Extracellular Oligosaccharides and Low- M_r Polysaccharides Containing (1→2)- β -D-Glucosidic Linkages from Strains of *Xanthomonas*, *Escherichia coli* and *Klebsiella pneumoniae*

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One strain each of *Xanthomonas campestris* and '*Xanthomonas phaseoli*', three strains of '*Xanthomonas oryzae*', and five strains each of *Escherichia coli* and *Klebsiella pneumoniae* were found to produce a mixture of (1→2)- β -D-glucosaccharides and/or low- M_r (1→2)- β -D-glucans in their culture media. The saccharides from the strains of *Xanthomonas* were all composed of unbranched, linear (1→2)- β -D-glucosaccharides with degrees of polymerization (DPs) of 8 to about 20, and a cyclic (1→2)- β -D-glucan (DP16) containing one (1→6)-linkage and one α -linkage. The saccharides produced by the five strains of *E. coli* and four strains of *K. pneumoniae* were glucans with branches at O-6, and DPs of 10 to 15, whereas one strain of *K. pneumoniae* produced unbranched linear (1→2)- β -D-glucosaccharides with DPs of 6 to about 20.

INTRODUCTION

(1→2)- β -D-Glucans, which were found in strains of the genera *Agrobacterium* (Putman *et al.*, 1950; Gorin *et al.*, 1961; York *et al.*, 1980; Hisamatsu *et al.*, 1982) and *Rhizobium* (Dedonder & Hassid, 1964; Zevenhuizen & Scholten-Koerselman, 1979; Abe *et al.*, 1982; Amemura *et al.*, 1983), were thought to occur only rarely until Amemura *et al.* (1985) demonstrated the presence of oligo- and polysaccharides consisting of β -(1→2)-linked D-glucosyl residues in seven strains of *Acetobacter*. Oligosaccharides linked by β -(1→2)- and β -(1→6)-bonds have also been isolated from the cell envelope of *Escherichia coli* K12 (Schneider *et al.*, 1979) and the presence of similar compounds was suggested in the cell membranes of other Gram-negative bacteria (Schulman & Kennedy, 1979).

Many bacteria excrete polysaccharides into their culture media. Since these polysaccharides generally make the media viscous, they can be detected easily and recovered by simple methods such as precipitation with alcohol. However, solutions of (1→2)- β -D-glucan and related poly- and oligosaccharides have low viscosity and because of their low M_r are precipitated only by a high concentration of alcohol. Possibly for these reasons, they have not been studied so extensively as high- M_r polysaccharides. In this work we have examined the polysaccharides and oligosaccharides in the culture filtrates of 11 species of bacteria.

METHODS

Organisms. The bacteria used in this work are listed in Table 1. Strains with an IFO number were obtained from the Institute for Fermentation, Osaka, Japan (IFO). The other strains were stock cultures in this laboratory.

Media. The media used to examine the production of polysaccharides and oligosaccharides had the following composition per litre: glucose, 40.0 g; $(\text{NH}_4)_2\text{HPO}_4$, 1.5 g; KH_2PO_4 , 1.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; NaCl, 10 mg; CaCl_2 , 10 mg; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 10 mg; CaCO_3 , 5.0 g (pH 7.0). The same medium supplemented with 0.1% yeast extract was used for strains that did not grow well.

Culture conditions. Samples (5 ml) of seed cultures were inoculated into 95 ml medium in 500 ml conical flasks with baffles. The cultures were shaken at 150 r.p.m. at 30 °C for 6 d on a rotary shaker.

Abbreviations: DP, degree of polymerization; ODS, octadecylsilane silica; TFA, trifluoroacetic acid.

Preparation of cells, polysaccharides and oligosaccharides. The culture broth was centrifuged at 56 000 *g* for 30 min to precipitate the cells and insoluble CaCO_3 . The CaCO_3 was removed by washing the precipitate with 1 M-HCl and the remaining insoluble material (cells) was washed with water and dehydrated with acetone.

The supernatant of the culture broth was mixed with ethanol (2 vols) and centrifuged to remove a high- M_r fraction including acidic polysaccharide. The supernatant was then concentrated to about 10 ml, again mixed with ethanol (4 vols), and the resulting precipitate was collected by centrifugation (low- M_r fraction), dissolved in water and applied to a column of DEAE-cellulose equilibrated with 1 mM-KCl. The column was washed with 1 mM-KCl and the effluent containing neutral materials was collected, concentrated to a small volume and passed through a column of Sephadex G-25 to remove salts and contaminating glucose. Sugar-containing fractions were collected, concentrated to a small volume and lyophilized (low- M_r neutral fraction). Materials adsorbed to DEAE-cellulose were eluted with 0.2 M-KCl and a low- M_r acidic fraction was prepared from the effluent by chromatography on Sephadex G-25 and lyophilization as described above.

The high- M_r fraction was dissolved in water to give a 0.2% (w/v) solution and an appropriate amount (5–50 ml, depending on the amounts of polysaccharide produced) of 10% (w/v) cetylpyridinium chloride solution was added to precipitate the acidic polysaccharide. The precipitate was collected by centrifugation, washed with water and dissolved in 10% (w/v) NaCl. Ethanol (2 vols) was added to the solution and the resulting precipitate was collected by centrifugation, washed with ethanol/water (6:1, v/v) and with ethanol and dried *in vacuo* (acidic polysaccharide). From the supernatant obtained after removal of the cetylpyridinium chloride-acidic polysaccharide complex, a neutral polysaccharide fraction was prepared by precipitation with ethanol (2 vols).

High-performance liquid chromatography (HPLC). Analyses were performed with a 6000A solvent-delivery system, a model U6K injector (both from Water Associates, Milford, Mass., USA), and a model SE-41 differential refractometer (Showa Denko, Tokyo, Japan). Amino-bonded (ERC-NH-1171, 200 $\times$ 6 mm i.d., Erma Optical Works, Tokyo, Japan) and ODS (YMC-Pack AL-312, 150 $\times$ 6 mm i.d., Yamamura Chem., Kyoto, Japan) columns were used. Chromatographic conditions for (1 $\rightarrow$ 2)- β -D-glucans and (1 $\rightarrow$ 2)- β -D-glucosyl-oligosaccharides have been reported (Koizumi *et al.*, 1983, 1984, 1985).

Methylation analysis. Materials were methylated with sodium methyl sulphoxide as described by Hakomori (1964). The methylated samples were hydrolysed with 2-M-trifluoroacetic acid (TFA) at 100 °C for 6 h and analysed as alditol acetates by gas-liquid chromatography (GLC) in a GC7A chromatograph (Shimadzu, Kyoto, Japan) fitted with a flame ionization detector and a column (2 m $\times$ 3 mm i.d.) of 0.3% OV275/0.4% GEXF1150 on Shimalite W (Wako Pure Chem., Osaka, Japan), as described by Hisamatsu *et al.* (1980). Methylated sugars were identified by mass spectrometry and by comparison of their retention times with those of standard sugars.

Nuclear magnetic resonance (NMR) spectrometry. ^1H -NMR and ^{13}C -NMR spectra (360–135 MHz and 90–56 MHz, respectively) were recorded in a Bruker AM-360 spectrometer at 70 °C with sodium 4,4-dimethyl-4-silapentane-1-sulphonate (DSS) as an internal standard. Samples were dissolved in D_2O at a concentration of 0.3% (w/v). Spectra were assigned by comparison with those for sophoro-oligosaccharides studied previously (Takai *et al.*, 1984). Non-selective polarization transfer spectroscopy (Doddrell & Pegg, 1980) was also used to assign ^{13}C spectra.

Preparation of standard sophorosaccharides. The cyclic (1 $\rightarrow$ 2)- β -D-glucans used for reference purposes were prepared from culture filtrates of *Agrobacterium radiobacter* IFO 12665, as described previously (Hisamatsu *et al.*, 1983). A mixture of standard sophoro-oligosaccharides and polysaccharides was obtained by partial hydrolysis of cyclic (1 $\rightarrow$ 2)- β -D-glucan with 0.2 M-TFA at 100 °C for 100 min.

RESULTS

Thirty-three strains of Gram-negative bacteria (three of *Flavobacterium*, four of *Cytophaga*, 11 of *Xanthomonas*, nine of *Escherichia coli* and six of *Klebsiella pneumoniae*) were examined for production of extracellular poly- and oligosaccharides. None of the strains produced appreciable amounts of low- M_r acidic fraction (acidic oligosaccharides) or high- M_r neutral polysaccharide. Six strains of *Xanthomonas* produced acidic polysaccharides, whereas five strains of *Xanthomonas*, five strains of *E. coli* and five strains of *K. pneumoniae* produced a low- M_r neutral fraction. The low- M_r neutral fraction was not detected in media of strains of '*Xanthomonas citri*', *Flavobacterium* and *Cytophaga*. The amounts of the low- M_r neutral fraction and acidic polysaccharide produced are shown in Table 1. The acidic polysaccharide was not studied further.

Samples of the low- M_r neutral fractions from the media of each of the 15 strains were methylated, hydrolysed and analysed by GLC. The main methylated sugar detected in all the samples was 3,4,6-tri-*O*-methyl-D-glucose (Table 2). This indicates that the fractions were composed mainly of (1 $\rightarrow$ 2)-linked D-glucans or D-glucosyl-oligosaccharides. The samples from

Table 1. Quantification of low- M_r neutral fraction and acidic polysaccharide by strains of *Xanthomonas*, *E. coli* and *K. pneumoniae*

The following strains did not produce a low- M_r neutral fraction or acidic polysaccharides: '*Flavobacterium lutescens*' IFO 3084*, *F. capsulatum* IFO 12533*, *F. okeanokoites* IFO 12536*, *Cytophaga heparina* IFO 12017, *C. arvensicola* IAM 12646, *C. arvensicola* IAM 12648, *C. arvensicola* IAM 12650, *Xanthomonas oryzae* IFO 12000*, '*X. citri*' IFO 3781*, '*X. citri*' IFO 3829*, '*X. citri*' IFO 3835*, *Escherichia coli* IFO 3301, *E. coli* IFO 3302, *E. coli* IFO 12062, *E. coli* IFO 13168, and *Klebsiella pneumoniae* IFO 3320. Names in inverted commas do not appear on the Approved Lists of Bacterial Names (Skerman *et al.*, 1980) or their supplements.

Strain	pH	Cells (mg ml ⁻¹)	Low- M_r neutral fraction† (mg ml ⁻¹)	Acidic poly- saccharide† (mg ml ⁻¹)
<i>X. campestris</i> IFO 13551*	6.51	2.11	0.110	11.90
' <i>X. phaseoli</i> ' IFO 13553	5.68	1.67	0.319	1.09
' <i>X. phaseoli</i> ' IFO 13554*	6.70	3.57	0	1.21
' <i>X. oryzae</i> ' IFO 3312*	7.02	0.59	0.918	0.75
' <i>X. oryzae</i> ' IFO 3510*	5.87	0.50	0.243	1.02
' <i>X. oryzae</i> ' IFO 3827*	6.21	2.02	0	1.63
' <i>X. oryzae</i> ' IFO 3833*	5.73	1.10	0.951	0
<i>E. coli</i> IFO 3044	4.85	1.68	0.294	0
<i>E. coli</i> IFO 3806	4.74	2.00	0.363	0
<i>E. coli</i> IFO 12433	4.54	1.47	0.072	0
<i>E. coli</i> IFO 13500	4.50	2.10	0.052	0
<i>E. coli</i> IFO K12	4.36	2.29	0.041	0
<i>K. pneumoniae</i> IFO 3317	4.65	1.48	0.026	0
<i>K. pneumoniae</i> IFO 3318	2.97	1.56	0.018	0
<i>K. pneumoniae</i> IFO 3319	3.11	1.85	0.033	0
<i>K. pneumoniae</i> IFO 3321	3.18	1.40	0.020	0
<i>K. pneumoniae</i> IFO 3512	2.79	1.43	0.030	0

* The culture medium for these strains was supplemented with 0.1% yeast extract.

† Expressed as amounts of sugar assayed by the phenol/H₂SO₄ method (Dubois *et al.*, 1956) with glucose as standard.

Xanthomonas also contained 3–5% of 2,3,4,6-tetra-*O*-methyl-D-glucose derived from non-reducing terminal D-glucosyl residues and 2,3,4-tri-*O*-methyl-D-glucose derived from (1→6)-linked D-glucosyl residues, but no di- or mono-methyl sugar derived from branching sugar residues; the samples from *E. coli* and *K. pneumoniae* contained few if any (1→6)-linked D-glucosyl residues and more branch points and terminal D-glucosyl residues. The low- M_r neutral fraction from *E. coli* K12 had a particularly highly branched structure. Of the low- M_r neutral fractions from strains of *E. coli* and *K. pneumoniae* only that from *K. pneumoniae* IFO 3321 had no branch points.

Characterization of the low- M_r neutral fractions from *Xanthomonas* strains

The specific optical rotation $[\alpha]_D^{20}$ values (*c* 0.5, water) of the low- M_r neutral fractions were *X. campestris* IFO 13551, -3.5° ; '*X. phaseoli*' IFO 13553, $+4.3^\circ$; '*X. oryzae*' IFO 3312, 0° ; '*X. oryzae*' IFO 3510, $+8.0^\circ$; and '*X. oryzae*' IFO 3833, 0° . These low values suggested a predominance of β -glycosidic linkages.

When the low- M_r neutral fractions were subjected to HPLC on an NH₂-bonded silica column, the elution profiles of the five samples were similar (Fig. 1); there was a major peak with a retention time of 25 min preceded by seven medium-sized peaks. The sample from *X. campestris* IFO 13551 contained different compounds, which were eluted later in broad peaks. The retention times of the seven peaks coincided with those of sophoro-octaose (DP8) to sophorotetradecaose (DP14), but the retention time of the major peak was distinct from that of sophoropentadecaose (DP15), although the positions of the two were very similar. Several peaks corresponding to sophorohexadecaose (DP16) and higher sophorosaccharides (up to about DP20) were observed.

Table 2. Methylation analysis of low- M_r neutral fraction from strains of *Xanthomonas*, *E. coli* and *K. pneumoniae*

Retention time (min) . . .	Methylated sugar (mol. %)*					
	2,3,4,6-Glc	3,4,6-Glc	2,3,4,-Glc	3,4-Glc	Mono-methyl sugar†	
	14.1	25.2	30.0	50.9	74.1	77.5
Primary fragments in mass spectrum (m/e)						
117	+		+			
129		+		+		
161	+	+	+			
189		+	+	+		
205	+					
<i>X. campestris</i> IFO 13551	3.7	92.6	3.7	0.0	0.0	0.0
' <i>X. phaseoli</i> ' IFO 13553	4.6	92.6	2.8	0.0	0.0	0.0
' <i>X. oryzae</i> ' IFO 3312	4.6	92.3	3.1	0.0	0.0	0.0
' <i>X. oryzae</i> ' IFO 3510	3.5	93.8	2.7	0.0	0.0	0.0
' <i>X. oryzae</i> ' IFO 3833	2.9	92.6	4.5	0.0	0.0	0.0
<i>E. coli</i> IFO 3044	19.3	56.3	3.1	15.5	0.0	0.0
<i>E. coli</i> IFO 3806	26.4	59.9	0.7	13.0	0.0	0.0
<i>E. coli</i> IFO 12433	23.6	56.3	0.0	17.4	0.0	2.5
<i>E. coli</i> IFO 13500	22.9	72.3	0.0	4.8	0.0	0.0
<i>E. coli</i> K12	34.5	37.6	1.3	8.9	6.8	11.0
<i>K. pneumoniae</i> IFO 3317	22.2	63.2	0.0	14.6	0.0	0.0
<i>K. pneumoniae</i> IFO 3318	13.1	82.4	0.0	4.5	0.0	0.0
<i>K. pneumoniae</i> IFO 3319	7.9	90.3	0.0	1.8	0.0	0.0
<i>K. pneumoniae</i> IFO 3321	7.6	91.7	0.0	0.0	0.0	0.0
<i>K. pneumoniae</i> IFO 3512	11.1	85.8	0.0	3.1	0.0	0.0

* The values are the means of two independent experiments. 2,3,4,6-Glc = 2,3,4,6-tetra-*O*-methyl-D-glucose, and so on.

† Not identified.

To obtain further information on the structure of the low- M_r neutral fractions, we collected the two fractions indicated by bars in Fig. 1(c) (3312-A and 3312-B), measured their specific optical rotation ($[\alpha]_D^{20}$) and performed methylation analysis (Table 3). The low $[\alpha]_D^{20}$ values (-0.75° and -3.75°) indicate that β -glycosidic linkages predominated in both fractions. Fraction 3312-A contained no 2,3,4-tri-*O*-methyl-D-glucose derived from (1 $\rightarrow$ 6)-linked D-glucosyl residues and the ratio of 3,4,6-tri-*O*-methyl-D-glucose to 2,3,4,6-tetra-*O*-methyl-D-glucose was 11.1. Thus this fraction consisted of β -(1 $\rightarrow$ 2)-linked D-glucosaccharides with an average DP of 12. The methylated sugars in fraction 3312-B were 3,4,6-tri-*O*-methyl-D-glucose and 2,3,4-tri-*O*-methyl-D-glucose in a ratio of 14.8:1. The small amount (0.6%) of tetra-*O*-methyl-D-glucose in this fraction may have been derived from sophoropentadecaose (DP15), expected to be present as a contaminant. The absence of a non-reducing terminal suggested that this glucan had a cyclic structure. Also a reducing terminal was not detected after reduction of this fraction with NaBH₄, followed by acid hydrolysis and GLC analysis. These results strongly support the idea that fraction 3312-B consisted of a cyclic β -D-glucan composed of one (1 $\rightarrow$ 6)-linked glucosyl and 15 (1 $\rightarrow$ 2)-linked glucosyl residues.

The ¹H-NMR spectrum of fraction 3312-B (Fig. 2) shows the presence of a doublet signal at 5.13 p.p.m. ($J_{1,2}$ 3.5 Hz) assigned to an α -linkage and many doublet signals between 4.6 and 5.0 p.p.m. ($J_{1,2}$ 7.7–7.9 Hz) derived from β -linkages. Measurements at other temperatures confirmed that the solvent signal (DOH) did not overlap any other peak. In the expanded spectrum of the anomeric proton region, 15 doublet signals assigned to β -linkages are clearly resolved. The integral ratio of α - to β -anomeric protons was 1:15. The ¹³C-NMR spectrum also gave one α -anomeric and 15 β -anomeric signals at 101 p.p.m. and between 103 and 106 p.p.m.,

Fig. 1. HPLC of the low- M_r neutral fractions of (a) '*X. oryzae*' IFO 3833, (b) '*X. oryzae*' IFO 3510, (c) '*X. oryzae*' IFO 3312, (d) '*X. phaseoli*' IFO 13553, and (e) *X. campestris* IFO 13551. Chromatographic conditions: column, ERC-NH-1171; eluent, acetonitrile/water (56:44, v/v); flow rate, 1 ml min⁻¹; temperature, ambient. The number beside each peak indicates its DP. The fractions indicated by bars were collected.

Table 3. Methylation analysis and $[\alpha]_D^{20}$ values of fractions 3312-A and 3312-B obtained from the low- M_r neutral fraction of '*X. oryzae*' IFO 3312

Fraction	Methylated sugar (mol %)*			$[\alpha]_D^{20}$ (c 0.4, water)
	2,3,4,6-Glc	3,4,6-Glc	2,3,4-Glc	
3312-A	8.2	91.3	0.5	-0.75
3312-B	0.6	93.1	6.3	-3.75

* The values are the means of three independent experiments.

respectively (Fig. 3). In the spectrum signals derived from C-3 and C-5 of an α -D-glucosyl residue can be seen at 74.6 p.p.m. There is a signal at 76.3 p.p.m. derived from a C-2 that is not part of a glucosyl bond, and another at 70.3 p.p.m. from a C-6 participating in a glucosyl bond. The C-6 assignment was confirmed by a non-selective polarization transfer (INEPT) technique, showing the characteristic, CH₂ negative signal (Fig. 3).

Thus, from methylation and reducing-terminal analyses and NMR spectrometry we concluded that the glucan of fraction 3312-B was an unbranched cyclic (1→2)- β -D-glucan with DP16 containing one α -D-glucosyl residue and one (1→6)-linked D-glucosyl residue.

Characterization of the low- M_r neutral fractions from strains of *E. coli* and *K. pneumoniae*

The low- M_r neutral fractions from the five strains of *E. coli* were subjected to HPLC on an NH₂-bonded silica column. The samples from strains IFO 3044, IFO 3806 and K12 gave similar elution patterns with several peaks (Fig. 4a, data for strain IFO 3806 only). The samples from strains IFO 12433 and IFO 13500 also gave similar chromatograms to that shown in Fig. 4(a), although overlapping broad peaks of some other compounds were present.

On a reversed-phase ODS column the preparation from strain IFO 3806 gave more than 10 peaks (Fig. 4b), although only four were obtained on the NH₂-bonded silica column. The seven fractions indicated by bars (fractions 3806-A to 3806-G) were collected. Fraction 3806-A did not contain sugar as determined by the phenol/H₂SO₄ reagent (Dubois *et al.*, 1956). The retention

Fig. 2. ^1H -NMR spectrum of fraction 3312-B obtained from the low- M_r neutral fraction of '*X. oryzae*' IFO 3312 (D_2O , 70°C). Above is a scale expansion of the anomeric region with enhanced resolution. DSS; reference signals of sodium 4,4-dimethyl-4-silapentane-1-sulphonate.

Fig. 3. ^{13}C -NMR spectrum of fraction 3312-B obtained from the low- M_r neutral fraction of '*X. oryzae*' IFO 3312 (D_2O , 70°C). Above are an expansion of the anomeric region and an INEPT sequence of the C-4 and C-6 region.

Fig. 4. HPLC of the low- M_r neutral fractions of (a, b) *E. coli* IFO 3806 and (c) *K. pneumoniae* IFO 3321. Chromatographic conditions for (a) and (c) were as for Fig. 1. Chromatographic conditions for (b): column, YMC-Pack AL-312; eluent, methanol/water (4:96, v/v); flow rate, 1 ml min⁻¹; temperature, ambient. The fractions indicated by bars were collected. For explanation of numbers beside peaks in (a), see text. The number beside each peak in (c) indicates its DP.

Table 4. *Methylation analysis of fractions 3806-B to 3806-G obtained from the low- M_r neutral fraction of E. coli IFO 3806*

Fraction	Methylated sugar (mol. %; molar ratio in parentheses)*		
	2,3,4,6-Glc	3,4,6-Glc	3,4-Glc
3806-B	14.8 (1)	85.2 (5.8)	0.0 (0)
3806-C	19.4 (2.4)	72.6 (9.1)	8.0 (1)
3806-D	23.6 (3.2)	61.6 (8.3)	14.8 (2)
3806-E	25.8 (3.1)	57.8 (7.0)	16.4 (2)
3806-F	32.2 (4.5)	46.4 (6.5)	21.4 (3)
3806-G	32.0 (4.4)	46.2 (6.4)	21.8 (3)

* The values are the means of three independent experiments.

times of fractions 3806-B and -C on the NH₂-bonded silica column were identical with those of peaks 1 and 2, respectively, in Fig. 4(a); the retention times of fractions 3806-E and -F were both identical with that of peak 3. Although fractions 3806-D appeared as a single peak in the effluent from the ODS column, it separated into two peaks with the retention times of peaks 2 and 3 on the NH₂-bonded silica column. Fraction 3806-G apparently gave two peaks on the ODS column, but only one peak, corresponding to peak 4, on the NH₂-bonded silica column. Thus, the four peaks in the effluent from the NH₂-bonded silica column (Fig. 4a) contained at least eight different compounds.

Table 5. *D*P_s of fractions 3806-B to 3806-G determined by reduction of their reducing end, followed by hydrolysis and GLC

Fraction	Glucose:Glucitol*	DP	DP estimated by methylation analysis (no. of branch points)
3806-B	6.3:1	7	7 (0)
3806-C	11.2:1	12	12 (1)
3806-D	10.5:1	11 or 12	13 (2)
3806-E	10.0:1	11	12 (2)
3806-F	12.0:1	13	13 or 14 (3)
3806-G	13.0:1	14	13 or 14 (3)

* The values are the means of two independent experiments.

The six saccharide fractions from the ODS column were each methylated and the methylated sugars were analysed by GLC (Table 4). The data show that the fractions were composed of terminal D-glucosyl and (1→2)-linked D-glucosyl residues with branching D-glucosyl residues at O-6, except fraction 3806-B, which had no branch point.

The DP_s of these glucosaccharides determined by reducing-end analysis were 7 for fraction 3806-B and 11 to 14 for the other fractions (Table 5). These DP_s are similar to those estimated by methylation analysis (Table 4), the slight differences probably being due to heterogeneity of the samples, especially fraction 3806-D (Table 5). The ¹H-NMR spectra of the six samples demonstrated that the glucosidic linkages in the saccharides were all β (data not shown). The numbers of branch points per molecule were one for fraction 3806-C, two for fractions 3806-D and -E, and three for fractions 3806-F and -G.

Five samples of the low-*M_r* neutral fractions from *K. pneumoniae* were analysed by HPLC. The elution profile of the samples from strain IFO 3321 (Fig. 4c) was superimposable on those of sophorosaccharides obtained by partial acid hydrolysis of cyclic (1→2)-β-D-glucan of *Agrobacterium radiobacter* IFO 12665. The first peak corresponded to sophorohexaose (DP₆), whereas the last detectable peak corresponded to DP₂₂. When the sample was mixed with a partial hydrolysate of cyclic (1→2)-β-D-glucan and injected on the column, the peaks of the two samples coincided exactly. Moreover, the methylated sugars from the sample of strain IFO 3321 were those from unbranched (1→2)-linked D-glucosaccharides (Table 2). Thus, the low-*M_r* neutral fraction of strain IFO 3321 was confirmed to be composed of a mixture of linear sophorosaccharides with DP_s of 6 to about 22.

The elution profiles of the low-*M_r* neutral fractions from the other *K. pneumoniae* strains (IFO 3317, 3318, 3319 and 3512) were similar to those of the low-*M_r* neutral fraction of *E. coli* strains (data not shown). Only the low-*M_r* neutral fraction of *E. coli* IFO 3806 was studied in detail, but the preparations from the other strains of *E. coli* and *K. pneumoniae* might be expected to consist of the same family of oligo- or low-*M_r* polysaccharides, judging from the data obtained by methylation analysis and the patterns on HPLC, i.e. a mixture of several (1→2)-β-D-glucosaccharides of similar molecular sizes with branching at O-6. The preparations may differ in their extents of branching and the distribution of molecular sizes.

DISCUSSION

Of the 33 strains of Gram-negative bacteria tested, 15 strains of *X. campestris*, '*X. phaseoli*', '*X. oryzae*', *E. coli* and *K. pneumoniae* produced a mixture of extracellular oligosaccharides and/or polysaccharides, which contained mainly or exclusively (1→2)-β-D-glucosidic linkages in their low-*M_r* neutral fractions.

The low-*M_r* neutral fractions from strains of *Xanthomonas* contained a series of linear sophorosaccharides with DP_s of 8 to about 20. These products were similar to those from strains of *Acetobacter* studied previously (Amemura *et al.*, 1985), although the DP_s of sophorosaccharides in the latter strains were between 6 and about 40. However, a characteristic of the

Xanthomonas products was that they contained a cyclic (1→2)- β -D-glucan (DP16) with a (1→6)-linkage. This cyclic glucan was found to contain one α -linkage per molecule by NMR studies. Cyclic (1→2)- β -D-glucans have been found in many strains of *Agrobacterium* and *Rhizobium*, but this is the first report of their production by members of other genera.

The low- M_r neutral fractions from strains of *E. coli* and *K. pneumoniae* were a mixture of branched glucans linked by β -(1→2)- and β -(1→6)-bonds with DPs of 10 to 15. These low- M_r glucans have similar structures to membrane-derived oligosaccharides from strains of *E. coli* K12, reported by Schneider *et al.* (1979). The latter oligosaccharides contain 10 to 12 D-glucosyl residues linked by β -(1→2)- and β -(1→6)-bonds with highly branched structures, and also contain non-carbohydrate moieties of *sn*-1 glycerophosphate, phosphoethanolamine and succinic acid (van Golde *et al.*, 1973). However, the saccharides obtained in this work were neutral, without charged moieties. The membrane-derived oligosaccharides were suggested to be localized in the periplasmic space. Therefore, it is probable that the only carbohydrate moiety is released into the medium.

Extracellular (1→2)- β -D-glucans or oligosaccharides have thus been found in the genera *Agrobacterium*, *Rhizobium*, *Acetobacter*, *Xanthomonas*, *Escherichia* and *Klebsiella*. The molecular forms (cyclic, linear or branched) and the distribution patterns of DPs are characteristic for the genera. The principal structures of the glucans from *Escherichia* and *Klebsiella* are the same, judging from the results of methylation analysis and HPLC, although they may differ in precise structure.

The functions of (1→2)- β -D-glucans in bacteria are unknown, but Abe *et al.* (1982) demonstrated that cyclic (1→2)- β -D-glucan from *R. trifolii* 4S influences symbiosis in the leguminous host plant. Kennedy (1982) provided evidence that membrane-derived oligosaccharides from *E. coli* are synthesized in the periplasmic space to maintain a high osmotic pressure when the cells are transferred to medium of low osmolarity. Cyclic (1→2)- β -D-glucans of *Agrobacterium* and *Rhizobium* have been found not only in the culture medium but also on the cell surface, probably in the periplasmic space (Zevenhuizen & Scholten-Koerselman, 1979; Abe *et al.*, 1982). These families of saccharides in the periplasmic space of Gram-negative bacteria may participate in the regulation of osmolarity, and over-produced saccharides may be released into the culture medium.

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REFERENCES

- ABE, M., AMEMURA, A. & HIGASHI, S. (1982). Studies on cyclic β -1,2-glucan obtained from periplasmic space of *Rhizobium trifolii* cells. *Plant and Soil* **64**, 315–324.
- AMEMURA, A., HISAMATSU, M., MITANI, H. & HARADA, T. (1983). Cyclic (1→2)- β -D-glucan and the oligosaccharide repeating-units of extracellular acidic polysaccharides produced by *Rhizobium*. *Carbohydrate Research* **114**, 227–285.
- AMEMURA, A., HISAMATSU, M., KOIZUMI, K. & UTAMURA, T. (1985). Occurrence of extracellular (1→2)- β -D-glucans and (1→2)- β -D-gluco-oligosaccharides in *Acetobacter*. *Journal of General Microbiology* **131**, 301–307.
- DEDONDER, R. & HASSID, W. Z. (1964). The enzymatic synthesis of a (β -1,2)-linked glucan by an extract of *Rhizobium japonicum*. *Biochimica et biophysica acta* **90**, 239–248.
- DODDRELL, D. M. & PEGG, D. T. (1980). Assignment of proton-decoupled carbon-13 spectra of complex molecules by using polarization transfer spectroscopy. A superior method to off-resonance decoupling. *Journal of the American Chemical Society* **102**, 6388–6390.
- DUBOIS, M., GILLES, K. A., HAMILTON, J. K., REBERS, P. A. & SMITH, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry* **28**, 350–356.
- VAN GOLDE, L. M., SCHULMAN, H. & KENNEDY, E. P. (1973). Metabolism of membrane phospholipids and its relation to a novel class of oligosaccharides in *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* **70**, 1368–1372.
- GORIN, P. A. J., SPENCER, J. F. T. & WESTLAKE, D. W. S. (1961). The structure and resistance to methylation of 1,2- β -glucans from species of *Agrobacterium*. *Canadian Journal of Chemistry* **39**, 1067–1073.
- HAKOMORI, S. (1964). A rapid permethylation of glycolipid, and polysaccharide catalyzed by methylsulfinyl carbanion in dimethyl sulfoxide. *Journal of Biochemistry* **55**, 205–208.
- HISAMATSU, M., ABE, J., AMEMURA, A. & HARADA, T.

- (1980). Structural elucidation of succinoglycan and related polysaccharides from *Agrobacterium* and *Rhizobium* by fragmentation with two specific β -D-glycanases and methylation analysis. *Agricultural and Biochemical Chemistry* **44**, 205–208.
- HISAMATSU, M., AMEMURA, A., MATSUO, T., MATSUDA, H. & HARADA, T. (1982). Cyclic (1 $\rightarrow$ 2)- β -D-glucan and the octasaccharide repeating-unit of succinoglycan produced by *Agrobacterium*. *Journal of General Microbiology* **128**, 1873–1879.
- HISAMATSU, M., AMEMURA, A., KOIZUMI, K., UTAMURA, T. & OKADA, Y. (1983). Structural studies on cyclic (1 $\rightarrow$ 2)- β -D-glucans (cyclosophoraoses) produced by *Agrobacterium* and *Rhizobium*. *Carbohydrate Research* **121**, 31–40.
- KENNEDY, E. P. (1982). Osmotic regulation and the biosynthesis of membrane-derived oligosaccharides in *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* **79**, 1092–1095.
- KOIZUMI, K., OKADA, Y., HORIYAMA, S., UTAMURA, T., HISAMATSU, M. & AMEMURA, A. (1983). Separation of cyclic (1 $\rightarrow$ 2)- β -D-glucans (cyclosophoraoses) produced by *Agrobacterium* and *Rhizobium*, and determination of their degree of polymerization by high-performance liquid chromatography. *Journal of Chromatography* **265**, 89–96.
- KOIZUMI, K., OKADA, Y., UTAMURA, T., HISAMATSU, M. & AMEMURA, A. (1984). Further studies on the separation of cyclic (1 $\rightarrow$ 2)- β -D-glucans (cyclosophoraoses) produced by *Rhizobium meliloti* IFO 13336, and determination of their degrees of polymerization by high-performance liquid chromatography. *Journal of Chromatography* **299**, 215–224.
- KOIZUMI, K., UTAMURA, T. & OKADA, Y. (1985). Analysis of homogeneous D-gluco-oligosaccharides and -polysaccharides (degree of polymerization up to about 35) by high-performance liquid chromatography and thin-layer chromatography. *Journal of Chromatography* **321**, 145–157.
- PUTMAN, E. W., POTTER, A. L., HODGSON, R. & HASSID, W. Z. (1950). The structure of crown gall polysaccharide. *Journal of the American Chemical Society* **72**, 5034–5027.
- SCHNEIDER, J. E., REINHOLD, V., RUMLEY, M. K. & KENNEDY, E. P. (1979). Structural studies of the membrane-derived oligosaccharides of *Escherichia coli*. *Journal of Biological Chemistry* **254**, 10135–10138.
- SCHULMAN, H. & KENNEDY, E. P. (1979). Localization of membrane-derived oligosaccharides in the outer envelope of *Escherichia coli* and their occurrence in other gram-negative bacteria. *Journal of Bacteriology* **137**, 686–688.
- SKERMAN, V. B. D., MCGOWAN, V. & SNEATH, P. H. A. (1980). Approved lists of bacterial names. *International Journal of Systematic Bacteriology* **30**, 225–420.
- TAKAI, Y., SAWADA, M. & AMEMURA, A. (1984). Analyses of sophoro-oligosaccharides using ^1H , ^{13}C -two dimensional NMR. *Proceedings of 7th Japanese Carbohydrate Symposium, Osaka, August, 1984*, p. 10.
- YORK, W. S., MCNEIL, M., DARVILL, G. & ALBERSHEIM, P. (1980). Beta-2-linked glucans secreted by fast-growing species of *Rhizobium*. *Journal of Bacteriology* **142**, 243–248.
- ZEVENHUIZEN, L. P. T. M. & SCHOLTEN-KOERSELMAN, H. J. (1979). Surface carbohydrates of *Rhizobium*. I. β -1,2-Glucans. *Antonie van Leeuwenhoek* **45**, 165–175.