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Structure and conformation of a novel genetically engineered polysaccharide P2

Ian J. Colquhoun, Andrew J. Jay,* John Eagles, Victor J. Morris, Kirstin J. Edwards, Annette M. Griffin, Mike J. Gasson

Institute of Food Research, Norwich Research Park, Colney, Norwich NR4 7UA, UK

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Abstract

A new exocellular polysaccharide (P2) has been produced by the manipulation of a glycosyl transferase gene (*aceP*) involved in the biosynthesis of the polysaccharide acetan by the bacterium *Acetobacter xylinum* strain CKE5. The P2 polysaccharide has been studied by methylation analysis, reductive cleavage, and ^{1}H and ^{13}C NMR spectroscopy. The data are consistent with the structure predicted when the *aceP* gene is deactivated:

The effect of cooling on proton NMR line width indicates a coil–helix transition in P2 at about 70 °C.

Keywords: Polysaccharide; *Acetobacter xylinum*; *aceP* gene; NMR; Methylation

1. Introduction

The synthesis of polysaccharides or oligosaccharides by chemical, or combined chemical–enzymatic methods remains a challenging task.[1,2] Bacteria provide a cell factory for the production and export of polysaccharides and the fermentation technology for such systems is well developed. The fact that bacteria can evolve strains with new exocellular polysaccharide structures[2] suggests that it is possible to modify the biosynthetic pathways for polysaccharide production. There is a question as to the extent to which it may be possible to engineer metabolic pathways to synthesise desired polysaccharide structures.[3] For branched bacterial polysaccharides there is the possibility of synthesising oligosacchar-

* Corresponding author. Tel.: +44-1603-255000; fax: +44-1603-507723.

E-mail address: jay@bbsrc.ac.uk (A.J. Jay).

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ides as branches for subsequent cleavage by chemical or enzymatic means. This paper describes studies on the production of a modified bacterial polysaccharide produced within the model bacterial system *Acetobacter xylinum*. The research represents the first steps on the route to the design of carbohydrates in bacterial systems.

A. xylinum was chosen as a model system for several reasons. Firstly, certain *A. xylinum* strains produce a polysaccharide called acetan (Fig. 1(a))[4,5] which is similar in structure to the commercially important polysaccharide xanthan (Fig. 1(b)), produced by *Xanthomonas campestris*.[6] Secondly, the biosynthetic pathways for production of polysaccharides in both bacteria are very similar.[7] Indeed, the first four steps in the assembly of sugars in the repeat unit are identical in both bacteria, and the polysaccharides thus possess a conserved structural region (Fig. 1), excluding non-carbohydrate substituents. Thus, these systems provide an ideal environment for examining the ability to delete or substitute genes, with a view to manipulating the structure of polysaccharides. Finally, the genes involved in the biosynthesis of xanthan have been identified in collections of natural and induced mutant strains.[8–10] This has provided the basis for the rational isolation of the cluster of genes involved in acetan biosynthesis.[11–15]

The first step in the modification of the polysaccharide structure will involve the deletion or inactivation of biosynthetic genes. It has been established through chemical mutagenesis studies that *A. xylinum* will produce variants of the acetan structure with truncated side-chains.[16] This paper reports the detailed structural characterisation of a new engineered polysaccharide P2, which has a modified acetan structure containing a truncated side-chain, and has been produced by inactivation of the *aceP* gene, which encodes for a glycosyl transferase involved in acetan biosynthesis.[17] NMR studies, used to determine the structure of the chemical repeat unit, have also provided evidence in favour of a helical structure for the polysaccharide and a temperature induced helix–coil transition in solution. The genetic manipulation of the biosynthetic pathway used to produce the new polysaccharide P2 is described in detail elsewhere, together with a preliminary structural characterisation of the polysaccharide P2.[17]

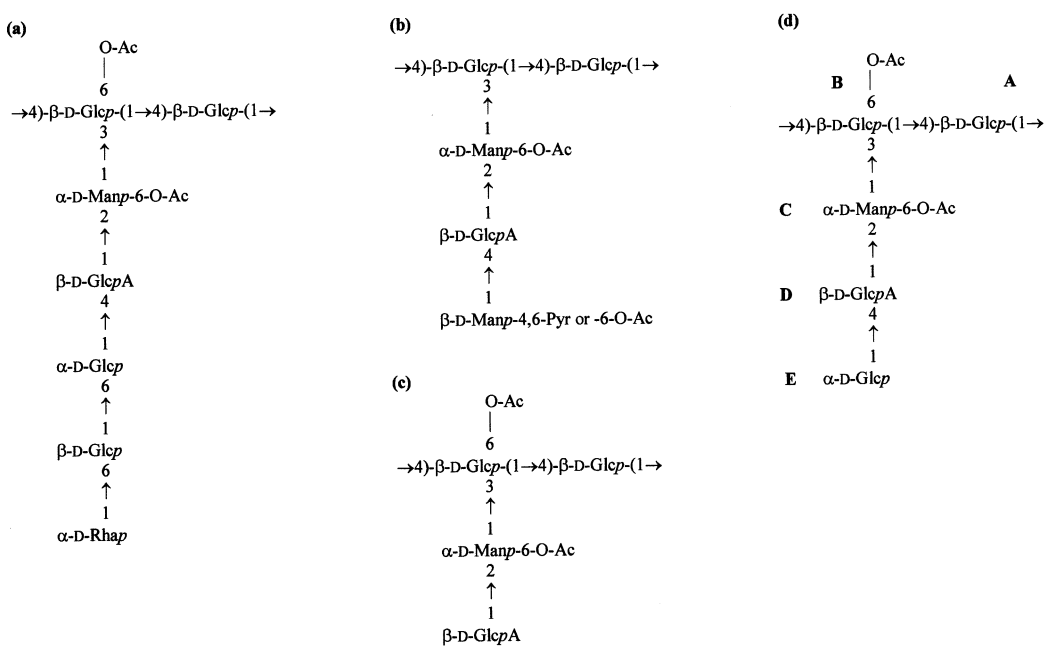

Fig. 1. The repeat unit of related bacterial polysaccharides: (a) acetan; (b) xanthan; (c) CR1/4; and (d) P2 (proposed). The labelling of residues **A**–**E** is referred to in the results and discussion. Ac = acetyl; Pyr = pyruvate ketal.

Table 1
Linkage analysis of polysaccharide from *A. xylinum* strain P2 [a]

Sugar residue	Mole ratio		
	PMAA	PMAA(c-red)	red-cl
t-Glc	1.8	1.8	1.3
1,2-Man	0.83	2.5	1.6
1,4-Glc	1.0	1.0	1.0
1,3,4-Glc	0.5	0.68	1.1
1,4-GlcA		0.95	2.1

[a] PMAA, partially methylated alditol acetate; c-red, carboxy-reduced; red-cl, reductive cleavage derivative.

2. Results and discussion

Linkage analysis.—The polysaccharide acetan is known to comprise of a repeat heptasaccharide unit, whilst the inactivation of *aceP* should result in the expression of a polysaccharide with a pentasaccharide repeat unit.

Preliminary chemical analysis had shown that the composition of the new polymer differed from that of acetan, most notably in the absence of rhamnose. Linkage analysis, using conventional methylation analysis and reductive cleavage[18] showed the presence of the expected sugar residues (Table 1).[17]

The linkage analysis using reductive cleavage was particularly useful for several reasons. Firstly, it confirmed the qualitative composition obtained by conventional methylation analysis. Secondly, the retention times and the electron ionization mass spectra (EI-MS) of the partially methylated alditol acetates (PMAAs) of terminally linked Glc and Man are nearly identical, whereas the retention times of the 1,5-anhydroalditol derivatives are distinct and identified the terminal sugar unambiguously as *t*-Glc (Table 2). Thirdly, ring sizes were confirmed as pyranose, except for the glucuronic acid residue which is known to rearrange to the furanose derivative during reductive cleavage.[19] Finally, the recovery of residues as 1,5-anhydroalditol derivatives gives molar ratios closer to unity since there is less degradation than with acid hydrolysis. The rhamnose component present in acetan has not been identified in any of the linkage analyses.

Absolute configuration.—The absolute configuration of the constituent sugars of carboxy-reduced P2 polysaccharide was determined, as trimethylsilylated (−)-2-butyl glycosides, to be D-glucose, D-glucuronic acid (as glucose) and D-mannose.

NMR spectroscopy.—Complete ^{1}H and ^{13}C NMR assignments were reported previously for the deacetylated CR1/4 polysaccharide and for the side chain units (except GlcA) of deacetylated acetan.[4] Whereas both those polysaccharides gave reasonably well-resolved NMR spectra at 95 °C in water, no useful spectra of either native or deacetylated P2 could be obtained until after a sonication treatment had been applied to the solutions.[20] Following this treatment, ^{1}H and ^{13}C spectra of P2, comparable in quality to those of the other polysaccharides, were obtained at 95 °C. NMR spectra of deacetylated P2 will be discussed first, then the differences seen for the acetylated or native polysaccharide will be

Table 2
GC–MS data for partially methylated 1,5-anhydroalditols of P2 polysaccharide

RT (min)	MS ions (*m*/*z*)	Compound	Sugar residue
10.27	101, 71, 75, 88, 99, 143, 175, 102, 111, 115	1,5-anhydro-2,3,4,6-tetra-*O*-methyl-D-glucitol	*t*-Glc*p*
18.44	71, 101, 87, 102, 102, 111, 147, 129, 88, 143, 171, 203	2-*O*-acetyl-1,5-anhydro-3,4,6-tri-*O*-methyl-D-mannitol	1,2-Man*p*
19.75	58, 97, 87, 129, 143, 59, 71, 75, 85, 171, 103, 111, 145, 203	4-*O*-acetyl-1,5-anhydro-2,3,6-tri-*O*-methyl-D-mannitol	1,4-Glc*p*
27.53	97, 58, 129, 171, 69, 87, 111, 142, 74, 184, 231	3,4-di-*O*-acetyl-1,5-anhydro-2,6-di-*O*-methyl-D-mannitol	1,3,4-Glc*p*
31.32	71, 58, 87, 131, 101, 99, 129, 142, 156, 172, 202	methyl 2-*O*-acetyl-3,6-anhydro-4,5-di-*O*-methyl-L-gulonate	1,4-Glc*p*A or 1,5-Glc*f*A

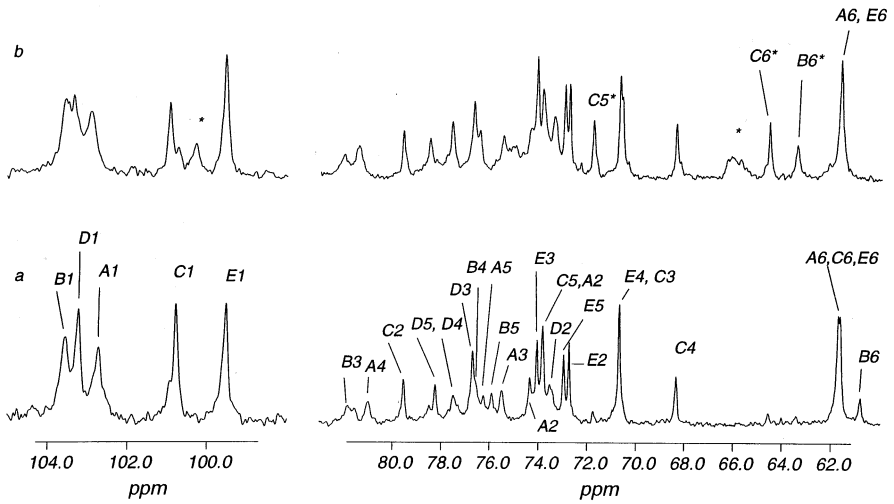

Fig. 2. 100 MHz ^{13}C NMR spectra (carbohydrate region) of (a) deacetylated (b) native P2 polysaccharide in D_2O at 95 °C. **B**6*, **C**6* and **C**5* indicate signals from units containing *O*-acetyl groups (see text). An asterisk (*) indicates impurity signals (unknown identity but easily distinguished from polysaccharide signals since the latter disappear on cooling, but impurity signals remain).

noted. Fig. 1(d) shows the proposed structure of P2, in which the sugar residues are referred to as **A**–**E**, respectively.

Fig. 2(a) shows the ^{13}C NMR spectrum of deacetylated P2. Compared with the spectrum of deacetylated CR1/4, the anomeric region shows an additional signal, **E**1 at δ 99.6, but there are no anomeric signals corresponding to the (1,6)-β-Glc or *t*-α-Rha units seen in acetan. Integration gives values of 3(**A**1 + **B**1 + **D**1):1(**C**1):1(**E**1) for the signals in the anomeric region. This supports the five sugar repeating unit proposed for P2, and the anomeric chemical shift is consistent with that expected if α-Glc is the third (terminal) unit of the side-chain. A more detailed investigation, leading to a complete assignment, was prompted when it was seen that the anomeric region of the ^1H spectrum of deacetylated P2 was more complex than expected. The origin of most additional signals was made evident by examination of a series of 2D NMR spectra (COSY, double RELAY, HOHAHA and NOESY), which allowed the proposed structure of P2 to be confirmed, but also revealed the presence of other closely related polysaccharides. The ^{13}C assignments were obtained with the help of a 2D carbon detected C/H correlation experiment.

The ^{13}C signal at δ 99.6 was correlated to the ^1H signal at 5.40 ppm and the remaining chemical shifts for this sugar unit, which were readily obtained from the 2D experiments (Table 3), are completely consistent with the identification of unit **E** as *t*-α-Glc.[21] A second network of chemical shifts was established for a sugar unit starting from the next upfield strong anomeric ^1H signal at δ 5.26, and comparison with previous results for CR1/4 and acetan shows that this must be unit **C**, (1,2)-α-Man, which is present in all three polysaccharides. Most of the remaining ^1H NMR information is summarised in the HOHAHA spectrum (Fig. 3), where the correlations within sugar units can be seen starting with (from left to right) the **B**1, **D**1, **A**1 and **C**2 signals. The assignments in Fig. 3 were confirmed in the usual step-wise fashion from COSY and double RELAY experiments. In the NOESY experiment inter-ring cross-peaks were detected linking protons **E**1/**D**4 (very weak), **D**1/**C**2 (and **D**1/**C**1), **C**1/**B**3, **B**1/**A**4 and **A**1/**B**4. Apart from the first of these, the pattern of cross-peaks was just as described previously for CR1/4 polysaccharide.

The chemical shifts in Table 3 are, in fact, very similar for rings **A**, **B** and **C** to those reported[4] for the corresponding rings in CR1/4. The ^1H and ^{13}C shifts for **D**4 are quite different, as expected, since the *t*-GlcA in CR1/4 has become a (1,4)-linked unit in P2. However, there is a more subtle difference in that the ^1H chemical shift of **D**1 (δ 4.49) is displaced so that **D**1 is slightly downfield of **A**1 (δ 4.48) in P2 whereas in CR1/4 the anomeric signal for the *t*-GlcA was upfield of

A1 at δ 4.45. Similar behaviour was noted[4] in comparing acetan with CR1/4. However an additional group of cross-peaks correlated to δ 4.45 can be seen in the HOHAHA spectrum of P2 (Fig. 3). These have chemical shifts identical to those determined for the *t*-GlcA unit in CR1/4, and it is concluded therefore that the sample contains some CR1/4 polysaccharide (which has a two-sugar side-chain) in addition to the main component P2 with a three-sugar side-chain. Another series of cross-peaks is evident in Fig. 3, correlated to a signal at δ 4.16, which itself is linked to an anomeric signal at 5.31 in both the COSY and HOHAHA spectra (not shown). The signal at δ 4.16 is therefore assigned as H-2 of a mannose ring: the remaining shifts in the network (including the anomeric signal) are very similar to those of ring **C** but the greatest difference is seen at C-2 (δ 4.16 versus δ 4.31). A

Table 3
^{1}H and ^{13}C chemical shifts of deacetylated P2

Code	Residue		Chemical shifts (δ) [a]					
			1	2	3	4	5	6
A	(1,4)-β-D-Glc*p*	^{1}H	4.48	3.36	3.65	3.57	3.57	3.81, 4.01
		^{13}C	102.6	74.4 [a]	75.5	81.0 [b]	76.3	61.7
B	(1,3,4)-β-D-Glc*p*	^{1}H	4.60	3.40	3.80	3.88	3.68	3.81, 3.98
		^{13}C	103.6	73.8 [a]	81.8	76.6	75.9	60.8 [c]
C	(1,2)-α-D-Man*p*	^{1}H	5.26	4.31	3.86	3.73	3.97	n.d. [d]
		^{13}C	100.8	79.6	70.7	68.4	73.9 [c]	61.7 [c]
D	(1,4)-β-D-Glc*p*A	^{1}H	4.49	3.43	3.74	3.82	3.84	
		^{13}C	103.3	73.5 [a]	76.7	77.5 [b]	78.2 [b]	174.6
E	*t*-α-D-Glc*p*	^{1}H	5.40	3.52	3.69	3.39	~3.73	~3.74, 3.82
		^{13}C	99.6	72.8	74.1	70.8	73.0	61.7

[a] Assignments may need to be interchanged for those shifts with the same superscript letter.
[b] Not seen in the C/H correlation spectrum.
[c] For acetylated P2 the ^{13}C chemical shifts are δ(**B**6) = 63.4, δ(**C**6) = 64.6, δ(**C**5) = 71.8.
[d] n.d., not determined.

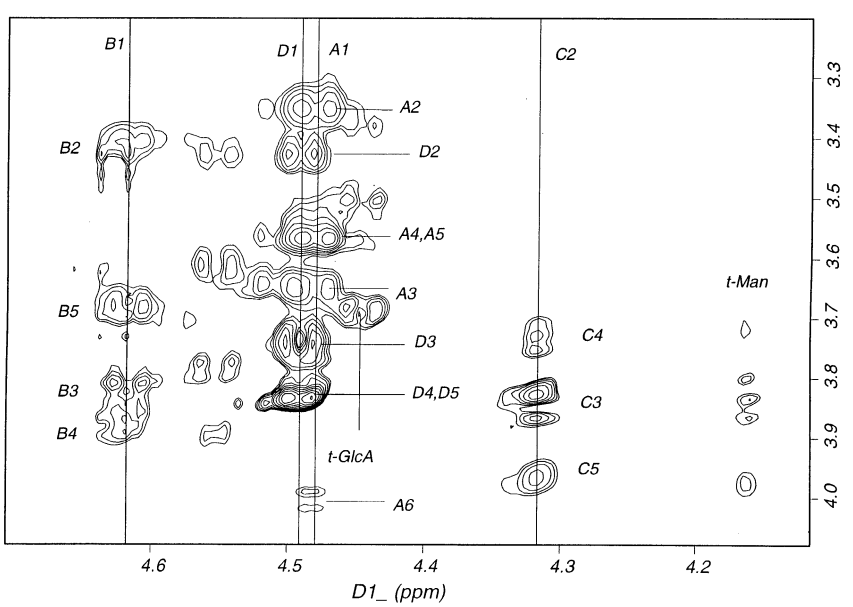

Fig. 3. 400 MHz HOHAHA spectrum (part) of deacetylated P2 (D_2O, 95 °C). Vertical lines indicate chemical shifts on the *D*1 axis of signals **B**1, **D**1, **A**1 and **C**2. Annotations and horizontal lines indicate position on *D*2 axis of correlated signals. Two series of peaks associated with *t*-GlcA and *t*-Man units (i.e., not from P2, see text) are also indicated.

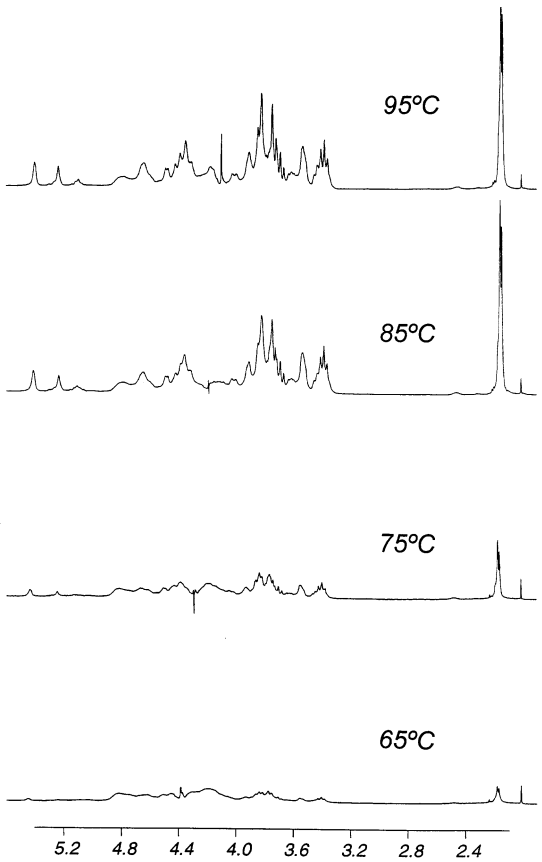

Fig. 4. 400 MHz ^{1}H NMR spectra of native (acetylated) P2 at temperatures between 65 and 95 °C.

plausible explanation for such a difference is that the mannose ring responsible for this minor set of peaks is not linked at the 2-position, i.e., it forms part of the polysaccharide with just one sugar unit (*t*-Man) in the side-chain. The presence of the polysaccharide is less certain than in the case of CR1/4 since, unlike CR1/4, there has not been an opportunity to examine the polysaccharide in a pure form. It is not clear whether the polysaccharides with shorter side-chains than P2 were present from the outset or were degradation products resulting from the conditions needed (sonication, high temperature) to obtain good quality spectra.

In the spectra of native P2, pairs of signals with equal intensity were seen at 2.15, 2.16 (^{1}H, total integral 7 relative to 1 for **A**1) and 21.05, 21.09 (^{13}C). These methyl group signals indicate the presence, in two locations, of *O*-acetyl substituents in the native polysaccharide, as was found for acetan and CR1/4. Peaks at δ 63.4, 64.6 are seen in the ^{13}C spectrum of native P2 but not in the deacetylated sample (Fig. 2). Signals were seen[4] at exactly the same chemical shifts in the spectra of acetylated acetan and CR1/4 and were assigned to **B**6* and **C**6*, respectively, where the asterisk indicates the presence of an *O*-acetyl group, linked to C-6 of rings **B** and **C**. **B**6* and **C**6* are displaced downfield with respect to **B**6 and **C**6 in the deacetylated polysaccharide and the near-complete acetylation at both sites can be seen by comparing the intensity of the **C**6* signal with other signals of the **C** unit and by noting the absence of a **B**6 signal (δ 60.8) in Fig. 2(b). The pattern of acetylation is therefore the same in P2, CR1/4 and acetan.

Fig. 4 shows proton NMR spectra collected at different temperatures for native P2 solutions. It can be seen that lowering the temperature leads to line broadening and, due to the experimental conditions used to collect the spectra, loss of area under the peaks. Such changes have been observed previously for the parent polysaccharide acetan[22] and attributed to coil–helix transition on cooling. The data shown in Fig. 4 are consistent with a coil–helix transition at about 70 °C. At temperatures below 65 °C the broadening is too large and the NMR spectra become featureless. The structural similarity between P2, xanthan, acetan and CR1/4 suggest that at low temperatures P2 adopts the helical structure common to xanthan, acetan and CR1/4.[23–26] The suggested helix-coil transition for P2 is consistent with the extensive experimental evidence[27–30] for a helix-coil transition in the structurally related polysaccharide xanthan gum.

The polysaccharide P2 (Fig. 1(d)) prepared by deletion of part of the acetan side-chain is effectively a modified xanthan structure (Fig. 1(b)) in which the terminal mannose is replaced by a terminal glucose residue. The same structure, ignoring acylation, could be produced by inactivating the gene for the glycosyl transferase, which inserts the terminal mannose residue on the xanthan structure, and introducing the glycosyl transferase for adding the terminal glucose residue. This heterologous expression of a foreign gene would demonstrate the feasibility of engineering a new polysaccharide structure by addition rather than deletion or inactivation of genes.

There is evidence that *A. xylinum* (acetan) genes can complement xanthan mutants[30] suggesting that 'foreign genes' can function when they are involved in the synthesis of a structure conserved in both bacterial polysaccharides. The insertion of the terminal glucose is stereochemically acceptable in P2 and hence the xanthan–acetan family provides a model system for testing heterologous expression for engineering of polysaccharide structures.

3. Experimental

Production and isolation of P2 polysaccharide.—The isolation of the *A. xylinum* strain CKE5, inactivation of the *aceP* gene, growth, isolation and purification of the polysaccharide P2 are described in detail elsewhere.[17]

Linkage analysis.—The linkage sites of all the sugar residues were determined by methylation analysis. The cetyltrimrthylammonium bromide-purified polymer (5.6 mg) was dispersed in dry DMSO by flushing with Ar and standing at 20 °C for 16 h. It was methylated by sequential addition of powdered NaOH (0.5 g) and iodomethane (4 mL).[31,32] After dialysis against deionized water, the methylated polymer was dried, extracted into (1:1) $CHCl_3$–CH_3OH, split into three parts, and placed in three screwcap tubes, which had been presilylated by treatment with dichlorodimethylsilane for 4 h. These were dried in a vacuum over P_2O_5 overnight.

One sample was reduced with $LiBDEt_3$ in THF (Super Deuteride, Aldrich) at 64 °C for 4 h.[33] Excess reagent was treated with isopropanol, water, then 1 M H_3PO_4 to pH 5, followed by filtration on glass fibre paper and washing with (1:1) $CHCl_3$–CH_3OH. This was dried into a clean tube. This and one of the other methylated samples were hydrolysed and converted to partially methylated alditol acetates (PMAAs) using TFA hydrolysis,[34] $NaBD_4$ reduction and acetylation with Ac_2O and *N*-methylimidazole (NMIM).[35]

The third methylated sample was subjected to reductive cleavage by adding 1 mL of the following mixture: 9 mL of CH_2Cl_2, 0.2 g CaH_2, 0.8 mL Et_3SiH and 1.0 mL TMSOTf, (which had been allowed to settle). As an added precaution, this reagent was used in a glove box filled with Ar. The sample was left to react for 20 h at 20 °C, after which an additional 1 mL of reagent was added, and sonicated for 1 h to disperse unreacted solid. Excess reagent was treated with CH_3OH (4 mL), then about 3 mL of Amberlite IRA-420 resin (HCO_3^-) in CH_3OH. The liquid part was dried and acetylated with (3:0.5:0.5 mL) Ac_2O–AcOH–NMIM at 30 °C for 30 min. This was extracted with H_2O–CH_2Cl_2 in the usual way to yield partially methylated anhydroalditol acetates.

The PMAAs and anhydroalditol derivatives were analysed by GC using the temperature program: 55 °C (2 min), +45 °C/min (1.9 min), 140 °C (2 min), +2 °C/min (35 min), 210 °C (40 min). The PMAAs were identified by measuring their retention times relative to *myo*-inositol hexaacetate, and comparing the relative retention times to those of external standards. A mixture of standards for each sugar was prepared by deliberate undermethylation of the methyl glycosides.[36] The flame ionization detector (FID) signal was used to measure peak areas which were calculated as relative molar quantities using effective carbon response factors.[37] 1,5-Anhydroglucitol and 1,5-anhydromannitol were synthesised according to the method of Ness et al.[38] These were similarly undermethylated and acetylated to produce mixtures of standards, whose retention times were used to identify anhydroalditol derivatives.

The identities of PMAAs were diagnostically confirmed from their EI-MS.[39] The mass spectra of the partially methylated anhydroalditol acetates were compared with those of the standards.[40] GC–MS analysis was performed 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.

Carboxy-reduction of P2 polysaccharide.—The polysaccharide was carboxy-reduced[41] by treating a 4% dispersion in water (0.5 mL) with MES buffer (pH 4.7, 0.5 mL) and CDC (100 mg) for 45 min. Imidazole (0.5 M, pH 7.0, 1 mL) and $NaBH_4$ (60 mg) were added at 0 °C. After 16 h this was neutralised with AcOH (100 μL), dialysed against water and dried.

Absolute configuration.—The absolute configuration of the sugars in carboxy-reduced P2 eps was determined from their trimethylsilylated (−)-2-butyl glycosides.[42] After hydrolysis in TFA (2 M, 120 °C, 1 h), the dried hydrolysate was treated with (−)-2-butanol (Aldrich, 100 μL) and AcCl (7 μL) under Ar at 80 °C (4 h). After adding Ag_2CO_3 (20 mg) and MeCN (0.5 mL) the filtrate was treated with BSA (Aldrich, 50 μL), MeCN (200 μL) and TBAF (Sigma, 2 μL) (Johnson, 1992) at 40 °C (1 h). After brief centrifugation, the supernatant was analysed by GC on an SPB-1 column (Supelco, 0.3 mm × 30 m) using the temperature program: 55 °C (2 min); 45 °C/min; 140 °C (2 min); 210 °C (10 min).

O-*Deacylation of polysaccharide.*—The CTAB-purified EPS (30 mg) was dissolved with heating (90 °C, 2 h) in 30 mL water. After cooling on ice, 0.1 M NaOH was added until the pH had increased from 4.9 to 12.0. This was left at 1 °C for 16 h. The pH was adjusted to 6.5 with 0.1 M HCl. The solution was dialysed exhaustively against deionized water, was concentrated to about 20 mL, then frozen and lyophilized (yield: 30 mg).

NMR.—NMR experiments were carried out on 2% solutions of P2 (native or deacetylated) in D_2O at 95 °C. Spectra were recorded on a JEOL GX-400 spectrometer operating at 400 MHz (^1H) or 100 MHz (^{13}C), using 5 mm o.d. tubes for the ^1H and H–H 2D correlation experiments and 10 mm o.d. tubes for the ^{13}C and C–H 2D correlation experiments. Mixing times of 100 and 400 ms were used in the HOHAHA and NOESY experiments, respectively. Other parameters for the 2D experiments were as reported previously.[4]

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