-mL fractions were collected.

Identification of the per-O-alkylated oligosaccharide-alditols and per-O-alkylated oligosaccharide methyl glycosides in the l.c. fractions. — The l.c. fractions obtained *via* partial hydrolysis of the per-*O*-methylated, carboxyl-reduced polysaccharide were analyzed by g.l.c.–e.i.-m.s., or by e.i.-m.s. using the direct-inlet probe. Pure, partially *O*-methylated, partially *O*-ethylated oligosaccharide-alditols were analyzed for their glycosyl-linkage composition as described[13]. The l.c. fractions obtained *via* base-catalyzed degradation of the per-*O*-methylated polysaccharide were analyzed by g.l.c.–e.i.m.s. The major product isolated was also analyzed by direct-inlet c.i.-m.s., using ammonia as the reactant gas. The major product isolated was also analyzed for its glycosyl-linkage composition as described[13].

G.l.c. was performed by using splitless injections on an SE-30 capillary column (25 m) and a temperature program starting at 160° for 2 min, rising from 160 to 220° at 30° per min, and, finally, from 220 to 330° at 8° per min.

The temperature of the probe for direct-inlet m.s. was increased in ~2 min from room temperature to 345°, and kept at the higher limit for 2 min. Spectra were recorded with a Hewlett–Packard model 5985 mass spectrometer, at 70 eV with a source temperature of 150°.

^{1}H-N.m.r. spectroscopy. — Per-*O*-alkylated oligosaccharide-alditols (20–100 μg) and per-*O*-alkylated oligosaccharide methyl glycosides (~50 μg) were dissolved in deuteriochloroform (99.8 atom% of D). ^{1}H-N.m.r. spectra were recorded with a Nicolet 360-MHz, Fourier-transform, n.m.r. spectrometer, with experimental parameters as described[12]. Chemical shifts were assigned relative to internal chloroform at δ 7.26.

RESULTS AND DISCUSSION

Glycosyl composition of the polysaccharide, and the absolute configurations of its glycosyl residues. — Glycosyl-composition analysis of the *Klebsiella* polysaccharide demonstrated that it contains fucose and glucose in the ratio of 1:2. The presence of glycosyluronic acid residues was indicated by the colorimetric assay. These results were confirmed by glycosyl-linkage composition-analysis of the methylated, carboxyl-reduced polysaccharide (see later). The glucosyl and glucosyluronic residues were found to be in the D. and the fucosyl residues in the L, configuration by the method of Gerwig *et al.*[9].

TABLE I

GLYCOSYL-LINKAGE COMPOSITION[a] OF THE ACIDIC POLYSACCHARIDE SECRETED BY *Klebsiella aerogenes* TYPE 54 STRAIN A3

Glycosyl residue	*Determined positions of O-methyl groups*	*Deduced points of attachment of glycosyl residues*	*R.t.*[b]	*Mole %*	
				A	B
Fucosyl	2,4	3	0.53	25.6	25.3
Glucosyl	2,3,4,6	terminal	0.55	36.1	24.2
Glucosyl	2,6	3,4	0.77	38.3	26.3
Glucosyl	2,3	4[c]	0.83	—	24.0

[a]The polysaccharide was methylated and hydrolyzed, and the products reduced and acetylated (column A); or, the polysaccharide was methylated, carboxyl-reduced, and hydrolyzed, and the products reduced, acetylated (column B), and analyzed by g.l.c.–m.s. [b]Retention times relative to that of hexa-*O*-acetyl-*myo*-inositol as unity. [c]This residue has two deuterium atoms at C-6 and, therefore, originates from a glucosyluronic residue.

Determination of the glycosyl-linkage composition. — Glycosyl-linkage composition-analysis of the *O*-methylated polysaccharide showed that the polysaccharide isolated consists of O-3-linked fucosyl residues, terminal glucosyl groups, and (3→4)-linked (if in the pyranoid form) glucosyl residues (see Table I, column A). The lower than stoichiometric value for the fucosyl residues (see later) can be rationalized by the assumption that some of the glycosidic linkages of the glucosyluronic residues were not hydrolyzed; such linkages are known to be resistant to acid hydrolysis.

Glycosyl-linkage composition-analysis of the per-*O*-methylated, carboxyl-reduced polysaccharide showed, in addition to the glycosyl residues already mentioned, (4→6)-linked (if in the pyranoid form) glucosyl residues. G.l.c.–m.s. analysis demonstrated that all of the (4→6)-linked glucosyl residues contained two deuterium atoms at C-6. Consequently, these residues originated from 4-linked glucosyluronic residues. The four different glycosyl residues of the *O*-methylated, carboxyl-reduced polysaccharide were found to be present in the ratios of 1:1:1:1 (see Table I, column B), which is consistent with a tetrasaccharide repeating-unit.

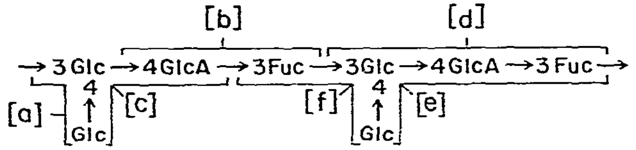

Fig. 1. A summary of the structurally characterized, oligosaccharide fragments isolated from the polysaccharide secreted by *K. aerogenes* type 54 strain A3. (Each fragment is labelled by a letter that identifies the per-*O*-alkylated oligosaccharide-alditol or per-*O*-alkylated oligosaccharide methyl glycoside derived from the fragment.)

Preparation, separation, and analysis of partially O-*methylated, partially* O-*ethylated oligosaccharide-alditols.* — The ethyl groups introduced after partial hydrolysis of the per-*O*-methylated, carboxyl-reduced polysaccharide, and reduction of the fragments obtained, serve as markers to identify where other glycosyl residues were attached in the intact polysaccharide. However, the ethyl group at O-6 of carboxyl-reduced glycosyluronic residues is an exception; this potential ambiguity is avoided by the labeling of C-6 of such glycosyluronic residues with two deuterium atoms that are introduced when deuterated reagents are used for the carboxyl reduction.

Relatively mild conditions for partial hydrolysis of the per-*O*-methylated, carboxyl-reduced polysaccharide were selected in an attempt to isolate per-*O*-alkylated oligosaccharide-alditols containing the acid-labile glycosidic linkage of the fucosyl residues. Although no fragment containing the fucosidic linkage was isolated, the mild hydrolysis resulted in the isolation of the per-*O*-alkylated tetrasaccharide-alditol [e] (see Fig. 1).

Partially *O*-methylated, partially *O*-ethylated oligosaccharide-alditols [a], [b], [c], [d], and [e] (see Fig. 1) were all isolated in pure form after l.c. fractionation. The per-*O*-alkylated oligosaccharide-alditols were analyzed by e.i.-m.s. (see Table II), and by ^{1}H-n.m.r. spectroscopy (see Table III). The A- and J-series[16] of fragment-ions present in the e.i.-mass spectra, together with the results of the original, glycosyl-linkage composition-analysis, established the identity and sequence of the glycosyl residues of each per-*O*-alkylated oligosaccharide-alditol following principles outlined in earlier publications[12,17,18]. The J_1 fragment-ions of per-*O*-alkylated oligosaccharide-alditol [c] at *m/z* 354, and per-*O*-alkylated oligosaccharide-alditol [e] at *m/z* 528, in conjunction with the A and J fragment-ions, give the sequence of the glycosyl residues in [c] and [e], and establish that a (terminal) glucosyl group is attached to O-4 of the (3→4)-linked glucosyl residue in both of the per-*O*-alkylated oligosaccharide-alditols. The anomeric configuration of each glycosidic linkage was made evident by ^{1}H-n.m.r. analysis of the purified, per-*O*-alkylated oligosaccharide-alditols.

Two per-*O*-alkylated disaccharide methyl glycosides having virtually identical e.i.-mass spectra were also isolated by the sequence of reactions already described. The per-*O*-alkylated, disaccharide methyl glycoside isolated in highest yield was analyzed by ^{1}H-n.m.r. spectroscopy (see Table III), and found to be the β anomer of

TABLE II

L.C. AND G.L.C. RETENTION TIMES AND DIAGNOSTIC IONS FROM E.I. MASS SPECTRA OF THE PARTIALLY METHYLATED, PARTIALLY ETHYLATED OLIGOSACCHARIDE-ALDITOLS AND PARTIALLY METHYLATED, PARTIALLY ETHYLATED OLIGOSACCHARIDE METHYL GLYCOSIDES

Oligosaccharide	*Fragment*[a]	*R.t.*[b] *(l.c.)*	*R.t.*[c] *(g.l.c.)*	*Diagnostic ions from the e.i. mass spectra:* m/z *value of ion (origin of ion, % of base peak)*
Et ↓ 3 Glc→4Glc→	[a]	17.5	6.6	338 (J_1, 4), 278 (J_2, 15), 219 (A_1, 9), 187 (A_2, 70)
Et→4GlcA→3Fuc→	[b]	25.6	6.2	294 (J_1, 45), 234 (J_2, 8), 249 (A_1, 7), 217 (A_2, 80)
Et ↓ 3 Glc→4Glc→4GlcA→	[c]	30.9	13.1	558 (abJ_1, 1), 498 (abJ_2, 1), 354 (aJ_1, 8), 280 (aJ_2, 35), 219 (cA_1, 8), 187 (cA_2, 90)
Et ↓ 3 Et→4Glc→4GlcA→3Fuc→	[d]	48.1	12.7	528 (abJ_1, 1), 454 (abJ_2, 2), 294 (aJ_1, 33), 234 (aJ_2, 7), 247 (cA_1, 12), 215 (cA_2, 10), 201 (cA_2, 83)
Et ↓ 3 Glc→4Glc→4GlcA→3Fuc→	[e]	40.9	—	672 (abcJ_2, 0.2), 528 (abJ_1, 2), 454 (abJ_2, 2), 294 (aJ_1, 15), 234 (aJ_2, 6), 219 (dA_1, 10), 187 (dA_2, 100)
Et ↓ 3 Glc→4Glc→OMe	[a]	10.9	6.5	293 (aJ_1, 52), 233 (aJ_2, 51), 219 (bA_2, 7), 187 (bA_2, 72)
Et→3Fuc→3Glc→OMe 4 ↑ Glc	[f]	—	11.5	497 (b′aJ_1, 12), 423 (b′aJ_2, 0.4), 467 (baJ_1, 2), 407 (baJ_2, 0.5), 219 (b′A_1, 9), 187 (b′A_2, 74), 203 (bA_1, 15), 171 (bA_2, 2), 157 (bA_2, 8)

[a] See Fig. 1. [b] Retention times (in min) on a Partisil 5 or Zorbax, ODS column, as described in the Experimental section. [c] Retention times (in min) on an SE-30 column, as described in the Experimental section.

TABLE III

^{1}H-N.M.R. CHEMICAL-SHIFTS AND COUPLING CONSTANTS OF THE ANOMERIC PROTONS OF ISOLATED PARTIALLY METHYLATED, PARTIALLY ETHYLATED OLIGOSACCHARIDE-ALDITOLS AND METHYL GLYCOSIDES

Oligosaccharide	*Fragment*[a]	*Chemical shift*[b] (δ)	*Observed* $J_{1,2}$ *(Hz)*	*Assigned anomeric configuration*
Et→4GlcA→3Fuc→	[b]	5.18	3.1	α
Et ↓ 3 Glc→4Glc→4GlcA→	[c]	4.32	7.8	β
		4.48	7.8	β
Et ↓ 3 Et→4Glc→4GlcA→3Fuc→	[d]	4.30	7.8	β
		5.19	2.9	α
Et ↓ 3 Glc→4Glc→4GlcA→3Fuc→	[e]	4.30	8.3	β
		4.33	7.9	β
		5.19	3.5	α
Et ↓ 3 Glc→4Glc→OMe	[a]	4.15[c]	8.3	β
		4.30	8.0	β
Et→3Fuc→3Glc→OMe 4 ↑ Glc	[f]	4.15[c]	8.0	β
		4.28	7.6	β
		5.42	4.0	α

[a] See Fig. 1. [b] Relative to the signal from internal chloroform, at δ 7.26. [c] The signal from H-1 of the branched glycosyl residue.

the partially *O*-methylated, partially *O*-ethylated disaccharide methyl glycoside derived from fragment [a] (see Fig. 1).

Some of the per-*O*-alkylated oligosaccharide-alditols were analyzed for their glycosyl-linkage composition. The results of these analyses unambiguously confirmed the structure of each per-*O*-alkylated oligosaccharide-alditol.

*Base-catalyzed degradation of the per-*O*-methylated polysaccharide.* — The formation of methyl glycoside fragments (see earlier) may be attributed to base-catalyzed degradation during methylation of the polysaccharide[19]. On further base-catalyzed degradation, partially *O*-methylated oligosaccharide methyl glycosides are released. After derivatization, processing of the reaction mixture, and l.c. fractionation, two products were isolated that gave virtually identical e.i.-mass spectra (see fragment [f] in Table II). The major product isolated was further analyzed by direct-inlet, c.i.-m.s. (ammonia) and by ^{1}H-n.m.r. spectroscopy (see Table III). The c.i. mass spectrum showed a prominent (M + 18) ion at m/z 660. The major product isolated was also analyzed for its glycosyl-linkage composition. The results showed that the major product is the β anomer of the partially *O*-methylated, partially *O*-ethylated trisaccharide methyl glycoside derived from fragment [f] (see Fig. 1), which contains the acid-labile, glycosidic linkage of the fucosyl residues.

Per-*O*-alkylated trisaccharide methyl glycosides obtained by base-catalyzed degradation of the per-*O*-methylated polysaccharide, with subsequent ethylation, were isolated in low yields. Methyl glycoside formation during methylation of the polysaccharide was, therefore, only a minor side-reaction. The amounts isolated were, however, more than sufficient for complete structural characterization of fragment [f].

Ring form of the glycosyl residues. — It is evident from the methylation analysis that the terminal glucosyl group and the 3-linked fucosyl residue must be in the pyranoid form. Glycosyl-linkage composition-analysis of per-*O*-alkylated oligosaccharide-alditols, where the (3→4)-linked glucosyl residue and the 4-linked glucosyluronic residue appeared at either the alditol end (fragments [a] and [c]), or at the nonreducing terminus (fragments [b] and [d]), demonstrated that these residues are also in the pyranoid form.

^{1}H-N.m.r. spectroscopy. — The anomeric configurations of all glycosidic linkages present in the polysaccharide were determined by evaluating the data obtained from the ^{1}H-n.m.r. analysis of the per-*O*-alkylated oligosaccharide-alditols and methyl glycosides. Both glycosidic linkages of per-*O*-alkylated trisaccharide-alditol [c] were found to be in the β-anomeric configuration, and the glycosidic linkage of per-*O*-alkylated disaccharide-alditol [b] was found to be in the α-anomeric configuration (see Table III). In the preponderant, per-*O*-alkylated trisaccharide methyl glycoside derived from fragment [f], the terminal glucosyl group is in the β-anomeric configuration, and the signal at δ 4.15 can be assigned to the β-anomeric linkage of the methyl glycoside[20] (compare with the per-*O*-alkylated disaccharide methyl glycoside [a] in Table III). Consequently, the fucosyl residue of fragment [f] must be in the α-anomeric configuration (see Table III).

Sequence of the glycosyl residues in the polysaccharide. — The fragments listed in Table II provide a number of ways in which to elucidate the sequence of the glycosyl residues in the polysaccharide. According to the glycosyl-linkage composition-analysis, the isolated polysaccharide contains four different glycosyl residues in equal amounts. The high yield of per-*O*-alkylated tetrasaccharide-alditol [e], obtained by mild hydrolysis of the per-*O*-methylated and carboxyl-reduced polysaccharide and subsequent ethylation, indicates that the fucosyl residue is 3-linked to the branch-point glucosyl residue. All other linkages present in the polysaccharide appear once in per-*O*-alkylated tetrasaccharide-alditol [e], and, thus, per-*O*-alkylated tetrasaccharide-alditol [e] represents the repeating-unit of the polysaccharide.

There is only one way of combining the isolated per-*O*-alkylated oligosaccharide-alditols and methyl glycosides to arrive at the structure of the polysaccharide.

No per-*O*-alkylated oligosaccharide fragment was detected that contradicts the structure now presented, namely, **1**. This structure, in which the branched glucosyl residue is (3→4)-linked, with the fucosyl residue attached to O-3, differs from that proposed earlier, in which the branched glucosyl residue was (4→6)-linked, and the fucosyl residue was attached to O-6. The anomeric configuration of the fucosidic linkage, which is now known to be α, was not determined in the earlier work.

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NOTE

After this manuscript was written, we learned that G. G. S. Dutton and E. H. Merrifield, of the University of British Columbia, Vancouver, B.C., had determined, independently and by substantially different methods*, that the capsular polysaccharide of *Klebsiella* type 54 has the sequence of glycosyl residues reported herein.

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