Structures and Properties of Gellan Polymers Produced by Sphingomonas paucimobilis ATCC 31461 from Lactose Compared with Those Produced from Glucose and from Cheese Whey

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The dairy industry produces large quantities of whey as a by-product of cheese production and is increasingly looking for new ways to utilize this waste product. Gellan gum is reliably produced by Sphingomonas paucimobilis in growth media containing lactose, a significant component of cheese whey, as a carbon source. We studied and compared polysaccharide biosynthesis by S. paucimobilis ATCC 31461 in media containing lactose, lactose (5 to 30 g/liter), and sweet cheese whey. We found that altering the growth medium can markedly affect the polysaccharide yield, acyl substitution level, polymer rheological properties, and susceptibility to degradation. Depression of gellan production from lactose compared with gellan production from glucose (approximately 30%) did not appear to occur at the level of synthesis of sugar nucleotides, which are the donors of monomers used for biosynthesis of the repetitive tetrasaccharide unit of gellan. The lactose-derived biopolymer had the highest total acyl content; the glucose- and whey-derived gellans had similar total acyl contents but differed markedly in their acetate and glycerate levels. Rheological studies revealed how the functionality of a gellan polysaccharide is affected by changes in the acyl substitution.

The microbial exopolysaccharides (EPS) are a class of high-value polymers that have many industrial applications (36). Large amounts of one EPS, gellan gum, are synthesized by Sphingomonas paucimobilis ATCC 31461 (19, 35), and this compound is used in the food and pharmaceutical industries, as well as other industries (9, 30). The repeating unit of this linear heteropolysaccharide is composed of D-glucose, (D-Glc), D-rhamnose (D-Rha), and D-gluconic acid (D-GlcA), is the tetrasaccharide (3′→3′)-β-D-GlcP(1→4)-β-D-GlcAp(1→4)-β-D-GlcP(1→4)-α-L-Rhap(1→) (17, 34). The native polysaccharide is partially esterified; the 1,3-D-Glc residue can be linked to l-glycerate at C-2 and/or to acetate at C-6, and there is 1 mol of glycerate per repeating unit and 0.5 mol of acetate per repeating unit (21). Acyl substituents affect the rheology of gels, and decylation of native gellan results in a change from soft, elastic, thermoreversible gels to harder, more brittle gels. Using variants of gellan containing both glycerate and acetate, no substituents, and only an acetate substituent, Jay et al. confirmed that gylcerate substituents are responsible for the significant changes in rheology observed after decylation of gellan (18). These results confirmed both a prediction based on X-ray studies and results obtained in rheological studies of chemically decylated gellan (2, 9).

Although the production yields, compositions, structures, and properties of bacterial EPS are genetically determined, it is possible to influence these factors by modifying culture conditions, such as temperature (22, 28), dissolved oxygen tension (23, 24), and growth medium composition (i.e., the concentration of cations [25, 29] and the carbon source used [6, 8, 33]). S. paucimobilis ATCC 31461 is able to grow with lactose (35), and previous observations indicated that this strain is able to produce a large amount of highly viscous EPS directly from lactose. In the present study we examined gellan gum production in basail medium containing glucose or lactose at concentrations ranging from 5 to 30 g/liter. Using media containing 2% (wt/vol) lactose or 2% (wt/vol) glucose, we examined the effects of carbon source on the specific activities of all of the gellan-biosynthetic enzymes necessary for the formation of the sugar nucleotides UDP-glucose, UDP-gluconic acid, and dTDP-1-rhamnose, which are the monomer donors during biosynthesis of the repetitive tetrasaccharide unit of gellan (26), and on the chemical composition, structure, and properties of the gellan polysaccharides produced. In this work we also assessed whether sweet cheese whey, provided by a Portuguese dairy, could be used as a fermentation medium for gellan gum production and whether its biological oxygen demand (BOD) could be reduced. Although cheese whey is frequently used as an animal feed, centralization of production has created a need for an alternative way to dispose of and valorize this substance. Whey is a nutrient-rich medium; in particular, sweet whey contains approximately 5% lactose, 0.2% lactic acid, and 1% protein, as well as fat, minerals, and vitamins (40). Proper disposal of this product has long been a concern to the dairy industry. The most desirable way of handling this waste is to utilize it as a substrate for the production of useful products; some of these products, bacterial EPS, have recently received some attention (40).

MATERIALS AND METHODS

Bacterial strain and growth conditions. S. paucimobilis ATCC 31461 was maintained in agar-containing S medium, which contained (per liter of distilled water) 10 g of Na2HPO4, 5 g of KH2PO4, 1 g of K2SO4, 1 g of NaCl, 0.2 g of MgSO4·7H2O, 0.01 g of CaCl2, 0.081 g of FeSO4·7H2O, 1 g of Casamino Acids (Difco Laboratories, Detroit, Mich.), 1 g of yeast extract (Difco), 20 g of glucose, and 20 g of agar. The defined media used for gellan production were based on S medium; some of these media contained glucose (5 to 30 g/liter), and in some of them the glucose was replaced by lactose (5 to 30 g/liter). Overnight liquid cultures in S medium (100 ml) in shake flasks (250 ml) that were incubated at 30°C with orbital agitation (250 rpm) were used to prepare the inocula. The
cultures were centrifuged, and the pellets were resuspended in growth media containing different concentrations of the two carbon sources in order to obtain initial culture optical densities at 640 nm (OD640) of 0.2 ± 0.01. Strain ATCC 31461 cells were grown aerobically in an orbital shaker (250 rpm) in basal S media containing glucose or lactose as the carbon source (at concentrations of 5, 10, 15, 20, and 25, and 30 g/liter). The amount of gellan produced was determined by determining the dry weight (24 h, 80°C) of the precipitate recovered after centrifugation of culture medium at 0.3. The amount of gellan produced after centrifugation was determined by determining the glucose contents by the method described by Dubois et al. (14). The levels of uronic acids were determined by the 3-hydroxyphenyl method (15), which was calibrated with glucuronic acid (Sigma). The levels of 6-deoxyhexose were determined by determining the levels of thiamine by the thiochrome method (3, 12).

The neutral sugar contents of the gellan samples were determined by acid hydrolysis with 2 M trifluoroacetic acid (Aldrich, Gillingham, United Kingdom) (4), derivatization to alditol acetates (1), and gas chromatography (GC) analysis. The flame ionization detector signal was used to measure peak areas, which were calculated by determining the relative molar quantities with effective carbon response factors (38). The identities of PMAs were diagnostically confirmed on the basis of their electron impact ionization mass spectra (7) by performing an analysis of the ratio of the peaks at a ratio of 0.01. Strain ATCC 31461 depolymerizing activity, approximately 90% of the total, was determined by using the glucose-oxidase method (16). The neutral sugar contents of the gellan samples were determined by acid hydrolysis with 2 M trifluoroacetic acid (Aldrich, Gillingham, United Kingdom); the source temperature used was 200°C, and the ionization potential was 70 eV. NMR spectroscopy. 1H (400 MHz) nuclear magnetic resonance (NMR) spectra were recorded on a model GX-400 spectrometer (JEOL Ltd., Tokyo, Japan) at 95°C. 1% gellan solutions in D2O (in 5-mm-wide-diameter tubes) were used for 1H one-dimensional NMR experiments. 1H spectra with acceptable signal-to-noise ratios for determinations of acetate and glycerate levels could be obtained with an extract of 100 g sample. The 1H spectrum was recorded relative to tetramethylsilane by using sodium 3-trimethylsilylpropanoate (Al- drich) (1H, 0 ppm) in D2O as a secondary external reference. Data processing was carried out by using the Felix 95.0 software (Molecular Simulations, San Diego, CA). The level of the sugar was determined by integrating to obtain the peak areas at d1.12 + d2.13 (CHO2 in acetate) and d1.26 + d1.27 (CHO2, 1.4-Rha) and measuring the ratio of these areas. The level of glycerate substitution on the 1,3-Glc residue was determined from the ratio of the three times the peak area at d5.11 residue (H-1, 1.4-Rha without glycerate substitution) to the area of the Rha methyl.

Rheological characterization of gellan polysaccharides. Gellan samples were purified and converted into the tetramethyl ammonium (TMA) forms in order to compare their rheological properties. Solutions prepared from the freeze-dried polymers were passed through a TMA Dowex ion-exchange column (H+ Dowex; BDH, Poole, United Kingdom) to convert the carrageenans to TMA. The solutions were then dialyzed exhaustively against distilled water and freeze-dried. The resulting TMA gellans were dissolved in water at twice the required concentration and then diluted while they were hot withKC1 or water as required. The hot solutions were poured into 50-mm-diameter cylindrical molds and left overnight prior to testing. For rheological tests we used a model 3250 mechanical spectrometer (Instron Corporation, Canton, Mass.) operated in the parallel-plate mode with a diameter of 25 mm. Solutions were tested with constant rotation, while the gels were subjected to oscillatory shear over a range of frequencies (strain 0.01). For the gels the molds were glued to the lower platen.

Successtibility of gellan polymers to S. paucimobilis ATCC 31461 depolymerizing activity. The gellan type polysaccharides produced in the different media and decayed gellan (Gelrite; Schweizerhall, South Plainfield, N.J.) were used at a concentration of 0.75% (wt/vol) to solidify a semisynthetic growth medium containing salts, 0.1% (wt/vol) yeast extract, and 0.1% (wt/vol) casein hydrolysate as a nitrogen source (37). The sphingomyelinase activity was determined by measuring the amount of sphingomyelinase activity as if you were reading it naturally.
medium, and the plates were incubated for 5 days at 30°C. The liquifying effects of bacterial growth on the different gellan-containing media were compared.

RESULTS

Gellan production from glucose or lactose by *S. paucimobilis* ATCC 31461. The industrial gellan-producing strain *S. paucimobilis* ATCC 31461 was able to produce an EPS directly from lactose (Fig. 1 and 2). Several batch cultures were grown for 4 days at 30°C in basal S media containing lactose or glucose at concentrations ranging from 5 to 30 g/liter. The growth kinetics in lactose- and glucose-containing media were similar after the first 48 h of incubation. After 48 h the concentrations of gellan (ethanol precipitate) that could be recovered from the cultures were maximal as the result of entry into the stationary phase (Fig. 2; data not shown). Significant residual concentrations of the sugars remained unused in media in which the initial sugar concentrations were greater than 10 g/liter at the stationary phase (Fig. 1 and 2), indicating that another nutrient limited growth. Maximal EPS production and maximal broth viscosity were observed when the initial concentrations of glucose and lactose were greater than 15 g/liter. Interestingly, despite the fact that the concentration of gellan polymer that was produced from glucose was higher than the concentration of gellan polymer that was produced from lactose (14 and 9 g/liter, respectively), the viscosity of the lactose-containing broth was significantly greater than the viscosity of the glucose-containing broth for all of the initial sugar concentrations examined (Fig. 1 and 2). To understand these results, we compared the chemical compositions, structures, and rheological properties of the gellan polymers produced after 48 h of incubation in media containing 2% (wt/vol) glucose and in media containing 2% (wt/vol) lactose. Additionally, we compared the specific activities of the gellan-biosynthetic enzymes involved in formation of the sugar-activated precursors of gellan polymers in cell extracts prepared from cells grown in glucose-containing media and in lactose-containing media.

Gellan-biosynthetic enzymes in lactose- or glucose-grown cells. Cells of *S. paucimobilis* ATCC 31461 were grown in medium containing 2% (wt/vol) glucose and in medium containing 2% (wt/vol) lactose, and the specific activities of enzymes involved in the synthesis of sugar nucleotides were similar, although not identical, in the two cultures (Fig. 3). For most of the enzymes examined (PGI, PGM, UGP, and UGD) the specific activities did not correlate with gellan-specific production in the two media. In fact, the enzyme specific activities were slightly higher in lactose-grown cells, while gellan-specific production (associated with the amount of ethanol precipitate isolated per unit of OD$_{640}$ at the early stationary phase) was lower in lactose-containing medium than in glucose-containing medium. However, the TRS activity in lactose-grown cells was slightly less than the estimated TRS activity in glucose-grown cells, and the TGP specific activities were apparently identical in the two types of cells (Fig. 3).

Gellan production from cheese whey. The concentrations of lactose and lactic acid in the sweet cheese whey used in this study were 52 and 0.5 g/liter, respectively. With undiluted whey (pH 7.0), the culture broth viscosity did not increase during incubation. It is possible that the undiluted cheese whey included metal ions or other compounds at concentrations that...
inhibited gellan production without drastically affecting cell growth. Whey diluted 1:4 to 1:5 with water produced maximal levels of EPS (approximately 7 g/liter after 2 to 3 days of incubation), and this was accompanied by a substantial increase in broth viscosity (Fig. 4). Greater dilution resulted in decreases in the final concentration of EPS produced (Fig. 4). The percentage of reduction in the initial BOD5 was maximal (1:5) when whey diluted 1:5 was used, while greater dilution resulted in lower gellan concentrations and with no increase in the percentage of BOD5 removed (Table 1). Surprisingly, culture viscosity values, which were maximal after 2 to 4 days of incubation, decreased very substantially when preparations were incubated for 2 more days (Fig. 4). Such drastic decreases in broth viscosity did not occur during prolonged cell incubation (up to 7 days) in basal S medium containing lactose or glucose as the carbon source (Fig. 2; data not shown). However, a slight decrease in the early-stationary-phase broth viscosity was also detected after 12 days of incubation (data not shown). These results are consistent with the finding that different gellan-related polymers are susceptible to S. paucimobilis ATCC 31461 depolymerizing activity. Indeed, the susceptibility of the whey polymer to degradation by S. paucimobilis ATCC 31461 was confirmed, while the gellan samples obtained from lactose-containing and glucose-containing preparations were apparently not affected; however, Gelrite was more susceptible to bacterial enzyme degrading activity than the whey-derived polymer was (data not shown).

Chemical compositions and structures of gellan polysaccharides produced from lactose, glucose, or cheese whey. The results of our chemical and structural analysis of the gellans produced after 48 h of growth in defined medium containing lactose or glucose as the carbon source or in diluted (1:5) cheese whey after 72 h of incubation indicated that the gellans had similar primary carbohydrate structures (Table 2). The ratio of Glc to Rha in the neutral sugar analysis was higher than the ratio of Glc to Rha in the linkage analysis. This may have been due to partial degradation of free rhamnose under hydrolysis conditions, while the methylated sugar may have been less labile. It is not clear why the product obtained from whey did not exhibit such a difference. Reduction of uronic acid in the methylated samples reduced the resistance of certain glycosidic linkages to hydrolysis and thus may also have reduced selective degradation of some residues. Therefore, although the linkage analysis results were only semiquantitative, they were probably more reliable. Glucuronic acid appeared to be underreduced in the methylation analysis, since colorimetry indicated that it accounted for about one-fifth of the dry matter in all three samples. The sample from the culture grown in the presence of glucose also contained about 0.5 mol of a terminal sugar, either t-Glc or t-Man (which coeluted). The same peak was present in the lactose- and whey-grown samples, but it was much smaller. Since no true branched residue was observed, this terminal sugar may have been an artifact of degradation. A very small quantity of 1,3-linked mannose was detected in the glucose-grown sample. Complete structural characterization by two-dimensional NMR was not considered necessary, since such a characterization had been done previously for the polysaccharide produced from glucose (18). However, one-dimensional NMR gave useful information concerning the levels of acetate and glycerate, which were found to vary in the three polysaccharides examined (Fig. 5 and Table 2).

Rheological properties of gellan polymers. Figure 6 shows the results of a comparison of the viscosities of the three purified gellan polymers which we analyzed. The sample produced with whey had the highest bulk viscosity, while the sample produced with glucose had the lowest shear viscosity. The viscosity values appeared to be directly related to the level of glycerate present (Table 2). Figure 7 shows that the lactose-grown sample, which had the highest total acyl content (mole proportion, ~1.3 [Table 2]), yielded the lowest modulus. The glucose- and whey-grown samples had similar total acyl contents (mole proportion, ~1.1 [Table 2]) and their acetate and glycerate levels differed markedly, but the moduli of these samples were similar and higher than the modulus of the lactose-grown sample.

DISCUSSION

Gellan polysaccharides can be produced by the industrial strain S. paucimobilis ATCC 31461 in a laboratory-defined production base medium containing lactose (2%, wt/vol), although the yields are only around 70% of the yields obtained with glucose-containing medium. The results of the chemical and structural analysis of the two gellan samples indicated that they have the same primary carbohydrate structure, but the levels of acetate and glycerate are different. The gellan polysaccharide produced from lactose in sweet cheese whey diluted 1/5 with water also differed from the other two polysaccharides in the nature of the noncarbohydrate acyl substitution. Gellan modification may be strictly regulated and may depend not only on the enzyme activities that catalyze the corresponding biosynthetic steps but also on the intracellular concentrations of the acyl activated precursors, which may vary depending on cell metabolism in the different growth media. (16, 36).

The different acylation patterns affected the rheological properties of the three polymers obtained. The comparison of the viscosities of the three purified gellan polymers analyzed (Fig. 6) indicated that the sample produced from whey had the highest bulk viscosity, while the sample produced from glucose had the lowest shear viscosity. The viscosity values appeared to be directly related to the level of glycerate present (Table 2).

**TABLE 1. Concentration of gellan produced**

<table>
<thead>
<tr>
<th>Cheese whey dilution</th>
<th>Gellan (g/liter)</th>
<th>Residual BOD5 (mg/liter)</th>
<th>BOD5 removed (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:4</td>
<td>7.9</td>
<td>26,000</td>
<td>52</td>
</tr>
<tr>
<td>1:5</td>
<td>7.2</td>
<td>18,750</td>
<td>66</td>
</tr>
<tr>
<td>1:6</td>
<td>6.4</td>
<td>18,000</td>
<td>67</td>
</tr>
</tbody>
</table>

*Expressed as the concentration of the ethanol precipitate isolated from the culture broth after 72 h of growth at 30°C and 250 rpm in different dilutions of cheese whey media. The residual BOD5 of cell-free cultures and the consequent percentage of BOD5 removed is also indicated. Initial whey BOD5 was 30,000 ± 1,500 mg/liter. The relative standard deviation for three independent growth experiments was less than 10%.*
be directly related to the level of glycerate present (Table 2), as demonstrated previously (2, 18, 31). While the molecular analysis of X-ray fiber diffraction data suggested that glycerate alone is important in determining the gellan association and rheology, the results of studies of chemically modified gellans suggested that the rheology and conformation depend on both the level of acetate and glycerate substitution (18, 31). As shown in Fig. 7, the lactose-derived polymer, which had the highest total acyl content (mole proportion, \(0.90\) [Table 2]) yielded the lowest modulus, and the glucose- and whey-grown samples (which had similar total acyl contents [mole proportion, \(0.86\), as shown in Table 2] but markedly different acetate and glycerate levels) had similar moduli, which were higher than the modulus of the lactose-grown sample. The similarity of the modulus values obtained for the glucose- and whey-grown samples suggests that glycerate and acetate play significant roles in controlling polymer association and gelation, as does the total level of acyl substitution.

The lower yield of gellan from lactose than from glucose can hardly be explained on the basis of the levels of most of the enzymes that produce the activated sugar precursors for gellan gum polymerization, which were identical in lactose-grown cells and glucose-grown cells or were slightly higher in lactose-grown cells than in glucose-grown cells. Only the level of the TRS was depressed in lactose-grown cells. TRS and UGD are thought to be more specific enzymes for sugar precursor formation and to limit gellan biosynthesis (26), but the UGD specific activity was also lower in glucose-grown cells. Based on the overall results of the enzyme assays and the fact that the lactose- and glucose-derived gellans have similar primary carbohydrate structures, the control of gellan synthesis from lactose or glucose does not appear to take place at the level of nucleoside-sugar phosphate synthesis.

Direct fermentation of sweet cheese whey diluted 1:5 with water by \(S.\) paucimobilis ATCC 31461 resulted in production of approximately 7 g of EPS per liter and in a 70% reduction in the initial BOD\(_5\). We anticipate that supplementation of the medium with noncarbon nutrients and/or the use of whey permeate obtained after separation of a marketable protein concentrate may result in interesting valorization of this waste and in a reduction in its BOD.

The marked reduction in the high viscosity values of cheese whey-containing medium observed during the early stationary phase of \(S.\) paucimobilis ATCC 31461 growth when incubation

### Table 2. Chemical and NMR analysis of gellan polysaccharides, produced in basal S medium with glucose (G) or lactose (L) as the carbon source, or in diluted (1:5) cheese whey (W)

<table>
<thead>
<tr>
<th>Gellan sample</th>
<th>Carbohydrate as Glc (% [wt/wt])</th>
<th>Uronic acid as GlcA (% [wt/wt])</th>
<th>6-Deoxy-hexose as Rha (% [wt/wt])</th>
<th>Molar ratio of neutral sugars (Rha:Glc:Man)</th>
<th>Molar ratio of glycosidic linkages</th>
<th>Molar ratio of acyl substituents</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1:2.8:0.17</td>
<td>1</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td>1:2.4:0.20</td>
<td>1</td>
<td>0.51</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>1:2.4:0.15</td>
<td>1</td>
<td>0.53</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td>1:2.3:0.10</td>
<td>1</td>
<td>0.46</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1:2.1:0.08</td>
<td>1</td>
<td>0.46</td>
</tr>
</tbody>
</table>

FIG. 5. 400-MHz \(^1\)H NMR spectra (90°C) of \(S.\) paucimobilis gellan polysaccharides. S(G), cells grown in glucose-containing medium; S(L), cells grown in lactose-containing medium; S(W), cells grown in cheese whey-containing medium. Signal assignments: a, Rha H-1 (no glycerate); b, Rha H-1 (glycerate on 1,3-Glc); c, acetate CH\(_3\); d, Rha CH\(_3\).
other polysaccharide lyases active against acylated gellan exhibit negligible activity against the native acylated gellan. Other polysaccharide lyases active against gellan lyases active against alginate are also strongly inhibited by the presence of O-acetyl or other acyl groups on the polymeric substrates (11, 20). The suggested high levels of resistance of the polysaccharides produced in lactose- or glucose-containing media to the depolymerization of this type of gellan.

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