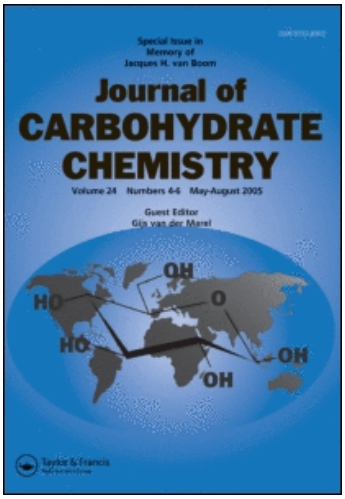

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A (1→4)-ß-D-GLUCURONAN EXCRETED BY A MUTANT OF THE *RHIZOBIUM MELILOTI* M5N1 STRAIN[1]

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ABSTRACT

A mutant of the *R. meliloti* M5N1 strain has been selected. This strain, *R. meliloti* M5N1 CS (NCIMB 40472), excretes an extracellular material composed of 2-*O*-Ac-β-Glc*p*A, 3-*O*-Ac-β-Glc*p*A, 2,3-di-*O*-Ac-β-Glc*p*A and three species of β-Glc*p*A residues 1→4 linked. For the culture conditions used, the weight average molecular weight of the polymer varied in the range of $6 \times 10^4 < \overline{Mw} < 4 \times 10^5$. High molecular weight glucuronate forms thermoreversible gels at 5 g L^{-1}. In the presence of divalent cation such as Ca^{2+} or trivalent cations such as Cr^{3+} or Fe^{3+}, cross linking of the polymer occurs. This polysaccharide is the first exocellular (1→4)-ß-D-glucuronan produced by a *R. meliloti* strain.

INTRODUCTION

Bacteria of the Rhizobiaceae family are known to produce complex mixtures of exopolysaccharides from simple glucans to elaborate heteropolysaccharides.[2] Chemical mutagenesis (NTG) was performed on the *R. meliloti* M5N1, a strain which produces a succinoglycan, several mutants were obtained. Among them, the strain M5N1 CS (NCIMB 40472), producing a glucuronan was selected[3] for further studies.

Until now glucuronans have been detected in the cell wall and in the extracellular material of mucorales,[4-5] or can be partially obtained by oxidation of cellulose.[6] The present paper describes the isolation, structural studies and rheological properties of the glucuronan excreted by the M5N1 CS mutant strain of *R. meliloti*.

RESULTS AND DISCUSSION

The polysaccharide (EPS) produced by the *R. meliloti* M5N1 mutant strain was isolated from the broth medium by microfiltration and then purified by ultrafiltration. The yield was: 2 g L^{-1} / 24 h with a biomass of 3 x 10^{10} cells mL^{-1}. As the EPS was not soluble in a 1M sulfuric acid solution, and due to the strong resistance towards acid hydrolysis, the polymer was reduced before being submitted to an acidic hydrolysis. Glucose was the only component detected after analysis of the hydrolysate by HPLC. A positive value for the signal obtained from this component using a polarimetric detector on line with a refractometric detector, indicated the component was D-glucose.

After *O*-methylation and CF_3COOH hydrolysis of the reduced EPS, the resulting sugars were reduced and acetylated. GLC analysis revealed the presence of a single component having the same retention time as that of 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-glucitol. This result suggested that the glucuronic acid (GlcA) residues are 1→4 linked.

The ^1H NMR spectrum of native polymer revealed a complex spectral pattern in the ring proton region and signals in the 2 ppm region characteristic of *O*-acetyl groups. These results clearly indicated that the glucuronan was partly acetylated.

The ^1H NMR spectrum obtained with the deacetylated polymer (by NaOH treatment (pH > 8), at ambient temperature, > 2 hours) revealed only five protons of a uronic acid. Comparison of deacetylated EPS chemical shifts (δH-1 = 4.53, δH-2 = 3.36, δH-3 = 3.61, δH-4 = 3.69, δH-5 = 3.85) with those of β–glucuronic acid (δH-1 = 4.67, δH-2 = 3.29, δH-3 = 3.52, δH-4 = 3.59, δH-5 = 3.92) confirms that this new polysaccharide is formed by glucuronic acid β 1→4 linked units ($J_{1,2}$ =7.9 Hz for β-Glc*p*A and 7.7 for deacetylated exopolysaccharide).

The spectrum of the native polymer also had an envelope of overlapping multiplets in the region 3.0-4.0 ppm. Downfield to 4.3 ppm, several broad signals could be assigned to protons linked to C-1 or C-*O*-Ac. The peaks were assigned using a combination of COSY and relayed COSY experiments, and by observing the chemical shift variations induced by *O*-acetylation.[7-8] Proton chemical shifts for an 3-*O*-Ac-Glc*p*A residue agree with those reported by Buchanan *et al.*[9] and Canter-Cremer *et al.*[10] Integration of resonances in the downfield region (4.3-5.2 ppm),

TABLE 1. Experimental and Calculated[a] ^{1}H NMR Chemical Shifts at 80 °C of the Ring Proton in the Native Polysaccharide.

residue	H-1	H-2	H-3	H-4	H-5
β-Glc*p*A	4.40	3.22	3.52	3.66	4.02
β-Glc*p*A	4.44	3.25	3.57	3.70	3.99
β-Glc*p*A	4.53	3.36	3.61	3.74	3.96
β-2-*O*-Ac-Glc*p*A	4.69	4.69	3.78	3.87	4.06
	(4.71)	(4.77)	(3.80)	(3.77)	(3.86)
β-3-*O*-Ac-Glc*p*A	4.61	3.48	4.97	3.92	3.88
	(4.63)	(3.54)	(5.10)	(3.88)	(3.94)
β-2,3-di-*O*-Ac-Glc*p*A	4.80	4.80	5.10	3.96	3.96
	(4.81)	(4.96)	(5.29)	(4.04)	(3.95)

a. calculated chemical shifts are in parentheses.

upfield region (3.1-4 ppm) and acetyl region (1.9-2.2 ppm) of native exopolysaccharide ^{1}H NMR spectrum, provides the degree of substitution per residue. In Table 1, the calculated chemical shifts for various protons are reported; the values were determined by using ^{1}H chemical shift variations[7] induced by acetylation of β-glucopyranoside at ambient temperature. Calculated values agree with experimental values obtained from 1D and 2D spectra. Overlapping of H-4 and H-5 signals makes signal assignment ambiguous.

The relative proportions of *O*-acetylated species were determined by integration of the downfield signals. Signal areas were determined by studies of integrals or by peak deconvolution. From preliminary studies, variation of the proportion of acetyl residues had been detected and found to be dependant on fermentation conditions. For one sample, molar proportions of 19% 3-*O*-acetyl, 13% 2-*O*-acetyl, 25% 2,3-di-*O*-acetyl β-**D**-Glc*p*A and 39% of unacetylated residues were estimated.

Methyl signals assignments were determined from spectra obtained for EPS with different degrees of acetylation. The resonances at 2.14 ppm and 2.01 ppm are respectively attributed to the methyl of acetyl groups linked to C-2 in 2-*O*-acetyl residues and in 2,3-di-*O*-acetyl residues. Signals at 2.09 ppm and at 2.07 ppm are assigned respectively to the methyl of acetyl groups linked to C-3 in 3-*O*-acetyl residues and 2,3-di-*O*-acetyl residues.

The molecular weight of the EPS was measured by size exclusion chromatography (SEC). The results indicate a weight average molecular weight ($\overline{Mw}$)

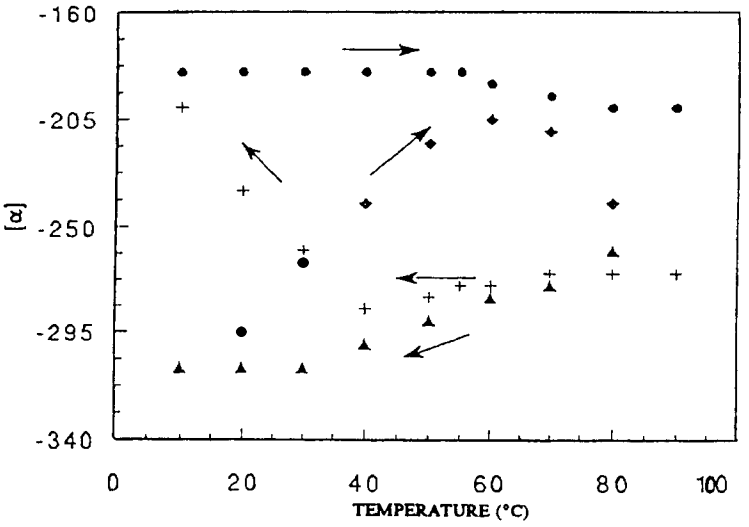

FIG.1. Effect of temperature on the optical rotation $[\alpha]^{300}$ of polysaccharide solutions (0.5 g L^{-1}) in 1M NH_4Cl or 1M NaCL (♦ , ▲ EPS-NaCl solutions; • , + EPS-NH_4Cl solutions; → rise and ← decrease of temperature).

TABLE 2 . Determination of Young Modulus (E)[a] of polymer Gels Formed in the Presence of Trivalent or Divalent Cations.

Cation	Cr^{3+}	Fe^{3+}	Ca^{2+}
E x 10^{-4}	12.63	13.70	1.87

a. E expressed in N/m^2.

in the range of: $6 \times 10^4 < \overline{Mw} < 4 \times 10^5$. From the data obtained by SEC, a viscosity law was obtained: $[\eta]=2.10^{-2} \times \overline{Mw}^{0.9}$ with $[\eta]$ the instrinsic viscosity in mL g^{-1} and $\overline{Mw}$ the molecular weight.

When concentrations of high molecular weight ($\overline{Mw} > 3 \times 10^5$) glucuronan solutions are greater than 5 g L^{-1}, a thermoreversible gel is obtained at room temperature. The same phenomenon is observed with EPS solutions inferior to 5 g L^{-1} when ionic strengh is increased; when the temperature is 15 °C < t < 90 °C we observed a change in optical rotation attributed to a conformational transition dependant on the electrolyte used and on the concentration tested (FIG. 1).

In the presence of divalent or trivalent cations, cross linkages between the EPS molecules are formed instantaneously. With trivalent cations, higher strengh gels are

TABLE 3. Evolution of Young Modulus (E)[a] on Heating at 100 °C a Ca-Gel Obtained by Dialysis of a 10 g L^{-1} EPS Solution against 0.34 M $CaCl_2$.

	Time (hours)				
	1	2	3	4	24
Ca-gel in H_2O	1.20	1.10	1.10	0.90	destroyed
Ca-gel in $CaCl_2$ 0.34.M	1.30	1.30	1.30	1.25	1.20

a. E x 10^{-4}expressed in N/m^2.

formed. The E modulus of gels obtained with Cr^{3+} or Fe^{3+} and polymer at 10 g L^{-1} is approximately 7 times larger than the values obtained with Ca^{2+} gels (Table 2).

The Ca gels obtained are thermally stable in 0.34 M $CaCl_2$ (Table 3); in pure water, the gel swelled before being destroyed due to osmotic effects.

EXPERIMENTAL

EPS Production and Purification. The mutant strain *R. meliloti* M5N1 CS cultivated on RC medium,[11] supplemented with sucrose (1%) synthesized the extracellular material. The EPS production was studied according to a method described previously.[12]

EPS was isolated from the broth by microfiltration (with pore size of 0.2 μm) and purified by ultrafiltration (with 100,000 NMWCO membrane), the polymer was dried under vacuum at ambient temperature.

Reduction and Hydrolysis. The glucuronic residues were reduced by the action of *N*-cyclohexyl-*N'*[β-(*N*-methyl morpholino) ethyl] carbodiimide-*p*-toluene sulfonate at pH 4.75 and sodium borohydride at pH 7 (to prevent alkaline hydrolysis) according to a method described previously;[13] the reduction was repeated twice. The reduced EPS was hydrolysed in 70% sulfuric acid (2 mL) for 30 min at room temperature, then diluted with water (10 mL) and kept overnight at 100 °C. The mixture was neutralized with $BaCO_3$ before concentration and filtration.

Methylation. Methylation of the reduced EPS was carried out by the Hakomori method.[14] The methylated polysaccharide was recovered by dialysis. After hydrolysis and conversion of the aldoses into *O*-acetylated-*O*-methylated alditols, the solution was analyzed by gas liquid chromatography (GLC).

Determination of Constituents. Separation of neutral monosaccharides was achieved by high performance liquid chromatography (HPLC) on a Waters instrument

using a CHO-682 column from Interchim (France) with water as eluent at 85 °C. The detection system was an analytical IOTA refractive index detector (Jobin-Yvon, France) in series with a Perkin-Elmer model 241 polarimeter equipped with a 80 μL microflow cell operating at 365 nm with 0.05 degree full scale.

Characterization of Exopolysaccharides. The EPS were characterized by size exclusion chromatography (SEC) at room temperature using a modified Waters 150C apparatus with multidetection: a differential refractometer for concentration, a capillary viscometer for intrinsic viscosity [η], and a low angle light scattering detector (CHROMATIX CMX 100) for molecular weight.[15] Two aqueous gel filtration columns, B-804 and B-805 from Shodex, were applied in series with 0.1 M $NaNO_3$ as eluent. The samples were filtered through a 0.22 μm filter before injection. The injected volumes were 200 μL (1 g L^{-1}), the flow rate was 1 mL min^{-1}.

NMR Experiments. All NMR spectra were recorded with a Bruker AM-300 spectrometer equipped with an aspect 3000 computer for solutions in D_2O (about 10 g L^{-1}). ^1H NMR spectra were obtained using a spectral width of 2.5 KHz and a 16 K data block. The solvent peak (HDO) was partially suppressed under a 180°, t, 90° pulse sequence (t = 3 s) with 7 s recycle time.[16-17] The chemical shifts were referenced to internal sodium 3 (trimethylsilyl)propionate d_4.

Two dimensional homonuclear COSY and COSY with one step relayed coherence transfer were recorded using the standard Bruker software.

Optical Rotation Measurement. Solutions were prepared by mixing EPS solutions (0.5 g L^{-1}) heated at 85 °C with 1M NH_4Cl or 1M NaCl solutions heated at 85 °C; the mixture was cooled to 10 °C and then heated to 85 °C. The temperature was controlled by a Haake circulating water bath. During the decrease and rise of temperature, optical rotation [α] was measured with a Fica spectropolarimeter model 1b operating at 300 nm with a 5 cm thermostated cell.

Elastic Modulus Measurements. A 10 g L^{-1} solution of the sodium glucuronate heated at 60 °C was poured into a dialysis tubing (diameter = 14 mm) and immersed in a 0.34 M solution of $CaCl_2$, $FeCl_3$ or $CrCl_3$ at 60 °C during 5 h and kept overnight at room temperature. The gel formed was introduced into a cylindrical mould, cut into small cylinders (height = 17 mm) and immersed in a 0.34 M $CaCl_2$, $FeCl_3$ or $CrCl_3$ solution. The mechanical properties of the gels were obtained by compression between parallel plates on a Instron 4301 instrument, at a constant rate of deformation (25 mm min^{-1}) at ambient temperature. The elastic modulus were calculated on the linear region of the stress-strain diagram (lengh of deformation < 1mm).

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REFERENCES

1. Presented at the *XVIth International Carbohydrate Symposium* Paris, France, July 5-10, 1992.

2. A. Heyraud, M. Rinaudo and B. Courtois, *Int. J. Biol. Macromol.*, **8**, 85 (1986).

3. J. Courtois, B. Courtois, A. Heyraud, Ph. Colin-Morel and M. Rinaudo, Fr. Pat. 9202510, 1992.

4. H. Tsuchihashi, T. Yadomae and T. Miyazaki, *Carbohydr. Res.*, **111**, 330 (1983).

5. G.A. De Ruiter, S.L. Josso, I.J. Colquhoun, A.G.J. Voragen and F.M. Rombouts, *Carbohydr. Polym.*, **18**, 1 (1992).

6. A. Cesaro, F. Delben, T.J. Painter and S. Paoletti inV. Crescenzi, I.C.M. Dea and S.S. Stivala, *New Developments in Industrial Polysaccharides*; Gordon and Breach Science Publishers, New York, 1985.

7. P. E. Janson, L. Kenne and E. Schweda, *J. Chem. Soc. Perkin Trans. I.* , 377 (1987).

8. J. Haverkamp, H. Van Halbeek, L. Dorland, J. F. G. Vliegenthart, R. Pfeil and R. Schauer, *Eur. J. Biochem.*, **122**, 305 (1982).

9. C.M. Buchanan, K.J. Edgar, J.A. Hyatt and A.K. Wilson, *Macromolecules*, **24**, 3050 (1991).

10. H.C.J. Canter-Cremers, K. Stevens, B.J.J. Lugtenberg C.A. Wyffelman, M. Batley, J.W. Redmond, M.W. Breedveld, and L.P. Zevenhuizen, *Carbohydr. Res.*, **218**, 185 (1991).

11. B. Courtois, J. P. Hornez, J. Courtois and J.C. Derieux, *Ann. Microbiol. (Paris)* **134**, 141 (1983).

12. J. Courtois, P. Pheulpin, A. Heyraud and B. Courtois, *J. Gen. Appl. Microbiol.*, **36**, 215 (1990).

13. C. Bouffar, and A. Heyraud, *Food Hydrocolloïds*, **1**, 559 (1987).

14. S. Hakomori, *J. Biochem.*, **55**, 205 (1964).

15. B.Tinland, J. Mazet and M. Rinaudo, *Macromol. Chem. Rapid Comm.*, **9**, 69 (1988).

16. H. Grasdalen, B. Larsen and O. Smidsrød, *Carbohydr. Res.*, **68**, 23 (1979).

17. K.M. Varum, M.W. Anthonsen, H. Grasdalen and O. Smidsrød, *Carbohydr. Res.*, **211**, 17 (1991).