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Microbial exopolysaccharides—structural subtleties and their consequences

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Microbial extracellular heteropolysaccharides are mainly linear molecules to which side chains of varying length and complexity are attached at regular intervals. Examination of 'families' of microbial exopolysaccharide with closely related structures enables us to determine the effect of minor (or major) changes to structure on the physical properties of these macromolecules. Changes may also be seen in some of the biological properties of the polysaccharides, such as susceptibility to enzymes; interaction with antibodies or lectins; and capacity and specificity of ion binding. Acetyl groups frequently assert very marked effects on the properties of microbial polysaccharides. The presence or absence of an *O*-acetyl or pyruvate (ketal) group on each oligosaccharide repeat unit can greatly alter the properties of a number of exopolysaccharides.

Introduction

Microbial extracellular heteropolysaccharides and especially those obtained from numerous bacterial species are generally considered to be of uniform structure and are also frequently thought to be of fairly limited polydispersity. In many cases this belief may be correct, yet the methods used for the growth of the micro-organisms and the isolation of their extracellular products, may produce polymers with loss of some substituents or with material which has been to some extent degraded through chemical or biological action. In some preparations these alterations may have no significant effect on the bulk properties of the polysaccharides, but in others sufficient changes may have occurred to cause marked alterations to their solution rheology and to other characteristics. The major differences seen between these bacterial products and the plant polysaccharides which find such wide industrial applications lies in the lack of extensive branching in the microbial polymers, as they are composed of essentially linear molecules to which side chains of varying length and complexity are normally attached at regular intervals. Such side-chains are usually limited to mono-, di-, tri- or at most pentasaccharides and only rarely are they themselves branched.

Through careful control of culture conditions, through the use of enzymes or through use of mutants, it has proved possible to prepare microbial polysaccharides with altered structures. Mild chemical degradation procedures have also proved useful. It has also been noted that different microbial species or strains may produce a range of polysaccharides with close structural similarities. Examination of these diverse products provides several interesting 'families' of polysaccharide structures and enables us to determine the effect of minor (or major) changes to structure on the physical properties of these macromolecules. Such relatively minor differences may also greatly change the biological properties of the polysaccharides in respect to their susceptibility to enzymes or their interaction with antibodies or lectins, while a further property which may be altered is the capacity and specificity of ion binding. As well as differences in carbohydrate structure, the range of possible structures is further extended by the presence or absence of various acyl groups.

Homopolysaccharides

1,3β-D-Glucans and 1,4β-D-Glucuronans

Several homopolymers belonging to this group are available and provide an example of the effects which single β-D-glucosyl or β-D-gentobiosyl side-chains confer on the linear macromolecule. Thus *curdlan* the linear molecule produced by several bacterial species is a unique polymer with interesting physical properties including a triple-helix conformation, but little biological activity. In contrast, *scleroglucan* and related fungal polysaccharides carrying the glucosyl side-chains have been widely claimed to belong to the group of macromolecules classified as 'biological response modifiers' and inhibit tumorogenesis (ref. 1). Equally curdlan forms gels in the absence of ions and shows interesting transitions at two different temperatures, while scleroglucan and related β-D-glucans, although yielding viscous aqueous solutions, do not form gels.

An interesting new bacterial homopolysaccharide is the 1,4-β-linked poly-D-glucuronic acid produced by a mutant *Rhizobium* strain (refs. 2,3). This polymer has been the subject of a number of studies which have revealed some intriguing gelation properties similar to other polyuronic acids. Like bacterial alginates, the polymer is acetylated and the deacetylated product yielded a crystalline fibre pattern indicative of two-fold screw symmetry. It has been suggested that the high acetyl content of the native polymer promotes interchain as sociations and aggregate formation. As with bacterial alginates, the acetyl groups appear to inhibit junction zones in the presence of multivalent ions (ref. 4).

Heteropolysaccharides

The simplest group of heteropolysaccharide structures are the galactoglucans produced by a very diverse group of bacteria. These polymers possess a disaccharide repeat unit together with acetyl, succinyl or pyruvate substituents. As yet, studies have been mainly confined to structural determination rather than physical parameters, but they do provide a range of relatively simple structures in which the effects of various acyl groups may be studied..

XM6 and related *Klebsiella/Enterobacter* polysaccharides

A group of closely related structures are found in the polysaccharides of *Enterobacter* XM6, *Klebsiella aerogenes* serotype 54 and *Escherichia coli* serotype 27. In each case, the major structural repeat unit is a tetrasaccharide in which a glucosyl residue on the main-chain trisaccharide carries a monosaccharide substituent (ref. 5). In the *E.coli* polysaccharide this is a β-D-galactosyl residue while in the other strains it is a β-D-glucosyl residue. Three possible variants exist in the Klebsiella polymers - they may be non-acetylated, they may carry an O-acetyl group on every alternate tetrasaccharide repeat unit, or they may have an acetyl group on each repeat unit, carried on the fucosyl residue (refs. 6,7). Surprisingly, all the polymers within this group can be hydrolysed by a polysaccharide depolymerase initially obtained from a bacteriophage lytic for one of the *K. aerogenes* type 54 strains (ref. 8). This enzyme degrades all the substrates to tetrasaccharides or octasaccharides. The major differences in physical properties are seen when the effect of cations on the non-acetylated XM6 polysaccharide is examined. This polymer interacts with various monovalent and divalent cations to yield a very sharp sol-gel transition at a temperature of 30°C with virtually no hysteresis (ref. 9). No gelation was observed with any of the other polysaccharides in this group - the acetylated *Klebsiella* products or the *E.coli* K27 polymer. However, alkaline treatment of the *Klebsiella* polysaccharides removed the O-acetyl groups and yielded products the behaviour of which mimicked that of XM6. Approximately 30% more Ca^{2+} was bound to XM6 than to the mono-acetylated *Klebsiella* polysaccharide (ref. 10). Thus the presence of even one acetyl group on each alternate repeat unit was sufficient to impede ion binding and prevent gelation. Fibre X-ray diffraction clearly showed the similar helical structures in the XM6 and *Klebsiella* polymers and the inhibitory effect of the acetyl group on *O*-2 of fucose residues on association between molecules (ref. 11).

Bacterial alginates

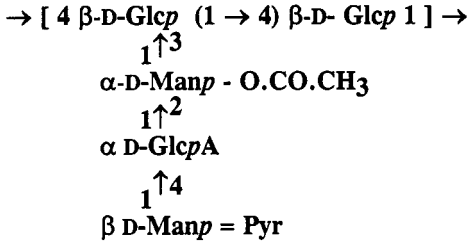
Although a number of bacterial species are capable of synthesising polysaccharides which can correctly be described as alginates, most work has been on EPS from *Azotobacter vinelandii* or *Pseudomonas aeruginosa, Pseudomonas fluorescens* and *Pseudomonas putida.* The alginate from *Azotobacter* most closely resembles marine algal products, containing homopolymeric and mixed sequences of D-mannuronic and L-guluronic acids and differing only in the presence of *O*-acetyl groups on some D-mannuronosyl residues. (Table 1). It is however, normally of much larger molecular mass than the chemically extracted, commercial algal-derived material. By contrast the *Pseudomonas* alginates all lack poly-guluronic acid blocks and contain only single guluronosyl residues. Again they may be singly or doubly acetylated on D-mannuronosyl residues. As the work of Smidsrød and others (e.g. ref. 12) on algal alginates has clearly shown the role of polyguluronic acid sequences in the chelation of divalent ions such as Ca^{2+} and Sr^{2+}, it might thus be expected that the *Pseudomonas* products would therefore lack the capacity and specificity for ion binding found in the algal material. Investigations in our laboratory failed to confirm this. As expected, the native, acetylated Pseudomonas alginates bound Mg^{2+} and Ca^{2+} only slightly, but after chemical deacetylation revealed enhanced ion binding with preference for Ca^{2+} over Mg^{2+}. The ion binding was still lower than that found in *Azotobacter* alginate preparations which also showed lack of specificity and capacity until the *O*-acetyl groups present on the original products had been removed. None of the *Pseudomonas* alginate preparations formed gels with divalent cations. Delben *et al.* (ref. 13) indicated that even a modest amount of acetyl substitution in bacterial alginates could produce significant conformational effects.

All the bacterial alginates mentioned above were high molecular weight products with considerable viscosity in solution. Unlike the algal polymers, in their natural acetylated forms they were generally poor substrates for alginate lyases whether with specificity for poly-D-mannuronic or poly-L-guluronic sequences. Alkali treatment to remove the acetyl groups rendered the bacterial polysaccharides susceptible to some of the enzymes although degradation was less than that observed with the lower mass algal products. Thus the acetyl groups present on bacterial alginates confer both resistance to enzymes and greatly reduce the ion binding capacity even though they are not attached to the guluronic acid residues which are considered to play the major role in the specificity and capacity for divalent ion binding.

Xanthan

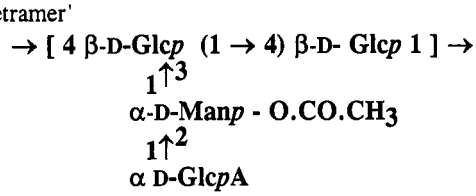
Xanthan, the polysaccharide synthesised by most strains and pathovars of *Xanthomonas campestris*, has probably been studied more than any other bacterial exopolysaccharide. Even today, years after the initial structural studies of Jansson *et al.* (ref. 14) and Melton *et al.* (ref. 15), further intricacies of its physical structure are being reported and it is clear that some of the physical properties depend on the mode of preparation of the polysaccharide (ref. 16). The original commercial product, together with variants altered in their acylation patterns, provide excellent models for examination of the role of pyruvate ketals and *O*-acetyl groups on the terminal β-linked mannosyl side-chain residue and the internal α-linked residue respectively. Products from mutants strains and the polymer termed 'acetan' from strains of *Acetobacter xylinum* provide further related polymer structures. All these polymers share a basic cellulose main chain structure to which side-chains are attached on alternate D-glucosyl residues (Fig. 1). The side-chains bear a strong similarity to the trisaccharide repeat units found in the linear structures of EPS from *Klebsiella* K5 (ref. 17); *E. coli* K55 (ref. 18) and *Pseudomonas* "gingeri" (ref. 19) and xanthan lyases which degrade the side-chains of xanthan show some activity against these two polymers (I.W.Sutherland, unpublished results). The longest side-chains, pentasaccharides, are seen in the acetan structure, while mutants of xanthan carrying α-D-mannosyl residues only, can be obtained. O-acetyl groups are present on the side-chains of many of the xanthan structures and on the main-chain of acetan. They clearly play very important roles in determining both transition temperatures and interaction with other molecules as well as susceptibility or otherwise to enzymic degradation. In the normal ordered structure, xanthan is not hydrolysed by the endo-1,4-β-D-glucanases which degrade carboxymethylcellulose (ref. 20). Susceptibility to such enzymes requires exposure of several adjacent main-chain glucose residues through the absence of

Fig. 1 The structure of the exopolysaccharides (Xanthan) from *Xanthomonas campestris* and acetan from *Acetobacter xylinum* wild type and mutants

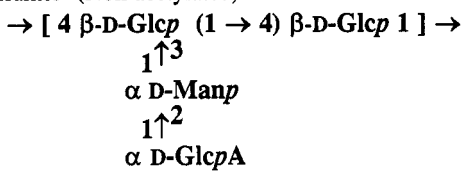


Typically the internal α-mannosyl residue is fully acetylated but only c. 30% of the β-mannosyl termini are ketalated.

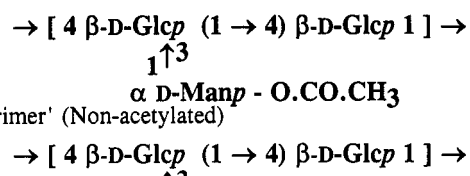
'Polytetramer'



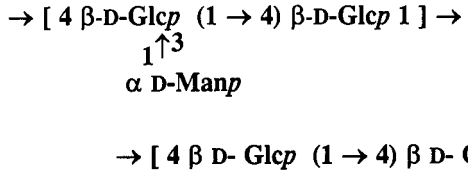
'Polytetramer' (Non-acetylated)



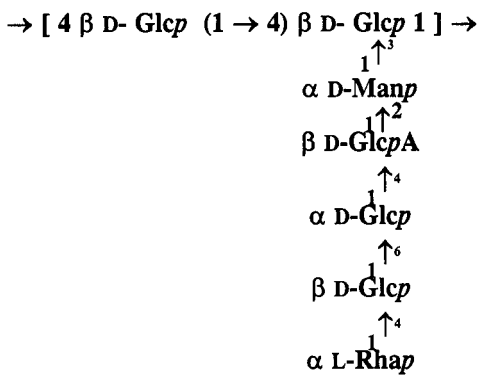
'Polytrimer'



'Polytrimer' (Non-acetylated)



Acetan



Typically the polymer carries 2 *O*-acetyl groups on each repeating unit, one of which is possibly on a main-chain glucose residue

side-chains (ref. 21). The very marked effects of acylation on transition of xanthan from order to disorder were clearly demonstrated in the studies of Shatwell *et al.* (ref. 22), which also revealed the stabilising effect of increasing salt concentration resulting in greatly increased transition temperature. Dentini *et al.* (ref. 23) remarked on the strong destabilising effect which the pyruvate groups had on the Ca^{2+}-induced ordered structure of xanthan and which they attributed to unfavourable electrostatic contribution. Very similar effects were noted when the polysaccharide succinoglycan was examined before and after removal of the charged succinyl half-esters and pyruvate ketals. (ref. 24). Loss of the terminal mannose residue results in polysaccharide with lower viscosity than wild type material but the deacetylated polytetramer (Fig. 1) had increased viscosity (ref. 25). it was suggested that the differences observed in viscosity of these polymers derived from conformational differences resulting from higher side-chain flexibility in the wild type.

In the interaction between xanthan and locust bean gum, studies by Shatwell *et al.* (ref. 26) revealed that the majority of xanthans tested formed a relatively strong gel network with LBG except in the case of a xanthan which was heavily acetylated and very low in pyruvate. Deacetylation of this polymer significantly increased gel strength - G' increasing by almost 250% (from 930 to 3300) and tan δ fell. Xanthan defective in the terminal β-mannosyl side-chain residue formed a much weaker gel than any of the wild type gels tested and G' was very low (620). From these results it was concluded that the presence of *O*-acetyl groups had an inhibitory effect on copolymer gelation while the terminal mannose residue on the trisaccharide side-chain played an important role in the interaction between xanthan and LBG. This was recently supported by the finding in our laboratory, that acetan and LBG also form gels. However, these were much weaker than most xanthan-LBG interactions, resembling those of the polytetrasaccharide xanthan polymer BD9. The acetan was of comparable molecular mass to wild type xanthan, but the truncated xanthan material was almost certainly lower in mass. Thus the results for this material are not strictly comparable with the other values. Ojinnaka *et al.* (ref. 27) initially reported that deacetylation of acetan was necessary to achieve gelation with LBG and Konjac mannan, the glucose residues in the latter yielding stronger associations; later study did reveal weak interaction with the acetylated polysaccharide (V.J.Morris, personal communication). While the presence of the acetyl groups in acetan does not inhibit helix formation, deacetylation did stabilise the helical conformation.

The role of side-chains in gelation induced by Cr^{3+} has also been studied (ref. 28). Partial acid hydrolysis was used remove the terminal mannose residues without significant reduction in molecular mass. The terminal β-D-mannose had little influence on such gelation and its removal, or the partial removal of the trisaccharide side-chains had minimal effects on gelation. Acetan did not show similar gelation unless sugars were removed from the side-chains by partial acid hydrolysis. It was therefore suggested that gelation resulted from interaction between the Cr^{3+} ions and glucuronic acid residues in which the longer and bulkier side-chains of acetan suppressed interaction through steric hindrance.

Gellan

The series of structures to which gellan, welan, rhamsan and other polysaccharides from *Sphingomonas* spp. belong also provides examples of the effects of minor changes. Gellan only forms weak or rubbery gels unless the acyl groups are removed, most of the inhibitory effect being due to the L-glyceryl residues rather than the *O*-acetyl groups which stick out from the helix and do not interfere with the packing arrangement stimulated by the presence of cations. Deacylated gellan yields hard and brittle gels which find applications for food and other uses. Commercial gellan preparations contained sufficient Ca^{2+} and Mg^{2+} to permit gelation, but conversion to the monovalent cation form yielded material which resembled alginates in their gelation when the monovalent ions were displaced by divalent ions (ref. 29). Gellan solutions with excess divalent cations present form firm gels when cooled below the setting temperature and remelting proved difficult (ref. 30). This behaviour contrasted with that found with gels formed in the presence of monovalent ions. Although other members of the gellan family of bacterial polysaccharides do not normally form gels, all possess double helical morphology despite the various substituents and side-chains present (ref. 31). However, unlike xanthan and acetan, the side-chains of welan promote the stability of

the helix rather than interfering. Even rhamsan with its gentibiosyl side-chains adopts the double helix conformation. The helices cannot associate in exactly the same manner as in gellan and the high viscosity in solution may result from non-specific interactions between main chains and side-chains. The different side-chain structures found in the gellan family also greatly affect their susceptibility or resistance to various gellan lyases; the acyl groups present in native gellan are sufficient to cause almost complete inhibition of enzyme action, presumably through steric hindrance at the enzyme binding or catalytic site (ref. 32).

Thus, gellan and related polysaccharides with their structural similarities and complexities provide a further group of polysaccharides, the structure of which may show further subtle differences due to the presence of either acyl groups or substituent monosaccharides. As this group are of considerable commercial interest, they may well provide the main focus of interest in the 'post-xanthan' era.

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Table 1. Composition and diad frequencies of some bacterial and algal alginates .

Source	F_G	F_M	F_{GG}	F_{MM}	$F_{GM,MG}$	Acetyl
L. hyperborea	0.665	0.335	0.558	0.228	0.107	0
M. pyrifera	0.41	0.59	0.24	0.42	0.17	0
A. vinelandii 73	0.561	0.439	0.372	0.25	0.189	11%
A. vinelandii 206	0.08	0.92	0.03	0.87	0.05	24%
P. fluorescens	0.4	0.6	0	0.2	0.4	3%
P. putida	0.37	0.63	0	0.26	0.37	3%
P. aeruginosa B	0.16	0.84	0	0.68	0.16	37%