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CHEMICAL AND STRUCTURAL ANALYSIS OF THE CAPSULAR POLYSACCHARIDE FROM *Escherichia coli* O9:K28(A):H⁻ (K28 ANTIGEN)*

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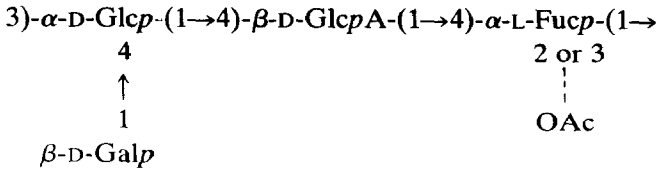
ABSTRACT

The structure of the capsular polysaccharide from *Escherichia coli* O9:K28(A):H⁻ (K28 antigen) has been determined by using the techniques of methylation, periodate oxidation, and partial hydrolysis. N.m.r. spectroscopy (^1H and ^{13}C) was used to establish the nature of the anomeric linkages. *O*-Acetyl groups were determined spectrophotometrically and were located using methyl vinyl ether as a protective reagent.

The polysaccharide is comprised of repeating units of the tetrasaccharide shown (three-plus-one type) with 70% of the fucosyl residues carrying an *O*-acetyl substituent.



This structure resembles that of *E. coli* K27 (refs. 6,14) and has the structural pattern of *Klebsiella* K54 polysaccharide (refs. 15,16).

INTRODUCTION

E. coli and *Klebsiella* bacteria are both members of the family Enterobacteriaceae but although the former is of the greater medical interest[1,2] little is known about the chemical structure of the different antigens of the genus. The accepted serotyping scheme is based on the identification of surface "K", somatic "O", and flagellar "H" antigens[3], and approximately 100 "K", 164 "O" and 56 "H" antigens are currently recognized[3].

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The K antigens of *E. coli* can be divided into three groups (A, B, and L), all of which comprise acidic polysaccharides. Distinctive features of the A antigens are that they occur only together with O8 and O9 antigens, that they form thick capsules, and that they are said to be free of amino sugars[4]. The extracellular A antigens of *E. coli* bear a strong similarity to the K antigens of *Klebsiella*[4]. This paper presents the structure of the K antigen of *E. coli* K28, and represents the first report by our laboratory of a systematic investigation of this species.

RESULTS AND DISCUSSION

Isolation and characterization. — The capsular (K28) polysaccharide was obtained from cells grown on Müller–Hinton agar. The isolation and purification of the polysaccharide were achieved as previously described[5] for several *Klebsiella* species. The purified product obtained after Cetavlon precipitation had $[\alpha]_D$ −18.2° (*c* 1.03, water). It was shown to be heterogeneous by gel permeation chromatography, having two components with molecular weights of 9×10^6 (30% by weight) and 450,000 (70% by weight) respectively; the weight average molecular weight $\bar{M}_w$ was 3×10^6. The polysaccharide was found to be homogeneous after mild alkali treatment ($\bar{M}_w$ = 350,000, based on calibration with dextrans). This is a well-known phenomenon for *E. coli* K antigens associated with O groups O8, O9 and O101. Capsular (K) antigens belonging to this group form very viscous aqueous solutions. Treatment with dilute alkali reduces the viscosity drastically[6,7]. These findings indicate the presence of inter-chain ester linkages between carboxyl groups of hexuronic acid constituents and hydroxyl groups of sugar residues[6,7]. The presence of acetate groups could also contribute to the formation of aggregates, since the removal of acetate yields a homogeneous polysaccharide.

The K28 polysaccharide is composed of D-glucose, D-galactose, D-glucuronic acid, and L-fucose. It does not contain D-galacturonic acid and D-mannose as was earlier thought[8]. The presence of glucose, galactose, fucose, glucuronic acid, and an aldobiuronic acid in the acid hydrolyzate of the polysaccharide was observed by paper chromatography. Determination of the neutral sugars as the alditol acetates showed the ratio of fucose, galactose, and glucose to be 0.45:1:0.76. The carboxyl-reduced polysaccharide[9] gave fucose, galactose, and glucose in the ratio of 1:1.1:1.58, indicating that the uronic acid is glucuronic acid.

^{1}H-N.m.r. spectroscopy. — The ^{1}H-n.m.r. spectrum of the *E. coli* K28 polysaccharide indicated the repeating unit to be a partially acetylated tetrasaccharide (see Table I).

The spectrum exhibits a signal at δ 1.3 which arises from the CH_3 group of L-fucose. Two singlets at δ 2.15 and δ 2.18 are due to the presence of $OCOCH_3$ groups. The presence of twin signals can be attributed to the location of acetyl groups on both O-2 and O-3 of fucose (see later). In the spectrum of deacetylated *E. coli* K28 polysaccharide those signals were absent. In the anomeric region four signals were detected. The signal at δ 4.48 is assigned to β-linked galactose by

TABLE I

^{1}H-N M R DATA FOR *Escherichia coli* K28 POLYSACCHARIDE

Polysaccharide	*Chemical shift (p.p.m. δ)*[a]	*J*[b] *(Hz)*	*Number of protons*	*Assignment*
Native	5.41	b	2.0	α-Glc α-Fuc
	4.93	b	1.0	β-GlcA
	4.86	b		
	4.48	b	1.0	β-Gal
	4.44	b	1.0	ring
	2.18	s	~1	CH_3 of *O*-acetyl
	2.15	s		
	1.30	b	3.0	CH_3 of α-Fuc
Native, after autohydrolysis (overnight, 100°)	5.41	s	1.0	α-Glc
	5.39	s	1.0	α-Fuc
	4.94	8.0	1	β-GlcA
	4.83	8.0		
	4.48	8.0	1.0	β-Gal
	4.42	8.0	1.0	ring
	2.18	s	1.4	CH_3 of *O*-acetyl
	2.15	s		
	1.30	6.0 ($J_{5,6}$)	3.0	CH_3 of α-Fuc
Native, after reduction of COOH	5.43	s	2.0	α-Glc
	5.37	s		α-Fuc
	4.95	b	0.76	β-Glc
	4.85	b		
	4.47	b	2.0	β-Gal ring
	2.20	s	0.6	CH_3 of *O*-acetyl
	2.16	s		
	1.31	b	3.0	CH_3 of α-Fuc
Deacetylated	5.41	s	1.0	α-Glc
	5.39	s	1.0	α-Fuc
	4.83	8.0	1 0	β-GlcA
	4.48	6.5	1.0	β-Gal
	4.42	6.5	1.0	ring

[a]Relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS), measured against internal acetone (δ 2.23). [b]Key: b = broad, unable to assign accurate coupling constant; s = singlet.

comparison with the spectrum of the disaccharide **N1** (see above). Two signals at δ 4.86 and δ 4.93 belong to the β-glucosyluronic acid residues. The twinning is attributed to the presence of *O*-acetyl groups on the adjacent fucose, since, after deacetylation, the two signals are replaced by a 1-proton doublet at δ 4.83. A broad signal at δ 5.41 represents the two anomeric protons of α-L-fucose and α-D-glucose. The signal at δ 4.44 is due to a ring proton and is unchanged after deacetylation. Generally, the ^{1}H-n.m.r. spectrum was not well resolved due to the extreme

viscosity of the solution. The quality of the spectrum was improved after autohydrolysis of the polysaccharide (overnight, 100°). A good spectrum was also obtained after reduction of the viscosity of the polysaccharide by deacetylation.

^{13}C-N.m.r. spectroscopy. — The ^{13}C-n.m.r. spectra of the native and *O*-deacetylated K28 polysaccharides (see Table II) differ by the presence, in the former, of signals arising from CH_3CO (21.39 p.p.m.) and $CH_3C{=}O$ (175.62 p.p.m.). Deacetylation of the polysaccharide caused only slight changes in the positions of the signals for the anomeric carbons (see Table II). Carbons 2 and 3 of the fucosyl residue exhibit a downfield shift ($\Delta\delta$ = 2.9 p.p.m. for C-2 and 2.67 p.p.m. for C-3) in the native polysaccharide due to the presence of acetate at these positions. Once the acetate is removed the signals for C-2 and C-3 of fucose shift upfield and are in agreement with the literature values[10]. These data, together with ^1H-n.m.r. findings, suggest that acetyl groups are located on O-2 and O-3 of the α-L-fucosyl residues, and explain the twinning[11] of the anomeric signal assigned to β-D-glucuronic acid. These conclusions were reinforced by the results of periodate oxidation of the native and deacetylated polysaccharides. The positons of the *O*-acetyl groups were confirmed by replacing the *O*-acetyl groups with stable *O*-ethyl groups, using methyl vinyl ether as a protective reagent (see later).

Methylation analysis. — Methylation of the K28 polysaccharide, followed by

TABLE II

^{13}C-N M R DATA FOR THE NATIVE AND *O*-DEACETYLATED *E. coli* K28 POLYSACCHARIDE

Native polysaccharide		*O-Deacetylated polysaccharide*	
Chemical shift (p.p.m. δ)[a]	*Assignment*[b]	*Chemical shift (p.p.m. δ)*[a]	*Assignment*[b]
102.79	C-1 β-GlcA	103 91	C-1 β-GlcA
	C-1 β-Gal	102.81	C-1 β-Gal
99.30	C-1 α-Fuc	99 32	C-1 α-Fuc
99.26	C-1 α-Glc		C-1 α-Glc
76 94	C-3 β-GlcA	77 97	C-3 β-GlcA
76.89	C-4 α-Fuc	76.88	C-4 α-Fuc
75.91	C-5 β-Gal	75.93	C-5 β-Gal
74.34	C-3 α-Glc	74.40	C-3 α-Glc
73.74	C-4 β-GlcA	73 74	C-4 β-GlcA
73.46	C-2 β-GlcA	73.43	C-2 β-GlcA
73.33	C-4 β-Gal		C-4 β-Gal
72.27	C-4 α-Fuc	72 21	C-4 α-Fuc
72.14	C-3 α-Fuc	69 47	C-3 α-Fuc
72.10	C-2 α-Fuc	69 19	C-2 α-Fuc
69 11	unassigned	67.57	unassigned
62.28	C-6 α-Glc, β-Gal	62.26	C-6 α-Glc, β-Gal
21.39	CH_3 of acetate		
16.02	CH_3 α-Fuc	16.12	CH_3 α-Fuc
15.97		16.06	

[a]Downfield from Me_4Si, measured against internal acetone (δ 31.07). [b]The assignments were made by comparison with literature values (ref 10), but must be considered tentative.

hydrolysis, derivatization of the products as alditol acetates, and g.c.-m.s. analysis, gave the values shown in Table III, column I. These results indicated that the polysaccharide consists of a repeating tetrasaccharide unit having a branch on glucose, with galactose as the terminal group of the branch. By reduction of the methylated polysaccharide (see Table III, column II), the proportion of 2,3-di-*O*-methylfucose was increased, and 2,3-di-*O*-methylglucose was formed, indicating that glucuronic acid is linked through O-4, and that it is joined to fucose. Methylation analysis of carboxyl-reduced polysaccharide showed the presence of 2,3,6-tri-*O*-methylglucose, derived from reduction of the carboxyl group of the glucuronic acid (see Table III, column III).

Partial hydrolysis. — Partial hydrolysis of the native polysaccharide with acid was followed by separation of the acidic and the neutral fractions by ion-exchange chromatography. The neutral fraction contained monosaccharides and a disaccharide (**N1**). The acidic fraction contained an aldobiouronic acid (**A1**). On the basis of their n.m.r. spectral data (Table IV) and their methylation analyses (Table V), the structures of these compounds were shown to be as follows:

A1 β-Glc*p*A-(1→4)-Fuc

N1 β-Gal*p*-(1→4)-Glc

Periodate oxidation. — Smith degradation of the native polysaccharide followed by methylation and hydrolysis showed the presence of 2,3,4-tri-*O*-methylfucose, 2,3,4,6-tetra-*O*-methylglucose and 2,4,6-tri-*O*-methylglucose (see Table III, column IV). These results show that terminal galactose and the glucuronic acid were completely oxidized. The fucosyl linkage was hydrolyzed during the Smith degradation, with the consequent formation of 2,4,6-tri-*O*-methylglucose, proving that fucose and glucose are engaged in a 1→4 linkage. The proportion of 2,3,4-tri-*O*-methylfucose found indicates that 70% of the fucose survived periodate oxidation. This result suggests that 70% of the fucosyl residues are acetylated at O-2 or O-3 and are thus protected against oxidative degradation. In confirmation of this, the deacetylated polysaccharide showed a significant increase in the amount of fucose oxidized by periodate (see Table III, column V).

Quantitative determination of O-*acetyl groups.* — The percentage of *O*-acetyl groups present in *E. coli* K28 polysaccharide was determined spectrophotometrically[12]. In one batch it was found that about 70% of the fucosyl residues carried an *O*-acetyl substituent. The acetate groups could be easily removed by mild alkali treatment. These findings are in very good agreement with the results obtained by periodate oxidation of the native and deacetylated polysaccharides. The partial loss of *O*-acetyl groups during the high temperature n.m.r. experiments is indicative of the lability of the *O*-acetyl groups located on the fucosyl residues.

Location of the O-*acetyl groups.* — *O*-Acetyl groups were located[13] by reac-

TABLE III

METHYLATION ANALYSIS OF *Escherichia coli* K28 POLYSACCHARIDE AND DERIVED PRODUCTS

Methylated sugar[a] *(as alditol acetate)*	*Mole percent*[b]				
	I[c]	*II*[c]	*III*[c]	*IV*[c]	*V*[c]
2,3,4-Fuc	—	—	—	42	—
2,3-Fuc	23	28	26	—	21
2,3,4,6-Glc	—	—	—	20	—
2,3,4,6-Gal	37	22	24	—	—
2,4,6-Glc	—	—	—	38	—
2,3,6-Glc	—	—	18	—	—
2,6-Glc	40	26	31	—	79
2,3-Glc	—	23	—	—	—

[a]2,3,4-Fuc = 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylfucitol, etc. Determined on an OV-225 column programmed for 4 min at 180°, then increasing at 2°/min to 230°. [b]Values are corrected by use of the effective carbon-response factors given by Sweet *et al.*[27]. [c]I, original polysaccharide; II, compounds from $LiAlH_4$ reduction of methylated *E. coli* K28; III, carboxyl-reduced polysaccharide; IV, product from Smith degradation of the original polysaccharide; V, product from periodate oxidation of the deacetylated polysaccharide

TABLE IV

N M R DATA FOR OLIGOSACCHARIDES DERIVED FROM PARTIAL HYDROLYSIS OF THE *Escherichia coli* K28 POLYSACCHARIDE

Compound	^1H				^{13}C	
	Chemical shift (p.p m. δ)[a]	*J (Hz)*	*Number of protons*	*Assignment*[b]	*Chemical shift (p.p m δ)*[c]	*Assignment*[b]
GlcA$\overset{1\ 4}{\underset{\beta}{}}$Fuc-OH	5.24	2.7	0.3	4-Fuc—OH (α)	103 89	GlcA—(β)
A1	4.63	5.4	0.7	4-Fuc—OH (β)	97 04	4-Fuc—OH (β)
	4.54	8 0	1.0	GlcA—(β)	93.11	4-Fuc—OH (α)
	4.29	q	1.0	H-5 of Fuc	16.23	CH_3 of Fuc
	1.33 1.29	6.75 6.75	3.0	CH_3 of β-Fuc-OH CH_3 of α-Fuc-OH		
Gal$\overset{1\ 4}{\underset{\beta}{}}$Glc-OH	5.23	4	0.4	4-Glc—OH (α)	103 83	Gal—(β)
N1	4.67	7	0.6	4-Glc—OH (β)	96.62	4-Glc—OH (β)
	4.45	7.5	1.0	Gal—(β)	92.68	4-Glc—OH (α)

[a]Chemical shift relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS), measured against internal acetone (δ 2.23). [b]The numerical prefix indicates the position at which the sugar is substituted; the α or β, the configuration of the glycosidic bond, or the anomer in the case of a reducing terminal sugar residue. [c]Downfield from Me_4Si, measured against internal acetone (δ 31 07)

TABLE V

ANALYSIS OF THE OLIGOSACCHARIDES FROM PARTIAL HYDROLYSIS OF *Escherichia coli* K28 POLYSACCHARIDE

Oligosaccharide	*Sugar*	*Molar proportion*	*Methylation analysis*	*Molar proportion*
A1	Fuc	1.0	—	—
	Glc (GlcA)	0.8	—	—
N1	Gal	1.0	2,3,4,6-Gal	0.95
	Glc	0.9	2,3,6-Glc	1.00

tion of the polysaccharide with methyl vinyl ether and an acidic catalyst, followed by ethylation analysis of the product. It was found that either O-2 or O-3 in the α-L-fucosyl residues was ethylated, but no 2,3-di-*O*-ethylfucose was obtained.

CONCLUSIONS

From the sum of these experiments the complete structure of the capsular polysaccharide from *Escherichia coli* K28 may be written. This structure closely resembles that of the capsular antigens from *E. coli* K27 (refs. 6,14) and it has the same pattern as that of the capsular polysaccharide from *Klebsiella* K54 (refs. 15,16).

EXPERIMENTAL

General methods. — The instrumentation used for infrared and n.m.r. spectroscopy, g.l.c., and g.c.-m.s. has been described previously[17]. Spectrophotometric measurements were made with a Perkin–Elmer 552A u.v./vis. spectrophotometer. Circular dichroism measurements were made with a Jasco J-500A automatic recording spectropolarimeter equipped with a quartz cell of path length 0.02 cm. Paper chromatography was conducted by the descending method, using Whatman No. 1 paper and the following solvent systems (v/v): (1) 18:3:1:4 ethyl acetate–acetic acid–formic acid–water, (2) 8:2:1 ethyl acetate–pyridine–water, (3) 2:1:1 1-butanol–acetic acid–water. Spots were visualized with silver nitrate, or by spraying with *p*-anisidine hydrochloride in aqueous 1-butanol and heating the papers for 5–10 min at 110°. Preparative paper chromatography was performed by the descending method using Whatman No. 3 MM paper and solvent 3. Analytical g.l.c. separations were achieved in stainless steel columns (1.8 m × 3 mm), with nitrogen as the carrier gas at a flow rate of 20 mL/min. The columns used were: (A) 3% of SP-2340 on Supelcoport (100–120 mesh), and (B) 3% of OV-225 on Gas Chrom Q (100–120 mesh). Ion-exchange chromatography, for the separation of neutral from acidic oligosaccharides, was performed in a column (2 × 28 cm) of

Bio-Rad AG1-X2 (formate) resin (200–400 mesh). The neutral fraction was eluted with water, and the acidic fraction with 10% formic acid.

Preparation and properties of E. coli *K28 capsular polysaccharide.* — A culture of *E. coli* K28 (Professor S. Stirm, Giessen), was first grown on sucrose-rich medium as previously described for *Klebsiella*[5]. Growth was not satisfactory, and the yield of polysaccharide was very poor. In order to find suitable growth conditions six different media were tried: (1) trypticase soy agar (BBL), (2) Luria broth (Difco), (3) nutrient broth (Difco), (4) nutrient broth plus yeast extract (Difco), (5) beef heart infusion (Difco), and (6) Müller–Hinton agar (BBL). Sodium chloride improves the growth of *E. coli*[18] and was used in the preparation of all six media (0.5% w/v). The streaked plates were incubated at 37° overnight. The best results were obtained on Müller–Hinton agar. For the production of polysaccharide the organism was grown in three small trays (30 cm × 50 cm) each containing 1.5 L of Müller–Hinton agar with 5 g of NaCl added. One tray was filled with water to serve as a humidity source[19].

Each tray was layered with an activity growing liquid culture of *E. coli* K28 bacteria, obtained by the inoculation of 50 mL of Müller–Hinton broth with a single fresh colony of *E. coli* K28 and further incubation of the resulting solution for 5 h at 37°. The trays were left in the incubator for 5 d, then 200 mL of slime was collected. The purification procedure was as previously described[20,21]. The yields per batch were: acidic polysaccharide, ~400 mg; neutral polysaccharide, ~100 mg. Three different batches of the polysaccharide were prepared.

The molecular weight of the polysaccharide was determined by gel permeation chromatography in the laboratory of Dr. S. C. Churms, University of Cape Town, South Africa. The native polysaccharide was shown to be heterogeneous, but it became homogeneous after mild alkali treatment ($\overline{M}_w$ = 350,000). N.m.r. spectroscopy (^1H and ^{13}C) was performed on the original and the deacetylated K28 polysaccharide. The principal signals and their assignments are recorded in Tables I and II respectively.

Deacetylation of the polysaccharide. — The polysaccharide was dissolved in 0.01M NaOH and stirred overnight at room temperature. The product was dialyzed against tap water and freeze dried. The completeness of deacetylation was checked by ^1H-n.m.r. spectroscopy.

Hydrolysis of the polysaccharide. — Hydrolysis of a sample (4 mg) of K28 polysaccharide with 2M trifluoroacetic acid (TFA) for 18 h at 95°, removal of the acid by successive evaporations with water, and paper chromatography using solvents 1 and 2 showed the presence of fucose, glucose, galactose, glucuronic acid, and an aldobiuronic acid. The quantitative sugar analysis of the carboxyl-reduced polysaccharide was performed as previously described[22]. The alditol acetates of fucose, glucose, and galactose were identified by g.l.c. (column A, programmed for 4 min at 195°, and then increasing at 2°/min to 260°), and found to be present in the ratios of 1:1.1:1.58.

Circular dichroism measurements[23]. — Glucose was proved to be of the D-

configuration by circular dichroism measurements made on glucitol hexaacetate. Fucose was assigned the L-configuration from c.d. measurements on fucitol pentaacetate. The absolute configuration of the galactose was established enzymatically by the positive action of D-galactose oxidase[24] on the hydrolysis product of the polysaccharide.

Methylation analysis. — The capsular polysaccharide (60 mg) in the free acid form, obtained by passing the sodium salt through a column of Amberlite IR-120 (H^+) resin, was dissolved in dry dimethyl sulfoxide (6 mL) and methylated by the Hakomori procedure[25]. The product, recovered after dialysis against tap water, was not completely methylated (hydroxyl absorption in the i.r. spectrum). It was dissolved in chloroform and subjected to Purdie methylation[26] with methyl iodide and silver oxide. This treatment yielded a fully methylated polysaccharide (57 mg). A portion of this product (5 mg) was hydrolyzed with 2M TFA, the sugars were reduced with sodium borohydride, the alditols were acetylated with 1:1 acetic anhydride–pyridine, and analyzed by g.l.c. in column C. Carboxyl reduction of the fully methylated polysaccharide (12 mg) was accomplished with $LiAlH_4$ in anhydrous oxolane, and the eventual alditol acetates were analyzed by g.l.c. and g.c.-m.s. in column C. Carboxyl-reduced K28 polysaccharide was obtained by the technique described previously[9].

Partial hydrolysis. — The K28 polysaccharide (514 mg) was dissolved in 125 mL of 0.01M TFA and the solution was heated for 32 h on a steam-bath. After removal of the acid by successive evaporations with water, an acidic and a neutral fraction were separated on a column of Bio-Rad AG1-X2 ion-exchange resin. The acidic fraction was separated by preparative paper chromatography (solvent 3) to give 45.7 mg of a pure aldobiuronic acid (**A1**). Paper chromatography of the neutral fraction showed fucose, glucose, galactose, and a neutral disaccharide, which was isolated by preparative paper chromatography (solvent 3) to give 21 mg of **N1**. ^{1}H-N.m.r. spectral data were recorded for each oligosaccharide, and sugar analyses were performed as follows. The acidic oligosaccharide was treated with 3% HCl in anhydrous methanol for 18 h on a steam bath. The methyl ester methyl glycoside obtained was reduced with sodium borohydride in anhydrous methanol, followed by hydrolysis with 2M TFA, reduction to the alditols, and acetylation with 1:1 acetic anhydride–pyridine. The alditol acetates obtained were analyzed by g.l.c. in column A (195° for 4 min, and then increasing at 2°/min to 260°). The neutral oligosaccharide was hydrolyzed, and analyzed similarly. The methylation of compound **N1** was accomplished by the Hakomori procedure[25].

Periodate oxidation. — A solution of K28 polysaccharide (21.5 mg) in water (10 mL) was mixed with 0.03M $NaIO_4$ (10 mL) and the mixture was stirred in the dark at room temperature (23°) for 6 d. After ethylene glycol (2 mL) was added, the polyaldehyde was reduced with $NaBH_4$, the solution was neutralized with 50% AcOH, dialyzed overnight, and freeze dried to yield the polyalcohol (15 mg). A portion of this (4.2 mg) was hydrolyzed with 2M TFA overnight on a steam bath and converted into alditol acetates. Analysis by g.l.c. in column A showed fucitol,

galactitol and glucitol in the ratios of 0.72:0.10:1. The remainder of the material was treated with 0.5M TFA for 48 h at room temperature. The product (10.7 mg) was methylated by the Hakomori procedure, hydrolyzed with 2M TFA overnight on a steam-bath, and converted into alditol acetates. G.l.c. analysis, conducted in column B, showed the presence of of 2,3,4-tri-*O*-methylfucose, 2,3,4,6-tetra-*O*-methylglucose and 2,4,6-tri-*O*-methylglucose in the ratios of 0.70:0.35:0.65.

Periodate oxidation of the deacetylated polysaccharide was performed similarly. A fraction of the product (1.6 mg) was analyzed for constituent sugars, and the remainder of the material was methylated by the Hakomori procedure. Conversion of the partially methylated sugars into alditol acetates, and g.l.c. thereof in column B, showed the pesence of 2,3-di-*O*-methylfucose and 2,6-di-*O*-methylglucose in the ratios 0.25:1.00.

Location of O-acetyl groups. — *E. coli* K28 polysaccharide (17.6 mg) and *p*-TsOH (5 mg) were dried overnight under vacuum and dissolved in dry dimethyl sulfoxide (10 mL). Methyl vinyl ether (3 mL) was added to a frozen solution, and the reaction mixture was brought to 23° and stirred for 4 h. Then a second portion of methyl vinyl ether (3 mL) was introduced in a similar manner. The clear, red solution obtained was placed on a Sephadex LH-20 column (58 × 1.5 cm), the column eluted with acetone (with slight suction), and the product was collected and concentrated. Half of the residue was ethylated[25] and the product, a dark red-orange oil, was dialyzed (cut off 3,500) overnight against tap water. Extraction of the dialysate with chloroform gave the ethylated, acetal-protected polysaccharide, a portion of which was hydrolyzed with 2M TFA overnight on a steam-bath and converted into alditol acetates. G.l.c. analysis, conducted in column B (210°, isothermal) showed the presence of 2-*O*-ethylfucose (16.9%), 3-*O*-ethylfucose (23.8%), fucose (12.7%), galactose (25.1%), and glucose (21.6%). These results were confirmed by g.c.-m.s., which was performed on a Kratos MS80RFA instrument, using a DB-225 capillary column (150° for 1 min, and then increasing at 10°/min to 210°).

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