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Structural and Rheological Properties of the Extracellular Polysaccharides from *Bacillus polymyxa*

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SUMMARY

The extracellular polysaccharides of Bacillus polymyxa *(Strain NCIB 11429) have been fractionated to give a minor neutral polysaccharide (N-1), a minor acidic polysaccharide (A-1) and a major acidic polysaccharide (A-2). Analysis of the components of the A-2 polysaccharide gave glucose (41%), mannose (26%), galactose (15%), glucuronic acid (14%) and pyruvate (4%). Methylation analysis and* ^{1}*H-NMR spectroscopy suggest that the repeating unit is branched and contains at least three* α*-linked and four* β*-linked sugar residues. The total extracellular polysaccharide preparation displays high solution viscosities at low concentrations with a high yield stress and forms thermally-reversible gels at concentrations greater than 0·5% (w/v). The rheological characterisation has shown that the A-2 polysaccharide is responsible for the characteristic rheological properties and that it adopts a rigid, ordered conformation in solution resulting in the formation of a weak gel network. Both specific ionic interactions and other non-covalent forces such as hydrogen bonding contribute to the stability of this network.*

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INTRODUCTION

Many bacteria have the ability to produce polysaccharides which lie outside the cell wall (Sutherland, 1977). These extracellular polysaccharides may be in the form of a capsule attached to the cell wall or may be secreted as a slime into the growth medium. One of the most actively studied extracellular polysaccharides is that from *Xanthomonas campestris*, known as xanthan. The versatile rheological properties of this polysaccharide have led to its widespread technological exploitation (Sandford, 1979). This rheological behaviour of xanthan has been rationalised in terms of the ordered conformation adopted and the consequent intermolecular associations (Morris, 1977).

Due to the unique success of xanthan, a considerable interest now exists in the microbiology and chemistry of other extracellular microbial polysaccharides. An extracellular anionic heteropolysaccharide which forms very viscous aqueous solutions at low concentrations has previously been isolated from *Bacillus polymyxa* (Ninomiya & Kizaki, 1969). It was composed of glucose, mannose, galactose and glucuronic acid in the molar ratios of *c.* 3:3:1:2 and contained pyruvate. A different strain of *B. polymyxa* was reported to produce a fucose-containing extracellular polysaccharide consisting of glucose, galactose, mannose and fucose in the molar ratios of *c.* 6:4:3:2 (Fumino *et al.*, 1979). Apparently it was devoid of uronic acid.

Our particular interest in the extracellular polysaccharides from *Bacillus polymyxa* (Strain NCIB 11429) arose when examination by high-performance gel permeation chromatography indicated that the material was not homogeneous but contained two components. Preliminary examination of the material by NMR relaxation to obtain the spin–spin relaxation time, T_2, also provided evidence for two components. One component had a T_2 in the millisecond region, typical of a flexible disordered polysaccharide, and the other had a T_2 in the microsecond region, indicative of an ordered structure. Furthermore the extracellular polysaccharide preparation, when dissolved in water, exhibits temperature dependent yield stress solution and gel-forming properties, depending on the concentration used. An understanding of the molecular origins of such interesting rheological properties requires a knowledge of the primary chemical structure. In this paper we report the isolation and properties of two chemically

distinct anionic extracellular polysaccharides from *B. polymyxa* strain NCIB 11429.

MATERIALS AND METHODS

Culture and fermentation of *B. polymyxa*

The strain of *B. polymyxa* (strain number NCIB 11429) was isolated from sea water and maintained on agar plates. The mucoid colonies which appeared on the plates were cultured for 2 days in shake flasks containing the following medium: sucrose, 5% w/v; yeast extract powder (Oxoid), 0·5% w/v; K_2HPO_4, 0·25% w/v; $MgSO_4.7H_2O$, 0·1% w/v; urea, 0·15%; trace element solution, 0·4% w/v. The composition of the trace element solution was: $MnSO_4.4H_2O$, 0·3% w/v; $FeSO_4.7H_2O$, 0·9% w/v; $ZnSO_4.7H_2O$, 1·8% w/v; $CuSO_4.5H_2O$, 0·08% w/v; $CoCl_2.6H_2O$, 0·09% w/v; conc. H_2SO_4, 0·5% w/v. The shake flask culture (400 ml) was then transferred to a 28 litre New Brunswick fermenter containing 20 litres of medium (as above) which had been sterilised at 100°C for 2 h and cooled. During fermentation the temperature was maintained at 30°C, the pH at 6 and the stirrer speed at 200 rpm. After about 40 h the broth started to become noticeably viscous and the viscosity increased during the next 24 h to give a final viscosity of 250 poise (shear rate 1 s^{-1}) at 25°C. Fermentation was terminated after 70 h and at this stage the broth contained polysaccharide at 10 g $litre^{-1}$ and cells at 0·7 g $litre^{-1}$.

Isolation of the extracellular polysaccharides

The fermentation broth was diluted with 2 volumes of water, heated to 30–35°C and centrifuged at this temperature for 90 min at 16 000 *g*. A compact pellet of cell material was formed and the polysaccharide solution was separated by decantation. Isopropanol (IPA) was then added to a final concentration of 60% v/v with continuous mixing. The precipitated polysaccharide was removed by filtration through a Whatman 41 filter paper after the bulk of the solvent liquor had been decanted off. The precipitate was washed by stirring in a further volume of 60% v/v IPA and recovered as before. The polysaccharide was dried at 40°C and milled to give a fine white powder.

Fractionation of the extracellular polysaccharides

The polysaccharide was fractionated using cetyltrimethylammonium bromide (CTAB). Typically, 5% w/v aqueous CTAB (100 ml) was slowly added to a 0·1% w/v solution of the polysaccharide (200 ml). A white precipitate immediately formed and the mixture was left at room temperature for 60 min. Following centrifugation (12 000 rpm, 30 min), the supernatant was separated and polysaccharide precipitated from it by the addition of ethanol (5 vol.). This neutral polysaccharide was dissolved in water, dialysed and freeze-dried to give the N-1 polysaccharide (14 mg). The CTAB–polysaccharide complex was stirred with 2 M NaCl (100 ml) for 16 h at room temperature. The mixture was then centrifuged (12 000 rpm, 30 min) and the supernatant which was recovered was treated with ethanol (5 vol.) to precipitate the polysaccharide. This was subsequently redissolved, dialysed and freeze-dried to give the A-1 fraction (36 mg). The remaining CTAB–polysaccharide complex, which was in the form of a soft gel, was stirred in ethanol saturated with calcium chloride (100 ml) overnight at room temperature. The mixture was centrifuged (12 000 rpm, 30 min) and the resulting pellet was rewashed with the calcium chloride solution for a further 60 min. Following centrifugation, the pellet was redissolved in water (occasionally, slight heating was required) and precipitated with 5 volumes of ethanol. The precipitate was dissolved in water, dialysed and freeze-dried to give the A-2 polysaccharide (150 mg).

Ion-exchange chromatography of total polysaccharide and of the A-1 fraction

Polysaccharide was chromatographed on a column (1 × 50 cm) of DEAE-cellulose (Whatman DE52) anion-exchange resin. The polysaccharide (100–200 mg) was prepared for chromatography by dialysing overnight against 50 mM Tris-HCl buffer material. The column, operating under gravity at a flow rate of 20 ml h^{-1}, was first eluted with buffer (350 ml) and then with a continuous gradient formed by buffer (200 ml) and buffer (200 ml) containing 0·5 M NaCl. Fractions (2 ml) were collected and the presence of polysaccharide was ascertained by optical rotation, and the content of NaCl by conductivity. Chromatography of total polysaccharide gave two peaks which were isolated as follows. The front and centre of the first peak was isolated as Peak

1, the front and centre of the second peak as Peak 2A, the back as Peak 2B and the trailing tail as Peak 2C. Two peaks were also obtained when A-1 was chromatographed. The mobility of these peaks indicate that A-1 is a mixture of acidic polysaccharides, with no neutral polysaccharides present. The front and centre of the first peak was isolated as Peak 1 and the centre and back of the second peak as Peak 2. The combined fractions were dialysed and freeze-dried.

Constituent analysis

Polysaccharide (2–5 mg) was hydrolysed with 0·25 M TFA (1–2 ml) in a sealed tube at 100°C for 16 h. The contents were then transferred to a 50 ml pear-shaped flask and acid was removed by rotary evaporation. For paper chromatography, a portion of the hydrolysate dissolved in water was applied to Whatman No. 1 paper, chromatographed in ethyl acetate–pyridine–water (10:4:3) and the sugars visualised using the silver nitrate–sodium hydroxide stain. A ninhydrin stain was used to detect any amino sugars which might be present: the paper was stained with 0·1% (w/v) ninhydrin in *n*-butanol and heated at 100°C for 10 min. Neutral sugars were quantified by g.l.c. of the alditol acetates which were prepared as described by Sloneker (1972). G.l.c. analysis was carried out on a Perkin-Elmer GCF17 instrument fitted with a flame ionisation detectors. Data was collected on a Trilab Model II computer. Separations were performed on glass columns (1·8 m × 2 mm) containing 10% SP2340 on 100/120 Supelcoport at 220°C.

Carboxyl-reduction of uronic acid was achieved by two consecutive treatments according to the method of Taylor & Conrad (1972). Uronic acid was also identified by g.l.c. of the trimethylsilyl derivatives of the derived aldonolactones (Perry & Hulyalkar, 1965). Chromatography was on a glass column (4·6 m × 3 mm) containing 10% OV-17 on Chromosorb WHP80-100 at 190°C.

Colorimetric assays were used to quantify uronic acid (Bitter & Muir, 1962) and pyruvate (Sloneker & Orentas, 1962).

Methylation analysis

In order to effect complete dissolution of the polysaccharide (both acid and salt forms) in dimethyl sulphoxide, the vial was agitated for 18 h in an ultrasonic bath at room temperature. The native polysaccharide was

then methylated according to Hakomori (1964). As the final product showed minor hydroxyl group adsorption in the infrared spectrum, a Purdie treatment with silver oxide and methyl iodide (Hirst & Percival, 1965) was carried out. However, minor adsorption at 3600 cm^{-1} was still evident.

Methylation of the carboxyl-reduced polysaccharide was by the Hakomori procedure. A portion of this material was kept for analysis. The remainder was used to locate the pyruvate substituents. The carboxyl-reduced and methylated polysaccharide was hydrolysed under mild conditions (50% v/v acetic acid for 90 min at 100°C) to remove the modified pyruvic acid residues (Jansson *et al.*, 1979) and remethylated.

All the methylated samples were analysed as their partially methylated alditol acetates (Jansson *et al.*, 1976). G.l.c. was on a packed column (2·7 m × 4 mm) at 220°C. G.l.c.-mass spectrometry was performed on a VG Micromass 12F instrument linked to a Perkin-Elmer Sigma 3B gas chromatograph via a jet separator. The electron impact (E1) source was operated at an energy of 70 eV, an emission of 200 μA and an ion-source temperature of 200°C.

^{1}H-NMR spectroscopy

For high resolution NMR, solutions (1–3% w/v) of the polysaccharide in D_2O were run at 80°C using a Bruker WP200 spectrometer (200 MHz). Chemical shifts (δ values) were measured relative to 4,4-dimethyl-4-silapentane-1-sulphonate (DSS) as internal (capillary) standard.

To improve the resolution of the NMR spectra, the polysaccharide was depolymerised using a mild acid hydrolysis. Polysaccharide (40 mg) was transferred to a pre-drawn hydrolysis tube (1 × 14 cm). Water (4 ml) was added and the polysaccharide allowed to hydrate for 2 h at room temperature. TFA (4 ml of 0·2 M) was then added, the tube was sealed and the contents hydrolysed for 30 min in a boiling water bath. The hydrolysate was neutralised with sodium hydroxide, dialysed overnight and freeze-dried to give 33 mg depolymerised polysaccharide.

Rheological measurements

Samples were measured at 25°C using both steady and oscillatory shear on the Rheometrics mechanical spectrometer (RMS-605) using the

ST-10 transducer with 50 mm diameter cone and plate (cone angle 0·04 rad). In the experiment demonstrating the effect of temperature on viscosity, heating and cooling was effected using a Haake circulating water bath.

Rotation viscosity measurements were made on a Haake Rotovisco, using an MV1 rotor assembly operating at 25°C.

The different salt forms of the polysaccharide were prepared by ion-exchange chromatography on Amberlite IR-120 resin as follows. The resin (H^+ form) was pre-treated by washing with 1 M HCl (10 vol.) and then with distilled, deionised water to neutral pH. Columns (3 × 15 cm) were prepared and converted to the required salt form by eluting with a 1 M solution (10 vol.) of the relevant chloride salt form. The columns were finally washed with 10 volumes of distilled, deionised water. Polysaccharide (200 mg) was applied as a 0·1% w/v solution and allowed to pass through the column at a flow rate of 10 ml min^{-1}. The solutions were then dialysed and freeze-dried. The degree of conversion to the required salt form was checked by elemental analysis and found to be virtually 100%.

To investigate the effect of urea on rheological properties, solid urea was added to 1% w/v polysaccharide preparation to a concentration of 4 M and dissolved by stirring.

Intrinsic viscosity

Due to the polyelectrolyte nature of the polysaccharide, it was necessary to ensure a constant ionic strength during viscosity measurements. In order to achieve this, polysaccharide (0·1% w/v in 0·2 M NaCl) was dialysed for 48 h against 0·2 M NaCl (containing 0·02% w/v sodium azide). The dialysate was then used for subsequent dilutions. A range of concentrations (0·1–0·03%) was made up with relative viscosities in the range 2·0–1·2 and viscosities were measured at 25°C using the Contraves Low Shear 30 viscometer. The intrinsic viscosity was determined (Morris & Ross-Murphy, 1981) by the Huggins and Kraemer treatments.

Optical rotation

Samples were filtered prior to measurement by passing through a 0·45 μm Millipore filter. Measurements were made using a Perkin-Elmer

241 MC polarimeter with a 2 cm cell (sufficient light could not be transmitted through the solutions in a 10 cm cell). The temperature was controlled using a Haake circulating water bath.

RESULTS AND DISCUSSION

Total extracellular polysaccharide was obtained by alcohol precipitation from the fermentation broth. On hydrolysis, the polysaccharide preparation yielded fucose, mannose, galactose and glucose in the molar ratios of *c.* 0·2 : 1·5 : 1·0 : 2·2. Uronic acid was also present. The ^{1}H-NMR spectrum was not well resolved but did show a complex anomeric region with signals indicative of both α- and β-linked residues (δ 4·5–5·6), two doublet signals arising from the CH_3 protons of 6-deoxy sugars (δ 1·3 and 1·2) and singlet signals typical of pyruvate (δ 1·5) and O-acetyl substituents (δ 2·1). As extracellular microbial polysaccharides tend to be composed of relatively simple repeating units, the very complex structure indicated by this analysis suggested that the polysaccharide was heterogeneous.

Because the preliminary characterisation had indicated the presence of acidic sugars, further purification was undertaken using the ability of cetyltrimethylammonium bromide (CTAB) to complex anionic polysaccharides (Fig. 1). Polymeric material which was not precipitated by CTAB was isolated as the neutral fraction N-1. The precipitated complex was only partially disrupted by the use of excess sodium chloride. The use of 4 M NaCl at 37°C overnight did not fully disrupt the complex, and for convenience, 2 M NaCl at room temperature overnight was chosen as the standard treatment. Polysaccharide which was made soluble in this way was isolated to give the first acidic fraction A-1. Together, the N-1 and A-1 fractions accounted for *c.* 25% of the total extracellular polysaccharides. The complex, which had the form of a soft gel, was successfully disrupted by treating it with ethanol saturated with calcium chloride. The calcium salt of the polysaccharide was subsequently dissolved in water and isolated as the second acidic fraction A-2. A preliminary characterisation of the three fractions is shown in Table 1. The two acidic polysaccharides are clearly different. Fucose, which is a constituent of the A-1 polysaccharide, is completely absent from A-2. This is evident from the sugar analysis shown in Table 1 and also from a comparison of the ^{1}H-NMR spectra of A-1 and A-2. The

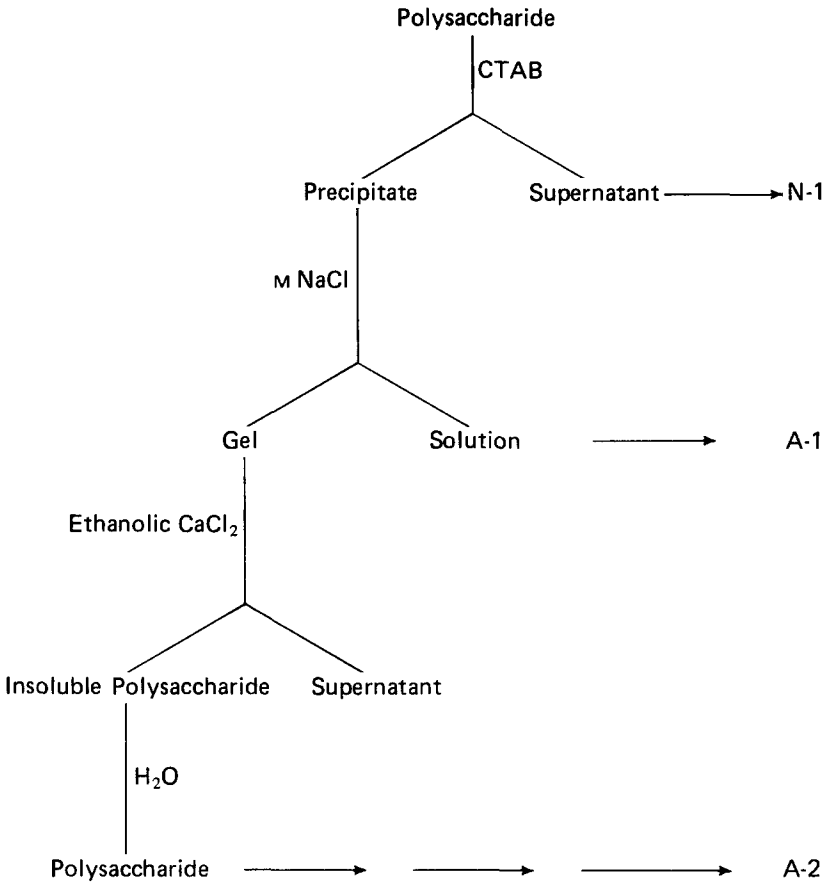

Fig. 1. Fractionation of *B. polymyxa* extracellular polysaccharides. Details of the fractionation are given in the text while the yields obtained and a characterisation of the fractions are shown in Table 1.

^{1}H-NMR spectrum of the A-1 polysaccharide shows two doublets at δ 1·20 and δ 1·31, which we have attributed to the CH_3 group of fucose. No such signals are present in the ^{1}H-NMR spectrum of the A-2 polysaccharide, but this spectrum does contain a singlet signal at δ 1·45 which is typical of the CH_3 group of a pyruvate substituent.

Thus the strain of *B. polymyxa* used in this study produces two structurally-different acidic extracellular polysaccharides. There is preliminary evidence to suggest that A-1 is itself heterogeneous and consists

TABLE 1
Preliminary Characterisation of the Polysaccharides Obtained by a Fractionation of *B. polymyxa* Extracellular Polysaccharide Preparation

Fraction	*Relative amount (%)*	$[\alpha]_D$	*Neutral sugar composition (%)*				*Uronic acid (%)*
			Fucose	*Mannose*	*Galactose*	*Glucose*	
N-1	7	+18°	–	0·8	1	0·3	–
A-1	18	+10°	1·2	0·7	1	1·7	22
A-2	75	+67°	–	1·7	1	2·7	13

of a mixture of non-pyruvylated A-2 and a fucose-containing acidic heteropolysaccharide (Dea & Madden, 1985). This conclusion was based on a separation of A-1 into two components by chromatography on DEAE-cellulose and characterisation of these components by g.l.c. of the sugar residues liberated by acid hydrolysis and by ^{1}H-NMR spectroscopy of the intact polysaccharides. Although the production of more than one extracellular polysaccharide by a pure bacterial strain is not usual, it does seem from recent literature that it is more common than was once believed. For example, a strain of *Xanthomonas juglandis* has been reported to produce two extracellular polysaccharides, one similar in composition to xanthan and the other composed of glucose and rhamnose (Evans *et al.*, 1979). Extracellular polysaccharides differing in composition to the xanthan of the wild-type have been isolated from mutants of *X. campestris* (Whitfield *et al.*, 1981). *Rhizobium meliloti* has been shown to produce two different extracellular acidic polysaccharides (Yu *et al.*, 1981) while the extracellular mucilage of *Beijerinckia mobilis* contains small quantities of a neutral polysaccharide and a major acidic polysaccharide (Cooke & Percival, 1975). Strains of *Alcaligenes faecalis* var. *myxogenes* can produce the β1,3 glucan curdlan in addition to a succinoglycan-type heteropolysaccharide (Harada, 1977). Similar behaviour has been found in certain *Agrobacterium* species (Harada, 1977; Hisamatsu *et al.*, 1977) and in a strain of *R. trifoli* (Ghai *et al.*, 1981).

It is interesting to note that, as already mentioned in the Introduction, there are reports of an extracellular acidic polysaccharide from *B. polymyxa* with a sugar composition similar to A-2 (Ninomiya &

Kizaki, 1969) and also of a fucose-containing extracellular polysaccharide from a different strain of the same organism (Fumino *et al.*, 1979). Surprisingly, this latter polysaccharide was not reported to contain any uronic acid. In this paper we deal mainly with our studies on the A-2 polysaccharide. This is because it was the major extracellular polysaccharide isolated and it alone formed very viscous solutions and gels at low concentrations.

Chemical characterisation of fraction A-2

The polysaccharide had $[\alpha]_D + 67°$ and contained the neutral sugars mannose, galactose and glucose in a molar ratio of *c.* 1·7 : 1 : 2·7. After carboxyl reduction the molar ratio of mannose, galactose and glucose was 1·9 : 1 : 3·7, suggesting one mole of glucuronic acid in the repeating unit. The ^{1}H-NMR spectrum of A-2 was not well resolved and in order to improve the resolution the polysaccharide was depolymerised by a mild acid hydrolysis. Anomeric signals integrating for at least seven different protons could then be distinguished in the spectrum. Two signals, integrating for three protons, at δ 5·36 (1H) and δ 5·18 (2H) were assigned to α-linked residues on the basis of their chemical shifts and small coupling constants. At least four residues were judged to be β-linked due to their larger coupling constants ($J_{1,2}$ 7–8 H_3) and to their chemical shifts at δ 4·83 (2H), 4·70 (1H) and 4·53 (1H). The signal at δ 1·45 was attributed to the CH_3 of pyruvate. In a separate experiment, this substituent was found to be present at a level of 3·7% (Sloneker & Orentas, 1962). Although both sugar analysis and inspection of the ^{1}H-NMR spectra suggest that A-2 is composed of heptasaccharide repeating units, the evidence from methylation analysis is not as conclusive.

Complete methylation of the A-2 polysaccharide was difficult to achieve. The polysaccharide was not readily soluble in dimethyl sulphoxide and extensive ultrasonication was required to dissolve it. Both the salt and free acid forms of the native polysaccharide were methylated according to the Hakomori method (Hakomori, 1964) and, in addition, a Purdie methylation (Hirst & Percival, 1965) of a Hakomori methylated sample was also carried out. In all three cases, evidence for minor hydroxyl group absorption was observed in the infra-red spectra. There were no significant differences between the three samples in the identity or relative amounts of the partially methylated alditol acetates obtained.

TABLE 2
Methylation Analyses of Native and Modified A-2 Polysaccharide

Methlyated sugar[a]	T^b	T^c	*Mole %*		
			I^d	*II*	*III*
2,3,4,6-Man	1·00 (0·97[e])	0·99	13·1	11·6	14·9
2,3,4,6-Glc	1·00 (1·00[e])	1·00	12·2	9·3	12·9
2,3,4,6-Gal	1·20	1·19	–	–	3·4
2,4,6-Glc	1·82	1·82	23·3	19·7	22·9
2,4,6-Man	1·90	1·90	8·0	9·8	4·7
2,3,6-Man	2·03	2·03	11·5	8·8	9·7
2,3,6-Glc	2·32	2·32	11·5	8·4	7·5
2,6-Man	3·08	–	4·7	6·0	6·5
2,6-Gal	3·16	3·14	7·7	7·2	6·5
2,6-Glc	3·36	3·38	–	12·5	9·8
2,3-Gal	4·73	4·70	6·0	4·7	1·0
2-Glc	6·65	6·60	2·0	2·0	0·2

[a] 2,3,4,6-Man = 2,3,4,6-tetra-O-methylmannose, etc.
[b] Retention time of the corresponding alditol acetate relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylglucitol on an OV-225 column at 170°C.
[c] Literature value (Jansson *et al.*, 1976).
[d] Polysaccharide: I, native; II, carboxyl-reduced; III, carboxyl-reduced, methylated, partially hydrolysed and remethylated.
[e] Retention time relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylglucitol on an OV-225 SCOT column at 220°C.

The partially methylated alditol acetates from methylated native polysaccharide (I) and carboxyl-reduced polysaccharide (II) are shown in Table 2. The complex array of methylated sugars may be due to severe undermethylation, as indicated by the presence of small amounts of 2-O-methylglucose, rather than to the complexity of the structure itself. However, this methylated sugar could also arise from substitution of, for example, a 3-linked glucose with pyruvate. The polymer is clearly branched, containing both mannose and glucose as terminal sugars. The appearance of 2,6-di-O-methylglucose in the carboxyl-reduced polysaccharide suggests that the glucuronic acid occurs as a branch point rather than as a pyruvylated terminal residue.

In order to locate the positions of the pyruvate substituent and to circumvent the problems of analysis associated with such substitution, carboxyl-reduced polysaccharide was methylated, hydrolysed using mild conditions, remethylated and converted to the partially methylated alditol acetates (Table 2, column III). This procedure has been reported to readily cleave the hydroxyisopropylidene ketals without significant glycosidic cleavage (Jansson *et al.*, 1979). The extent of depyruvylation was difficult to assess by ^{1}H-NMR. The significant reduction in 2,3-di-O-methylgalactose coincided with the emergence of 2,3,4,6-tetra-O-methylgalactose, indicating that a terminal galactose residue is substituted with pyruvate at O-4 and O-6. However, the amount of terminal galactose is low and from this analysis would not appear to be present on every repeating unit. The decrease in the level of 2-O-methylglucose could be due to a more complete methylation of the polysaccharide or it could be related to an apparent increase in 2,4,6-tri-O-methylglucose.

The A-2 polysaccharide is clearly a branched anionic molecule containing at least three α-linked and four β-linked sugars. It has not proved possible to fully elucidate the structure of the repeating unit but there is evidence to suggest that it contains mannose (1 mole) and glucose (1 mole) as terminal residues, glucuronic acid (1 mole) and mannose (1 mole) as branch points and glucose (2 moles) linked through O-3. A terminal galactose residue, not present in every repeating unit, contains the pyruvate substituent. In addition 4-linked mannose (1 mole) and 4-linked glucose (1 mole) may also occur. Although we have striven to obtain a pure sample of the A-2 polysaccharide, it is conceivable that the complex series of peaks evident in the methylation analysis is due to the presence of small amounts of a second polysaccharide rather than a consequence of the structural complexity of the polysaccharide.

Rheological properties and conformation of the extracellular polysaccharides

Solutions of the total polysaccharide mixture are very viscous at low concentrations. For example, 0·5% w/v of the polysaccharide has a viscosity of 86 poise at a shear rate of 1·1 s^{-1}, which compares with a viscosity of 40 poise for 0·5% w/v xanthan at this same shear rate. At concentrations above 0·5% w/v, *B. polymyxa* polysaccharide forms

thermally-reversible gels. Gelation of the polysaccharide can occur in the absence of added salt although an increase in the ionic strength or the addition of specific cations does lead to an increase in gel strength. This study was continued in an attempt to relate the observed rheological characteristics to the underlying molecular conformation and intermolecular interactions. While these studies were mainly on the total extracellular polysaccharide mixture, there is evidence to show that it is the A-2 polysaccharide which is the active component.

The response of polysaccharide solution dynamic viscosity to increasing ionic strength is shown in Fig. 2. There is a definite increase in dynamic viscosity with increasing ionic strength, with some tendency towards a plateau. This is not the sort of behaviour expected for a typical polyelectrolyte where an increase in ionic strength leads to a screening of the ionic charges resulting in a contraction of the coil and a

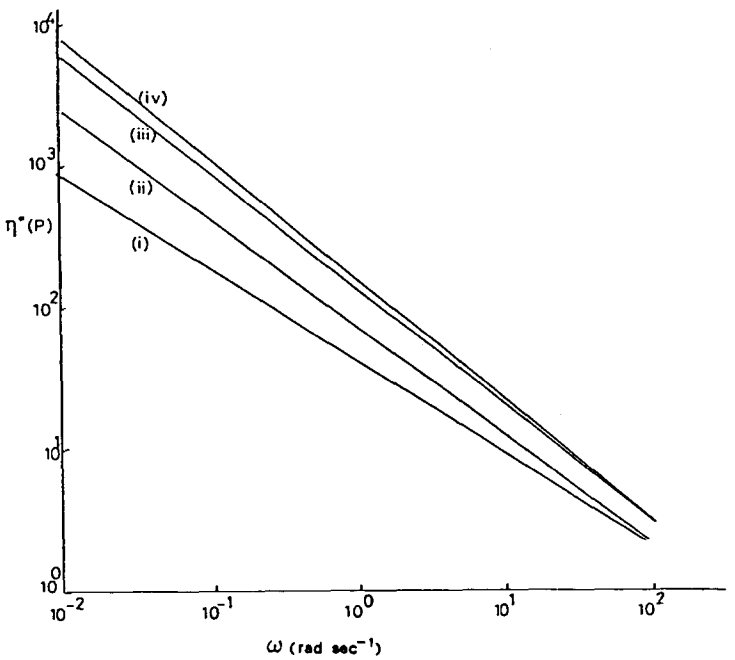

Fig. 2. Variation in solution dynamic viscosity of *B. polymyxa* extracellular polysaccharides (1% w/v) with ionic strength (I) of NaCl. The conditions are (i) no added salt, (ii) $I = 0{\cdot}01$, (iii) $I = 0{\cdot}02$, and (iv) $I = 0{\cdot}05$.

consequent decrease in solution viscosity (Smidsrod & Haug, 1971). However, it is behaviour consistent with an association of ordered chains with very limited, if any, flexibility (Morris, 1977). The increase in the dynamic viscosity is due to a reduction in electrostatic repulsions between the extended, ordered molecules thereby allowing a greater degree of intermolecular association, as for xanthan (Morris, 1977) and the extracellular polysaccharides from certain *Arthrobacter* species (Jeanes *et al.*, 1965; Jeanes, 1974). The shape of the flow curve is itself indicative of a degree of association of conformationally ordered molecules as, clearly, there is no evidence of the Newtonian plateau typical of random coils interacting only by physical entanglement (Morris *et al.*, 1981). In such weak gel systems which are set up by a non-covalent association of ordered chain segments, flow can only occur once a finite stress has been reached. Using a stress–relaxation technique, the yield stress of 1% (w/v) *B. polymyxa* extracellular polysaccharide was measured as 54 dynes cm^{-2}.

The effect of specific cations on the flow behaviour of the extracellular polysaccharides can be seen in Fig. 3. Four different salt forms, sodium (Na^+), potassium (K^+), calcium (Ca^{2+}) and tetramethylammonium (Me_4N^+), were examined. For each salt form, the dynamic viscosity increases with increasing ionic strength, as discussed above. The final viscosities reached are broadly similar, although the Me_4N^+ form may be an exception. However, there appear to be differences in the rate at which this final viscosity is approached. For example, the Ca^{2+} form shows a faster rate of approach than the K^+ form implying that Ca^{2+} has a greater effect on intermolecular association. Further evidence for the occurrence of specific cationic interactions is apparent by comparing the flow curves of the different salt forms in water only. Not only are there differences in viscosity, but the shapes of the flow curves also differ. In particular, for the K^+ and Me_4N^+ ions the curves appear to shown evidence of a Newtonian plateau suggesting that these ions are tending to inhibit intermolecular association or, indeed, destabilising the ordered structure itself. In addition to the importance of specific ionic interactions, non-covalent interactions are also relevant, since the addition of urea to 1% w/v total polysaccharide results in an immediate decrease in viscosity (Fig. 4). A similar observation has been made for xanthan (Frangou *et al.*, 1982). Urea disrupts water structure, and therefore would affect any hydrogen bonding, hydrophobic interaction and specific ionic interactions involved in the stabilisation of

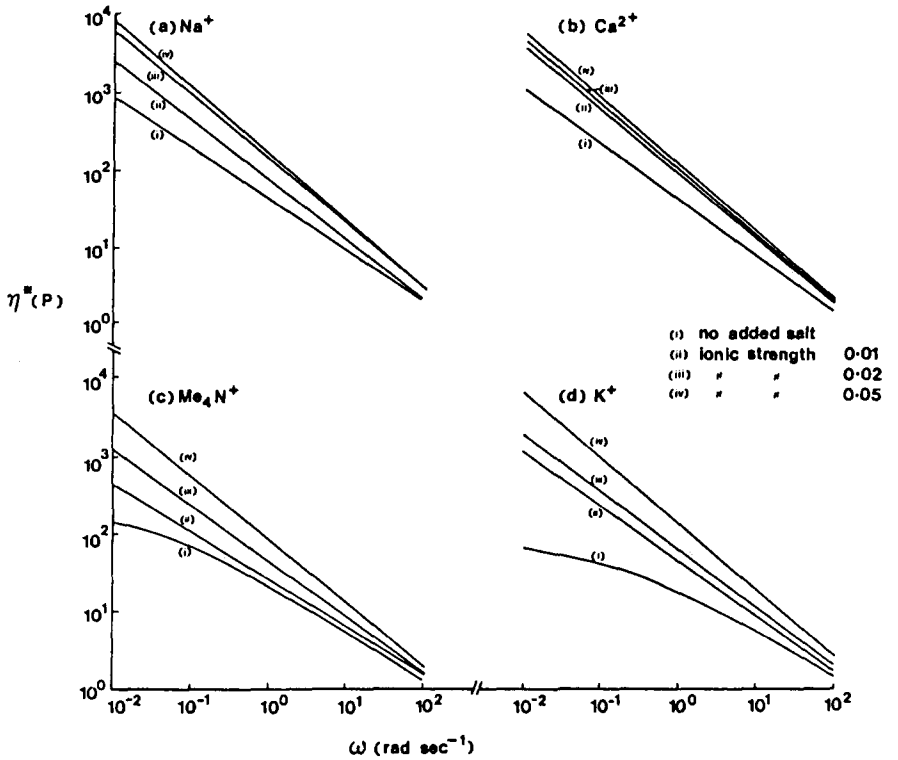

Fig. 3. Effect of specific cations on the flow behaviour of *B. polymyxa* extracellular polysaccharides (1% w/v).

the *B. polymyxa* polysaccharide ordered structure. Recent studies on the nature of the intermolecular interactions in xanthan have shown that there is a contribution both from hydrogen bonding and from ionic interactions (Ross-Murphy *et al.*, 1983).

The weak intermolecular associations were further characterised using small deformation measurements (Morris & Ross-Murphy, 1981). The degree of solid-like character is given by the rigidity modulus, G', the liquid-like nature of the material by the viscosity modulus G'', while the overall response to the applied deformation is monitored by the dynamic viscosity η^*. The mechanical spectrum of a 1% w/v total polysaccharide preparation from *B. polymyxa* and the pure A-2 fraction at the same concentration is shown in Fig. 5. For both samples

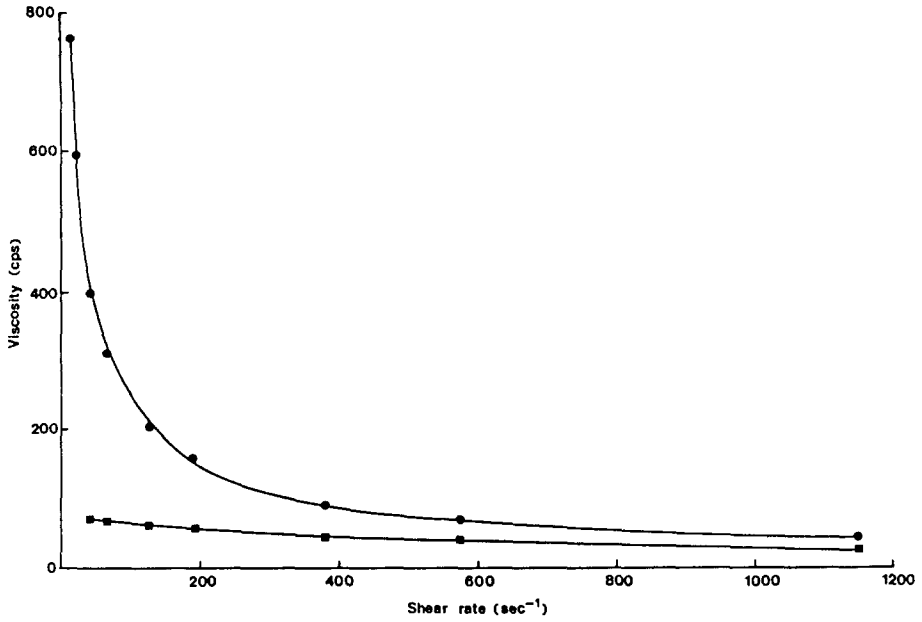

Fig. 4. Effect of urea on viscosity of *B. polymyxa* extracellular polysaccharides (● 1% w/v polysaccharide in water; ■ 1% w/v polysaccharide in 4 M-urea).

G' is greater than G'' at all frequencies and shows little frequency dependence. This type of behaviour is typical of ordered gel networks with solid-like characteristics (Morris & Ross-Murphy, 1981). The similarity between the A-2 fraction and the total polysaccharide preparation is apparent, thus emphasising the fact that it is the A-2 component which is the active one rheologically. It is not clear why the total polysaccharide preparation has slightly higher moduli; it might indicate some rheological synergism between the different components.

At a concentration of 1% w/v in 50 mM NaCl the total polysaccharide preparation exists as a weak non-supporting gel. At this same concentration in water only, the sample appears as a mixture of gel islands. This difference is reflected in the mechanical spectra (Fig. 6). For 1% w/v total extracellular polysaccharide in the absence of added salt, G' and G'' are dependent on the frequency of oscillation and at low frequencies (10^{-1} radsec^{-1}) the moduli converge, reflecting a tendency of the 'network' to rearrange to accommodate the applied strain. In the

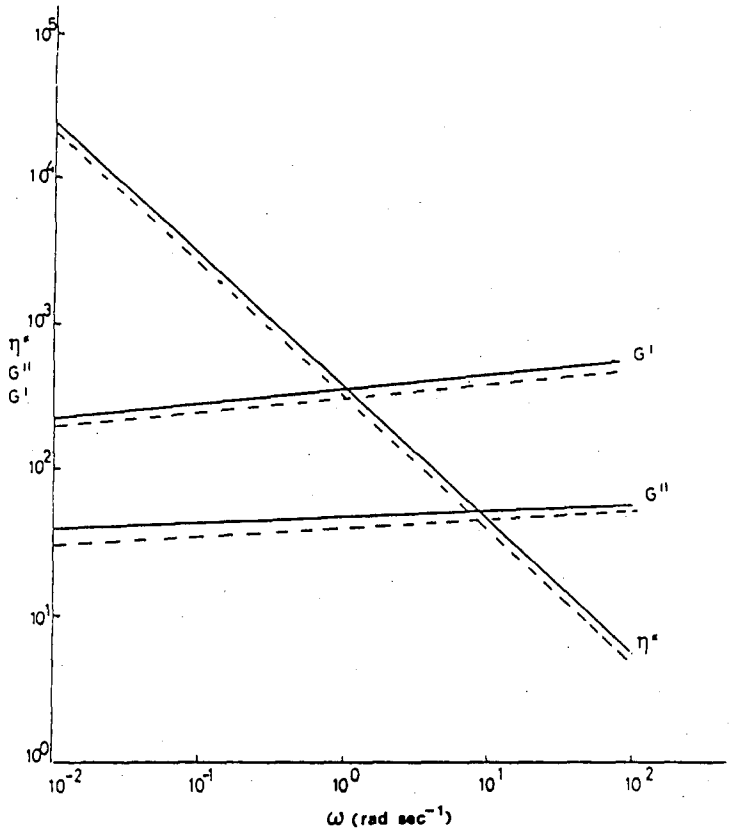

Fig. 5. Mechanical spectrum of the total extracellular polysaccharide preparation from *B. polymyxa* (——) and the A-2 fraction (– – –). Both are 1% w/v in 50 mM NaCl. The frequency (ω) is in radians s^{-1}, while G' and G'' are in dyne cm^{-2} and η^* is in poise.

presence of salt the moduli are virtually independent of frequency, as already discussed. The nature of the gels formed by the extracellular polysaccharide mixture at a range of concentrations in the presence and absence of salt has been qualitatively evaluated (Table 3). In the presence and absence of 0·3 M NaCl, a self-supporting gel is obtained at concentrations of 1% w/v and above. Gel formation will also occur in the absence of salt if a sufficient concentration of polysaccharide (e.g. 2% w/v) is used. All these gels are thermally-reversible. This sensitivity of the gel network to heat is evident from an examination of the

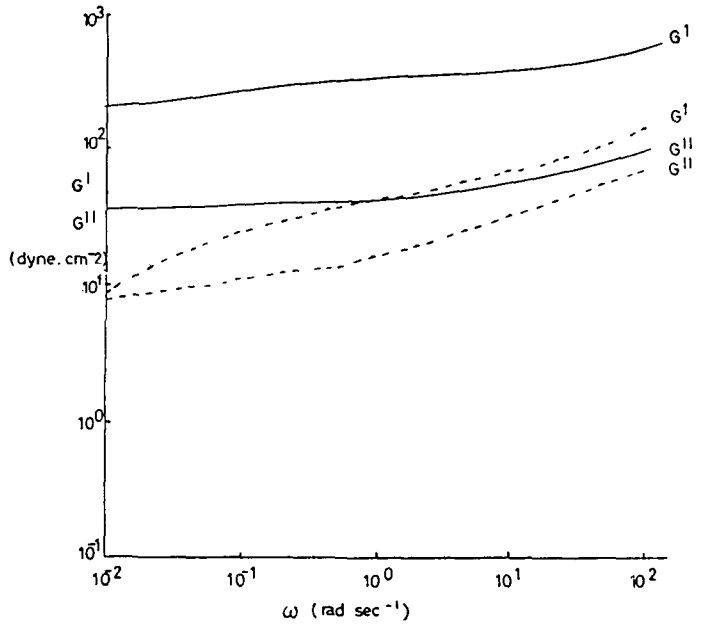

Fig. 6. Frequency dependence of G' and G'' for the total extracellular polysaccharides of *B. polymyxa* (1% w/v) in water only (– – –) and in 50 mM Nacl (——).

TABLE 3
Ability of the Total Polysaccharide Preparation to Form Gels

Polysaccharide concentration (% w/v)	*Medium*	*Nature of gel*
0·5	Water	Gel islands
1·0	Water	Weak gel/gel islands
2·0	Water	Weak self-supporting gel
3·0	Water	Strong self-supporting gel
0·5	0·3 M NaCl	Weak gel/gel islands
1·0	0·3 M NaCl	Medium self-supporting gel
2·0	0·3 M NaCl	Strong self-supporting gel
3·0	0·3 M NaCl	Strong self-supporting gel

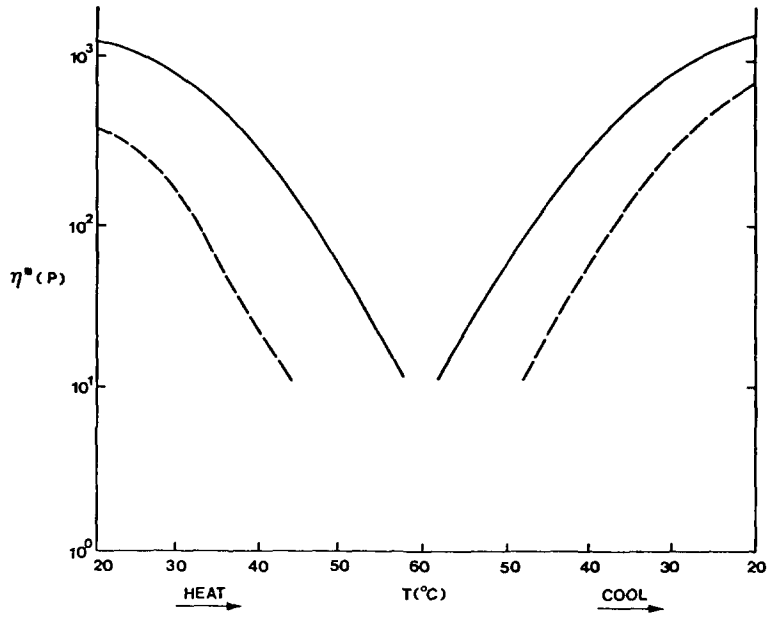

Fig. 7. Temperature dependence of viscosity of the extracellular polysaccharides of *B. polymyxa* (1% w/v) in 10 mM NaCl (– – –) and 50 mM NaCl (——). The frequency (ω) used was 10^{-1} radians s^{-1}. At higher temperatures (*c.* 45-60°C) no measurement of η^* could be obtained due to the decrease in viscosity.

temperature dependence of viscosity (Fig. 7). A significant loss of viscosity occurs at temperatures as low as 40°C. However, this is fully reversible and on cooling the network structure is re-estabilished.

Changes in polysaccharide conformation are frequently accompanied by large changes in optical rotation. As a result single-wavelength optical rotation measurements have been used in other polysaccharide systems to monitor thermally-induced order–disorder transitions (e.g. Dea *et al.*, 1972). In these cases the co-operative nature of the conformational transition imparts a sigmoidal shape to the temperature course of the optical rotation. The temperature dependence of optical rotation for a 1% gelling concentration of total polysaccharide preparation from *B. polymyxa* was therefore examined, and no discontinuity in optical rotation was observed. This could indicate that the ordered structure remains stable over the temperature range examined, and that the melting of the gel with increase in temperature is due to a disruption

of intermolecular association. However, over the same temperature range there is a marked change in the intensity of the high resolution NMR spectrum, indicating a stiffening (ordering) of the polymer chain on gelation. It is therefore more likely that the average conformation of the A-2 polysaccharide in the disordered state is similar to that of the ordered state, thereby resulting in constant optical rotation values. A similar observation has been reported for the gelation of amylose (Dea, 1982).

CONCLUSIONS

Due to the fortuitous stability of the cetyltrimethlylammonium-polysaccharide complex formed during the fractionation of the total extracellular polysaccharide mixture, two acidic polysaccharides were obtained in addition to small amounts of a neutral polysaccharide. The ability of a pure bacterial strain to produce two structurally-different extracellular polysaccharides is unusual but from an examination of recent literature it is not uncommon. The neutral polysaccharide is thought to be a cell wall contaminant. A-2, the major acidic polysaccharide, is a complex heteropolysaccharide with a branched structure. It adopts a rigid, ordered conformation in solution and the subsequent intermolecular interactions of these rigid molecules lead to the formation of a weak gel network which is stabilised both by specific ionic interactions and by non-covalent forces. The A-2 polysaccharide is responsible for the rheological properties of the total polysaccharide mixture. At low concentrations, the total polysaccharide preparation has high solution viscosities with a high yield stress and at concentrations greater than 0·5% w/v in the presence of salt, thermally-reversible gels are formed. In the absence of salt, gel formation will also occur if a sufficient concentration of polysaccharide (e.g. 2% w/v) is used.

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