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Analysis of structure and function of gellans with different substitution patterns

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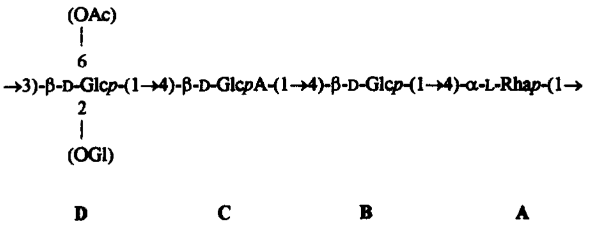
Chemical mutagenesis or exposure to antibiotic stress of *Sphingomonas paucimobilis* ATCC 31461 and R40 have been used to isolate mutants producing modified gellan gum polysaccharides. N.m.r. and conventional carbohydrate analysis methods have been used to characterise these polysaccharides. The ^{1}H and ^{13}C n.m.r. spectra of gellan gum have been fully assigned and the anomeric regions have been shown to be very sensitive to the type and location of non-carbohydrate substituents. Analysis of the gellan gum mutants suggests that they differ in the nature of acetate and glycerate substitution. Such gellan-related polysaccharides have been used to test the selective effect of acyl substituents on the gelation of gellan gum. © 1997 Elsevier Science Ltd. All rights reserved.

INTRODUCTION

Gellan gum is a linear, anionic heteropolysaccharide based on a tetrasaccharide repeat unit (Jannson et al., 1983; O'Neill et al., 1983). The native polysaccharide is partially esterified: the 1,3-D-Glc residue can be linked to L-glycerate at C-2 and/or acetate at C-6 (Kuo et al., 1986). In the structure shown, (OAc) and (OGl) refer to partial substitution by acetate and L-glycerate, respectively. The residues are lettered as referred to later in the text.



$$\rightarrow 3)\text{-}\beta\text{-D-}\underset{\substack{2\\|\\(\mathrm{OGl})}}{\overset{\substack{(\mathrm{OAc})\\|\\6}}{\mathrm{Glc}p}}\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{A-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow$$

Gelation occurs in the presence of both monovalent and divalent cations (Grasdalen and Smidsrød, 1987; Tako et al., 1989; Sanderson, 1990; Moritaka et al., 1992). Non-carbohydrate acyl substituents affect the rheology of the gels, and deacylation of native gellan results in a change from soft, elastic thermo-reversible gels to harder, more brittle gels (Moorhouse et al., 1981). Early X-ray diffraction studies of oriented fibres prepared from gellan gum gels suggested a 3-fold helical structure (Carroll et al., 1982), independent of the species of monovalent counter-ion present (Attwool et al., 1984). Oriented fibres prepared from deacylated gels gave highly crystalline patterns (Carroll et al., 1983; Miles et al., 1984; Chandrasekaran et al., 1988a), whereas the X-ray patterns from native gellan gels were less crystalline (Upstill et al., 1986), suggesting that acylation affects side-by-side association and crystallisation. The latest X-ray studies suggest that both deacylated and native gellan structures are isomorphous forming a left-handed 3-fold parallel double helix (Chandrasekaran et al., 1988b; Chandrasekaran and Thailambal, 1990; Chandrasekaran et al., 1992).

It has been shown that the acetate substituents, which sit on the outside of the helix, do not prevent interhelix association. However, the bulky L-glycerate substituents alter hydrogen bonds within, and between the double helices,

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and sterically mask the uronate groups, altering the binding of cations such as K^+. Gelation of gellan involves two steps; adoption of a helical structure leading to the formation of branched filaments, followed by association of these basic units into a fibrous gel network (Gunning et al., 1996). Thus, Chandrasekaran et al. (1992) argue that the inhibition of cation binding reduces interhelical association and alters the rheological properties of the gels. In order to demonstrate the selective effects of the acyl substituents it is necessary to study gellans with different types and levels of acyl substitution. Treatment with aqueous KOH at 40°C has been used to selectively remove acetate from native gellan and to control the level of remaining glycerate (Baird et al., 1992). The brittleness of gels (with Ca^{2+}) was found to correlate with glycerate more than with acetate content. Further rheological studies on high acyl and on acetate-depleted gellan have further indicated that glycerate primarily influences double-helix formation (Morris et al., 1996). Acetate, sited on the periphery of the double-helix, was implicated in blocking helix–helix aggregation (Morris et al., 1996).

Chemical mutagenesis and antibiotic stress are also well accepted methods for producing variations in the structure of extracellular polysaccharides. They may thus provide a means for obtaining gellans with altered acetate and glycerate substitution, not possible by chemical modification.

Although the synthesis of extracellular polysaccharides has been reported to be unstable in different Gram-negative bacterial species, the appearance of colonial variants with respect to mucoid morphology during cultivation of the industrial gellan-producing strain of *Sphingomonas paucimobilis* ATCC 31461 (formerly *Pseudomonas elodea*) (Pollock, 1993) is a very low-frequency phenomenon (Martins et al., 1996). However, during Cu^{2+}-stressed cultivation, the appearance of mutant cells defective in gellan synthesis dramatically increased (Richau et al., 1997). Interestingly, the spontaneous antibiotic-resistant sub-population, that emerged after extended incubation of gellan-producing *Sphingomonas paucimobilis* strains in antibiotic-supplemented growth medium, gave rise to colonies with a mucoid morphology different from the original morphology. The exopolysaccharides synthesized by four of the many mucoid colonial variants isolated were analysed in the present work, and subtle changes in the structure of gellan were found. Mutants synthesizing gellan-modified polysaccharides were also obtained by chemical mutagenesis, selection being also based on the distinct mucoid morphology of their colonies. Screening for structural changes in these gellan-related exopolysaccharides is difficult. N.m.r. provides a powerful method for screening such mutants.

This article describes for the first time the full assignment of the ^1H and ^{13}C n.m.r. spectra of intact gellan, and the use of such data, coupled with wet chemical methods, to screen mutated gellans. Structural characterisation of such gellan-related exopolysaccharides has made it possible to use them to investigate the predicted selective effect of acyl substituents on the rheology and gelation of gellan gum.

EXPERIMENTAL METHODS

Strains and growth media

The gellan gum-producing strain *Sphingomonas paucimobilis* (formerly *Pseudomonas elodea*) ATCC 31461 (Pollock, 1993) and the spontaneous rifampicin-resistant gellan-producing strain *S. paucimobilis* R40 (Fialho et al., 1991; Martins et al., 1996), were used to derive mutant-producing gellan-related polysaccharides. Liquid growth media used were S medium or LB medium. S medium contained, per litre of distilled water: 10 g Na_2HPO_4, 3 g KH_2PO_4, 1 g K_2SO_4, 1 g NaCl, 1 g casamino acids (Difco, USA), 1 g yeast extract (Difco), 0.2 g $MgSO_4 \cdot 7H_2O$, 0.01 g $CaCl_2 \cdot 2H_2O$, 0.001 g $FeSO_4 \cdot 7H_2O$, 20 g glucose. LB medium contained: 10 g peptone (Difco), 5 g yeast extract, 5 g NaCl per litre of distilled water. Solid media were prepared by the agarization of liquid media (2%, w/v) of agar (Iberoagar, Portugal). When used, the antibiotics rifampicin and kanamycin (Sigma, USA; solubilized in ethanol and water, respectively) were filter sterilized and added to liquid and solid media.

Isolation of mutants

Mutants MJ11, MJ13 and MJ23 were derived from *S. paucimobilis* R40 by chemical mutagenesis during an experiment carried out to isolate non-producing mutants, used to search for complementations of deficiencies by the introduction of a genomic library from *S. paucimobilis* R40 (Fialho et al., 1991; Monteiro et al., 1992). For mutagenesis, an overnight culture of *S. paucimobilis* R40 in LB medium was diluted (1:20) with fresh S medium and incubated at 30°C with agitation until late-exponential phase of growth (5×10^8 viable cells per ml). The culture was centrifuged and cells were washed with sterile saline (0.9% NaCl) solution and resuspended in 5 ml of a buffer consisting of 0.2 M Tris–HCl, pH 7.0, 70 mM K_2HPO_4, 28 mM KH_2PO_4, 7.5 mM $(NH_4)_2SO_4$ and 0.4 mM $MgSO_4$. Five or 10 μl of ethyl methanesulfonate (Sigma, USA) were added per ml of this cell suspension, and incubation proceeded for 1 h at 30°C without agitation. Cells were then washed twice with saline, resuspended in S medium and incubated overnight at 30°C with agitation. Culture was diluted and spread on S medium agar plates, which were incubated at 30°C for 1 week. Mutants were screened based on the distinct mucoid morphology of their colonies as compared with that of the wild-type strain. Non-producing or low-producing mutants were isolated at a frequency of approximately 4×10^{-4}. Mucoid colonies morphologically different from the wild type (for example, larger colonies or colonies exhibiting a wrinkled appearance, a more or less fluid mucus, a different brightness, a depression in the centre, etc.) were retained for further study. Among these variants were strains MJ11, MJ13 and MJ23. The MJ23 colony was pale yellow as compared with the wild-type bright yellow colonies. When this colony was streaked onto S plates, several non-pigmented colonies spontaneously arose. Amongst these was mutant JB3. Strains MJ11, MJ13 and JB3 exhibited a stable phenotype with respect to mucoid morphology and pigmentation of colonies formed in S solid medium.

Mutants CRS2, POLIS and RPS were isolated among the rifampicin ($10\ \mathrm{mg\ l^{-1}}$)-resistant population spontaneously derived from *S. paucimobilis* ATCC 31461. Their identification was also based on the distinct mucoid morphology of colonies formed on S plates with $10\ \mathrm{mg\ l^{-1}}$ of rifampicin, by cells of *S. paucimobilis* ATCC 31461. These were grown until late exponential phase and then exposed to $10\ \mathrm{mg\ l^{-1}}$ of rifampicin for an additional period of 30 h of incubation at 30°C with orbital agitation ($250\ \mathrm{rev\ min^{-1}}$). Morphological variation of mucoid colonies was not observed when *S. paucimobilis* ATCC 31461 was grown and plated in media lacking rifampicin.

Strain SBM1 is a spontaneous kanamycin-resistant mutant derived from the kanamycin-sensitive R40. It was isolated based on the distinct morphology of that colony among those that grew on S agar with rifampicin ($40\ \mathrm{mg\ l^{-1}}$) and kanamycin ($25\ \mathrm{mg\ l^{-1}}$) when cells of *S. paucimobilis* R40 were plated.

Preparation and purification of exopolysaccharides

Cells from overnight liquid pre-cultures of the different strains grown at 30°C in S medium were used to inoculate 200 ml of S medium in 1 l Erlenmeyer flasks to give an initial OD (640 nm) of 0.2 ± 0.02. Cultures were incubated at 30°C with orbital agitation ($250\ \mathrm{rev\ min^{-1}}$). After 50 h of growth, cultures entered the stationary phase and the exopolysaccharide produced was recovered by the addition of 2 vol. of cold ethanol 95% (v/v), followed by several washings of the precipitate with ethanol (Martins and Sá-Correia, 1994; Martins et al., 1996). The ethanol precipitates isolated were redissolved in water, dialysed against distilled water for 48 h and lyophilized.

Chemical analysis

The total carbohydrate content of the polysaccharide was determined as glucose according to Dubois et al. (1956). The level of uronic acids was determined using the 3-hydroxybiphenyl method (Filisetti-Cozzi and Carpita, 1991), calibrated using glucuronic acid. The level of 6-deoxyhexose was determined as rhamnose using the thiocarbamide method (Dische and Shettles, 1948; Baird and Smith, 1989).

Neutral sugars in the gellan samples were determined by acid hydrolysis in 2 M trifluoroacetic acid (Blakeney et al., 1983), derivatization to alditol acetates (Albersheim et al., 1967) and analysis by GC. This was performed on an HP 5890 series II gas chromatograph, with a Thames Rtx-225 column ($0.32\ \mathrm{mm} \times 30\ \mathrm{m}$). The carrier gas was helium at $3.0\ \mathrm{ml\ min^{-1}}$ and the temperature program was: 180°C (1 min), $2\mathrm{°C\ min^{-1}}$ (12.5 min), 205°C (30 min). 2-Deoxyglucose (200 μg) was added as an internal standard and derivatives of external sugar standards were used to identify analytes and calibrate response factors.

The linkage sites of all the sugar residues were determined by methylation analysis. Samples were methylated by sequential addition of powdered sodium hydroxide and iodomethane (Ciucanu and Kerek, 1984; Needs and Selvendran, 1993). After dialysis against deionized water the samples were dried, extracted into $CHCl_3/CH_3OH$ (1:1), dried and reduced with $LiBDEt_3$ in THF ('Super Deuteride', Aldrich) for 2 h (York et al., 1985). They were then partitioned into CH_2Cl_2 with water, dried, hydrolysed and converted to partially methylated alditol acetates (PMAAs) using TFA hydrolysis (Blakeney et al., 1983), $NaBD_4$ reduction and acetylation with acetic anhydride and *N*-methylimidazole (Albersheim et al., 1967). Borate was removed prior to acetylation by neutralizing excess $NaBD_4$ with 200 μl acetic acid, co-evaporating with 4×1 ml methanol, then adding 100 μl of water, then acetylating. The PMAAs were analysed by GC using the temperature program: 55°C (2 min), $45\mathrm{°C\ min^{-1}}$ (1.9 min), 140°C (2 min), $2\mathrm{°C\ min^{-1}}$ (35 min), 210°C (40 min). Analytes were identified by measuring their retention times relative to *myo*-inositol hexaacetate, and comparing the relative retention times to external standards (Doares et al., 1991). The FID signal was used to measure peak areas which were calculated as relative molar quantities using effective carbon response factors (Sweet et al., 1975). The identities of PMAAs were diagnostically confirmed from their electron-ionization mass spectra (Carpita and Shea, 1989) by analysis on an identical GC in series with a Fisons Analytical Trio 1S mass spectrometer, using a source temperature of 200°C and an ionization potential of 70 eV.

N.m.r. spectroscopy

^1H (400 MHz) and ^{13}C (100 MHz) n.m.r. spectra were recorded on a JEOL GX-400 spectrometer at 95°C. One percent of gellan solutions in D_2O (in 5 mm o.d. tubes) were used for ^1H one-dimensional n.m.r., and for DQF-COSY, NOESY and HOHAHA experiments. ^1H spectra with acceptable signal to noise ratio for determination of acetate and glycerate levels could be obtained in 100 scans (about 5 min). Gellan solutions (2%; in 10 mm o.d. tubes) were used for ^{13}C and C/H correlation experiments. Chemical shifts are reported relative to TMS using TSP (^1H, 0 ppm) and dioxan (^{13}C, 67.4 ppm) in D_2O as secondary external references. 2D H/H correlation experiments were carried out in phase-sensitive mode with spectral widths of 2500 Hz in both dimensions and matrix size $2048\ (t_2) \times 256\ (t_1) \times 2$. The phase-sensitive C/H correlation spectra were acquired into a $4096\ (t_2) \times 32\ (t_1) \times 2$ data matrix with spectral widths (covering the carbohydrate region only) of 5555 Hz (^{13}C) and 1000 Hz (^1H). Approximately 2000 scans per t_1 value (depending on the complexity of the sample) were required to detect all signals of interest in the C/H correlation spectra. Data processing was carried out using Felix 95.0 software (Molecular Simulations).

Rheological characterisation of gellan samples

The tetramethylammonium (TMA) forms of JB3, CRS2 and deacylated CRS2 were prepared in the following manner: 150 mg of JB3 was dissolved in 200 ml of water at 95°C with vigorous stirring for 20 min. Whilst still hot, the sample was centrifuged (4500 × *g*, 5 min) to remove insoluble matter, eluted through a column of Dowex-50WX8 (H) resin, adjusted to pH 7.0 with 0.1 M TMA hydroxide, dialysed exhaustively against deionised water and lyophilized. After centrifugation the CRS2 sample was split into two samples. The first sample was ion-exchanged into the TMA form as described above. The second sample was deacylated with 0.1 M NaOH at 1°C for 24 h followed by adjustment to pH 6.5 with dilute HCl, dialysis and conversion to the TMA salt form.

The gellan samples were dispersed in distilled water in a sealed tube by heating at 90°C for 30 min. After cooling to room temperature these samples were centrifuged (5000 × *g*, 1.5 h) to clarify them prior to the calculation of polymer concentration by dry matter measurements. The 0.4% gellan gels were prepared by addition of KCl (to a total concentration of 0.06 M) to the hot sol, pouring these sols into 50 mm diameter moulds and allowing the covered samples to cool to room temperature. Gels were allowed to stand overnight prior to rheological studies. The gels were characterised using an INSTRON 3250 mechanical spectrometer in the parallel plate configuration (plate diameter 40 mm). The gels in perspex moulds were fixed in the rheometer by gluing the underside of the mould to the lower platen. Gels were tested under oscillatory shear (strain 0.01) over a range of frequencies (0.1–10 Hz) and to a range of strains (0.001–0.5) at 1 Hz.

RESULTS AND DISCUSSION

Growth and isolation of mutants

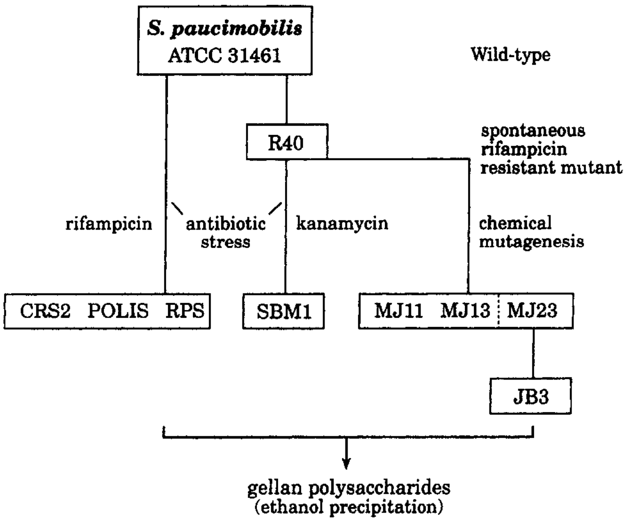

Fig. 1. Schematic representation showing the production of mutants from which gellans were isolated for study.

Mutant strains of *S. paucimobilis* were produced according to the scheme shown in Fig. 1. Nine polysaccharides, including the wild type, were isolated for structural and rheological studies (MJ23 was not used).

Chemical analysis

Colorimetric methods (Table 1) indicated that most of the purified gellans comprised 40%–50% by dry weight of carbohydrate (as glucan), 15%–20% uronic acid (as glucuronan), and 25%–30% 6-deoxyhexose (as rhamnan). Mutants CRS2 and RPS gave notably lower results. Monosaccharide analysis by GC as alditol acetates (neutral sugars only) indicated a Glc:Rha ratio in the mutant gellans of about 1.8–2.2, compared with 1.9 found for the wild strain. A small amount of Man (0.1–0.2 mol) was also detected in all samples. This initially appears to suggest variation in sugar composition amongst the mutants. However, some underestimation of Glc may occur due to incomplete hydrolysis of glycosyl linkages to the uronic acid.

Linkage analysis was performed by methylation, reduction of the uronic acid, and GC–EI-MS of the subsequently formed partially methylated alditol acetates (PMAAs). Deuterium labelling was used in all reduction steps. Four

Table 1.
Chemical analysis of gellan polysaccharides

Gellan mutant	Carbohydratee as Glc (%, w/w)	Uronic acid as GlcA (%, w/w)	6-Deoxyhexose as Rha (%, w/w)	Mole ratio of neutral sugars			Mole ratio of glycosidic linkages			
				Rha	Glc	Man	1,4-Rha*p*	1,3-Glc*p*	1,4-Glc*p*	1,4-Glc*p*A
W-T	44.7	27.6	29.8	1	1.86	0.20	1	1.07	0.99	0.55
CRS2	31.6	14.9	14.3	1	2.23	0.09	1	1.08	0.97	0.45
JB3	49.5	15.7	22.1	1	2.26	0.10	1	1.12	1.12	0.87
MJ11	49.5	17.9	27.0	1	2.03	0.22	1	0.76	0.98	0.48
MJ13	46.9	16.7	26.8	1	1.90	0.17	1	1.07	1.01	0.48
POLIS	50.7	18.3	26.6	1	2.21	0.16	1	1.06	1.04	0.40
R40	41.2	18.2	25.5	1	2.19	0.11	1	1.03	0.92	0.40
RPS	13.6	8.4	18.1	1	1.77	0.11	1	1.14	0.90	0.34
SBM1	43.6	16.7	25.5	1	1.80	0.09	1	1.15	1.06	0.59

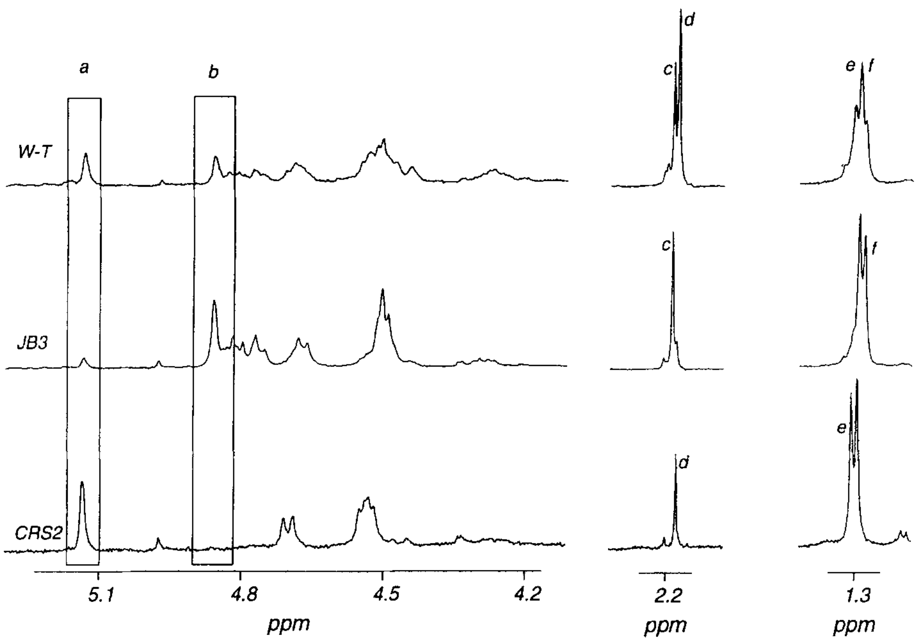

Fig. 2. 400 MHz ^{1}H n.m.r. spectra (95°C) of wild-type (W-T) and mutant gellan polysaccharides showing the anomeric region and methyl resonances. Signal assignments (see text): (*a*) A1 (no glycerate); (*b*) A1 (glycerate on **D**); (*c,d*) Ac Me (with, without glycerate also on **D**); (*e,f*) A6 (without, with glycerate, respectively).

components were derived from all the mutants (Table 1): 1,4,5-tri-*O*-acetyl-1-deuterio-2, 3-di-*O*-methylrhamnitol; 1,3,5-tri-*O*-acetyl-1-deuterio-2,4-di-*O*- methylglucitol; 1,4,5-tri-*O*-acetyl-1-deuterio-2,3-di-*O*-methylglucitol; and 1,4,5,6-tri-*O*-acetyl-1,6,6-trideuterio-2,3-di-*O*-methylglucitol. These correspond to the expected residues in gellan gum, i.e. 1,4-linked Rha*p*, 1,3-linked Glc*p*, 1,4-linked Glc*p* and 1,4-linked Glc*p*A, respectively. The relative proportion of 1,4-Glc*p*A is lower than 1, possibly due to under-reduction of the uronic acid. The proportion of 1,3-Glc*p* was initially very low, but recovery was improved when the linkage analysis was repeated including removal of borate prior to acetylation. No component was found corresponding to 1,4-Man*p*, as might be expected if substitution for Rha had occurred, as in related polysaccharides (Chandrasekaran and Radha, 1995). Close inspection of the mass spectrum of the 1,3-Glc*p* component showed evidence of some coeluting 1,2-Man*p* derivative for several of the samples, but this did not appear in the n.m.r. spectra (see below). The absolute configuration of sugars was not determined.

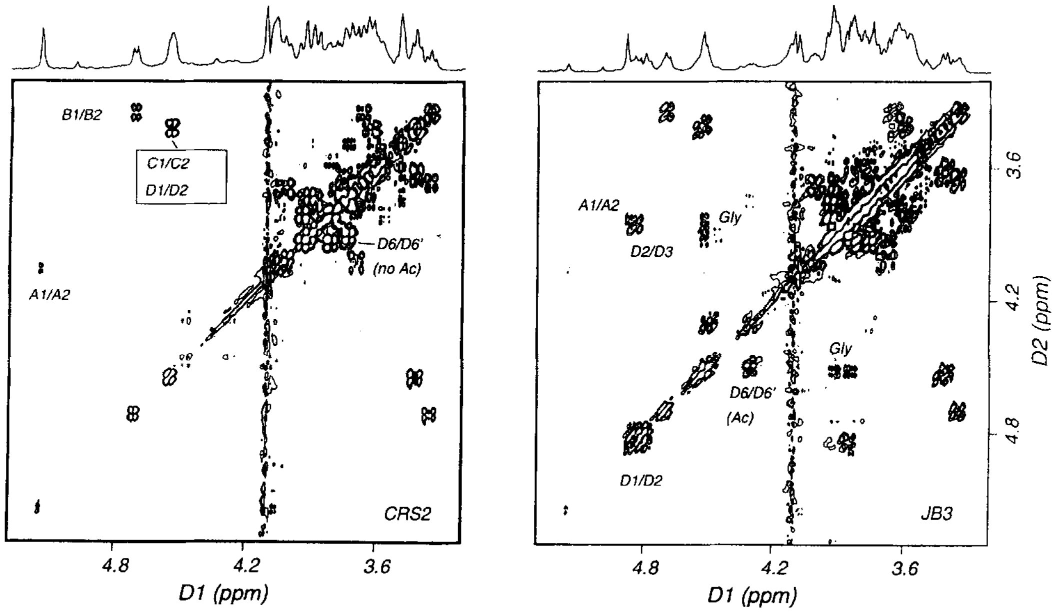

Fig. 3. 400 MHz COSY spectra of CRS2 and JB3 (95°C). Cross peaks involving anomeric protons are labelled together with peaks which show major shifts on substituent addition (Gly, cross peaks involving bound glycerate).

N.m.r. spectroscopy

The presence of *O*-glycerate and *O*-acetate substituents in native gellan was first demonstrated by Kuo et al. (1986). They generated oligosaccharides from the polymer by treatment with liquid hydrogen fluoride and used n.m.r. and FAB-MS to determine the identity and location of the substituents. HF attacks the linkage between rings **A** and **D** (coding shown above), but leaves the substituents in place. The substituents were found to be at *O*-2 (L-glycerate) and *O*-6 (acetate) of the resulting non-reducing end residue, **D**. They reported that the substitution pattern of gellan may be heterogeneous but were unable to define the substitution patterns due to limitations in the methods available at that time. In the present approach all the n.m.r. studies were carried out directly on the intact polysaccharides (1%–2% solutions in D_2O at 95°C). ^1H n.m.r. spectra obtained under these conditions allow rapid screening of gellan polysaccharides from mutant strains for any compositional alterations.

Preliminary examination of the ^1H n.m.r. spectra of gellan samples from the wild-type and all the mutant strains revealed only three main spectral types, illustrated by the three spectra in Fig. 2. For one typical case (CRS2) the downfield region of the spectrum ($\delta = 4.2$–5.2) was very similar to that of commercial gellan samples which have had both substituents removed by alkali treatment during processing. (Observation of a signal at $\delta = 2.14$, however, showed that a small amount of acetate was still present in CRS2). The region contained doublet signals from the four anomeric protons, two of which (**C**1 and **D**1) were closely overlapped at $\delta = 4.54$. The downfield region was considerably more complex for other gellans represented in Fig. 2 by wild type (W-T) and JB3, the spectral changes being associated with the addition of substituents.

^1H and ^{13}C signals for the different gellan types were assigned (Table 2) using a combination of 2D n.m.r. proton–proton and carbon–proton correlation experiments. COSY experiments on CRS2 and JB3 are shown in Fig. 3, and illustrate the origin of the additional signals observed in the downfield region for JB3. The signals are attributable to downfield displacements of carbohydrate proton chemical shifts on addition of a neighbouring substituent, as well as to one signal from the glycerate itself.

The results of the 2D experiments explained the spectral differences between the wild-type gellan and the various mutants. These were entirely due to changes in the amounts of substituents present, not to any changes in backbone repeating unit or in the locations of substituents. The large downfield ^1H chemical shift displacements seen in Table 2 for **D**2 (addition of glycerate) and **D**6 (addition of acetate) confirmed that the substituents were at the same positions as determined previously (Kuo et al., 1986).

As well as these direct effects of substituent addition on the n.m.r. parameters of the Glc ring, **D**, some indirect effects were observed on the neighbouring rings. In particular, the chemical shift of the Rha anomeric proton (**A**1) showed a considerable upfield shift ($\delta = 5.15$–4.88) when glycerate was present on ring **D**. The glycerate level in each sample could readily be determined by integration (or curve fitting, as used here) of the two Rha anomeric signals. The relevant signals, *a* and *b*, have been highlighted in Fig. 2,

Table 2. ^1H and ^{13}C chemical shifts for gellan polysaccharides

Residue		Chemical shift δ							
		1	2	3	4	5	6		6′
No substituents									
(1,4)-α-L-Rha*p*	^1H	5.15	4.06	4.00	3.70	4.03	1.32		
A	^{13}C	101.43	71.34	71.14	82.16	68.28		17.68	
(1,4)-β-D-Glc*p*	^1H	4.73	3.37	3.64	3.61	3.58	3.18		3.95
B	^{13}C	103.81	74.67	75.35	79.82	75.75		61.34	
(1,4)-β-D-Glc*p*A	^1H	4.54	3.40	3.64	3.75	3.89	—		
C	^{13}C	102.96	73.70	75.35	81.57	76.35		175.1	
(1,3)-β-D-Glc*p*	^1H	4.54[a]	3.37	3.62	3.48	3.49	3.73		3.92[b]
D	^{13}C	103.17	74.67	83.35	69.15	76.88		61.79	
With glycerate[c]									
(1,4)-α-L-Rha*p*	^1H	4.88	3.83	3.92	3.67	4.01	1.30		
A ^{13}C		106.64	71.42	71.06	81.92	68.47		17.62	
(1,4)-β-D-Glc*p*	^1H	4.69	3.34	3.64	3.64	3.56	3.80		3.93
B ^{13}C		103.76	74.66	75.34	79.61	75.71		61.34	
(1,4)-β-D-Glc*p*A	^1H	5.54	3.40	3.65	3.73	3.73	—		
C ^{13}C		103.17	73.78	75.21	81.02	77.44		175.1	
(1,3)-β-D-Glc*p*	^1H	4.78[d]	484	3.85	3.58	3.54	3.76		3.94[e]
D ^{13}C		100.41	75.22	81.95	69.59	76.87		61.64	

Notes:

[a] With acetate, δ $^1H = 4.55$; δ $^{13}C = 103.54$

[b] With acetate, δ $^1H = 4.28, 4.48$; δ $^{13}C = 64.30$; Ac Me: δ $^1H = 2.14$; δ $^{13}C = 20.96$

[c] Bound glycerate, δ $^1H = 4.52, 3.92, 3.84$; δ $^{13}C = 72.61, 63.72$ (free glycerate, δ $^1H = 4.04, 3.80, 3.70$; δ $^{13}C = ?, 65.2$)

[d] With acetate, δ $^1H = 4.78$; δ $^{13}C = 100.69$

[e] With acetate, δ $^1H = 4.30, 4.50$; δ $^{13}C = 64.11$; Ac Me: δ $^1H = 2.16$; δ $^{13}C = 20.93$

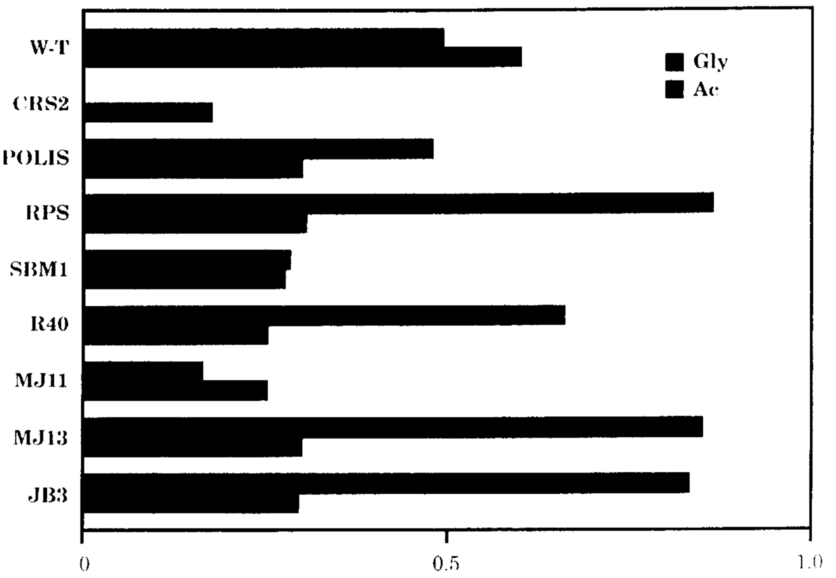

Fig. 4. Content of L-glycerate and acetate (relative to 1 sugar unit) in wild-type and mutant gellan polysaccharides.

which shows that for the three samples displayed the relative glycerate levels are CRS2 (zero), wild type (intermediate), JB3 (high). Results for these and the other samples are shown in Fig. 4. The acetate content was also determined (relative to the total area of the two Rha H-1 signals) from integration of the two methyl signals centred at $\delta = 2.15$ and shown in Fig. 2. Results for the three samples displayed were CRS2 (low), wild-type (high) JB3 (intermediate) and the acetate contents for all the samples are again shown in Fig. 4. Both the acetate and the Rha methyl signals were sensitive to the presence of glycerate in ring **D**, and these signals could be assigned since the glycerate levels were known. For acetate the signals are at $\delta = 2.16$ (glycerate present, marked *c* in Fig. 2) or $\delta = 2.14$ (no glycerate, marked *d*); for the **A6** doublets the corresponding chemical shifts are $\delta = 1.32$ (no glycerate, peaks marked *e*) and $\delta = 1.30$ (glycerate present, doublet *f*).

The measurements of substituent content shown in Fig. 4 indicated that the acetate content of all the mutants was only about half that found in the wild type. There appeared to be wide variations in the glycerate content, but examination of the ^1H spectra showed signals attributable to free glycerate in all samples which were low in bound glycerate. Although the amounts of free glycerate were difficult to determine exactly from the n.m.r. spectra, the actual variations in total glycerate would be much less than the variations in bound glycerate shown in Fig. 4. The origin of the free glycerate is not known, but it seems likely that it was originally bound to the polysaccharide. It was established that the short periods of heating needed to dissolve the samples and obtain the ^1H spectra did not lead to any loss of substituents. Heating for longer periods, especially at higher pH (e.g. pH 8) did lead to some loss of substituents, glycerate being removed more readily than acetate. For example overnight heating (at 95°C, pH 8) led to loss of 60% of the bound glycerate and 25% of the bound acetate.

The ^{13}C spectra allow cases to be distinguished where the **D** ring carries no substituents, one substituent (of either type) or both substituents. This is illustrated in the spectra of the anomeric region shown in Fig. 5. For W-T gellan, the ^{13}C spectrum was obtained after the sample had been modified by prolonged heating (several days at 95°C). This procedure led to a total loss of the glycerate but retention of the acetate substituent. The presence of the acetate at *O*-6 in ~50% of the **D** rings results in a downfield displacement of about half the intensity of one of the anomeric signals (compare 'W-T' with CRS2, peaks *e* and *b*). The assignment of **C**1 and **D**1 ^{13}C signals (peaks *b* and *c*) by the usual $^{13}C/^1H$ correlation method is ambiguous since the two attached protons have the same chemical shift in unsubstituted gellan. Hence it is also difficult to know which of these two anomeric signals is affected by addition of acetate: either is plausible. A provisional assignment can be made by comparing the behaviour of the **D**1 resonances which are displaced upfield when glycerate is present (JB3). In JB3 two separate resonances are observed and can easily be assigned by $^{13}C/^1H$ correlation: the two **D**1 signals are at $\delta = 100.41$ (glycerate only, peak *h*) and $\delta = 100.69$ (both glycerate and acetate, peak *g*). Assuming that the addition of acetate has the same effect when glycerate is not present, the **D**1 signals were assigned as $\delta = 103.54$ (acetate only, peak *e*), $\delta = 103.17$ (no substituent, peak *b*). The intensities in Fig. 5 for JB3 also require that the **C**1 δ is changed from 102.96 to 103.17 in the presence of glycerate. The ^{13}C δ of **A**1 is not affected by presence of acetate, but shows a small downfield displacement when glycerate is present (peaks *d*, *f* in Fig. 5). Assignments are summarised in Table 2.

The effects of substituent addition on chemical shifts of anomeric carbons and linkage carbons, such as **C**4, may be attributed to the changes in the average conformation of the glycosidic linkages. Addition of glycerate (which is located at *C*-2, between the two linkage positions of ring **D**) might

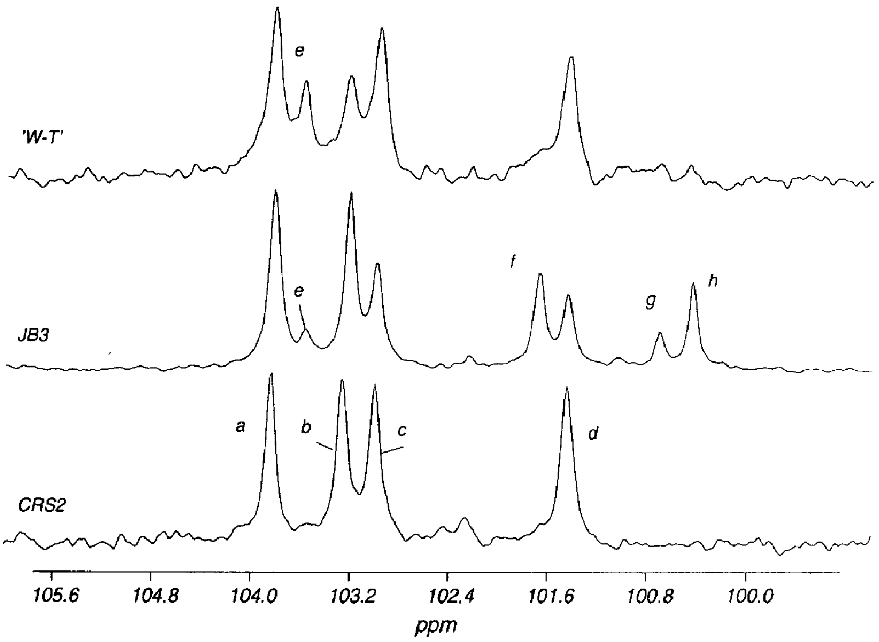

Fig. 5. 100 MHz ^{13}C n.m.r. spectra (95°C) of modified wild-type ('W-T') and mutant gellan polysaccharides. The 'W-T' sample differs from that in Fig. 1 through loss of most of the glycerate. Signal assignment (see text for details): (*a*) **B1**; (*b*) **D1**; (*c*) **C1**; (*d*) **A1** (all with no substituent); (*e*) **D1** (with Ac); (*f*) **A1** (with glycerate); (*g*) **D1** (with Ac + glycerate); (*h*) **D1** (with glycerate).

be expected to cause somewhat greater changes than addition of acetate. The n.m.r. experiments reported here were made with gellan in the disordered form. In the ordered form, X-ray data show that glycerate does alter certain glycosidic linkages (Chandrasekaran et al., 1992).

Rheological characterisation

In this study, variants of the gellan structure have been selected in order to assess the selective effects of different acyl substituents on gelation of gellan gum. The wild-type and the isolated mutants can be divided into groups with similar substitution patterns. Representative samples of these groups have been chosen for study. Rheological studies were made on CRS2, a gellan containing a moderate level of acetate substitution but no detectable glycerate substituents, and on JB3, a gellan containing both acetate and glycerate (0.3 and 0.8 mol per repeat unit, respectively). Data on these two gellan variants were compared with data obtained on chemically deacylated CRS2. In order to eliminate effects on the gelation due to differing ionic composition of the gellan variants after isolation from the broths, the gellans were all converted into the tetramethylammonium (TMA) form prior to gelation study. The gels

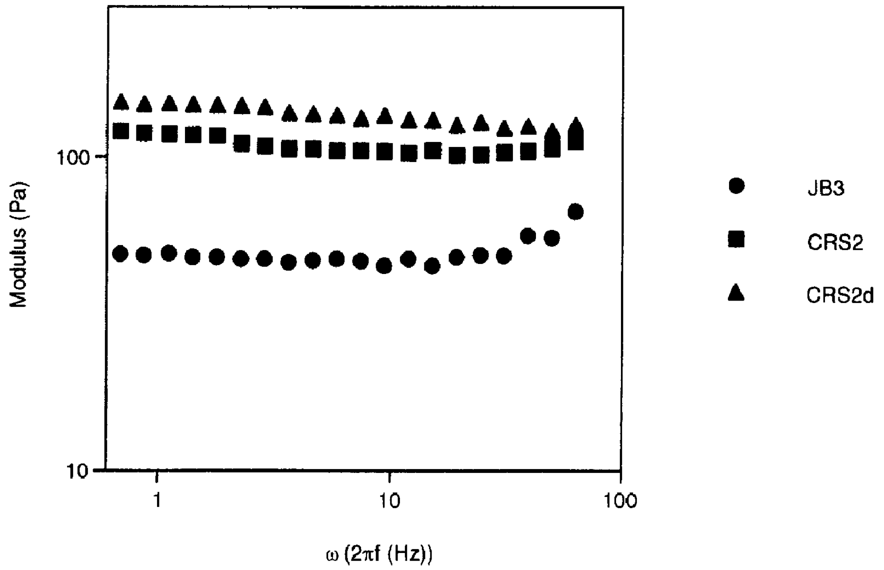

Fig. 6. Frequency dependence of the shear modulus for gellan gels (0.4%, w/v) in 0.06 M KCl.

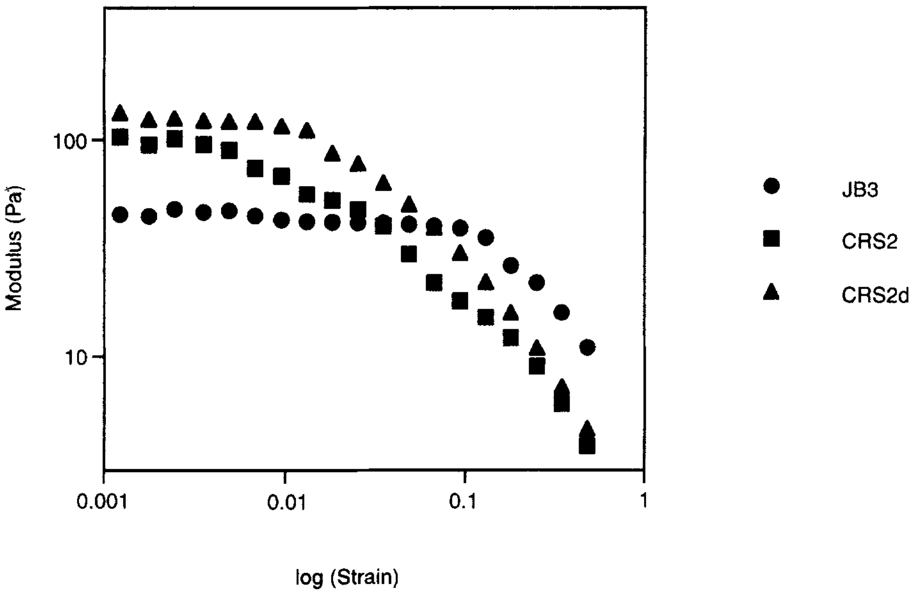

Fig. 7. Strain dependence of the shear modulus for gellan gels (0.4%, w/v) in 0.06 M KCl.

were compared by determining the shear modulus of the gels. This parameter was chosen because it is only sensitive to the molecular weight of the polysaccharide when this becomes very small. Preliminary studies of the viscosity of the TMA gellans at high temperature, prior to gelation, revealed no significant differences in molecular weight.

Figs. 6 and 7 show the effect of non-carbohydrate substituents on 0.4% (w/w) gels prepared in 0.06 M KCl. Fig. 6 shows that the shear modulus (G^1) is independent of frequency. The phase angles for all systems was less than 5°, indicating the formation of elastic gels. The gellan variant (JB3) containing both acetate and glycerate substituents produces the weakest gels. Sample CRS2, which contains no glycerate, produces stronger gels and deacylation of CRS2 produces a further small increase in modulus. These data confirm that both acetate and glycerate influence G^1 but the major effect is due to glycerate substituents. As well as influencing the stiffness of the gels, acyl substituents also alter the brittleness of the gels. This is indicated in Fig. 7 where the strain dependence of the modulus is used to illustrate fracture of the gel network. Whereas JB3 gels can withstand strains up to 10% without structural breakdown, CRS2 and deacylated CRS2 gel structures break down at strains of the order of 0.7%. Once again, the predominant effect appears to involve glycerate rather than acetate substituents. The data for the CRS2 mutant gellan, with acetate-only substitution, complement previous studies which used gellans with glycerate-only substitution (Baird et al., 1992; Morris et al., 1996).

CONCLUSIONS

The ^1H and ^{13}C n.m.r. spectra of gellan gum have been fully assigned. Variants of the gellan structure were produced by mutants of the industrial gellan gum-producing strain *S. paucimobilis* ATCC 31469, and of its mucoid rifampicin-resistant spontaneous mutant R40, prepared by chemical mutagenesis or antibiotic stress. N.m.r. together with wet chemical analysis methods were used to screen these variants, demonstrating that they differ in their acetate and glycerate contents. The anomeric region has been found to be very sensitive to type and location of the substituents and can be used to identify 1,3-linked Glc residues containing both substituents, either substituents or no substitution. The anomeric region of one of the mutants shows that it is possible for all four types of substitution to occur at the 1,3-Glc residue, within a single polymer sample. Variants of the gellan structure containing both glycerate and acetate, no substituents and, for the first time, only acetate substitution have been used to demonstrate the selective effects of different substituents on rheological properties. These results confirm both predictions from X-ray studies and results from rheological studies on chemically deacylated gellan, that it is the glycerate substituents which are responsible for the significant changes in rheology observed upon deacylation of gellan gum.

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